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The role of receptor tyrosine kinases in mediating glioblastoma resistance to radiotherapy and temozolomide

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ABSTRACT

Glioblastoma is the most common and aggressive form of malignant glioma. Currently, despite treatment with surgery followed by radiotherapy and the chemotherapeutic agent temozolomide (TMZ), mean patient survival time is approximately 12 months and the 5-year survival rate is close to 0%. A key factor for the dismal prognosis is tumour recurrence post-treatment which is largely due to: 1) the infiltrative nature of glioblastoma rendering complete resection impossible and 2) glioblastoma cell resistance to radio-chemotherapy. In this thesis we aimed to investigate the cellular mechanisms of receptor tyrosine kinases in conferring resistance to therapy.

We first performed a literature search and found that almost all studies that advocated for the utility of targeting RTKs in overcoming treatment resistance did not employ both therapeutic agents comprising standard therapy – radiotherapy and TMZ. We next generated an *in vitro* glioblastoma resistant model via short-term treatment with radiotherapy and TMZ and found that these cells had down-regulated RTK activity in addition to down-regulated protein and gene expression of the commonly altered and studied epidermal growth factor receptor (EGFR) and MET receptor. After generating an *in vitro* glioblastoma recurrent model via long-term treatment we demonstrated that the surviving sub-population of cells also displayed down-regulated EGFR and MET expression compared to treatment naïve cells. Furthermore, we also showed that the resistant cell population already pre-exists within the parental population which suggests the possibility of pre-emptively targeting the inherently resistant population.

Interestingly, we also observed differential microRNA expression in radiotherapy- and TMZ-treated cells and, specifically, found that miR-221 confers resistance to glioblastoma cells and is capable of down-regulating EGFR expression. We validated this relationship in a human cohort of 105 primary and 36 recurrent glioblastoma patients, showing a significant inverse relationship between miR-221 and EGFR. Consistently, we showed that high miR-221 and low-EGFR expression at recurrence is associated with a poorer prognosis.

Lastly, we investigated the relevance of epithelial to mesenchymal transition markers after observing that migration rates were maintained in resistant cells despite low EGFR and MET. Both N-Cadherin and CD44 were found to be highly expressed in treatment-resistant cells

and the down-regulation of AKT activity with wortmannin led to reduced levels of EMT markers, suggesting that AKT is a regulator of key EMT transcription factors that are specific to N-Cadherin and CD44.

The thesis gains it significance by providing an explanation to the failure of RTK inhibitors in the glioblastoma clinic by suggesting that standard radio-chemotherapy down-regulates RTK activity and expression, thereby diminishing any theorised benefit of targeting RTKs. Furthermore, the thesis advocates for microRNAs to be crucial regulators of therapy resistance, potential biomarkers and targetable molecules for the clinic.

DECLARATION

This declaration is to certify that:

i. The thesis comprises only their original work towards the PhD except where indicated in the preface
ii. Due acknowledgement has been made in the text to all other material used
iii. The thesis is fewer than 100,000 words in length, exclusive of table, maps, bibliographies and appendices

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PREFACE

The experimental data described in this thesis comprises only my work, except for the following, obtained in collaboration:

- The temozolomide resistant U87, U251 and U118 cell lines were generated by Dr Rodney Luwor and Dr Stanley Stylli

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Thank you to my parents for being the best parents they could possibly be – may God reward you both.

And may God reward all those who have helped me.

I thank God and acknowledge that all affairs, including my blessings and failings are in the Hands of God. Studying cell biology always reminds me how glorious Your creation is.

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LIST OF ABBREVIATIONS

*	p-value ≤ 0.05
**	p-value ≤ 0.01
***	p-value ≤ 0.001
AKT	Protein Kinase B
APE	Apurinic/apyrimidine endonuclease
ATP	Adenosine triphoshphate
BBB	Blood brain barrier
BER	Base excision repair
CD44	Cluster of differentiation 44
CDK4	Cyclin-dependent kinase 4
CNS	Central nervous system
CSC	Cancer stem cell
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DSB	Double strand break
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
Exo1	Exonuclease 1
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GLUT1	Glucose transporter 1
GSC	Glioma stem cell
HA	Hyaluronic acid
HGF	Hepatocyte growth factor

HIF	Hypoxia inducible factor
HR	Hazard ratio
IDH1	Isocitrate dehydrogenase 1
IL-	Interleukin-
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LOH	Loss of heterozygosity
MAPK	Mitogen-activated protein kinase
MET	Hepatocyte growth factor receptor
MGMT	O6-methylguanine DNA methylatransferase (also referred to as O6-alkylguanine DNA alkyltransferase)
MMP	Matrix metalloproteinase
MMR	Mismatch repair
miRNA	MicroRNA
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NF-kB	Nuclear factor kappa light chain enhancer of activated B cells
N.S	Not significant
Oct4	Octamer binding transcription factor 4
OLIG2	Oligodendrocyte transcription factor
PARP	Poly ADP ribose polymerase
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PDGFR-A	Platelet-derived growth factor a
PDGFR-B	Platelet-derived growth factor b
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol (4,5)-triphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PTEN	Phosphatase and tensin homolog
qRT-PCR	Quantitative reverse transcription polymerase chain reaction

RB	Retinoblastoma
RNA-seq	RNA sequencing
RT	Radiotherapy
RTK	Receptor tyrosine kinase
SGZ	Subgranular zone
SH2	SRC-homology 2
SOX2	Sex determining region Y-box 2
Src	Sarcoma family kinases
SSB	Single strand break
STAT-	Signal transducer and activator of transcription
SVZ	Subventricular zone
TCGA	The Cancer Genome Atlas
TGF	Transforming growth factor
TGF-B	Transforming growth factor beta
TIMP3	Metalloproteinase inhibitor 3
Tks	Tyrosine kinase substrate with (1, 2, 3 etc.) SH3 domains
TMZ	Temozolomide
TNF	Tumour necrosis factor
TP53	Tumour protein 53
TRAIL	TNF-related apoptosis-inducing ligand
U/D	Undetermined
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WHO	World Health Organisation
Wnt	Wingless-related integration site

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CHAPTER 1:
INTRODUCTION

PART 1: Defining glioblastoma

1.1 Epidemiology

A glioma refers to a subtype of primary brain tumours and is the most common type of tumours of the central nervous system (CNS), accounting for approximately 80% of all malignant brain tumours [1]. Glioblastoma is a type of glioma, and the most aggressive and the deadliest malignant primary brain tumour. In fact, almost 50% of malignant primary brain and CNS tumours as well as over 50% of all gliomas are glioblastomas [1]. In Australia, the incidence rate is approximately 3.96 cases per 100,000 but increases to 14.42 cases per 100,000 for the 65 years or older group [2]. Glioblastoma is slightly more frequent in males than females with an approximate 1.6:1 predominance [2]. It is also primarily a disease of the elderly with data from the United States showing the median age for glioblastoma patients to be 64 years and the incidence rate is highest in the 75-84 years old group [1]. Younger age is also a predictive factor for treatment response given that the present 2-year survival rate is around 45% for patients between 20-44 years old while it is only 27%, 14% and 3% for the 45-64, 65-79 and 80+ age categories, respectively. Similar trends are also observed regardless of the treatment era [1, 2].

Data from the United States also suggests that the incidence rate is higher for White Americans compared to other racial categories such as Afro-Americans, Asians and American Indians – for example, the incidence rate for Whites is approximately 2.5 times higher than Afro-Americans [3]. Whether a similar observation regarding racial differences is true for Australia is unclear. The frontal, temporal, parietal and occipital lobes of the brain are the common brain regions that glioblastoma is located compared to the cerebellum [3].

There is currently no consensus regarding glioblastoma aetiology rendering preventive therapies ineffective, though ionising radiation is regarded as one of more definitive examples for increasing the likelihood of glioblastoma [4]. The aggressiveness of glioblastoma can be noted by the low median survival time of only 2-4 months for patients with best supportive care only [5, 6]. However, recent advancements in therapy have improved the hazard ratios compared to early 1990s and currently the median survival time is 12-15 months [7]. To illustrate this, the 2-year survival rate was approximately 7% of all diagnosed cases for 1993-1995, 13% for 2002-2004 and 17% for 2005-2007. In fact, the

hazard ratios have improved for all patients since 2005 compared to cases from the 1990s, except for the 80+ age category [7].

1.2 The problem of classifying glioblastoma

Gliomas were first reported as far back as the beginning of the 19th century under different names - such as fungus medullary in German, encephaloide in French and medullary sarcoma in English - based solely on morphology [8]. The term glioma itself was first coined in a study by the pathologist Rudolf Virchow in 1865, almost a decade after he initially described the presence of a homogenous group of cells in the CNS called glia [9-11]. Virchow, with the macroscopic and microscopic tools available, proposed two main groups of brain tumours - the glioma and the sarcoma – according to their descriptive features [12]. To Virchow, gliomas were large tumours with similar appearance to normal brain tissue, presenting slow tumourogenic evolution, derive from glial cells and heterogeneous in terms of histology [8]. In contrast, Virchow defined cerebral sarcomas as more vascularised, proliferative and invasive together with a larger and higher number of fusiform shaped cells [8]. Hence, this may be the first attempt at filing gliomas into low grade glioma, as referred to the former category by Virchow, and high grade glioma, as referred to the latter category by Virchow.

The rest of the 19th century and the beginning of the 20th century witnessed the segmentation of glial cells with descriptions of astrocytes by Cajal and Lenhossek, followed by Hortega describing oligodendrocytes and microglial cells [13, 14]. This set the future foundations for the ordering of varying forms of observed gliomas according to the proposed cell of origin or histological similarities with certain types of glial cells. Hence, astrocytomas are named for their origin is thought to stem from astrocytes, albeit malignant astrocytomas resemble precursor forms of the astrocytes, and oligodendrogliomas are thought to originate from oligodendrocytes. Glioblastoma was formerly referred to as spongioblastoma multiforme; termed so due to the proposed cell of origin for the tumour mass to be the spongioblast, not glial cells because of the lack of resemblance between the tumour and healthy glial cells or other gliomas; and multiforme for its highly heterogeneous appearance [15]. It was not until the seminal study by Cushing and Bailey that the term glioblastoma multiforme was coined [16]. This study is regarded as the first major project to

classify and order brain tumours based on histology and prognosis, and culminated in subdividing glioma into ten categories with glioblastoma occupying a separate category [17, 18]. The classification system developed by Bailey and Cushing depended on the predominant cell type found in the glioma, though it was noted that gliomas aren't necessarily composed of a single type of cell, and the duo made the link between clinical impact and the category of glioma [15, 16, 19]. A limitation of this histological approach to classification is not only the scant attention given to the possibility that a particular anatomical presentation does not necessarily entail a specific biological behaviour but also that the atypical feature that is common of glioma cells makes it difficult for a connection to be made with mature glial cells; differing tumours may be grouped under the same category [20]. Consequently, if the aim for a glioma classification system is taken to be a reliable prognostic tool a method beyond a purely histological approach is required.

The next development in the classification of glioblastoma was distinguishing primary (pGBM) from secondary (sGBM) glioblastoma. Scherer in 1940 defined sGBM as being derived from pre-existing astrocytomas, having relatively minor necrosis and less morbid clinically due to slower evolutionary development compared to pGBM which developed *de novo* [21]. Commenting on this observation, which at this time was a peculiarity, Scherer claimed that the majority of astrocytomas eventually spontaneously transformed to glioblastoma [22]. After ruling out the possibility that surgery was the cause of sGBM and noting the inadequacy in morphology as a distinguishing marker separating pGBM from sGBM, the question of causality was posed but left unanswered.

The next few decades was absent of any major advance in glioblastoma classification though there were attempts to improve the classification system such as by Netsky et al., who characterised glioblastoma specimens according to age, gender, symptoms, tumour location and surgery status [23]. The developmental stages in glioma research divided into the macroscopic approach prominent in the early 1800s, the use of the microscope as witnessed in the works of Virchow in the mid 19th century and finally the adoption of histological techniques were successes if only in regards to our very basic understanding of glioblastoma. Nonetheless, the elusiveness of a comprehensive characterisation of glioma, and glioblastoma in particular, remained and resulted in the failure of great leap in improving survival outcome.

1.2.1 WHO classification

Today, the most regarded and well known glioma classification system is by the World Health Organization (WHO), developed first in 1979 [24, 25]. In the first edition glioblastoma was placed under the category of 'Poorly Differentiated and Embryonal Tumours' [26]. Not until in the 2nd edition in 1993 was glioblastoma filed under 'Astrocytic Tumours' – more than half a century after Scherer first categorised glioblastoma as an astrocytoma - where it remains till today in the 2016 WHO classification, and given a malignancy grade of IV, defined by : nuclear abnormalities, mitotic activity, endothelial proliferation and necrosis [26, 27].

The main impetus behind the shift in category was the accumulation and spread of molecular genetic evidence. According to the 1979 WHO classification of gliomas, glioblastoma is defined as "An anaplastic, highly cellular tumor consisting of fusiform cells, small, poorly differentiated round cells or pleomorphic cells alone or in varying combinations. [They display] necrosis, pseudopalisading, fistulous vessels and vascular endothelial proliferation, hemorrhage and invasive growth and usually prominent features [28, 29]". According to this definition glioblastoma has the features of anaplastic astrocytoma with the addition of necrosis.

Such a description may provide a working definition but visual criteria for a clinically important task as classification breeds a problematic level of subjectiveness and arbitrariness on the part of the observer. The resulting inter-observer variation introduces inconsistencies across independent observations and increases the probability of misidentifying tumour grades or distinguishing between two glioma types.

This was quite evidently the case in a 1997 study assessing the utility in relying upon histology for glioma classification [30]. Histological slides of various forms of glioma were reviewed by four neuropathologists and the histology characteristics were recorded according to cellularity, cytological atypia, the presence of mitoses and necrosis, and microvascular proliferation [30]. It was noted that at least one of the four neuropathologists mischaracterised oligodendrogliomas or oligoastrocytomas with anaplastic astrocytomas and vice versa around one-third of the time, while differentiating a grade II astrocytoma from a grade III astrocytoma or glioblastoma was another layer of difficulty.

Pseudopallisading necrosis, a hallmark of glioblastoma, was also found to be present in grade IV oligodendrogliomas [30]. Furthermore, the interpretation of the scale of the histological feature under assessment was an additional hurdle. Though the presence of a certain histological feature can be identified with reasonable consensus the scale of the degree of any particular feature was low. For example, although consensus for the presence of hypercellularity and microvascular proliferation reached approximately 97% and 62%, respectively, agreement on the scale of both of the corresponding elements dipped to 51% and 36% [30].

The magnitude of discordance regarding the degree of the histological element (and in the case of microvascular proliferation, the very presence of it) represents the elusiveness of an objective scientific definition for various qualitative biological features [30]. This is unsurprising given that dependency on purely qualitative data subjects the human interpretation of visual phenomena to relativity and disallows any avenue for precise quantification. Objective thresholds distinguishing between high cellular atypia and low cellular atypia or thick vasculature and thin vasculature or hypercellularity and hypocellularity were absent and are contributors to interminability [30]. Finally, histological approaches do not reveal the underlying biology of the tumour mass thereby diminishing the ability to infer patient prognosis from a particular diagnosis and explains the bifurcation between specific presentations of pathology, such as identical levels of necrosis, and the clinical course of a glioblastoma patient.

1.2.2 After histology: The rise of molecular analysis

Over the course of almost 15 years research progress in the field of glioma research, such as evidence from immunocytochemistry, partially revealed the molecular characteristics of gliomas. During this period accumulating evidence indicated that p53 mutations were part of the essence of astrocytomas. p53 gene mutations are present in approximately one-third of low grade (WHO grade II) astrocytomas [31]. Similar rates of p53 mutations are also observed in higher grade (WHO grade III) astrocytomas [31, 32].

Furthermore, a 1992 study found that around one-third of glioblastomas possess p53 mutations, a strikingly similar rate to p53 mutations found in lower grade astrocytomas in other studies [32]. This was followed by confirmatory evidence from separate laboratories

within the year [33, 34]. Additionally, studies conducted concurrently - for example those by Ohgaki et al. - found that p53 mutations in non-astrocytic tumours were rare [35, 36]. Only 12% of oligodendrogliomas that were sampled possessed p53 mutations; 53% of all oligodendrogliomas were WHO grade III but none showed detectable p53 mutations [36]. None of the four WHO grade III anaplastic ependymomas, none of the twelve circumscribed pilocytic astrocytomas(compared to diffuse astrocytomas such as anaplastic astrocytoma and glioblastoma) and only 11% of WHO grade IV medulloblastomas carried p53 mutations [35, 36]. Taken together, these studies were suggestive of the common roots shared between grade II-III and glioblastoma. Briefly, the p53 protein, approximately 393 amino acids, can function as a transcription factor and as mentioned, a dysfunctional p53 is observed in glioblastoma [37]. The key overall function of p53 as a transcription factor is to act as a checkpoint, whereby the induction of cell cycle arrest and apoptosis is caused by p53 signalling. Recent studies have placed a genetically altered p53 signalling pathway, consisting of CDKN2A, MDM2 and TP53, as a common occurrence in glioblastoma at a rate of approximately 85% [38]. The tumour suppressive role of p53 is activated in response to various stimuli, such as DNA damage, activation of tumour promoters and excessive growth factor signalling [37].

What these studies have in common is the molecular biology technique that allowed for such characterisations and paradigm shifts to occur. Polymerase chain reaction, invented in 1982, was used by all studies as part of the process to detect single strand conformation polymorphisms, thus signifying the beginning of a new era in glioblastoma research [39]. From then and till now the focus within the field of glioblastoma research has been on the molecular characteristics of the disease which was expected to create improved classification systems and better match clinical presentation with prognosis prediction.

1.3 Primary and secondary glioblastoma

With the advances in molecular biology Scherer's hitherto dormant conceptual distinction between pGBM and sGBM was reopened. The landmark study that once again brought back Scherer's observation was conducted by Watanabe et al. in 1996 [40]. Samples from 19 pGBM patients were identified according to whether a clinical history of less than three months was evident and absent of any histological evidence of a lesser grade astrocytoma.

30 sGBMs were identified after evidence of the patients being operated on for low grade or anaplastic astrocytoma. Epidermal Growth Factor Receptor (EGFR) and p53 expression was assessed via immunohistochemistry. The majority of pGBM patients (63% or 12 out of 19) displayed EGFR over-expression compared to only 10% of sGBM patients. In contrast, all but one sGBMs had higher p53 accumulation as opposed to 37% of pGBMs. p53 mutations were characteristic of sGBM, with two-thirds possessing them, while around 90% of pGBMs lacked it. Only one out of all forty-nine glioblastomas assessed presented both EGFR over-expression and p53 mutations indicating that the two genetic alterations belong to mutually exclusive glioblastoma subtypes. What this suggests is that the previous studies connecting mutated p53 to glioblastoma were in fact not alluding to an essential general characteristic of glioblastoma but a specific subtype. Furthermore, p53 mutations leading to p53 accumulation may be an important early event for malignant astrocytoma transformation, a genetic mark that is carried over throughout all stages in glioblastoma development. Given that EGFR is associated with aggressive tumour proliferation the link between EGFR and pGBM matches the aggressive clinical presentation of pGBM and the slower growth of sGBM [41]. Of further significance is EGFR amplification being a purported feature of *de novo* or pGBM rather than sGBM raises the possibility of EGFR amplification being an exclusive glioblastoma marker; thereby minimising the likelihood of confusing glioblastoma for a lower grade astrocytoma.

Another positive aspect to this study by Watanabe et al. is that it unified the major findings from preceding studies into a clearer conceptual scheme. Watanabe et al. (1996) was the first to order glioblastoma into pGBM and sGBM according to the molecular characteristics of EGFR expression and p53 mutation [40]. Previously, glioblastomas were already examined for loss of heterozygosity (LOH) on chromosome 17, which contains the gene coding for p53, and EGFR amplification [42]. Notably, EGFR amplification was linked to the absence of LOH17; the absence of EGFR amplification was linked to LOH17 [42]. The following year researchers showed that a subset of glioblastoma had LOH17 and identified that these patients also possessed p53 mutations [43]. What was thought to be *de novo* glioblastoma also had the feature of EGFR amplification and a lack of p53 mutations and LOH17 [43]. However, the methodology of both these previous studies, though alluding to a molecular distinction between two subsets of glioblastoma, lacked any clinical criteria to sort patients.

Consequently, they were unable to conclude, for example, that glioblastomas with LOH17 and p53 alterations were indeed the result of previous lower grade astrocytoma or that the presentation of *de novo* glioblastoma was contingent upon EGFR amplification; only a link between older age with EGFR amplification and younger age with p53 alterations was noted [42]. Indeed, it was Watanabe et al. (1996) that sorted glioblastoma patients according previous clinical operative history and that identified older age to be associated with pGBM and younger patients to be associated with sGBM [40].

Subsequent studies studying the characteristics of pGBM and sGBM ensured that patients were sorted according to prior histological evidence, or the lack of it, of pre-existing lower grade astrocytoma. A population based study assessing 715 glioblastoma patients showed that pGBM to be more frequent (95% vs. 5%) and a marker for worse prognosis compared to sGBM (mean survival time 4.7 months vs. 7.8 months) [44]. This study also restated the pre-eminence of p53 mutations in sGBM with 65% of sGBM possessing it (though 28% of pGBM also had p53 mutation) and confirmed that EGFR amplification to be a feature of pGBM with 36% of pGBM displaying it compared to only 8% of sGBM. The approximately 35% overall rate of glioblastoma EGFR amplification was similar to other studies - von Deimling also noted that around 40% of glioblastoma contained amplificated EGFR – which laid the foundations for mutated EGFR to become a key malignancy event in glioblastoma [42, 44].

However, several incongruences between studies that blur a clear distinction and inadequacies in the pGBM vs. sGBM conceptual scheme require commenting upon. LOH10 is consistently found to be a feature of glioblastoma with studies showing rates from almost 40% to high as 70% [42-45]. Nonetheless the status of LOH10 - noted by studies as a possible marker for either progression from less lesions or pGBM - as a distinguishing tool is not forthright [43, 45]. It was observed that EGFR amplification, thought to be common in pGBM, was exclusively linked to LOH10 [42]. Specifically, LOH10q was associated with EGFR amplification [44]. Indeed, the occurrence of LOH10q was found to be similar between pGBM and sGBM [44]. Furthermore, Fugisawa et al. showed 54% of sGBM compared to 47% of pGBM showed LOH10q as measured by PCR-based microsatellite analysis [45]. It has been suggested that this confusion may be due to loss of the entire chromosome 10 being more common in pGBM while partial loss of chromosome 10, namely LOH10q, to be more

common in sGBM [46]. Nonetheless, even this response has been disputed in a separate study that utilised fluorescence in situ hybridization and reported that in a set of 44 pGBMs and 20 sGBMs complete loss of chromosome 10 occurred in 79% of pGBM and 70% of sGBM [47].

The clinical value of LOH10 has also been questioned. Chromosome 10 hosts the tumour suppressor gene PTEN with its loss ex hypothesi leading to poorer survival [48-50]. Indeed, evidence has been put forth indicating that LOH10q is predictive of shorter glioblastoma patient survival compared to those without LOH10q, with mean survival times of 7.7 months and 9.3 months, respectively [44]. Balaesaria et al. in 1999 concluded that any level of chromosome 10 loss in high grade astrocytoma (34 WHO grade III patients and 49 glioblastoma patients) was tied to significantly less favourable survival outcome, though when taking glioblastoma alone this difference did not reach significance ($p=0.26$) [51]. Another study which analysed 25 newly diagnosed glioblastoma patients also did not find a statistical significance in survival times between patients with chromosome 10 loss and those without such alteration; a p-value of 0.06 though may be due to the smaller sample size [52]. Finally, in a cohort of 17 glioblastoma patients, which included one sGBM with chromosome 10 loss and another with chromosome 10 disomy, comparative mean patient survival time between the chromosome 10 monosomy group of 13 patients and the chromosome 10 disomy group of 4 patients was 10.9 months and 11.5 months, respectively; the result therefore was not significant [53].

Moreover, EGFR amplifications, initially hoped to be a highlight of pGBM, was later to be found to occur in pGBM too infrequently [40]. In a particular study , though EGFR amplification was absent in sGBM, EGFR amplification was only presented in 29% of pGBM [45]. Although EGFR amplification is more common in pGBM rather than sGBM, for genetic characteristics to be considered as hallmark or incorporated into a possible criteria as a diagnostic marker for pGBM it is reasonable to assert that the relative occurrence of such genetic alteration needs to capture at least the majority of the patients within the subset. As this requirement is lacking as far as EGFR amplification is concerned we must look beyond for a more unifying characteristic if we are to maintain the pGBM/sGBM distinction for diagnostic and prognostic purposes.

1.3.1 IDH1 mutation

The clinical significance of classifying glioblastoma into either pGBM or sGBM was questionable at best. At worse, it was merely used as a concept for theoretical pondering. Approximately ten years since the burst of studies in the late 1990s, that attempted to distinguish pGBM from sGBM, had to pass before genomic analysis revealed the potentiality of IDH1 being a hallmark of sGBM [54]. This paradigm shifting study was significant due to the high scale genomic analysis which sequenced 20,661 protein coding genes to elucidate glioblastoma genetic alterations and the use of Illumina sequencing to detect DNA copy number alterations [54]. The study was the first to show that IDH1 mutations were frequently mutated in sGBM; 83%, 7% and 11% was the rate of IDH1 mutation in sGBM, pGBM and overall glioblastoma, respectively [54]. Furthermore, IDH1 mutation indicated a significantly better survival rate compared to IDH1 wildtype (median survival time 3.8 years versus 1.1 years) [54]. However, although 105 tumour samples were available for study, only 6 were classified sGBM sanctioning caution regarding the conclusions that can be inferred.

In the same year Balss et al. confirmed the status of IDH1 as a sGBM marker [55]. A total of 685 brain tumours, which included 99 pGBM and 8 sGBM, were subject to IDH1 amplification via PCR prior to sequencing [55]. Interestingly, 74%, 62% and 88% of WHO grade II, anaplastic astrocytoma and sGBM displayed mutated IDH1, respectively, indicating both the possible connection between IDH1 and early malignancy and IDH1 as a hallmark of sGBM; again, only 7% of pGBM were IDH1 mutated [55].

Nobusawa et al. also restated the significance of IDH1 in glioblastoma classification [56]. From a total of 407 glioblastoma patients only 36 carried IDH1 mutations; however 22 out of 30 sGBM and only 3.7% of pGBM were positive. Interestingly, as with other studies, IDH1 mutations were specific to the region codon 132 [54-56]. Again, IDH1 mutation was indicative of a significantly favourable survival outcome with mean survival time for IDH1 mutation carriers being 27.1 months compared to 11.3 months [56]. The relationship between IDH1 mutation and favourable survival outcome has been validated in other studies [57, 58].

Taken together, IDH1 mutation appears to be a promising marker for sGBM and prognosis prediction. It may be argued that mutated IDH1, though it may be present in the majority of sGBM so far analysed, is not part of the essence of sGBM given that it is absent in some cases of sGBM; thereby calling into question the relevance of the pGBM and sGBM distinction. Indeed, it has been reported in a 2018 preliminary study with a small sample size (n=4) that IDH1 mutation in sGBM can be as low as 50% [59]. Moreover, when the number of sGBM samples were increased to as high as 30 patients the number of IDH1 mutated sGBMs decreased to almost two-thirds of the sGBM sample size, a number below the rate that has been reported in other studies with a lower sGBM sample size [54-56]. Therefore, positing IDH1 as the hallmark for sGBM cannot be concluded necessarily. An alternative explanation, however, is that the reported sGBMs that did not carry mutated IDH1 and those pGBMs that were IDH1 mutation positive may have been misclassified. Given that IDH1 mutated pGBMs have a genetic signature similar to IDH1 mutated sGBM; and given that IDH1 mutated sGBMs showed a WHO grade II precursor lesion; and lastly, given that sGBMs negative for IDH1 mutation being shown to derive from WHO grade III gliomas, this explanation cannot *a priori* be ruled out [55, 56, 60, 61]. Nonetheless, the vast majority of glioblastoma being pGBM, better methods are required to better characterise the tumour biology.

1.4 The four subtypes

In 2005 The Cancer Genome Atlas (TCGA) project begun and glioblastoma was the first cancer studied by the new project [62]. This initiative aimed to conduct large-scale genome sequencing to reveal the molecular characteristics and the genetic abnormalities of glioblastoma – and other cancers – to eventually create a classification system that would better inform researchers and clinicians about the individuality, along with the associated biological behaviour, of each glioblastoma [62]. The utility of such expansive data was shown in 2009 which defined several genes and signalling pathways that are key to glioblastoma tumourigenesis, such as the EGFR/MET-PI3K-AKT, the MET-Ras/PI3K and the CDKN2A-p53 signalling axes [63]. This conclusion was induced from the TCGA according to the levels of gene amplification, mutation and homozygous deletion, as the case with CDKN2A [63]. TCGA data revealed the importance of receptor tyrosine kinases (RTK) genetic abnormalities in glioblastoma such as EGFR, amplified in 45%; PDGFRA, amplified in 13%;

MET, amplified in 4%; and ERBB2, amplified in 8%. It was observed that 86% of all glioblastomas possessed genetic abnormalities in the RTK-PI3K pathway, thereby centring this signalling pathway in glioblastoma research [63].

Using this foundational study as a proof of concept, the influential paper by Verhaak et al. put forth a new classification system for glioblastoma [64]. After analysing the expression of 1740 genes in 202 glioblastoma samples it was revealed that four subtypes could be distinguished. A gene signature consisting of a total of 840 genes, with 210 genes in each subtype, was identified. These results were further validated in an independent validation cohort consisting of 260 glioblastomas. The subtypes were termed: Classical, Pro Neural, Neural and Mesenchymal. The defining genetic alterations of each subtype as described by the authors have been summarised in Table 1 [64]. Interestingly, each of the subtypes displayed genetic signatures associated with distinct cell types. The genetic signature of Classical subtype was more closely associated with murine astrocytes; the Pro Neural more closely associated with oligodendrocytes; the Mesenchymal group was similar to the cultured astroglial and immortalised cell lines' signature; and the Neural group resembled oligodendrocytic and astrocytic differentiation and neuron enrichment genetic signatures [64].

In fact, the aforementioned study was preceded by a similar project that profiled 76 high-grade gliomas with DNA microarray [65]. Three subtypes, labelled Proneural, Proliferative and Mesenchymal, were distinguished according to prognosis and a 35-gene signature [65]. The data was further validated by an independent set consisting of only glioblastoma samples. Mesenchymal and Proliferative groups were mostly seen in glioblastoma samples rather than the Proneural dominated WHO grade II astrocytomas. Similar to Verhaak et al., the Proneural subtype trended towards a favourable survival outcome and is comprised of younger patients [65]. Consistently, the Proneural class correlated with the genetic signature of normal neural tissue and the Proneural phenotype was similar to that seen after culturing neural stem cell lines [65]. The Mesenchymal class was closely associated with tumour recurrence and the genetic signatures of bone, smooth muscle, endothelial, dendritic and cultured human fetal astrocyte cells [65]. The Proliferation class exhibited stronger proliferative markers such as TOP2A, EGFR amplification, AKT activity and Ki-67 positivity compared to other groups; though the Ki-67 index was higher in the vasculature of

Mesenchymal tumours, a feature absent in the other classes, indicating that this group is more infiltrative [65].

It is unclear the level of relevancy that can attributed to a particular characteristic of each subtype and how much causal efficacy there can be attached to a genetic alteration. It is possible that the numerous features that help in the classification are not of any, or not of significant, value and accidental to a certain degree. For example, the relatively favourable survival outcome for patients of the Proneural group has been shown to be derived from the Glioma CpG island methylator phenotype (GCIMP) [38]. This has also been validated in another study showing DNA methylation to be a favourable factor towards survival, in particular to GCIMP-positive Proneural glioblastomas [66]. In fact, non-GCIMP Proneural glioblastomas showed a worse survival outcome compared to the aggressive Mesenchymal subtype [38]. This sheds light upon the need to better characterise causality and look beyond a purely descriptive analysis and towards the prescriptive. Certainly, it does not entail from the discussion thus far that the attempts to classify glioblastoma are futile – as far as clinical significance is concerned. But it is indicative of an element of arbitrariness in the process of categorisation. How much significance is applied to a certain genetic alteration - there could very well be other, more relevant, genes that have been lost or amplified but its effect may have been silenced by gene expression noise - and how broadly or narrowly the categories are defined - is it a Proneural glioblastoma or a GCIMP positive glioblastoma? - requires further research. To take the example from the previous study, the existence of a distinct Proneural group is questionable and may not be an objective biological reality but a mask over other, prognostically relevant, glioblastoma strata [38].

An additional question raised for this type of classification system is that these purported, largely distinct, glioblastoma groups may not have evolved through mutually exclusive pathways as implied but are part of a continuum. Recently, Ozawa et al. provided evidence that non-GCIMP glioblastomas of a Proneural nature are precursors to the Mesenchymal subtype [67]. Though non-GCIMP glioblastomas were similar to Proneural GCIMP glioblastoma, as evident with transcriptome analysis, the non-GCIMP cohort displayed alterations frequent in other subtypes, such as chromosome 7 amplification and loss of chromosome 10 [67]. When mathematic modelling was utilised to identify the possible evolution of tumour development it was deduced that chromosome 7 amplification and loss

of chromosome 10 were initial genomic events for all non-GCIMP subtypes [67]. NF1 is a tumour suppressor that is highly mutated or deleted in Mesenchymal glioblastomas while these alteration are almost absent in the Proneural group [64, 68]. PDGFRA-expressing tumours in mice had NF1 knockdown leading to a shift towards the Mesenchymal subtype characterised by expression of Mesenchymal markers such as CD44 and activated STAT3. Consequently, the authors come to the conclusion that the "primordial tumors are Proneural in character with the other subgroups evolving from them" [67].

The molecular classification data that has been accumulating in recent times has informed the latest WHO classification of tumours of the CNS. Previous WHO classifications relied on histology, microscopic features and lineage-associated proteins to define the different types of glioma, largely based on phenotype [28]. The histogenic system is largely inherited from the Cushing and Bailey studies but the principle itself - tumours can be classified according to naked observation and comparison with normal tissue - goes far back as Muller in 1838; thus standard glioma classification has shifted from a centuries old paradigm [18, 69, 70]. After a meeting of neuropathologists in Haarlem several recommendations with consensus were put forth so that WHO classification system is reformed. One such recommendation was that phenotypic characteristics are coupled with genotypic characteristics such that "molecular information should be incorporated into the definitions of some diagnostic entities" [71]. CNS tumours are now diagnosed with specific nomenclature indicating a molecular marker; for example, a specific case of glioblastoma is categorised as glioblastoma, IDH-wildtype or glioblastoma, IDH-mutant [27]. Furthermore, diffuse astrocytic and oligodendroglial tumours, including glioblastoma but excepting diffuse midline glioma, are to be diagnosed as IDH-wildtype, IDH-mutant or not specified [27]. Therefore, IDH status has now become a gold standard molecular marker for glioblastoma sorting. A variant of glioblastoma has also been added to the WHO classification system called 'glioblastoma with primitive neuronal component' which contains primitive cells displaying neuronal differentiation and sometimes "MYC or MYCN amplification" [27]. Lastly, another variant, the small cell glioblastoma, is to be characterised according to EGFR amplification [27]. The 2016 WHO classification, therefore, is the culmination of years of molecular biology studies characterising glioblastoma pathogenesis. However, with IDH-status the only hallmark currently used more research is clearly in need.

The questions that have been raised with the problem of glioblastoma stratification, such as clinical relevance, and indeed some of the inconsistencies that have been brought up in the models discussed so far, may be at least partially answered within the context of both the heterogeneous intra-tumoural and inter-tumoural nature of glioblastoma [72]. In order to adequately discuss molecular age of glioblastoma research an understanding of both the treatment currently available for glioblastoma and the discussion surrounding the origin cell of glioblastoma is required; and it is the latter that will be discussed first.

Table 1-1: Proposed subtypes of glioblastoma

A) According to Verhaak et al. [64]

Classical	Proneural	Mesenchymal	Neural
Ch 7 amplification + Ch 10 loss paired, 100% rate	Locus 4q12 amplification, altered and over-expressed PDGFRA	NF1 mutated, deleted, low expression	Neuron marker expression: NEFL, SYT1, GABRA1
EGFR amplification rate = 97%	IDH1 mutation	Over-expression of MET and CHI3L1	
EGFR over-expression	P53 mutation	High CD44	
12/22 EGFRvIII or point mutation	Lack of paired Ch 7 amplification + Ch 10 loss, rate 54%	High NF-kB signalling	
Lack p53,PDGFRA, IDH1 mutation	Oligodendrocyte marker OLIG2		
CDKN2A deletion			
Over-expression of Notch, Sonic Hedgehog and Nestin			
Median age: 55.7 years	Median age: 51.8 years	Median age: 57.7 years	Median age: 63.8 years
Median Survival (TCGA vs. validation set: 12.2 vs. 12.2 months	Median Survival (TCGA vs. validation set: 11.3 months vs. 16.2 months	Median Survival (TCGA vs. validation set: 11.8 months vs. 15 month	Median Survival (TCGA vs. validation set: 13.1 months vs. 15.0 months

B) According to Phillips et al. [65]

Proneural	Proliferation	Mesenchymal
Mostly WHO Grade III, 31% GBM	20% were GBM	49% were GBM
36% of GBM necrosis	Lack necrosis	7.5% of GBM necrosis
Mean Age = 49 years	Mean age = 50.7 years	Mean age= 40.5 years
Ch10 gain	PTEN loss	Over-expressed VEGF/VEGFR PTEN loss
Lower AKT activation	Ch7 amplification, Ch10 loss	Ch7 amplification, Ch10 loss
	EGFR amplification	EGFR amplification

1.5 The cancer stem cell model

As discussed, glioma classification systems have sorted different varieties of gliomas according to the assumed type of glial cell while glioblastoma classification is central to understanding the underlying tumour biology. It may be, though, that glioblastoma can be classified according to the cell of origin that initiates the tumour. Consequently, if the origin cell is discovered then it is possible to develop models that can predict cell behaviour subject to varying selective pressures. Furthermore, the varying proposed classes of glioblastoma have possibly arisen from independent origins or a single origin cell may be the initiator. Consequently, the origin cell is of significant research interest as it may be the link between a normal cell and the tumour initiating cells. One possible theory for the origin cell of glioblastoma is differentiated mature astrocytes. The alternative conceptual scheme situates the neural stem cell at the centre of glioblastoma initiation.

1.5.1 Astrocyte de-differentiation as the origin of glioblastoma

Historically, astrocytes have been thought to be a source of origin of glioma both due to similarities in morphology, as discussed previously, and more recently due to molecular characteristics. The rationale is as follows. Mature, differentiated astrocytes proliferate via symmetric cell division which makes astrocytes fulfill a prerequisite for cellular transformation. It has also been established that glial fibrillary acidic protein (GFAP) is highly expressed in mature astrocytes of the CNS, associated with astrocyte proliferation and is the prototypical marker for astrocytes [73]. In agreement, one study observed that all glioblastoma samples assessed were reactive to GFAP with immunohistochemistry [74]. Interestingly, all oligodendrogliomas assessed were negative for GFAP [74]. Furthermore, tumour volume in high grade glioma patients correlated with GFAP expression [75]. However, the relationship between GFAP levels and grade of glioma is contradictory with results showing either that GFAP is higher in lower grade gliomas compared to glioblastoma or decreased in lower grade glioma compared to glioblastoma [74, 75]. Therefore, the link between glioblastoma and astrocyte mediated via GFAP expression gives some credence to astrocytes being the glioma initiator.

Liu et al. attempted to determine whether PDGFRa over-expression in p16-negative/CDKN2A-negative mouse astrocytes leads to gliomagenesis [76]. When these cells,

which were cultured *in vitro,* were transplanted into mice brain invasive glioma was observed [76]. These tumours that were harvested from mouse brain showed expression of the astrocyte marker GFAP and the neural and progenitor stem cell marker Nestin [76]. Consistently, immunohistochemistry staining for the oligodendrocyte marker CNPase appeared negative [76].

A 2011 study first established cell cultures derived from primary murine astrocytes which were knocked out for p53 and PTEN leading to Akt expression, and transduced to drive EGFRvIII expression [77]. It was noted that primary astrocytes with knocked out p53 and PTEN leads to induction of tumours after intracranial implantation [77]. Furthermore, tumours were characteristically high grade glioma – cytologically similar to WHO grade III astrocytomas - with necrosis present, meaning that tumours are glioblastoma [77]. This induction was accelerated after EGFRvIII expressing primary astrocytes were added [77]. Again, this study also noted that these induced gliomas expressed GFAP and Nestin [77].

In a 2013 study, Radke et al. isolated primary astrocytes, determined by GFAP expression, from one day old C57BL/6 mice that expressed p53+/+ or p53+/- or p53-/- [78]. Upon harvesting the brain and culturing the cells, a majority astrocyte population was obtained in culture which was transduced with Akt or/and c-Myc. Significantly, when p53-/- astrocytes that expressed both Akt and c-Myc were injected into C57BL/6 mice tumour formations were observed that resembled tumours that form after injection of GL261 glioma cell line into mice [78]. Interestingly, late passage astrocytes that were transduced with Akt and c-Myc eventually displayed expression of stem cell marker CD133 suggesting that astrocyte dedifferentiation into progenitor stem cells may be the glioma initiator [78].

1.5.2 Neural stem cells as the origin of glioblastoma

The alternative paradigm for elucidating the glioblastoma cell of origin is the neural stem cell model. The term stem cells refers to a group of cells that have the potential to self renew and differentiate to generate mature cells that form the tissues which make up an organ. Recently, stem cell biology has been of increasing interest in glioblastoma research as it is possible that normal stem cells give rise to tumour cells and tumours themselves possess a population of cancer stem cells. These cancer stem cells are able to proliferate potentially infinitely and populate the tumour bulk [79-81].

Neural stem cells and glial progenitor cells – progeny of neural stem cells and precursors to cells of glial origin such as astrocytes and oligodendrocytes – have been known to exist throughout varying brain regions such as: the subventricular zone, dentate gyrus, subcortical white matter, cortex, corpus callosum and periventricular white matter [79].

Specifically, the subventricular zone of the lateral ventricles and the subgranular zone of the dentate gyrus in the hippocampal formation are the regions of neurogenesis in the adult mammalian brain [82]. GFAP is a marker for neural stem cells in the subventricular zone and subgranular zone, while Nestin, along with a number of other markers such as Sox2, is found in both neural stem cells and progenitor cells [82]. Unipotent progenitor cells can also express specific markers allowing for identification, such as oligodendrocyte progenitor cells expressing PDGFRa and Olig1 [82].

Gliomas and neural stem cells both have high migration, high proliferation and activation of signalling molecules such as EGFR, Wnt and Nestin suggesting that transformed precursor cells carry certain markings belonging to their origin cells [82]. This raises the hypothesis that glioma stem cells are progeny of normal neural stem cells and the link between the matured glioblastoma and the cell of origin.

Studies have suggested that neural stem cells found in the subventricular zone are the cell-of-origin for astrocytomas. Mice harbouring p53 and NF1 deletions developed GFAP-positive astrocytomas of various grades, including glioblastoma, with the origin of the majority of the tumours being associated with the subventricular zone - as assessed by magnetic resonance imaging and immunofluorescence – and express Nestin [83]. Furthermore, a subset of GFAP-expressing cells in the subventricular zone was found to express the EGFR and a highly proliferative neural progenitor-like population [84]. Upon EGF stimulation this subset displayed increased invasion and proliferation [84].

Two studies, conducted almost simultaneously, provide some of the stronger evidence for neural stem cells being the glioma cell of origin rather than differentiated mature astrocytes. First, Llaguno et al., using mouse models, concluded that neural stem cells can be origin cells for glioma [85]. The researchers utilised the cre-lox system with tamoxifen administration for temporal control and preferred Nestin promoter, as opposed to a GFAP promoter, to more specifically target neural and progenitor without differentiated

astrocytes. All embryonic and adult tamoxifen-treated Nestin-cre-ERT2 mice harbouring p53/PTEN/Nf1 deletions developed high grade gliomas similar to glioblastoma [85]. The astrocytomas that developed were immunoreactive for GFAP, Nestin and Olig2, and highly proliferative as assessed with Ki-67. Furthermore, the transformed neural stem cells were found in areas adjacent to the subventricular zone, such as the cortex and striatum, suggesting migration. Significantly, injection of viral cre-mediated tumour suppressor inactivation did not lead to adult astrocytoma formation except in the subventricular zone [85].

The second, a 2010 study by Jacques et al., inferred that neural stem cells alone, rather than mature astrocytes in the parenchyma, were responsible for glioma initiation regardless of the location in the brain [86]. This group of researchers deleted Retinoblastoma protein, p53 and PTEN by using a cre-lox system with Adeno GFAP-cre [86]. After injection of Adeno-cre controlled by GFAP promoter into the lateral ventricles of mice with homozygous deletions of Retinoblastoma and p53 or Retinoblastoma, p53 and PTEN or PTEN and p53, tumours were developed suggesting that GFAP-expressing cells in the subventricular zone allows for glioma initiation [86]. Significantly, astrocytes derived from the forebrain with mutated Retinoblastoma, PTEN and p53 and recombined with Adeno-cre *in vitro* before transplantation into the striatum resulted in no tumours being formed [86].

Consequently, it is possible that neural stem cells are the origins of glioma. If this is the case it will mean that deactivation of tumour suppressors in neural and/or progenitor stem cells leads to the glioma formation. Nonetheless, another study developed a transgenic model consisting of mutated Ras and p53 knockout harbouring mice, aiming to specifically target neural stem cells and mature astrocytes [87]. The brains of these hGFAP-Cre/KrasG12D/p53f l/f 1 mice were then examined with immunohistochemistry and immunofluorscence and showed high grade glioma phenotypes. It was observed that one particular group of cells that presented with high grade glioma phenotype expressed higher levels of GFAP and glioma stem cell markers such as Olig2, Sox2 and Bmi-1; Nestin, another stem cell marker, was prominent in this phenotype but also expressed in other glioma phenotypes to a lesser degree [87]. When tumours were analysed in 17-day transgenic mice, glioma-phenotype cells in the subventricular zone were immunoreactvive for Nestin, Sox2 and Olig2 while tumours in other regions – which had different phenotypes - expressed Nestin but not Sox2

and Olig2 [87]. Ghazi and colleagues harvested and cultured cells from the brains of neonatal hGFAP-Cre/KrasG12D/p53f l/f 1 [87]. To sort for neural stem and progenitor cells the subventricular zone was dissected while astrocytes were derived from the cortex; the transgenic astrocytes developed neural stem cell marker expression for Sox2, Nestin, Bmi-1 and Olig2 [87]. Upon transplantation of these genetically manipulated cultured neural stem cells or astrocytes development of tumours were noted but showed differences in tumourogenic characteristics such as invasiveness. The authors concluded that the differentiation status of the transformed cell determines, at least partially, the glioma phenotype [87].

The aforementioned studies provide evidence supporting the hypothesis that gliomagenesis can originate from mature astrocytes or neural stem cells, although common limitations that are found across these studies ought to be mentioned. First, GFAP is commonly regarded as a standard astrocyte marker and indeed researchers have used GFAP to select for mature astrocytes [78]. However, GFAP is also expressed in neural progenitor cells which raise doubts on the validity of relying solely upon GFAP for astrocyte isolation [88-90]. Furthermore, the models of cell utilised in cell-of-origin studies are mainly embryonic or post-natal cells which may differ in terms of biological behaviour from glioblastoma, an adult disease [87, 91]. Another source for caution is that astrocytic behaviour and characteristics are thought to differ between *in vitro* and *in vivo* [92]. For example, it is has been reported that transcriptome profiles vary between cultured astrocytes and *in vivo* astrocytes, although detailed analysis of *in vivo* astrocytes require overcoming the hurdle of maintaining the microenvironment [93]. Lastly, the models used in the studies above - with conditional knockouts and oncogenic mutations - to study glioma lineage do not necessarily replicate the molecular characteristics of clinical glioblastoma and, more specifically, the conditions in which gliomagenesis begins. Therefore, more studies taking into consideration the limitations discussed are required to further understand the role of mature astrocytes in glioblastoma initiation. If it is the case that mature astrocytes are the cause, or partially contribute, to the initiation of glioblastoma it may have relevance in the glioblastoma classification project. It may well be, as Ghazi et al. noted, that glioblastoma can be classified according to the origin tumour initiating cell which explains both the

heterogeneous molecular and morphological characteristics as well as the varying clinical outcomes.

PART 2: The Stupp Protocol

1.6 Current treatment of glioblastoma

1.6.1 Resection

Successful removal of brain tumour was first accomplished by William Macewen in 1879 and the first removal of a glioma took place in November 25, 1884 by Alexander Bennet and Rickman Godlee [16, 94]. In the immediate following decades neurosurgery was a depressing endeavour due to high morbidity rates, with surgical mortality rates ranging from 30-50% [95]. However, with Cushing's research into neurosurgical methods, in particular his focus on intracranial pressure, the surgical mortality was decreased by approximately 45% [96]. Advances in surgical tools such as the operating microscope, introduced to neurosurgery in 1957, cortical mapping and functional magnetic resonance imaging has improved the surgical outcomes [97]. Today, blue light illumination after 5-aminolevulinic acid ingestion by the glioma patient and intraoperative angiography has allowed neurosurgery to greatly reduce complications [97]. Indeed, a 2016 analysis of over 16,300 cases of malignant brain tumour surgery showed only a 0.9% mortality rate [98].

Surgical resection of the tumour is the front-line therapy for glioblastoma [99]. This has brought added focus upon surgery's role on glioblastoma survival. A retrospective review of 360 primary glioblastoma patients highlighted the clinical importance of neurosurgical techniques on glioblastoma survival independent of extent of resection [100]. Survival times were lower in patients who had neurological complications - including acquired language and motor deficits - arising from neurosurgery compared to patients without any surgery induced neurological deficits; median survival and 2-year survival rates were approximately 9 months and 0-8%, respectively, in the former group, compared to 12.8 months and 23%, respectively, in the latter group [100].

The extent of surgical resection of the tumour is commonly categorised as either gross total resection or partial resection. Gross total resection is achieved if complete removal of the

tumour is judged to have occurred as measured by the contrast enhancement imaging such as post-operative magnetic resonance imaging [101]. A partial resection is when only incomplete resection has occurred, again by comparing pre- with post-operative imaging [102]. Total resection, if defined by 100% of tumour removal, is impossible for two broad related reasons. Firstly, a verdict of total resection is judged macroscopically and does not take into consideration the presence of glioblastoma cells at the microscopic level [103, 104]. Secondly, the infiltrative nature of glioblastoma means that the tumour cells at the outer ring of the larger overall central mass is consolidated with normal brain regions which cannot be surgically removed unless severe neurological deficits is the outcome [100, 104]. Indeed, it is established that glioblastoma recurs within a margin of approximately 4cm from the site of resection [104].

The predominant study that made this link was the 2001 study by Lacroix and colleagues [105]. In this study the histological, Karnofsky Performance Scale pre- and post-operative images, age, necrosis and other clinical data of 416 patients were retrospectively analysed and tumour volume was quantified with magnetic resonance imaging [105]. Crucially, it was observed that glioblastoma with equal to or more than 98% of resection had significantly longer survival time compared to a resection of less than 98%; median survival time was 13.8 months and 8.8 months, respectively [105]. When only treatment naive patients were included – which amounted to 233 patients in total – median survival time was 13 months and 10.1 months, respectively and reaching statistical significance [105]. The authors divided the entire cohort of 416 patients into four groups according to the presence of tumour necrosis, with the presence of necrosis considered as unfavourable; patient age which itself was divided into three groups: less than 45 years, between 45 and 64 years and equal to or more than 65 years, with the higher age group considered to be unfavourable; and the Karnofsky Performance Scale, with score of equal to or more than 80 considered more favourable [105]. After the sorting of patients based on the favourable status according to this criteria it was reported that patients who belonged to the most unfavourable clinical group but had equal to or more than 98% resection survived longer than patients in the same clinical status group but had less than 98% of the tumour removed; though the result was not significant the median survival times were 8.6 months and 7.8 months, respectively [105]. The survival difference according to resection levels in

the group of patients with the most favourable clinical status was also not significant [105]. However, in the second most favourable and second most unfavourable group, as judged according to clinical presentation of necrosis, age and Karnofsky Performance Scale, showed a significant survival difference with greater than or equal to 98% resection being favourable to survival [105]. This last analysis was not conducted for treatment naive patients. For this reason the possibility that the exposure to other treatment has played a role in the survival differences and that the significance of the survival differences that is claimed to have been due to the extent of resection cannot be ruled out.

The Lacroix et al. study remained as the pivotal study - for at least a decade and perhaps till now - justifying the surgical attempts to maximally remove the tumour when possible. Several other analyses conducted subsequently verified this conclusion [106-108]. For example, Sanai and colleagues, when assessing the extent of resection of 500 cases of newly diagnosed glioblastoma concluded that along with age and Karnofsky Performance Scale, the post-operative tumour volume were all significant predictors of overall survival [109]. In fact, as little as 78% of tumour resection level was seen to have a significant positive relationship with overall survival – the median survival for this group was 12.8 months whereas the overall median survival was 12.2 months – while resection levels of greater than 90% and 100% led to median survival time of 13.8 months and 16 months, respectively [109]. The extent of resection required to lead to a significant survival difference differs between Lacroix et al and Sanai et al; the former claims that 89% resection is required for glioblastoma patients and 96% resection is required for treatment naive patients but the latter study claims only 78% resection is required [109]. Lastly, the same research group as the 2001 Lacroix et al. study showed that in a cohort of 1229 glioblastoma patients complete resection led to a 15.2 month median survival time compared to 9.8 months after less than complete resection; however, this was a result obtained without taking into account whether the patients were newly diagnosed or recurrent [110]. 752 patients in the cohort were treatment naive and 539 and 213 of these patients underwent complete resection and at least 78% resection, respectively [110]. Median survival time in the treatment naive group was 10.4 months and 17.9 months after partial resection and complete resection, respectively [110].

Taken together there is strong evidence suggesting that maximal safe resection provides on overall clinical benefit for glioblastoma patients. However, it must be noted that studies supporting this conclusion incorporate patients that have also undergone adjuvant therapy, most commonly radiotherapy. Therefore, a true causal relationship between resection level and survival cannot be clearly uncovered since a possibility remains that the survival times reported is at least partially contingent upon therapies other than surgical removal of the tumour. It follows from then that those patients that are predisposed to respond favourably to a particular form of adjuvant therapy – or those patients that are less responsive for that matter – introduce an element of selection bias. Secondly, tumour location may play a decisive role in determining the extent of resection thus, a limitation and masking factor. Resection can be rather limited in eloquent brain regions such as the visual cortex; therefore, it may not be extent of resection per se but tumour location that is the significant prognostic variable [111].

1.6.2 Radiotherapy

Due to impossibility of surgical resection other treatment modalities are required for glioblastoma treatment. Radiotherapy for glioblastoma as standard treatment was suggested in a 1966 study showing the 6-month and 1-year survival rates for surgery plus radiotherapy to be 75% and 29.2%, respectively, and for surgery alone to be 11.9% and 5.1%, respectively [112]. Though it was a series of studies conducted mainly in the 1970s that established radiotherapy as a key therapeutic tool in glioblastoma management [113-116]. The first large randomised trial, evaluating the efficacy of the antineoplastic antibiotic called mithramycin, consisting of 96 glioblastoma patients reported median survival times of 15, 33 and 36 weeks for the post-operative untreated group, patients that received less than 50Gy and patients that received more than 50Gy of radiotherapy, respectively; however the total number of patients for this comparison was a total of 44 patients [116]. Even though mithramycin showed no significant affect on survival the group that received more than 50Gy radiotherapy had an overall longer survival time compared to patients that received a lower dose or no radiotherapy but mithramycin alone [116]. It must be noted however that radiotherapy scheduling and dosage was not accounted for and not randomised meaning the results reported was suggestive, albeit strongly [116]. Notably, this

study was the first multi-institutional clinical trial testing the efficacy of a drug for the treatment of anaplastic glioma.

Two years after the former trial the same research group, the Brain Tumor Study Group, evaluated the efficacy of the nitrosourea 1,3-bis(2-chloroethyl)-1-nitroscourea (hereafter called BCNU but also called carmustine) in a group of 222 anaplastic glioma patients [114]. Patients were divided into four groups: BCNU alone, radiotherapy alone, radiotherapy plus BCNU and 'best conventional care', meaning no BCNU or radiotherapy [114]. Unlike their previous report, patients during this clinical trial were given set doses of radiotherapy – 50Gy in 5 fractions per week over 5 to 6 weeks [114]. Though BCNU was found to have no significant efficacy in improving survival (there was only a modest increase when compared to the untreated group), the radiotherapy group - consisting of 68 patients - showed a median survival time of 36 weeks, the longest survival time in the study and significantly longer than the untreated and BCNU groups' median survival time of 14 and 18.5 weeks, respectively [114].

Another randomised study conducted by the Brain Tumor Study Group involved 358 malignant glioma patients, 84% of which were diagnosed with glioblastoma; all patients were allocated to received radiotherapy alone, BCNU plus radiotherapy, semustine alone or semustine plus radiotherapy [115]. The radiotherapy regimen was a total dose of 60Gy in 30-35 fractions of 171-20 rads, 5 days per week. All groups that received radiotherapy had significantly longer survival times than semustine alone but no significance was detected when comparing the three groups that received radiotherapy with each other [115]. Interestingly, though not reaching statistical significance a modest improvement in median survival times was observed in the BCNU plus radiotherapy group compared to radiotherapy; median survival time for radiotherapy alone was 36 weeks as with the previous study and BCNU plus radiotherapy median survival time was 51 weeks [115]. Thus, the authors made the conclusion that radiotherapy should be part of the standard glioblastoma treatment protocol and BCNU to be the chemotherapeutic agent of choice [115]. These keystone studies also established that maximum total irradiation dose of 60Gy should be administered as both a safety precaution and due to effectiveness [113-116].

1.6.3 Chemotherapy

1.6.3.1 Pre-TMZ era

Summarising and evaluating all the clinical trials in the lead up to the incorporation of Temozolomide (TMZ) as the cornerstone chemotherapeutic agent for glioblastoma is an arduous task and largely irrelevant for the current endeavour of providing a rational justification for our research project. Consequently, only a brief account of the pre-TMZ chemotherapeutic era will be given.

The previous studies by the Brain Tumor Study Group showed promising results regarding the effectiveness of BCNU, an alkylating drug belonging to the class of lipid soluble, blood-brain-barrier-crossing nitrosoureas compounds. Indeed, throughout the 1990s nitrosoureas were the preferred class of agents for treatment [117]. The list of nitrosoureas includes BCNU, lomustine and nimustine, though other nitrosoureas such as semustine have been part of clinical trials.

Meta-analysis by Fine et al. in 1993 comparing the efficacy of radiotherapy alone with radiotherapy plus chemotherapy showed that across the 16 studies analysed the median survival for the former group to be 9.4 months and 12 months for the latter group, which was a significant difference [118]. However, the report did not provide an adequate account as it neglected the fact that a number of these studies consisted of a large proportion of Grade III astrocytomas which may introduce an intolerable level of bias. When only the studies that consisted of mainly glioblastoma patients, defined so when more than 75% of patients were glioblastoma-positive, the number of studies that are available to analyse decreases to six. The median survival time for the radiotherapy alone and the radiotherapy plus any nitrosourea group reduces to 8.5 months and 11.5 months, respectively. The radiotherapy plus nitrosoureas group survival time was longer compared to radiotherapy alone in three studies. Therefore, the data in this meta-analysis does suggest a therapeutic benefit for the addition of nitrosoureas to the standard surgery plus radiotherapy treatment regimen.

Another meta-analysis published in 2002 surveying 12 randomised controlled trials which involved treatment regimens consisting of at least one nitrosourea compound concluded that the 1-year and 2-year glioblastoma survival rate increases on an absolute level from

35% to 41% and 9% to 13%, respectively [119]. The overall increase in survival is equivalent to 2 months [120]. Considering that the total number of glioblastoma cases included in the meta-analysis reached 1900 the increase in survival rate is reasonably representative and the results are modest [119]. A randomised phase 3 clinical trial in 2003 - consisting of 240 primary malignant glioma cases of which 207 were glioblastoma - combined BCNU wafers with surgery and post-operative radiotherapy and observed that this treatment regimen led to a median survival time of 13.9 months compared to 11.6 months in the placebo group [121]. When taking only glioblastoma cases in the analysis median survival time for BCNU-treated group was 13.4 months and 11.4 months in the placebo group; however this result was not significant [121].

1.6.4 Temozolomide (TMZ)

TMZ was first synthesised and identified by the research team led by Malcolm Stevens as a 3-methyl mitozolomide analogue and given the name CCRG 81045 [122]. Using a variety of test systems which involved murine tumour models it was observed not only that TMZ showed similar activity to DTIC – which is poorly metabolised in humans to the active metabolite MTIC resulting in clinical failure – but the most potent of all analogues tested [122]. Mitozolomide itself was tested for efficacy against a number of cancers, such as colorectal, breast, malignant melanoma and small cell carcinoma, but intolerable levels of toxicity, myelosuppression and lack of effectiveness ended its promotion to first-line therapy [123-128]. The clinical development of TMZ was furthered with reports of absence of cumulative toxicity, ease of handling compared to mitozolomide, penetration of the blood brain barrier (BBB), positive tissue distribution, clinical responses and clinical improvement in a Phase I trial consisting of glioma patients [129].

A number of clinical studies regarding TMZ and high-grade gliomas were published in the 1990s and reported positive toxicity profile. It was in these trials that the dosage scheduling was set to 150-200 mg/m^2/day for 5 days a week for 4-weeks; this dosage scheduling is now part of the gold standard treatment regime [130-133]. However, it was two Phase II trials testing TMZ efficacy in recurrent glioblastoma that boosted TMZ potentiality. The first, published in 2000 and consisting of 225 patients, reported that the 6-month progression free survival for TMZ to be 21% and only 8% for those administered procarbazine [134]. Meanwhile the 6-month survival rate was 60% for TMZ and 44% for procarbazine [134].

Brada et al. in 2001 also confirmed the effectiveness of TMZ in a Phase II trial consisting of recurrent glioblastoma; the 6-month progression free survival rate, the primary endpoint, was 18% [135]. Comparison with procarbazine - at the time a preferred option for recurrent malignant high grade glioma after nitrosourea-based treatment – showed TMZ to be more effective [136]. Thus, TMZ, though only showing a modest clinical efficacy, became the rated and approved recurrent glioblastoma treatment of choice.

Finally, in 2005, a single study by a research team led by Roger Stupp led to TMZ being incorporated into the gold standard treatment regimen for primary glioblastoma alongside surgical resection and radiotherapy [133]. To summarise this landmark study a 573 glioblastoma cohort were divided into two groups: Radiotherapy alone or radiotherapy plus TMZ. Radiotherapy consisted of 2Gy per fraction daily for 5 days per week over a period of 6 weeks; the total dose was 60Gy. TMZ was administered at a dose of 75mg/m^2/day for 7 days a week concomitantly with radiotherapy for 6 weeks then after a 4 week break a further adjuvant therapy with TMZ 5 days a week for 28 days at a dose of 150mg/m^2 to 200mg/m^2. It was observed that the median survival time with radiotherapy plus TMZ was 14.6 months and with radiotherapy alone was 12.1 months. The 2-year survival rate was 26.5% and 10.4% for the former and latter, respectively. Lastly, median progression free survival was 6.9 months for the former and 5 months for the latter. These significant results promoted TMZ to gold standard and the treatment combination – surgical resection, radiotherapy and TMZ – as well as the treatment scheduling became known universally as the Stupp protocol.

Two features of these trials are in need of remarking. First, it is observable from the Stupp study that TMZ efficacy is similar to the survival benefits previously reported with BCNU. However, the favourable toxicity profile with TMZ and the severe toxicity profile with BCNU led to the preference of the former over the latter; it must be noted BCNU never gained universal acceptance for this characteristic [120]. Therefore, it is not necessarily the superiority of TMZ over BCNU or other nitrosoureas, strictly with respects to overall survival time, but the tolerability of the agent. This of course is an essential characteristic if combined therapy regimens are to be tested in the future. Furthermore, the ease of oral administration over wafers is another positive.

Secondly, patient selection bias may have distorted and over exaggerated the efficacy of TMZ that was reported in the Stupp study. The reason for this caution is that the group treated with radiotherapy alone reported a median survival time of 12.1 months – a survival time that was higher than previous clinical trials consisting of a 'radiotherapy alone' group and a difference that can be as large as 3.6 months [115, 118, 137]. This was made partially aware to the readers by the authors of the 2005 Stupp et al. paper themselves as they wrote "The outcome for patients treated with radiotherapy alone in our trial compares favourably with the outcome in other trials... these criteria may have served to exclude patients with the worse prognosis, who may not benefit from any therapy" [133]. What the authors did not state was the obvious logical consequence given that the favourable survival time for radiotherapy alone was the result of patient selection bias – the favourable survival time for the radiotherapy plus TMZ group is also a result of patient selection bias. Taken together, caution must be attributed when assessing the efficacy of TMZ based on this report and a need for improved chemotherapeutic regimens remains. Nonetheless, the Stupp, 2005 paper has become part of the foundation of glioblastoma research and paradigm defining.

1.6.4.1 Metabolism of TMZ

TMZ is a lipophillic 194 dalton molecule part of the imidazotetrazine family that is orally administered and acts as a prodrug [138]. Its oral administration is contingent upon its stability at acidic pH but under the physiological pH conditions of circulation TMZ is converted to MTIC which reacts with H_2O to form AIC and methyldiazonium cation [139]. The environment of brain tumours being more alkaline than healthy brain tissue further aids the TMZ metabolic process and, recently, glioblastoma peritumoral pH was found to be slightly alkanilic at pH 7.20 [140, 141]. MTIC however is stable in alkaline conditions and unstable in acidic conditions meaning that the TMZ metabolic reactions is initiated and processed in a narrow pH range [142, 143].

It is this second product of MTIC breaking down that is the active agent of TMZ as it methylates the DNA at the N7 position of guanine, N3 position of adenine and O6 position of guanine; the methyl lesion at the O6 position of guanine being the main source of TMZ cytotoxicity although the other two methyl lesions have minor, secondary cytotoxic roles [139, 144]. A property of DNA upon exposure to methylation agents is that guanine rich

areas are at risk of methylation, mainly middle guanines of guanine-consecutive runs. Furthermore, guanine enriched areas leads to widening of the major grow and increased functional group steric accessibility [145, 146]. Consequently, approximately 70%, 9% and 6% of the lesions formed are N7-MeG, N3-MeA and O6-MeG, respectively [142, 145, 146].

1.6.4.2 Can TMZ cross the blood-brain-barrier (BBB)?

TMZ has the property of BBB penetration. Reports have been published that cerebrospinal fluid concentrations are as high as 30-40% of plasma concentrations in rhesus monkeys after TMZ infusion as measured by reverse-phase high-pressure liquid chromatography [147]. The percentage compared to plasma concentration was reduced to around 20% in 9L glioma rat models [148]. Similarly, pharmokinetic analysis with glioblastoma patients revealed a 20% cerebrospinal fluid TMZ concentration of plasma concentration. Cerebrospinal fluid TMZ levels reached 6.1±1.2 mg/L/hr when TMZ was administered at 200 mg/m^2/day [149]. This is crucial given that TMZ is converted to MTIC after BBB penetration - the latter lacking this penetrative capacity – and if this capacity is important for glioblastoma treatment [150]. However, whether this is a property of importance for glioblastoma treatment is controversial.

Along with the molecular biology and intra-tumoural heterogeneity, the third characteristically issue of importance in glioblastoma research is the BBB [151]. The question to what extent the BBB is a problem in glioblastoma therapy is of crucial importance as it 1) defines the scope of therapy available to be tested clinically and 2) serves a rational justificatory role for *in vitro* and animal model work. Although this project that we are undertaking does not require addressing this controversy in detail, a brief summary will be provided.

The BBB refers to an interface between the vasculature and the brain that is essential for the maintenance of homeostasis [152]. The three dominant cell types of the BBB – endothelial cells, pericytes and astrocyte end-feets – work to prevent circulating molecules from unrestricted entry into the brain [152]. Specifically, specialised endothelial cells - which differ due to tight junctions, absence of fenestrations and infrequent picocytic vesicular transport - impede hydrophilic molecules but lipophillic molecules (i.e. O_2 and CO_2) can diffuse unrestricted [152]. In glioblastoma, functional computerised tomography scans have

shown that the BBB is permeable [153]. In contrast to the tumour-free region which does not take up the iodinated contrast material due to the intact BBB, areas surrounding the glioblastoma mass are easily delineated [153]. K_1 constants, a measure of the blood-to-tissue transfer, in high grade astrocytomas are up to fifteen times more than the tumour-free areas [154]. The permeability of this interface is a product of high vascularisation, abnormal angiogenesis with structural and functional deformity in blood vessels that is mediated by vascular endothelial growth factor receptor (VEGFR) signalling [155, 156].

This suggests that chemotherapeutic agents for glioblastoma are not required to possess the physiochemical characteristics needed to penetrate the BBB, such as high lipophillicity and small molecular weight and size - characteristics that TMZ possesses [157]. Indeed, it has been shown, using high performance liquid chromatography and mass spectroscopy, that high concentration of the anti-EGFR agent gefitinib is found in patient glioblastoma tissue leading the authors to proclaim that high drug concentrations are not required and Agarwal et al. to comment that this study suggests the BBB "does not influence delivery in glioma" [158, 159]. However, the breakdown of the BBB is not homogenous and the invasiveness of glioblastoma makes the tumour cells at the outer-edge invisible to contrast enhancement, meaning the tumour burden is also present within an intact BBB [160, 161]. This is evident with non-enhancing FLAIR signals being detected in glioblastoma [162]. It follows that the penetration of the BBB is reaffirmed as a conceptual barrier and any therapeutic regimen must take this into consideration. TMZ possessing BBB penetrative capabilities perhaps explains its effectiveness compared to other agents that have been trialled [157]. In addition to its positive structural characteristics, TMZ increases BBB permeability via down-regulation of the ATP-binding cassette transporter P-glycoprotein - which is positioned on the luminal side of the BBB and acts as part of an efflux system - through inhibition of the Wnt-B-Catenin pathway [163]. These characteristics are absent in other non-penetrative drugs. For example, doxorubicin, a cytotoxic agent, in *in vitro* studies was found to be clinically ineffective due to poor penetration of the BBB [164]. Or the proteosome inhibitor bortezomib, which was shown to be futile in a Phase II recurrent glioma trial that can be explained by previous data reporting poor penetrative ability [165, 166]. In conclusion, it is evident that the BBB is indeed an obstacle for glioblastoma treatment and innovative technologies are required to increase permeability [167-170].

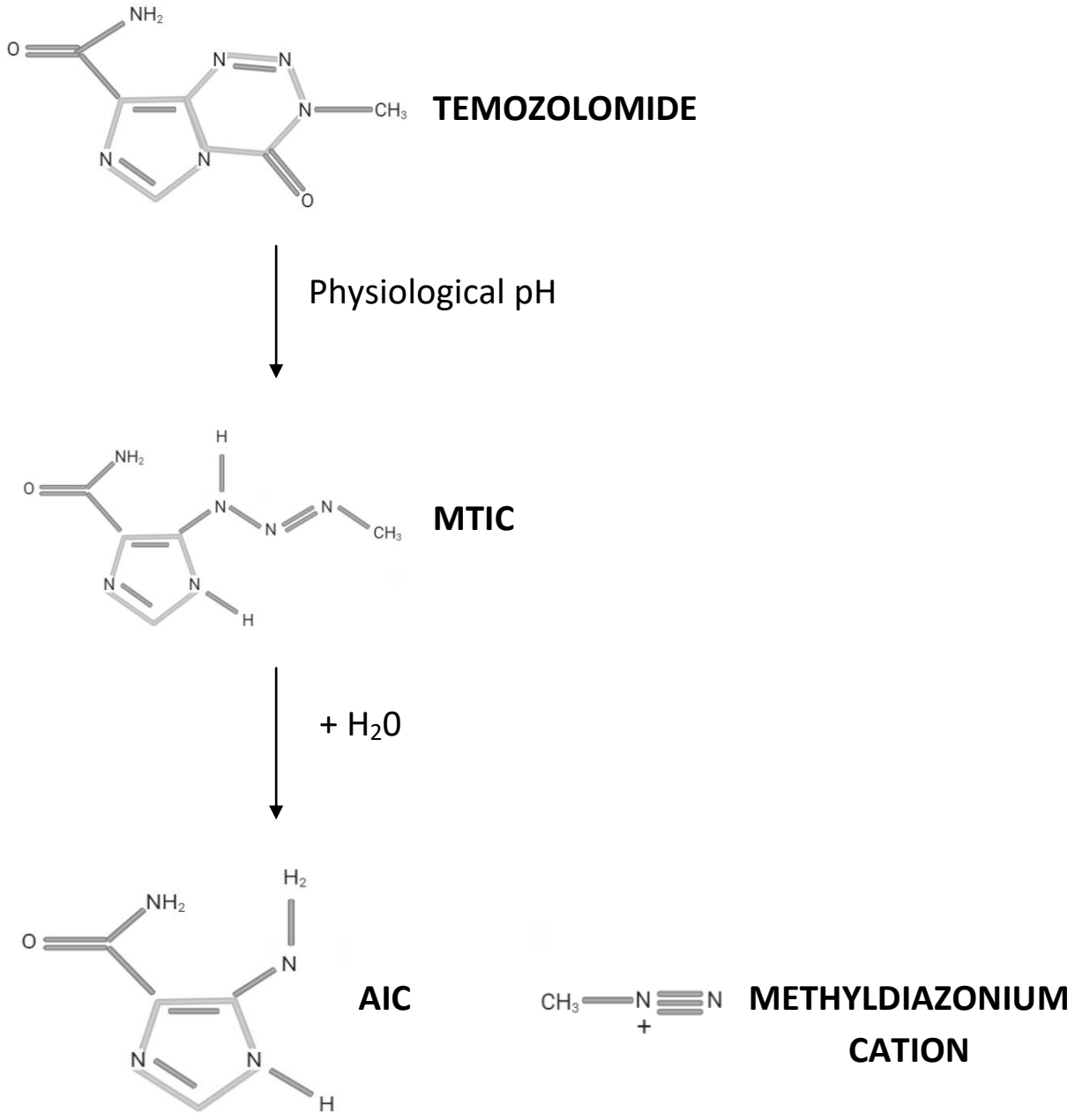


Figure 1-1: Schematic representation of the metabolism of TMZ. Under physiological pH conditions temozolomide (TMZ) is converted to its active form MTIC. In reacting with H_2O, MTIC converts to AIC and the methyldiazonium cation, the latter of which possesses the methyl group that typically forms DNA lesions producing N7-MeG, N3-MeA and O6-MeG.

1.7 Molecular mechanisms of TMZ

A detailed layout of the cellular and molecular mechanisms of TMZ currently remains lacking. This is a consequence of the complications of cause and effect studies which place significance upon a particular signalling mechanism but cannot rule out that another pathway is equally or more significant for TMZ efficacy. One such example where this problem can be partially illuminated, at least on conceptual basis, is the search for the cytotoxic methyl lesion formed by TMZ. Earlier it was mentioned that the predominant position taken in the literature is that the most cytotoxic lesion formed by TMZ is the O6-MeG. For this position to be justified we must first at least have evidence that invalidates the other methyl lesions' role as the main trigger for apoptosis or cell cycle arrest.

1.7.1 The dominant cytotoxic lesion formed by TMZ

The dominant position in the literature is that the O6-MeG lesion is the most cytotoxic and significant lesion formed by TMZ. Administration of the methylating agent DTIC agent leads to low levels of O6-MeG and N7-MeG levels were 10-fold higher in the liver, kidney and lungs; though brain DNA damage was found to be low re-iterating the incapacity of DTIC to cross the BBB [171]. N7-MeG lesions leave apurinic sites that are known to be cytotoxic and mutagenic due to inefficient site repair and mismatch pairing of the opposing nucleotide[172]. However, equating the frequency of N7-MeG with being the most cytotoxic is claimed to be falsified by Abbot et al. who detected no non-complementary nucleotide binding in Escherichia coli DNA after treatment with dimethyl sulphate – a methylation agent that selects for N7-MeG [173] . On the contrary, it was treatment with N-methyl-N-nitrosourea which led to O6-MeG lesions and subsequent non-complementary nucleotide binding, with other studies confirming this [173-175].

Forthright evidence from studies on the superior cytotoxicity of O6-MeG over other methylation lesions formed by TMZ or other methylating agents in cancer, let alone glioblastoma, seems elusive. Meshina and colleagues' conclusion (in a highly respected and expansive 2006 literature review focussing on DNA alkylation damage that was published in the esteemed Chemical Reviews journal) that the N7-MeG lesion "is relatively innocuous" is derived from a 1996 research article that by itself shows no evidence warranting that conclusion; in fact the cited paper adopts an agnostic position by making clear that data

regarding the cytotoxic role of N7-MeG is lacking and the consequence of such a lesion in unknown [176, 177].

A more recent literature review - published in the well-regarded Nature Reviews Cancer journal – also makes the exact same argument as Meshina et al. by stating “By itself, (N7-MeG) does not possess any mutagenic or cytotoxic properties but it is prone to spontaneous depurination to form apurinic/apyrimidinic sites that are toxic and mutagenic” before citing a 2004 review in support [144, 178]. Putting aside the fact that no direct evidence was cited in support, the cited review paper does echo the same sentiment regarding the lack of cytotoxicity of N7-MeG but cites in support a 1974 research paper that, unfortunately, studied the consequence of methylation treatment in RNA-containing bacteriophage; an unideal model, as the research authors themselves stated, given that a single-strand nucleic acid lesion may not necessarily translate to the double stranded DNA [144, 179].

Marchesi, in his review article that focussed on the mechanisms and DNA repair systems related to triazene compounds, also makes this often repeated claim but cites a 2002 review article [180, 181]. It takes another two articles to finally reach the original research paper – a review paper from 1996 and the original research article published in 1970 [182, 183]. A few comments are in order regarding this quartet. First, the original 1970 research paper – coincidentally by the same author as the 1996 review paper – was based on work on E.coli DNA and the damage induced upon it by the methylation agent N-methyl-N’-nitro-N-nitrosoguanidine. The authors infer from the observation that N7-MeG not being enzymatically excised – N3-MeA and O6-MeG were the first and second most excised lesion after treatment – that the lesion itself is perhaps inert [183]. And again, the same author echoes the same sentiment 26 years later suggesting that the “Persistence of damage, as with (N7-MeG), could indicate the inert nature of the adduct” [182]. It is certainly plausible according to evolutionary dynamics that the persistence of a lesion, and in this case the N7-MeG lesion, equates to inertness; however, this conclusion is suggestive and does not falsify the opposing claim. Secondly, though other methylating agents may be functionally similar to TMZ different outcomes cannot be ruled out until experiments with TMZ are planned. Thirdly, experimental evidence from bacteria may not necessarily translate to more complex systems such as found in humans.

Perhaps the stronger evidence for the relative inertness of N7-MeG comes from animal studies targeting the components of the base excision repair system which repairs the lesions N7-MeG and N3-MeA [184]. When Alkylpurine-DNA-N-glycosylase (APNG) - a key enzyme part of the BER pathway that facilitates N3-MeA and N7-MeG removal - was knocked out in murine embryonic stem cells, viable APNG-deficient mice were generated [185]. Afterwards, cell survival of primary embryonic fibroblasts cell lines established from both wild-type and APNG-knockout mice did not differ after treatment with TMZ nor did the decrease in number of leukocytes differ after BCNU treatment [185]. Interestingly, bone marrow cells of myeloid lineage that were Aag-knockout and consequently lacked N3-MeA DNA glycosylases were found to be more resistant to methyl methanesulfonate treatment [186]. Previous work by Engelward et al. showed that the loss of Aag – which led to silencing of N3-MeA DNA glycosylase - in mice cells are sensitised to BCNU, mitomycin C and the N3-MeA selective Me-Lex [187, 188]. These are therefore inconsistent results but also contradict the part of the dominant paradigm of TMZ mechanism. While Engelward's work showed methylation by Me-Lex and methyl methanesulfonate confers sensitivity upon Aag -/- mice, Roth concluded that Aag -/- myeloid progenitor bone marrow cells were resistant to TMZ, Me-Lex and methyl methanesulfonate. This is perhaps due to the difference in the biological models as Engelward used primary mouse embryo fibroblasts and mouse embryonic cells unlike Roth [186-188]. Crucially however, it is known that Aag can also excise N7-MeG raising the possibility that consequence of Aag -/- is at least partially contingent upon unrepaired N7-MeG [189].

Recent evidence within the glioblastoma literature specifically leads us to further doubt the dominant paradigm. Loss of the transcription factor GATA4 was observed in approximately 60% of glioblastoma and drives glioma formation but transient transfection for GATA4 re-expression was involved with down-regulated APNG, TMZ-induced apoptosis and reduced glioblastoma cell line viability independent of MGMT status [190]. Furthermore, direct siRNA-induced APNG knockdown also sensitised glioblastoma cells to TMZ more than MGMT-knockdown though combining APNG- and MGMT-knockdown sensitised the glioblastoma cells higher than single siRNA treatments [190]. In a panel of various glioma cell lines treated with TMZ concentrations from 50uM to 250uM the glioblastoma cell line T98G – which expresses high MGMT and APNG – was the most resistant; the MGMT-null

and APNG-null A172 was the most sensitive; and there was no significant difference between U251 (MGMT-null and APNG-high) and C6 (MGMT-high and APNG-low). Importantly, A172 cells transfected to express APNG or MGMT led to resistance to 100uM TMZ [191].

Taken together, there is conclusive evidence of cytotoxicity after the O6-MeG lesion and this is in line with the consensus position. However, in contrary to the often assumed position in the literature there is convincing evidence attributing a cytotoxic role to the lesions N7-MeG and N3-MeA after the use of methylating agents, including TMZ.

1.7.2 How does MGMT function?

TMZ is termed a S_N1-type methylating agent since it can form lesions at the nitrogen atoms on the rings of the DNA in addition to the extracyclic oxygen atoms [178]. MGMT, a small 22kDa enzyme spanning 207 amino acids, is capable of removing and repairing the DNA adducts at the O^6 of guanine [192]. This repair mechanism is a stoichiometric reaction that involves MGMT directly transferring the methyl group to a cysteine residue located in its activation site leading to MGMT inactivation and degradation via the ubiquitin pathway. The reason for the self-destructiveness of MGMT is thought to be the strong MGMT-CH_3 covalent bonding that inhibits MGMT from demethylating.

Experimental evidence indicates that MGMT rotates target nucleotides in order to gain access to the methyl lesion [193]. Two domains make up the MGMT protein – the 19kDa C-terminal domain and the N-terminal domain – of which the C-terminal contains the critical residues for the removal of methyl groups while the N –terminal may be required for the former domain's active configuration and MGMT transcription post-removal [194, 195]. The recognition complex of MGMT protein, led by Arg128, binds to the minor groove causing widening of the minor groove; the DNA molecule itself rotates approximately 15^o away from MGMT allowing the Cys145 residue of the MGMT active site to interact and complete the removal of the methyl lesion [193]. Essentially, the Arg128 residue rotates the nucleotide and replaces the vacant space with itself allowing the methylated nucleotide to be removed from the major groove end [193]. The structural interactions of the recognition complex selects for guanine while selection for the Me-G lesion is thought to be three times more than guanine, perhaps due to the hydrophobic properties induced by methylation and

Cys145 affinity to the methyl lesion [193, 196]. Secondly, the thiol functional group and the low pKa value of Cys145 confers high reactivity [197]. Thirdly, it has been suggested that the methylation at O^6 distorts the structure of guanine and the DNA duplex, increasing accessibility to the methyl group [198].

Methylated MGMT is rapidly degraded via the ubiquitin pathway after treatment with methylating agents such as O^6-benzylguanine (O6-BG). One such mechanism for ubiquitination is the alterations in structural configurations after the S-alkyl adduct is formed increases specificity towards the ubiquitin E3 ligase and the E2 ubiquitin-conjugating enzyme. Indeed, treatment with the proteosome inhibitor MG132 prevents methylated MGMT degradation [199]. Other studies using murine and human tumour models have also postulated this [176, 200].

MGMT in normal conditions serves as a tumour suppressor. Rats administered with methyl-nitrosourea and treated with the MGMT inhibitor O6-BG had reduced times in developing tumours [201]. In addition, transgenic mice that over-expressed the E.coli MGMT gene were less susceptible liver carcinogenesis from alkylating carcinogens, such as dimethylnitrosamine [202]. Tsuzuki et al. showed, utilising MGMT-gene knockout mice models, that MGMT -/- mice appeared normal, though minor weight loss was reported [203]. The 2-week survival rate of MGMT -/- knockout mice treated with 10 mg/kg of N-methyl-N-nitrosourea was 0% while MGMT +/+ mice treated with the same agent but with 100 mg/kg dose all survived past 3 months. It must be noted that the treated MGMT -/- mice developed severely damaged internal organs, dramatic decrease in the size of spleen and thymus, dysfunctional hematopoiesis and hypocellular bone marrow [203].

1.8 Mechanism of resistance to TMZ

1.8.1 Mismatch Repair System

The failure of MGMT to remove O6-MeG lesions leads to a series of reactions called the Mismatch Repair (MMR) system and eventually apoptosis, meaning that the cytotoxicity of the O6-MeG is dependent upon a functional MMR system [204]. During replication and upon TMZ-induced O6-MeG DNA polymerase mispairs the O6-MeG with a thymine residue triggering the MMR system to remove the thymine. Repeating this futile cycle eventually

leads to double-strand breaks and cell death in a p53-dependent manner [205]. The MMR system has been extensively studied in E.coli and the steps involved are: 1) The MutS homodimer detects the methyl lesion and recruits the homodimer MutL 2) This ATP-dependent complex activates the endonuclease MutH that is needed for DNA cutting close-by to the lesion 3) UvrD helicase unravels the ends of the cut strand which allows digestion of a specific portion of the DNA strand up until the lesion is removed 4) DNA polymerase III synthesises the remaining gap before DNA ligase completes the process by joining the strands together [180]. However, in methylated systems ataxia replication protein A and telangiectasia and Rad3 related-interacting is localised to the DNA damaged region followed by phosphorylation of checkpoint CHK1 kinase in the second cell cycle immediately after methylation treatment [206]. Alternatively, it has been suggested that after recognition of the DNA damage the MutS complex recognises and signals for cell-cycle arrest and apoptosis [206]. Ultimately, O6-MeG activates caspase- and mitochondrial-mediated apoptosis [207]. Caspases-2, -3, -8 and -9 increase after methylation treatment while expression of mitochondria-inhibiting signallers Bcl-2 and Bcl-Xl inhibited O6-MeG cytotoxicity; mitochondria death pathway is therefore an essential pathway for O6-MeG cell death [207].

Whether TMZ induces apoptosis or only cell cycle arrest in glioblastoma cells is debateable. TUNEL assays showed an increase in TUNEL-positive U87MG spheroids 2 days after 20ug/ml TMZ; though senescence, as indicated b lysosomal staining was also detected [208]. Other studies have reported that TMZ does not lead to MMR-induced apoptosis but p53- and p21-dependent G2M cell cycle arrest and subsequent senescence in glioblastoma cell lines, including U87MG [209]. The difference in result may be due to the latter using FACS sorted sub-G1 DNA cells to quantify apoptosis throughout the 10 days the U87MG monolayer cells were treated with TMZ; the sub-G1 population was significantly lower than G2M population of cells and did not alter throughout the treatment course [209]. Furthermore the latter study treated cells with 100uM for only 3 hours before TMZ-free incubation throughout the 10-day period while the former study, although TMZ treatment was also approximately 100uM, incubated the cells with TMZ for either 2 days or 7 days [208, 209]. Importantly, both studies fail to extend the methods used for apoptosis detection. TUNEL assays, and DNA fragmentation methods in general, are not specific for apoptosis and may also lead to

detection of necrosis; consequently other detection techniques should be utilised in tandem [210-212].

Evidently, a functional MMR system is required for cytotoxicity to methylating agents [213]. MMR-deficient Chinese hamster ovary and colorectal carcinoma cells are able to tolerate N-methyl-N-nitrosourea-induced O6-MeG rendering these cells DNA damage tolerant rather than DNA damage resistant, as with the case of MGMT [214, 215]. Indeed, malignant brain tumour cells were found to be resistant to TMZ treatment after MMR dysfunction [216]. Furthermore, MMR- and MGMT-deficient glioblastoma cell line A1235 was insensitive to TMZ as high as 200uM while MMR-positive/MGMT-deficient A1235 cells were only sensitive to TMZ treatment at 10uM [217].

1.8.2 Base excision repair

The Base Excision Repair (BER) is a mechanism that functions as a remover and repairer of DNA lesions, such as alkylations, oxidations, deaminations and depurinations, a maintainer of genomic stability and is implicated in a number of biological processes such as ageing and neurological disorders, in addition to cancer [218].

A number of DNA glycolysases exist in the BER system but four are specifically for the removal of incorrect pairing of thymine and one is involved in removing alkylated nucleotide bases (see detailed overview [218, 219] . These monofunctional enzymes slice the base-sugar N-glycosyl bond. Apurinic endonuclease recognises the consequential apurinic site and cleaves the apurinic site leaving 3'hydoxyl and 5'phosphate termini [184]. DNA polymerase β, which recognises the 3' hydroxyl termini as a substrate, also functions as a lyase to separate the sugar from the 5'phosphate before this gap is filled and glued by DNA ligase [184].

The DNA glcosylase 3-Methylpurine DNA glyosylase, otherwise known as alkyladenine DNA glycosylase (AAG) is the BER glycosylase that is required for the recognition and repair of alkylated lesions, including N3-MeA and N7-MeG; therefore, two out of the three major lesions committed by TMZ [184]. Proof of concept studies have shown inhibition of DNA polymerase β leads to TMZ sensitivity and initiation but incomplete BER triggers TMZ-induced cytotoxicity [220].

So it has been reasoned that unrepaired N7-MeG and N3-MeA is required for TMZ sensitisation and it follows that an efficient BER is critical for TMZ-resistance [221, 222]. Methoxyamine is capable of inhibiting BER by competing with apurinic endonuclease for abasic site binding [223]. There have been papers regarding the efficacy of methoxyamine to overcome TMZ resistance, such as a 2011 study by Goellner et al which based its result on cell line LN428, which suggested potential benefit of a BER inhibition-based therapy [224, 225]. Furthermore, apurinic endonuclease inhibitors also demonstrate cytotoxicity in glioblastoma cell lines [226]. The only clinical trial - a Phase II trial - testing methyoxyamine, in combination with TMZ, on recurrent glioblastoma was pre-maturely brought to an end due to lack of responses in patients (ClinicalTrias.gov Identifier NCT02395692).

1.8.3 MGMT and resistance

Consistently, MGMT expression correlates with TMZ resistance in glioblastoma cells. A panel of 16 tumour cell lines - including 12 glioma and 4 carcinoma cell lines - that were subject to 250uM TMZ *in vitro* for 24 hours (the authors termed these surviving cells as 'resistant') had elevated MGMT protein expression though MGMT-methylation status varied; four resistant glioblastoma cell lines had low or undetectable MGMT-methylation [227]. The MGMT-positive T98G was the most resistant cell line out of a panel of six glioblastoma cell lines. Only in combination with O6-Benzylguanine did T98G show sensitivity after TMZ treatment as shown by increase in cells at the G2-M cell cycle phase. This indicates cell cycle arrest while apoptosis was not detected via TUNEL assay though the exact treatment conditions with TMZ plus MGMT inhibitor was not stated clearly [228]. This was confirmed by Hermisson et al. after inhibition of MGMT in MGMT-positive T98G cells and MGMT over-expression in MGMT-deficient LN229 cells conferred sensitivity and resistance to TMZ, respectively [229].

Glioma stem cells are thought to be key mediators of chemoresistance [230]. TMZ-resistant CD133-positive glioblastoma cells – and as such defined as glioma stem cells – had elevated levels of MGMT expression [231]. MGMT levels in glioblastoma stem cell-like cells – generated by adding EGF and bFGF daily onto stem cell culture medium and defined by expression of Nestin and Sox2 – that were subject to 50uM TMZ for 4 hours was found to be inversely correlated with treatment-induced cell death, as measured by propidium iodide uptake[232]. When MEK-ERK signalling axis was inhibited MGMT levels followed and

remained suppressed even after the removal of the MEK-ERK inhibitor agent. It was also reported that p53 up-regulation is associated with MEK-inhibition-induced MGMT suppression [232]. Given that the PI3K-AKT axis is also linked to the MEK-ERK axis and elaborate signalling mechanism tied to TMZ resistance can be mapped out [233]. Indeed, recently in 2017 it was reported that inhibition of the PI3K-AKT pathway by treatment with BKM120 suppressed the MGMT over-expression observed after TMZ treatment in glioma cells; although BKM120 monotherapy did not significantly reduce MGMT level suggesting again the TMZ-dependent MGMT relevancy [234]. In contrary, after 6 months of TMZ treatment of glioblastoma cell line U87 the resistant cells were MGMT-negative though epithelial to mesenchymal transition (EMT) and AKT signalling increased [235]. Given that parental U87 is MGMT-negative and TMZ-resistant U87 remained so, it is plausible that resistant characteristics can be intrinsic rather than adaptive [236].

Other signalling molecules are also associated with MGMT regulation. Kohsaka et al. generated TMZ-resistant U87 by exposing the parental line to 'low dose of TMZ' (what concentration is unclear) such that cell viability IC50 for the resistant line was 150uM and 40uM for the parental line [237]. STAT3 levels were higher in resistant U87 but in contradiction to Yi et al., Kohaska reported TMZ-resistant U87 had elevated levels of MGMT [235, 237]. Upon inhibition of STAT3 activity MGMT levels was decreased in resistant cell lines suggesting that MGMT may be regulated by STAT3 [237]. The mechanism at play in STAT3-MGMT signalling was not found to be transcriptional regulation of the MGMT gene as MGMT mRNA remained the same but a proper to explanation towards why is lacking by the authors [237]. Furthermore, the tumour suppressor miR-198 has also been implicated in MGMT regulation with down-regulation of the micro-RNA (miRNA) was found in glioblastoma patient samples and cell lines. Importantly, the luciferase reporter assay verified bioinformatics data postulating that MGMT to be a direct target. Stable expression of miR-198 down-regulated MGMT and led to sensitisation of glioblastoma cell line U138 and primary glioblastoma cell line [238]. Another recent study showed four miRNAs – miR-181d-5p, miR-127-3p, miR-409-3p and miR-124-3p – when transfected into the MGMT-positive TMZ-resistant T98G glioblastoma cell line MGMT levels were down-regulated [239]. The pro-angiogenic receptor tyrosine kinase VEGFR-1 and -2 was found to be higher in MGMT-positive U87 and T98G cells, although further signalling studies were not conducted

[240]. Beyond these studies, however, there is limited data elucidating the signalling mechanisms involved in MGMT-mediated chemoresistance. For example, a search with the keyword 'microRNA' or 'AKT' or 'EGFR' or 'STAT3' or 'ERK' or 'receptor tyrosine kinase' followed by 'MGMT' and 'glioblastoma' only resulted in 49, 31, 113, 15, 1 and 19 research papers, respectively - the majority not necessarily focussing on signalling mechanisms. This is quite surprising given the clinical relevance of MGMT.

1.8.3.1 Mice models

Glioblastoma orthotopic xenograft models are also consistent with the MGMT-Resistance thesis. Two studies led by Kitange showed this connection using glioblastoma xenografts. In a 2009 study 13 xenografts were derived from glioblastoma patients with known MGMT status and developed into an orthotopic tumour models which showed that MGMT methylated models had better overall survival after TMZ treatment; although two tumour models which were negative for MGMT methylation and a single MGMT-positive tumour model were sensitive and resistant to TMZ treatment, respectively [241]. Next, a such termed TMZ-resistant glioblastoma xenograft model was established after injection of patient samples led to mice generating flank tumours and treated with clinically relevant dose of 66 mg/kg/day for 3 days until resistance to 120 mg/kg/day for 5 days was reached [242]. 3 out of the 5 TMZ-resistant tumours showed up-regulated MGMT gene and protein expression levels compared to the parental tumours. MGMT up-regulation was found not to be overly connected with a change in the promoter methylation status of CpG islands as assessed with MS-PCR; although histone modifications via increased H3Kp-ac was suggested as the mechanism for increased MGMT expression in TMZ-resistant lines [242]. Interestingly, xenograft-derived TMZ-resistant lines that possess MMR dysfunction are not MGMT-deficient while MGMT-over-expressing lines did not present MMR mutations suggesting these two resistance mechanisms are mutually exclusive [242].

1.8.3.2 Clinical data

Clinical data showing MGMT status to be associated with patient survival was what drove MGMT to be a key mechanism in glioblastoma. In 2004, Paz and colleagues reported that in a sample of 92 glioma patients – almost one-third having MGMT-methylated promoters but only 15 out of 92 being glioblastoma – reported that two-thirds of methylated glioma

patients that received first-line TMZ therapy presented a partial or complete response to therapy compared to 25% of unmethylated patients [243].

However, it was the highly well-received, paradigm shifting paper by Hegi et al. in 2005 that instilled MGMT status as a resistance mechanism [244]. Out of the 206 glioblastoma patients in the cohort 45% were positive for MGMT promoter methylation. A methylated promoter resulted in a median survival time of 18.2 months compared 12.2 months for negative methylation status. Furthermore, patients that were positive for MGMT methylation that received both radiotherapy and TMZ treatment had a median survival time of 21.7 months compared to 15.3 months in the radiotherapy alone group. Patients with unmethylated MGMT promoter who were treated with radiotherapy plus TMZ had a median survival time of 12.7 months – notably lower than what was reported in the Stupp study – and 11.8 months for those treated with radiotherapy alone [244]. Subsequent studies also validated Hegi's conclusion [245, 246]. For example, Lakomy et al. showed that in addition to suppressed miR-195 and miR-196 levels, unmethylated glioblastoma patients had lower median survival times (13 months) compared to methylated patients (22.5 months) in a Stupp protocol-treated cohort [247]. Indeed, two meta-analyses concluded that MGMT promoter methylation was significantly favourable for survival, although an overall median survival time was not reported perhaps due to the heterogeneity in the literature [248, 249].

Contrarily, Combs et al. reported that MGMT methylation provided no overall survival benefit in the 127 newly diagnosed glioblastoma patients treated with radiotherapy plus TMZ; though a benefit was observed in older patients with an age over 60 years [250]. A cohort of 80 Portuguese glioblastoma patients treated with the Stupp protocol showed no significant overall survival benefit according to MGMT status, though a trend towards favourable survival – a median survival time of 16 and 13 months for methylated and unmethylated MGMT, respectively – was reported [251]. Glioblastomas treated with ACNU, cisplatin and radiotherapy also showed no survival difference between unmethylated and methylated MGMT status [252].

1.8.3.3 MGMT clinical trials

Given that MGMT functions stoichiometrically it is theoretically sound to postulate that increased or prolonged doses of TMZ can deplete MGMT stores and prolong patient survival, in particular the MGMT-positive group. Alternatively, MGMT inhibitors in combination with alkylating agents may be therapeutically beneficial.

A 2002 Phase II trial with carmustine plus O6-benzylguanine (O6-BG) inhibitor observed notable toxicities and no responses in 15 glioblastoma patients that were resistant to nitrosoureas [253]. In 2009, a Phase II trial consisting of 34 recurrent glioblastoma patients only noted one patient to respond to a combination therapy of TMZ plus O6-BG inhibitor; although 16% recurrent anaplastic gliomas were responders [254]. In 2006 an Italian study which administered TMZ on a 3 weeks on/1 week off basis at a dose of 75 mg/m^2/day reported no survival difference regarding MGMT methylation status in a group of recurrent glioblastoma patients [255]. A 2013 Phase II trial that scheduled a dose-intense TMZ treatment regimen for recurrent glioblastoma showed no overall survival benefit [256]. Specifically, recurrent patients were treated with 100 mg/m^2/day TMZ for 21 consecutive days of a 28 day cycle for 12 cycles or until progression. The unmethylated MGMT group median survival time was only 11.7 months though consistently methylated MGMT median survival time was 22.3 months forcing the authors to depressingly conclude that the 'efficacy results were disappointing. [256] A 2014 Phase II trial attempted to follow the rationale though a 7 days on/ 7 days off TMZ treatment schedule was employed in a group of recurrent high-grade glioma patients, which included 40 glioblastoma patients. The treatment regimen was administration of 150 mg/m^2/day for 7 consecutive days for one week and no treatment the next in a 28 day cycle for a total of 12 cycles [257]. No survival difference was observed between the methylated and unmethylated glioblastoma patients [257]. Finally in the same year a commentary by van den Bent and Taal resorted to questioning the faith in dose-intense TMZ treatment in recurrent glioblastoma after a series of failed clinical trials [258]. Even in a cohort of 833 newly diagnosed glioblastoma patients, a dose-dense TMZ regimen - consisting of a standard concomitant component for 6 weeks before randomisation into either standard adjuvant therapy or a dose-dense schedule of 75-100 mg/m^2/day for 1-21 days per 28-day cycle for 12 cycles - showed no significant difference in survival between the groups. The former group's median survival was 16.6

months while the latter group's median survival time was 14.9 months. Consistently, MGMT methylation-positive patients had an overall favourable median survival time of 21.2 months compared to 14 months in unmethylated patients. Importantly, however, a dose-dense schedule did not lead to improved survival in MGMT-expressing patients as their median survival time was 13.3 months while MGMT-expressing patients receiving standard therapy showed a median survival time of 14.6 months; a total of 517 unmethylated patients were part of the study [259].

1.8.4 PARP

Poly(ADP-ribose) polymer (PARP) is a group of enzymes that catalyse transfer of ADP-ribose units from NAD+ to acceptor proteins [260]. Early studies dating back to 1980 have postulated it with DNA repair after observations of increase in PARP levels after DNA damage [261]. PARPs are associated with functional DNA repair and recognise therapy-induced DNA single and double-strand breaks [262]. Both PARP1 and PARP2 (hereby collectively referred to as PARP) repair DNA single-strand breaks while PARP1 additionally repairs DNA double-strand breaks and replication fork damage, thereby collectively working to repair the DNA damage caused by both radiotherapy and TMZ [263]. This redundancy has been demonstrated with mice models in which only a double-knockout led to a lethal phenotype [264].

PARP has been suggested to be a key BER enzyme [264]. Indeed, PARP inhibition can reinstate TMZ sensitivity after MMR-deficiency [265]. PARP binding sites have been reported in DNA damage checkpoint and recognition proteins such as p53, cyclin-dependent kinase inhibitor $p21^{CIP1/WAF1}$, DNA ligase III, XRCC1, which facilitates single-strand and double-strand repair and BER process, and MSH6, which is a eukaryotic homologue of MutS that binds with MSH2 [266, 267]. Therefore, PARP may not necessarily only be involved in BER – as implied by Campalans et al. - but a key component in various DNA damage response systems [268]. In fact the exact role of PARP is a matter of controversy with contrary evidence existing indicating that PARP is not required for BER and single-strand DNA breaks repairs [269, 270].

A detailed mechanistic narrative of PARP is yet to be established although a broad understanding is available. Upon DNA damage, for example those induced by methylating

agents, PARP1 binds the area to be repaired signalling for other components of DNA repair systems such as XRCC1 to be recruited on-site [269]. Subsequent auto-ribosylation causes the now negatively charged PARP to remove itself from the DNA site [271].

Given the apparent importance of PARP in DNA repair, PARP inhibition has been reasoned to be an effective therapy. One such inhibitory mechanism has been designed to inhibit the formation of poly ADP-ribose chains by competing with the substrate NAD+, thereby disabling PARP's ability to disassociate from the DNA but also deplete vital NAD+ cellular stores [272]. This is precisely the mechanism of PARP inhibitors that have been developed such as olaparib (AZD2281), veliparib (ABT-888) and niraparib (MK-4827) [273-275].

ABT-888 efficacy for the treatment of glioblastoma exhibited potential in pre-clinical models. Indeed, pharmokinetic analysis has shown the BBB penetrative ability of AGT-888 [276]. Combining radiotherapy (between 1Gy to 6Gy) and TMZ (between 5uM to 10uM for 2 hrs) with the PARP inhibitor ABT-888 to treat T98G, LN18, U87 and U251 showed to be the most effective in reducing cell viability when compared to single agent treatment [277]. Two studies published between 2007 and 2008 treated orthotopic 9L rat glioma (gliosarcoma) models with combined TMZ and ABT-888. One study observed a reduced tumour volume by 63% compared to 19% with TMZ alone, while the second reported similar results with a tumour growth reduction of 52-67% [276, 278]. Furthermore, glioblastoma xenograft models treated with a combination of radiotherapy, TMZ and ABT-888 reported an enhanced survival benefit, although subsequent treatment with ABT-888 against TMZ-resistant xenograft lines showed inefficacy [279, 280]. Similarly, positive indications were reported to the efficacy of other PARP inhibitors, such as olaparib and talazoparib in *in vitro* and *in vivo* glioblastoma studies [281, 282].

Despite promising results from pre-clinical studies with ABT-888 the hematological toxicology reports from glioblastoma clinical data after its administration in combination with radiotherapy and TMZ was intolerable [283]. This result is made the more disappointing for PARP-based glioblastoma therapy given that other PARP inhibitors such as olaparib, rucaparib and talazoparib – all large molecules - have shown very limited efficacy in glioblastoma models and poor BBB penetration, the latter due to being substrates for the drug efflux pump ABCB1 [284-287]. Niraparib, however, is capable of BBB penetration but

only one Phase I study has been conducted – in 2012 – but its administration was also followed by adverse events and lack of response hampering advancing its development [288].

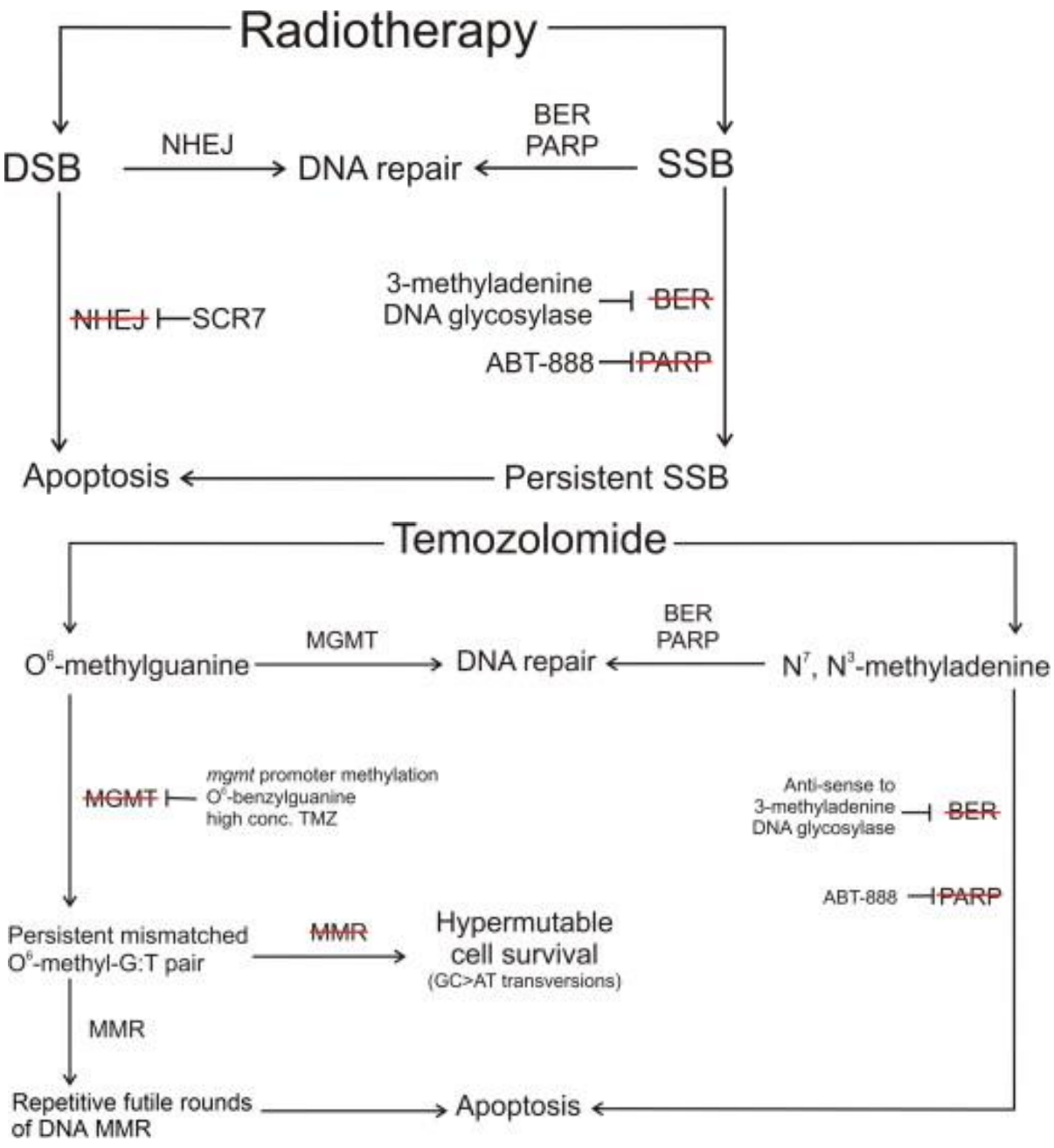


Figure 1-2: Mechanisms of action of radiotherapy and TMZ (schematic sourced from Atkins et al. [205]). Both radiotherapy and TMZ induce DNA lesions that lead to apoptosis. Radiotherapy induces both single and double strand breaks that, if let unrepaired, conclude in cell death. The process from radiotherapy and TMZ to apoptosis allows for opportunities for therapeutic interventions that supplement initial treatment.

PART 3: Overcoming chemo-radiotherapy resistance with targeted therapy

1.9 The oncogene addiction model

A prevailing theory of cancer progression is the oncogene addiction model [289-291]. This model has formed the major underpinning for targeted receptor tyrosine kinase (RTK) therapy for cancer in general, including glioblastoma. To understand the persistence to hold on to the rationale of targeted RTK therapy, therefore, requires an understanding of the oncogene addiction model.

Although earlier studies may have hinted towards supporting the oncogene addiction model, the first evidence to be cited as support for this paradigm was a 1995 study. Here it was reported that inhibiting cyclin D1 led to cyclin D1-amplified and over-expressed oesophageal cancer cells displaying normal cell phenotype, but cyclin D1 expression remained higher than non-amplified tumourogenic cells [292]. When elaborating upon the model Weinstein and Joe argued that although cancer progression is propelled by a wide scope of genetic and epigenetic alterations that accumulate, the malignant character of cancer is able to be inhibited by the "reversal of only one or a few of these abnormalities" [290]. In fact, the contingency of cancer progression is argued to rest on "one or a few genes". Furthermore, *de novo* oncogene mutations are taken to suggest the dependency of malignant cells on these oncogenes. Indeed, this line of reasoning has been adopted for the argument that EGFRvIII in glioblastoma is an ideal therapeutic target because the mutation is largely specific to glioblastoma and confers an aggressive malignant phenotype in mice models [293].

What the model entails - by advocating that only a single or a few genetic alterations are sustaining malignancy - is that a large segment of alterations that have been accumulated during cancer progression either as a result of prior disruptions or those that arise *de novo* are inconsequential in terms of being targeted. When accepting these assumptions a problem arises when categorising the nature of a particular genetic alteration or an oncoprotein: the difficulty to decipher between genuine oncogene dependency and a 'secondary' alteration of relatively lesser significance or insignificance. In other words, it

does not follow that identifying commonly altered genes or an over-expressed oncoprotein, as well as unique genetic alterations, accurately represent the state of oncogene addiction of a particular tumour type. This problem seems to have also been in the minds of Weinstein and John as they, too, posed with the difficulty in characterising the "specific state of oncogene addiction" [290]. However, the problem was only brushed over with the solution proposed limited to gene silencing with siRNA to establish both cause and effect, and the significance of an oncoprotein to tumourogenic capacity.

Nonetheless, the oncogene addiction model certainly appears to find confirmation in several different cancer types. Specific oncogene or oncoprotein-driven signalling pathways represents an attractive target for discriminating against non-malignant cells and depriving malignant cells of critical tumourogenic drivers. In several cancer types - non-small cell lung, breast and pancreatic cancer - have successfully trialled tyrosine kinase inhibitors (TKIs) that inhibit the phosphorylation of RTKs [294-296]. Such success has thus far left wanting in glioblastoma but a continued focus remains on developing novel therapeutic agents and strategies targeting RTKs, regarded as one of the key drivers of tumourigenesis in glioblastoma [38].

1.10 Introducing receptor tyrosine kinases

A protein kinase was first identified in 1959 and described as demonstrating enzymatic activity by transferring a phosphate from an ATP molecule to a tyrosine residue on the protein. A RTK is essentially composed of a glycosylated extracellular binding domain, a hydrophobic transmembrane domain and a cytoplasmic domain; the third part consisting of a juxtamembrane region and a protein tyrosine kinase, regulatory region that is available for phosphorylation and a carboxy-terminal region. Consequently, upon ligand binding a RTK must be able to transmit the signalling down to the cytoplasmic domain by bypassing the transmembrane domain [297]. Subsequent to ligand binding the RTKs, otherwise existing as monomers and single polypeptides, oligomerise – usually in the form of a dimer - and a structural shift takes place allowing for auto-phosphorylation to occur in the tyrosine residues of the cytoplasmic domain. The phosphorylation takes place in both the activation site of the kinase domain leading to kinase activity, and in the tyrosine residues found in the

juxtamembrane and carboxy-terminal region which acts as binding sites for downstream signalling molecules containing specific recognition domains [297].

Recent structural analyses advanced the earlier simplistic ligand-mediated RTK activation process. Currently, there are various types of RTK activation known. First, RTK activation can occur when a ligand interacts with both RTKs and becomes the dimer interface such that RTK dimerisation takes place without RTK-RTK contact. This is the method in which the ligand nerve growth factor (NGF) activates the TrkA RTK [298]. Secondly, dimerisation can be both ligand- and receptor-induced [299]. For example, a stem cell factor (SCF) molecule binds to the D1, D2, D3 extracellular binding domains of a single KIT molecule [299]. Subsequently, a KIT-bound SCF molecule joins together two KIT molecules leading to dimerisation[299]. This is followed by D4-D4 and D5-D5 interaction and contact between the opposing KIT molecules leading to a conformation change and ultimately auto-phosphorylation[299]. A similar mechanism is also proposed for PDGFR and FGFR activation [299, 300]. Alternatively, receptor dimerisation can be ligand-independent as the case with EGFR and the ErbB family [301]. The EGFR interacting with a single EGF molecule with its D1 and D3 extracellular domains undergoes a conformational change, followed by receptor-mediated dimerisation via the D2 domains and phosphorylation [302]. In addition to post-ligand binding dimerisation that has been discussed other RTKs such as insulin receptor predominately exist in dimers even prior to ligand binding though it is the ligand binding event that either stabilises dimerisation or converts an inactive dimer to the active state via conformational change [303, 304].

In addition to RTKs a large group of non-receptor tyrosine kinases exist such as Janus kinases, Src and Abl which are essential for RTK signal transduction [305-307]. Auto-phosphorylation of ligand-binding- and dimerisation-induced tyrosine residues on the juxtamembrane and carboxy-terminal domains on the RTK act as recruitment sites for these non-receptor tyrosine kinases which recognise specific phosphotyrosines [308]. Though non-receptor tyrosine kinases lack extracellular binding domains and transmembrane domains they do possess tyrosine residues available for phosphorylation and domains for protein, lipid and DNA interactions, such as the Src homology 2 (SH2) domain found on Src and Abl and the focal adhesion-binding domains found on Fak which binds to phosphotyrosines on the RTK [309-311]. A bound non-receptor tyrosine kinase itself can be

available for phosphorylation by RTK leading to non-receptor tyrosine kinases recruiting other downstream signalling molecules. For example, the SH2 domain of PLCy recognises the auto-phosphorylated state of carboxy-terminal tail domain of Fibroblast Growth Factor Receptor (FGFR) 1 leading to phosphorylation of PLCy by FGFR1 [312]. Other non-receptor tyrosine kinases, such as Jaks, can phosphorylate the RTK and provide binding sites for downstream signalling molecules such as STATs [313].

RTKs are required for a number of crucial cellular processes. Animal studies have revealed these diverse biological roles of RTKs. During embryonic development the ErbB family is required for formation of cardiac trabeculae, proper cardiac contractility and axon guidance[314] [315]. In fact, embryogenesis completion is dependent on ErbB4 expression with aborted foetuses showing retarded innervation in the CNS [314]. Cardiomyocyte proliferation is dependent upon both ErbB2 and ErbB4 expression [315]. Vasculogenesis is impaired by Flt-1 knockout leading to abortion of development, though endothelial cell differentiation remains normal. [316]. In contrast, mice defective in TrkB and TrkC receptors completed embryogenesis, developed normal hippocampus, cortex and thalamus brain regions but severe inner ear abnormalities were present[317]. The Eph family of RTKs are required for axonal guidance, motor neurons innervations of limb muscles, neuron-astrocyte synaptic communication, angiogenesis, gastrulation and neural crest migration [318-322]. Astrocytic production of TGFa can stimulate LHRH receptor dound on astrocytes and EGFR stimulation allows glial cells to release prostaglandin E2 allowing for mammalian onset of puberty [323].

Perhaps one of the first connections made between RTKs, specifically EGFR, and oncogenesis was in a 1977 paper by Fabricant et al. who reported a high number of EGF and NGF binding in a series of cancer cell lines and in a 1982 paper by Zenisek and Fernandez-Pol in which they reported increased phosphorylation of membrane-bound molecules upon EGF stimulation in adenocarcinoma cell line LoVo [324, 325]. In 1984 Ulrich and his team linked a viral oncogene with a mutated aberrantly expressed EGFR in the epidermoid carcinoma cell line A431[326]. In oncogenic studies since, RTKs have been at the forefront of research programmes from at least the late 1970s; over 300,000 articles are generated in a search on Scopus with the keywords ‘receptor tyrosine kinase’ and ‘cancer’. This is no surprise given that growth factor signalling is largely dependent upon RTK activation and an essential

characteristic of cancer cells is autonomous dysregulated cell growth [327]. Consequently, an exhaustive coverage of RTKs in cancer is beyond capacity although the key themes related to both RTKs and glioblastoma research paradigms will be discussed.

As we shall illustrate shortly the signals generated by the RTK is contingent upon a series of signalling pathways leading to cellular proliferation, metabolism, migration, invasion, apoptosis, autophagy and other processes such as DNA damage repair [327-332].

1.10.1 RTK signalling in cancer

Sequencing of the human genome has revealed that there are slightly over 90 known tyrosine kinases; 58 of which are RTKs divided into 20 subfamilies and 32 non-receptor tyrosine kinases divided into 10 families [333, 334]. Given that all cellular processes are caused by signal transduction of various modes it follows that RTK signalling is a possible mechanism for such processes. Indeed, earlier studies from the 1980s showed tyrosine phosphorylation to be related to cell growth control and retroviral oncogene products to have phospho-tyrosine activities [335]. It has been suggested – such as by Hunter and Blume-Jensen – that more than half of all known RTKs are consistently either mutated and over-expressed in human malignancies [336]. Although RTK aberration leads to dysregulation in cell growth and proliferation its ability to suppress cell death and inhibition of cell-cycle progression allow the cancer cells to acquire oncogenic characteristics [337]. Therefore, RTK regulation is required for preventing oncogenesis through maintaining a balance between the various processes, such as apoptosis, proliferation and growth, but its aberrant expression and activity that leads to its designation as an oncogene [337]. Gain of oncogenic RTK gene mutations and its associated signalling molecules are regarded as a primary cause of malignant transformation. These oncogenic mutations liberate RTK activity from the otherwise strong regulation in normal conditions by allowing evasion of the inhibitory cellular mechanisms [336]. Sources for the RTK-induced oncogenic transformation include: chromosomal translocations and other genomic rearrangements, gain-of-function or deletion mutations and also over-expression of the RTK which causes increased dimerisation due to increased RTK membrane-bound concentrations [336]. To better illustrate the oncogenic connection between RTK expression and dysregulated cellular processes a number of RTKs and associated downstream signalling molecules that are fundamental to glioblastoma biology shall be discussed.

The interest in RTKs being a druggable target for cancer therapy has led to the development of small molecule inhibitors termed tyrosine kinase inhibitors (TKIs), also referred to as RTK inhibitors. This class of drugs essentially competes with the transferable phosphate found in ATP for the ATP binding region on the tyrosine residues of the RTK. In fact, agents such as erlotinib, gefitinib, lapatinib, afatinib, axitinib, cabozantinib and crizotinib, all belong to the TKI class of drugs [338]. In contrast, monoclonal antibodies, such as cetuximab and nimotuzumab, compete with the ligand for the extracellular ligand binding domain [339]. The promise and the clinical efficacy of these strategies to inhibit the RTKs in glioblastoma shall be discussed shortly.

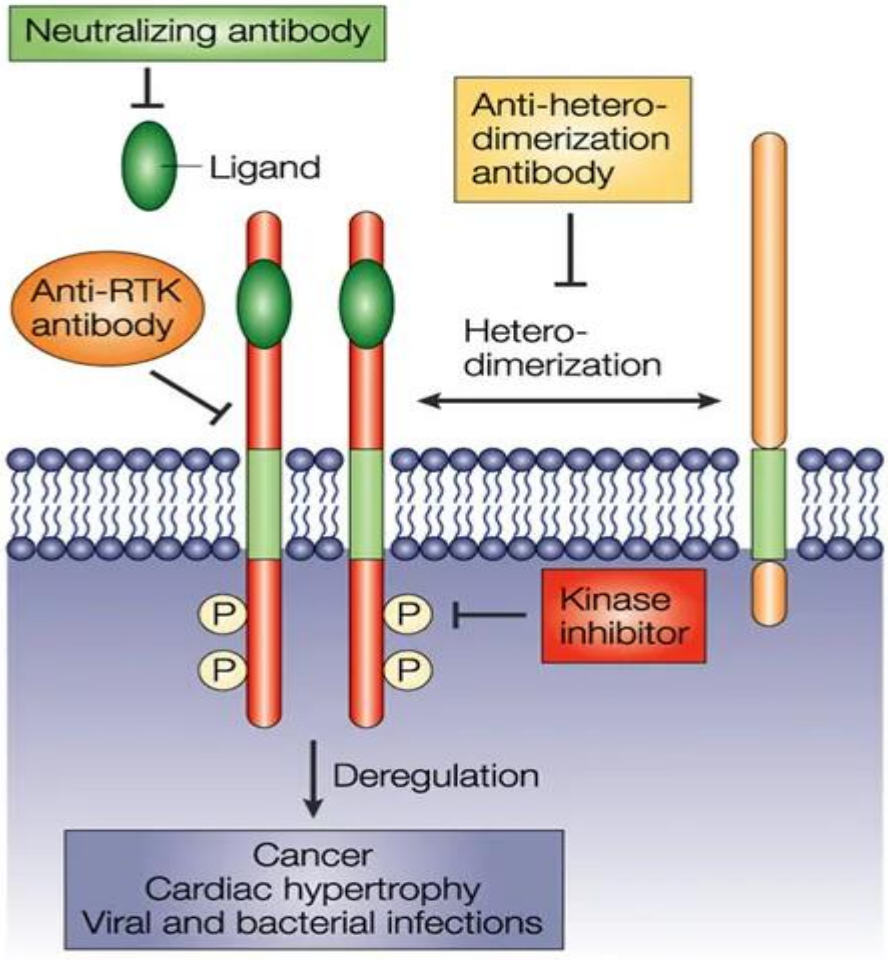


Figure 1-3: Various strategies to inhibit RTK signalling (schematic sourced from [340]). The structure and mechanism of RTKs allows for exploitation by several therapeutic strategies. Antibodies can disrupt heterodimer formation and ligand binding can be disrupted with binding to the ligand or ligand-binding site. Furthermore, kinase inhibitors can inhibit the phosphorylation of the intracellular component.

1.11 Platelet-derived growth factor receptor

1.11.1 Structure and activation

Platelet-derived growth factor (PDGF) derives its name from studies in the 1970s that revealed a serum growth factor for mesoderm-derived cells, such as glial cells, that is sourced from platelets [341]. Shortly thereafter it was revealed that PDGF stimulation induced phosphorylation of a protein RTK which is now called the PDGF receptor (PDGFR) [342]. The PDGF family of ligands is composed of four chains all existing as disulfide-bonded dimers - PDGF-AA, PDGF-BB, PDGF-CC, PDGF-DD and the heterodimer PDGF-AB – which are 30kDa large. These PDGF chains are encoded by separate genes on separate chromosomes, namely chromosome 7, 22, 4 and 11, respectively. The four chains contain a growth factor domain of around 100 amino acids. PDGF binds to the two RTKs that make up the PDGFR group- PDGFR-a and PDGFR-b. PDGF chains vary in their affinities for PDGFR type binding. The PDGF-A, PDGF-B and PDGF-C have high selection for PDGFR-a while PDGFR-b has high affinity for PDGF-B and PDGF-D [343-346].

The PDGFR is a class III RTK. Class III members are identified as sharing sequence homology and structurally by 5 immunoglobulin-like repeats in the extracellular domain of which the first three outer repeats are thought to be responsible for ligand binding; a transmembrane domain, a juxtamembrane domain, 2 intracellular tyrosine kinase domains separated by a kinase insertion, of which the second domain possesses the activation loop allowing for downstream signalling molecules to bind after PDGFR activation; and a C-terminal domain tail [347]. The auto-inhibition state of class III RTKs requires the juxtamembrane domain to block the active site between the N-terminal tail, found in the extracellular domain, and the C-terminal thereby preventing the activation loop on second tyrosine kinase domain to shift to an active configuration [348]. Ligand binding causes dimerisation and the rotation of the 5 components of extracellular domain leading to phosphorylation of the tyrosine residues found in the tyrosine kinase domain and the juxtamembrane [348]. The latter's phosphorylation liberates its inhibitory role. Bound downstream substrates are phosphorylated triggering a signalling cascade [349]. Other RTK members of this class in addition to PDGFR-a and PDGFR-b include c-Kit, FLT3 and CSF1R [349]. Furthermore, the

phosphorylated-juxtamembrane-dependent activation is also observed in other RTKs such as ephrin receptor (EphR) and insulin receptor (IR) [350].

The tyrosine kinase domain of RTK is highly conserved and transfers the γ-phosphate from the ATP molecule to the hydroxyl group of a downstream signalling protein [347]. In the case with PDGFR, the kinase insertion also provides for a space for protein docking [347]. Activated PDGFR recruits signalling molecules containing SH2 and phophotyrosine-binding domains (PTB), phosphorylating these signalling molecules and further recruiting other molecules with SH2, PTB, SH3 and plekstrin homology domains [348]. Consequently, rat sarcoma protein (Ras), mitogen-activated protein kinases (MAPK), phophoinositide 3-kinase (PI3K), phospholipase Cγ (PLCγ), (janus kinus (Jak), signal tranducers and activators of transcription (STAT) and (nuclear factor kappa-light chain enhancer of activated B-cells (NF-kB) can be activated via this up-stream signal [347].

1.11.2 PDGF-PDGFR signalling in glioblastoma

PDGF stimulation growth factor-free serum culture acts a growth factor in normal glial cells by inducing DNA replication and cell proliferation and PDGF is secreted by astrocytes for gliomagenesis and CNS development [351, 352]. Intracranial injection of the v-sis oncogene of simian sarcoma virus, a PDGF-B retroviral homologue, develops malignant gliomas in marmosets that are histologically similar to human gliomas [353, 354]. When the drug suramin, which inhibits PDGF-PDGFR activity by binding to PDGF, was administered to glioblastoma cells a decrease in DNA synthesis, tumour colony formation and malignant morphology was observed [355, 356]. Consistently, mutated PDGFR-b - lacking tyrosine kinase domains but retaining dimerisation ability - that were transfected in to C6 rat glioma cells led to slower cell growth in both *in vitro* and *in vivo* models [357]. Thus, these early preliminary studies shifted the status of PDGF-PDGFR from a mere growth factor responsible for normal glial function to a potential oncogene that drives gliomagenesis.

Quantitative gene expression analysis of glioblastoma cell lines and primary tissue revealed that PDGF and PDGFR to be highly expressed relative to normal brain tissue [358]. In fact all glioma cell lines assessed in a panel of 11 cell lines showed mid-to-high PDGF and PDGFR expression which are consistent with earlier studies; the majority concomitantly expressing PDGFR-a and PDGFR-b [358, 359]. Earlier studies, too, using southern blot analysis showed

that 8% of high-grade gliomas have amplificated PDGFR-a gene levels of between 18-35 fold change (EGFR gene amplification was only 18% but amplified at 4-19 fold times) but no evidence of amplificated PDGFR-b was obtained [353]. Furthermore, western blot analysis showed that PDGFR protein expression was elevated in almost 25% of high-grade gliomas [353].

Deregulated PDGF/R activity is largely attributed to over-expression and gene amplification and constitutively active receptor mutations are rare [360]. Genomic analysis has established aberrant PDGFR status as a driver of glioblastoma. Indeed, PDGFR is the second most amplified gene found in glioblastoma after EGFR with TCGA data showing amplification rates for PDGFR-a to be ~11% - but the rate can be as high as ~17% - and is a genetic event unique for glioblastoma in the glioma family [361, 362]. Although whether PDGFR deregulation belongs to a particular subgroup is a matter of debate. DeAngelis et al. argues that PDGFR over-expression is associated with sGBM [363]. To make this assertion evidence that PDGF over-expression in astrocytes is an early oncogenic event leading to low-grade astrocytomas is brought up; an assertion repeated by in papers by Behin, Kleihues, Ohgaki and others [363-365].

The basis for this argument most likely arose from Hermanson and colleagues' paper assessing PDGFR-a expression in glioblastoma samples; it was indeed this paper that was fundamental to DeAngelis' argument [366]. An analysis of 67 astrocytomas showed that PDGFR-a gene expression to correlate with glioma grade with the highest levels found in glioblastoma. Furthermore, though only 16% of glioblastoma samples possessed PDGFR-a gene amplification gliomas of lower grades showed no indications of amplification. Only LOH17p correlated with PDGFR-a mRNA expression. All astrocytic tumours with LOH17p showed high PDGFR-a gene expression while 56% of astrocytomas lacking LOH17p showed high PDGFR-a gene expression. Indeed, LOH17p correlated with PDGFR-a expression in glioblastoma. Given that mutated p53 was associated with both LOH17p and PDGFR expression and LOH17p was associated with lack of EGFR amplification it was perhaps incorrectly inferred that PDGFR expression is a characteristic of sGBM [366]. However, high PDGFR-a expression is a feature of more than half of all EGFR amplificated gliomas [366]. Other studies have also suggested the sGBM-PDGF correlation with immunohistochemistry showing stronger staining for PDGF in sGBM than pGBM and, in particular high PDGF-AB

was found to be exclusive to sGBM [367]. The association between EGFR amplification (30% frequency rate) and PDGFR-a amplification (21% frequency rate) was observed in a cohort of 47 pGBM but PDGFR-a amplification was also observed in the majority of sGBM [368]. More recent data from the Ivy Glioblastoma Atlas Project has also weighed into the debate and revealed that wild-type IDH1 glioblastomas have higher PDGF expression in addition to older patient age and poorer survival [369].

We can infer from the data available that PDGFR over-expression is an early oncogenic event and is characteristic of gliomas and glioma grade; however PDGFR amplification being more present in anaplastic astrocytomas than lower grade gliomas suggests that it is a late event (28% vs 5%) [370]. Although, PDGF/R status appears contradictorily as a characteristic to both pGBM and sGBM and it is more likely that dysregulated PDGF/R is required for the pathogenesis of glioblastoma in general rather than a specific subtype. Alternatively, a more radical opinion can be formed in that the very distinction of pGBM and sGBM can be rather clinically irrelevant and the data only appears contradictory if the existence of pGBM-sGBM distinction is assumed.

1.11.3 PDGF/PDGFR and glioblastoma tumour growth and survival

PDGF-mediated signalling has been an area of interest in a variety of malignancies including non-small cell lung cancer, breast cancer, prostate cancer, leukemia, non-Hodgkin's lymphoma [371-375]. In fact sorafenib and regorafenib, which inhibits the RTKs vascular endothelial growth factor receptor (VEGFR) -1, -2 and -3 and PDGFR in addition to Raf kinase, was shown to be of significant benefit and FDA-approved for the treatment hepatocelullar carcinoma [376, 377]. In addition, imatinib, another PDGFR inhibitor, is used for the treatment of myelogenous leukemia and gastrointestinal stromal tumours [378, 379].

PDGF-induced glioma formation has been established in a number of studies paving the way for more elaborate experiments designed to understand the significance of PDGF/R signalling [360, 380]. In glioma, siRNA-induced inhibition of PDGFR-b reduces the proliferation rate and increases apoptosis of C6 glioma cells both *in vitro* and *in vivo* [381]. The non-receptor tyrosine kinase ACK, which contains a SH2 andSH3 domain, is activated and phosphorylated at the Y635 site after PDGF stimulation, in addition to EGF and insulin

stimulation [382]. This causes AKT1 phosphorylation (pAKT) at the tyrosine 176 site (Tyr-176) in the kinase domain due to ACK-AKT stable complex formation mediated through the ACK kinase domain, the AKT pleckstrin domain and Tyr-176 [382]. The Tyr-176-pAKT transports to the plasma membrane leading to AKT activation via phosphorylation at Ser473/Thr308 site and suppression of apoptosis and cell-cycle progression [383]. In glioblastoma cells PDGF stimulation leads to AKT activation after ACK-PDGFR binding and PDK1 activation [384]. A signalling cascade is initiated as a consequence eventually leading to AKT-mediated positive regulation of cell cycle regulators such as B-catenin and cyclin D1 [384]. Consistently, U87 cells with inactivated ACK injected subcutaneously into mice developed tumours slower than wild-type ACK glioblastoma cells and PDGFR-b activation correlates with ACK expression in human glioblastoma samples [384]. PDGFR-ACK-PDK1-mediated AKT activation is independent of PI3K regulation which has also been established as a link between PDGFR and AKT signalling [385]. Indeed, inhibition of PDGFR with AG1433 – an inhibitor that most likely deactivates PDGF-induced phosphorylation of PDGFR - led to reduced growth in patient-derived glioblastoma cell line and increased apoptosis marked by caspase 3, 8 and 9 activation [386-389]. Constitutively active PDGFR-a mutant has persistent PI3K and MAPK signalling pathway activation but PDGFR inhibition – with the ATP competitors imatinib or PTK787 – reduces AKT and ERK activation in glioblastoma-derived cells [362, 390, 391]. This is consistent with other studies in which after the PDGFR antagonist CT52923 was used to treated five glioma cell lines (A172, SF188, U251, C6 and T98G) a decrease in AKT and ERK activation was induced [358, 392].

Surprisingly, very little data is available on the connection between PDGFR and STAT3 in glioblastoma. A search on Scopus with the keywords ‘PDGFR’, ‘STAT3’ and ‘glioma’ generated only 8 papers and substituting ‘glioma’ for ‘glioblastoma’ only generated 6 papers. In PubMed the former search query generated 10 and the latter generated only 9 articles. This is certainly a large gap in the literature given that STAT3 is considered a key regulator in not only glioblastoma tumourigenesis and treatment resistance but in cancer overall [393-395]. The papers that have studied the PDGFR-STAT3 signalling axis have been published only relatively recently. A 2016 study by Cenciarelli and colleagues investigated whether blocking PDGFR-a expression in glioma stem cells suppressed intracellular oncogenic signals [396]. First, the neurosphere protocol was employed to isolate cancer

stem cells from glioblastoma specimens and stem cells marked with higher GFAP expression; however a detailed molecular signature is not provided [397]. Notably, patient-derived glioblastoma stem cells have higher PDGFR-a levels were characteristic of peripheral tumour stem cells rather than tumour core stem cells [398]. PDGF-AA stimulation of patient-derived glioblastoma stem cells leads to higher STAT3 activation via phosphorylation at its Y705 site, which is usually phosphorylated by Jak and is essential for STAT3's role as transcription regulator [396] [399], in addition to EGFR and ERK activation is also increased after PDGF stimulation; all increased activation was more pronounced in peripheral cancer stem cells than tumour core stem cells [396]. Consistently, use of shRNA-PDGFR-a or crenolanib – a kinase RTK Class III inhibitor of FLT3 and PDGFR – down-regulates these downstream signalling pathways and up-regulates the pro-apoptotic Rb1 [396, 400]. This is in line with a even more recent paper concluding that inhibition of the RNA polymerase II activator and cell cycle progression kinase cyclin-dependent kinase 7 (CDK7) causes lower PDGFR-b and STAT3 activity in glioblastoma cells; however, considering that other RTKs, such as EGFR and MET, FGFR and AXL , were also down-regulated after inhibition a PDGFR-STAT3 cannot be asserted [401]. These reports are consistent with Kim et al. (published 2012) and Xia et al (published 2018). The former study, after showing that PDGFR-a is only expressed in a subset of glioblastoma but PDGFR-b is more commonly expressed in glioma stem cells, noticed that the stem cell maintenance role PDGFR-b is dependent upon STAT3 activation with supporting evidence showing shPDGFR-b inducing reduced pSTAT3 [402]. The latter study demonstrated that knockdown or down-regulation of leucine-rich repeats and immunoglobulin-like domain 2 (LRIG2) – LRIG2 promotes EGFR-mediated PI3K-AKTsignalling – decreases PDGFR-b expression in U87 cells and glioblastoma patient-derived samples; LRIG2 over-expression enhances PDGF-BB induced PDGFR activity and proliferation in U87 cells, in addition to pSTAT3 and pAKT levels *in vivo* [403, 404].

1.11.4 PDGFR in response to radiotherapy or TMZ

Perhaps even more surprising is the relatively scarce literature regarding the link between PDGFR and TMZ-resistance in glioblastoma. That heavy effort has been invested in trialling PDGFR inhibitors in the glioblastoma clinic (as we shall discuss shortly) adds to the urgent calls in the need for further studies elucidating the connection. A Scopus search with keywords 'PDGFR', 'Temozolomide' and 'glioma' only generates 23 results and once 'glioma'

is substituted for ‘glioblastoma’ the results list increases to 30; subtracting reviews from the results list cuts down the literature to 10 and 20 results, respectively.

The vast majority of papers published interrogating the PDGFR-TMZ link are related to clinical trials. Nonetheless, it has administration of perfosine, a AKT inhibitor, in combination with TMZ further reduces the proliferative capacity of a PDGF-driven glioma *in vivo* model compared to TMZ alone [405]. Recently, an *in vivo* rat C6 glioma model was radiosensitised after si-PDGFR-b treatment although the efficacy with TMZ was beyond their research aims. [406].

Given the dearth in literature on PDGFR-mediated therapy resistance in glioblastoma it is not surprising that the anti-PDGFR therapy or a therapeutic that includes anti-PDGFR has lacked efficacy. For example, the use of sunitinib on recurrent high-grade gliomas – including 16 glioblastoma patients (76.1 of total patients) showed no efficacy with not a single patient showing an objective response and all presenting tumour progression [407]. Another study also studying recurrent glioma – 51 cases were glioblastoma – tested imatinib but observed limited efficacy as only 3 glioblastoma patients showed a partial response and 6-month progression free survival was 16% [408]. Similar poor results were replicated in other clinical trials using PDGFR inhibitors such as with the selective PDGFR-a binding Medi-575 and multi-kinase inhibitors dasatinib and sorafenib [409-412].

Table 1-2: List of clinical trials with multi-tyrosine kinase inhibitors, including PDGFR

DRUG	Treatment	PATIENT TYPE	Comment 1	Comment 2	BBB?	REFERENCE
SUNITINIB	Sunitinib only 37.5 mg/day	Recurrent Glioblastoma	No responses	All tumours progressed	Yes [413]	[407]
IMATINIB	Imatinib only 600-1000 mg/day	Recurrent Glioblastoma	3/51 showed partial response	PFS = 16%	No [414]	[408]
SUNITINIB	Sunitinib + lomustine	TMZ-refractory, recurrent WHO Grade II and anaplastic glioma	1/13 showed complete response	Median PFS = 1.8 months 6-PFS = 15%		[415]
VATALANIB (PHASE I)	RT +TMZ+VATALANIB	Newly diagnosed Glioblastoma	Drug no longer manufactured	Trial stopped		[416]
Medi-575	Medi-575 25 mg/kg	Recurrent Glioblastoma	6-PFS = 15.4% Media PFS = 1.4 months	No response MST = 9.7 months		[409]
Sorafenib	Sorafenib 400mg Tmz 40 mg/m2 (metronomic)	43 recurrent Glioblastoma	6-PFS = 26% 5/43 showed partial response	MST = 7.4 months 48% showed no response	Yes [417]	[411]
Dasatinib	Dasatanib 100-150mg x2 day	77 recurrent Glioblastoma	Median OS =7.9m Media PFS = 1.7m	6-PFS = 6%	Yes[418]	[410]
Sorafenib	Sorafenib 200mg/2xd + temsirolomus 20mg/weekly	Recurrent Glioblastoma	7/41 6-months progression-free Median PFS = 1.9 months -2.6 months	MST = 3.9 months – 6.3 months High toxicity		[412]

1.12 Epidermal growth factor receptor

1.12.1 The EGFR family and structure

The first RTK group to have been described in cancer was the EGFR and all members in this group belong in the Class I family of RTKs [336, 419, 420]. The Class I family is defined by the presence of two cysteine-rich regions in the extracellular domain [336]. The EGFR family consists of four members - EGFR or erbB1 (hereafter called EGFR), erbB2 (aka HER2), erbB3 (aka HER3) and erbB4 (aka HER4) - and 11 known ligands bind to one or more of these receptors with varying affinities; except the structurally different ErbB2, which is considered to have no ligand-binding role but mainly as the favoured dimerisation partner formation, suggesting that ErbB2 to have a key role in dimer formation [421, 422].

ErbB3 is considered an impaired kinase making EGFR and ErbB4 the main drivers of downstream signalling [423]. ErbB ligands contain an EGF-like domain of approximately 60 amino acids (EGF is made of exactly 53 amino acids) except neuregulin which consists of approximately 200 amino acids.

Depending on which ligand has bounded with the receptor and which homo- or hetero-dimer is formed may determine the nature of the downstream signalling pathway activation [424]. For example, though the MAPK pathway is activated by all ErbB ligands the PI3K directly binds with ErbB3 and ErbB4 containing dimers [425]. Meanwhile ErbB2 containing heterodimers are thought to be the most potent downstream signalling activators while ErbB homodimers are thought to be less transformative than heterodimers [425].

1.12.2 The EGFR life cycle

Since EGFR amplification does not necessarily correlate with EGFR transcription, it is possible that EGFR over-expression is caused by up-regulation of transcription factors that bind to the EGFR promoter. Importantly, the EGFR promoter does not have the canonical TATA box and the CCAAT box; however, it has several GC box sequence elements at its 5'-regulatory sequence [426]. These GC boxes are binding sites where transcription factors promote EGFR transcriptional activation. For this reason, transcription can be initiated at different sites of the promoter. In addition, there are 3 enhancer elements: enhancer 1 is located upstream near the start of translation +1 (ATG) and enhancer 3 is found upstream

from positions -1409 to -1109 bp; enhancer 2 is downstream located in the intron 1 at positions +1788 to +2318 bp and it is near a polymorphic region of CA dinucleotide repeats, which exclusively functions when there is an upstream element on enhancer 3, which suggests the complexity of the EGFR regulation [426, 427].

EGFR can regulate itself and increase its gene expression by promoting the expression of transcription factors specific to EGFR (ETF) [428, 429]. Many EGFR transcription factors, such as Sp1, MLTF, CTF, TFIIB and AP1, have been identified by using *in vitro* transcription systems in epidermoid carcinoma cell line, which over-express EGFR [430]. Interestingly, an *in vitro* experiment performed with human osteosarcoma cell lines by Ludes-Meyers JH et al. have shown that p53 is also an EGFR transcription factor, in which a mutant or wild type p53 is able to transcriptionally activate EGFR by different mechanisms [431]. Furthermore, many other EGFR transcription factors such as AP2, RPF-1, IRF-1, ERDBP-1, ETF, ETR, GCF2 and WT1 have been also found regulating EGFR expression in different tumour cell lines including breast cancer, leukemia and cervical cancer [430, 432-438].

The majority of these reported transcription factors have been confirmed in different tumours such as fibrosarcoma, gastric carcinoma and cervical cancer [439]. Most importantly, many of these transcription factors can work in a complex to regulate EGFR expression and can bind to different regions of the EGFR promoter such as Sp1, Ap1 and Ap2 [426, 427, 440]. To complicate our understanding of EGFR regulation and transcription, there is also evidence indicating that EGFR expression can be affected by oscillatory protein networks dependent of the biological clock [441].

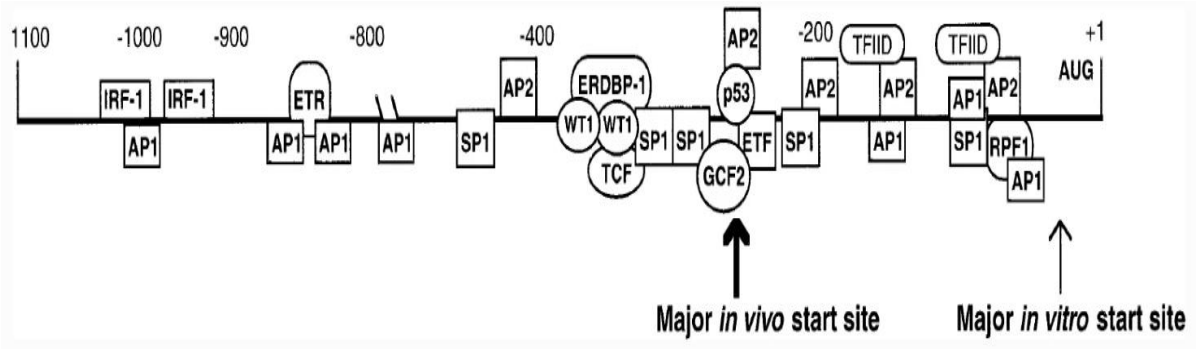


Figure 1-4: Schematic showing various EGFR transcription factors and their recognised region on the EGFR promoter (image sourced from [440]).

Furthermore, some other EGFR transcription factors have been evidenced in glioblastoma. Ou et al. identified a complex that consisted of Kindlin-2, Y-box binding protein 1 (YB1) and Beta-catenin that was responsible for enhancing EGFR transcription and promoting tumourogenic characteristics, such as invasion and proliferation [442]. Additionally, Mizuguchi et al. have recently found that ectopic viral integration site 1 (EVI1) transcription factor correlates with EGFR in glioblastoma cell lines A172 and YKG1, and most importantly, *in vitro* promoter reporter assays showed that EVI1 recognised the EGFR promoter region ranging from -377 to -266 bp to regulate EGFR expression [443].

Once EGFR is transcribed and before its transport to the cytoplasm, the precursor EGFR mRNA formed needs to be capped at the 5' end, spliced and polyadenylated at the 3' end. During this process the EGFR mRNA is bound to numerous proteins, which forms what is called - the messenger ribonucleoprotein complex (mRNP). This complex is then associated with nuclear pore proteins that allow the nuclear export of the mRNA, which is fundamental to ensure the functionality of the EGFR mRNA prior to translation in the cytoplasm [444]. Importantly, an alternative splicing of the precursor EGFR mRNA leads to many different EGFR variants that can have different activation levels inducing tumourigenesis [445-447].

In terms of translation, EGFR exon 1 has been established at +1 (ATG) initiation codon, however, EGFR translation has several starting codons at 5' region. EGFR mRNA is translated by the ribosomes of the rough ER following the canonical eukaryotic translation that has been divided in 3 highly regulated steps: initiation, elongation and termination [448] [449]. Nascent EGFR is inserted into the lumen of the ER and it is associated with chaperons to ensure adequate EGFR folding, such as head shock proteins [450]. Maturation of EGFR begins in the lumen of the ER, where it is post-translationally modified with N-glycosylations. N-glycosylation is essential for a correct conformational folding of the EGFR [451]. This involves moving a middle carbohydrate to an asparagine residue (N) within a typical aminoacidic consensus sequence N-X-S/T; in EGFR, there are 11 typical N-glycosylation consensus sequences and 4 atypical N-X-C [452, 453]. The processing of the carbohydrate chain and ligand binding activity happens in the ER [454]. In order to export the receptor to the plasma membrane, maturated EGFR moves through the secretory pathway in association with SEC23 and SEC24, which allow the interaction of the synthesised protein through the ER export signals with coat protein II (COPII) vesicles; these

COPII vesicles create regions free from ribosomes in the rough ER called ER exit sites (ERES) [455-458].

EGFR activation is not always dependent on ligand binding, in some cases inactive EGFR monomers can auto-activate themselves [459]. Since excessive ligand binding and EGFR signalling leads to tumourigenesis, cells have mechanisms to control and reduce EGFR activation such as through: receptor endocytosis, recycling or degradation. In this way, inactive receptors often travel across the endocytic route. From this point, receptors can be taken back to the plasma membrane when required or they can be degraded by lysosomes [460].

Even though EGFR ligand binding happens in the plasma membrane, activated EGFR remains most of the time in the intracellular region in the endocytic compartment. At low levels of EGFR ligands, activated EGFR is associated with clathrin-mediated endocytosis, also called CME [461, 462]. Inactive EGFR can also be transported through CME, however, EGFR phosphorylation increases CME by attraction of adaptor protein 2 complex (AP2), which recruits great levels of clathrin. This leads to EGFR clustering and rapid increase of the vesicle [463-465].

CME results in early Rab5 positive endosomes containing EGFR; after these endosomes mature, they move to the perinuclear area, where receptors are inactivated by high levels of phosphatase activity before receptor recycling [466]. On the other hand, at high levels of EGFR ligands, EGFR activation and increased phosphorylation there is a receptor ubiquitylation mediated by E3 ligase Cbl. This promotes receptor internalisation by clathrin-independent endocytosis (CIE). Importantly, in this case there is no receptor recycling but lysosomal degradation in the perinuclear region. Degradation is mediated by late endosomes positive for Rab7 in which receptors that have been ubiquitylated are sent from the endosomal membrane inside intra-luminal vesicles and forming an MVB or multi-vesicular body. Subsequently, there is a fusion between endosomes with lysosomes allowing EGFR degradation [465-467].

1.12.3 The extracellular domain and dimerisation

In 2001, Yarden et al. in his review of the ErbB signalling network did not contain a detailed overview of the mechanistic structure of EGFR activation [425]. Knowledge of the structure

of EGFR has progressed rapidly since then and accelerated since three papers - published between 2002-2003 – elucidating the crystal structure of ErbB families, dimerisation and mechanistic activation [468-470].

The extracellular domain of EGFR consists of two homologous ligand binding domains, called domain I and domain III, and the two characteristic cysteine-rich domains called domain II and domain IV. Domain II is considered the dimerisation bridge between one ErbB with another and mutations on the tyrosines in this domain disrupts ErbB dimer formation; domain IV is the second highest contributor but plays a minimal role compared to domain II and its deletion does not hamper dimer strength [471]. As mentioned previously, EGFR-ErbB dimerisation is receptor-mediated meaning that the ligand-binding which occurs by bivalent contact between domain I and III does not interact with dimer interface formed by domain II.

In the inactivated state domain II is buried within domain IV so that the dimerisation bridge is unexposed. Ligand binding breaks this auto-inhibited state by liberating the domain II that is trapped in domain IV and allowing for dimerisation between the domain II of two EGFR molecules. It has been suggested that this auto-inhibited state has a low threshold because mutated domain II-domain IV interaction sites failed to increase EGFR activation [472]. Nonetheless, it has been shown that EGFR can exist as a dimer – albeit unstably - without ligand-binding and these pre-ligand-bound dimers are more receptive to EGF binding [473, 474]. Due to the weakness of this auto-inhibitory mechanism it has been suggested by Kathryn Ferguson that the EGFR extracellular region exists in a dynamic state such that ligand binding traps the EGFR molecule within the time span that it exists in the non-auto-inhibited state [475]. Given that the domain II-IV interaction may not strongly influence the auto-inhibitory state of EGFR it is instead thought that the rigidity of the domain II-III interaction is to the extent that it is sufficient to work as an auto-inhibitory mechanism [476].

Furthermore disruptive mutations in the transmembrane domain do not influence the receptor signalling. Consistently, the major EGFR mutation in glioblastoma, the EGFRvIII with a truncated extracellular domain, leads to a constitutive active EGFR. Furthermore, mutations in the kinase domain of the EGFR have been also shown to lead to constitutively

active EGFR and the liberation from the auto-inhibitory state of the receptor [475]. As mentioned above the domain II position may be in a dynamic and shifting state. It follows from this that a domain II re-arrangement re-positions the relative relationship between domain I and domain III such that the otherwise widened gap between the two ligand-binding domains closes to an extent and made ideal for ligand binding [475].

EGFR activation requires a unique process that is termed an asymmetric kinase domain dimer which follows from ligand-binding, receptor-mediated dimerisation, rotation near or in the transmembrane domain and a structural conformational change so that the intracellular domains of two EGFR molecules are in close proximity. During the asymmetric kinase domain dimer interaction the N-lobe in the kinase domain of one EGFR molecule interacts with the C-lobe of another EGFR molecule's kinase domain followed by the former molecule being phosphorylated and activated by the latter. The now activated EGFR molecule then phosphorylates the activator EGFR molecule in its C-terminal tail. Lastly, the previously activator EGFR then acts as the receiver so that roles switch leading to two activated EGFR molecules in a single dimer [475].

Table 1-3 ERBB receptor family and corresponding ligand

GROUP	LIGAND	RECEPTOR BINDING
1	EGF, EPGN, TGF-a, AREG	EGFR
2	BTC, HB-EGF, EPR	EGFR & ErbB4
3	NRG-1,NRG-2	ErbB3 & ErbB4
4	NRG-3, NRG-4	ErB4

EGF: epidermal growth factor; EPGN: Epigen; TGF-a: transforming growth factor alpha; AREG: amphiregulin; BTC: betacellulin; HB-EGF: heparin binding EGF-like growth factor; EPR: Epiregulin; NRG: Neuregulin

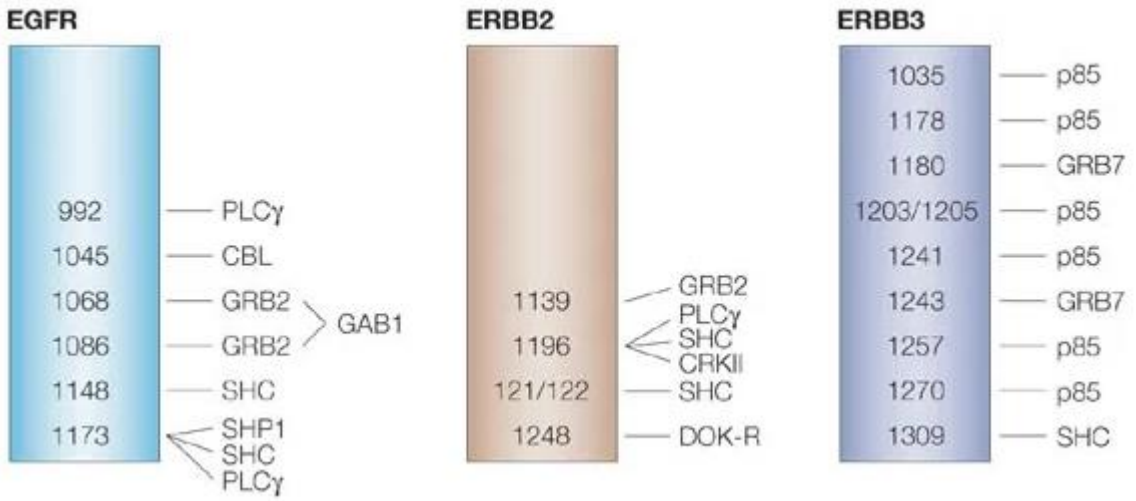


Figure 1-5: Phosphorylation sites and corresponding docking proteins on various members of the ERBB family of receptors (schematic from [477])**.**

1.12.4 EGFR in glioblastoma

The EGFR gene is located on chromosome 7 – gene locus p11.2- and the gene spans approximately 200kb with 30 exons and the EGFR protein full-length is 170kDa. [478, 479] Normal EGFR expression can range between 40,000-100,000 receptors/cell but over-expression is common in cancer [480]. Activated EGFR promotes a number of tumour-promoting cellular processes including cell proliferation, migration, transformation and anti-apoptosis [481-484].

EGFR/ErbB1 (hereafter called EGFR) has garnered increased attention in cancer research over the years. Aberrant EGFR activation has been established in lung cancer, breast cancer, pancreatic cancer, colorectal cancer and head and neck cancer [485-489]. Targeted EGFR therapy for the treatment of non-small cell lung cancer with the use of erlotinib and gefitinib; metastatic colorectal cancer with the use of the monoclonal antibody cetuximab; squamous cell carcinoma of the head and neck with the use of cetuximab; advanced pancreatic cancer with the use of erlotinib; and breast cancer with the dual EGFR and ErbB2 inhibitor lapatinib [490-494].

EGFR is one of the most well studied proteins in glioblastoma; a search on Scopus with the key terms ‘EGFR’ and ‘glioblastoma’ generates over 30,000 results, over 19,000 of them being research articles. This is perhaps largely due to reports that in glioblastoma EGFR

amplification is the most common amplified gene with amplification rates of ~32-40% and over-expression rates of ~60% [12, 56, 495, 496].

1.12.4.1 EGFR alteration initiates gliomagenesis

It can be reasoned that common alterations found across all fragments from a single tumour are early-event aberrations. This is premised upon the tumour mass being initiated by a unified source of tumour-initiating population. In contrast, aberrations present in a subset of tumour cells can be inferred to have arisen during late-event tumourigenesis. It follows that early-event aberrations are key drivers for tumour initiation and replicating an event would yield gliomagenesis more strikingly at least compared to models lacking such aberration. Use of this model allows us to better explain and understand the glioblastoma initiation. EGFR has been linked with the initiation and maintenance of glioblastoma with studies using glioma stem cells validating this model and a number of studies provide supporting evidence for the claim.

EGFR-positive glioblastoma stem cells derived from human glioblastoma samples initiated enhanced tumourigenicity compared to EGFR-negative glioblastoma stem cells when injected into mice models [497]. Furthermore, tumours isolated from EGFR-high or EGFR-low expressing glioblastoma mice models were larger compared to EGFR-negative and previously EGFR-negative cells that were injected into mice showed EGFR expression after resection. EGFR-negative glioblastoma stem cells require to up-regulated EGFR expression to regain tumourogenic capacity. [497]. Also, when glioblastoma mice models were generated with orthotopic xenograft tumours the EGFR-amplified glioblastoma samples that were cultured and then injected developed highly tumourogenic tumours but EGFR non-amplified cells did not form tumours or were initiated slower [498].

Glioblastoma surgery that is fluorescence-guided with 5-aminolevulinic acid allows for identification and sampling by reducing the likelihood of normal and necrotic tissue contaminating the sample [499]. This allowed Sottoriva et al. to source glioblastoma from 11 patients and observe intra-tumoural heterogeneity, in addition to the already known inter-tumoural heterogeneity. Patient-to-patient tumour samples vary so that a proportion contained MET amplification whereas others did not present such RTK aberrations but instead presented PDGFR-a or AKT or PIK3CA (which codes for the PI3K) amplification.

Furthermore, analysis on different fragments derived from the same original sample can also have variance; for example, PDGFR-a, MET and MDM4 amplification, in addition to PTEN, TP53 and RB1 deletion, are not homogeneous across the same tumour. [500]. Of note EGFR alterations, in particular gene amplification, were found to be an early-event glioblastoma driver – perhaps due to chromosome 7 alterations – and this conclusion was reached because all tumour fragments contained the aberration; commonality of the lesion is therefore indication of early-event gliomagenesis [500]. Along with EGFR alteration, MET and PTEN alteration was also identified as an early-event in glioblastoma while PDGFR-a alterations are thought to be mid-phase events. This is consistent with genomic analysis revealing that the only RTK lesion in 50% of all pGBM is EGFR alteration while only 7% of cases show that altered EGFR co-exists with other RTK alterations [501].

Further evidence shows that the common EGFR amplification occurs before the gain of the EGFRvIII mutation [502, 503]. Sorting EGFRvIII-containing samples showed heterogeneous expression of the mutation; at most 50% of the cells derived from EGFRvIII cell lines were EGFRvIII-positive. After culturing both EGFRvIII-positive and EGFRvIII-negative cells it was observed that the EGFRvIII-positive population re-instated EGFRvIII heterogeneity but cultured EGFRvIII-negative cells did not give rise to EGFRvIII-positive cells [503]. Interestingly, EGFRvIII-positive cells generated higher number of spheres and had a higher capacity for sphere-initiation. Other studies have also associated EGFRvIII expression with glioblastoma stem cells with reports of co-expression of CD133 along with EGFRvIII and disruption of the signalling of both inhibited tumourigenesis [503].

This suggests that EGFR expression to be linked with gliomagenesis and glioma initiation is dependent upon EGFR. If the neural stem cell model for the origin of glioblastoma is correct, therapy can be better targeted towards stem cells with tumourogenic potential without targeting non-cancerous tissue; indeed non-cancerous tissue also express stem cell markers such as CD133 but lack EGFR aberration [504, 505].

1.12.5 EGFRvIII

EGFRvIII mutations are frequent in glioblastoma; 20-30% of total patients and approximately 60% of EGFR-amplified cases carry the mutation. [293]. Other mutations are observed in the extracellular domain – such as point mutations G598V, A289V and T263P – that lead to

constitutive activity of EGFR but at lesser total frequencies at approximately 13.5%. [506]. The EGFRvIII mutation is the result of deletions of exons 2-7 (801 coding bases) leading to the deletions of the extracellular domain between amino acids 6-273 and glycine residue insertion between amino acids 5-274 [507]. This rearrangement results in a truncated receptor and its active conformation requires no ligand binding.

Some have argued that EGFRvIII signalling intensity relative to wild-type EGFR is debateable though increasing data shows that EGFRvIII signalling is significantly lower and approximately 10-20% of wild-type EGFR activity [293, 505, 508]. The weakness in signalling intensity is thought to reduce EGFRvIII internalisation and degradation and another source for its persistent signalling [508].

Given that the wild-type EGFR mediates dimerisation via the domain 2 of the extracellular domain whether EGFRvIII is capable of forming dimers or if it exists as activated monomer requires explanation. Earlier studies found no evidence of EGFRvIII dimerisation and concluded that such mechanism is not required for its signalling[509]. EGFRvIII co-precipitates with the wild-type EGFR suggesting that EGFRvIII forms heterodimers with the EGFR and, interestingly, the phosphorylation of wild-type EGFR that forms heterodimer with EGFRvIII is ligand independent [510] . Therefore, it can be thought that EGFRvIII trans-phosphorylates the wild-type EGFR. More recent data confirms that EGFRvIII dimerises. The truncated EGFRvIII has an unpaired cysteine residue at C307 in the extracellular domain and introducing a EGFRvIII carrying a mutation in this position disrupts the dimer forming ability reducing the phosphorylation activity of the mutated-cysteine-EGFRvIII [511, 512]. However, the observation of minimal EGFRvIII activation in the mutated variant indicates that EGFRvIII activity may persist in a monomer albeit in a vastly reduced manner [512]. Kancha and colleagues clearly replicated the previous studies showing EGFRvIII dimerisation. Significantly, they also reported that EGFRvIII auto-phosphorylation cannot as a monomer and activation requires asymmetric dimers being formed similar to wild-type EGFR. Indeed, introducing mutations in N-and C-terminal lobes of intracellular domain inhibited EGFRvIII signalling; ErbB3 was found to be a possible dimer partner for EGFRvIII signalling [513]. Taken together, it is clear that EGFRvIII forms dimer to convey signalling however the exact mechanical process and a detailed exposition on the dependency upon homo- or hetero-dimerisation for EGFRvIII activation is a matter of further research.

1.12.6 EGFR expression in tumour growth in *in vivo* models

A discussion on the down-stream signalling of EGFR will follow shortly but it suffices to note at this moment in our discussion that both EGFR and EGFRvIII expression has repeatedly been implicated in glioblastoma cell growth, proliferation, anti-apoptosis and migration via the PI3K-AKT, STAT3 and ERK signalling pathways [510, 514-522]. This correlation between EGFR and related down-stream signalling molecule activation has been also noted in tissue samples derived from glioblastoma patients and AKT and ERK pathway activation is more intense than found in anaplastic astrocytomas [523]. Stimulation of EGFR forms more aggressive tumours in glioblastoma murine xenograft models. Fan and colleagues showed that subcutaneous xenograft mice models bearing EGFR-expressing glioblastomas grew more aggressive tumours than the control; however, EGFR-EGFRvIII bearing mice carried the largest tumour burden 6 weeks post-injection[522]. In mice carrying intracranial EGFRvIII glioma xenografts anti-EGFR therapy via the monoclonal antibody mAB806 presented reduced tumour growth and angiogenesis in addition to increased apoptosis and survival time; the inhibitory effect of mAB806 is due to disrupting EGFR/EGFRvIII phosphorylation and not the degradation not the receptor [524]. Inhibition of EGFR via miR-34a and injecting glioblastoma cells in immune-deficient mice increased the tumour doubling time [525].

The above evidence collectively provided the rationale for designing a therapeutic regimen based on anti-EGFR. Until now a variety of treatment combinations of EGFR therapy and anti-EGFR agent have been trialled with underwhelming results. Out of 49 patients treated with EGFR inhibition only 9 patients had 25% reduction in tumour [526]. Comparison between responders and non-responders showed that 50% of patients with EGFRvIII lack a response [526]. Interestingly, clinical trials have shown that EGFR status does not predict the response to anti-EGFR based therapy [527, 528]. A possible reason for this is the lack of efficacy of EGFR inhibitors against EGFR activity in (recurrent) malignant glioma [529]. The majority of the clinical trials using erlotinib as part of or the sole agent in the therapeutic regimen consisted of recurrent glioblastoma patients. From these trials the best median survival time was 10.5 months and 6-month progression free survival was only 28%. The slightly improved results compared to other trials may be due to patient selection bias because the selected patients were previously heavily treated and enrolled after 2^{nd} or 3^{rd} progression in addition to the small sample size – this total number of 25 recurrent patients

makes this trial one the smallest in the literature [530]. In the trials the recruited newly diagnosed glioblastoma patients the best median survival time was 19.8 months (vs. 18 months in the historic control) which was an insignificant result [531].

Table 1-4: Previous glioblastoma clinical trials with EGFR inhibitors

AGENT	Patients	MST (months)	COMMENT 1	COMMENT 2	COMMENT 3	REFERENCE
ERL, RT, TMZ	97 pGBM	15.3	EGFR, EGFRvIII and PTEN not predictive. Significant toxicity.	No significant difference between historic controls	150mg ERL initially followed by 60Gy RT plus 75mg/m^2 TMZ plus ERL	[527] NCT00039494
Vorinostat + ERL+ TMZ	21 rGBM		Terminated due to toxicity			NCT01110876
ERL only	48 rGBM	9.7	EGFR amp survival = 8.6 months EGFR non-amp survival = 10.6 months	3/48 showed partial response 6-PFS = 20%	150mg – 300mg erlotinib until failure	[532] NCT00337883
Phase II ERL + sorafenib	56 rGBM	5.7	6-PFS = 14% PFS = 2.5 months	5% partial response 45% progressive disease	150mg once ERL 400mg twice S until failure	[533] NCT00445588
RT, TMZ, ERL, BVZ	59 pGBM	19.8 vs. 18 (control)	Median PFS = 13.5 months vs. 8.6 months		60Gy RT + TMZ 75mg/m2/d + ERL 150-200mg/day + BVZ 10mg/kg Adjuvant TMZ 200mg/m2/d + ERL + BVZ	NCT00525525 [531]
Sorafenib, TMS	18 rGBM		6-PFS = 0%			[534] NABTC-05-02
ERL + RT + TMZ	65 patients vs 128 HC	19.3 vs. 14.1 HC	Correlation between MGMT and survival	PFS = 8.2 months VS 4.9 months (HC) MGMT methylated MST = 25.5M MGMT positive MST = 14.6M	EGFR status not predictive	[528] NCT00187486
ERL, RT, TMZ	27 pGBM	8.6	Median PFS= 2.8 months	"unacceptable toxicity"		[535] NCT00274833
Phase I dasatinib + ERL	47 rMG		No response	1/47 patients progression free at 6 months		[536] NCT00609999
RT, TMZ, ERL, BVZ	48 pGBM	13.2	Only unmethylated patients selected	Median PFS = 9.2 months	RT + TMZ followed by ERL and BVZ	[537] NCT00720356
ERL PLUS	32 rGBM	8.45	6-PFS = 3.1%			[538]

SIROLIMUS			PFS = 6.9 weeks			NCT00672243
ERL, BVZ	25 rGBM	10.5	6-PFS = 28%	48% showed radio-graphical response but not associated with OS.		[530] NCT00671970
ERL OR TMZ OR CARMUSTINE	110 rGBM		6-PFS: ERL = 11.4% TMZ/CARMUSTINE = 24%	54 patients in ERL arm 29 patients in carmustine arm 27 patients in TMZ arm	EGFRVIII mutant in ERL arm had worse survival High AKT in ERL arm worse survival compared to low AKT	NCT00086879 [539]
ERL + TMS	42 rGBM		6-PFS = 13% MEDIAN PFS = 2 months	NO RESPONSE	EGFR STATUS NOT PREDICTIVE High ERK signalling associated with low PFS	NCT00112736 [540]
GEF	28 rHGG 16 rGBM	6.15	6-PFS = 14.3% PFS = 8.4 weeks	EGFR status not predictive		[541]
RT, GEF	96 pGBM	12	1-year survival: 54.2%	Non-significant survival difference	EGFR status not predictive	[542]
GEF	53 rGBM	9.85	6-PFS = 13%	Median PFS = 8.1 weeks	1-year survival = 35.6%	[543]
ERL + cediranib vs. cediranib only	38 rGBM	7.2 (cediranib + erlotinib) vs. 5.5 (cediranib only)	Median PFS = 3.6 months (cediranib + ERL) vs. 2.8 months (cediranib only)	8/19 had partial response in cediranib plus ERL arm	Recurrent patients previously treated with RT and TMZ	[544]

ERL = Erlotinib; GEF = Gefitinib; BVZ = Bevacizumab; GBM = Glioblastoma; rGBM = recurrent Glioblastoma; rMG = recurrent malignant glioma; pGBM = primary Glioblastoma; HGG = High grade glioma; HC = Historic control; MST = Median survival time; amp = amplification; PFS = progression free survival; 6-PFS = 6-month PFS; OS =Overall survival.

1.13 MET (also known as c-MET)

1.13.1 The structure of MET and its ligand

The cellular N-methyl-N'- nitroso-guanidine human osteosarcoma transforming gene, hereafter called MET, and named as such because it was first isolated using an osteosarcoma cell line (MET can also be referred to as c-MET and hepatocyte growth factor receptor (HGFR)) is a member of RTK Class 8 shared by two other members called RON and SEA [545, 546]. The 120kb MET gene is located on chromosome 7q21-q31 and expressed by epithelial cells of organs ranging from the liver to the pancreas and the prostate to the kidneys [547].

The initial MET transcript, spanning approximately 150kb, is glycosylated producing a 170kb precursor protein which is cleaved into a 140kDa beta-chain and a 50kDa alpha-chain [548]. These two chains exist as a hetero-dimer connected via a disulfide bond [548]. The alpha-chain is completely extracellular while the beta-chain spans the extracellular region, the transmembrane region and the intracellular domain [548]. The alpha-chain is completely composed of, and the beta-chain is partially composed of, a 500 residue semaphorin domain containing a cysteine-rich region [549]. The semaphorin domain in the beta-chain is followed by a 50 residue plexin-semaphorin-integrin domain, four immunoglobulin-plexin-transcription domains and the transmembrane region [549]. Finally, the intracellular tyrosine kinase domain is flanked by the juxtamembrane region, containing the tyrosine Y1003 which negatively regulates MET by attracting the ubiquitin ligase casitase B-lineage lymphoma, and carboxy-terminal sequences. This domain contains the catalytic tyrosines Y1230, Y1234 and Y1235 and the carboxy-terminal sequences contain the tyrosines Y1349 and Y1356 which recruits signal transducers post-MET activation. The sum of the functional domains total 1408 amino acid residues [549].

HGF, also known as scatter factor, is expressed by a 70kb gene located on chromosome 7q21.1 and the ligand that binds MET and secreted by mesenchymal and stromal cells [550]. This ligand is secreted as a single chain, inert precursor before protease-dependent cleavage produces an active HGF composed of alpha- and beta-chain linked with a disulphide bond [550]. The HGF alpha-chain is formed by a hairpin loop and four 'kringle' domains consisting of 80 amino acids each and connected with disulphide bonds [550].

The HGF-bound MET receptor results in homodimerisation and auto-phosphorylation of the tyrosine residues in the catalytic sites followed by phosphorylation of tyrosine residues in the carboxy-terminal sequences resulting in a docking site for SH2-containing signalling molecules such as growth factor receptor protein 2 (GRB2), SHC, CT10 oncogene homolog, phospholipase Cy, Src and PI3K – all bind to the phosphorylated tyrosine residues Y1349 and Y1356, though PI3K additionally binds to phosphorylated Y1313 also found in the carboxy-terminal sequences [551] [552]. GRB2-associated binding protein 1 (GAB1) can directly bind to, or indirectly through GRB2, and be phosphorylated by MET and acts as a recruiter for downstream effectors, a unique mechanism of MET signalling [553]. Downstream signalling pathways, such as the PI3K-AKT, MAPK, STAT3 and Wnt/beta-catenin pathways, are subsequently activated [554, 555].

1.13.2 The physiological role of MET

MET signalling is essential for hepatocyte survival, regeneration and proliferation [556]. Furthermore, HGF or MET embryonic gene knockout, causing disruption in myogenic precursor cell migration, is lethal in mice [557, 558]. In addition to embryonic cell growth and migration, the wound healing process is dependent upon MET signalling and cultured keratinocytes with mutated MET show suppressed closure compared to functional MET-containing keratinocytes [559]. Deficiency in the phophotyrosines residues located on the carboxyterminal region also leads onto embryonic lethality and limb muscle defects [560].

Aberrant MET activity, due to mutations altering the kinase domain, over-expression and gene amplification for example, has been found to occur in most solid tumour and haematological malignancies such as non-small cell lung cancer [555, 561, 562]. Oncogenic MET activation contributes to cell migration, proliferation, angiogenesis, invasion, anti-apoptosis and epithelial-to-mesenchymal transition [550, 554, 563].

1.13.3 Role of MET in glioblastoma

In glioblastoma, MET alteration is generally considered the third-most RTK aberration after EGFR and PDGFR. MET amplification is associated with higher grade gliomas, especially glioblastoma, and reportedly non-existent in lower grades while MET expression correlates with glioma grade [564] [565]. The amplification rate has been reported by Verhaak et al. in an analysis consisting of 500 samples to be 1.6%, %4 in an earlier TCGA analysis consisting of

206 glioblastomas and 5.1% in a 2015 study consisting of 137 glioblastomas – much lower than the EGFR or PDGFR amplification rates [38, 361, 564]. However, MET over-expression is relatively more common. Kwak et al. looking at 137 glioblastoma cases reported an over-expression rate of 13.1%; Kong et al. reported it in as much as 29% of a 62 patient cohort; a 2011 study analysing TCGA data from 202 patients repeated similar results showing over-expression of both HGF and MET to be found in 31.2% of cases; another 2011 study reported an over-expression rate of approximately 36%, though with a small cohort of 19 patients; Olmez et al. noted that, in a cohort of 68 patients, 27% showed no MET expression and 45% presented tumours in which at least 30% of cells carried MET expression [564, 566-568] [569].

1.13.4 MET in *in vivo* models of GBM

As described previously in our discussion glioblastoma stem cells are thought to be the drivers of tumour progression. Recent studies, almost entirely published in this decade, have connected MET signalling with glioblastoma stem cell maintenance. Perhaps the earliest study first demonstrated, in 2011, that glioblastoma neurospheres enriched for stem cells had higher MET activation and HGF stimulation leads to greater neurosphere formation[570]. In particular, MET activation was associated with the CD133-, Sox2- and Nestin-positive subpopulation and HGF-induced neurosphere formation was dependent on Nanog expression – later confirmed by another study showing *in vitro* models to have a subpopulation of CD133- and Sox2-positive, and MET expression independent of AKT activation [570, 571]. The signalling mechanism for MET-mediated glioblastoma stemness can possibly be due to MET-Wnt-CD133 axis as postulated by Kim et al [572]. Although MET expression correlated with tumour xenograft tumour formation, this efficiency was found to be independent of CD133-expression suggesting that MET signalling is upstream of CD133-induced stemness and the existence of CD133-independent GSC-related pathways [571]. Moreover, HGF stimulation of MET-positive neurosphere populations – indeed, consistently leading to co-expression of CD133, Nanog, Sox2 and Nestin - furthered the invasive, proliferative and migratory capacity *in vitro* [573]. In contradiction to Joo et al. in 2012, Tasaki and colleagues observed, in a more recent study in 2016, that human GSC-derived intracranial xenograft models formed tumour independent of MET expression which potentially questions previous conclusions of MET-initiated gliomagenesis; Jun et al in 2014,

for example, only saw tumour development after injection of 100 MET-positive GCSs and not 100 MET-negative GSCs [574, 575].

MET expression has been consistently associated with poor survival. A 2009 study first showed that the MET over-expression group had the lower median survival time of 11.7 months compared to 14.3 months in the low-expressing group [566]. Liu et al. reported the higher MET expression associated with worse progression-free survival and tumour recurrence [568]. This observation was repeated in two separate studies, in 2012 and 2014, which, in addition to also reporting a worse progression-free survival, noted that overall survival was significantly lower in MET-high glioblastoma patients – the former contained an undefined number MET-positive and MET-negative patients and the latter study consisted of 31 and 38 positive and negative MET patients, respectively [569, 571]. A recent 2016 study with a larger cohort, though with only 9 out 95 patients being MET-positive, validated association with worse overall survival [574]. Assumingly, the largest clinical evaluation hitherto, consisting of 186 glioblastoma patients – divided into a high-low ratio of 60:40 - concluded that high MET expression associated with poor overall survival in patients that survived for more than 8.5 months though the median survival time was not reported clearly[565]. In contradiction to these studies, Kwak et al in 2015, after observing no link between MET amplification and survival was able to conclude that MET over-expressing patients survived longer than low-expressing patients – consisting of 18 and 119 patients, respectively – though survival was atypically high at 35.7 months in the former group and 24 months in the latter group [564].

MET inhibition and knockdown – either through siRNA, tyrosine kinase inhibitors such as crizotinib, PTEN restoration, monoclonal antibodies - decreases *in vitro* glioblastoma proliferation, induces cell cycle arrest and clonogenicity [575-578]. In contradiction, inhibition of MET using SU11274, which targets the ATP-binding site of MET, was found to not decrease cell viability of EGFR-inhibition-resistant cells [579-581].

Intravenous delivery of si-MET reduced MET expression and tumour growth in glioblastoma xenograft mice models [571]. Furthermore, *in vivo* treatment with microRNA, monoclonal antibodies L2G7 or OA-5D5 and the tyrosine kinase inhibitors crizotinib or altiratinib

significantly reduced mice glioblastoma burden and may improve mice survival [577, 578, 581-585].

Nonetheless, despite promising pre-clinical data, phase II studies with the MET and multiple tyrosine kinase inhibitor cabozantinib failed to culminate into efficacy in recurrent glioblastoma patients both naïve to or pre-treated with anti-angiogenic therapy [586, 587]. Furthermore, two phase II studies with the HGF monoclonal antiobody rilotumumab – also known as AMG 102 - also did not sufficiently produce positive responses or increase survival in recurrent patients either pre-treated with Bevacizumab or in combination with Bevacizumab [588, 589]. A 2017 phase II study treated recurrent glioblastoma patients with the HGF monoclonal antibody onartuzumab in combination with Bevacizumab but observed no survival benefit compared to Bevacizumab alone [590]. Of note, a common occurrence in these studies is adverse reactions to therapy potentially limiting the dosage to be administered. Taken together MET inhibition in the glioblastoma has failed hitherto and further studies are required with other MET inhibitors that can be safely administered in high doses.

1.14 RTK-driven signalling pathways

1.14.1 The STAT3 pathway

Signal transducer and activator of transcription (STAT) proteins belong to a family of signalling protein transcription factors that map downstream of ligand induced receptor signalling including EGFR, PDGFR, MET and interleukins [591]. Upon phosphorylation of its tyrosine residue, the phosphorylated tyrosine residue STAT of a monomer interacts with the SH2 domain of another monomer leading to an active dimer. From there, the active STAT dimer translocates into the nucleus, recognizing specific DNA sequences to activate gene transcription [592]. The STAT family all approximately 750-850 amino acids in size, comprises of seven members including: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. Members of the STAT family are known to be associated with tumourogenic processes, such as cell cycle progression, proliferation, apoptosis regulation and invasion [591].

Due to it being stimulated by oncogenic ligand-induced signalling and association with several signalling tumourogenic pathways, one particular member of the STAT family, the STAT3, is a heavily studied protein that is a well established oncogene. The cytoplasmic latent STAT3 is demonstrated to be phosphorylated upon stimulation EGF, HGF, PDGF and other ligands, including cytokines such as IL-6 [395]. As touched upon previously, ligand:receptor binding initiates a signalling cascade. Commonly, the phosphorylation of STAT3 is induced by ligand:receptor binding followed by receptor dimerisation, and recruitment and phosphorylation of tyrosine kinases (of which the most commonly studied are JAKs), leading to STAT3 recruitment to the activating tyrosine kinase via SH2 domain interaction and STAT3 phosphorylation [593]. Activated STAT3s typically form homo-dimers - hetero-dimers with STAT1 are also possible - before translocation to nucleus to regulate transcription of genes, typically oncogenes in the context of cancer. Non-phosphorylated STAT proteins can also form dimers and induce transcription, possibly via interaction with other transcription factors [395, 593].

Due to the large size of the STAT dimer, nuclear translocation requires support from subunits, such as importin-a5, to interact with specific amino acid residues found on the STAT molecule. The defining role of STATs is gene transcription mediated via its DNA binding domain. Each STAT protein regulates transcription differently owing to its DNA binding domain and ability to recruit specific transcription co-activators – the STAT3 DNA binding domain is situated at the centre of the STAT3 molecule and spans from amino acid residue 319 to 573 [591]. Upon entering the nucleus, STAT proteins are prone to de-phosphorylation by phosphatases, such SH2-containing phosphatase 1 or 2, which lead to nuclear export to the cytoplasm. Therefore, given that STAT binding to DNA is reversible, it is possible that a constitutively activated STAT protein remains nuclear to increase gene transcription [591]. Other mechanisms of nuclear STAT3 negative regulation are dysfunctional DNA binding proteins occupying DNA sequences or interacting with functional STATs, in addition to transcription and expression of suppressors of cytokine signalling (SOCS).

Given that in glioblastoma upstream signalling molecules, such as RTKs, are commonly up-regulated and genetically altered, the consequent constitutive STAT3 activity is capable of driving the progression and increasing the aggressiveness of glioblastoma Indeed, the activation of STAT3 via EGFR and MET signalling is well established in glioblastoma [570,

594, 595]. The majority of studies evaluating STAT3 activity using clinical glioblastoma specimens report positivity level of greater than 50% [596]. In contrast, Wang et al. reported STAT3 activation to be only present in 8.6% in a cohort consisting of 70 patients [597]. This contrary observation may be due to differences in the controls used since Wang's control were normal brain samples derived from surgical resections of glioblastoma STAT3. Another proposition for the dissimilarities between the reports may be due to the more diluted pSTAT3 antibody used by Wang. Nonetheless, STAT3 activation along with its key signalling driver IL-6 appears to have increased activity in glioblastoma as assessed with clinical samples.

STAT3 activity has increasingly been associated with resistance to radiotherapy or TMZ. Ashizawa et al. developed a TMZ-resistant U87 cell line by incrementally exposing the parental population with up to 150uM TMZ and maintained at 100uM TMZ after 20 passages [598]. The TMZ-resistant U87 cell line was sensitive (measured by apoptosis and proliferation assays) to the STAT3 inhibitor STX-0119 in both *in vitro* and *in vivo*, which disrupts STAT3 dimer formation [598]. Lee and colleagues generated TMZ-resistant U251 and U373 cell lines with incremental TMZ doses over a span of 2 months, the TMZ doses reaching up to 800uM TMZ [599]. This group demonstrated that the TMZ resistance displayed by these variants was MGMT-independent and TMZ resistance was reversed with STAT3 inhibition using siRNA [599]. Interestingly, along with increased STAT3 protein expression and phosphorylation of STAT3 at Tyr705 residue and Ser727 residue was found to be decreased and increased, respectively, in both resistant cell lines compared to the parental cell lines[599]. Recently, it was shown that RRAD, a Ras-related GTPase, is highly up-regulated in glioblastoma patients and linked to a worse prognosis [600]. Western blot analysis demonstrated that RRAD transfection in LN229 increased EGFR and STAT3 (Y705) activity[600]. Furthermore, up-regulation of RRAD increased EGFR nuclear translocation and resistance to TMZ in LN229 cells [600]. Similarly, U87 TMZ resistant cells was established by exposing U87 cells to a "low dose (*undefined by these authors*) of temozolomide in culture media for 3 weeks" [237]. The U87 TMZ-resistant cell line had increased STAT3 protein and gene expression, along with MGMT,IL-2, IL-6 and IL-10 protein expression [237]. Interestingly, inhibition of STAT3 with STAT3 inhibitor VI decreased MGMT expression, suggesting that STAT3 increases MGMT transcription [237].

Han et al. in 2016 used Cpd188 to inhibit STAT3 Y705 phosphorylation and SH2-mediated binding [394]. They claimed, using cell line U251 had high pSTAT3 levels compared to U87, and was more sensitive to treatment with radiotherapy combined with TMZ and Cpd188 than U87, though comparative statistical analysis was not performed or shown [394]. STAT3 inhibition combined with radiotherapy and TMZ treatment using U251 cells also led to increased E-cadherin expression and down-regulated VEGF, MMP-2 and EphA2, thereby decreasing migration, invasion and EMT markers [394]. *In vivo* analysis using U251 intracranial mice models showed that a combined STAT3, TMZ and radiotherapy regimen led to an 83-fold decrease in tumour burden; but this combination significantly increased mice survival compared to control and radiotherapy alone, and radiotherapy plus TMZ[394].

1.14.2 The AKT pathway

AKT is a serine/threonine kinase implicated in cancer and part of the PI3K-mTOR tumourogenic signalling pathway. The PI3K (also known as class 1 phosphatidylinositol 3-kinase) is a lipid kinase downstream of RTKs and activated by RTK-induced phosphorylation of scaffolding proteins that go on to bind and activate the PI3K molecule [601]. This leads to generation of PIP3 via PI3K-induced phosphorylation of phosphatidylinositol-3,4,5-triphosphate (also known as PIP2), a process that can be inhibited and reverted by the tumour suppressor phosphatase and tensin homolog (PTEN) [602]. Subsequently, AKT and PDK1 bind to the PIP3 molecule allowing for PDK1-induced phosphorylation of AKT at the activator loop region of threonine 308[601]. AKT then phosphorylates TSC2, part of a TSC1-TSC2 binding complex, thereby inhibiting the GTPase activating function of TSC2 and alleviating Rheb-GTP to phosphorylate mTORC1, and leading to activation of the downstream 4E-BP1 and S6 kinases [601].

AKT is also known as protein kinase B (or PKB) and include three isoforms named AKT1, AKT2 and AKT3 [602]. Structurally similar to protein kinase A/G/C family of kinases, AKT consists of an amino terminal pleckstrin homology domain important for plasma membrane translocation and PIP3 binding, a central kinase domain and carboxyl terminal domain which includes the hydrophobic motif that contains residues prone to phosphorylation - a requirement for the full activation and carried out by mTORC2, auto-phosphorylation or other kinases [602]. It must be noted that although AKT signalling can be activated via EGF, PDGF and HGF ligand stimulation via PI3K, it is possible for PI3K-independent signalling

mechanisms to initiate AKT activity. Non-receptor tyrosine kinases such as Ack1, Src and PTK6 are able to stimulate AKT activity by recruiting AKT to the plasma membrane and phosphorylating AKT in RTK-dependent or independent manners [603].

Cell proliferation, migration, invasion and anti-apoptosis, in addition to a number of other pro-tumourgenic processes such as protein synthesis and metabolism, have all been implicated in AKT signalling in glioblastoma [604, 605]. This is expected give that PTEN mutations are common in glioblastoma, with up to 40% of all glioblastoma cases thought to carry PTEN alterations [64, 361]. Furthermore, epigenetic mechanisms and up-regulated upstream signalling are possible factors that can contribute to increased AKT activation in glioblastoma.

Inhibition of AKT has been commonly proposed as a strategy to sensitise glioblastoma cells to radiotherapy or TMZ. Combining si-Src, upstream of AKT, or si-AKT with TMZ administration produces greater cytotoxicity in pre-clinical experiments as well as suppression of tumour growth in LN229 and U87 subcutaneous xenograft mice models [606]. U87 cells with a retroviral construct containing AKT displayed resistance to TMZ-induced cell cycle arrest via down-regulation of the DNA damage induced Chk2, in a PTEN-independent manner [607]. Furthermore, TMZ-induced senescence, measured by beta-galactosidase activity, was decreased and colony formation capacity was increased by inducible AKT expression [607]. Also, inhibiting the PI3K pathway radiosensitises glioblastoma cell lines, such as U251 [608]. PI3K/AKT inhibitors in clinical trials are limited for glioblastoma, though phase II clinical trials with mTOR inhibitors, such as frontline therapy with everolimus in combination with radiotherapy and TMZ or sirolimus combined with erlotinib in recurrent patients, have yielded no favourable outcomes [538, 609, 610]. These unfavourable outcomes were reported despite pre-clinical mice models showing strong potential for success in targeting the PI3K/mTOR pathway combined with TMZ administration [611, 612].

1.14.3 The ERK pathway

The last major downstream signalling pathway that we shall discuss in this section is the Ras-Raf-MEK-ERK pathway (hereby referred to as the ERK pathway). Ras, a GTP-binding protein can initiate the ERK pathway, along with the PI3K-AKT pathway, upon activation of

RTKs, including EGFR, MET and PDGFR, and recruitment by scaffolding proteins [613-615]. Subsequently, Raf is activated by translocating to the plasma membrane, allowing for the phosphorylation of MEK, a type of Mitogen-activated protein kinase (MAPK) kinase [616]. Finally, ERK, a type of serine/threonine kinase called MAPK, is phosphorylated by MEK. The pERK translocates to the nucleus to stimulate nuclear transcription factors, including STAT3, to promote the progress of cell cycle and inhibit apoptosis [616].

MAPK signalling pathways can be divided into four: ERK, JNK, ERK5 and p38 MAPK. ERK, also known as extracellular signal-regulated kinase 1 and 2, is encoded by the genes ERK1 (known as MAPK3 and a 44kd protein product) and ERK2 (known as MAPK1 and a 42kd protein product); both of which are structurally and functionally similar [617]. MEK-induced phosphorylation of ERK leaves a phosphorylated threonine and tyrosine residue at its activation loop [617].

Relative to the significance of STAT3 and AKT signalling, the reports on the role of ERK in TMZ- and radiotherapy-treated glioblastoma is limited. Nonetheless, from the reports that exist, contradictory positions are present regarding the role of ERK in response to therapy. It has been shown to regulate MGMT expression with ERK knockdown decreasing MGMT levels and sensitizing glioblastoma cells to TMZ [232]. In contrast, ERK pathway inhibition with U0126 did not increase sensitivity of primary glioblastoma cell lines to TMZ, in contrast to PI3K-AKT inhibition with LY294002 [618]. Stepanenko et al. generated long-term TMZ-treated U251 cell lines by exposing the cells to 25uM TMZ twice in a week, 50uM TMZ twice in a week and then 100uM TMZ twice per week for either 5 weeks, to generate one U251 variant, or 10 weeks, to generate another U251 variant. Interestingly, the two U251 variants differed in pERK protein levels with the shorter-term treated variant displaying higher pERK levels [619]. This tension between ERK as pro-tumourogenic and ERK as a tumour suppressor is also echoed in other cancers in general [620]. Therefore, further research is required to better characterize the role of ERK in radiotherapy- and TMZ-resistance in glioblastoma.

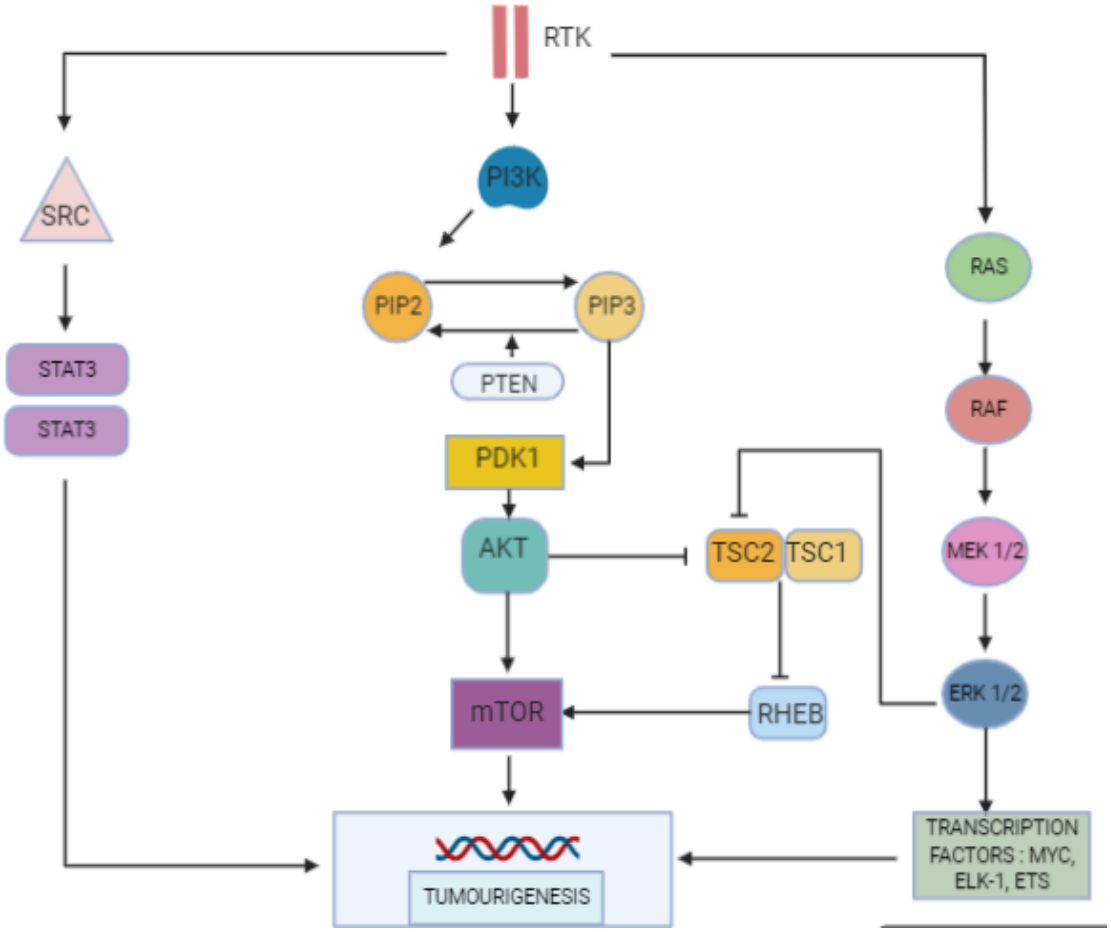


Figure 1-6: RTK downstream signalling pathways (schematic adapted from [601, 621, 622]). Once activated, RTKs initiate signalling cascades that lead to one or several cellular processes, such as cell growth, proliferation, migration and invasion. Three common downstream pathways include the STAT3 pathway which involves STAT3 dimer forming and translocating to the nucleus to initiate transcription of target genes; the PI3K-AKT pathway leading to mTOR activation; and the ERK pathway. In the cancer context these pathways have been shown to activate tumourogenic cell mechanisms.

1.15 The controversy over the role of autophagy

1.15.1 Introducing autophagy

Autophagy, derived from Greek and loosely translates to 'self eating', refers to a cellular recycling process that ends with lysosome-mediated degradation of proteins and other constituents of the cell allowing for the re-use of the products for ATP production, protein synthesis and other vital cell processes [623]. There are three types of autophagy: macroautophagy, chaperone-mediated autophagy and macroautophagy [623]. For the purposes of this present study, only macroautophagy, the major and common type of autophagy, will be referred to as autophagy. To briefly summarise, autophagy is initiated by the formation of an isolation membrane which elongates until the two ends of the membrane fuse to form the double-membrane autophagosome. In the process of membrane ends fusing cytoplasmic material is trapped within the autophagosome. Finally, the autophagosome fuses with a lysosome to form an autolysosome that degrades the contents within the autophagosome [623]. Autophagy is now well accepted as crucial mediators of a wide range of homeostasis mechanisms such as the ageing process; starvation adaptation; regulators of cell death; removal of long-lived or damaged organelles and proteins; removal of intracellular pathogens via xenophagy; and alleviation of oxidative stress [624]. As we shall see, autophagy has become an important, but controversial, focus in cancer research and its exact role in glioblastoma remains to be further studied.

There remain extensive gaps in the knowledge detailing autophagosome formation. The mechanism leading up to the initiation and source of the membrane that forms the phagophore and the fusion process forming the autophagosome is unknown. Nonetheless, a general, yet incomplete, overview can be described. Given that autophagosome formation has been consistently observed near the endoplasmic reticulum, a common position found in the literature is that the source of the initial membrane is a structure associated with the endoplasmic reticulum called the omegasome [623, 625]. Much of the advances in autophagy research have been propelled by the discovery and characterization of autophagy related genes (ATGs) using yeast and, as we shall see, these genes are highly conserved in mammals and involved across the autophagy process [626].

Regardless of the origin of the initial phagophore membrane, the site at which elongation takes place is called the phagophore assembly site (PAS) in which ATG proteins involved phagophore formation and elongation are found. Two complexes are crucial for autophagosome formation: the ULK1 (shortened from Unc-51 like autophagy activating kinase 1) complex and the class III PI3K complex I (PI3K complex) [625]. The ULK1 complex is part of the autophagic initiating complex which comprises of ULK1 and ULK2, in addition to ATG13, FIP200 and ATG101. FIP200 (mammalian orthologs for yeast ATG11 and ATG17) and ATG13 are considered to function as a scaffold at the PAS and exist in a complex regardless of autophagy-inducible conditions. ULK1 activation can be liberated upon autophagy-inducible conditions, such as the inactivation of mTOR signalling, leading to ULK1 phosphorylation. The ULK1 is recruited by ATG13 to the FIP200 scaffold. The second complex, the PI3K complex, includes VPS34, VPS15, Beclin 1 (ATG6 in yeast), ATG14L and NRBF2 (ATG38 in yeast). An activated ULK1 phosphorylates Beclin 1 leading to the activation of the second complex. In addition, VPS34 and AMBRA1 (shortened from Autophagy and Beclin 1 Regulator 1), which is associated with the PI3K complex, are also able to be phosphorylated by ULK1. It is the function of ATG14L to mediate the translocation of PI3K complex to the PAS [625].

1.15.2 Autophagy markers: LC3 and p62

ATG9 is a membrane-bound associated protein that is recruited to the PAS after autophagy induction. This ATG is suggested to be a crucial regulator of autophagy given that the amount of ATG9 recruited during the initial stages of phagophore formation is likely a limitation factor [625]. In addition, PI3K complex generates PI3P leading to the recruitment of WD repeat domain phosphoinositide-interacting (WIPI) proteins, most notably WIPI2. All the above-mentioned proteins involved in autophagy may theoretically serve as markers of autophagy induction. However, LC3 is the most commonly used marker for autophagy induction. LC3 has three isoforms: LC3A, LC3B and LC3C (collectively referred to as LC3 from now). Upon transcription, LC3-I is formed after premature LC3 is cleaved at the C-terminal glycine by ATG4B [627]. After autophagy induction, the C-terminal glycine residue of LC3-I is conjugated to the amino group of phosphatidylethanolamine (PE) in an ATG-mediated process, leading to the formation of the mature LC3-II (now referred to as LC3) [627]. The LC3 protein is found on autophagosome double membranes pre-lysosome fusion and is

crucial for phagophore formation, elongation and the fusion of both ends of the membrane [627]. LC3 is cleaved by ATG4B at the outer membrane of the autophagosome membrane and the inner membrane bound LC3 is degraded by the lysosome. This, therefore, serves as an ideal marker for the number of autophagosomes that have been generated in autophagy-inducible conditions [627]. Upon lysosome fusion, the LC3 is degraded along with the autophagosome; therefore, increases in LC3 levels do not necessarily signal the up-regulation of functioning autophagy but only the increase in autophagosome numbers [627]. Lysosome inhibition is then required to assess whether LC3 increases upon stimulus is due to autophagy induction and not due to non-autophagic processes or dysfunctional autophagy that inhibits the fusion between the autophagosome and lysosome [627]. Lastly, p62 which has been suggested to be transferred into the autophagosome by membrane-bound LC3 is also another autophagy marker [628]. The ubiquitous accumulation of p62 is indicative of defective autophagy and the decrease in p62 levels suggests autophagy activity [628].

1.15.3 The role of EGFR in autophagy

As mentioned previously, autophagy can be activated by a number of stimuli. RTK signalling has been frequently associated with autophagy activity in cancer and particular attention has been attributed to the relationship between EGFR and autophagy. For example, EGFR inhibitor resistance in metastatic colorectal cancer is known to be induced by autophagy activation, potentially raising the question of combined EGFR and autophagy inhibition as a therapeutic strategy [629]. The observation of EGFR inhibition leading to autophagy activation has also been reported in other cancer, including non-small cell lung cancer [630]. This suggests that EGFR activity has autophagy inhibitory capacities. Indeed, Levine's group recently showed that phosphorylation of EGFR and subsequent endocytosis and EGFR internalization leads to EGFR-mediated inhibition of Beclin 1 via phosphorylation, autophagy suppression and increased tumour growth in non-small cell lung cancer [631]. In a panel of epithelial carcinoma cell lines, the inhibition the EGFR-ERK signalling pathway was shown to increase autophagy activity [632]. Interestingly, STAT3 and p62 has been suggested to increase the transcription of autophagy genes [633]. As we have seen previously this contradicts the role of STAT3 as oncogene if it is a given that autophagy is a tumour suppressive process but consistent with the tumour suppressive role of p62 as we shall note

in the next section of this chapter. These results are unsurprising given that autophagy is commonly a starvation-induced mechanism while RTKs, in particular EGFR, are growth factor signalling enhancers. Nonetheless, controversy remains regarding the role of EGFR in autophagy. EGFR has been reported to activate autophagy in a kinase-independent manner. Recently it was demonstrated that a non-phosphorylated EGFR associates with LAPTM4B, a positive regulator of the PI3K-AKT pathway, on endosomes during starvation [634]. In turn, this complex activates autophagy via EGFR binding the autophagy inhibitor Rubicon and liberating Beclin 1[634].

In glioblastoma, too, EGFR inhibition, with the use of the TKI ZD6474 which also can inhibit VEGFR, increases autophagy in a manner that dependent upon down-regulation of PI3K-AKT signalling [635]. This result is confirmed by other studies, such as Eimer et al. demonstrating that erlotinib induces autophagy in U87 cells and the combined erlotinib and autophagy inhibition (with si-LC3) can induce apoptosis and is potentially a therapeutic strategy [636]. The relationship between EGFR and autophagy within the context of radiotherapy or TMZ treatment has also previously been described. Recently, Palumbo et al. showed that silencing EGFR mRNA translation can decrease migration and increase radiosensitivity in U373 and T98G, in addition to increasing TMZ sensitivity[637]. Importantly, silencing the ATG7 increased clonogenic capacity of si-EGFR and radiotherapy treated glioblastoma cells [637]. Consistent with Palumbo's thesis advocating the role of autophagy as a pro-death or tumour suppressing mechanism, Tini et al. in 2015 reported that a low EGFR and high Beclin 1 expression profile in a cohort of glioblastoma patients (n=117) treated with radiotherapy and TMZ carried a favourable prognosis (along with low EGFR expression alone when compared to high EGFR expression) [638].

These reports appear to contradict the previously cited research as it suggests that autophagy stimulates cell death mechanisms post-irradiation, post-TMZ therapy or after EGFR inhibition. The controversy regarding the categorisation of autophagy in glioblastoma biology and its relationship with EGFR remains to be further studied. Surprisingly, only two other related studies are generated in a literature search on Scopus with the key terms 'EGFR', 'glioblastoma', 'temozolomide' and 'autophagy'. Choi et al. demonstrated that 6Gy irradiation combined with 25uM TMZ for 24 hours led to the increased expression of LC3 in U251 cells[639]. Furthermore, combining HSP90 inhibition with DMAG-17 - thereby

inhibiting EGFR and AKT signalling – with radiotherapy alone or combination with TMZ increased LC3 expression which may contradict Palumbo and Tini as this opens the possibility that therapy may increase autophagy [639]. However, it remains to be seen whether resistance to radiotherapy and TMZ is mediated by autophagy.

1.16 MicroRNAs

1.16.1 Biogenesis

The pioneering work by Victor Ambros and colleagues in the early 1990's first described a gene product of approximately 22 nucleotides in length called lin-4 which had anti-sense complementarity with regions found on the 3' untranslated region of the lin-14 gene [640] [641]. The lin-4 RNA molecule was observed to suppress the translation of lin-14 to regulate larval development of C.elegans. With these studies lin-4 has become recognised as the first microRNA (miRNA) to be discovered. We had to wait several more years, until 2000, for the discovery of let-7, found also in C.elegans, and the next microRNA to have been discovered - let-7 was later observed in humans and other species. MiRNAs during this period were termed small temporal RNAs, due to their involvement in development, and were known to be conserved, endogenously expressed small RNAs derived from an arm of a stem-loop precursor. Only after the realisation that miRNAs were regulators of processes other than development due to the cell-type specific expression profile was the name miRNA coined [642].

MiRNA genes are commonly found in distinct and isolated regions of DNA sequences though a substantial proportion are found within the introns of non-miRNA pre-mRNA sequences - perhaps up to 25% - suggesting a regulatory relationship that exists between protein production and miRNA-mediated negative regulation. Clusters of miRNAs are also found within a primary transcript, such as the miR-15/16 cluster found on chromosome 13, and can lead to the production of multiple miRNAs after the transcription of single DNA sequence region [642].

MiRNAs are commonly transcribed by RNA polymerase II, with a minority that a within Alu-repetitive elements being transcribed by RNA polymerase III [643]. The pri-miRNA molecule that is transcribed contains a 5-cap structure, polyadenylated and is hair-pinned shaped

with less than perfect base pairing compatibility [643]. Clustered miRNAs are considered to be containing within a single pri-miRNA transcript. The pri-miRNA undergoes nuclear modification by a Microprocessor complex containing RNase III type endonucleases called Drosha and a double stranded RNA-binding domain protein DGCR8/Pasha which cleaves the pri-miRNA to produce a shorter hair-pinned shaper stem loop RNA structure called pre-miRNA (or precursor miRNA) that is approximately 70 nucleotides in length. Due to staggered RNase III cleavage a small two nucleotide long 3' overhang is found in the pre-miRNA with 5' phosphate. Finally, the pre-miRNA 3' overhang is identified and is exported from the nucleus by exportin-5 with the process mediated by Ran-GTP [643].

The cytoplasmic pre-miRNA is further processed by a second RNase III called Dicer, in combination with the cofactor TAR RNA binding protein (also referred to as TRBP), resulting in the cleavage of the stem loop and a miRNA:miRNA duplex [643]. TRBP recruits the Argonaute protein Ago2 and together with Dicer produces the RNA-induced silencing complex (RISC). The relatively unstable 5' end strand of the pre-miRNA is incorporated into the RISC and 3' end is commonly, though not always, disposed. A functioning miRNA therefore requires RISC as a guide for miRNA binding a target mRNA. The final product is an approximately 22 nucleotide long single stranded RNA molecule miRNA [643].

1.16.2 Mechanism of action

The function of miRNA is to recognise, bind and suppress the translation of target mRNA. miRNA:mRNA interaction leads to the cleavage of mRNA and is aided by RISC associated Argonaute proteins [642]. The interaction between miRNA and mRNA is mediated by the degree of complementarity between the miRNA sequence and the sequence of target mRNAs, and is not stoichiometric such that a single miRNA can target multiple mRNAs [644]. Commonly, it is the 3' untranslated region of mRNA that is targeted by the miRNA. It must be noted though that translational inhibition can occur via degradation and, commonly, repression due to mRNA deadenylation and decapping. Furthermore, complete complementarity is not required with imperfect binding sufficient for the endonuclotyic-mediated mRNA suppression being far the most common occurrence [644]. A region within the miRNA strand, called the seed sequence (or seed region), typically ranging across nucleotides 2 to 8 is the reason for this and the crucial mediator of miRNA:mRNA binding [644].

Apart from deadenylation and decapping other mechanisms of miRNA suppression of mRNA translation have been proposed. Several steps during mRNA translation are prone for miRNA-mediated repression[643]. The m^7GpppN at the 5' terminal cap of mRNA, recognised by the eukaryotic translation factor eIF4F during translation, is considered to be needed for miRNAs to bind mRNA. Generally, disruption of either end of the mRNA, the 5' cap and 3'poly A tail, are prone for miRNA-mediated repression [643]. Furthermore, immunoprecipitation analysis showing that the eIF6 and 60S ribosomal subunit on one hand and the tri-complex RISC components on the other immunoprecipitate together suggest the presence of eIF6 to also be a key requirement. MiRNA may also target the mRNA during post-initiation of translation. For example, evidence showing that miRNA and mRNA are found together in polysomes may suggest the miRNAs inhibit the elongation stage of translation [643].

Other than inhibition of mRNA translation, miRNA-mediated mRNA degradation and destabilisation are other mechanisms in which protein synthesis can be negatively regulated by miRNA [643]. Exosome-mediated shortening of the poly A tail of mRNA in a 3' to 5' direction or removing of the m^7GpppN cap by exonuclease XRN1 in a 5' to 3' direction are two mechanisms of mRNA degradation. mRNAs are finally degraded by structures called P-bodies which contain mRNA degrading enzymes [643]. It is suggested that miRNA-bound mRNAs lead to recruitment of the deadenylation complex CCR4-NOT and consequent shortening from the 3' end. Additionally, miRNA-bound mRNA may lead to the recruitment of proteins involved in decapping complexes, such as DCP1 and DCP2, thereby shortening in a 5' direction and, finally, mRNA degradation [643].

1.16.3 The significance of microRNAs in glioblastoma

Given the role of miRNAs to be a non-coding RNA capable of negatively regulating the translation of mRNAs increased interest has been paid to the potential of miRNAs to serve as part of a therapeutic strategy in cancer. The rationale behind this proposal is rather simple: miRNAs, by suppressing either oncogenes or tumour suppressors, are regulating the tumourogenic pathways and development [645]. The significance of miRNAs in glioblastoma was first noted in 2005 when Chan and colleagues identified miR-21 as an anti-apoptotic miRNA that is up-regulated in glioblastoma cell lines and silencing miR-21 can increase caspase-dependent apoptosis [646]. The study was almost simultaneously with another, by

Ciafre et al., identifying a miRNA expression signature in glioblastoma - consisting of down-regulated miR-128, miR-181a, miR-181b and miR-181c, in addition to up-regulated miR-221 – that are altered [647]. The large gap between the time of miRNA discovery and these glioblastoma studies are indicative of the rather underappreciated status of miRNAs as key regulators of disease. Since these pioneering studies miRNA have been recognised as key modulators of glioblastoma - there are now well over 1,000 research articles associated with both miRNA and glioblastoma. Furthermore, the roles of the miRNAs that these initial studies proposed have been extensively confirmed. Both miR-21 and miR-221 are regarded as oncomiRs, in cancer let alone glioblastoma [648-652]. In fact increased plasma miR-21 has now been argued as a non-invasive biomarker for glioblastoma diagnosis [653]. The category of oncomiRs, first coined by Esquela-Kerscher and Slack in 2006, refers to miRNAs that function in the context of cancer as inhibitors of the translation of tumour suppressor genes [654]. In contrast, another category of miRNAs, termed tumour suppressor miRNAs, are considered to target a range of oncogenes to negatively regulate tumourogenic pathways leading to cell proliferation, invasion, migration and anti-apoptosis [655]. It shall be noted that some authors, such as Esquela-Kerscher, refer to all cancer associated miRNAs as oncomiRs, regardless of their specific function in cancer [654].

In glioblastoma, miRNAs have been shown to modulate canonical signalling pathways downstream of RTKs, either by directly regulating RTKs or downstream signalling molecules. PDCD4 is downstream of the PI3K-AKT pathway and is inhibited by S6K due to mTOR signalling. PDCD4 has been shown to increase p21-mediated cell-cycle arrest. MiR-21 in glioblastoma has been shown to directly target the PDCD4, as well as other positive regulators of p21, such as p63, JMY, DAXX, TP53BP2 and TOPORS [656]. In contrast to the oncomiR miR-21, Shah's group showed recently that miR-7 up-regulation with transfection down-regulates EGFR expression and AKT activity - an observation already previously reported by Kefas et al. - but increases the death receptor 5 to stimulate TRAIL-induced apoptosis [657, 658]. Sensitisation to TMZ-induced apoptosis can be achieved with miR-182 up-regulation with transfected mimics, while miR-182 delivery to intracranial glioma mice models reduced tumour burden [659]. The miR-182-induced TMZ sensitisation mechanism reported may be via miR-182 down-regulating MET expression [659]. Another miRNA, miR-562, was recently shown to down-regulate MET and AKT signalling, thereby also suppressing

glioblastoma cell proliferation [660]. Canonical downstream signalling pathways may also up-regulate miRNA expression. STAT3 activity directly stimulates miR-182-5p expression to increase cell proliferation, migration and invasion of glioblastoma cells [661].

Cellular resistance to radiotherapy or TMZ mediated by miRNAs has also been reported. For example, in 2010, increasing miR-21 was shown to suppress TMZ-induced caspase-mediated apoptosis in U87 cells – perhaps the first report linking miRNAs to therapy resistance in glioblastoma – while radio-resistance is strengthened with miR-21 mediation of PDCD4 [662, 663]. U251 cells made resistant to TMZ, after 100uM exposure over two weeks and validated with survival assays, were shown to over-express miR-195, miR-455-3p and miR-10a. Combining the inhibition of miR-10a or miR-455 with TMZ overcame TMZ resistance, an effect not seen when inhibiting either miRNA alone [664]. The tumour suppression role of miR-181 family has been further validated with Li et al. demonstrating the sensitisation of TMZ-resistant U87 glioma stem cells after miR-181b over-expression [665]. Similarly, resistance to radiotherapy can increase with up-regulation of miR-221/222, possibly mediated by miR-221/222 stimulating the AKT signalling pathway [666]. Meanwhile, miR-338-5p may increase radio-sensitivity in glioblastoma cell lines by directly targeting proteins associated with the DNA damage response [667].

Overall the link between miRNAs and therapy resistance in glioblastoma has been understudied. A Scopus search with key terms ‘microRNA’, ‘resistance’, ‘radiotherapy’ and ‘glioblastoma’ only generates approximately 90 articles, perhaps many of these articles being unrelated to the topic. A Scopus search with key terms ‘microRNA’, ‘resistance’, ‘radiotherapy’ and ‘glioblastoma’ only generates approximately 90 articles, perhaps many of these articles being unrelated to the topic. Similarly, a Scopus search with key terms ‘microRNA’, ‘resistance’, ‘temozolomide’ and ‘glioblastoma’ only generates approximately 100 articles, and also likely that many of these articles being irrelevant to the topic. Importantly, the combined effect of radiotherapy and TMZ, a clinically relevant treatment regimen, has largely been overlooked in regards to miRNA expression and mediated responses. Additionally, the role of miRNAs in mediating RTKs expression and signalling within the context of cellular resistance to radiotherapy and/or TMZ has also been overlooked. As we shall see, this gap in the literature shall be addressed in **chapter 5** of this thesis.

MiRNA expression profiles are increasingly being advocated as signatures of treatment response and prognosis. An eight miRNA signature was recently proposed to be predictive of glioblastoma recurrent patient response to bevacizumab [668]. In this analysis, using a final cohort of over eighty patients, it was reported that miR-124a, miR-202, miR-7 and miR-222 are up-regulated in poorer surviving patients but down-regulated in patients that responded to bevacizumab [668]. In contrast, miR-363, miR-630, miR-663 and miR-204 were down-regulated in poor surviving patients but up-regulated in bevacizumab responders [668]. The potential for a miRNA expression profile to differentiate primary from recurrent glioblastoma has also been trialled recently, consisting of miR-10b, miR-21, miR-181b, miR-181c, miR-195, miR-221 and miR-222, though no significant difference was observed – possibly due to the small 15-patient cohort and authors deciding to relativise the miRNA expression levels according to the median expression of either primary or recurrent tumours [669]. Furthermore, miRNA expression signatures may also be potentially used for indicating response to radiotherapy and TMZ therapy in the clinic, such as miR-132, miR-518b, miR-524-5p and miR-566 [670, 671]. However, although immense effort has poured into postulating miRNA gene expression signatures as clinical biomarkers, no one signature has yet been confirmed as a clinically relevant classifier capable of consistently stratifying glioblastoma patients [672]. Future studies are required to better model miRNA expression profile changes across tumour development stages. Additionally, a likely reason for the lack of reproducibility of miRNA expression signatures is likely due to researchers failing to differentiate biologically relevant miRNA interactions with a specific mRNA and the functionally possibly interaction between miRNA and mRNA. In other words, the mere fact that a particular miRNA binds and suppresses the translation of a particular mRNA does not necessitate that the interaction is crucial to the tumour biology.

1.16.4 The previous status of miR-221 in glioblastoma

The role of miR-221 in glioblastoma has previously been described in recent papers. To truly contextualise our novel proposals that have been established in this present study **(**see **chapter 5)**, we need to first turn to this literature that is available. Therefore, in this section, prior to discussing the significance of our proposals pertaining to miR-221, we will describe the role of miR-221 in glioblastoma that is already known.

1.16.4.1 The role miR-221 in other cancers

The miR-221 and miR-222 family of miRNAs has been gaining interest in cancer research in recent times. Both miR-221 and miR-222 are paralogous miRNAs located in the Xp11.3 chromosomal region and share the same seed region sequence but differ with regards to the rest of the RNA sequences(miR-221: GenBank AJ550425.1, miR-222 GenBank AJ550426.1). With ovarian cancer cell lines Farsani et al. reported that miR-221/222 targets PTEN and high miR-221/222 promotes cisplatin resistance – inhibition of these two miRNAs down-regulated AKT activity and re-sensitised the cells to chemotherapy [673]. Similarly, in hepatocellular carcinoma cell lines miR-221 positively regulated the stem cell and epithelial-mesenchymal transition associated marker CD44 [674]. A recently published meta-analysis evaluated the prognostic value of miR-221 in a number of malignancies - including breast, ovarian, bladder, laryngeal, thyroid, lung, colon, renal, gastric and prostate cancer, but excluding glioblastoma [675]. It was concluded in this study that miR-221 is significantly associated with worse overall and progression-free survival [675]. To date, there are no exhaustive reviews detailing the role of between miR-221/222 in glioblastoma and, therefore, in this review, we will report on the latest data pertaining to miR-221/222 in glioblastoma.

1.16.4.2 MiR-221 in glioblastoma versus normal tissue

The overwhelming evidence thus far suggests the miR-221/222 cluster to be associated with an unfavourable prognosis. However the status of miR-221/222 relative to normal brain tissue is contentious. In perhaps the earliest study, Ciafre et al. demonstrated that miR-221 was significantly up-regulated in glioblastoma compared to normal brain tissue [647]. Furthermore, miR-221 was up-regulated in 5 out of 10 glioblastoma samples, when compared to peripheral tissue of paired patients [647]. Ciafre showed, for the first time using a panel of seven glioblastoma cell lines, that miR-221 was over-expressed in all glioblastoma cell lines compared to normal brain tissue [647]. This is supported by other studies such as Conti et al. in a study consisting of 10 glioblastoma patients and by Jin and colleagues who showed that in a cohort of 49 glioblastoma patients, serum miR-221 levels was significantly increased compared to cancer-free controls [651] [676]. Swellam et al. also confirmed this observation by demonstrating that miR-221 and miR-222 was up-regulated in glioblastoma patients compared to normal healthy tissue, from a cohort consisting of 20

subjects in each group prognosis [677]. A recent study showed that miR-221 levels in both glioma tissue and glioma-derived exosomes are higher than in normal samples [678]. On the contrary, Visani et al. in 2014, observed lower miR-222 and unchanged (insignificant decrease in) miR-221 levels in glioblastoma patient samples compared non-neoplastic tissue [679]. Supporting this is Slaby and colleagues who also showed low miR-221 and miR-222 expression in glioblastoma samples compared to non-neoplastic brain tissue [680].

1.16.4.3 MiR-221 correlates with glioma grade

Compared to the lack of consensus regarding miR-221/222 glioblastoma expression relative to normal brain tissue, there does appear to be a consensus regarding miR-221/222 correlation with glioma grades. In a study consisting of 9 low grade glioma and 13 high grade glioma, miR-221 was found to correlate with glioma grade [681]. Two other studies also showed that miR-221 correlates with glioma grade and the highest was observed with glioblastoma [651, 682]. These results were recently validated by Yang et al. concluding the miR-221 levels were found to positively correlate with glioma grade as measured in glioma tissues and glioma-derived exosomes [678]. Taken together, the high levels of miR-221 in glioblastoma relative to lower grades of glioma indicate that miR-221 is a biomarker for the level of malignancy and associated with the increased aggressiveness of glioblastoma.

1.16.4.4 MiR-221 and patient survival

It has been previously reported that a high miR-221 and miR-222 expression glioma patients is associated with highly invasive tumours, as measured by fractional anisotropy values, indicating that clinical glioblastoma tumourigenesis is driven by miR-221/222 [681]. Nonetheless, the role of miR-221/222 in glioblastoma patient survival is currently debatable. For example, Lakomy et al. noted that although miR-221 and miR-222 are down-regulated in glioblastoma compared to normal tissue, the differential expression was not a prognostic factor [247]. In contrast, Hua et al., who also noted miR-221 and miR-222 to be lower in glioblastoma compared to normal tissue, reported that a high miR-93 and low miR-221 or high miR-19a and low miR-222 expression profile significantly predicted a favourable survival outcome [683].

The majority have concluded that miR-221/222 is associated with worse prognosis. Srinivasan et al. also confirmed miR-221 to be down-regulated in glioblastoma compared to

normal brain tissue but found high miR-221 to be associated with poorer prognosis within the glioblastoma subset [684]. Responders to radiotherapy and TMZ have been shown to have had lower levels of miR-221 and miR-222 compared to non-responders. Though this indicates a trend towards poorer survival in treated patients, this difference was not significant [680]. In another cohort consisting of 36 glioma patient-derived samples, high miR-221 or miR-222 was significantly associated with poorer survival, although the cohort consisted with a variety of glioma grades with an undefined number of glioblastoma patients [681]. Also, supporting a correlation between high miR-221 with poor survival are Swellam and colleagues, who measured serum miRNA levels. In this study they showed that high miR-221 was associated with older age - indicating that miR-221 is more common in primary glioblastoma - and, miR-221 and miR-222 was found to be significantly reduced in chemoradiotherapy treated patients [677]. Indeed, high miR-221 and miR-222 was associated with poorer survival and progression-free survival [677]. Recently, analysis of a cohort of 56 glioblastoma patients with unmethylated MGMT promoters, reported that high miR-221 was associated with a worse prognosis but such an association was not observed with methylated MGMT glioblastoma [685]. This report has been validated in a separate glioblastoma patient cohort that observed in short-term surviving patients, defined by survival times of less than 15 months, MGMT levels inversely correlate with miR-221 expression [686].

1.16.4.5 MiR-221 and radiotherapy and chemotherapy resistance

Studies that are *in vitro* have also reported on the association between miR-221 and MGMT. In glioblastoma cell lines with low MGMT, miR-221 levels are relatively higher. The tumour-initiating U87, LN18 and LN229 cells, compared to the high MGMT expressing T98G cells, had higher miR-221 levels [687]. Consistently, luciferase reporter assays have verified that MGMT is a target for miR-221 [686]. However, Quintavalle et al. reported that this mechanism sensitises glioblastoma cell lines to TMZ therapy, potentially contradicting clinical studies and miR-221 as a driver for TMZ resistance [686]. Consistent with this, miR-221 transfection sensitised glioma cell lines T98G and LN428 to 300uM TMZ exposure for 24 hours. In addition, it was postulated that in T98G cells miR-221 over-expression increases caspase-3 activity upon TMZ treatment, leading to greater apoptosis [686]. Contradicting Quintavalle's results is a 2012 paper authored by Chen and colleagues. This paper found

that as-miR-221/222 transfected LN308, U251 and U87 cells were sensitised to 10uM-50uM TMZ after an undisclosed treatment period. Furthermore, the caspase 3/7 activation and increased apoptosis rates observed in as-miR-221/222 alone treated cells were compounded with additive to TMZ therapy [688]. The role of miR-221 as a chemo-resistance modulator has also been supported by a recent study in which 48 hours of 100uM TMZ treatment combined with as-miR-221 reduced SHG-44 viability [678].

Interestingly, miR-221, by targeting connexin 43, perhaps promotes U251 cell viability, invasion and cell cycle progression [689]. Additionally, as-miR-221/222 transfection was able to increase the rate of apoptosis as measured by annexin-V labelling – though the time span was undisclosed [689]. This appears to contradict Munoz et al. who concluded that by driving connexin 43 levels, the EGFR induces TMZ resistance; however, the apparent incongruence may be due to the difference in cell lines since Munoz only utilised the U87 and T98G and not the U251[690]. Additionally, miR-221 may have separate roles in promoting glioblastoma tumourigenesis depending upon experimental cellular contexts. Therefore, the exact miR-221 signalling pathways in a TMZ context may differ in an untreated context.

Glioblastoma stem cells are regarded as drivers of chemo-radiotherapy resistance that are capable initiating gliomagenesis. Aldaz et al. investigated the role of miR-221 and miR-222 in the differentiation of glioblastoma stem-like initiating cells, identified by nestin expression. Upon differentiation of this cell type it was observed that the expression levels of miR-221, miR-222 and miR-21 were increased along with increases in GFAP or the neuronal marker TUJ1 and decreases in nestin. Furthermore, upon inhibition of miR-221 or miR-222 the differentiated glioblastoma cells reverted to the glioma initiating profile [691]. Therefore, these results may suggest that targeting miR-221 and miR-222 in glioblastoma may lead to the selection of chemo-radiotherapy resistant subpopulation of cells at tumour recurrence. However, further studies are required. Taken together, whether miR-221 and miR-222 is involved in TMZ-resistance remains controversial and requires further study.

Xie et al. showed that miR-221 was highly expressed in BCNU-resistant glioma cells. SWO38-derived BCNU-resistant cells showed higher miR-221 levels compared to the relatively BCNU-sensitive U87 and U251 cell lines [692]. Furthermore, U87 and SWOZ1 – also derived

from the SWO38 glioma cell line – transfected with miR-221 mimic were more resistant BCNU treatment, while miR-221 inhibition sensitised both cell lines to BCNU[692]. The authors also demonstrated that miR-221 negatively regulates PTEN protein expression (though not mRNA levels) and, consistently, increases activation of the PTEN downstream signalling molecule AKT [692].

Up-regulation of miR-221 has also been linked to radio-resistance in glioblastoma. Li et al., in 2014, showed that miR-221 transcription was induced by c-Jun after radiotherapy leading to DNA-PK expression and PTEN-independent AKT activation. Radio-sensitivity may be re-established via AKT-inhibition mediating miR-221 down-regulation or miR-221/222 anti-sense therapy. Furthermore, radiotherapy plus anti-miR-221/222 treatment led to reduced tumour growth compared to either treatment alone in an *in vivo* mice xenograft model [666].

1.16.4.6 MiR-221 and *in vivo* tumour growth

The U251 cell line is the most commonly used cell line to generate xenograft tumours in mice models in the study of miR-221/222 in glioblastoma. A U251 subcutaneous xenograft mice model, treated with as-miR-221 or as-miR-222 inhibited tumour growth, and the effect is compounded with combined as-miR-221/222 therapy [693]. This observation, that as-miR-221 or as-miR-222 reduces *in vivo* U251 tumour growth, has repeatedly been validated [681, 694] [695]. The inhibition of miR-221/222 of *in vivo* U251 tumour growth has also been associated with increased DNA fragmentation and Bax expression compared to scrambled and non-transfected controls, indicating that as-miR-221/222 initiates apoptosis to slow tumour growth [695]. Yang et al., however, recently moved away from the U251 *in vivo* model and showed SHG-44 cells transfected with miR-221 and injected subcutaneously in to mice had increased tumour growth [678]. Taken together, although the role of miR-221/222 is understudied using different *in vivo* models, results thus far suggest as-miR-221 and as-miR-222 therapy may slow tumour growth and increase apoptosis.

1.16.4.7 MiR-221 and cell signalling

The majority of *in vitro* studies have focussed upon the mRNA targets of miR-221/222 and have associated miR-221 and miR-222 with conferring proliferative capacities to glioblastoma cell lines. For example, high miR-221 expression in U87 relative to T98G was

associated with low PTEN and high AKT activation [687]. Moreover, as-miR-221 or as-miR-222 disrupted U87 colony formation but up-regulating miR-221 or miR-222 increased T98G colony formation [687]. The $p27^{kip1}$ (p27) is a tumour suppressor known to inhibit glioblastoma growth and invasion, and its over-expression has been shown to cause cell cycle arrest and suppression of cell migration. Earlier studies have demonstrated that miR-221/222 inversely correlate with p27 in glioblastoma cell lines and that p27 is a direct miR-221/222 target [696] [697]. It was later shown that CPEB1 can compete with miR-221 in binding to the 3'UTR of p27, thereby disrupting miR-221/222 seed sequences in pairing with the target p27 sequence [698]. U251 cells with up-regulated miR-221/222 suppressed p27 levels while as-miR-221/222 induced cell cycle arrest and inhibition of proliferation [693]. Consistently, immunohistochemistry analysis of U251 xenograft tumours showed that as-miR-221/222 up-regulated p27 and had lower Ki-67 [693]. Finally, Galardi et al. concluded that the transcription factors NF-kB and c-Jun stimulate miR-221 and miR-222 transcription [699]. To elaborate, after bioinformatics predicted transcription factor binding sites upstream of miR-221/222 to pair with NF-kB and c-Jun, and, indeed, inhibiting NF-kB phosphorylation or c-Jun depletion reduced miR-221/222 and increased p27 levels in U87 cells, as measured by qRT-PCR[699].

Another signalling mechanism proposed by which miR-221 stimulates proliferation and inhibits apoptosis is through its targeting of PUMA. Upon inhibition of miR-221 or miR-222 the apoptosis levels increased in U251 and LN229 as demonstrated by annexin-V labelling, in addition to increased caspase 3/7, increased Bax, decreased Bcl2 and collapse in the integrity of mitochondrial membrane potential – the most marked increased observed when as-miR-221 was combined with as-miR-222 [695]. Western blot and luciferase assay, showed that the anti-apoptotic function of miR-221 and miR-222 may be mediated via targeting of PUMA, further supported by an inverse correlation between miR-221/222 and PUMA in an analysis with 40 glioma patient samples [695]. Perhaps in contradiction to the studies thus far was an earlier study by Medina et al. in 2008. Here it was reported that serum-deprived T98G cells transfected with miR-221/222 mimic underwent cell death and, on the other hand, as-miR-221/222 transfected cells were morphologically similar to untreated controls [700]. A potential reason, other than the difference in cell lines, could be that in the previous studies cells were not serum-deprived; thereby miR-221/222 may have

opposing roles under starvation conditions and the exact mechanism involved requires further study.

MiR-221/222 has also been postulated as a modulator of glioblastoma invasion and migration signalling, for example by directly targeting phosphatase μ, a cell surface protein tyrosine capable of inhibiting glioblastoma migration and invasion[687, 701]. Also, transfection of miR-221/222 mimic using U251 and LN229 increased migration and invasion rates which was abrogated by as-miR-221/222 treatment. The increased tumourigenesis correlated with MMP-2/9 expression in both cell lines. Furthermore, miR-221/222 was found to negatively regulate and target TIMP3 and TIMP2 in U251 and LN229, with TIMP3 and TIMP2 up-regulation abrogating miR-221/222-induced invasion and migration [681, 702]. By miR-221 directly targeting DNM3 SHG-44 cell proliferation and migration increased and, indeed, DNM3 over-expression abrogated the tumourogenic capacity of miR-221 expression in SHG-44 [678]. Using cell lines U87 and U251, Cai et al. showed that inhibition of miR-221 suppressed migration and invasion, proposing that miR-221 can promote glioblastoma migration and invasion via targeting of SEMA3B [703].

Lastly, miR-221 was recently associated with glioblastoma angiogenesis. Specifically, Xu et al. demonstrated that as-miR-221/222 treated U251 cells showed reduced MMP-2/9 and VEGF protein and mRNA expression in both *in vitro* and *in vivo* U251 models [694]. The mechanism by which miR-221 mediated these effects was proposed to be via directly targeting SOCS3. Indeed, up-regulation of SOCS3 abrogated the miR-221/222 driven invasion and promotion of MMP-2/9 and VEGF expression [694]. Furthermore, up-regulating miR-221/222 in U251 increased Jak2 and STAT3 activity which was abrogated via SOCS3 expression [694].

Table 1-5: List of validated miR-221/222 targets in glioblastoma

TARGET	REFERENCE
MGMT	[686]
PTEN	[673]
SOCS3	[694]
p27	[696]
PUMA	[695]
Connexin 43	[689]
TIMP3	[681]
Phosphatase μ	[687]
DNM3	[678]
SEMA3B	[703]
TIMP2	[702]

1.17 Epithelial to mesenchymal transition

The most basic definition of epithelial to mesenchymal transition (EMT) is a biological process culminating in the phenotypic conversion of epithelial cells to mobile mesenchymal cells, after the epithelial cell detaches its basal surface from the basement membrane. Within the context of cancer, however, a partial transition in which the cancer cell remains in a state of flux between the two endpoints can be referred to be undergoing EMT. During EMT, cancer cells develop aggressiveness, increased migration and invasive capacity, display altered morphology and become metastatic. After metastasising from the primary site of the tumour a reverse process called mesenchymal to epithelial transition (also referred to as MET) is initiated such that the metastatic tumour resembles the epithelial nature of the tumour cells residing within the primary site [704].

EMT has been paid increasing attention and has become a key biochemical process in cancer subtypes, leading to EMT being regarded as a key tumour resistance mechanism and of therapeutic interest. Classically, EMT is recognised molecularly by a so-called 'Cadherin switch' with E-Cadherin down-regulation and N-Cadherin up-regulation [705]. Certain transcription factors, miRNAs, expressed mRNA transcripts and proteins can be categorised as EMT-like associated markers and used as an identification tool to detect the initiation of EMT [706]. In addition to hypoxia other signalling pathways, such as those driven by Wnt, TGF-B and Notch, are also key regulators but for the purpose of our present study the

discussion will be refined to the role of RTKs in EMT[706]. Some of the main EMT-associated markers will be summarised below.

The Cadherin switch is considered the master regulator of EMT. Several mechanisms may lead to the modulation of Cadherin balance, such as deactivating mutations and epigenetic alterations via methylation of the promoters [705]. The switch involves the two cadherins and transmembrane glycoproteins E-Cadherin and N-Cadherin, which can also be regulated by several transcription factors commonly belonging to the TWIST, SNAIL and ZEB families [705]. EMT-associated transcription factors are able to suppress E-Cadherin expression by binding to E-box sequences of the E-Cadherin gene promoter [705]. Furthermore, the E-Cadherin suppressing transcription factors, such as TWIST1, ZEB1, ZEB2, Snail1 and Snail2, can be expressed by upstream signalling led by WNT and b-Catenin pathways, receptor tyrosine kinases and TNF-TNFR that activate downstream signalling cascades and other transcription factors, namely Elk-1, EGR1 and NF-kB [705]. In contrast, N-Cadherin up-regulation stimulates EMT and its up-regulation accompanies E-Cadherin down-regulation for cell-cell adhesion loss.

The Cadherin switch is not a feature in glioblastoma due to the almost absent levels of E-Cadherin and therefore, EMT in the strict sense is not considered to take place in glioblastoma but rather an EMT-like state is suggested. This is unsurprising given that E-Cadherin is majorly expressed in epithelial tissues and the Cadherin switch is a feature of carcinomas. Furthermore, contradictory data regarding the significance of N-Cadherin in high grade glioma complicates the narrative. Previous studies, for example, have suggested N-Cadherin to not correlate with invasion or even inversely correlate [707, 708]. Moreover, recent findings concluded there to be no prognostic significance regarding E- and N-Cadherin expression in glioma and the Cadherin switch was not indicative of glioma grade [709].

Vimentin belongs to the intermediate filament protein family and is a type III intermediate filament expressed in mesenchymal cells [710]. A protein approximately 57 kDa in size, Vimentin is regarded as a key EMT marker as it is expressed in epithelial cells undergoing EMT, notably in carcinomas [710]. In a Chinese cohort, low Vimentin in high grade glioma patient samples was associated with favourable prognosis and better response to TMZ in

glioblastoma specifically, suggesting a link between chemotherapy resistance and Vimentin expression in clinical glioblastoma [711]. This finding was later validated in a separate study which also showed that Vimentin inhibition led to decreased glioblastoma cell migration and invasion *in vitro* [712]. Lastly, the direct relationship between RTKs and Vimentin is relatively understudied; however, in epithelial carcinomas it well established that MET expression is able to induce Vimentin over-expression [713].

CD44 is a transmembrane glycoprotein receptor that commonly binds to the hyaluronic acid (HA) ligand, though can bind less specifically to other ligands such as collagen, osteopontin and fibronectin, and can range between 80-200 kDa in size [714]. Upon CD44:HA binding, conformational changes takes place to allow for scaffolding proteins to bind to the CD44 intracellular regions, therefore similar to the mechanism in which RTK signalling is initiated [714]. CD44 is relevant in cell-cell interaction and matrix adhesion and is expressed in the standard form called CD44s in both normal and cancer cells [714]. In cancer, however, several CD44 variants are expressed after exon insertions at the membrane proximal extracellular region and named CD44v isoforms v1 to v10, allowing for greater HA affinity and tumourigenicity [714].

CD44 can interact with several RTKs, including the EGFR family, PDGFR and MET, and the HA-bound CD44 can regulate RTK phosphorylation. For example, CD44 variants bind to the MET ligand HGF, leading to MET auto-phosphorylation via CD44-MET interaction [715]. Furthermore, in breast cancer cells a correlation between CD44v8-v10 expression and EGFR was evident [716]. These studies suggest a link between EGFR or MET with CD44 and possibly directly related to tumour progression.. Interestingly, variants of CD44 have been found to bind several growth factors such as VEGF, FGF and HGF [717]. Subsequent downstream signalling pathways and pro-tumourigenic and anti-apoptotic cascades are activated rendering CD44 involvement in cancer invasion, proliferation and migration [717]. These downstream signalling molecules include the ERK and PI3K-AKT signalling pathways [717].

In glioblastoma, CD44 has been noted to correlate glioma grade; however, whether CD44 is of prognostic value is controversial [718]. Lower CD44 surprisingly was shown to be associated with an unfavourable prognosis for both primary and recurrent glioblastoma

patients while other studies have shown significant association between CD44 and prognosis; therefore, the prognostic value of CD44 requires further study within the context of glioblastoma [718-720]. This differs from epithelial carcinomas such as ovarian, colorectal and breast cancer, again stressing the difference between EMT in glioblastoma and other cancer categories [721-723].

Lastly, several miRNAs have also been demonstrated to be regulators of EMT. ZEB1 and ZEB2 have been shown to be targets of miR-200c leading to EMT inhibition [724]. MiR-300 is another EMT negative regulator with increased miR-300 leading to suppression of TWIST1 [724]. In addition, oncomiRs, such as miR-221/222, can also stimulate b-Catenin to up-regulate EMT and cancer aggressiveness [724].

1.18 Justification and rationale for the project

Glioblastoma is the deadliest malignant brain tumour and the mean survival time has remained approximately 12 months despite aggressive treatment. Currently, the gold standard therapy is the so-called Stupp Protocol which consists of treatment with radiotherapy and TMZ which follows the surgical resection, the extent of the resection being dependent on a case by case basis. The almost impossible accomplishment of complete resection is a factor that contributes to tumour recurrence post-therapy, in addition to radio-chemotherapy failing to penetrate to target the residue tumour. Another factor contributing to recurrence is therapy resistance; an overarching phenomena describing the cellular molecular mechanisms allowing an individual malignant cell to survive exposure to radio-chemotherapy.

A number of resistance mechanisms have been identified in glioblastoma. MGMT driven resistance is a well-studied and widely accepted resistance mechanism with promoter methylation depleting the MGMT enzyme levels and hindering the DNA repair process. Other mechanisms, as discussed in detail in **Chapter 1**, can include other DNA repair mechanisms such as the mutated MMR and higher expression of base excision repair components.

Currently, the two biomarkers commonly utilised in determining a personalised treatment regimen are age and karnofsky score – neither are molecular markers. [725]. Research is,

therefore, primarily focussed on theorising and validating potential molecular biomarkers for individualised therapy in glioblastoma. One group of biomarkers are the RTKs - a class of membrane receptors that are well known to elicit oncogenic signalling in several cancers, including glioblastoma. Our project largely rested on an expansive body of literature detailing the oncogenic role of RTKs and the significance of RTK signalling in key tumourogenic processes such as: proliferation, invasion, migration, angiogenesis, DNA repair, anti-apoptosis and metabolism. Since elucidation of the genomic landscape of glioblastoma showed that RTK deregulation – as well as associated signalling molecules - to be a common genetic alteration, the RTK as a class of signalling proteins has been attributed a key place in research. The subsequent consequence was the gradual development of a RTK-based paradigm which has been sustained by decades of *in vitro* and *in vivo* research centring RTKs in key gliomagenesis signalling pathways. Indeed, RTKs have become enticing molecular targets for therapy; the development of targeted therapeutic agents has been trialled in glioblastoma clinical trials has largely yielded unfavourable results.

Certain scholars, writing after the failure of selective RTK therapy using erlotinib and gefitinib, have suggested alternative strategies and proposed potential reasons for the failure. For the latter, poor BBB penetration, redundant pathways and the intrinsic heterogeneity of glioblastoma are deemed essential hurdles for a RTK-based therapy to overcome before any success can be garnered [726]. As for the former, a rational combinatorial therapy that takes into account redundancy and resistant mechanisms is now increasingly being voiced as the future for targeted therapy [727].

We initially performed a literature review critically evaluating the origin of the RTK-based paradigm and the experimental data and methodologies employed that have been essential for the formations of the assumptions and rationale behind targeting RTKs in glioblastoma. Surprisingly, we found that the almost all studies on the role of RTKs have a major blindside that potentially lead to repercussions in the clinic. RTKs have largely been studied in treatment-naïve glioblastoma cell and knockout studies that concluded the critical role of certain RTKs have been studied in this or similar conditions, such as in hypoxia. Only a minority have utilised a radiotherapy or TMZ in the evaluation of RTKs in gliomagenesis and no studies have combined both. Given that clinical glioblastoma is exposed to both radio- and chemo-therapeutic genotoxic insults, and the utilisation of RTK inhibitors is in

combination of the gold standard, it follows that RTK research in glioblastoma hitherto does not have the necessary components representing clinical glioblastoma and we can expect, certainly, any rationale that supported the RTK paradigm to be essentially faulty during translation.

In this project we proposed an alternative approach: combining radiotherapy and TMZ to evaluate the RTK associated mechanisms to be found in glioblastoma. We also proposed an alternative explanation for the failure of RTK-based therapy: yet identified RTK expression and regulation, and associated signalling pathway activation profiles contribute to radio-chemotherapeutic resistance rendering previously utilised RTK inhibitors clinically insufficient.

MiRNA research has been gathering pace recently as evidence continues to suggest that this class of non-coding RNAs are potential targets for cancer therapeutics. Due to their ability to bind and disrupt mRNA translation, miRNAs are direct post-transcription gene expression regulators and indirectly, via mRNA binding, modulate signalling pathways. As a consequence of experimental data validating hypotheses that miRNAs can function as tumour suppressors or oncomiRs, miRNA gene therapy is now put forth as an alternative therapeutic strategy which can be realised via several methods, such as antisense oligonucleotides or synthesised miRNA mimics [728].

Relative to the RTK, the role of miRNAs in glioblastoma is a nascent field – the first report on the oncogenic role of miRNAs in glioblastoma was a 2005 study on miR-21 [646]. Since then, researchers have gradually elucidated the biological relevance of individual miRNAs in glioblastoma by being concerned with identifying mRNA targets and the accompanying effect on tumourigenesis, in addition to generating miRNA signatures that can predict prognosis. However, this endeavour not only has many unanswered questions but unformulated questions. The relevance of miRNAs within the context of tumour resistance and recurrence is overlooked in glioblastoma. Therefore, in this project we aimed to address this gap in the literature by pre-emptively proposing that miRNAs are key regulators of the differential RTK expression profile driving radio-chemotherapy resistance.

CHAPTER 2:
METHODS & MATERIALS

2.1 Cell lines

Primary glioblastoma cell lines #4, #15, #20, #28, #35, #39 and #41 are from the Royal Melbourne Hospital (RMH) and obtained by Dr James Dimou. These cells were modified into adherent monolayer culture from non-adherent neurosphere culture. U87, LN229, U251, U118, U138, were purchased from American Tissue Culture Collection.

All cell lines were grown as a adherent maintained in DMEM (which contains 4.5g/L D-glucose, L-Glutamine sodium pyruvate) supplemented with 1% penicillin/streptomycin (consists 10,000 units/mL penicillin, 10,000 ug/mL streptomycin) and 5% fetal bovine serum. Cells were incubated at 37^{o}C in 10% atmospheric CO_2.

2.1.1 Generation of TMZ-resistant cell lines

Parental #15, #20 and #41 cells were treated with 5Gy radiotherapy and 1000uM TMZ. The cells were then incubated for 7 days, followed by a 3-5 day period in which cells were left with TMZ-free media. After another round of radiotherapy and TMZ with the same doses and 7-day incubation period the cells were again left to incubate with TMZ-free media for 1-2 months – the period of time required for cells to proliferate and re-populate the 75cm^2 vent-capped flask.

The U87-R, U251-R and U118-R cells were generated by Dr Rodney Luwor and Dr Stanley Stylli. The parental U87, U251 and U118 cells were continuously treated over a time period of 6 months with increasing doses of TMZ (a minimum of 62uM and a maximum of 1000uM TMZ). The resistance was confirmed with cell viability assay (see cell viability section).

2.1.2 Single cell isolation assay

The U87-V and U251-V were derived from a single cell isolation assay. Briefly, 100uL of media was pipette into all the wells of a 96-well plate. 2000 cells/100uL/well were seeded using either the parental U87 or U251 cell line into all the wells of the first column of a 96-well plate. Subsequently, 100uL of media containing the cells were transferred from the first column and into the second column, leading to a total media volume of 200uL in all the wells of the second column. This was repeated across all columns of the 96-well plate. This was calculated to allow for approximately 1 cell and 3 cells to be seeded into all wells of the last column and second-last column. The wells were monitored to ensure a single-cell

derived colony was formed over a period of 2-4 months and cells from the colony were eventually transferred into 24-well, 12-well and 6-well plates, eventually being transferred into a 75cm^2 vent-capped flask.

2.2 Western blotting

Approximately 30,000 cells were seeded into a 6-well plate followed by overnight incubation. Untreated control cells were harvested when cells reached a confluency of approximately 70%. Treated cells were exposed to 5Gy radiotherapy and 1000uM TMZ 24 hours after seeding (100uM and 500uM TMZ). After the defined incubation of 7 days (unless otherwise stated) cells were harvested. Cells were lysed for 15 min with lysis buffer (50mM Tris [pH 7.4], 150mM NaCl, 1% Triton-X-100, 50mM NaF, 2mM $MgCl_2$, 1mM Na_3VO_4, protease inhibitor mix (complete mini, Roche) and phosphatase inhibitor (PhosStop, Roche)) on an orbital shaker. The lysate was centrifuged for 20 min at 4^{o}C and 13000 g. A protein estimation kit (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific) was used to achieve a final protein concentration of 1ug/uL. Whole cell lysates were prepared with reducing agent and sample buffer (Invitrogen) and boiled at 95^{o}C for 5 min.

Approximately 20ug of protein was loaded in to the wells of, and separated by, a 10% Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE, Invitrogen) run at 120 volts for 2 hrs. The gel containing the proteins was transferred onto nitro-cellulose membrane at 30 volts for 90 min. The membrane was blocked using skim milk powder for 1 hr followed by being incubated with a primary antibody overnight on an orbital shaker at 4^{o}C.

Membranes were rinsed and washed with Tris-buffered Saline and Tween 20 (TBST) for 3 rounds, each lasting 3-4 minutes. The appropriate secondary antibody diluted to 1:5000 with bovine serum albumin was placed over the membrane and left to incubate for at least 1 hr at room temperature on an orbital shaker. Signal intensity was detected using the ECL chemiluminescence detection kit (GE Healthcare) and x-ray film (Fujifilm). Signal intensity was measured using densitometry with Image J software.

2.3 Cell viability

2.3.1 RT+TMZ experiments

200 cells/well were seeded into 96-well plates and incubated overnight to allow the cells to adhere. The media for the control untreated plates was aspirated and fresh media was re-added. The plate to be treated was exposed to 5Gy radiotherapy and incubated for approximately 30 min. Subsequently, media was aspirated and fresh media with TMZ at the appropriate concentration was re-added into each well followed by incubation of the plate for the designated time period. After a designated incubation period CellTiter-Glo Luminescent Cell Viability assay (Promega) was used according to the manufacturer's instructions. 40uL of 1x CellTiter-Glo was added into each well and plates were left to incubate for 10-15 min at room temperature. 30uL of the lysate was transferred into a microplate reader, followed by reading of the microplate by a GLOMAX luminometer. The luminescent signal was indicative of the amount of ATP generated in each well and assumed to correlate with number of viable cells. The measurement of the untreated control plate was set at 100% in order to normalise the viability measurement.

2.3.2 Tyrosine kinase inhibitors

The protocol for the cell viability studies with TKIs are the same as found in 2.4.1 except that 1uM of a TKI was used for each experiment.

2.4 Quantitative real-time polymerase chain reaction (qRT-PCR)

Approximately 100,000 cells were seeded into each well of a 6-well plate and incubated overnight. The media was aspirated and fresh media was pipette into the well. In cases when cells are to be treated, the appropriate amount of an agent was pipette into the well to achieve a designated concentration and left to incubate for a designated time period. The RNeasy Mini Kit (Qiagen) was used according to the manufacturer's instructions to isolate total RNA. To verify the high quality of RNA and quantify the amount of RNA that has been extracted the NanoDrop ND-1000 (NanoDrop Technologies) was used. If the A_{260}/A_{280} and A_{260}/A_{230} ration was approximately 1.8-2.2 the RNA was considered to be of sufficient quality.

Reverse transcription (RT) was conducted using the High Capacity RNA-to-cDNA Kit (Applied Biosystems) according to the manufacturer's instructions; an equal amount of RNA was used for the RT to ensure no biases were introduced during the RT process. A maximum of 2ug of RNA was used and RT was conducted using the Thermocycler (Perkin Elmer) and PCR was for 1 cycle at 37^{o}C for 60 min, followed by 1 cycle of 95^{o}C for 5 min after which cDNA product was stored at -80^{o}C. All RNA samples were stored at -80^{o}C.

qPCR was initiated by forming a Master Mix containing 5uL Taqman Fast Advanced Master Mix (Applied Biosystems), 0.5uL of primer, 3.5uL nuclease-free water and 1uL of cDNA, for a total volume of 10uL which was pipette into a well of a 384-well plate in duplicate (Applied Biosystems). The 384-well plate was read using the ViiA 7 Real-Time PCR system (Applied Biosystems). The qPCR run was set as the following: an incubation period lasting 2 min at 50^{o}C; a polymerase activation period for 20 seconds at 95^{o}C; 40 cycles of PCR split with 1 second at 95^{o}C to denature the cDNA and 20 seconds at 60^{o}C annealing period. The internal controls used were GAPDH transcripts. The $2^{-\Delta\Delta CT}$ method was used to quantify the gene expression levels.

2.5 Human phospho-receptor tyrosine kinase array

100,000 cells were seeded into a 10cm Petri dish and incubated overnight. The plate designated to be treated was then irradiated with 5Gy and incubated for approximately 30 min after which the media was removed and cells were exposed to fresh media containing the appropriate concentration of TMZ. The treated plate was then incubated for 7 days. Cells were lysed and protein concentration was quantified (see **2.3**). A concentration of 300ug was used to prepare the cell lysates and the phospho-RTK levels were analysed using the Phospho-Receptor Tyrosine Kinase Array Kit (R&D Systems) according to the manufacturer's instructions. Signal intensity was detected and measured as with **2.3**.

2.6 microRNA transfection

The miR-221 mimic (cat# 4464066), miR-221 inhibitor (cat# 4464084), miRNA inhibitor negative control (cat# 4464076) and miRNA mimic negative control (cat# 4464058) were purchased from Ambion, USA, and prepared according to manufacturer's protocol. A final concentration of 25nM of miRNA mimic, inhibitor and both controls was used for the transfection. A 3:1 volume FuGENE (Promega):mimic/inhibitor ratio was chosen to be

optimal. 30,000 cells were seeded into each well of a 6-well plate with media containing the transfection agent and miRNA mimic/inhibitor. The next step was determined according to the experiment being conducted.

To determine whether miR-221 regulates EGFR expression, the cells were then incubated for 48 hrs before qRT-PCR **(**see **2.5)** was conducted. To determine whether increased miR-221 expression mediates treatment resistance, the 6-well plate designated as the plate to be treated was irradiated and incubated for 30 min. The media of both the untreated plate and treated plate was removed. TMZ-free media was pipetted into each well of the plate designated as the untreated plate and TMZ-containing media was pipetted into the wells designated as the treated plate. TMZ was administered at 1000uM and radiotherapy dose was 5Gy. A cell viability assay **(**see **2.4)** was conducted 1, 3 or 7 days after treatment.

2.7 *In vitro* wound healing assay

Approximately 100,000 cells were seeded into a 10 cm petri dish and incubated until 95-100% confluency was reached after which mitomycin was added into the dish containing media and cells at a final concentration of 10uM. Scratches across the dish were made with a p200 pipette tip and fresh Opti-MEM media (Thermo Fisher Scientific, MA, USA) was then replaced to remove debris before the dish was incubated. Phase-contrast images were taken at 0 and 24 hrs after the scratch. To process and acquire images, an inverted microscope (IX50, Olympus) and Leica Application Suite (LAS v4.5) were employed and ImageJ was used to quantify the rate of wound healing.

2.8 Patient survival analysis (RMH cohort)

RNA was first extracted from glioblastoma samples on formalin-fixed, paraffin-embedded (FFPE) slides by using PureLink FFPE Total RNA Extraction Kit (Invitrogen, cat# KI560-02) and the manufacturer's instructions were followed, including performing the DNA digestion step prior to reverse transcription. The RNA reverse-transcription and quantification was conducted as previously mentioned in section **2.5**. To evaluate the EGFR fold-change at recurrence, 'ΔCT recurrent – ΔCT primary' was calculated to obtain a ΔΔCT and the $2^{-\Delta\Delta CT}$ used to calculate the difference found in the recurrent tumour sample relative to primary tumour sample. GAPDH was used as an internal control. Similarly, to evaluate the miR-221 fold-change at recurrence, 'ΔCT recurrent – ΔCT primary' was calculated to obtain a ΔΔCT

and the $2^{-\Delta\Delta CT}$ used to calculate the difference found in the recurrent tumour sample relative to primary tumour sample. RNU6B was used as an internal control. An expression change of less than 30% was considered to have no change in gene expression.

To assess the correlation between EGFR and miR-221 expression using primary patient tumour samples the ΔCT value for EGFR and miR-221 was first calculated for each patient. These values were then plotted on a scatter plot and a Spearman r analysis was used to assess the degree of correlation and significance. The Spearman was appropriate because two out of the three tests - KS, Shapiro-Wilk and D'Agostino and Pearson omnibus - indicated that the data passed for normality.

Survival times were already available in our databases and previously were obtained from clinicians at the Royal Melbourne Hospital. Patients that were considered to have high and low EGFR or miR-221 expression were stratified; no patient with less than 30% expression difference between primary and recurrent was selected. A Kaplan Meier plot analysis was conducted using GraphPad Prism.

2.9 Bioinformatics

2.9.1 OncoLnc (TCGA)

TCGA gene expression data was obtained from using the OncoLnc database (www.oncolnc.org). For a given gene 'X', the gene ID was entered and 'GBM' was selected. Patients belonging to either the lower and upper 25^{th} or 35^{th} percentiles were chosen for the analysis.

2.9.2 MicroRNA target prediction

Three microRNA target prediction programs were used: TargetScan (www.targetscan.org), microRNA.org and miRDB (www.mirdb.org). For the TargetScan analysis the human species was selected before submitting the mRNA transcript of interest. All other parameters were default.

2.10 Nanostring

To determine the miRNA expression in commercial glioblastoma cell lines treated with radiotherapy and TMZ, nanostring was employed. Cell lines U87, U87-vIII, LN229, U251 and

U138 were seeded into a 150mm x 15mm petri dish (Corning Inc.) and the dishes designated to be the untreated control were incubated until 100% confluency was reached. Dishes designated to be treated were irradiated with 5Gy before incubation for 30 min followed by TMZ administration at 1000uM. The treated dishes were incubated for 7 days. All plates were then given to Dr. Rachel Koldej based at the ACRF Translational Research Laboratory, Royal Melbourne Hospital for nanostring analysis. The employed raw nanostring data used in this thesis was generated in 2014.

2.11 Statistical analysis

The statistical analyses for all western blots, qRT-PCR and cell viability assays was conducted with an unpaired, two-tail Student's t-test was used to test for significance and a minimum threshold of $p<0.05$ was chosen to determine significance. The following asterisks were used to indicate level of significance: * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$. The survival analyses from OncoLnc used a log-rank t-test to determine significance and data was displayed on a Kaplan-Meier plot. A Grubbs' test with the alpha value set at 0.05 was used to determine outliers for all analyses. For the survival analyses from the RMH recurrent cohort, both log-rank t-test and Gehan-Breslow-Wilcoxon test were used and the p-values are shown.

Table 2-1: List of all cell lines used

Cell line	Commercial/Primary/Recurrent	SOURCE
U87	Commercial	ATCC
U118	Commercial	ATCC
U138	Commercial	ATCC
LN229	Commercial	ATCC
U251	Commercial	ATCC
#4	Primary	RMH
#15	Primary	RMH
#20	Primary	RMH
#28	Recurrent	RMH
#35	Recurrent	RMH
#39	Primary	RMH
#41	Primary	RMH

ATCC = American Type Culture Collection; RMH = Royal Melbourne Hospital.

CHAPTER 3:
IDENTIFYING SIGNALLING MOLECULES AS BIOMARKERS FOR TREATMENT RESISTANCE

3.1 Introduction

Despite aggressive therapy glioblastoma patient survival time remains dismal and the current treatment regimen, after the addition of TMZ, only marginally improves the survival time achieved with radiotherapy alone. Key contributors to the observed poor survival are molecular mechanisms that confer resistance to therapy and tumour recurrence as discussed in Chapter 1. In fact, it has been reported that recurrence rates can be as high as between 72-83% in studies with a median follow-up of 17-19 months for patients treated with a TMZ-based regimen [729, 730]. Re-treatment with TMZ is an option upon recurrence with some benefit observed in clinical trials; however, this benefit may only be obtained in selected patients with MGMT-methylation and there is debate whether there is any reason for relapsed patients to be re-treated with TMZ [258, 731]. Clearly, other strategies and options are required to be put forth.

RTKs have been implicated as oncogenic drivers due to their collective role in promoting and deregulating proliferation, invasion, migration and cell cycle progression. As discussed earlier, several key RTKs have been thoroughly studied for their role in gliomagenesis but clinical trials utilising RTK inhibitors have generated unsuccessful results. This lays in stark contrast to the success of RTK-targeted therapies, such as cetuximab, erlotinib and gefitinib, in other cancers such as metastatic colorectal cancer, pancreatic and non-small cell lung carcinoma [493, 732].

In glioblastoma, focus on the failure of RTK targeted therapies has been multi-fold. Inefficient BBB penetrative ability, redundant RTK activation that allows bypassing of the targeted inhibition of the RTK, intrinsic heterogeneity in RTK signalling networks and deregulated downstream signalling, such as PTEN deletions, have been proposed as possible reasons for the apparent RTK resistance in clinical trials [594]. However, what is missing in these explanations is the critical clinical context that RTK targeted therapy typically resides – the role of radiotherapy and TMZ in conferring RTK therapy resistance. Indeed, writing recently in 2015 for the journal *Nature Reviews*, Furnari et al. correctly states, while commenting on possible mechanisms for RTK therapy resistance, that whether chemotherapy selects for EGFRvIII-positive (and mTORC2) glioblastoma cells is unknown

[501]. This succinctly highlights the gap in the literature regarding the contribution of standard therapy towards resistance to RTK inhibition.

As previously mentioned, EGFR inhibition with erlotinib and gefitinib, and MET inhibition with cabozantinib have yielded poor clinical results for glioblastoma patients. Given that extensive effort has been exerted elucidating the oncogenic role of EGFR and MET in glioblastoma, and EGFR and MET knockdown studies showed promising pre-clinical results, this utter failure requires urgent addressing. We propose that previous pre-clinical models studying resistance mechanisms activated in response to EGFR or MET targeting lack in clinical relevance because the roles of radiotherapy and TMZ treatment in contributing to said resistance were not accounted for.

Aims

Although RTK signalling and related downstream pathways have been extensively studied in glioblastoma, their significance in resistance to standard therapy has been largely overlooked hitherto. Given that RTKs have been found to be key drivers in maintaining tumourogenic signals it can be reasonably hypothesised that persistent activation of a number of RTKs maintain the survival of, and provide a mechanism for, the tumour cells to escape treatment-induced cytotoxic insults. It is possible that these post-treatment activated RTKs sustain the signalling required for key downstream pathways – such as the STAT3, AKT and ERK signalling axes – to sustain survival. Therefore, the aim for this chapter is:

To evaluate the differential expression of RTK and related downstream signalling pathways and identify activated RTKs in resistant glioblastoma cells.

This aim first requires us to generate an *in vitro* model of treatment resistance. This will be followed by analyses of multiple RTKs and downstream signalling pathways that are activated in treatment-resistant glioblastoma cells.

3.2 Results

3.2.1 Optimising treatment conditions and generating an *in vitro* short-term resistant model

In order to determine the ideal length of time to adopt for future studies in the making of an *in vitro* model of treatment resistance, multiple glioblastoma cell lines were exposed to 5Gy irradiation and 1000uM TMZ then incubated for 1-10 days ***(Figure 3-1)***. Cell viability was measured at the specified time by evaluating cell lines #15, #20, #39 and #41 (primary human glioblastoma cell lines), and #28 and #35 (recurrent human glioblastoma cell lines). All cell lines had reduced viability compared to untreated controls at all measured time points except #15 ***(Figure 3-1 A)***, which did not reduce in viability at the Day 1 time point, and #39 ***(Figure 3-1 C)***, which did not reduce in viability at both Day 1 and Day 3 points. Furthermore, all cell lines, except #39 and #41, had significant reduction in cell viability from Day 3 to Day 5. Since there was no significant reduction in viability across all cell lines between cells treated for 7 and 10 days when compared to 5 days, the Day 7 time point was considered to be short-term resistant to therapy. The use of the term 'short-term resistant' for our intent and purpose refers to the population of viable cells that persist after a specific treatment time point. Given that this time point was Day 7 it was decided that all future studies will adopt a treatment regimen of 5Gy irradiation plus 1000uM TMZ before incubation for 7 days, unless stated otherwise. All cell lines had a cell viability reduction of at least 80% at the Day 7 time point.

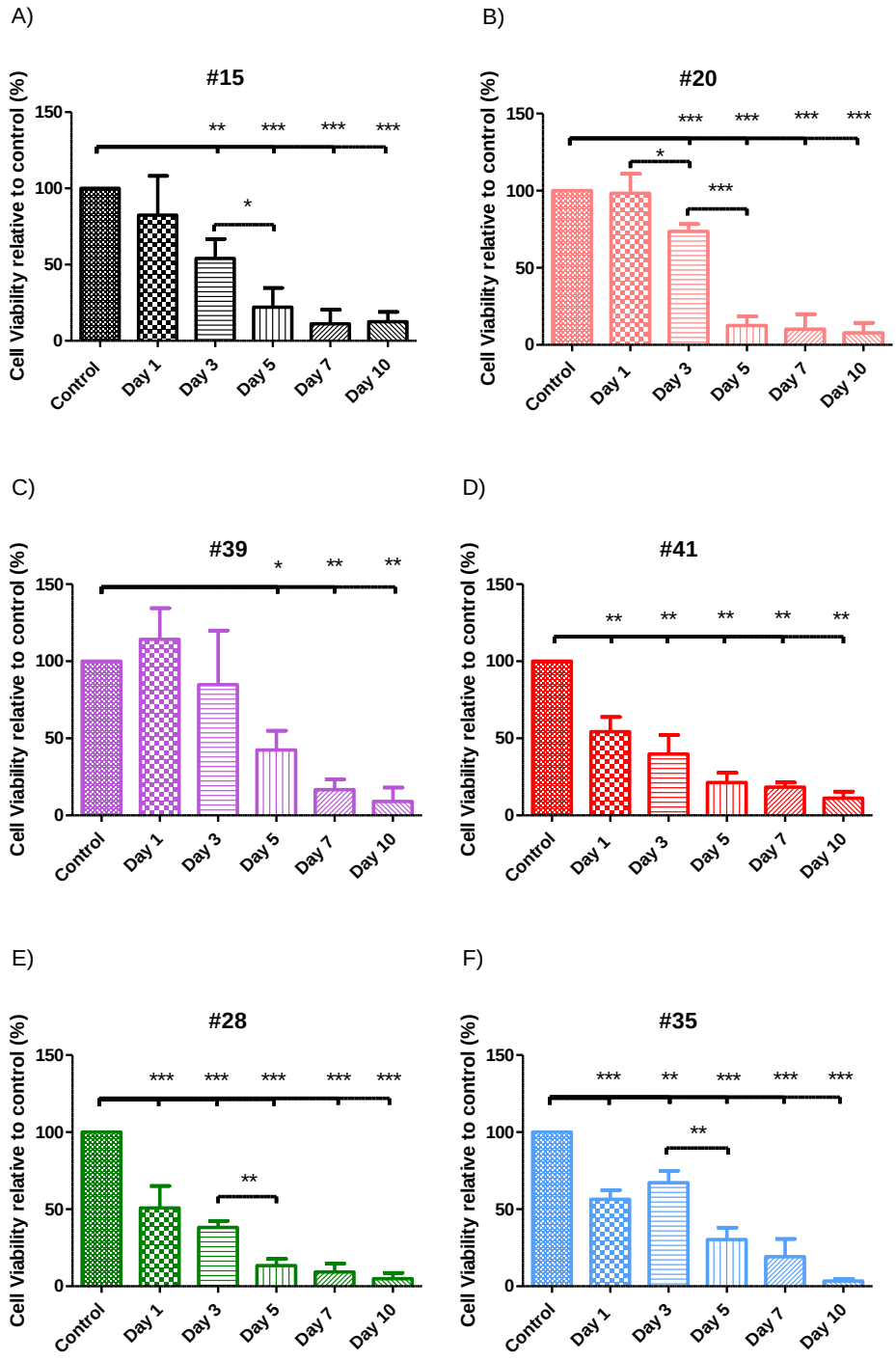


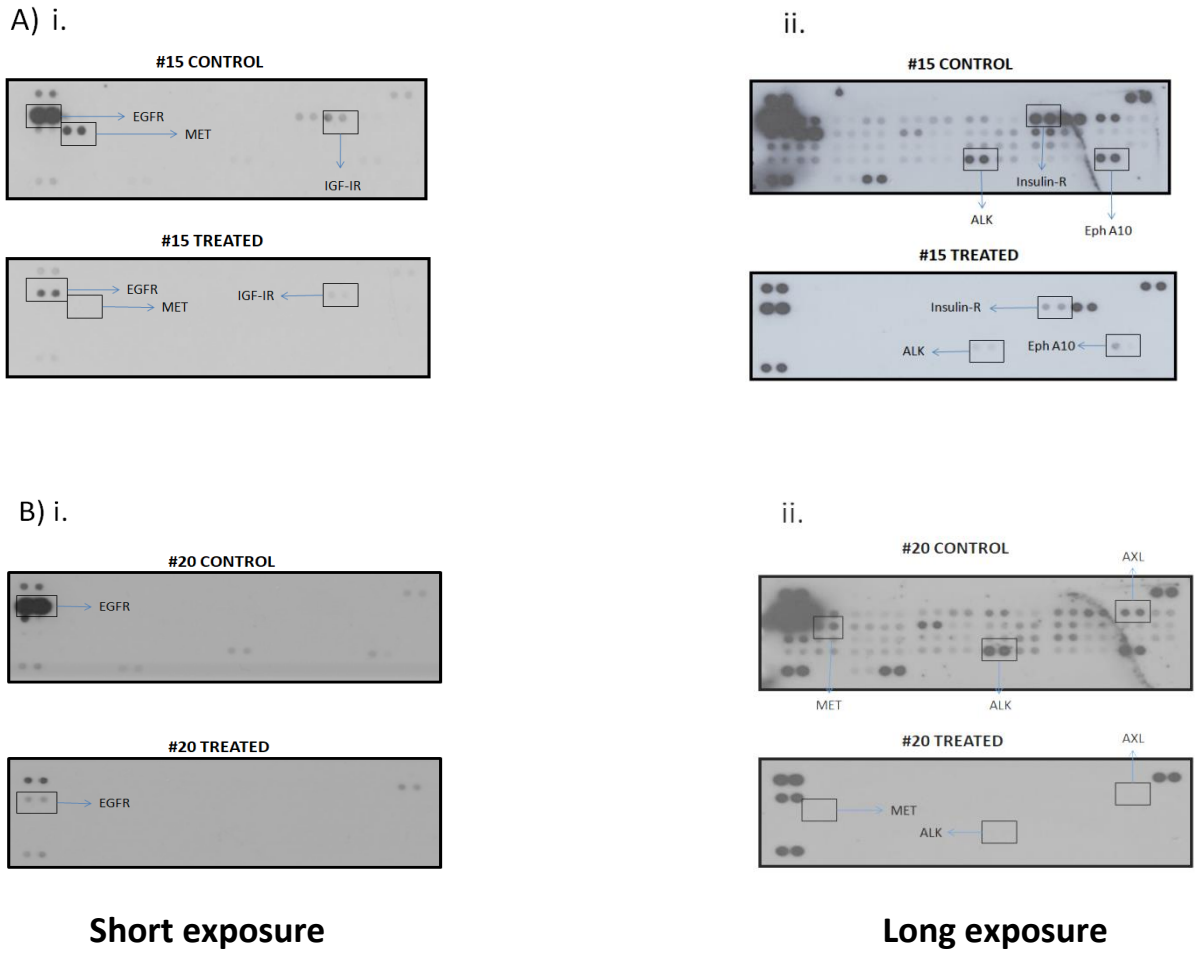
Figure 3-1: Optimisation of treatment protocol. Primary **(A)** #15,**(B)** #20, **(C)** #39 and **(D)** #41 and recurrent **(E)** #28 and **(F)** #35 glioblastoma cell lines were treated with 5Gy irradiation and 1000uM TMZ then incubated for either 1, 3, 5, 7 or 10 days before cell viability was assessed. Values are mean ±SD. N=3. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

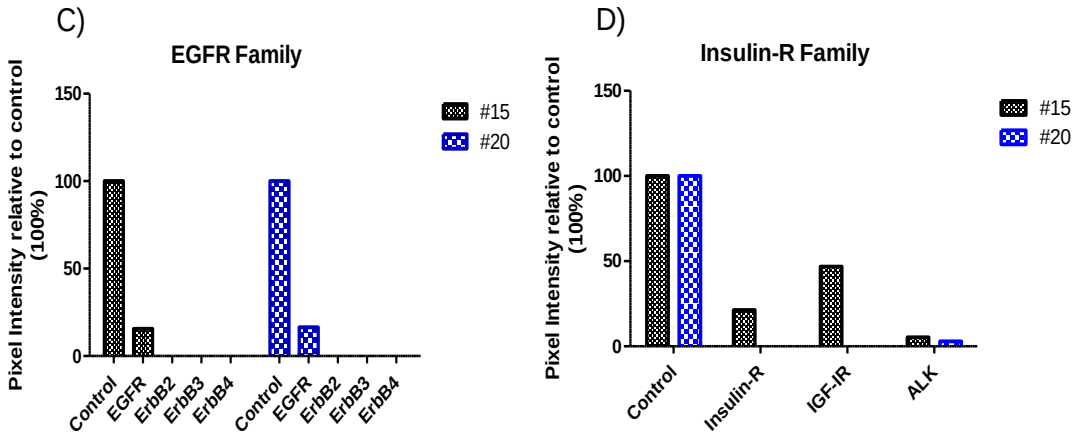
3.2.2 RTK expression is reduced in glioblastoma cell lines after radiotherapy and TMZ treatment

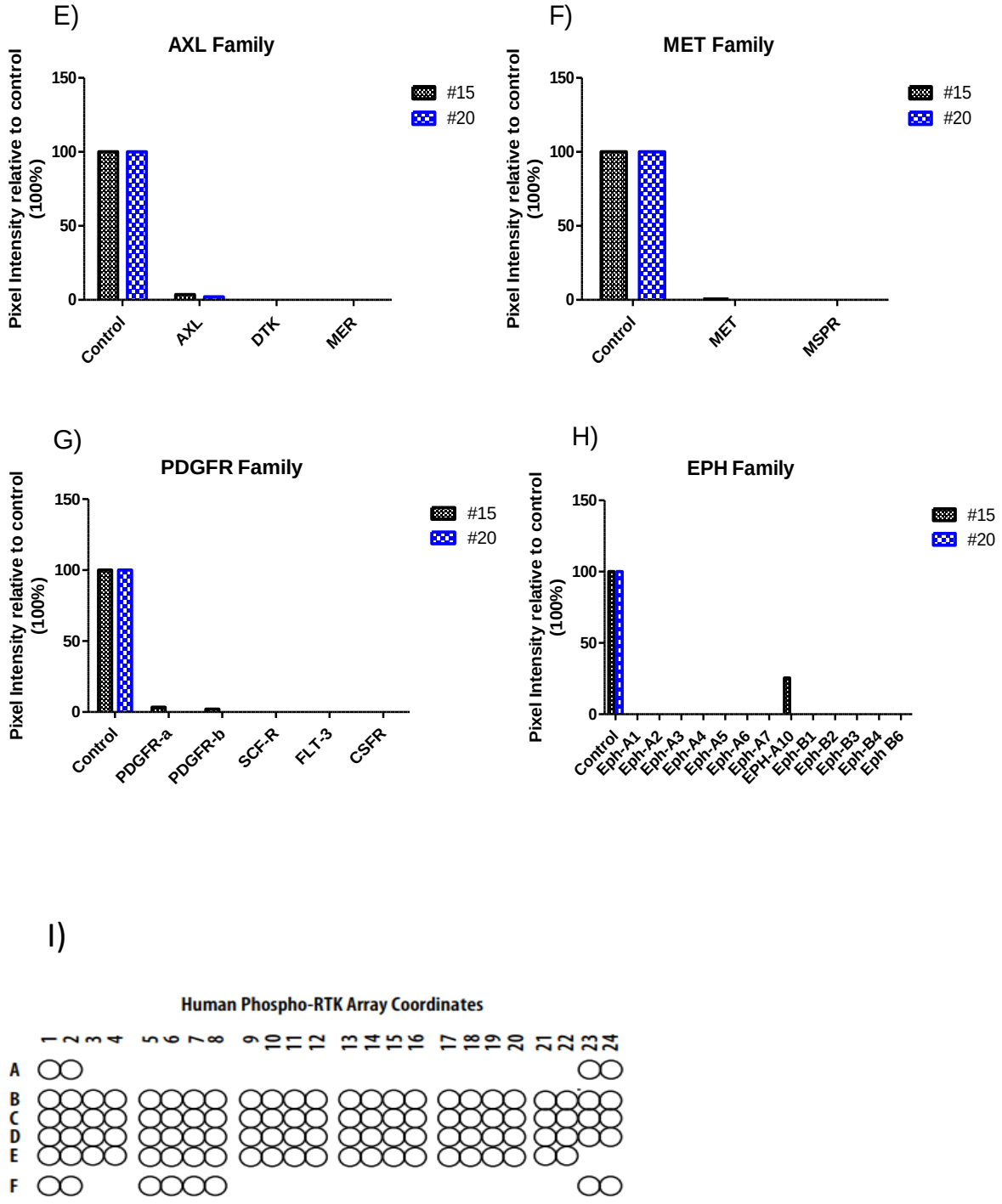
A phospho-RTK array was utilised to determine which RTKs changed in activity after short term treatment with radiotherapy and TMZ compared to control untreated cells. Primary glioblastoma cell lines #15 and #20 were chosen to be treated by 5Gy irradiation and 1000uM TMZ before incubation for 7 days. This array allowed for the observation of the phosphorylation level differences of 49 RTKs in the untreated control cells compared to the treated cells. Surprisingly, both #15 and #20 cells treated with radiotherapy and TMZ had reduced phosphorylation of all RTKs after 7 days **(*Figure 3-2*).** Both #15 and #20 treated cells had undetectable phosphorylated levels of all RTKs except the EGFR, ALK and AXL. The data in which the phospho-RTK levels were reduced in the treated cells such that it is undetectable is not shown. Only treated #15 had detectable levels of Insulin-R, IGF-R, MET, PDGFR family and EphA-10. Out of all the RTK families assessed only the Insulin-R family - consisting of Insulin-R, IGF-R and ALK - had detectable levels of activation in at least one of the cell lines used after treatment ***(Figure 3-2 D)***. Therefore, it was concluded that irradiation and TMZ reduced RTK activation in treated #15 and #20 cells.

Next, to verify our results obtained from the phospho-RTK array, western blot was utilised for assessment of both phosphorylated RTK levels and the total protein expression of RTKs. Given that both EGFR and MET are among two of the most studied and deregulated RTKs in glioblastoma, these two RTKs were chosen for further validation studies with two additional cell lines, the primary human glioblastoma cell line #41 and the established cell line LN229. Western blot verified that phosphorylated levels of the EGFR and MET were reduced in all cell lines treated; although, no detectable pEGFR was observed in treated and control LN229 ***(Figure 3-3 A-D)***. Interestingly, treated #20 pMET levels in the RTK array was undetectable, though western blot analysis showed a pMET reduction of 5.1-fold. The variance is possibly due to the difference in the two methods employed. Unexpectedly, total expression levels of the EGFR and MET were also down-regulated in all treated cells compared to the respective untreated controls ***(Figure 3-3 A-D)***. Furthermore, the MET and EGFR gene expression was down-regulated in both cell line #20 and #41 after treatment compared to untreated controls ***(Figure 3-3 I-J)***. Taken together, our data indicates that RTK activity is

reduced in short-term resistant glioblastoma cell lines through reduction in RTK mRNA expression.





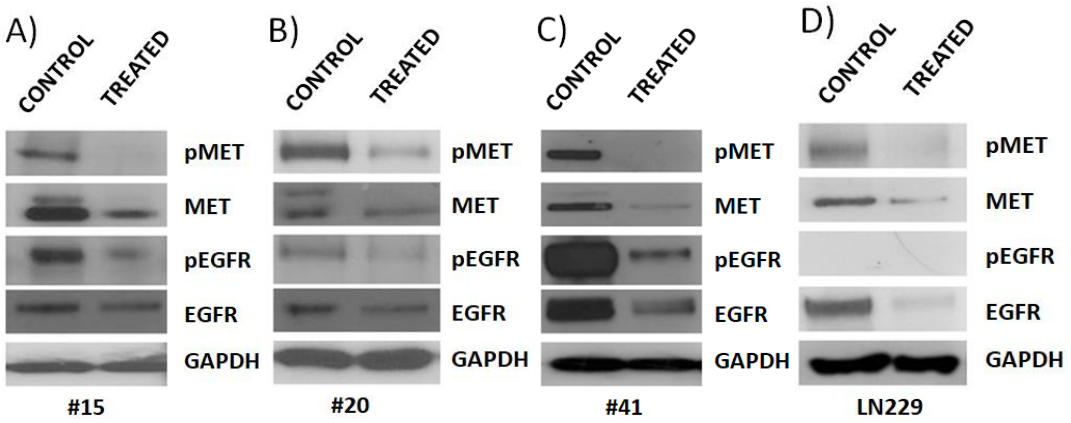

E)
AXL Family
Pixel Intensity relative to control (100%)
150
100
50
0
#15
#20
Control
AXL
DTK
MER
F)
MET Family
Pixel Intensity relative to control (100%)
150
100
50
0
#15
#20
Control
MET
MSPR
G)
PDGFR Family
Pixel Intensity relative to control (100%)
150
100
50
0
#15
#20
Control
PDGFR-a
PDGFR-b
SCF-R
FLT-3
CSFR
H)
EPH Family
Pixel Intensity relative to control (100%)
150
100
50
0
#15
#20
Control
Eph-A1
Eph-A2
Eph-A3
Eph-A4
Eph-A5
Eph-A6
Eph-A7
EPH-A10
Eph-B1
Eph-B2
Eph-B3
Eph-B4
Eph B6

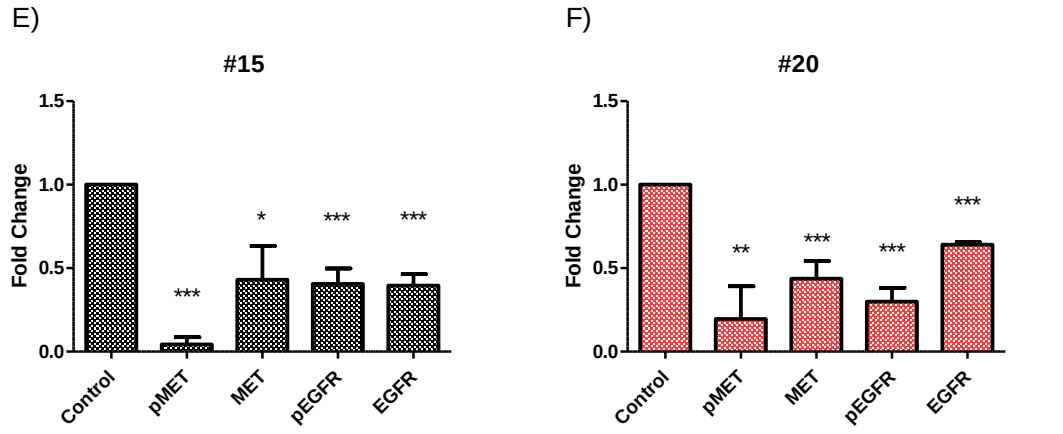
I)

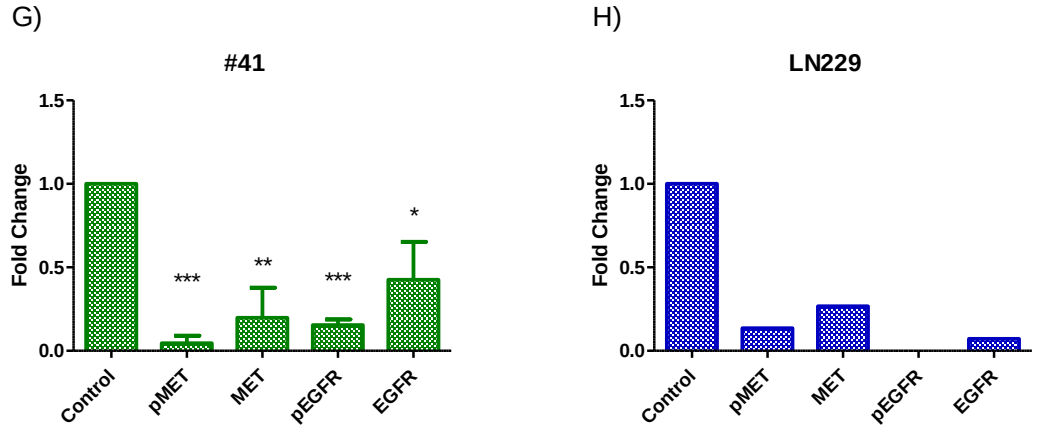
Human Phospho-RTK Array Coordinates
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
A
B
C
D
E
F

Coordinate	Receptor Family	RTK/Control	Coodinate	Receptor Family	RTK/Control
A1, A2	Reference Spots	——	D1, D2	Tie	Tie-2
A23, A24	Reference Spots	——	D3, D4	NGF R	TrkA
B1, B2	EGF R	EGF R	D5, D6	NGF R	TrkB
B3, B4	EGF R	ErbB2	D7, D8	NGF R	TrkC
B5, B6	EGF R	ErbB3	D9, D10	VEGF R	VEGF R1
B7, B8	EGF R	ErbB4	D11, D12	VEGF R	VEGF R2
B9, B10	FGF R	FGF R1	D13, D14	VEGF R	VEGF R3
B11, B12	FGF R	FGF R2α	D15, D16	MuSK	MuSK
B13, B14	FGF R	FGF R3	D17, D18	Eph R	EphA1
B15, B16	FGF R	FGF R4	D19, D20	Eph R	EphA2
B17, B18	Insulin R	Insulin R	D21, D22	Eph R	EphA3
B19, B20	Insulin R	IGF-I R	D23, D24	Eph R	EphA4
B21, B22	Axl	Axl	E1, E2	Eph R	EphA6
B23, B24	Axl	Dtk	E3, E4	Eph R	EphA7
C1, C2	Axl	Mer	E5, E6	Eph R	EphB1
C3, C4	HGF R	HGF R	E7, E8	Eph R	EphB2
C5, C6	HGF R	MSP R	E9, E10	Eph R	EphB4
C7, C8	PDGF R	PDGF Rα	E11, E12	Eph R	EphB6
C9, C10	PDGF R	PDGF Rβ	E13, E14	Insulin R	ALK
C11, C12	PDGF R	SCF R	E15, E16	——	DDR1
C13, C14	PDGF R	Flt-3	E17, E18	——	DDR2
C15, C16	PDGF R	M-CSF R	E19, E20	Eph R	EphA5
C17, C18	RET	c-Ret	E21, E22	Eph R	EphA10
C19, C20	ROR	ROR1	F1, F2	Reference Spots	——
C21, C22	ROR	ROR2	F5, F6	Eph R	EphB3
C23, C24	Tie	Tie-1	F7, F8	——	RYK
			F23, F24	Control (-)	PBS

Figure 3-2: p-RTK array showed RTK activation was decreased after treatment. Glioblastoma cell lines #15 and #20 were treated with irradiation and TMZ before RTK activity levels of 49 RTKs were assessed. Cell lines **(A)** #15 and **(B)** #20 were treated with 5Gy irradiation and 1000uM TMZ before RTK array was conducted. For both cell lines **(i)** show low exposure and **(ii)** shows high exposure scans. Densitometry analysis was conducted on all 49 RTKs and shown as **(C)** EGFR family **(D)** Insulin-R family **(E)** AXL family **(F)** MET family **(G)** PDGFR family **(H)** EPH family. The pixel intensity of the untreated array was set at 100% (and marked as control) and all kinase intensity values were normalised in the treated arrays. The target map is also shown in **(I)** and the table was taken from the product data sheet. N=1.


A)
CONTROL
TREATED
pMET
MET
pEGFR
EGFR
GAPDH
#15
B)
CONTROL
TREATED
pMET
MET
pEGFR
EGFR
GAPDH
#20
C)
CONTROL
TREATED
pMET
MET
pEGFR
EGFR
GAPDH
#41
D)
CONTROL
TREATED
pMET
MET
pEGFR
EGFR
GAPDH
LN229


E)
#15
Fold Change
1.5
1.0
0.5
0.0
***
*
***
***
Control
pMET
MET
pEGFR
EGFR
F)
#20
Fold Change
1.5
1.0
0.5
0.0
**
***
***
***
Control
pMET
MET
pEGFR
EGFR


G)
#41
Fold Change
1.5
1.0
0.5
0.0
***
**
***
*
Control
pMET
MET
pEGFR
EGFR
H)
LN229
Fold Change
1.5
1.0
0.5
0.0
Control
pMET
MET
pEGFR
EGFR

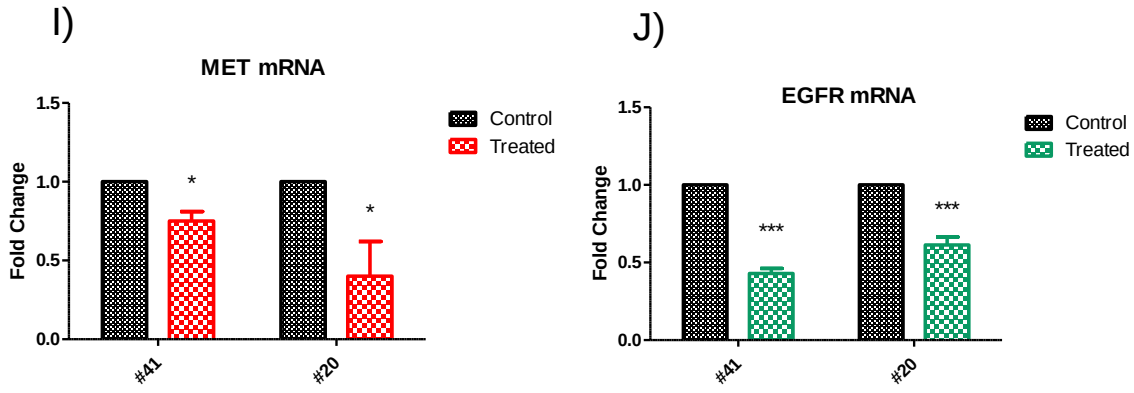
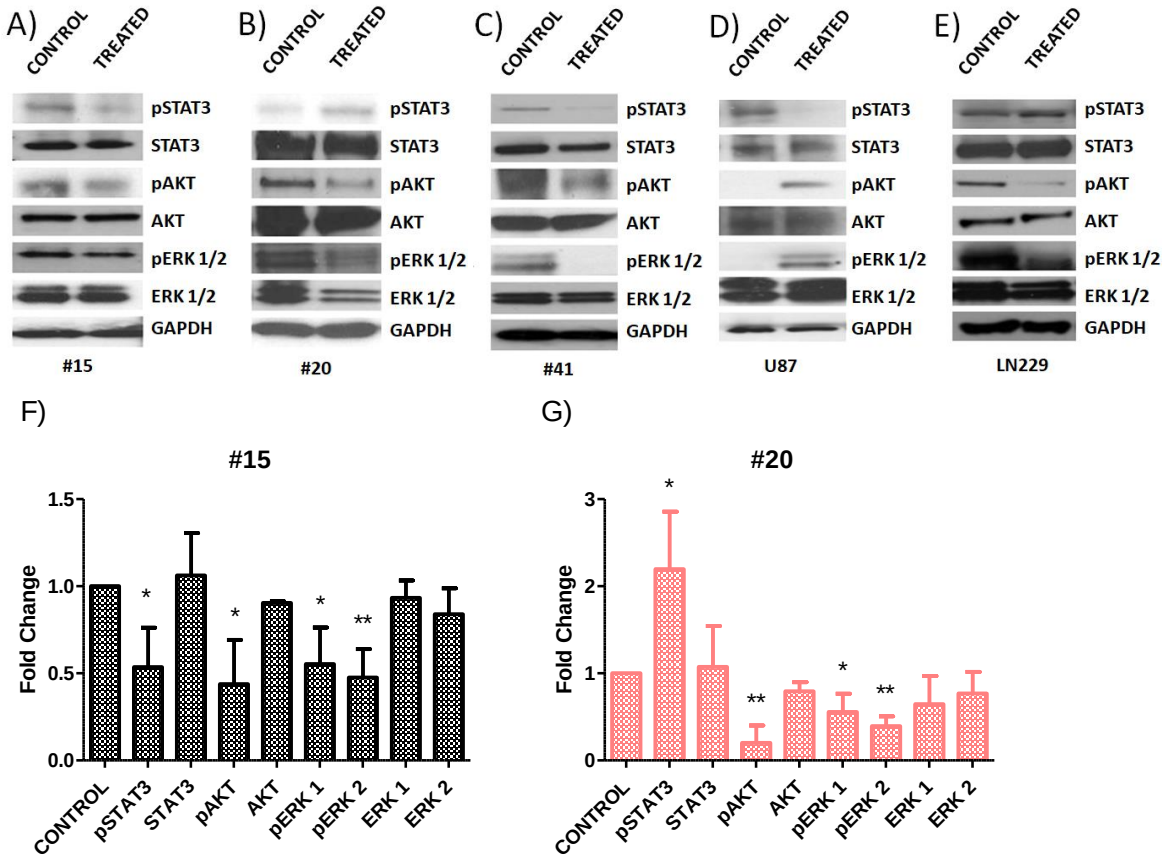

I)
MET mRNA
Fold Change
1.5
1.0
0.5
0.0
*
*
#41
#20
Control
Treated
J)
EGFR mRNA
Fold Change
1.5
1.0
0.5
0.0
***
***
#41
#20
Control
Treated

Figure 3-3: Validation of RTK array showed that MET and EGFR protein and gene expression is reduced after treatment. Primary glioblastoma cell lines **(A)** #15, **(B)** #20 and **(C)** #41 plus established cell line **(D)** LN229 were treated with 5Gy irradiation and 1000uM TMZ. pEGFR, EGFR, pMET and MET levels were analysed after 7 days. Band intensity was analysed and densitometries for **(E)** #15, **(F)** #20, **(G)** #41 and **(H)** LN229 are shown. The **(I)** MET and **(J)** EGFR mRNA expression levels decreased in cell lines #20 and #41 after treatment with irradiation and TMZ and incubation for 7 days. All values are mean ± SD; N=3.* $p<0.05$; ** $p<0.01$; *** $p<0.001$.

3.2.3 Downstream signalling pathways can persist after standard therapy regardless of down-regulated RTK gene expression

EGFR and MET activation increases downstream signalling pathways, including the STAT3, AKT and ERK pathways. As we have demonstrated that treatment with irradiation and TMZ led to reduced RTK activation and protein expression, we consequently hypothesised that the activation of downstream signalling molecules would also be reduced. To evaluate this, cell line #15, #20, #41, U87 and LN229 were treated (as previously) and both the phosphorylation and protein expression levels of both untreated and treated cells were analysed after 7 days. In all cell lines, except U87, pAKT and pERK levels were reduced after treatment compared to untreated controls **(*Figure 3-4*).** As for pSTAT3 levels, treated cells had reduced levels except for #20 and U87 which had significantly up-regulated STAT3 activation. Treatment did not lead to significant changes in the protein expression of any of the three signalling molecules assessed. This data suggests that glioblastoma cell lines that undergo treatment with radiotherapy and TMZ maintain persistent downstream signalling pathway activation despite lower RTK activity. Furthermore, which pathways are activated in short-term resistance cells is cell line-specific.



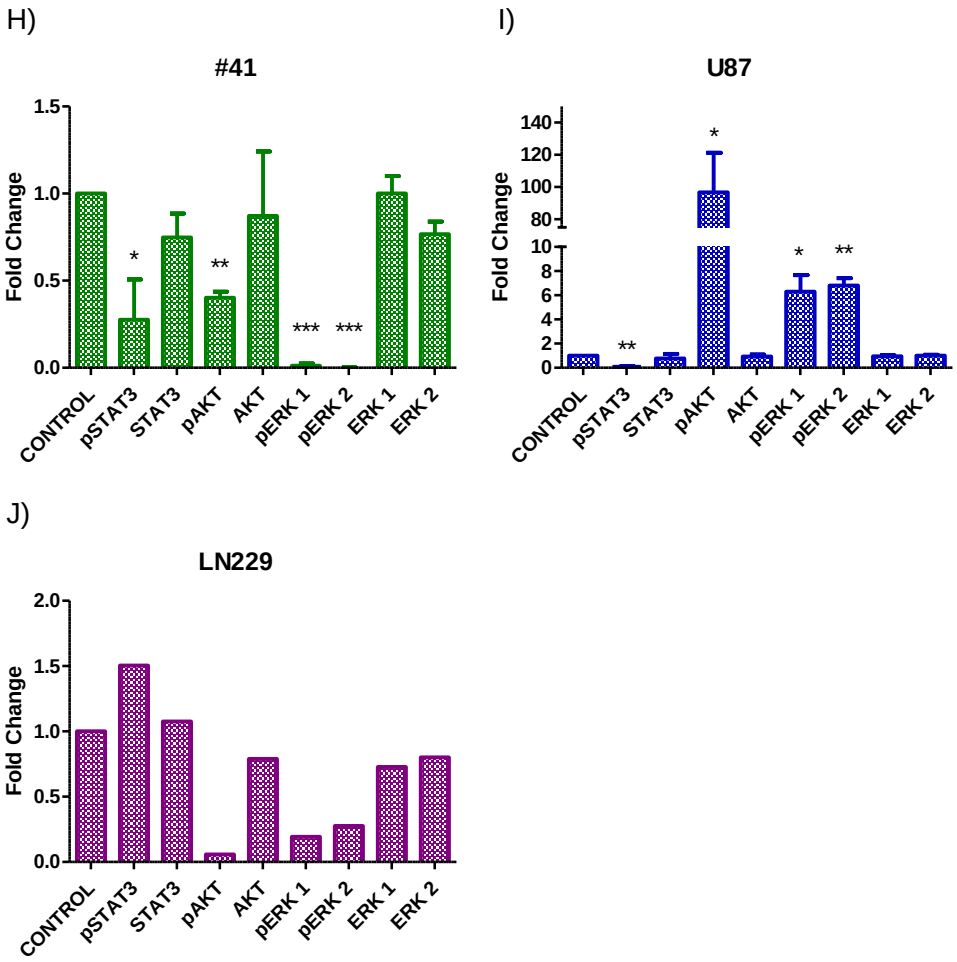


Figure 3-4: Differential downstream signalling pathway activation in glioblastoma cells after treatment. Cell lines (**A)** #15, **(B)** #20, **(C)** #41, **(D)** U87 and **(E)** LN229 were treated irradiation and TMZ before levels of signalling proteins of three key signalling pathways: STAT3, AKT and ERK. The levels of STAT3, AKT and ERK 1/2 activity differed across all cell lines. All cell lines had reduced STAT3 activation after treatment except #20 and LN229. All cell lines had reduced AKT and ERK 1/2 activation after treatment except U87. Protein fold change was assessed and densitometries for **(F)** #15, **(G)** #20, **(H)** #41, **(I)** U87 and **(J)** LN229 are also shown. Values mean ± SD. All are n=3: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.2.4 Changes in RTK expression and signalling pathway activation is both time- and dose-dependent

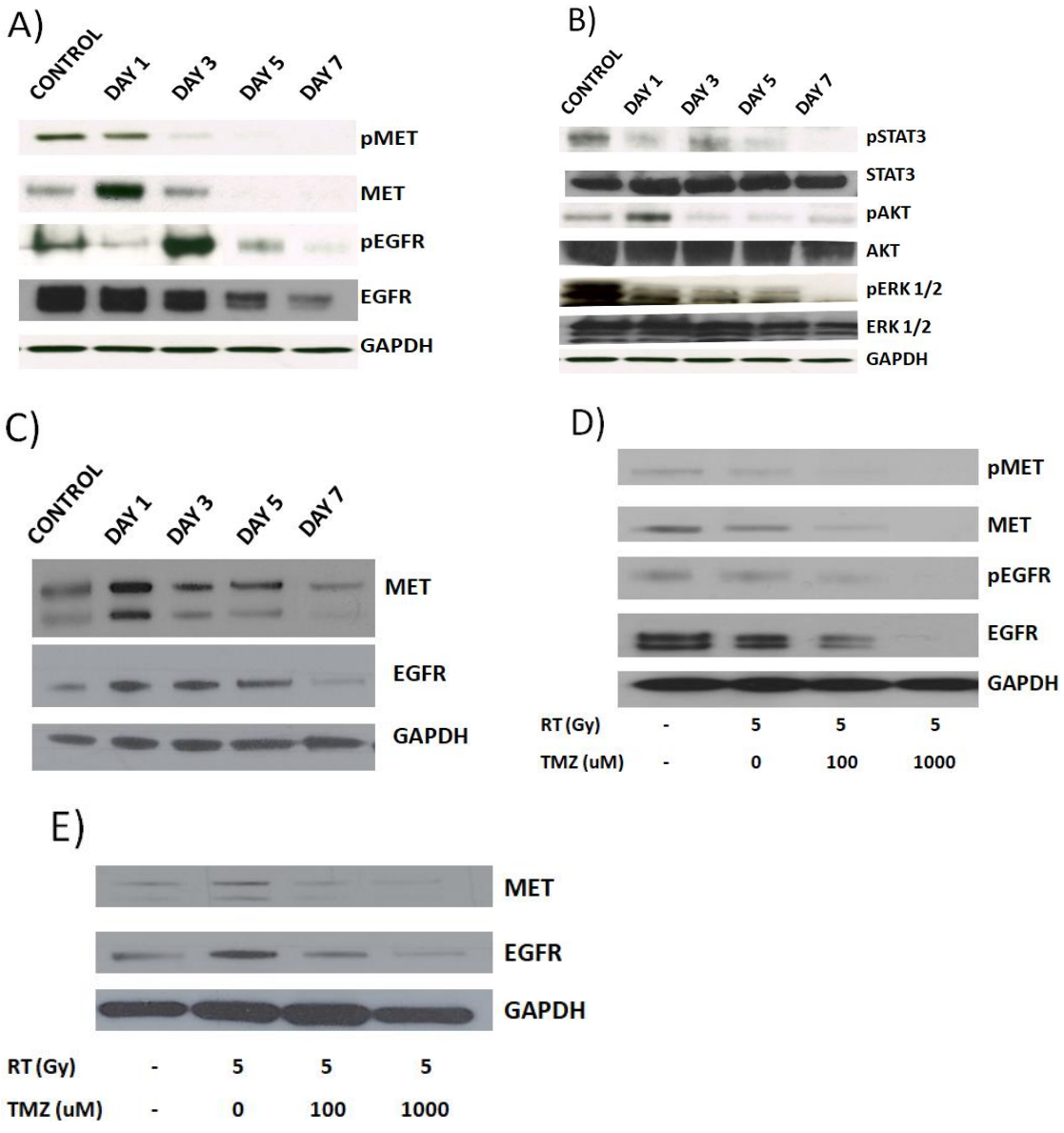
To determine when these changes in phoshphorylated and total protein expression occurred within the 7 day treatment period, time- and dose-dependent studies were performed. Both cell line #41 and #20 were exposed to 5Gy irradiation and 1000uM TMZ for either 1, 3, 5 or 7 days. The reduction in pMET and total EGFR levels in treated #41 was time-dependent. pMET levels were significantly reduced from Day 1 onwards and total EGFR expression was only significantly reduced at Day 5, although a gradual reduction was observed from Day 1 onwards. **(*Figure 3-5 A*).** MET protein expression was significantly increased in treated #41 at Day 1 before MET expression levels were reduced back to the untreated control levels. At Day 5 and Day 7 post-treatment cell line #41 MET expression was significantly lower than the untreated control. Interestingly, treatment reduced #41 pEGFR levels at Day 1 before a significant up-regulation at the Day 3 time point followed by reductions at Day 5 and 7. A similar pattern of activation was observed with pSTAT while pAKT levels increased at Day 1 after treatment before a decrease **(*Figure 3-5 B*).** ERK 1/2 activation gradually reduced from the onset of treatment at Day 1 to Day 7.

Similar to #41, treated #20 cells had significantly higher MET protein expression levels compared to untreated control before a reduction back to the levels observed in the untreated cells **(*Figure 3-5 C*).** EGFR protein expression levels were significantly higher in the treated #20 cells compared to the untreated control from Day 1 till Day 5 – only at the Day 7 time point was EGFR protein expression significantly reduced compared to the untreated control.

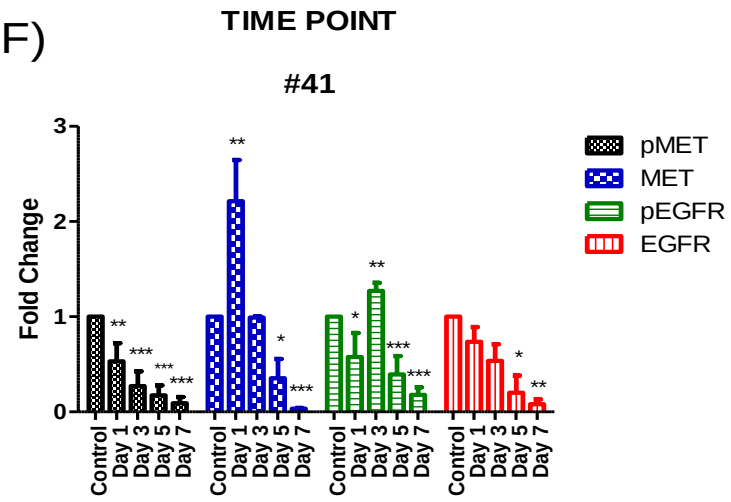
Next, for the dose-dependent study both cell lines underwent 5Gy radiotherapy only or in addition to either 100uM or 1000uM of TMZ, followed by an incubation period of 7 days. The reduction in MET activation and expression was dose-dependent in treated #41 cells; although radiotherapy alone led to a reduction in pMET and MET levels this was furthered with the addition of TMZ **(*Figure 3-5 D*).** Radiotherapy alone induced a significant increase in pEGFR with #41, but the supplementation with 100uM TMZ led to a reduction such that pEGFR was not significantly altered compared to the untreated control. Only administrating radiotherapy with 1000uM TMZ was pEGFR levels reduced significantly compared to the

untreated control. Lastly, EGFR protein expression was only reduced when radiotherapy was supplemented with TMZ.

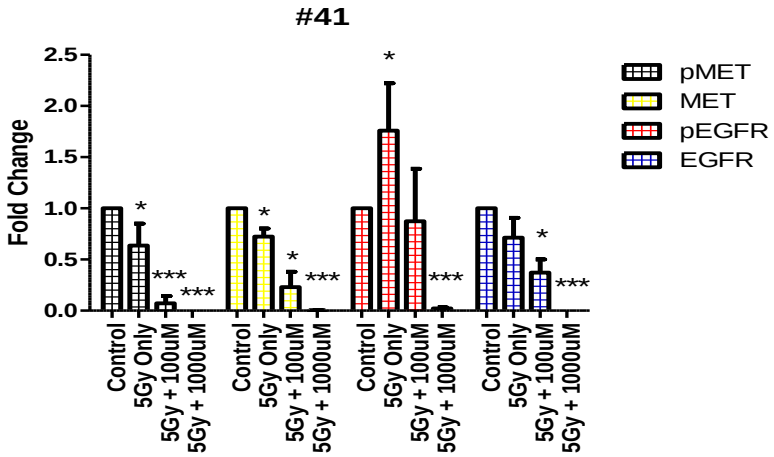
Both MET and EGFR protein expression significantly increased in #20 after treatment with radiotherapy alone; however supplementing radiotherapy with 100uM TMZ led to no change in MET and EGFR protein expression compared to the untreated control **(*Figure 3-5 E*).** Only radiotherapy plus 1000uM TMZ led to a significantly reduced MET and EGFR protein expression. To summarise, the change in protein expression and activation levels of MET and EGFR is both time- and TMZ dose-dependent in the selected glioblastoma cell lines.



F)


TIME POINT
#41
Fold Change
3
2
1
0
pMET
MET
pEGFR
EGFR
Control
Day 1
Day 3
Day 5
Day 7

G)


#41
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
pMET
MET
pEGFR
EGFR
Control
5Gy Only
5Gy + 100uM
5Gy + 1000uM

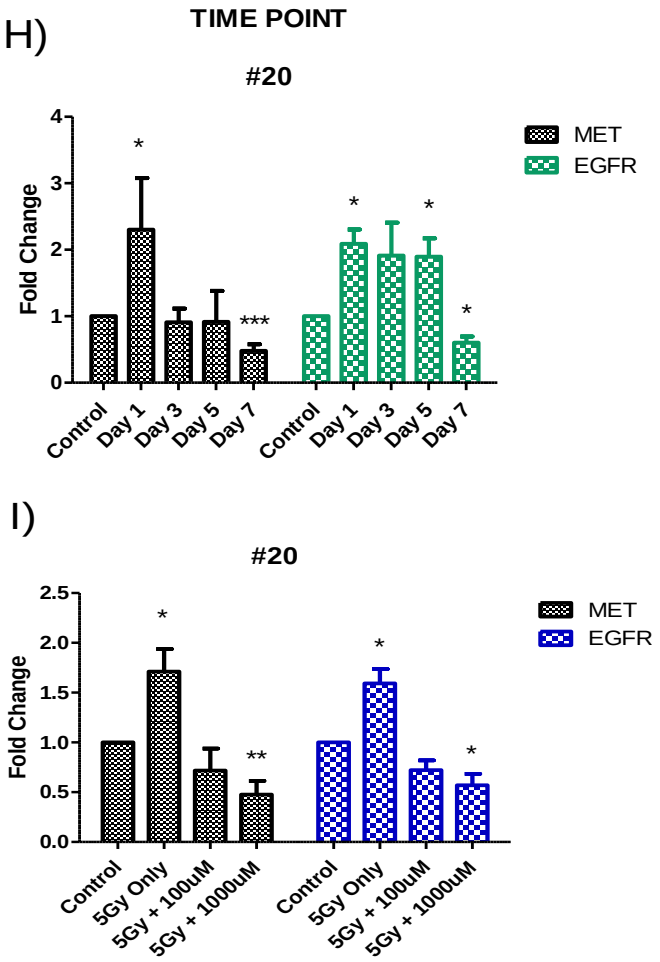


Figure 3-5: Change in MET and EGFR protein expression levels after treatment is both dose and time dependent. Cell line (**A)** #41 was treated with 5Gy irradiation and 1000uM TMZ before MET and EGFR expression was assessed 1, 3, 5 or 7 days after treatment. Next **(B)** #41 was treated with 5Gy irradiation and 1000uM TMZ and downstream signalling pathway activation was assessed at various time points after treatment. **(C)** Blot shows the time point study using cell line #20, only MET and EGFR expression was assessed **(D)** #41 MET and EGFR expression levels were assessed after 5Gy irradiation plus varying concentrations of TMZ. **(E)** Blot shows dose dependent study results using cell line #20, only MET and EGFR expression was assessed. Densitometry for **(F)** #41 time point, **(G)** #41 dose dependent study, **(H)** #20 time point and **(I)** #20 dose dependent are also shown. Values are mean ± SD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.2.5 LC3 is a potential biomarker for treatment resistance in glioblastoma

As EGFR activity has been linked to the induction of autophagy and as our results show that radiotherapy plus TMZ treatment reduces EGFR activity, we next evaluated whether autophagy is induced in treatment resistance. Treated #41 was incubated for 1, 3, 5 or 7 days before LC3 levels were assessed, whereas both treated #20 and #4 were incubated for7 days only **(*Figure 3-6*)**. Radiotherapy plus TMZ induced a significant increase in LC3 levels from Day 1 onwards to Day 7 in #41 **(*Figure 3-6 A*)**. LC3 protein levels peaked at Day 3 and was more than 20-fold higher compared to the untreated #41. The 7-day post-treatment LC3 protein expression in #20 was up-regulated compared to the untreated #20, which showed no detectable band **(*Figure 3-6 B*)**. Similarly, treated #4 cells showed significantly up-regulated LC3 levels compared to the untreated control **(*Figure 3-6 C*)**. Patient clinical data obtained from the TCGA via OncoLnc supported the inverse correlation observed in our short-term resistant cell lines. Specifically, a high EGFR-LC3A and high EGFR-LC3B co-expression had significantly association with favourable survival compared to low EGFR-LC3A and low EGFR-LC3B co-expression, respectively **(*Figure 3-6 F-G*)**. Taken together, autophagy-related gene expression confers poor survival in glioblastoma. Importantly, the treatment-induced down-regulation of EGFR is associated with high levels of the autophagic marker LC3, suggesting that the level of LC3 protein expression is a biomarker for treatment resistance in glioblastoma.

Clinical data was obtained from the TCGA server via OncoLnc, an online database that has stored gene expression from glioma patients. A number of autophagy-related genes were selected for gene expression analysis. Patients were stratified into the top and bottom 30th percentile of expression. It was observed that high ATG9B, WIPI2 and LC3A were significantly associated with poor survival in glioblastoma patients **(*Figure 3-7 A-C*)**. In addition, a high LC3A-WIPI2 signature was significantly associated with poorer survival compared to a low ATG9B-WIPI2 signature **(*Figure 3-7 D*)**. Furthermore, high-LC3A expressing glioblastoma patients had poorer survival rates compared to high-LC3A expressing low grade glioma patients; this was also the case when comparing low-LC3A expressing glioblastoma patients with low-LC3A expressing low grade glioma patients **(*Figure 3-7 E-F*)**.

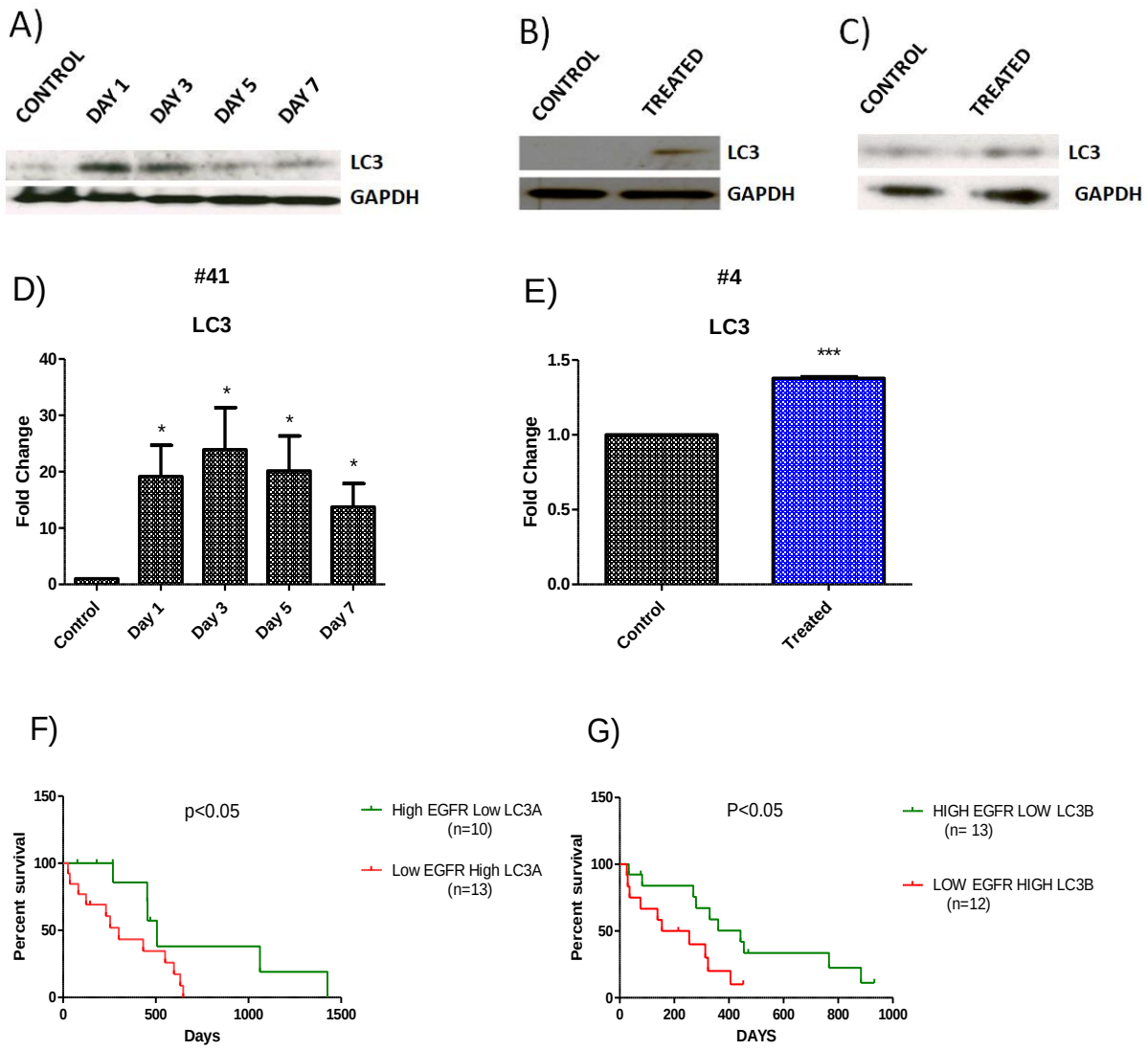


Figure 3-6: LC3 levels increases in response to irradiation and TMZ therapy and inversely correlates with EGFR expression. Cell line **(A)** #41 was treated with irradiation and TMZ before LC3 protein expression was assessed with western blot at various time points. Cell lines **(B)** #20 and **(C)** #4 were treated with irradiation and TMZ and LC3 protein expression was compared to the untreated counterpart 7 days after treatment. LC3 levels increased after treatment in both cell lines. Densitometry analyses for **(D)** #41 and **(E)** #4 is shown. Values are means ± SD. **(F)** Patient data from the TCGA database was obtained. High-EGFR expressing glioblastoma patients were cross-matched with low-LC3A expressing glioblastoma patients and survival rates were compared with low-EGFR and high-LC3A expressing glioblastoma patients. The latter group had significantly worse survival rates compared to the former group. The same type of analysis was conducted with LC3B-expressing glioblastoma patients and shown in **(G)**. High-LC3B/Low-EGFR expressing

glioblastoma patients had significantly worse survival compared to the Low-LC3B/High-EGFR expression patients. * p<0.05; *** p<0.001.

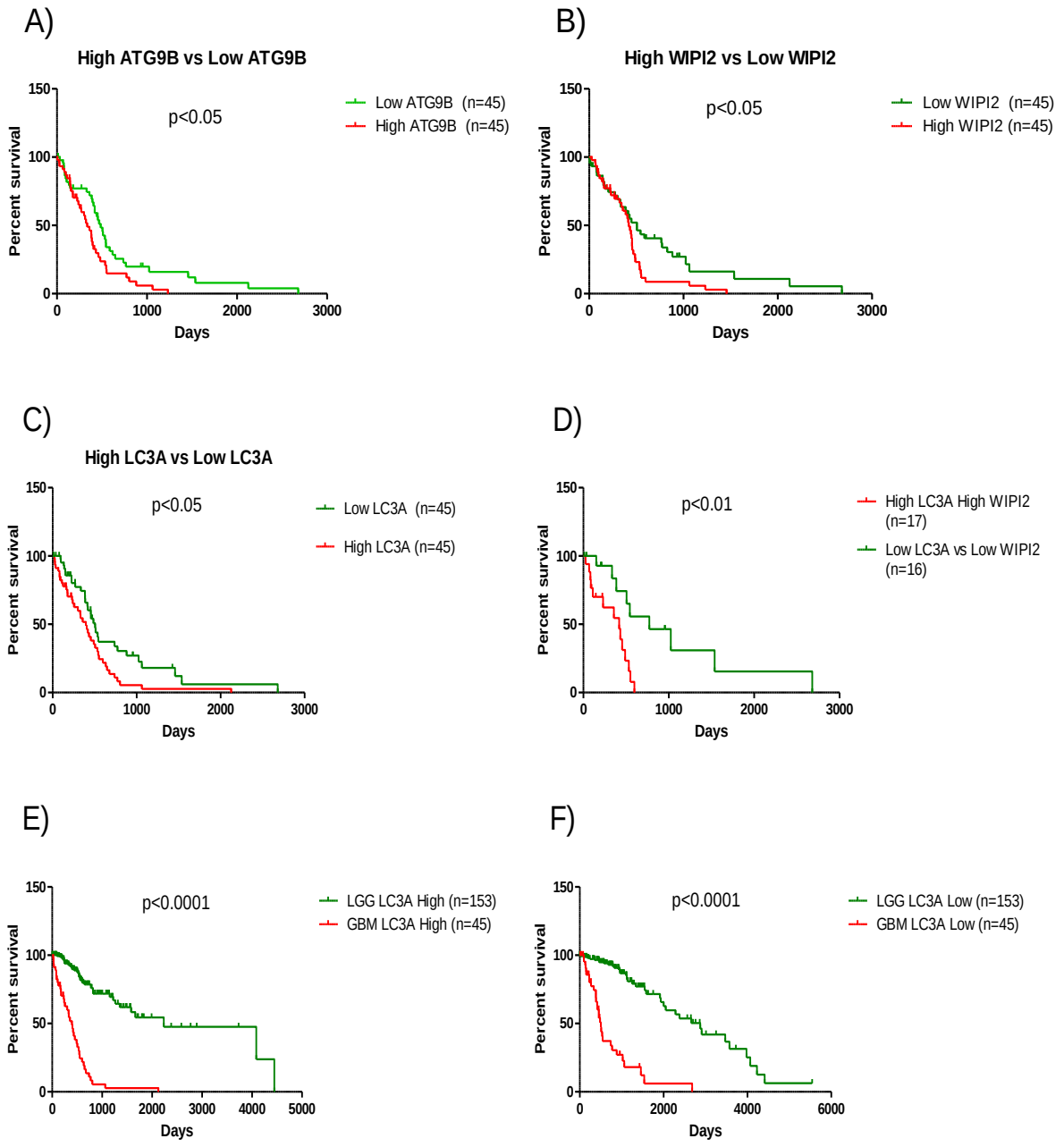


Figure 3-7: Autophagy-related genes are highly expressed in poor surviving glioblastoma patients. Bioinformatics data obtained from the TCGA showed that expression of autophagy-related genes correlates with poor survival. The top and bottom 30th percentile of **(A)** ATG9B, **(B)** WIPI2 and **(C)** LC3A expressing glioblastoma patients were selected and a survival curve analysis was performed. **(D)** High-expressing LC3A and high-expressing WIPI2 patients were cross-matched and patients belonging to both gene groups were included for

further analysis. The same was done for low-expressing LC3A and low-expressing WIPI2 patients. Survival curve analysis showed that high-expressing LC3A/high-expressing WIPI2 patients had significantly worse survival compared to low-expressing LC3A/low-expressing WIPI2 patients. **(E)** The survival rates of the top 30^{th} percentile of LC3A-expressing glioblastoma patients were compared to the top 30^{th} percentile of LC3A-expressing low grade glioma (LGG) patients. High-LC3A glioblastoma patients had worse survival compared to high-LC3A LGG patients. **(F)** The same type of analysis was conducted for low-LC3A expressing glioblastoma and LGG patients. Low-LC3A expressing glioblastoma patients had worse survival compared to low-LC3A expressing LGG patients.

3.3 Discussion

Even with an aggressive treatment regimen – commonly referred to as the Stupp protocol but may consist of varying combinations and schedules of radiotherapy and TMZ therapy – patient survival time is only marginally better than standard radiotherapy alone. It is largely agreed upon that a main impetus behind the clinical efficacy of TMZ being attenuated are resistance mechanisms that allow for tumourogenic signalling to persist despite the cytotoxic lesions formed by radiotherapy and TMZ. There is however, a lack of consensus for a hierarchical model signifying the importance of a particular class of signalling molecules. Nonetheless, RTKs have garnered considerable attention due to their collective role in signal transduction pathways in cancer, including glioblastoma. Glioblastoma requires a number of signalling-driven endpoints for its malignant characteristics, including but not limited to: proliferation, invasion, migration, angiogenesis, anti-apoptosis, cell cycle progression and DNA repair. RTK signal transduction pathways are an integral component for these signalling endpoints. The influence of a RTK-based therapeutic paradigm for glioblastoma has furthered due to the successes in such a paradigm after its adoption in several other cancers. A number of RTK agents have been trialled in the hope of complementing the Stupp protocol; however, to date, no single drug has successfully - and consistently - shown efficacy in either primary or recurrent glioblastoma patients. This lack of success signifies the urgency that is before us to identify novel biomarkers for treatment resistance so that future therapies can supplement the Stupp protocol by selectively targeting those very signalling pathways that provide the tumour an escape mechanism.

3.3.1 Down-regulation of key RTKs in radiotherapy- and TMZ-treated glioblastoma cells

We initially hypothesised that RTK-driven signalling will be activated as a response in surviving cell populations to stimulate downstream signalling pathways to promote survival and tumourigenesis. This hypothesis was consistent with previous studies using both protein and gene expression analysis detailing the critical role of RTKs in glioblastoma tumourigenesis. Our RTK screen, however, invalidated the strongly supported hypothesis which highlights the importance in the use of clinically relevant therapeutic regimens in pre-clinical studies. Using an *in vitro* model of treatment resistance we showed that down-regulation of RTKs is a feature in our cell lines after standard therapy with irradiation and

TMZ (***Figure 3-2***). This observation, that the activity levels of 49 RTKs were reduced in treated cells, suggests that redundant activation of RTKs was not triggered as a response.

Furthermore, EGFR and MET gene expression was also down-regulated after treatment (***Figure 3-3***). It has been demonstrated that berberine-induced down-regulation of EGFR causes senescence in glioblastoma cells [733]. This initially suggested that the surviving cells in our experiments may have preferentially shifted from a highly proliferative and tumourogenic state to senescence. We therefore assessed the activation of STAT3, AKT and ERK downstream pathways since these signalling molecules are known for promoting glioblastoma proliferation and progression (***Figure 3-5***). Surprisingly, however, in all cell lines except #15 at least one of the downstream signalling molecules was up-regulated in the treated cell population. As we may rule out alternative RTKs as potential drivers of sustained downstream pathway activity we propose here that RTK-independent mechanisms are driving STAT3, AKT or ERK signalling, thereby conferring resistance to the surviving cell population and sustaining the tumourogenic capacities of these cell lines. Speculatively, it is possible that interleukin receptor signalling is a source for the sustained signal transduction and, indeed, studies have highlighted the role of this class of receptors in activating the three assessed pathways [734] [735, 736] [737]. Although, ultimately, further study is required to elucidate these exact mechanisms for the observed sustained downstream pathway signalling.

3.3.2 Autophagy marker LC3 is up-regulated in treatment-resistant cells

EGFR activity is considered to have an inhibitory effect on autophagy induction and autophagy is known to be induced upon EGFR inhibition [738]. Furthermore, the role of autophagy in resistance is a matter of debate. For instance, Palumbo's works have suggested up-regulated autophagy to carry a sensitising effect towards therapy [637, 739]. Specifically, autophagy inhibition, via silencing of beclin-1 and ATG7, suppressed the combined cytotoxic effects of radiotherapy and TMZ in T98G cells [739]. In addition, autophagy inhibition in EGFR-silenced TMZ-resistant T98G glioblastoma cells rescued clonogenicity [637]. In contradiction, a more recent paper put forth evidence that inhibition of TMZ-induced autophagy triggers the apoptotic pathway [740]. Notably, the EGFR-AKT-

mTOR pathway is known to inhibit autophagy in various cancers, including glioblastoma, and combining AKT inhibition with EGFR inhibition augmented LC3II levels in glioblastoma cell lines [631, 741]. Therefore, considering the decreased EGFR signalling upon treatment with radiotherapy and TMZ in our cells we next aimed to analyse whether increased autophagy is associated with treatment resistance.

Given that our resistant #20 and #41 cells had lacking EGFR expression and down-regulated pAKT levels, we hypothesised that LC3 expression is increased in this resistant subpopulation **(*Figure 3-6*)**. TCGA data suggested that autophagy markers are associated with patient survival supporting, albeit inconclusively, the position that autophagy is a resistance mechanism **(*Figure 3-7*)**. Our hypothesis was validated when treated #4, #20 and #41 cells had increased LC3 levels compared to parental populations. However, time point LC3 analysis with #41 revealed that LC3 expression may be time-dependent as we observed peak levels at the Day 3 time point. Despite EGFR #41 post-treatment levels were the lowest at Day 7, coupled with the observation of an increase in pAKT at Day 1, LC3 levels did not peak at Day 7 nor were expression levels reduced at Day 1. This suggests that EGFR-AKT signalling does not directly inversely correlate with LC3 and autophagy can be induced independently of the EGFR-AKT activity. This is consistent with Sugita and colleagues demonstrating that non-EGFR expressing non-small cell lung cancer cell lines treated with gefitinib exhibit autophagy induction as indicated by increased LC3 expression [742]. Therefore, a decrease in EGFR activity or expression is not essential for autophagy induction, but rather autophagy activation as a cyto-protective mechanism is not limited to EGFR activity.

3.4 Conclusion

To conclude, in this chapter we have shown that resistant glioblastoma cells have reduced RTK activation. Furthermore, it was demonstrated that the protein and gene expression of EGFR and MET is also reduced; however, a reduction of RTK activity after treatment did not lead to down-regulation of STAT3, AKT and ERK 1/2 in all cell lines employed suggesting RTK independent mechanism maintain tumourogenic signal transduction. Our results demonstrated that this reduction in RTK expression was both time- and dose-dependent. Lastly, the autophagic marker LC3 was highly expressed in treated cells and may be a

potential biomarker for treatment resistance. In the next chapter we will explore whether similar molecular differences are seen with long term stably acquired resistance to radiotherapy and TMZ.

CHAPTER 4:
INVESTIGATING THE ROLE OF EGFR AND MET IN GLIOBLASTOMA RECURRENCE

4.1 Introduction

Glioblastoma recurrence is regarded to be inevitable despite aggressive treatment with radiotherapy and TMZ. Recurrence may be attributed partially to the difficulty in achieving complete resection or arise due to poor penetration of the therapy due to factors such as BBB penetration, bioavailability and tumour penetration; however, a treatment-resistant subpopulation is also likely to be at least partly responsible for tumour mass repopulation. These subtypes may contain specific genetic alterations conferring advantageous capabilities to withstand the otherwise cytotoxic insults induced by radiotherapy and TMZ. For the purposes of our study glioblastoma recurrence refers only to recurrence initiated by treatment-resistance.

Two dominant theoretical paradigms exist that attempt to explain glioblastoma recurrence initiated by treatment-resistance **(*Figure 4-1*)**. The first suggests that resistance develops through spontaneous random mutations such that it is regarded that the tumour mass possess inherently a resistant subpopulation at the onset of treatment [743]. In other words, the recurrent mass is a subtype selected for by standard therapy that possesses a genetic makeup that allows for being predisposed to resistance and initiating recurrence. The second suggests that recurrence is initiated by a subpopulation of cells that have acquired genetic alterations that arise from treatment [743]. In this model, resistance develops through an adaptation process in response to a specific condition, such as therapy [743]. Therefore, an account of recurrence requires an explanation in the origin of resistance and this question will be explored in this chapter.

Whether EGFR confers poorer survival in glioblastoma is controversial with contradicting reports. We have demonstrated in **Chapter 3** that short-term treatment with radiotherapy and TMZ led to reduced RTK activity and EGFR and MET gene expression. A possible avenue for deciphering the role of EGFR in resistance is to analyse the EGFR status upon recurrence. MET expression has been suggested to be a predictor of poor survival and progression-free survival [568, 569]. In addition, a study by Liu et al. reported recently that MET expression in recurrent glioblastoma is higher than primary glioblastoma and a net increase in the number of MET-positive patients at recurrence than primary tumours [568]. Although there is clinical data available reporting on the change in EGFR and MET status after recurrence there is a

gap in the literature studying the role of EGFR and MET in recurrence at the fundamental biological level. Therefore, we aim to study the role of EGFR and MET in both treatment resistance and recurrence. Given that a number of clinical trials that have unsuccessfully employed agents that target EGFR or MET in either newly diagnosed or recurrent glioblastoma in conjunction with TMZ, an account for such a failure is lacking in the literature. The aim in this chapter is to address this gap.

Aim

Given the above our aim for this chapter is to:

Elucidate the role of EGFR and MET in treatment-resistance initiated recurrence.

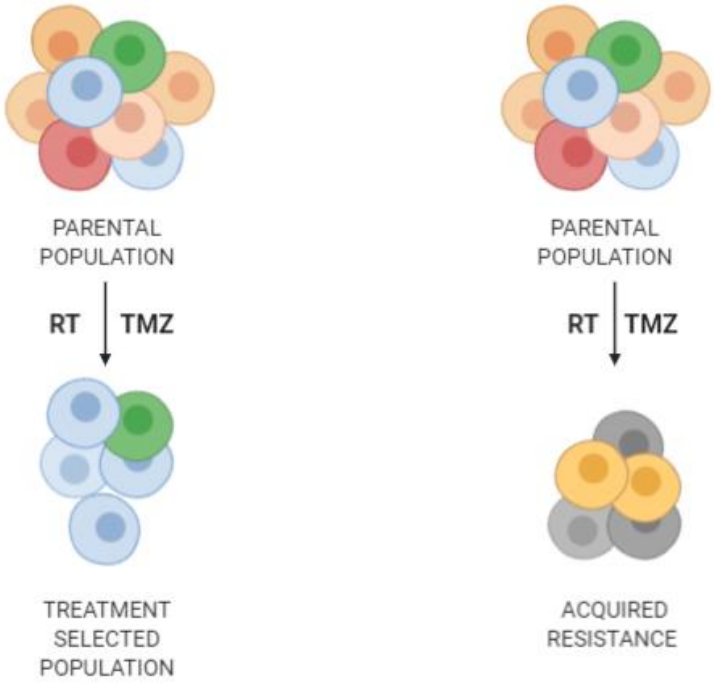


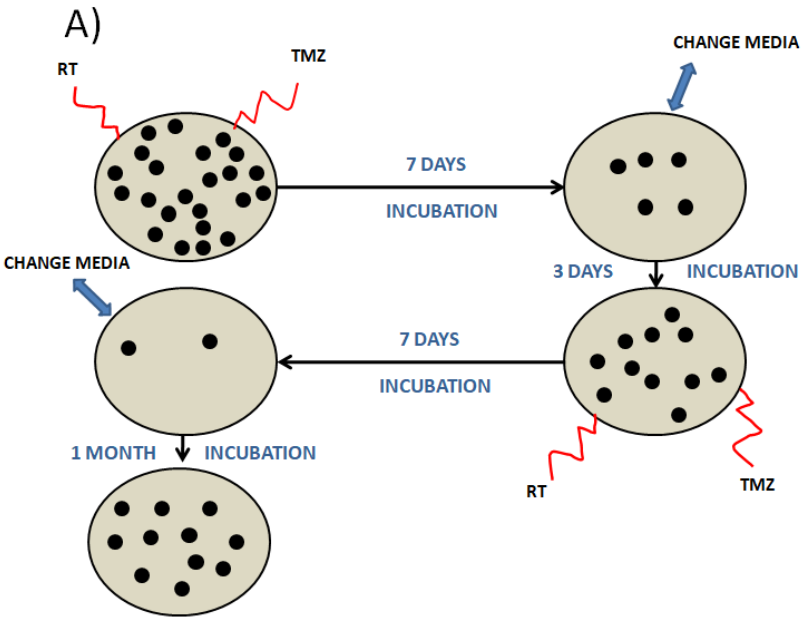
Figure 4-1: Origins of glioblastoma recurrence. Two broad theories attempt to explain the source for tumour recurrence in glioblastoma. One view is that an inherently resistant pre-existing subpopulation of tumour cells exists within the parental population and this resistant population is selected during therapy (left). The alternative explanation is that therapy induces a subpopulation to acquire resistance as a consequence of genetic and epigenetic alterations (right). RT = Radiotherapy; TMZ = Temozolomide.

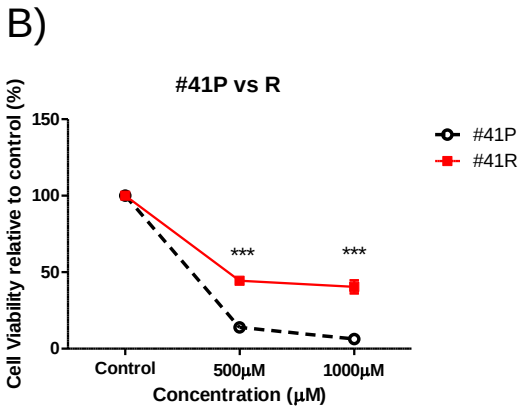
4.2 Results

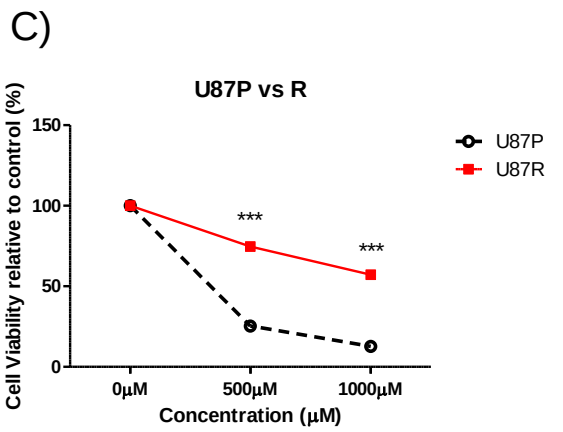
4.2.1 Generation of *in vitro* recurrent glioblastoma model

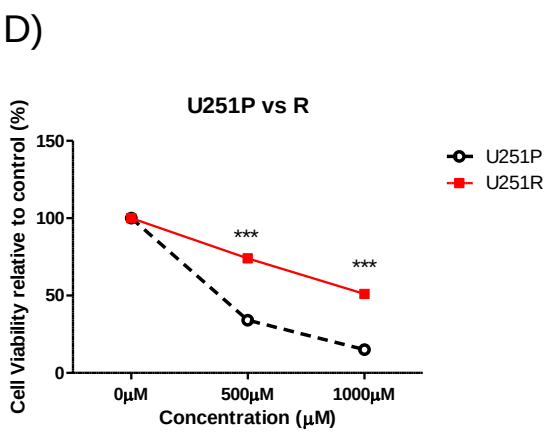
Currently, there is a lack in the number of studies that have utilised standard therapy in the generation of a recurrence model *in vitro*. Given that treatment resistance contributes to tumour recurrence in glioblastoma there is an urgent requirement to address this gap. Therefore, we first aimed to generate a recurrent *in vitro* model that potentially mimics the same features in recurrence found in the clinical tumour recurrence.

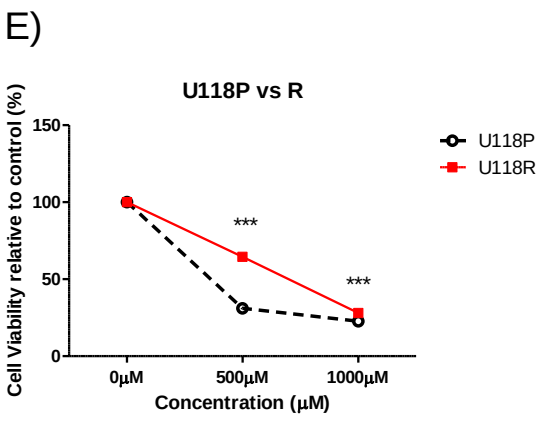
Primary glioblastoma cell lines #15, #20 and #41 were subject to 5Gy irradiation and concomitant TMZ at 1000uM followed by a 7-day incubation period. After a change in media and a 3-day treatment-free period the regenerated tumour population was again subject to the same treatment regimen of irradiation and TMZ which was followed by another 7-day incubation period **(*Figure 4-2 A*)**. Cells were re-cultured in TMZ-free media after the end of the second 7-day incubation period and allowed to repopulate the culture flask – a period of 2 months was required for this repopulation phase to be complete. This method allowed us to successfully generate a resistant #41 variant (#41R) **(*Figure 4-2 B*)**. #41 cell line resistance was tested by subjecting both the parental and resistant cell line to 5Gy irradiation plus either 500uM or 1000uM TMZ followed by a 7-day incubation period before a cell viability assay was conducted. The resistant #41 variant (#41R) was significantly more resistant to therapy compared to the parental cell line. However, a resistant #15 and #20 was unable to be generated (data not shown). Previously, a TMZ-resistant U87, U251 and U118 variant was generated in our laboratory (by Dr Rodney Luwor and Dr Stanley Stylli) after continuous exposure to TMZ for 6 months. The resistance of these three cell lines was tested by subjecting both parental and variant cells to 500uM and 1000uM TMZ for a 7-day incubation period before a cell viability assay was conducted. All three resistant variants were significantly more resistant at both TMZ doses compared to the corresponding parental cell line **(*Figure 4-2 C-E*)**. The #41R was significantly more proliferative compared to the parental cell line; after 5 days the #41R cell number was more than 15 times the Day 0 time point while the #41 parental cell line (#41P) was only above 5 times the Day 0 time point **(*Figure 4-2 F*)**.


A)
RT
TMZ
CHANGE MEDIA
7 DAYS
INCUBATION
CHANGE MEDIA
3 DAYS
INCUBATION
7 DAYS
INCUBATION
1 MONTH
INCUBATION
RT
TMZ


B)
#41P vs R
Cell Viability relative to control (%)
150
100
50
0
***
***
Control
500μM
1000μM
Concentration (μM)
#41P
#41R


C)
U87P vs R
Cell Viability relative to control (%)
150
100
50
0
***
***
0μM
500μM
1000μM
Concentration (μM)
U87P
U87R


D)
U251P vs R
Cell Viability relative to control (%)
150
100
50
0
***
***
0μM
500μM
1000μM
Concentration (μM)
U251P
U251R


E)
U118P vs R
Cell Viability relative to control (%)
150
100
50
0
***
***
0μM
500μM
1000μM
Concentration (μM)
U118P
U118R

F)

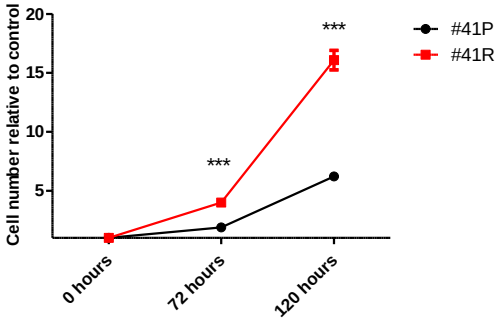


Figure 4-2: Treatment resistant cells were successfully generated. (A) Treatment protocol employed to generate treatment resistant #41 cells (#41R). Next Parental **(B)** #41 cells and their counterpart long-term treated cells were treated with 5Gy irradiation plus the indicated dose of TMZ. After incubation for 7 days #41R cells were significantly more viable than the parental cells at both doses of TMZ. **(C)** - **(E)** show the cell viability difference between long-term treated U87R, U251R and U118R and the respective parental controls. Cell viability was compared to corresponding untreated controls. **(F)** #41 resistant cells (#41R) were significantly more proliferative than the parental line. Cell viability was measured 3 and 5 days after seeding. The control was set as the viability measured one day after seeding for each respective cell line. All values are mean± SD; n=3; *** $p<0.0001$.

4.2.2 Glioblastoma recurrent cells lack EGFR and MET protein and gene expression, and are resistant to RTK inhibition

In chapter 3, short-term treated resistant cells showed decreased EGFR and MET protein and gene expression in addition to increased LC3 protein expression. To test whether this is also the case in the recurrent cells EGFR and MET activation and protein expression was assessed using both #41P and #41R. Both the #41P and #41R were treated with 5Gy irradiation and 1000uM TMZ before a 7-day incubation period. Interestingly, the untreated #41R cells showed a lack in both EGFR and MET protein expression **(*Figure 4-3 A*)**. Although we previously showed that short-term treated resistant cells have elevated levels of the autophagic marker LC3 the #41R cells had decreased LC3 compared to #41P **(*Figure 4-3 B*)**.

To quantify relative EGFR and MET mRNA levels in the resistant cells qRT-PCR was performed **(*Table 4-1*)**. We observed that #41R, U87R and U251R had undetectable levels of both EGFR and MET gene expression at the maximum cycle number 40. Table 4-1 lists the ΔCT values for EGFR and MET mRNA expression for #41 Parental (#41P), U87 Parental (U87) and U251 Parental (U251) cells as calculated by the equation ΔCT = EGFR CT value (or MET CT value) – GAPDH CT value. Furthermore, all resistant cell lines - #41 resistant (#41R), U87 resistant (U87R) and U251 resistant (U251R) cells - showed no detectable levels of EGFR and MET mRNA expression.

To rule out promoter methylation as the reason for reduced EGFR we used a demethylating agent. 10uM treatment with the demethylating agent azacitidine (also called 5-aza) for 48 hours showed no increase in EGFR gene and protein expression in #41R cells, indicating that EGFR promoter methylation may not be causing the reduced EGFR gene expression in #41R **(*Table 4-1 & Figure 4-3 C*)**.

The hypothesis that EGFR- and MET-lacking recurrent cells are resistant to RTK inhibitors was tested. Both #41P and #41R were treated with the EGFR inhibitors – erlotinib, gefitinib, afatinib and lapatinib – and crizotinib and cabozantinib which are multi-kinase inhibitor that also targets MET. Cells were treated with 1uM and incubated for 7 days before a cell viability assay was performed. #41R cells were more resistant to all six RTK inhibitors compared to #41P **(*Figure 4-4 A-F*)**. Interestingly, erlotinib, afatinib and lapatinib were the only agents that did not significantly reduce #41R cell viability when compared to the

untreated #41R control. Similarly, U87R was also more resistant to erlotinib and cabozantinib compared to the parental U87 (U87P) **(*Figure 4-4 G-H*)**. While erlotinib significantly reduced U87R cell viability compared to U87R untreated control, cabozantinib showed no such effect. Taken together, our data suggests that resistance to RTK inhibitors in recurrent cells is mediated by a loss of EGFR and MET expression.

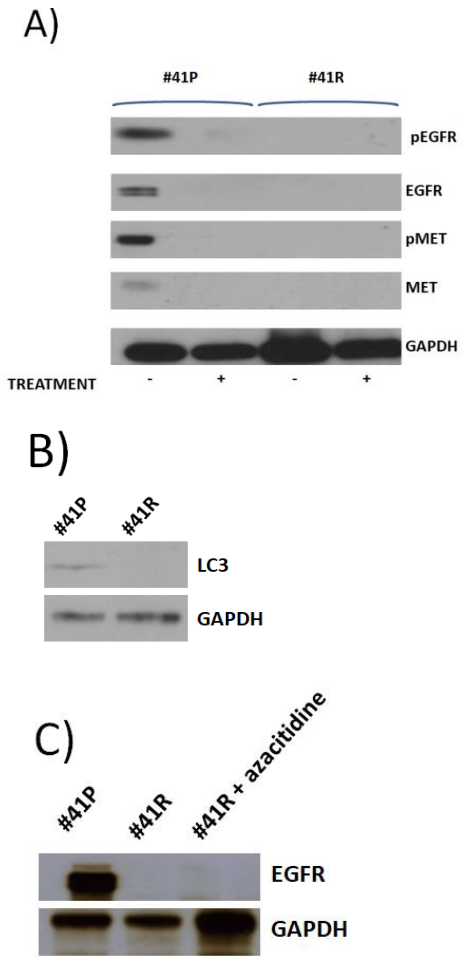


Figure 4-3: Recurrent cells lack EGFR, MET and LC3 protein expression. (A) Both #41P and #41R cells were treated with irradiation and TMZ before incubation for 7 days. As expected EGFR and MET protein expression was reduced in #41P cells; however #41R untreated cells lacked EGFR and MET protein expression. **(B)** Blot shows the levels of LC3 in both parental and resistant #41 cells. The #41R cells showed no detectable levels of LC3. **(C)** #41R was treated with 10uM of azacitidine and EGFR protein expression was assessed with western blot after 48 hours.

Table 4-1: EGFR and MET gene expression is reduced in resistant cell lines.

EGFR GENE EXPRESSION	#41P	#41R	#41R+ 5-aza	U87P	U87R	U251P	U251R
ΔCT value	8.56	U/D	U/D	8.43	U/D	19.10	U/D
SD	0.30			0.75		0.65	

MET GENE EXPRESSION	#41P	#41R	U87P	U87R	U251P	U251R
ΔCT value	8.01	U/D	8.10	U/D	17.07	U/D
SD	0.05		0.54		0.19	

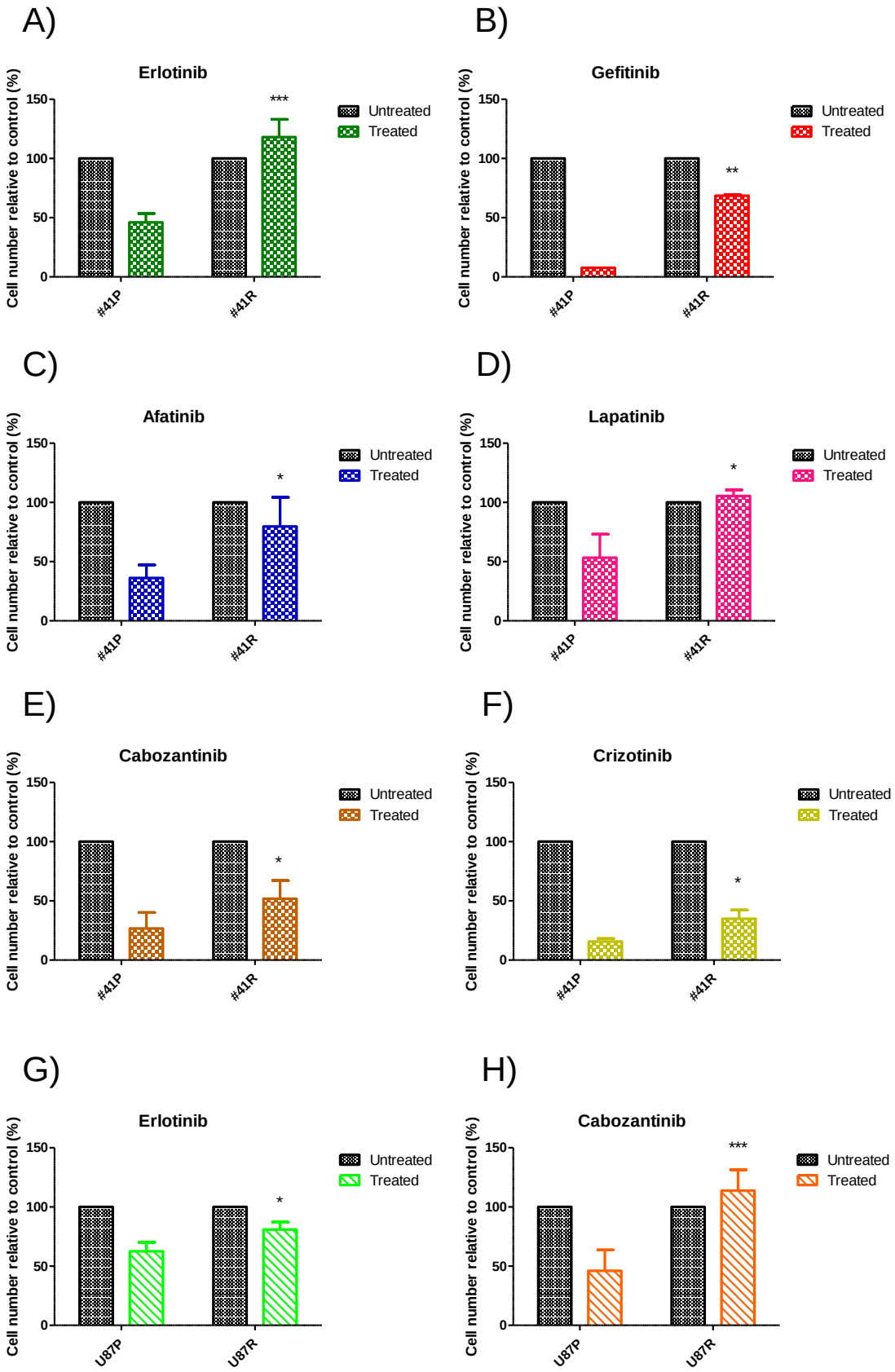
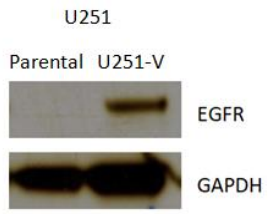

A)
Erlotinib
Cell number relative to control (%)
150
100
50
0
***
#41P
#41R
Untreated
Treated
B)
Gefitinib
**
C)
Afatinib
*
D)
Lapatinib
*
E)
Cabozantinib
*
F)
Crizotinib
*
G)
Erlotinib
*
U87P
U87R
H)
Cabozantinib
***

Figure 4-4: Resistant cells are resistant RTK inhibitors. Resistant cell lines #41 and U87 were treated with RTK inhibitors and cell viability was compared to the parental line. Cell line #41P and #41R were exposed to EGFR inhibitors **(A)** erlotinib, **(B)** gefitinib, **(C)** afatinib, **(D)** lapatinib, and MET inhibitors **(E)** crizotinib and **(F)** cabozantinib and cell viability was measured 72 hours later. #41R was significantly more resistant to all drugs compared to #41P. U87P and U87R were also treated with **(G)** erlotinib and **(H)** cabozantinib and cell viability was 72 hours later. U87R was significantly more resistant to both drugs compared to U87P. All values are mean ± SD; n=3; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

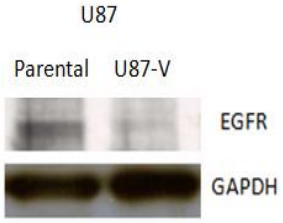
4.2.3 A low-EGFR expressing subpopulation of cells is pre-disposed to treatment resistance

To investigate whether a pre-existing subpopulation was inherently sensitive to treatment we performed a serial cell dilution to cultivate colonies originating from a single cell. After 3 months we were successfully able to obtain a single cell derived population of U251 (U251-V) and U87 cells (U87-V). When comparing the total EGFR expression of parental U251 with the U251-V we observed higher EGFR protein expression levels in the U251-V cells **(*Figure 4-5* A)**. Additionally, U251-V showed higher EGFR gene expression **(*Figure 4-5* B)**. Consistent with our previous findings, cell viability assays showed that the U251-V cells were significantly more sensitive to the treatment of 5Gy irradiation and 1000μM TMZ at both Day 3 and Day 7 **(*Figure 4-5* C)**. Conversely, U87-V displayed lower EGFR protein expression **(*Figure 4-5* D)** and EGFR gene expression **(*Figure 4-5* E)** compared to the parental U87 cells. When both U87 parental cells and U87-V cells underwent 5Gy radiotherapy plus 1000uM TMZ treatment U87-V cells were significantly more resistant to therapy compared to the parental population expression **(*Figure 4-5* F)**. Consistently with our previous results, subpopulation of cells with increased EGFR displayed greater sensitivity to erlotinib **(*Figure 4-5* G)**.

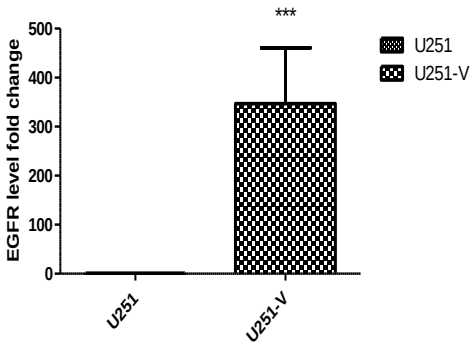
A)



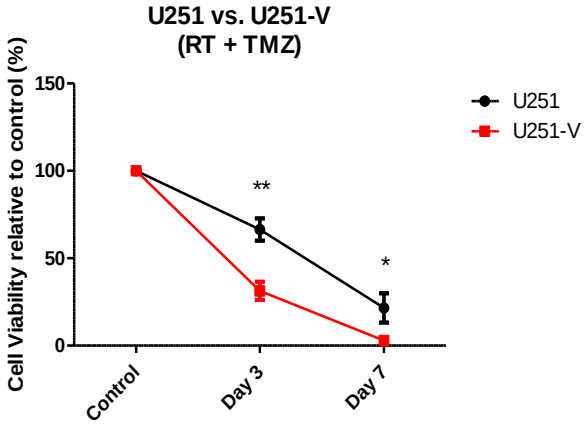
B)



C)



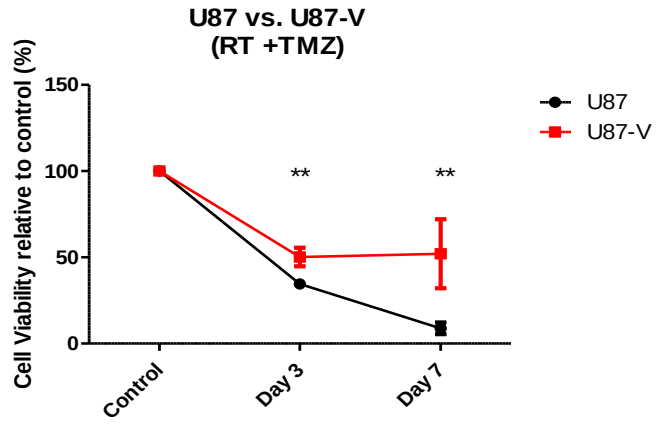
D)



E)

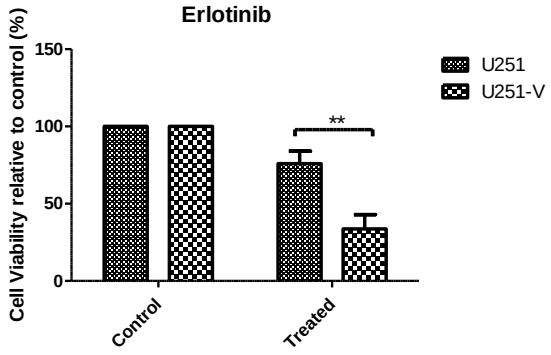
	U87	U87-V
ΔCT value	9.160	U/D
SD	0.150	

F)



G)

i)



ii)

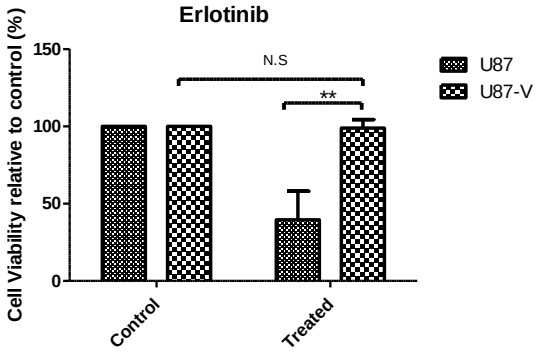


Figure 4-5: Subpopulation of low EGFR-expressing cells is more resistant to treatment. Colonies originating from either single U251 or U87 parental cells were generated after serial cell dilution. After 2-3 months the U251 colony expressing higher EGFR and a U87 colony expressing lower EGFR compared to the parental line was selected for further analysis. **(A)** Western blot analysis shows a difference in EGFR expression between parental U251 cells and the U251-V cells which originated from single U251 cell. Substantially higher EGFR expression was observed in the U251-V cells. **(B)** A subpopulation of U87 GBM cells (U87-V) expressing lower level of EGFR protein compared to the parental population. **(C)** Similarly, EGFR mRNA levels in U251-V were significantly higher compared to U251 parental population. **(D)** Shows the differences in cell viability between U251 parental and U251-V

after treatment. U251 parental and U251-V cells were treated with 5Gy irradiation and 1000μM TMZ before incubation for 3 and 7 days. U251-V cell viability reduced more than the parental after treatment at both time points and these differences were significant. **(E)** The U87-V population also displayed no detectable level of EGFR gene expression as quantified by qRT-PCR. **(F)** Cell viability assay showed that U87-V cells were significantly more resistant to radiotherapy and TMZ compared to the parental population. **(G)** High-EGFR expressing **(i)** U251-V and low EGFR-expressing **(ii)** U87-V were treated with 1uM of erlotinib. Cell viability assays showed U251-V and U87-V cell populations were significantly more sensitive and resistant to treatment compared to the corresponding controls, respectively. Values are means ± SD; N.S = not significant; RT = radiotherapy; *p<0.05, **p<0.01, ***p<0.001; n=3.

4.2.4 Human patient glioblastoma lose EGFR upon recurrence

Our results thus far suggest that a low-EGFR treatment-resistant subpopulation is selected for by radiotherapy and TMZ. To further validate this position human patient-derived recurrent glioblastoma cell lines were employed. Consistently, recurrent glioblastoma cell line #28 and #35 had decreased EGFR gene expression compared to both primary glioblastoma cell lines #41 and #20 **(*Figure 4-6 A*)**. This was also observed when analysing MET gene expression levels **(*Figure 4-6 B*)**.

Matched primary and recurrent samples from 36 different glioblastoma patients were available for analysis. We, therefore, analysed the gene expression of both primary and recurrent samples from each patient to evaluate frequency for loss of EGFR upon recurrence. Patients with differential EGFR gene expression of less than 30% between primary and recurrent samples were considered to have stable expression. Only two patients out of the entire 36-patient cohort were, therefore, considered to have stable expression **(*Figure 4-8 A*)**.

Before stratifying the cohort according to treatment received we observed that 12 and 22 patients had reduced and increased EGFR expression, respectively **(*Figure 4-7 A-B*)**. 7 and 8 patients from the respective groups had a change of greater than 5-fold. Interestingly, in this cohort low recurrent EGFR expressing patients had a worse prognosis, though this difference was not significant **(*Figure 4-7 C*)**.

To better associate recurrent EGFR expression, patient survival and treatment-resistant recurrence we selected patients who were treated with radiotherapy alone or TMZ alone or the Stupp Protocol from our recurrent cohort. We first plotted all patients that received any therapy in addition to resection **(*Figure 4-8 A*)**. A total number of 31 patients were selected for this analysis out of the original 36 patients. The remaining five patients did not receive radiotherapy or TMZ, four of these patients had an increase in EGFR expression and one had a decrease in EGFR at recurrence. From the 31 patients selected, 25 had received the Stupp Protocol regimen (81%). 9 patients from the Stupp Protocol group had reduced recurrent EGFR expression (36%), 1 patient had no change (4%) and 15 patients had increased EGFR expression (60%). The majority of the Stupp Protocol treated patients that lost EGFR expression had a greater than 5-fold decrease (6/9 patients, 66%) while Stupp Protocol

treated patients that had a 5-fold increase in EGFR were in the minority amongst patients that had increased EGFR (5/15 patients, 33%). A total of 3 patients were treated with radiotherapy only; 1 of these patients had no EGFR changes, 1 had an increase more than 5-fold and 1 had an EGFR expression decrease of less than 5-fold. Similarly, 3 patients received TMZ only; 2 of these patients had an EGFR increase of less than 5-fold at recurrence and 1 patient had a decrease of more than 5-fold.

Survival rates of patients that received any treatment in addition to surgical resection were analysed with high recurrent EGFR expression and low recurrent EGFR expression separated into two groups **(*Figure 4-8 B*)**. 18 patients were in the high EGFR expressing group and 10 patients were in the low EGFR expressing group (outliers were deselected). The analysis revealed that patients with decreased recurrent EGFR expression had a lower median survival time compared to patients with increased recurrent EGFR expression (13.7 months vs. 21.9 months) and a hazard ratio of 0.47 (High EGFR/ Low EGFR) represents a risk of 2.13 times more for the low EGFR expressing group compared to high expression. Similar results were also obtained when only patients treated with the Stupp Protocol were selected for the analysis **(*Figure 4-8 C*)**. The greatest difference between the two groups was observed when Stupp Protocol treated patients were divided into either the greater than 5-fold EGFR increase group or greater than 5-fold EGFR decrease group. The median survival time was almost double in the high EGFR expressing group compared that of the low EGFR expressing group (32.1 months vs. 16.6 months), and although the survival curves were not statistically significant according to two tests, the p-value was 0.07 while a hazard ratio of 0.28 demonstrates 3.57 times more risk for the low EGFR expressing patients compared to high EGFR expression **(*Figure 4-8 D*)**.

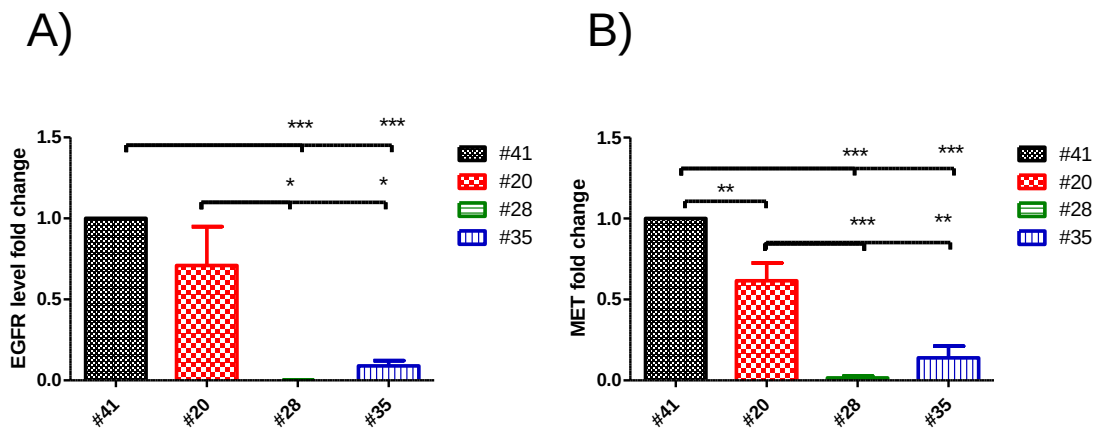
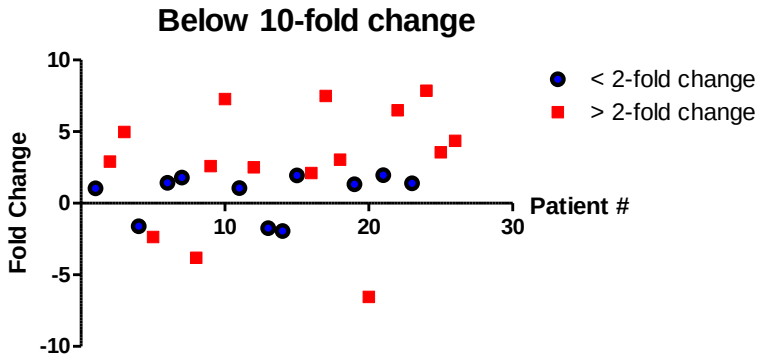


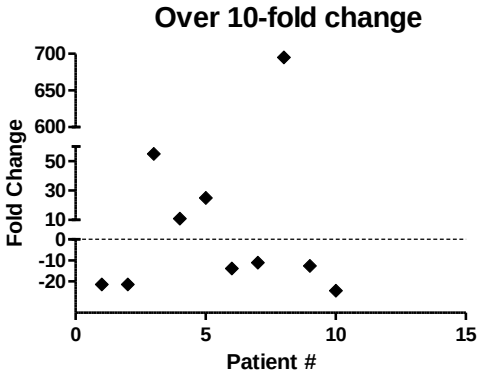
Figure 4-6: Recurrent glioblastoma cell lines have reduced EGFR and MET gene expression. Patient-derived recurrent glioblastoma cell lines #28 and #35, in addition to primary glioblastoma cell lines #20 and #41, had EGFR and MET gene expression levels quantified by qRT-PCR. **(A)** #20, #28 and #35 EGFR levels relative to #41 EGFR levels are shown. **(B)** #20, #28 and #35 MET levels relative to #41 MET levels are shown. All values are mean ± SD; *p<0.05, **p<0.01, ***p<0.001; n=3.

A)

i)


Below 10-fold change
Fold Change
Patient #
< 2-fold change
> 2-fold change
10
5
0
-5
-10
10
20
30

ii)


Over 10-fold change
Fold Change
Patient #
700
650
600
50
30
10
0
-10
-20
0
5
10
15

B)

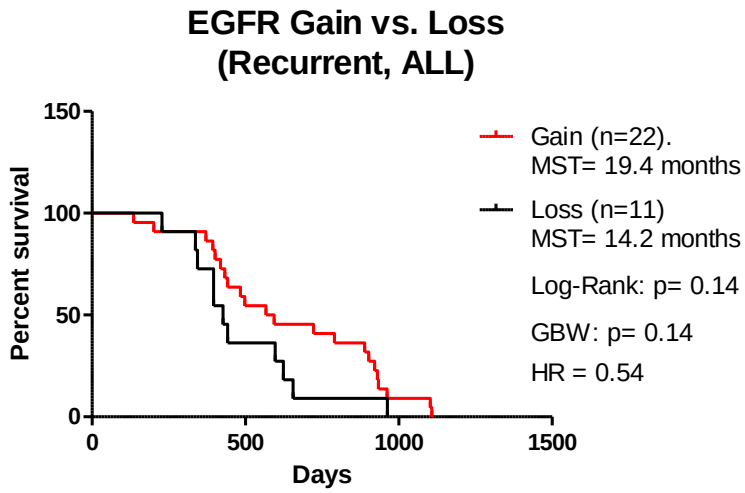
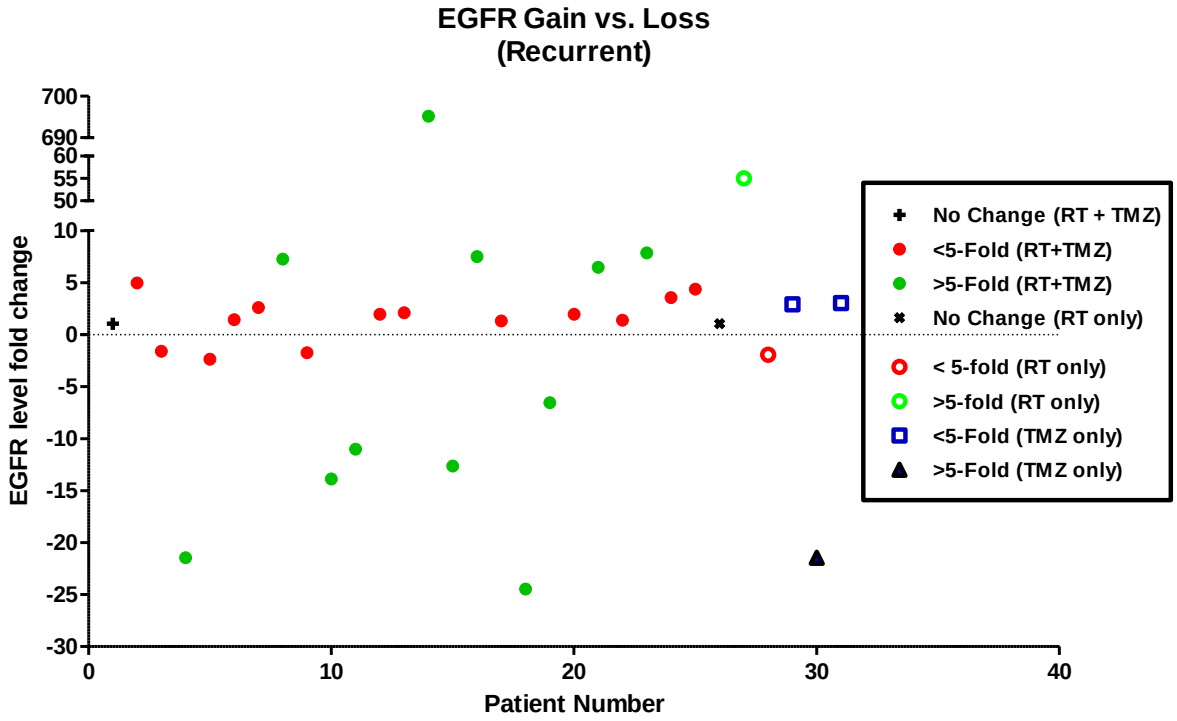
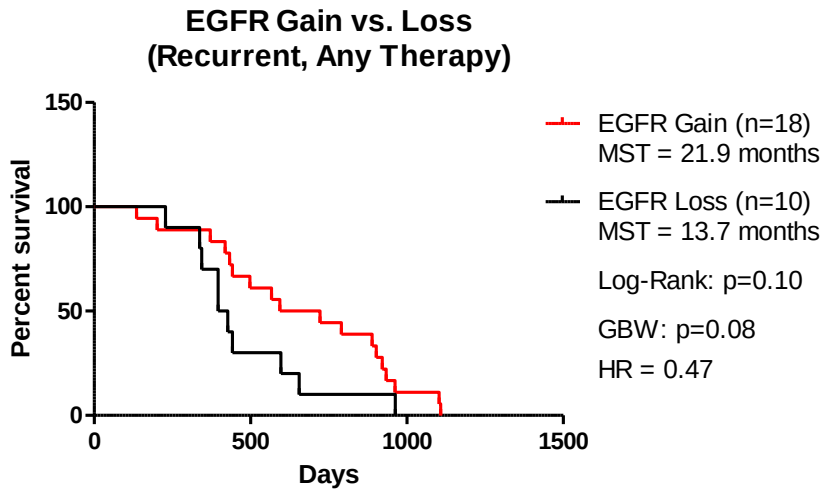

EGFR Gain vs. Loss
(Recurrent, ALL)
Percent survival
Days
Gain (n=22).
MST= 19.4 months
Loss (n=11)
MST= 14.2 months
Log-Rank: p= 0.14
GBW: p= 0.14
HR = 0.54
150
100
50
0
0
500
1000
1500

Figure 4-7: EGFR expression decreases in one-third of recurrent glioblastoma tumours. A) The EGFR expression in recurrent tumours relative to the corresponding paired primary tumours. 12 out of 36 glioblastoma recurrent tumours analysed presented a loss in EGFR expression. 2 patients had less than 10% increases in EGFR expression between primary and recurrent tumours. **(i)** Recurrent EGFR expression relative to primary tumour in which the fold-change is less than 10-fold. **(ii)** Recurrent EGFR expression relative to primary tumour in which the fold-change is greater than 10-fold. **B)** Kaplan-Meier plot presenting the survival rates of the group which gained EGFR expression upon recurrence and the group which had reduced EGFR expression upon recurrence. The survival cure was not statistically significant though the EGFR loss group had worse median survival time. MST = Median survival time. Log-Rank = Mantel-Cox method; GBW = Gehan-Breslow-Wilcoxon method; HR = Hazard Ratio (High:Low); n = number of patients.

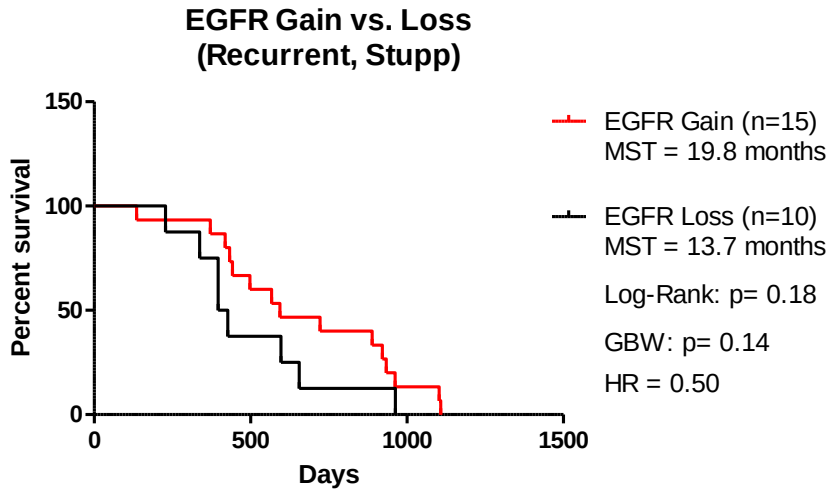
A)


EGFR Gain vs. Loss
(Recurrent)
EGFR level fold change
Patient Number
700
690
60
55
50
10
5
0
-5
-10
-15
-20
-25
-30
0
10
20
30
40
No Change (RT + TMZ)
<5-Fold (RT+TMZ)
>5-Fold (RT+TMZ)
No Change (RT only)
< 5-fold (RT only)
>5-fold (RT only)
<5-Fold (TMZ only)
>5-Fold (TMZ only)

B)


EGFR Gain vs. Loss
(Recurrent, Any Therapy)
Percent survival
Days
150
100
50
0
0
500
1000
1500
EGFR Gain (n=18)
MST = 21.9 months
EGFR Loss (n=10)
MST = 13.7 months
Log-Rank: p=0.10
GBW: p=0.08
HR = 0.47

C)



D)

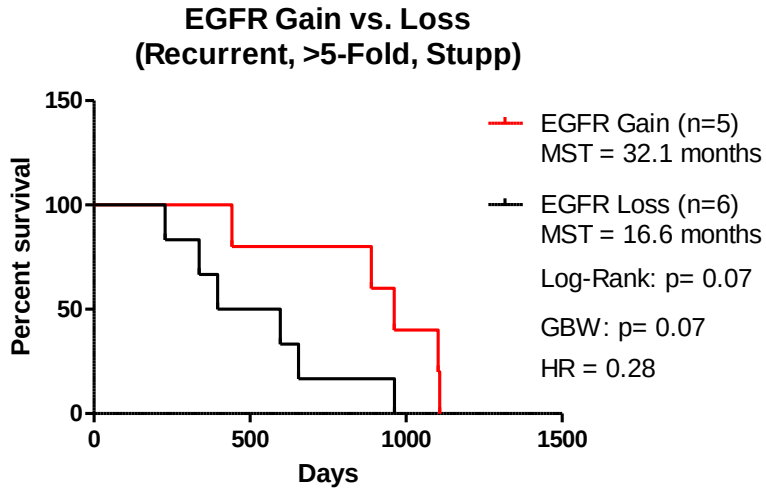


Figure 4-8: Subset of Stupp Protocol treated patients have decreased EGFR expression at recurrence and trend towards poorer survival. Recurrent patients that were treated with radiotherapy and TMZ were selected from the recurrent cohort. **(A)** Chart showing the EGFR expression changes in all patients from the recurrent cohort that were treated with radiotherapy only, TMZ only or the Stupp Protocol. **(B)** All patients that received radiotherapy alone or TMZ alone or in combination were grouped according to EGFR expression change. **(C)** The survival rates of patients treated with the Stupp Protocol that

had an increase in EGFR expression at recurrence were compared with patients who had decreased EGFR expression at recurrence. **(D)** The survival rates of patients treated with the Stupp Protocol that had a greater than 5-fold increase in EGFR expression at recurrence were compared with patients who had a greater than 5-fold decrease in EGFR expression at recurrence. MST = Median Survival Time; Log-Rank = Mantel-Cox method; GBW = Gehan-Breslow-Wilcoxon method; HR = Hazard Ratio (High:Low); n = number of patients.

4.3 Discussion

Glioblastoma recurrence is considered to be inevitable due to the limitations of current therapy and a significant contributor is treatment resistance initiating tumour recurrence. Currently, there is no consensus in the literature on a standard of biomarkers for both treatment resistance and recurrence. Moreover, supplementary therapeutic agents have not been successful in improving standard therapy calling in the need to better understand the mechanisms driving resistance-initiated recurrence.

4.3.1 Low EGFR and MET is an attribute of treatment resistance

As discussed previously in chapter 1 and 3, glioblastoma studies employing treatment resistance models are lacking which has limited our understanding in the genetic alterations conferring resistance and consequent recurrence. In this chapter we aimed to generate an *in vitro* recurrence model to further characterise the role of EGFR and MET in driving tumour resistance **(*Figure 4-2 A*)**. To the best of my knowledge, the model that generated a #41 *in vitro* recurrent cell line is a first in the glioblastoma literature **(*Figure 4-2 B*)**. Although previous publications have generated resistant cell lines (as previously mentioned) these did not adequately represent recurrence as it occurs in the clinic due to only utilising TMZ or radiotherapy. The U87R, U251R and U118R cell line differs from the #41R in this regard as the former cell lines were selected after 6 months of TMZ treatment and must be considered as TMZ-resistant cell lines rather than an *in vitro* representation of clinical recurrence **(*Figure 4-2 C-E*)**. Unfortunately, apart from #41R, we were not able to successfully generate a #15R or #20R. This may possibly due to the aggressive therapy employed in the protocol coupled with the relative sensitivity of the respective resistant subpopulation.

Our data shows that recurrent cell lines selected by either standard therapy or TMZ alone have decreased EGFR and MET expression which is consistent with chapter 3 that concluded resistant cells have low RTK activity **(*Figure 4-3*)**. Interestingly, cell lines #41R, U87R and U251R did not present a detectable level of EGFR and MET gene expression in the qRT-PCR analysis **(*Table 4-1*)**. Therefore, from the tools available to us – western blot and qRT-PCR - the conclusion that the surviving subpopulation does not express EGFR and MET can be

justified. Nonetheless, single-cell RNA sequencing is required for further studies to verify our results and the subsequent conclusion.

Our results, interestingly, suggest that recurrence does not require the re-activation of the key EGFR and MET RTKs and this was consistently observed even as late as the 30th culture passage (data not shown). Treatment with DNA methylation removal agent 5-azacitidine also did not rescue #41R EGFR and MET protein expression. This further supports the existence of other redundancy pathways maintaining tumourigenicity in the absence of EGFR and MET. Combined with the results from chapter 3 we can conclude that low EGFR and MET expression confers resistance to standard therapy and this suppression is maintained at recurrence.

4.3.2 A possible reason for the failure of RTK inhibition

As discussed previously, many clinical trials using RTK inhibitors have been unsuccessfully trialled in the glioblastoma clinic. Given that the RTK therapy paradigm is aimed to supplement the standard therapy involving radiotherapy and TMZ we explored the response in our recurrent models to RTK inhibition. We expected that our recurrent models are resistant to RTK inhibitors given the lack in EGFR and MET. Indeed, both #41R and U87R were resistant to monotherapy with EGFR and MET inhibitors compared to parental controls **(*Figure 4-4*)**. Interestingly, the effectiveness of erlotinib and gefitinib differs when administered towards #41R; although erlotinib was ineffective in reducing #41R viability, gefitinib treatment significantly led to a decrease in #41R compared to the untreated #41R. Although this may appear to be contradictory given that #41R lacks EGFR gene expression and both agents are EGFR specific, an empirically validated explanation is available. Gefitinib has been shown to have EGFR-independent mechanisms of action. For example, autophagy induction is still present in gefitinib-treated lung cancer cells lacking the EGFR [742]. Similarly, erlotinib anti-tumour activity has been observed to be independent of EGFR expression in lung cancer cells *in vivo* [744]. Therefore, it is plausible to suggest that the difference in effectiveness between EGFR inhibitors is due to the targeting of EGFR-independent. Furthermore, as we have seen, downstream signalling pathways are differentially activated in resistant cell lines and it follows that it is possible that gefitinib may target these pathways specifically linked to #41R tumourigenicity. The same line of reasoning can be utilised to explain the significant reductions in #41R cell viability after

crizotinib or cabozantinib treatment. Specifically, crizotinib and cabozantinib are both well-known multi-kinase inhibitors that are not restricted to targeting MET. Therefore, the reduction in #41R may be due to the re-expression of other RTKs upon recurrence though this requires further study. U87R subjected to cabozantinib did not reduce cell viability possibly due to down-regulation of the other targets of the agent. Taken together, we conclude that resistance to EGFR and MET inhibition can be attributed to radiotherapy and TMZ down-regulating EGFR and MET levels leading to the recurrent population to be inherently resistant to the supplementary RTK inhibitors.

4.3.3 Glioblastoma heterogeneity drives recurrence

Heterogeneity is a hallmark of glioblastoma such that 'glioblastoma multiforme' is a common naming alternative to glioblastoma. As our novel results have potentially led to a breakthrough in the RTK-based paradigm in glioblastoma we suggested that radiotherapy and TMZ combine to select for low EGFR expressing cells from which derives the low EGFR expressing recurrent population. To test this hypothesis we performed a serial cell dilution, a method employed to study heterogeneity [745, 746]. This led to the generation of single-cell derived high EGFR protein and mRNA expressing U251 in addition to a low EGFR protein and mRNA U87 variant **(*Figure 4-5 A-B and D-E*).** Consistent with our previous results the U251-V had increased sensitivity to both standard and EGFR inhibition therapy while the U87-V was resistant to both types of treatment **(*Figure 4-5 C and F-G*).** Our results re-emphasise, therefore, the heterogeneous nature of glioblastoma and its consequent challenge to targeted therapy. It must be noted, however, that our results do not necessarily invalidate the possibility of acquired resistance contributing to recurrence even though it supports the inherent resistance model. Nonetheless, the absence of our glioblastoma recurrent cell lines in establishing complete heterogeneity, as present in the parental population, indicates that acquired resistance may not be a significant factor as we would expect heterogeneity levels to be re-established.

4.3.4 Patient analysis

The relevance of EGFR with regards to survival is controversial with contradicting results [747, 748]. Similarly, EGFR status at recurrence differs with patients being reported to have gained or loss EGFR expression [749] [750]. To begin the investigation we first analysed both

EGFR and MET mRNA levels in patient-derived recurrent glioblastoma cell lines and compared expression levels with two primary glioblastoma cell lines. Consistently, we found that recurrent glioblastoma cell lines had significantly lower EGFR and MET mRNA levels **(*Figure 4-6 A-B*)**.

Next we obtained 36 recurrent patient tumour samples with paired primary samples from patients at the Royal Melbourne Hospital, Victoria, to investigate the role of EGFR at recurrence. We first divided the recurrent patient cohort according to whether they lost or gain EGFR expression at recurrence and observed that 12 recurrent had decreased EGFR **(*Figure 4-7*)**. Next we selected patients that received radiotherapy alone, TMZ alone or the Stupp Protocol, leading to the exclusion of five patients that did not receive any mode of treatment in addition to surgical resection **(*Figure 4-8*)**. Interestingly, from the cohort that received any additional treatment and the Stupp Protocol treated patients, 11 patients and 9 patients had reduced EGFR compared to the primary tumour sample, respectively. In other words, 75% of patients that lost EGFR expression at recurrence received the Stupp Protocol. We therefore suggest reduced recurrent EGFR may be a phenomenon more associated with the Stupp Protocol than other modes of treatment – a conclusion that is consistent with our pre-clinical data. The rate of EGFR loss at recurrence is higher than reported elsewhere, such as by van den Bent and colleagues, even after taking into account the difference in the set threshold defining expression change [749]. Finally, although the survival analysis did not produce a significant difference the hazard ratio consistently showed that low EGFR at recurrence carried an increased risk, with the worst risk in patients with greater than 5-fold EGFR decrease compared to the opposing group – the latter group, interestingly, also showed the most favourable median survival time compared to any other subdivided cohort. Increasing the sample size may produce a statistically significant outcome.

4.4 Conclusion

The results from our study suggest that standard therapy resistance and subsequent recurrence is due to radiotherapy and TMZ selecting for low EGFR and MET expressing cells. This leads to ineffectiveness of EGFR and MET inhibition as a mode of therapy and possibly the reason for the failure of the rationale behind targeting these proteins in the clinic

yielding positive results. Clinical specimens obtained from glioblastoma patients revealed that a substantially high number of patients lose EGFR expression at recurrence and have a modest poorer survival rate. In the next chapter we will evaluate the role of miRNAs in glioblastoma cell resistance to radiotherapy and TMZ, in addition to investigating miRNA-mediated suppression of EGFR and MET expression.

CHAPTER 5: ROLE OF MICRORNAS IN TREATMENT-RESISTANT GLIOBLASTOMA

5.1 Introduction

MicroRNAs (miRNAs) are non-coding, short single stranded RNA molecules, approximately 22 nucleotides in length, that are capable of regulating gene expression via direct binding to the mRNA. The association between miRNA and cancer was established relatively recently with a study in 2002 reporting that 13q14 chromosomal regions deleted in leukemia cell contain the genes for the tumour suppressors miR-15 and miR-16, which are commonly down-regulated [751]. Since then, miRNA analysis has been established and is a burgeoning field in cancer research on the basis that a miRNA-based therapeutic paradigm can re-establish a normative miRNA expression and, consequently, proteomic profile. Indeed, within the last decade this school of thought has blossomed and the potential of miRNA to overcome chemotherapeutic resistance is a main focus at both the basic science and clinical ends of precision medicine [752, 753].

Through miRNA binding directly to mRNAs protein translation is suppressed. Therefore, it is possible to divide miRNAs into two categories: tumour suppressors and tumour promoters. Tumour suppressors have complementary binding sites to the mRNAs of oncogenic drivers. For example, in glioblastoma, miR-16 target genes include the anti-apoptotic Bcl2, the cell cycle and proliferation promoter cyclin D1 and the pro-invasion and EMT-associated Sox5, with direct binding leading to anti-proliferation, pro-apoptosis and cell cycle arrest[754]. On the other hand, tumour promoting miRNAs binding activities are known to stimulate oncogenic drivers by inhibiting the translation of mRNAs that would otherwise suppress tumourigenesis. An example is miR-221 over-expression inducing a down-regulation of the migration suppressor PTPu [687]. Similarly, knockdown of miR-21, which is over-expressed in glioblastoma tissues, was first identified in glioblastoma to lead to caspase-dependent apoptosis [646]. Later miR-21 has been found to complementary bind to key apoptotic pathways and componenet such as the p53 pathway components the TGF-beta pathway and APAF1 in addition to promoting invasion and migration via enhanced Sox2 and beta-catenin signalling [755, 756].

In glioblastoma, EGFR signalling is promoted by or correlated with miRNAs such as miR-566, miR-200c and miR-148a [757, 758]. Up-regulating other miRNAs, such as miR-148a, miR-

219-5p and miR-133, are reported to induce EGFR suppression and activation of associated tumourogenic pathways [759, 760] [761]. MET is also regulated by miRNAs such as miR-562 and miR-144-3p [660]. However, literature regarding the MET-miRNA relationship is lacking. Using the Scopus database only 13 articles are generated after a search with the keywords 'c-MET', 'glioblastoma' and 'microRNA' and only 28 studies are generated if 'c-MET' is substituted out for 'MET'.

Aims

Currently, there is a substantial gap in the literature regarding the interplay between miRNA-mediated therapeutic resistance in glioblastoma and either EGFR or MET. To highlight this, a literature search in the Scopus database generates 88 studies in search consisting of 'EGFR', 'glioblastoma' and 'microRNA' but this filters down to 18 articles if 'temozolomide' is added on to the list of keywords during the search. Clearly, there is a need to further explore and expand our knowledge within this field of enquiry. Therefore, our aim in this chapter is to:

Examine the role of miRNA-mediated suppression of EGFR and MET in therapeutic resistance in glioblastoma.

5.2 Results

5.2.1 Differentially expressed miRNAs in radiotherapy and TMZ treated glioblastoma cell lines

To begin our investigation we first utilised Nanostring data generated previously using five glioblastoma cell lines: LN229, U118, U138, U87 and U87vIII. The five cell lines were subject to 5Gy radiotherapy plus 1000uM TMZ for 72 hrs before the expression of 800 miRNAs were analysed. Next, we sorted the miRNAs that were both differentially expressed in the treated cells compared to untreated control by more than 20% in at least one cell line and also were predicted by one of three miRNA bioinformatics database to target either EGFR or MET or both. The three miRNA bioinformatics databases used for our analysis were microRNA.org, miRDB and TargetScan.

Using this selection criterion allowed the formulation of a preliminary list composed of miRNAs that may potentially target both the EGFR and MET **(*Table 5-1*)**. The shortlisted miRNAs are as follows: miR-1266, miR-27b-3p, miR-520b, miR-197-3p, miR-148-3p, miR-301a-3p, miR-30c-5p and miR-589-5p. Only miR-27b-3p and miR-589-5p were not up-regulated by more than 1.5 fold in more than one cell line after treatment **(*Table 5-1 A*)**. The only miRNA to be predicted by at least two databases to target both EGFR and MET was miR-27b-3p, which has been previously validated to target MET. The other seven miRNAs were only predicted to target both genes by one database **(*Table 5-1 B*)**.

A total of seven miRNAs that target EGFR and not MET satisfied the selection criteria: miR-573, miR-1208, miR-574-3p, miR-874, miR-221-3p, miR-134 and miR-107 **(*Table 5-2*)**. MiR-573, miR-221 and miR-107 were up-regulated by over 1.5 fold compared to the respective untreated controls in more than one cell line **(*Table 5-2 A*)**. All miRNAs, except miR-1208, were predicted by at least two databases to target EGFR **(*Table 5-2 B*)**.

Seven miRNAs that target only MET and not EGFR satisfied the above selection criteria: miR-29a-3p, miR-181a-5p, miR-181c-5p, miR-182-5p, miR-1912, miR-34a-5p and miR-943 **(*Table 5-3*)**. From this list, miR-29a-3p, miR-181a-5p, miR-182-5p and miR-34a-5p were up-regulated by more than 1.5 fold in the treated cells of more than one cell line compared to the respective untreated control **(*Table 5-3 A*)**. Only miRNAs, except miR-29a-3p, miR-181c-

5p and miR-1912, were predicted to target MET by at least two databases **(*Table 5-3 B*)**. A list of all miRNAs that were up-regulated by more than 20% in the treated cells is given in ***Table 5-4***.

Table 5-1: MiRNAs that are predicted to target EGFR and MET according to at least one miRNA target bioinformatics database. (A) The expression fold change of each miRNA relative to the untreated control. **(B)** Shows the miRNAs that are predicted to target both EGFR and MET according to three miRNA target prediction softwares. The miRDB score and the TargetScan score given are the target rank and target context scores, respectively. *miR-520b: The EGFR miRDB score for miR-520f-5b was 74. The shown value for MET is for miR-520b-5p specifically. *miR-148b-3p: The Target context score of -0.24 shown is for miR-148-3p. *miR-301a-3p: The EGFR and MET Target context score of -0.04 and -0.41, respectively, is for miR-301-3p. *miR-30c-5p: The EGFR Target context score of -0.01 shown is for miR-30-5p specifically.*miR-589-5p: The MET miRDB score of 90 is for miR-589-3p. ‡: The miRNA family is poorly conserved across species as indicated by TargetScan. NA = Not available or not found.

A)

MicroRNA	Cell line + differential miRNA expression				
	LN229	U118	U138	U87	Δ-U87
miR-1266	1.43	1.67	1.03	0.91	2.33
miR-27b-3p	1.79	0.95	1.12	0.66	0.76
miR-520b	2.15	0.86	1.38	1.55	0.78
miR-197-3p	2.32	0.88	0.96	2.63	2.28
miR-148b-3p	1.19	1.03	0.98	1.67	2.97
miR-301a-3p	1.78	1.18	1.34	1.74	1.02
miR-30c-5p	1.84	1.53	0.21	1.02	1.17
miR-589-5p	1.53	1.24	0.97	1.22	1.30

B)

miRNA	miRSVR		miRDB		TargetScan		Validation
	EGFR	**MET**	**EGFR**	**MET**	**EGFR**	**MET**	
‡ miR-1266	-0.305	-0.176	NA	94	NA	-0.32	NA
miR-27b-3p	-0.94	-0.75	91	79	-0.11	-0.12	MET: [762, 763]
***miR-520b**	-0.17	-0.65	NA	86	NA	NA	EGFR: [764]
‡miR-197-3p		-0.55	NA	NA	-0.01	NA	NA
***miR-148b-3p**		-0.97 -0.49 -0.67	NA	72	-0.24	-0.19	NA
***miR-301a-3p**		-1.06	NA	93	-0.04	-0.41	NA
***miR-30c-5p**		-0.34	NA	NA	-0.01	NA	NA
‡miR-589-5p		-1.21	NA	*90	-0.07	NA	NA

Table 5-2: MiRNAs that are predicted to target EGFR according to at least one miRNA target bioinformatics database. (A) Differential miRNA expression relative to the untreated control. **(B)** MiRNAs predicted to target EGFR according to three miRNA target prediction softwares. ‡miR-574-3p: The TargetScan database indicates that this miRNA is poorly conserved across species. Δ: The miRNA family is conserved only among mammals**.***miR-134: The TargetScan and miRDB score shown is for miR-134-5p.

A)

MicroRNA	**Cell line + differential miRNA expression**				
	LN229	U118	U138	U87	Δ-U87
miR-573	2.65	1.06	2.80	0.83	0.86
miR-1208	1.23	2.39	1.05	1.14	1.12
miR-574-3p	1.24	1.27	1.06	1.24	1.40
miR-874	1.31	2.62	0.99	1.93	1.10
miR-221-3p	1.61	1.20	0.60	1.66	2.44
miR-134	1.12	1.24	1.93	0.74	0.78
miR-107	1.20	1.00	0.99	1.88	1.62

B)

MicroRNA	MiRSVR	miRDB	TargetScan	Validation
miR-573	-1.09 -0.12	80	NA	[765]
miR-1208	-0.85	NA	NA	NA
‡ miR-574-3p	-0.46	NA	-0.23	[766]
****miR-874**	-0.33	NA	-0.08	Via directly targeting DOR [767]
miR-221-3p	-0.27	NA	-0.27	Downstream of EGFR [768, 769]. Increased EGFR associated with increased miR-221 [770]
Δ * miR-134	-1.06	59	-0.42	[771] [772]
miR-107	-0.32	NA	-0.04	miR-107-5p: [773]

Table 5-3: MiRNAs that are predicted to target MET according to at least one miRNA target bioinformatics database. (i) The expression fold change of each miRNA relative to the untreated control. **(ii)** Shows the miRNAs that are predicted to target EGFR according to three miRNA target prediction softwares. *miR-181a-5p: TargetScan score shown is for miR-181-5p.*miR-34a-5p: The miRDB score of 80 shown is for miR-34b-5p; however the score of 98 is for miR-34a-5p. The TargetScan score of -0.63 is for miR-34a-5p and miR-34c-5p.

A)

MicroRNA	Cell line + differential miRNA expression				
	LN229	U118	U138	U87	Δ-U87
hsa-miR-29a-3p	2.14	0.83	0.92	2.69	2.33
hsa-miR-181a-5p	1.19	0.90	1.54	2.50	2.55
hsa-miR-181c-5p	1.10	1.31	1.07	1.22	0.71
hsa-miR-182-5p	1.43	1.79	0.52	1.62	0.62
hsa-miR-1912	1.47	1.24	0.94	1.52	0.95
hsa-miR-34a-5p	1.68	1.13	1.43	1.23	2.68
hsa-miR-943	1.74	1.09	1.02	0.50	1.30

B)

MicroRNA	MiRSVR	miRDB	TargetScan	Validation
miR-29a-3p	-0.65	NA	NA	NA
miR-181a-5p	-0.87	NA	-0.04	[774]
miR-181c-5p	-0.94 -0.87	NA	NA	NA
miR-182-5p	-0.52 -0.57	64	-0.30	NA
miR-1912	-0.80	NA	NA	NA
***miR-34a-5p**	-0.37 -0.92	80 (34b-5p) 98 (miR-34a-5p & 34c-5p)	-0.63	[775]
miR-943	-1.07	55	NA	NA

Table 5-4: List of miRNAs differentially over-expressed by ≥20% in 3 or more treated cell lines.

MicroRNA
miR-589-5p
miR-301a-5p
miR-197-3p
Mir-520b
miR-1266
miR-107
miR-874
miR-574p
miR-1912
miR-182-5p
miR-181a-5p
miR-34a-5p
miR-29a-3p
miR-221-3p

5.2.2 Differentially expressed miRNAs are associated with glioblastoma survival

The differentially expressed miRNAs from Table 5-4, showing the miRNAs that are up-regulated by more than 20% in the majority of cell lines, were further analysed using the TCGA survival data from OncoLnc. Patients belonging to either the 25th percentiles of high and low expressing groups or the 35th percentile of high and low expressing groups were analysed. The 25th percentile was chosen to select a patient cohort that excludes differential noise and the 35th percentile was chosen to incorporate a larger cohort, though ultimately preferring these two percentiles is arbitrary. The majority of these miRNAs had unavailable data pertaining to the relationship between glioblastoma patient survival rates or were insignificantly associated with survival **(*Table 5-5*)**. Except for miR-301a-3p, in which there was a significant association between favourable glioblastoma survival and high expression when patients were divided according to the 35th percentile **(*Figure 5-1 A*)**.

The following miRNAs were associated significantly with glioblastoma survival after patients were divided by both the 25th percentile and 35th percentile: miR-182-5p, miR-221-3p (miR-

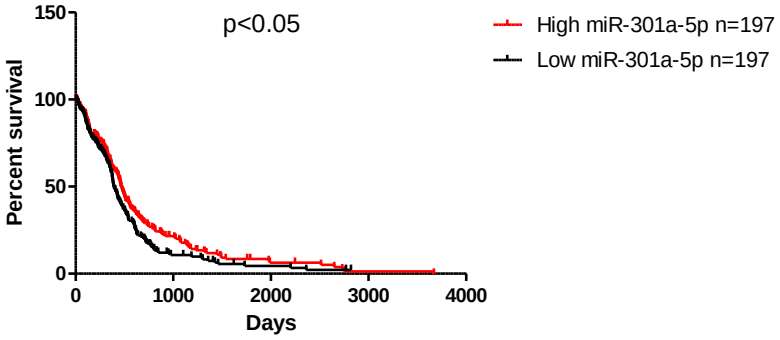
221), miR-34a-5p (miR-34) and miR-29a-3p (miR-29). High miR-182-5p expression was associated with favourable glioblastoma patient survival at both the 25th and 35th percentiles **(*Figure 5-1 B*)**. On the contrary, high miR-221, miR-34 and miR-29 expression was significantly associated with poorer glioblastoma survival at both the 25th and 35th percentiles **(*Figure 5-1 C-E*)**.

Therefore, miR-29, miR-34 and miR-221 were selected for further study as these miRNAs were up-regulated in the majority of our cell lines treated with radiotherapy and TMZ, predicted to target EGFR (as is the case with miR-221) or MET (as is the case with miR-29 and miR-34) and, lastly, high expression of these miRNAs are associated with poorer glioblastoma survival according to TCGA data. The binding sequences of these three miRNAs with their respective target genes are shown in ***Figure 5-2***. Both miR-29 and miR-221 are predicted to target a single region of MET and EGFR, respective, while miR-34 is predicted to target two regions of MET mRNA.

Table 5-5: List of miRNAs with unavailable survival data or with non-significant association with patient survival. Patients were stratified according to either top and bottom 25th percentile or 35th percentile. The p-value for each survival curve is given. If miRNA data is unavailable on OncoLnc '×' will be displayed.

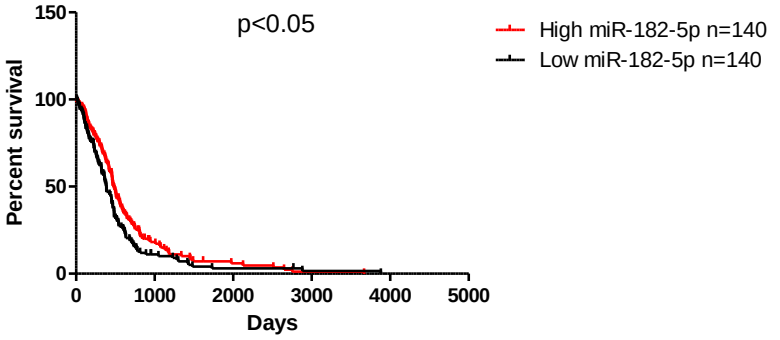
MicroRNA	Data available	p-value	
		25th percentile	35th percentile
miR-589-5p	×		
miR-301a-3p		0.17	0.04
miR-197-3p		0.38	0.12
Mir-520b		0.54	0.32
miR-1266	×		
miR-107		0.60	0.74
miR-874	×		
miR-574-3p		0.42	0.14
miR-1912	×		
miR-181a-5p		0.63	0.68

A)

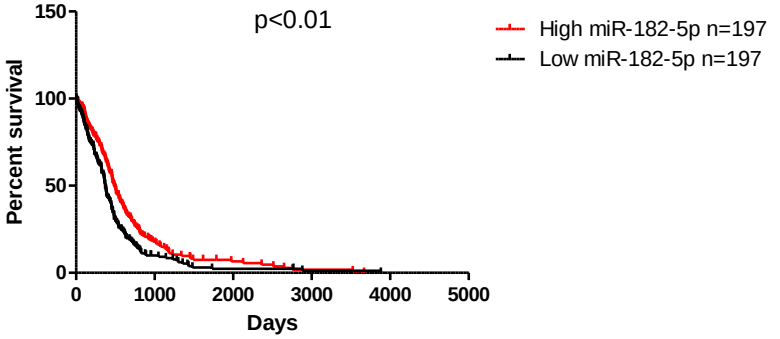

p<0.05
High miR-301a-5p n=197
Low miR-301a-5p n=197
Percent survival
150
100
50
0
0
1000
2000
3000
4000
Days

B)

(i)

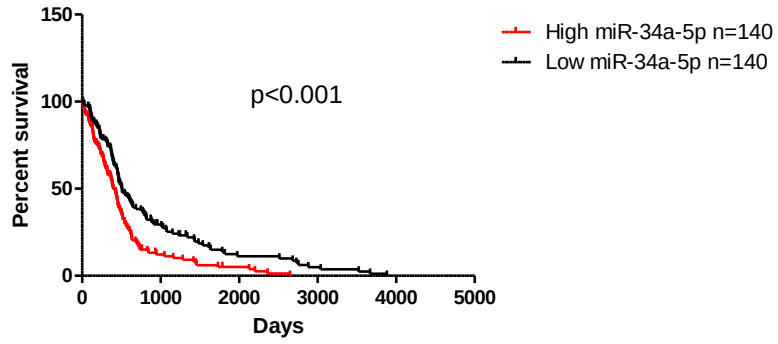

p<0.05
High miR-182-5p n=140
Low miR-182-5p n=140
Percent survival
150
100
50
0
0
1000
2000
3000
4000
5000
Days

(ii)

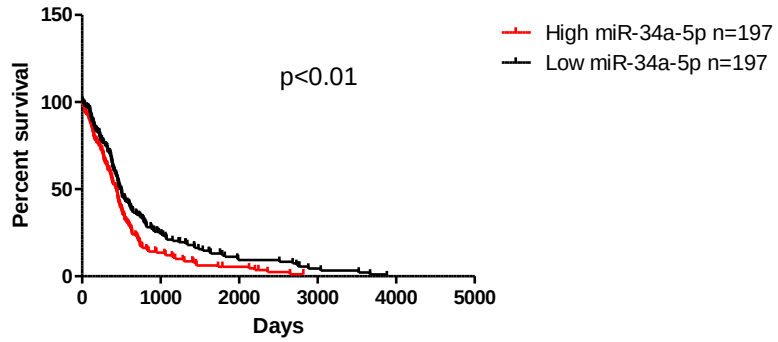

p<0.01
High miR-182-5p n=197
Low miR-182-5p n=197
Percent survival
150
100
50
0
0
1000
2000
3000
4000
5000
Days

C)

(i)

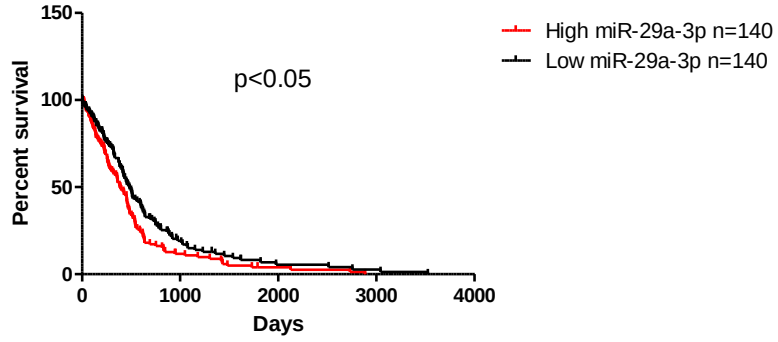

High miR-34a-5p n=140
Low miR-34a-5p n=140
p<0.001
Percent survival
Days
150
100
50
0
0
1000
2000
3000
4000
5000

(ii)

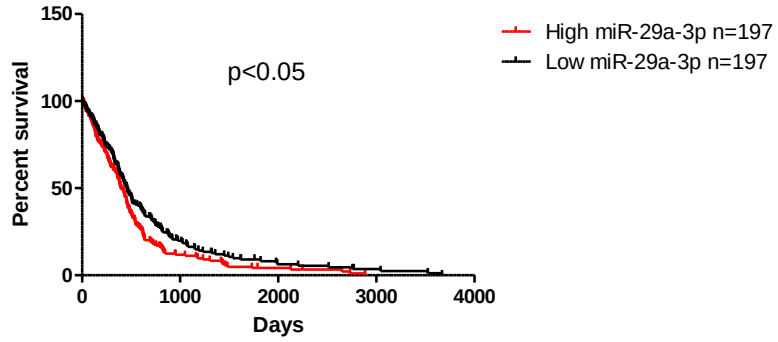

High miR-34a-5p n=197
Low miR-34a-5p n=197
p<0.01
Percent survival
Days
150
100
50
0
0
1000
2000
3000
4000
5000

D)

(i)

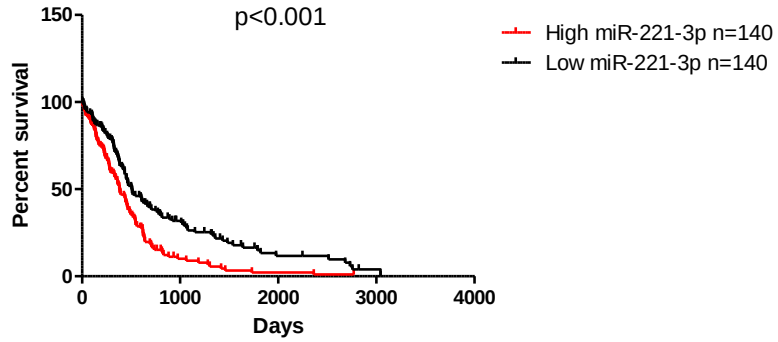

High miR-29a-3p n=140
Low miR-29a-3p n=140
p<0.05
Percent survival
Days
150
100
50
0
0
1000
2000
3000
4000

(ii)



E)

(i)



(ii)

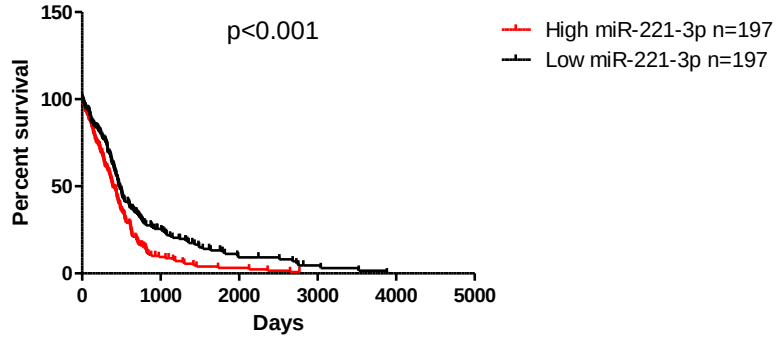
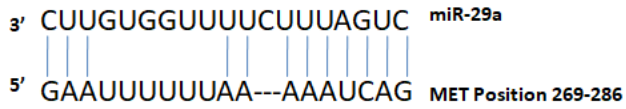


Figure 5-1: Poor survival is associated with increased expression of short-listed miRNAs. Shortlisted miRNAs that were over-expressed by in treated cells were further filtered according to whether expression was associated with patient survival. **(A)** Kaplan-Meier plot for 35th percentile high- and low-expressing miR-301a-5p. Low miR-301a-5p expression was associated with worse survival compared to high expression. **(B)** Kaplan-Meier plot for **(i)**

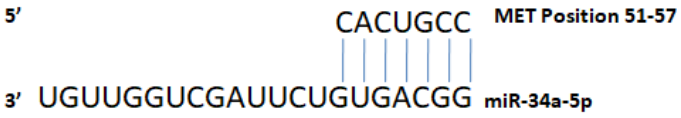
25^{th} and **(ii)** 35^{th} percentile high- and low-expressing miR-182-5p. Low expression of miR-182-5p was associated with worse survival compared to high expression. **(C)** Kaplan-Meier plot for **(i)** 25^{th} and **(ii)** 35^{th} percentile high- and low-expressing miR-34a-5p. High expression of miR-34a-5p was associated with worse survival compared to low expression. **(D)** Kaplan-Meier plot for **(i)** 25^{th} and **(ii)** 35^{th} percentile high- and low-expressing miR-29a-3p. High expression of miR-29a-3p was associated with worse survival compared to low expression. **(E)** Kaplan-Meier plot for **(i)** 25^{th} and **(ii)** 35^{th} percentile high- and low-expressing miR-221-3p. High expression of miR-221-3p was associated with worse survival compared to low expression.

A)

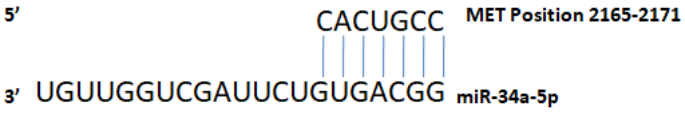


B)

(i)



(ii)



C)

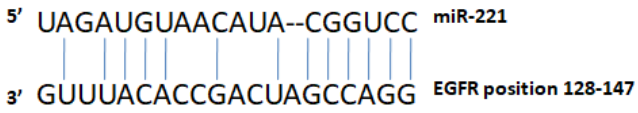


Figure 5-2: The base pairing between short-listed miRNAs and target genes. (A) miR-29a target sequence and potential binding site with MET (sourced from microRNA.org). **(B)** The two potential binding target areas of miR-34a with MET: **(i)** position 51-57 of MET and **(ii)** position 2165-2171 of MET as indicated by TargetScan (http://www.targetscan.org/vert_72/). **(C)** miR-221 target sequence and potential binding site with EGFR (sourced from microRNA.org).

5.2.3 The miR-34a is up-regulated in selected treatment resistant glioblastoma cells

The expression levels of miR-29a and miR-34a - both predicted to target the MET mRNA and considered tumour suppressors - were analysed using qRT-PCR in the high and low MET expressing parental #41 and treatment resistant #41R cell line, respectively. Both miRNAs were found to be significantly down-regulated in the resistant cells compared to the parental #41 **(*Figure 5-3 A*)**. However, when analysing the miR-34a levels in the resistant U87R and U87-V relative to the parental U87, miR-34a was found to be up-regulated **(*Figure 5-3 B*)**. Similarly, relative to the parental U251, miR-34a was up-regulated and down-regulated in the resistant U251R and the sensitive U251-V, respectively **(*Figure 5-3 C*)**. Therefore, in selected resistant cell lines with low MET expression, miR-34a is up-regulated and consistent with our hypothesis.

5.2.4 The miR-221 is highly expressed in resistant glioblastoma cells

Next, the expression levels of miR-221 was analysed in the high EGFR expressing #41 parental and the low EGFR expressing #41R variant using qRT-PCR. MiR-221 was significantly up-regulated in the #41R compared to the #41P **(*Figure 5-4 A*)**. Furthermore, miR-221 was also up-regulated in U118R and U251R compared to the respective parental controls **(*Figure 5-4 B*)**. Similarly, both the U87R and U87-V cell line (with reduced EGFR expression compared to the parental cells) showed up-regulated miR-221 levels **(*Figure 5-4 C*)**. MiR-221 was also highly expressed in #20 and #41 cells treated with 5Gy radiotherapy and exposed to 1000uM TMZ for 7 days compared to the respective untreated controls **(*Figure 5-4 D*)**. Therefore, miR-221 is up-regulated in all tested resistant glioblastoma cell lines and consistent with our hypothesis that the EGFR is negatively regulated by miR-221.

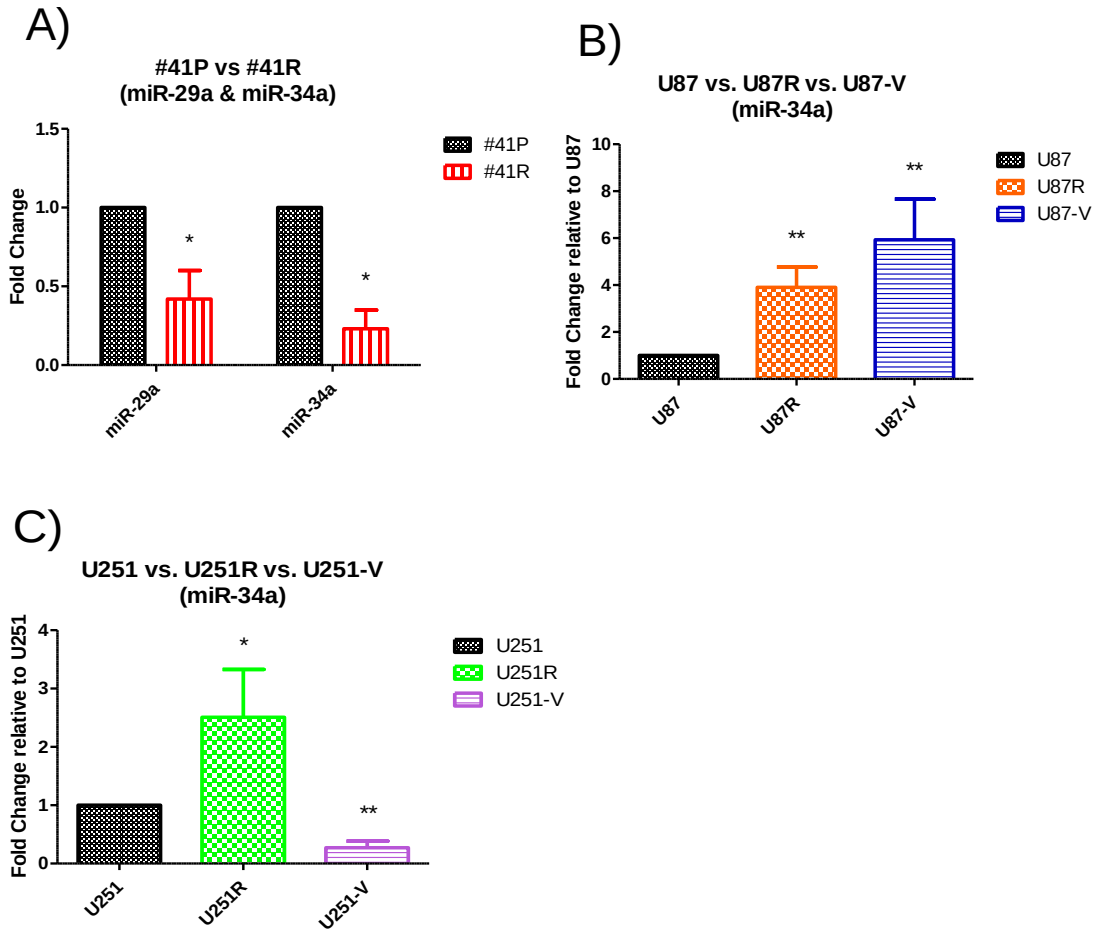


Figure 5-3: miR-34a is highly expressed in treatment resistant cell lines. Expression levels of miR-34a were analysed using qRT-PCR in several glioblastoma cell lines. **(A)** Relative to the #41P, miR-34a levels in the #41R was significantly up-regulated. **(B)** U87R and U87-V cells had up-regulated miR-34a relative to U87. **(C)** U251R and U251-V miR34a levels were up- and down-regulated relative to U251 cells. All values are mean ± SD; *p<0.05, **p<0.01; n=3.

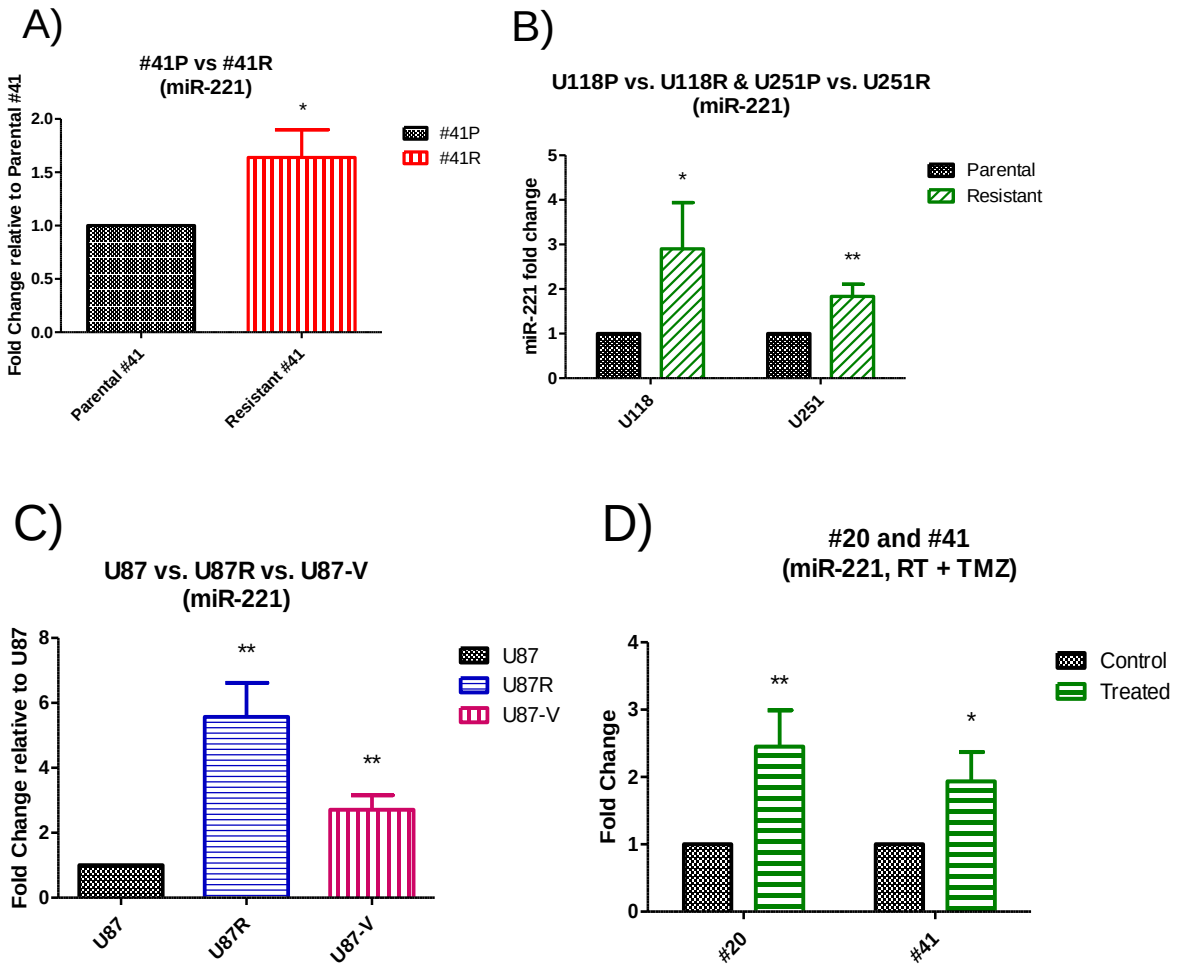


Figure 5-4: miR-221 is up-regulated in treatment resistant cell lines. (A) Shows expression levels of miR-221 in #41R relative to the parental cell line. **(B**) miR-221 expression levels in U118R and U251R relative to the corresponding parental cell line. **(C)** MiR-221 expression levels in U87R and U87-V relative to the parental U87. **(D)** #20 and #41 were treated with irradiation and TMZ, incubated for 7 days and miR-221 levels were quantified in the treated cells relative to the untreated control. All values are mean ± SD; *p<0.05, **p<0.01; n=3.

5.2.5 miR-221 regulates the EGFR expression in glioblastoma recurrent cells

Given that miR-221 was up-regulated in both the TMZ-resistant cell lines and the recurrent model #41R, we next examined whether miR-221 plays a role in mediating resistance to radiotherapy and TMZ. For this experiment #41R was selected as it was the only cell line generated resistant to both radiotherapy and TMZ and better replicates clinical resistance to treatment. First, miR-221 mimic (pre-miR-221) and the miR-221 inhibitor (as-miR-221) were transfected into the #41P and #41R, respectively **(*Figure 5-5 A*)**. In the #41P cells, transfected with pre-miR-221, miR-221 expression increased by 2 fold, relative to the scrambled control. Similarly, miR-221 levels in #41R cells, transfected with as-miR-221 sequence, displayed a reduction of 2 fold, relative to scrambled control.

However, after evaluating EGFR mRNA levels in both transfected cell lines, only the #41P transfected with pre-miR-221 led to a decrease in EGFR mRNA levels, by more than 2 fold as measured by qRT-PCR **(*Figure 5-5 B*)**. The EGFR mRNA levels in the #41R was not rescued and remained undetectable after transfection with as-miR-221 (data not shown), demonstrating that the EGFR gene may not be transcribed.

We next studied the relation between miR-221 over-expression, EGFR down-regulation and therapeutic resistance. #41P cells transfected with pre-miR-221 - and therefore had reduced EGFR expression - were significantly more resistant to therapy compared to the unscrambled and non-transfected controls over the 7 day assay time period **(*Figure 5-5*)**. There was no difference in cell viability between the non-transfected #41P cells and the negative scrambled control indicating that the reduction in cell viability after treatment to be independent of the transfection method. Therefore, we conclude that increased miR-221 down-regulates EGFR and increases therapeutic resistance in primary glioblastoma cells.

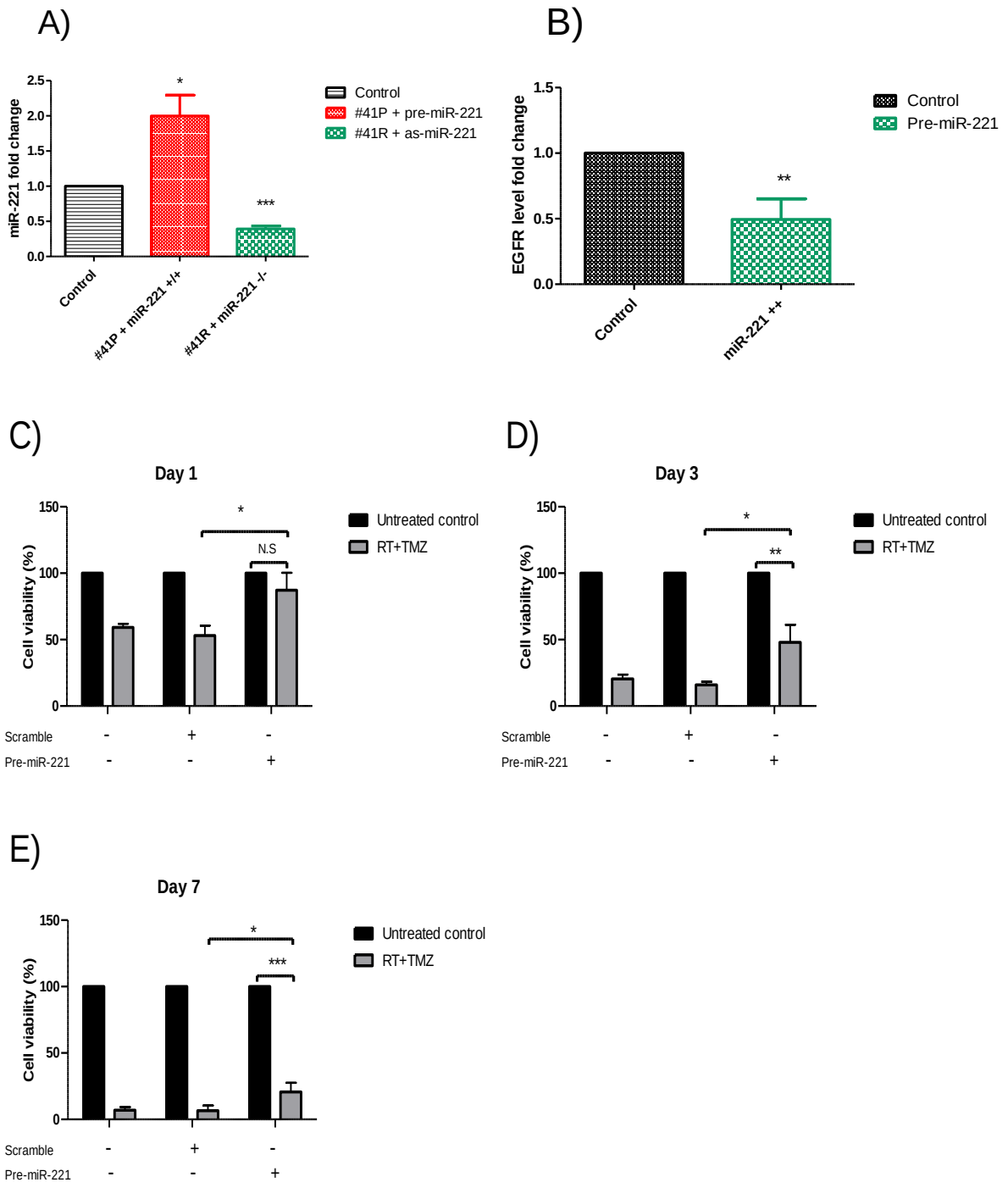


Figure 5-5: Up-regulating miR-221 leads to decreased EGFR levels and increased treatment resistance. (A) #41P and #41R were transfected with pre-miR-221 and as-miR-221. Pre-miR-221 and as-miR-221 treatment leads to significantly increased and decreased miR-221 levels, respectively. **(B)** Shows that pre-miR-221 reduces the level of EGFR expression in #41P. Next, the #41P non-transfected, scrambled-transfected and pre-miR-221 transfected cells underwent treatment with irradiation and TMZ. Cell viability was then measured **(C)** 1

day **(D)** 3 days and **(E)** 7 days after treatment. All values are mean ± SD; *p<0.05, ** p<0.01, *** p<0.001; n=3.

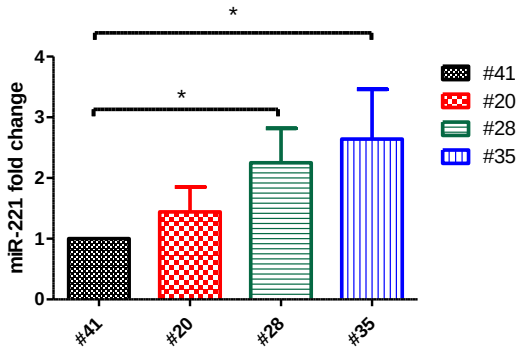
5.2.6 miR-221 is up-regulated in recurrent patient glioblastoma

Our results suggest that miR-221 suppresses EGFR expression leading to an increase of tumour resistance to radiotherapy and TMZ. We next compared miR-221 levels between patient-derived primary and recurrent glioblastoma cell lines. The primary cell lines #41 and #20 were chosen in addition to recurrent cell lines #28 and #35. We found that miR-221 was highly expressed in #28 and #35 compared to #41. Although both recurrent cell lines had higher miR-221 expression compared to #20, this difference was not significant **(*Figure 5-6 A*)**. It shall be recalled that this correlates with lower EGFR expression in both the recurrent cell lines compared to the primary cell lines as observed in **chapter 4.**

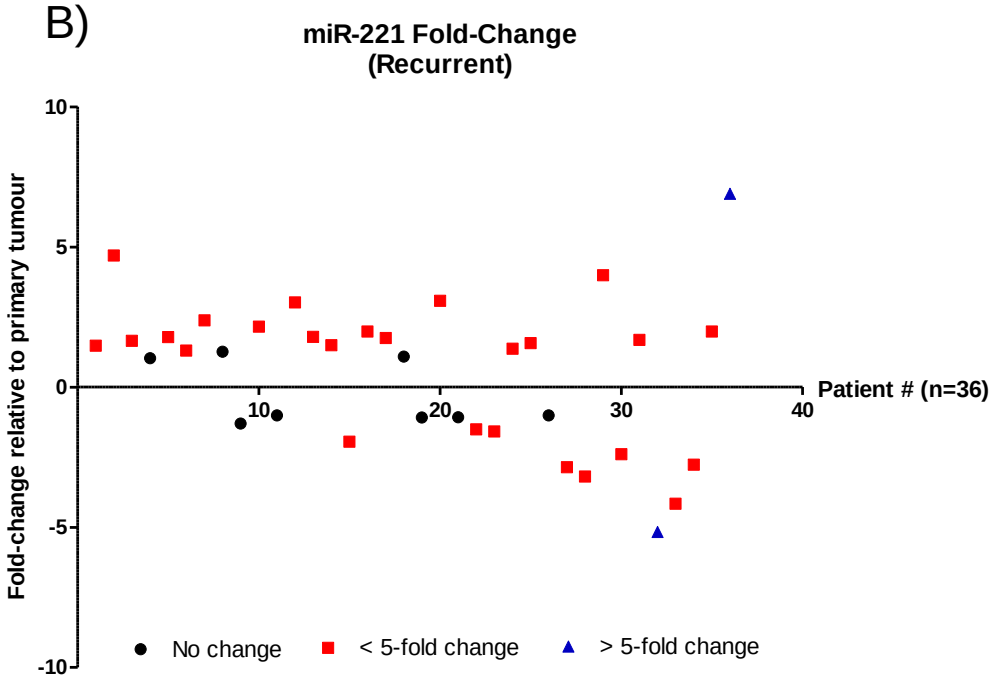
To validate the *in vitro* observation of miR-221 negatively regulating EGFR, a glioblastoma patient cohort (n=107) was used, which included the paired recurrent patient cohort from **chapter 4**. Using qRT-PCR, the expression levels of miR-221 (along with EGFR as was previously seen in chapter 4) was assessed. We first analysed the miR-221 levels in the recurrent patient cohort (n=36) without stratifying according to treatment. The majority of patients had increased miR-221 expression (19/36, 53%), eight patients had stable expression (22%) and nine displayed decreased expression (25%) **(*Figure 5-6 B*)**. In the cohort which consisted of patients that were treated with radiotherapy alone, TMZ alone or the Stupp Protocol (n=31), 25 patients were treated with the Stupp Protocol. 15/25 (60%) Stupp Protocol treated patients had increased miR-221 levels and 7/25 (28%) patients had decreased miR-221 levels **(*Figure 5-6 C*)**.

Next, we analysed the survival rates of the recurrent patient cohort according to miR-221 differential expression. In the non-stratified cohort (n=27) high recurrent miR-221 expressing patients had significantly worse prognosis (according to the Gehan-Breslow-Mantelcox test) compared to the low miR-221 expressing group **(*Figure 5-6 D*)**. A similar worse prognosis was also observed when patients that received any additional treatment (radiotherapy alone, TMZ alone or Stupp Protocol) were selected and sorted according to high and low miR-221 expression but this was not significant **(*Figure 5-6 E*)**. Similarly, when patients who were treated with the Stupp Protocol only were selected and sorted according to miR-221 expression, high recurrent miR-221 expressing patients had a worse but insignificant prognosis **(*Figure 5-6 F*)**.

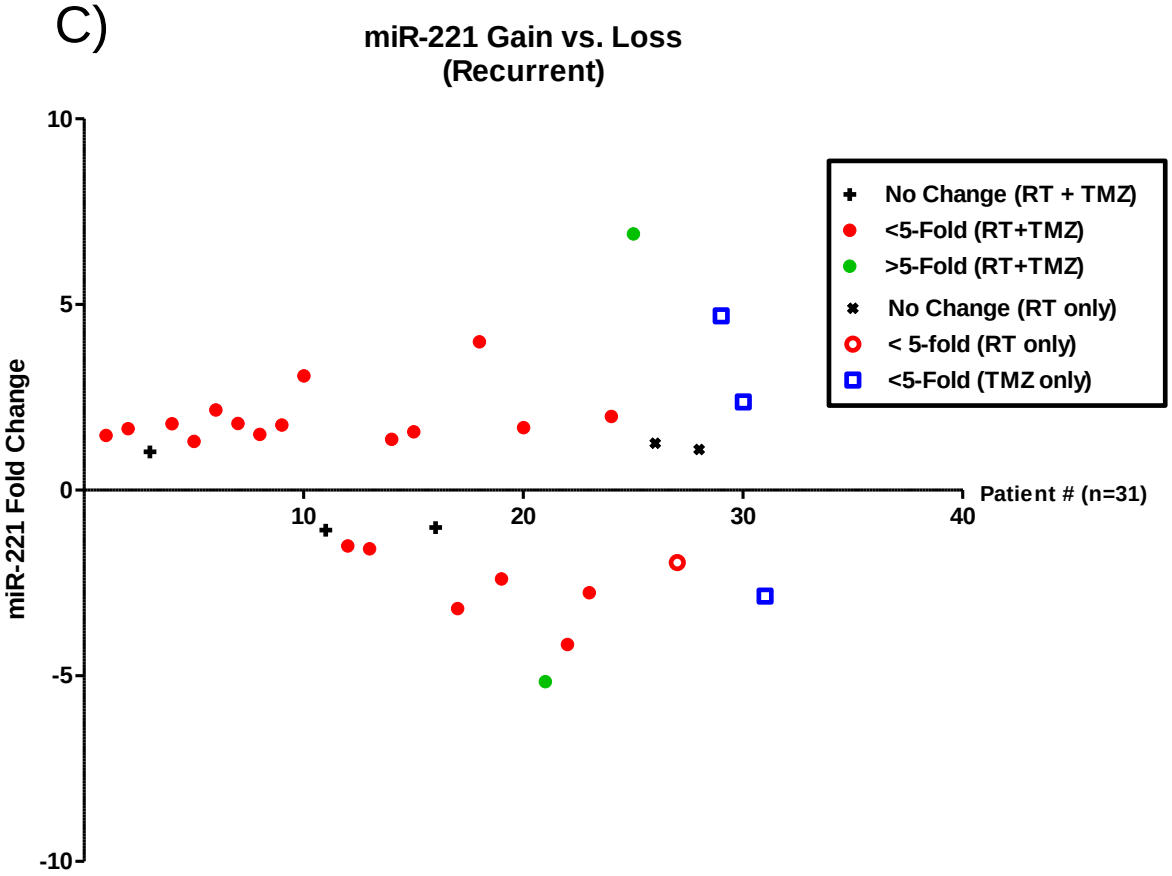
A)


*
*
4
3
2
1
0
miR-221 fold change
#41
#20
#28
#35
#41
#20
#28
#35

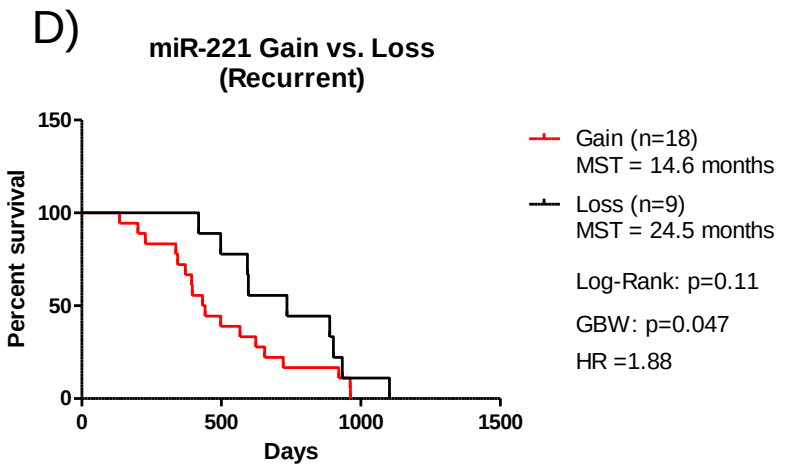
B)


miR-221 Fold-Change
(Recurrent)
10
5
0
-5
-10
Fold-change relative to primary tumour
10
20
30
40
Patient # (n=36)
No change
< 5-fold change
> 5-fold change

C)


miR-221 Gain vs. Loss
(Recurrent)
miR-221 Fold Change
10
5
0
-5
-10
10
20
30
40
Patient # (n=31)
No Change (RT + TMZ)
<5-Fold (RT+TMZ)
>5-Fold (RT+TMZ)
No Change (RT only)
< 5-fold (RT only)
<5-Fold (TMZ only)

D)


miR-221 Gain vs. Loss
(Recurrent)
Percent survival
150
100
50
0
0
500
1000
1500
Days
Gain (n=18)
MST = 14.6 months
Loss (n=9)
MST = 24.5 months
Log-Rank: p=0.11
GBW: p=0.047
HR =1.88

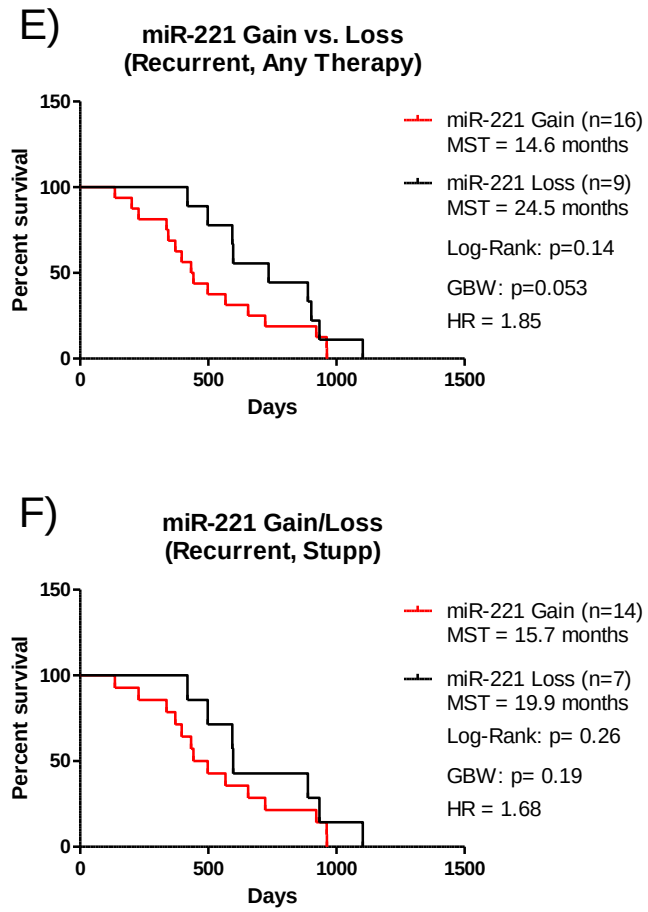


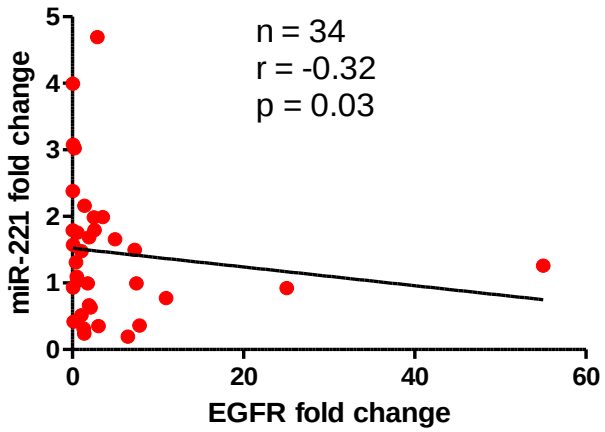
Figure 5-6: Recurrent glioblastoma have increased miR-221 levels. (A) MiR-221 levels in recurrent cell line #28 and #35, and primary cell line #20, relative to #41. Value shown is mean ± SD. **(B)** MiR-221 expression fold-change at recurrence for 36 patients. All values are relative to the levels of miR-221 in the paired primary tumour. **(C)** MiR-221 expression fold-change at recurrence for patients that received any additional therapy (radiotherapy or/and TMZ) to surgical resection. All values are relative to the levels of miR-221 in the paired primary tumour. **(D)** Survival curve after dividing patients who had increased miR-221 and decreased miR-221. One patient was excluded as an outlier. **(E)** Survival curve with recurrent patients who received any additional therapy (radiotherapy or/and TMZ) to surgical resection. Patients were divided according to high and low miR-221 expression at recurrence, relative to the paired primary tumour. **(F)** Survival curve with recurrent patients who only received the Stupp Protocol. Patients were divided according to high and low miR-221 expression at recurrence. MST = Median Survival Time; Log-Rank = Mantel-Cox method; GBW = Gehan-Breslow-Wilcoxon method; HR = Hazard Ratio (High:Low); n= number of patients.

5.2.7 miR-221 inversely correlates with EGFR in glioblastoma patients

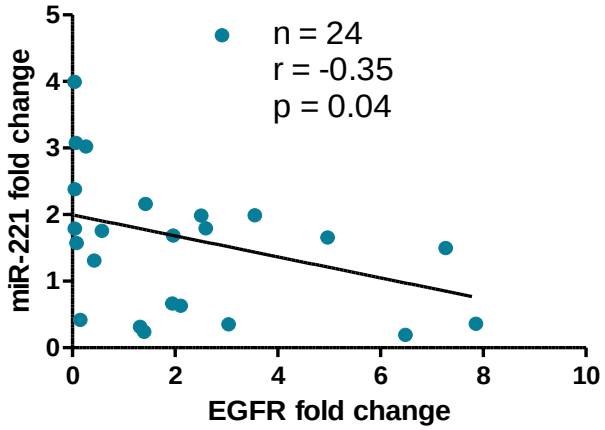
Using the same patient cohort further analysis was performed to evaluate the relationship between miR-221 and EGFR in glioblastoma patients. A cohort of 34 patients with both primary and recurrent samples was available for analysis. With each patient the fold-change levels of miR-221 and EGFR at recurrence, relative to the primary tumour, were matched and plotted on a scatter plot **(*Figure 5-7 A*)**. Data analysis revealed a statistically significant inverse correlation between miR-221 fold-change at recurrence and EGFR fold-change at recurrence. Furthermore, when only patients with a fold difference of greater than 30% were included, a similar significant inverse correlation was observed **(*Figure 5-7 B*)**. Primary tumours only were also assessed for a correlation between miR-221 and EGFR, and this cohort consisted of a total of 105 patients. A significant inverse correlation between miR-221 and EGFR was also observed **(*Figure 5-7 C*)**. Patients with a lower miR-221 delta CT (dCT) value (which inversely represents expression) had a higher EGFR dCT value; therefore, an increase in miR-221 expression in primary tumours correlates with a decrease in EGFR expression.

Lastly, recurrent patients were stratified accordingly into two groups. One group consisted of patients with an increase in miR-221 and a decrease in EGFR expression at recurrence, relative to the primary tumour. Patients who had higher miR-221 levels displayed a significantly worse prognosis compared to low miR-221 expressing patients in the Gehan-Breslow-Wilcoxon test and reached but did not cross the statistical significance threshold for the log-rank test **(*Figure 5-7 D*)**. When patients from the two groups were selected if they received any treatment (radiotherapy or/and TMZ) in addition to surgical resection **(*Figure 5-7 E*)** or who were treated with the Stupp Protocol **(*Figure 5-7 F*)**, high miR-221 expressing patients had a worse prognosis though the differences were not significant, perhaps due to the smaller sample size.

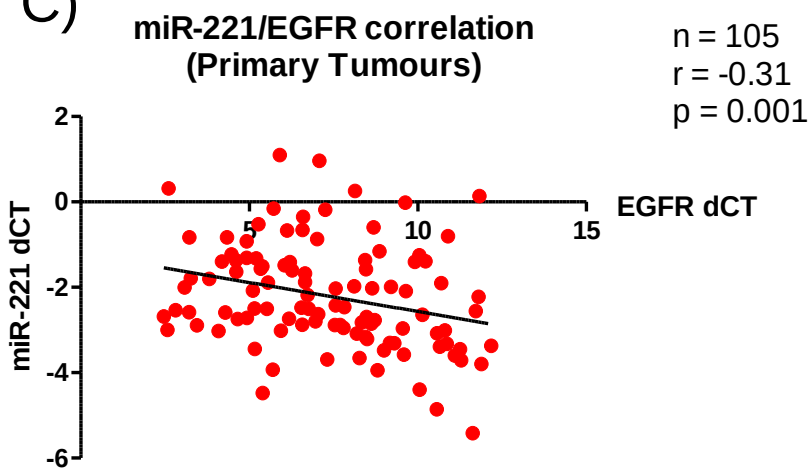
A)


miR-221/EGFR correlation
(Recurrent Tumours, ALL)
n = 34
r = -0.32
p = 0.03
miR-221 fold change
EGFR fold change
0
1
2
3
4
5
0
20
40
60

B)

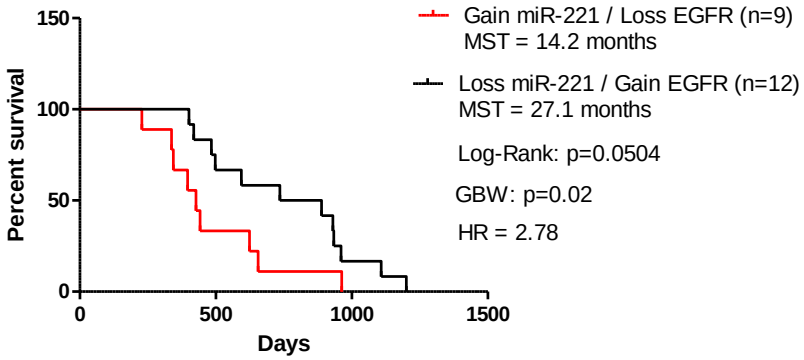

miR-221/EGFR correlation
(Recurrent Tumours, 30%)
n = 24
r = -0.35
p = 0.04
miR-221 fold change
EGFR fold change
0
1
2
3
4
5
0
2
4
6
8
10

C)


miR-221/EGFR correlation
(Primary Tumours)
n = 105
r = -0.31
p = 0.001
miR-221 dCT
EGFR dCT
2
0
-2
-4
-6
5
10
15

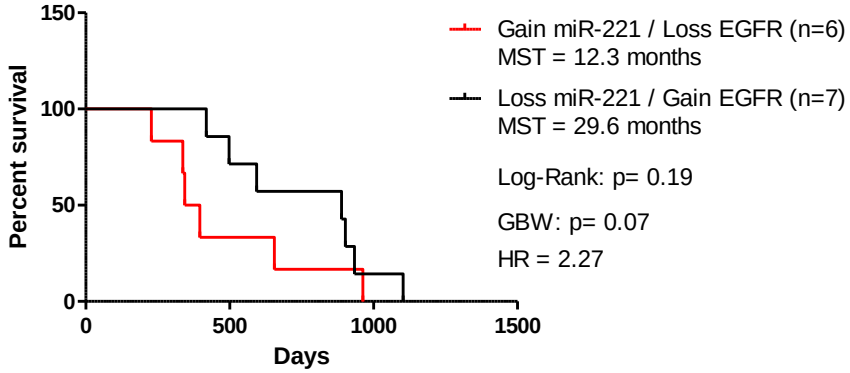
D)

Gain miR-221/Loss EGFR vs. Loss miR-221/Gain EGFR (Recurrent)



E)

miR-221 Gain / EGFR Loss vs. miR-221 Loss / EGFR Gain (Recurrent, Any Therapy)



F)

miR-221 Gain / EGFR Loss vs. miR-221 Loss / EGFR Gain (Recurrent, Stupp)

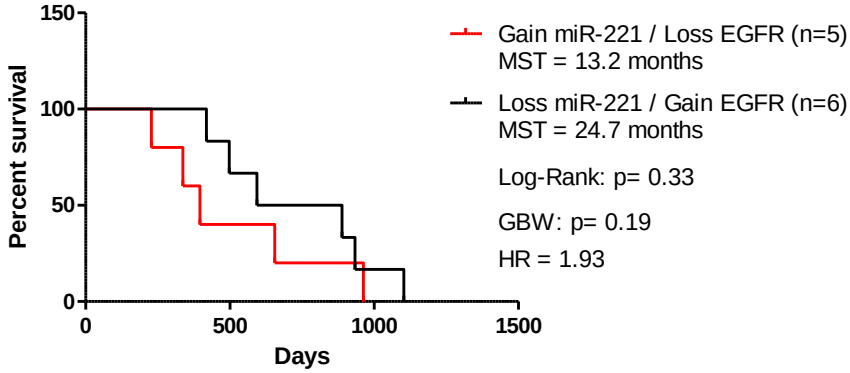


Figure 5-7: miR-221 expression inversely correlates with EGFR expression. (A) The fold-change of miR-221 and EGFR levels at recurrence of 34 patients compared to paired primary tumours. The inverse correlation between miR-221 and EGFR levels was significant. **(B)** Only patients with a fold-change in miR-221 and EGFR levels of greater than 30% were selected **(C)** EGFR and miR-221 expression in 107 primary tumours were assessed and dCT values were plotted. There was a significant inverse correlation between EGFR and miR-221. Two values were excluded due to being outliers. **(D)** Kaplan-Meier plot after separating patients who had increased miR-221 and decreased EGFR from decreased miR-221 and increased EGFR. **(E)** Kaplan-Meier plot after selecting recurrent patients who were treated with radiotherapy or/and TMZ in addition to surgical resection and separating patients who had increased miR-221 and decreased EGFR from decreased miR-221 and increased EGFR. **(F)** Kaplan-Meier plot after selecting recurrent patients who were treated with the Stupp Protocol and separating patients who had increased miR-221 and decreased EGFR from decreased miR-221 and increased EGFR. For the correlation plots: p= p-value; n= number of patients; r = spearman correlation. For the Kaplan-Meier plot: MST = Median Survival Time; Log-Rank = Mantel-Cox method; GBW = Gehan-Breslow-Wilcoxon method; HR = Hazard Ratio (High:Low); n= number of patients.

5.3 Discussion

Increasingly, miRNA regulation of tumourigenesis is becoming a main field of research into glioblastoma signalling akin to the earlier era in which the RTK first gained attention. MiRNAs are known as crucial gene expression regulators due to their role in complementarily binding to the transcribed mRNA, thereby silencing its translation. Since our previous chapters implicated the silenced EGFR and MET as key resistance mechanisms in glioblastoma we now posited the miRNA to possess a key mediatory role between the silenced RTK and resistance. MiRNA regulation of EGFR and MET is understudied perhaps due to the relative novelty of miRNA regulation as a resistance mechanism in glioblastoma and/or the traditional paradigm in which the RTK in particular EGFR and MET, was thought to be a driver in glioblastoma resistance. This provided further urgency to choose to study miRNA regulation as a means to resistance over other mechanisms, such as methylation profile, gene transcription regulators and compensatory signalling, whether it be via RTKs or otherwise.

In this chapter we have shown that miR-34a is up-regulated in a selected number of treatment resistant cell lines. This finding is largely contradictory to the dominant understanding of miR-34 in cancer. In several cancers, including lung, colon and prostate cancer, miR-34 is regarded as a tumour suppressor [645]. In fact, miR-34 mimic has been trialled in phase 1 clinical trial, although the study was prematurely discontinued due to adverse events [645]. Only three articles are generated on Scopus with the key terms ‘miR-34’ and ‘glioma’ and only one article is generated with key terms ‘miR-34’ and ‘glioblastoma’. In 2013, it was reported that rescuing miR-34 inhibits proliferation in pro-neural, PDGFR-amplified glioblastoma cell lines and miR-34 target PDGFR-a [776]. It is likely that miR-34 expression is cell line-specific and this may explain the differences in the three resistant cell lines in our study. Nonetheless, given that miR-34 is highly expressed in a number of resistant cell lines and down-regulated in the sensitive U251-V, it is possible that the role of miR-34 differs compared to other malignancies though further study is required.

Compared to miR-34, miR-221 is better understood regarding its role in glioblastoma. It is generally considered as an oncomiR, stimulating proliferation, invasion and tumourigenesis

[687, 703]. However, the role of miR-221 in glioblastoma is found to be controversial with conflicting accounts, perhaps because only recent studies have attempted to elucidate the mechanism. For example, miR-221 was found to correlate with glioma grade and as-miR-221 treatment has been shown to increase apoptosis and target tumour suppressors in glioblastoma cell lines [681, 687, 777]. Contradictorily, miR-221 was found recently to target MGMT to increase sensitivity to TMZ and caspase-3 activation [686]. In addition to the contradicting accounts, there is no report concerning the role of miR-221 in resistance to radiotherapy and TMZ. This chapter demonstrated that pre-miR-221 can increase resistance to radiotherapy and TMZ and, potentially, inhibition of miR-221 may be a potential therapeutic strategy for glioblastoma.

Our results support the categorisation of miR-221 as an oncomiR and we have demonstrated that treatment resistant cell lines have up-regulated miR-221 expression. Up-regulation of miR-221 was also observed in the majority of Stupp Protocol treated patients in a cohort consisting of recurrent patients. Notably, our data proposes that miR-221 mediates resistance via the down-regulation of EGFR. No account on the association between miR-221 and EGFR exists for glioblastoma, though there have been reports on a positive regulatory loop mapping EGFR upstream of miR-221 in breast and non-small cell lung cancer [769] [768]. Our report differs from these two previous papers given that pre-miR-221 was found to down-regulate EGFR and as-miR-221 led to an increase in EGFR, which both maps miR-221 upstream of EGFR and contextualising the association as a negative regulatory loop. We further validated these *in vitro* results in a cohort consisting of recurrent patients (n=36) and primary glioblastoma patients (n=105) and observed an inverse correlation between miR-221 and EGFR.

In this present study we also demonstrated that displaying low miR-221 expression at first recurrence is associated with a favourable patient prognosis; however only in the cohort that included all recurrent patients, which consisted of patients regardless of the treatment received, was a significant association observed. A similar trend of favourable prognosis for low miR-221 expressing patients was also observed when patients were selected according to the treatment received but, perhaps due to the reduced sample size, the difference was not significant. Also, the same conclusion was drawn when patients with high miR-221 and

low EGFR expression at recurrence were compared with low miR-221 and high EGFR expression – the cohort consisting of all available patients showed a significantly favourable prognosis for the latter group but upon selecting patients that received radiotherapy or/and TMZ only a trend towards a favourable prognosis was observed. Again, the requirement to decrease the sample size is likely the reason for our survival analysis not reaching statistical significance in the radio-chemotherapy treated cohort. Taken together, we propose that increased miR-221 and the consequent EGFR is a potential biomarker to determine the prognosis of recurrent glioblastoma patients.

5.4 Conclusion

In this chapter we have demonstrated that elevated miR-34 may be a resistance marker for a subset of glioblastoma cells. Furthermore, we have established miR-221 to be up-regulated in resistant and recurrent glioblastoma cells. The role of miR-221 in resistance and recurrence was demonstrated to be mediated by miR-221 down-regulating the expression of EGFR. Our data suggests that targeting miR-221 may be a potential treatment strategy to complement the Stupp Protocol due to targeting the pre-existing subpopulation of resistant glioblastoma cells. Future studies will require greater sample sizes to better elucidate the significance of an antagonistic relationship between miR-221 and EGFR in patient survival.

CHAPTER 6:
IDENTIFYING RTK-INDEPENDENT MECHANISMS OF TREATMENT RESISTANCE

6.1 Introduction

Epithelial-Mesenchymal transition (EMT) refers to a process by which a cell undergoes a transformation from a polarised epithelial cell associated with the basal membrane to a mesenchymal cell detached from the degraded basal membrane, such that migration and invasion capacities increase. Three subgroups of EMT have been proposed with specific characteristics noted in each. The first is observed during embryogenesis and organ development; the epiblast via EMT generates the primary mesenchyme. The second type concerns tissue regeneration and inflammation-mediated fibrosis; epithelial cells, lining organs such as the kidney and liver, can undergo EMT in conditions of chronic inflammation stress that passage through the basement membrane before taking on a fibroblast phenotype and relocate to the interstitium. The third, and which we shall concern ourselves with in this chapter, is associated with tumour progression and metastasis; highly proliferative epithelial cells acquire invasive capacities, detaching from the basal membrane and ushering in the metastatic stage of cancer progression. Up-regulation of certain pathways endow invasive and metastatic capacities that allow the cancer to colonise secondary sites by reverting to an epithelial phenotype via process known as mesenchymal-epithelial transition or MET (not to be confused with the RTK also called MET or c-MET).

The EMT process induces the neoplastic cell to alter its genetic and epigenetic profile. Several RTKs have been implicated in EMT along with associated downstream signalling pathways. These include MET, EGFR, PDGFR and FGFR which activate downstream pathways and transcription factors supportive of EMT [563, 706]. Several downstream pathways have also been implicated in EMT including the PI3K/AKT, STAT3 and MAPK/ERK pathways [778-780]. The EMT process can be defined according to an expression profile of specific markers of EMT that have been proposed, including: up-regulation of N-cadherin, Vimentin, CD44, B-Catenin, TWIST1, Snail, ZEB1 and Slug [706]. It is also well established that miRNA regulate EMT by targeting the EMT suppressors to promote motility and invasion or the EMT-inducing transcription factors, such as miR-200 binding ZEB1 [781]. Interestingly, miR-221 is recently being associated with EMT drivers in a number of different malignancies such as breast cancer and extrahepatic cholangiocarcinoma and hepatocellular carcinoma [782-784].

Chemotherapeutic resistance has been argued to be induced by EMT in several cancers, such as gefitinib-resistant non-small cell lung cancer, gemcitabine-resistant pancreatic cancer and paclitaxel-resistant ovarian carcinoma cells [785-787]. In the Verhaak et al. 2010 report, a subtype of glioblastoma called 'mesenchymal' was described which had the worst survival compared to all other described subtypes as well as relative absence in EGFR genetic alterations and an abundance in alterations in the neurofibromin 1 and PTEN genes, both pathways converging upon the AKT pathway [64]. EMT is associated with both radiotherapy and TMZ resistance, as both therapies can increase the expression of EMT pathway markers. Studies have shown that in radiotherapy-treated glioblastoma cells, the mesenchymal signature, including that which was defined by Verhaak, 2010, was enriched and another similar report has also been published [788, 789]. In T98G TMZ-treated cells, EMT markers, such as TWIST1, Snail, Vimentin and B-Catenin, were significantly up-regulated after 48 hours [790].

Aims

Given our previous chapters have demonstrated that resistance to radiotherapy and TMZ is exhibited by low MET- and EGFR-, and high miR-221-expressing cells, we next explored the possible role of EGFR- and MET-independent EMT in mediating resistance and recurrence in our *in vitro* models. Our aim was:

Exploring EGFR and MET-independent signalling of EMT markers in glioblastoma resistance and recurrence

6.2 Results

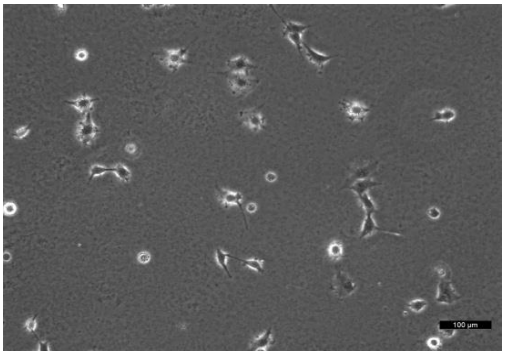
6.2.1 Treatment-resistant and recurrent glioblastoma cells present morphological alterations

To begin our investigation we first assessed the morphological differences between parental, resistant and recurrent cells. EMT is known, and expected, to induce morphological changes in its transitioning away from the parental cells function.

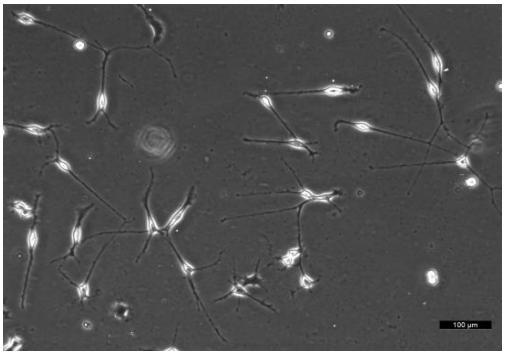
Both #41R and U87R cells were observed to have morphological alterations compared to respective parental controls **(*Figure 6-1 A-B*)**. These alterations include longer pseudopodia in the recurrent cell lines. Cell spreading was measured by quantifying both cell lengths (the angle providing the longest length possible was measured) and cell area. Both #41R and #U87R had significantly longer length and greater areas compared to respective parental controls **(*Figure 6-1 C-D*)**. Similar morphological alterations were also observed in #35, #41 and U87 cells treated with 5Gy radiotherapy and 1000uM TMZ for 7 days **(*Figure 6-1 E-F*)**. Take together, both recurrent and resistant cells present morphology consistent with EMT.

A) #41P

(i)

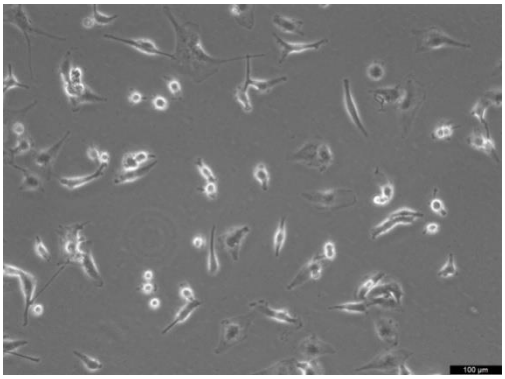


(ii)

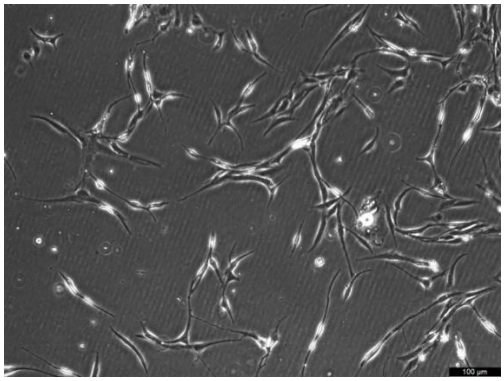


B) U87

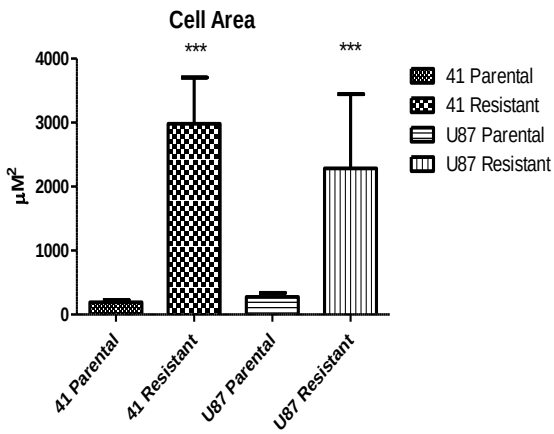
(i)



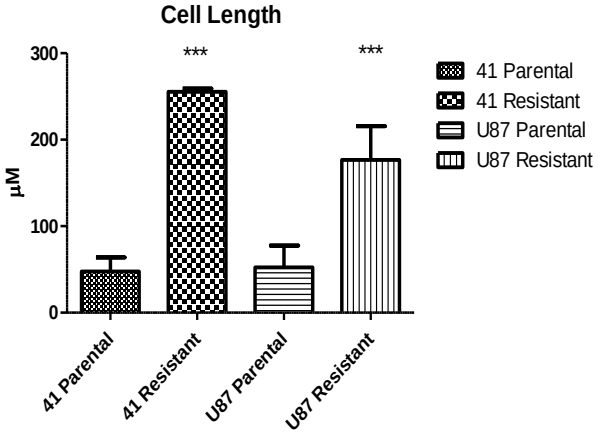
(ii)



C)

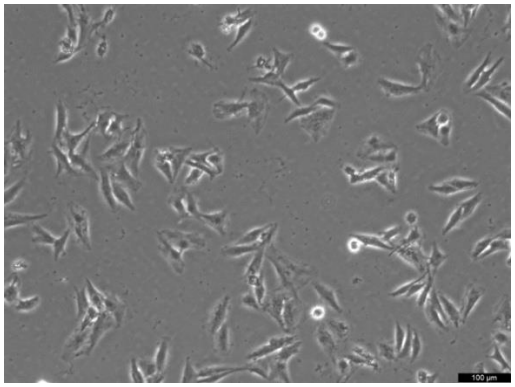


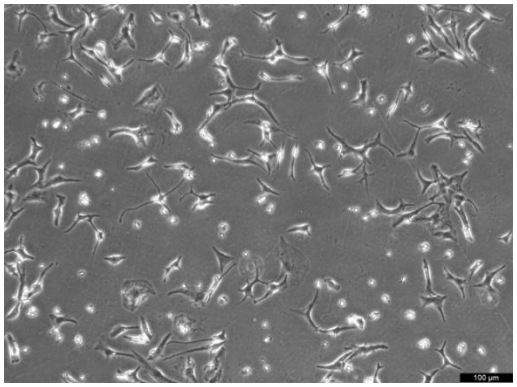
D)



E) #35

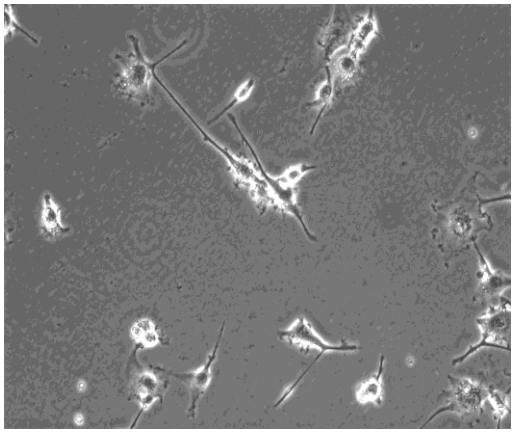
(i) **(ii)**





F)

(i) #41 treated **(ii) U87 treated**



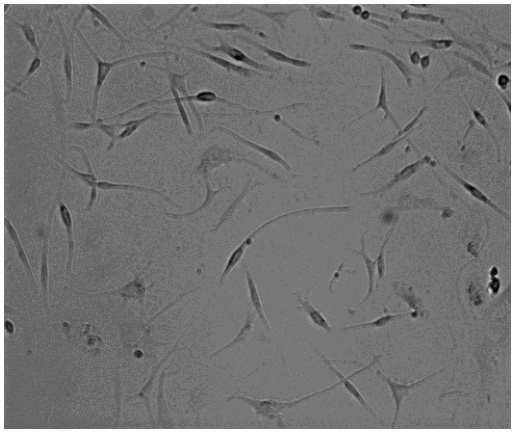


Figure 6-1: Treatment resistant cells obtain a fibroblast-like morphology. (A) (i) The morphology of #41P and **(ii)** #41R**. (B) (i)** The morphology of U87 and **(ii)** U87R. **(C)** Cell area of #41R relative to #41P and U87R relative to U87. **(D)** Cell length of #41R compared to #41P and U87R compared to U87. **(E)** A morphological comparison of **(i)** #35 untreated and (**ii)** #35 treated cells. **(F)** Short-term treated **(i)** #41 and **(ii)** U87 cells also change morphology.

6.2.2 Recurrent cell lines have EGFR- and MET-independent high capacity migration

EGFR signalling is known to promote glioblastoma migration and its inhibition can lead to migratory suppression [791]. Similarly, MET activation has also been implicated in glioma migration and invasion [792]. Given that our recurrent cell lines had decreased EGFR and MET signalling, in addition to high miR-221 which also increases invasion and migration in glioblastoma, we next investigated the migration capacity of these cell lines with a scratch assay [703]. Cells were seeded onto a 10cm dish and incubated until approximately 90% confluence was reached before a wound across the plate was generated and migration was assessed 24 hours later. Surprisingly, both U87R and #41R retained aggressive migratory capacities despite displaying reduced EGFR and MET expression compared to controls. U87R cells had higher migration capacity compared to the U87 parental line **(*Figure 6-2 A-B*)**. However, there was no difference in migration rates between the #41 parental and #41R cells. **(*Figure 6-2 C-D*)**. This suggests that EGFR and MET may not be necessary for migration in #41 cells or redundant pathways have been activated.

6.2.3 EMT markers are up-regulated in resistant glioblastoma cells

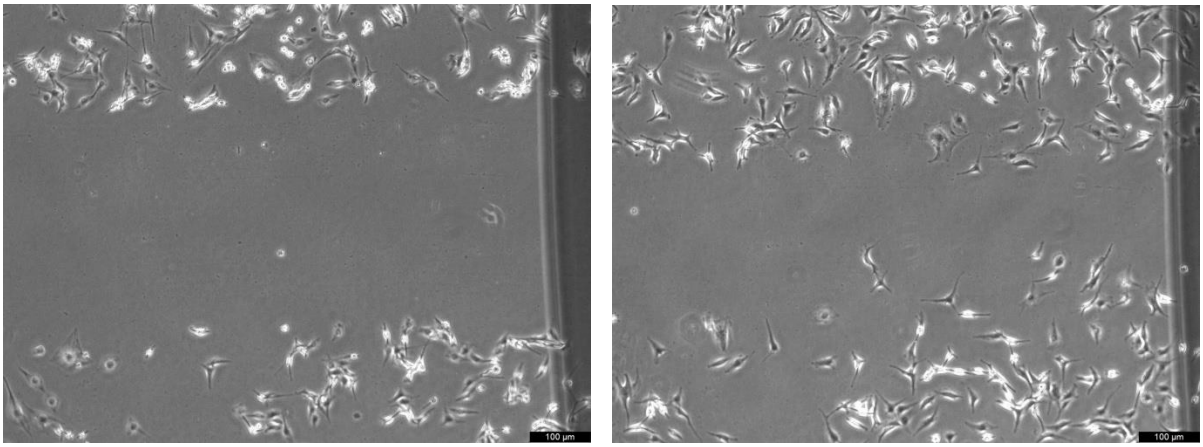
As our results have shown that recurrent and resistant cells are morphologically altered and retain migration capacity we next hypothesised that these cells have acquired a mesenchymal-like profile. To evaluate this we first assessed PDGFR expression and activity in #41P/R, U87P/R and U251P/R. The levels of PDGFR activity in both #41R and U251R was up-regulated compared to respective parental controls; although PDGFR was undetectable in U87 parental and U87R cells via western blot **(*Figure 6-2 F, J*)**. We also assessed downstream signalling molecules AKT and ERK. There was no difference in both pAKT and pERK1/2 levels when comparing #41P with #41R; however, pAKT was higher in U87R compared to the parental control **(*Figure 6-2 F, H*)**.

Next, the protein expression levels of several EMT markers were assessed. The levels of N-Cadherin, Vimentin and CD44 in #41P/R, U87P/R and U251P/R were analysed with western blot. Consistently, CD44 and N-Cadherin was more highly expressed in #41R, U87R and U251R cells compared to the respective parental controls; however, Vimentin was only up-regulated in U87R and U251R and down-regulated in #41R **(*Figure 6-2 G, I-J*)**. Additionally,

Oct3/4 expression was highly up-regulated in #41R compared to #41P. E-Cadherin was undetectable with western blot in all six cell lines used (data not shown). In summary, our results suggest that EMT markers are highly up-regulated in glioblastoma recurrent cells.

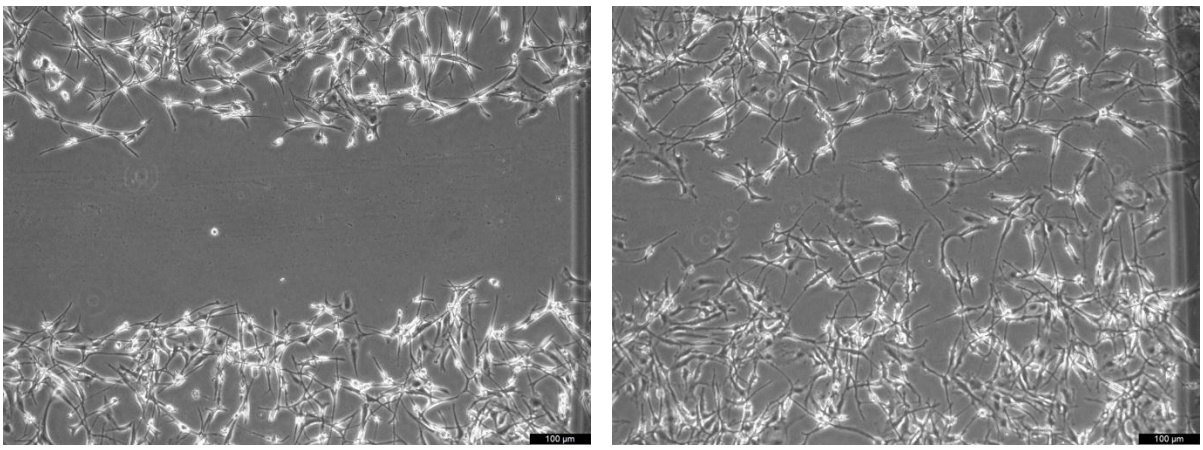
A)

(i) U87 Day 0 **(ii) U87 24 hours**



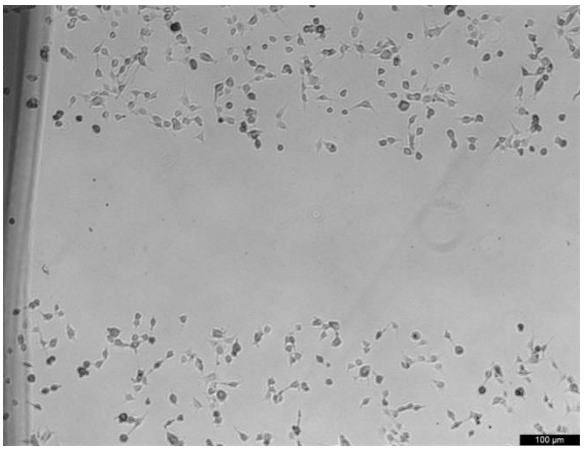
B)

(i) U87R Day 0 **(ii) U87R 24 hours**

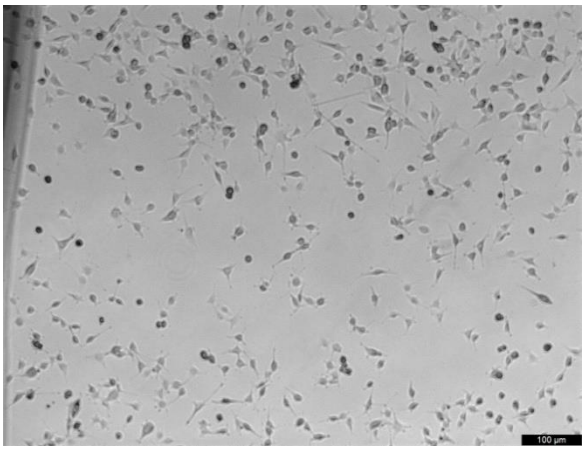


C)

(i) #41P Day 0

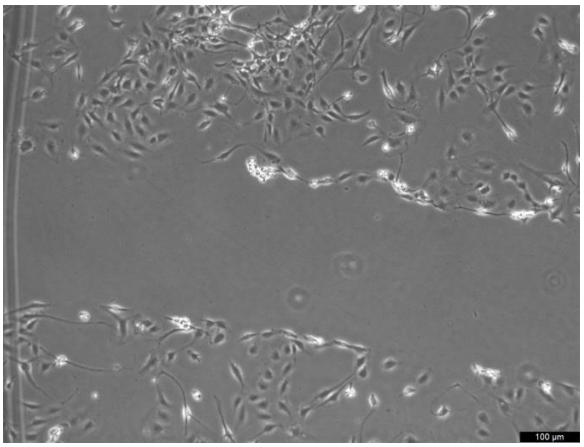


(ii) #41P 24 hours

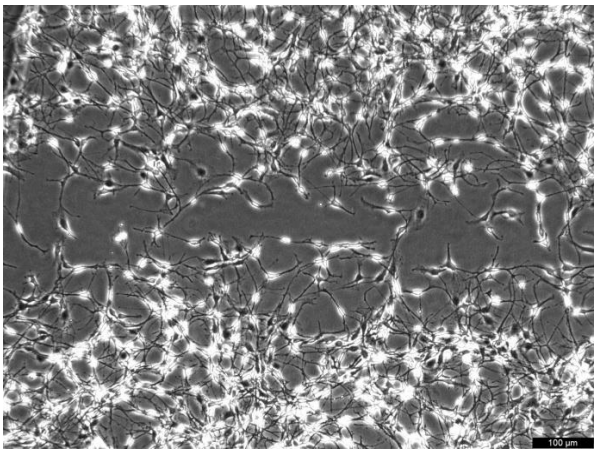


D)

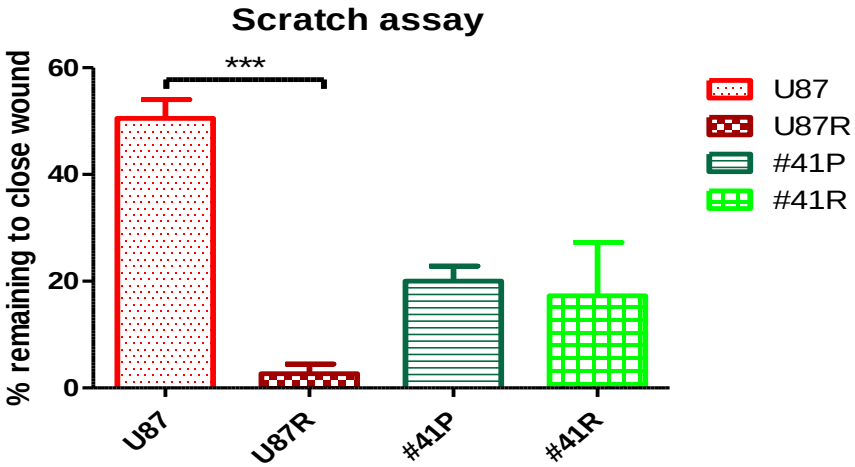
(i) #41R Day 0



(ii) #41R 24 hours



E)



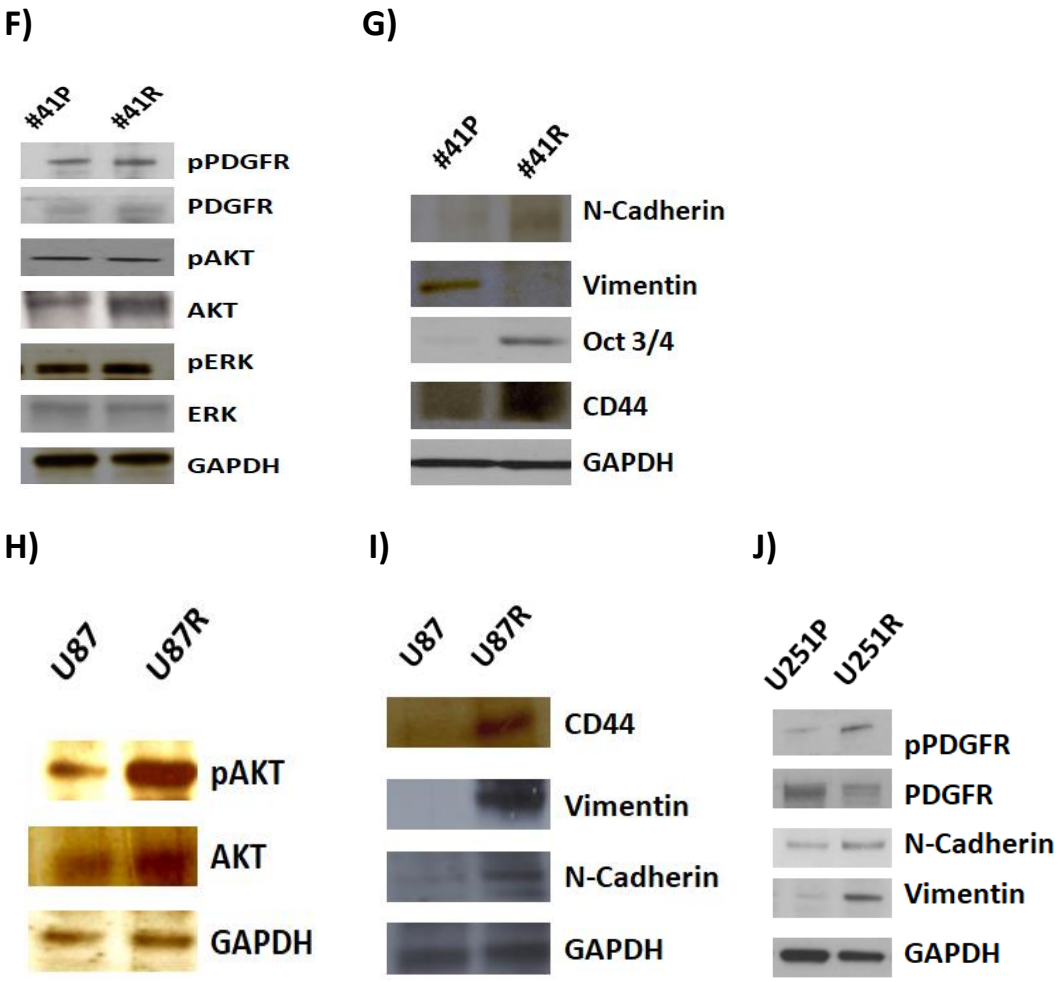


Figure 6-2: Resistant cell lines have persistent signalling related to EMT. Migration rate of **(A**) U87, **(B**) U87R, **(C)** #41P and **(D)** #41R was assessed by a scratch assay. The migration rate of U87R was higher than U87; however, there was no difference between #41P and #41R. **(E)** Graphical representation of scratch assay. All values are relative to the Day 0 wound gap which was set at 100%. **(F)** PDGFR and downstream signalling molecules AKT and ERK activation was detected in #41R. **(G)** Expression of EMT markers N-Cadherin and CD44 was detected in the #41R. Vimentin was down-regulated in the #41R relative to #41P. **(H)** AKT activation was increased in U87R relative to U87P, in addition to **(I)** EMT markers CD44, Vimentin and N-Cadherin. **(J)** U251R had increased levels of PDGFR activation in addition to increased expression of N-Cadherin and Vimentin, relative to U251P. *** denotes $p<0.001$; All values shown are mean ± SD.

6.2.4 AKT inhibition reverses EMT marker expression

As mentioned previously, AKT signalling has been suggested as an EMT driver in several cancers. However, at the time of writing, the relationship between AKT, and CD44, N-Cadherin and Vimentin in primary glioblastoma and treatment-resistance is understudied. #41R, U87R and U251R were subject to 10uM of wortmannin for 72 hours and the levels of the three EMT markers were assessed. pAKT was inhibited in all treated cells; furthermore the protein expression of three EMT markers were also reduced after treatment **(*Figure 6-3 A-C*)**. Therefore, we conclude that AKT signalling drives the activation of CD44, Vimentin and N-Cadherin in treatment-resistant glioblastoma cells.

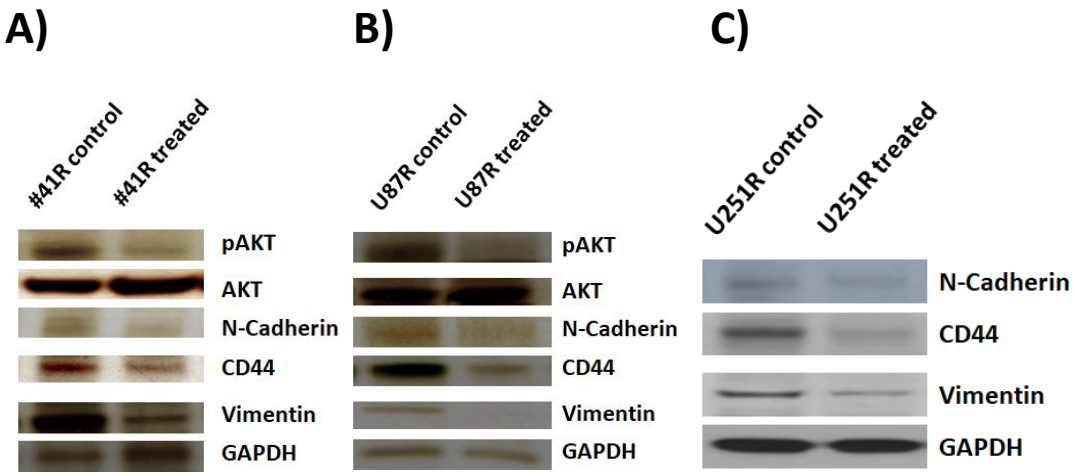


Figure 6-3: Inhibition of PI3K-AKT pathway activation down-regulated EMT markers. Both **(A)** #41R, **(B)** U87R and **(C)** were treated with the PI3K-AKT inhibitor wortmannin for 72 hours. Subsequently, the levels of N-Cadherin, CD44 and Vimentin were analysed in both the untreated control and treated cells. Treatment with PI3K-AKT inhibition was found to decrease the EMT-associated molecules N-Cadherin, CD44 and Vimentin.

6.3 Discussion

Glioblastoma is a highly aggressive, invasive and migratory neoplasm, and these characteristics render recurrence inevitable and complete resection improbable. EMT is transformation process undergone by neoplastic cells driven by genetic alterations. These transitioned cells are highly invasive and migratory, and a mesenchymal genetic signature has been proposed as a marker for both resistance and poor patient survival in cancer, including glioblastoma. However, EMT-like process in glioblastoma and its relationship with resistance to standard therapy is understudied. Furthermore, many studies have attributed EGFR and MET a key role in malignant EMT and considering both this attribution and our previous results we aimed to elucidate the significance of EMT markers in TMZ-resistant recurrent glioblastoma cells utilising our *in vitro* models.

6.3.1 Morphologically-altered glioblastoma resistant cells

We first observed the morphological changes that have occurred at both recurrence and in resistant cells. Consistent with our hypothesis - that EMT is driving glioblastoma resistance and recurrence – we observed that all recurrent and resistant cell lines have an elongated, pseudopodia-like, cell spreading phenotype. This morphological criterion to discern the activation of EMT has been utilised by other researchers, including Zhou and co-workers [793].

6.3.2 EGFR- and MET-independent migration and the role of PDGFR in EMT

Our results have shown that glioblastoma migration can be independent of EGFR and MET signalling, and recurrent cells retain migratory capacity despite loss of EGFR and MET. The presence of EGFR and MET being irrelevant to migration, as was seen when comparing the migration rates of #41P and #41R, largely goes against the dominant paradigm that currently exists in glioblastoma research [792, 794]. Potentially, the reactivation of AKT in #41R and the maintenance of migration rate may be attributed to the up-regulation of p-PDGFR compared to short-term treated #41cells – this up-regulation is likely a redundant pathway mechanism in response to EGFR down-regulation. The proposed mechanism is in need of experimental validation in glioblastoma; however, support for this proposal can be found when searching beyond the glioblastoma field. Non-small cell lung cancer cell lines

erlotinib resistance levels correlated with mesenchymal characteristics, such as higher Vimentin and ZEB1 and lower E-Cadherin [795]. Additionally, mesenchymal-like non-small cell lung cancer cells had increased pPDGFR-a and PDGFR-a [795]. Indeed, PDGFR expression is required for EMT maintenance in *in vivo* carcinoma mouse models and thought to drive EMT via the PI3K/AKT pathway [796]. Therefore, although further studies are required to validate the EGFR bypassing RTK signalling pertaining to glioblastoma EMT, our data is consistent with the larger scope of oncology research.

6.3.3 AKT regulation of EMT markers

Interestingly, although Vimentin was up-regulated in both U87R and U251R cells, it was down-regulated in #41R cells. Likely, this is due to the variability in the relevance of a single EMT marker in glioblastoma and given that glioblastoma EMT may differ from tumours of epithelial origins, such as non-small cell lung cancer and breast cancer. Additionally, EMT plasticity can allow for cells to revert to a MET-like state; therefore, it is possible that the down-regulation of Vimentin in #41R is paradigmatic of the fluid nature of EMT-MET process.

That Vimentin is a downstream substrate of AKT signalling is well-established in epithelial carcinomas and the interaction is thought to be via direct AKT binding to Vimentin causing the latter to phosphorylate [797-800]. However, N-Cadherin has been mapped upstream of AKT has evident when N-Cadherin transfection increased AKT activation in bladder cancer and silencing N-Cadherin inhibited AKT in melanoma and prostate cancer cell lines [801-803]. Only recently, in 2018, it was shown that inhibiting AKT using MK2206 in U373 and SHG44 cells down-regulated both Vimentin and N-Cadherin [804]. Our data is consistent with the literature supporting Vimentin regulation by AKT in epithelial cancers though offers a conflicting account by situating N-Cadherin downstream of AKT in glioblastoma.

Recently, Feng et al. inhibited Regulatory Factor X1 and observed a decrease in both CD44 and AKT activity in U87 and U251 cells [805]. To the best of my knowledge the positive regulatory role of AKT in CD44 expression has yet to been demonstrated, rendering our result novel in glioblastoma. On the contrary, stimulation of the CD44 variant isoform CDv66 can increase AKT activity in glioblastoma stem cell-like cells [806]. The difference between

this previous study and our result may largely be due to our model being representative of treatment-resistant recurrence and differences in cell lines.

6.4 Conclusion

To summarise the chapter, we have shown that treatment-resistant recurrent glioblastoma cells exhibit a morphological alteration and up-regulation of EMT markers. Furthermore, AKT was demonstrated as a positive regulator of EMT markers in glioblastoma and reactivation of PDGFR activity was shown to be a possible compensatory RTK signalling mechanism in response to down-regulation of EGFR and MET at recurrence.

CHAPTER 7: DISCUSSION, LIMITATIONS AND CONCLUSION

7.1 A novel treatment-resistant *in vitro* model

The failure in translating the rationale in targeting RTKs can perhaps be attributed to a number of factors: a lack of consensus with regards to a biomarker for treatment resistance in the glioblastoma literature; replicating *in vitro* data with an *in vivo* model; compensatory RTK activation; RTK-independent mechanisms sustaining tumourogenic signalling; and the lack of proper biological models leading to overstating or misplacing the significance of a particular mechanism. Importantly, as we have discussed previously in **Chapter 1**, there is a relative lack in studies investigating treatment resistance mechanisms, especially resistance to both radiotherapy and TMZ. As we have discussed and demonstrated in previous sections, though many research groups have utilised TMZ or radiotherapy to study the response in glioblastoma, these models are not models for the study of resistance by that very fact but, rather, models that study TMZ- or radiotherapy-induced mechanisms; these are treatment-induced models rather than treatment-resistant models.

To distinguish between these two models requires definitions. In this thesis we defined treatment-induced models as *models that study the regulation of mechanisms that are activated in response to treatment.* In contrast, a treatment-resistance model was defined as *models that study the mechanisms that confer a specific, surviving sub-population of cells with the capacity to survive treatment*. Treatment-induced models, such as those utilised by Munoz et al. and defined as "TMZ-resistant", fall short of meeting the criteria for being defined as treatment-resistance models as the former requires survival assays suggesting the remaining cells are viable and can withstand treatment [807]. Consequently, Qi and colleagues model does qualify as a bona fide treatment-resistance model particularly due to survival assays supporting the claim that the surviving cells can withstand the employed treatment conditions; therefore, their terming the surviving cells TMZ-resistant is also warranted [808].

In **Chapter 3** we aimed to address the problem of RTK signalling conferring treatment resistance – which requires evaluating the significance of RTKs in glioblastoma resistance to standard therapy – we first generated an *in vitro* model of radiotherapy- and TMZ-resistant glioblastoma. Our model was generated after treatment with both radiotherapy and TMZ for 7 days. Cell viability did not significantly reduce between Day 5 and Day7 post-treatment

in any of the cell lines unlike the significant reduction between Day 3 and Day 5. Furthermore, there was no significant reduction between Day 7 and Day 10 in any of the cell lines used in the study. Due to this reason the time point of 7 days was chosen for future studies and satisfies the criteria of treatment-resistance given that the surviving cells at Day 7 have demonstrated the capacity to withstand treatment. In other words, the lack of viability reduction from Day 5 to 7 and Day 7 to 10 implies the surviving population does not contain a sensitive sub-population; therefore the surviving population of cells at Day 7 is justified in being termed treatment-resistant at least to a short-term, one-off challenge. Although this follows that the Day 5 cell population can be justifiably be referred to as treatment-resistant, the Day 7 time point was chosen to eliminate the possibility of biological variance leading to carry-over sensitive cells from Day 3. The combination of radiotherapy with TMZ in the generation of treatment-resistance makes this treatment-resistance model a novel model. Using this model set a foundation for further studies to examine specific resistance mechanisms found in glioblastoma.

7.2 Justifying the 1000uM TMZ dose

We have emphasised and one of our main critiques of the current literature is that previous studies have not sufficiently incorporated a treatment regimen that replicates the treatment received by glioblastoma patients which has ultimately resulted in the hindering of translating pre-clinical findings. Throughout this thesis the treatment regimen for *in vitro* experiments was set at 5Gy irradiation and 1000uM TMZ, unless otherwise stated such as the dose-dependent and time point studies found in **Chapter 3**. Providing a proper justification for choosing these dosages has been largely ignored hitherto and requires addressing. Although our adoption for the 5Gy plus 1000uM TMZ treatment regimen was ultimately based on previous optimisation experimental data from our lab, a justification solely based on *in vitro* experiments is insufficient for the practical need to translate our findings into the clinic.

It shall be recalled that the standard radiotherapy dose for the Stupp Protocol is 2Gy daily for a period of 30 days, amounting to a total accumulative dose of 60Gy. In that regard, in terms of accumulative dosage, a one-off 5Gy radiotherapy treatment before an incubation period lasting 7 days is below the clinical dose. However, the generation of the #41R cell line

which received 2 fractions 5Gy over a period of 14 day is closer in terms of total dose **(**see **Chapter 4)**. Previous reports adopting a hypofractionated doses in which 5Gy daily fractions over a 2 week period reported no significant toxicities in human glioblastoma patients [809]. Therefore, though our 5Gy dose may not necessarily compose commonly adopted clinical treatment regimens, the 5Gy radiotherapy has demonstrated to be a clinically viable option.

The 1000uM dose of TMZ was previously found to be sub-lethal in our laboratory and that finding was again validated in our first experiment, as shown in **Chapter 3**. Therefore, the 1000uM dose was preferred over lower doses due to our cell lines showing high resistance to the latter rendering the deciphering between sensitive and resistant cells difficult. A problem arises, however, given it is regarded that the clinical relevant *in vitro* TMZ dose to range between 50-100uM – at least 10-fold lower than the commonly used dose in our project. This is indeed a limitation in our project. It shall be noted that our theoretical paradigm foundational for the explanation of resistance explains that the resistant cells are selected for rather than resistance being acquired. If this may be assumed to be true then it follows that the selected cell population that pre-exists is unaltered by the high dose adopted. Consequently, the difference between regimen consisting of high dose TMZ and a low dose TMZ is that the selection of cells in the latter regimen is more genetically heterogeneous compared to the former. In this way, the cell population that was selected in our experiments will indeed compose the resistant population if we were to adopt a treatment regimen consisting of 100uM TMZ.

7.3 The incoherence of targeted RTK therapy: Demonstration of a failed clinical strategy

The logical argument for the examination of RTKs in our project was as follows:

1. glioblastoma cells require pro-tumourogenic signalling for its persistence
2. RTKs have been found to stimulate such signalling and be dysregulated in glioblastoma
3. Glioblastoma maintains a pro-tumourogenic capacity post-treatment with radiotherapy and TMZ
4. Therefore, increased RTK activity contributes to glioblastoma cell survival post-treatment

We tested the validity of this conclusion in **Chapter 3** by first utilising a phospho-RTK array that screens the activity levels of 49 different RTK. Primary glioblastoma cell lines were subject to 5Gy radiotherapy and a 7 day exposure period with 1000uM TMZ before analysis with the phospho-RTK array. We observed that treatment resistant glioblastoma cells had reduced phosphorylated levels of all assessed RTKs. In addition, glioblastoma cell lines also displayed reduced EGFR and MET gene expression. But with the majority of the cell lines downstream signalling molecules – AKT, ERK and STAT3 – persisted with up-regulated activation. These findings appear to oppose a few of the most common accounts given pertaining to the functioning of RTK signalling networks: 1) a lack of redundant RTK activation, 2) the disposability of RTK for sustaining cell survival and 3) alternative upstream signalling for the continued oncogenic downstream pathways.

7.3.1 Significance in the lack of redundancy in RTK signalling networks

Previous studies and a prevailing account for the failure of RTK targeted therapy suggest that redundant RTK signalling allows the resistant cells to be independent of the targeted RTK. But these studies differ from ours from only focussing on RTK inhibition without combined radiotherapy and TMZ possibly leading to overstating the importance of such a mechanism and its contribution to resistance. However, in the time point studies found in **Chapter 3** the increase in EGFR activity on Day 3 after treatment in our cells being concurrent with down-regulation of MET activation is consistent with the compensatory signalling theory. This suggests that compensatory RTK signalling is an intermediate pathway rather than a contributor to radiotherapy and TMZ resistance.

Redundant signalling pathways as a means to tyrosine kinase inhibition ineffectiveness, as described in **Chapter 1**, was rapidly adopted after the groundbreaking study by Stommel et al. [810] Multiple RTKs were found activated in a panel of glioblastoma cell lines and only the inhibition of multiple RTKs reduced survival and PI3K/AKT pathway activation [810]. Furthermore, forced constitutive expression of wtEGFR or EGFRvIII can replace MET activation of PI3K, suggesting that inhibition of MET can still allow for sustained downstream signalling due to the EGFR [810]. Additionally, a similar redundancy relationship between EGFR and PDGFR has also been described previously in glioblastoma [811]. Down-regulation

of the EGFR can lead to up-regulation of other members of the EGFR family, IGF-1R, MET, PDGFR and AXL [398, 575, 812-814]. In fact, when reviewing EGFR-driven therapeutic resistance, Azuaje et al. recently noted RTK inhibition resistance to be due to "robust activation of survival pathways downstream of the EGFR" but more importantly also "other RTK signalling activation" [815].

To counteract this has led to the theory that a RTK-based therapeutic paradigm will require to target multiple RTKs simultaneously, hence the generation of agents such as crizotinib and cabozantinib [816]. Theoretically, it follows that an effective RTK inhibition strategy will require the targeting of all activated RTKs eliciting proliferative and other tumourogenic signals – a practically improbable endeavour with the current means available. Our data suggests such a strategy is not required and, even if clinically an option, will not resolve the problem of radiotherapy and TMZ resistance. We found that RTK redundancy has not been the driver for the resistance mechanisms which led to the failure of targeted RTK therapy as a means to complement the Stupp Protocol but the down-regulation of RTKs induced by combined radiotherapy and TMZ can instead reduce the efficacy of RTK inhibition.

7.3.2 Beyond EGFR and MET

Our data questions the view of centering the RTK family within the oncogenic addiction model. By demonstrating in **Chapter 3 and 4** that all 49 phospho-RTKs assessed had down-regulated in 7 day treated cell lines and reduced EGFR and MET gene expression, but persistent downstream signalling, we concluded that alternative RTK-independent signalling pathways are the sources for resistant cell survival.

For over a decade it has been argued that the addition of RTK inhibitors can potentiate the sensitisation effect of standard therapy. Much of the rationale for this predictivism stems from RTK alterations being common in glioblastoma. Another source for support is found in de-regulating RTK works that reported the causal role of RTK with regards to pro-tumourigenicity. These types of studies, both *in vitro* and *in vivo* based, provided the rationale groundwork for RTK-based therapies by demonstrating increased glioblastoma aggressiveness is a consequence of up-regulating certain signal transduction pathways that are contingent upon RTK signalling. Such examples are many in the literature. Ambrose et al. concluded that IGF-1R "is strictly required for the growth of T98G" after its inhibition

suspended growth induction stimulated by growth factors [817]. VEGFR inhibition with axitinib reduced growth of orthotopic glioblastoma models and increased the survival times of mice bearing U87-derived glioblastoma [818].

Many of the studies that have made claims for the translatability of their *in vitro* results have in fact attempted to juxtapose the clinical context in which there is a strong relationship between glioblastoma and the Stupp Protocol with the employment of a single component of the Stupp Protocol (radiotherapy or TMZ). Only a subset from this group qualifies to have focussed on treatment-resistance. A prototypical example was the recently published paper by Kessler et al [819]. Here it was concluded that VEGFR-2 expression is an attribute of a chemo-resistant subgroup of glioma because knocking down VEGFR-2 expression with shRNA sensitised glioblastoma cells to 10uM TMZ, although sh-VEGFR2 cells were not sensitised to 8Gy radiotherapy. Importantly, experiments combining radiotherapy with TMZ was absent which obscures the clinical significance of VEGFR-2 down-regulation [819]. Moreover, it was concluded that chemotherapeutic "resistance" is promoted by VEGFR-2. This is unfounded according to this thesis' definition given that Kessler never conducted any survival assay demonstrating that the surviving population at the one and only time point chosen – at 72 hours - is any less or more resistant to a later time point [819]. This is a crucial oversight as it does not follow that a surviving population at one particular time point is resistant to TMZ. As we have seen in this present study our own results suggest that the still sensitive whole population of cells that survived at Day 3 differs in RTK activity compared to the treatment-resistant Day 7 cells **(**see **Chapter 3)**.

The consequence of such an approach that only utilises a single component of standard therapy is that the cell population that is selected for does not adequately represent the population that is selected for during clinical treatment. In this way it is feasible to suggest that increased signalling of a particular RTK in a population selected for by TMZ treatment, such as VEGFR-2 as indicated by Kessler, is not clinically relevant unless patients are only being treated with TMZ, which is not the case. This position and our results are consistent with a 2018 paper showing that EGFRvIII protein expression is up-regulated with radiotherapy alone but reduces when combined with TMZ; however these authors did not link treatment resistance with EGFR [820].

7.3.2.1 Interleukin driven signalling as an alternative to EGFR and MET signalling

A possible mechanism for such RTK-independent activation of downstream pathways is cytokine receptor-mediated signalling through interleukins. These receptors, unlike RTKs, lack intrinsic enzymatic activity, but are found on the cell membrane and the ligand-receptor complexes can act in both a paracrine and an autocrine manner [821] [822]. The role of interleukin signalling in various cancers is well established. For example, IL-10 or IL-22 stimulation activates the STAT3, AKT and MAPK pathways in cancers including lung and breast cancer as well as osteosarcoma [734] [735, 736] [737].

Patient survival is lower in IL-6 amplificated glioblastomas and is well known to activate STAT3 [823]. In glioblastoma cell lines it is known that IL-6 stimulation can promote invasion and migration through up-regulation of pSTAT3 and the MAPK signalling and IL-6R knockdown disrupts tyr705 pSTAT3 levels [824, 825]. In renal cell carcinoma, *in vitro* treatment with tyrosine kinase inhibitors increased IL-6 levels stimulates AKT-mTOR pathway activation [826]. Data regarding the IL-6/AKT pathway in glioblastoma is lacking except for a recent paper which it was demonstrated that IL-6 phosphorylates AKT in glioblastoma cell line 05MG [827]. IL-22 treatment activated IL-22R-AKT and IL-22R-STAT3 – induced glioblastoma cell survival, though may inhibit pERK [828].

Further indication of interleukin-mediated compensatory signal transduction comes from RTK inhibitor-resistant malignant cells. Indeed, head and neck squamous cell carcinoma cell lines resistant erlotinib differentially expressed IL-6 and antagonising IL-6R reduced erlotinib-resistant tumour volume growth *in vivo*; IL-8 stimulation led to EGFR-inhibitor resistance in lung cancer cells [829] [830]. Although data regarding interleukin receptor signalling bypassing tyrosine kinase inhibitors in glioblastoma is lacking, it is known that radiotherapy alone can increase STAT3 activity [831]. Moreover, TMZ-resistant U87 cells were also found to have elevated IL-6 and STAT3 expression [237]. Therefore, it is likely that our treatment-resistant subpopulation have bypassed RTK-dependence with compensatory signalling mediated by interleukin receptors. However, further studies are required to validate this hypothesis.

7.3.2.2 CD44 as an alternative for EGFR- and MET-driven signalling

The role of CD44 in treatment resistance is not fully known in glioblastoma. We have shown in **Chapter 6** that CD44 was up-regulated in treatment resistant recurrent cell lines that expressed undetectable EGFR and MET gene expression - #41R, U87R and U251R. The CD44 is a transmembrane glycoprotein and a hyaluronan receptor that has been previously described to be oncogenic. Both an EMT and stem cell associated marker, CD44 is capable of driving signalling pathways to promote migration, invasion and proliferation. CD44 can form complexes with and drive STAT3 activity and stimulate the ERK and AKT pathway in cancer [832] [833]. This likely explains the persistent downstream signalling in our resistant cells in the absence of both EGFR and MET gene expression, and RTK activity.

CD44 is generally considered to be upstream of AKT, with inhibition of the former leading to the down-regulation of the latter. In **Chapter 6**, we showed that inhibiting AKT signalling can decrease CD44, thereby suggesting an alternative signalling pathway map in which both CD44 and AKT relate via a circulatory positive regulation loop. Although CD44 is upstream relative to AKT, this effect is most likely due to the inhibition of CD44 transcription factors promoted by AKT signalling. Indeed, AKT has been found previously to activate transcription factors in cancer, such as Sp1, EGR1, AP-1, ETS1 and NF-KB, which are positive regulators of CD44 transcription [834-838]. We therefore have described a novel interaction between CD44 and AKT in glioblastoma. We propose that CD44 is an attractive therapeutic target in treatment resistant glioblastoma cells since tumourogenic signalling pathways are likely sustained via CD44 activity after radiotherapy and TMZ reduces RTK signalling and protein expression.

7.3.3 Emphasising heterogeneity as an intrinsic resistance mechanism

By advocating and finding support with experimental validation that a pre-existing sub-population of low EGFR and high miR-221 expressing cells re-initiates treatment resistant recurrence supports the theoretical paradigm proposing that intrinsic heterogeneity drives therapeutic resistance in cancer. The traditional disputes found in the glioblastoma literature surrounding which single RTK needs to be inhibited are mostly as unwarranted as they are, demonstrably, unfruitful. Our data advocates instead for a combinatorial therapeutic regimen that pre-emptively targets the inherently resistant population of cells.

Sustaining RTK expression and down-regulating miR-221 may be a potential strategy to complement and increase the effectiveness of the Stupp Protocol.

7.3.3.1 Epigenetic mechanisms and loss of EGFR gene

Our data indicates that the radiotherapy and TMZ selects for a population of cells that have lost EGFR expression **(**see **Chapter 4)**. Interestingly, treatment with de-methylation agent azacitidine failed to rescue EGFR expression suggesting that the silencing of EGFR expression is not due to promoter methylation, but perhaps driven by either other epigenetic mechanisms or an EGFR sequence mutation that disrupts transcription. Furthermore, genomic instability, a hallmark of glioblastoma and cancer in general, did not result in re-gaining EGFR as we did not observe an up-regulation of EGFR in high passage number #41R. It must be stated that pPDGFR and pAKT levels were equivalent in #41P and #41R cells, which emphasises both the difference between treatment resistant and recurrent populations and the problem of genomic instability as the driver of re-instating tumour heterogeneity **(**see **Chapter 6)**. It is reasonable to suggest that the stability in the loss of EGFR and MET gene expression does not translate into losing the gene expression of other families of RTKs. Therefore, temporal heterogeneity, the idea that over time heterogeneity develops, may not rescue EGFR expression but perhaps will re-instate overall RTK driven tumourigenesis and signalling networks. The failure to observe the re-instating of EGFR gene expression (as well as MET) contradicts a previous report describing that a low EGFR expressing cell population re-expresses EGFR to re-state EGFR heterogeneity [503]. This is likely due to our study only selecting for cells that are resistant to radiotherapy and TMZ, and it is possible that a sub-population of cells in the previous study have lost the EGFR gene but were not selected for, and therefore, not detected.

7.3.3.2 Invalidating the acquired resistance model

Although we have argued that an intrinsically resistant sub-population drives resistance and subsequently supported this theory experimentally using single cell isolation assays, we must remain cautious since this does not invalidate the acquired resistance model. The strongest empirical data we have presented supporting the existence of intrinsic resistance is the generation of the resistant U87-V and sensitive U251-V, low and high EGFR expressing sub-populations, respectively; therefore, this serves as the best empirical test we have available for the intrinsic resistance model. The intrinsic model for resistance (also

sometimes referred to as the inherent resistance model in the present study) states that radiotherapy and TMZ (in the specific context of glioblastoma) serves as a selection pressure that leads to a resistant sub-population of cells repopulating the tumour population that initiates recurrence. Both the U87-V and U251-V cell lines demonstrate that the parental population is heterogeneous with regard to EGFR expression and these cell lines are resistant and sensitive, respectively. Nonetheless, it does not follow that the population isolated from the parental U87 and U251 cell lines are identical to the cell population selected after exposing the parental population to radiotherapy and TMZ. It remains possible that the low EGFR expressing population resistant to therapy had down-regulated EGFR and up-regulated miR-221 in response to treatment. It may be so that to invalidate one of the two resistant models studies requires utilising much more powerful methodologies that adopt a single cell genomic approach to track cellular genetic and proteomic alterations at the individual cell level.

7.4 Rethinking the significance of autophagy in glioblastoma

The role of autophagy in glioblastoma is controversial with reports suggesting autophagy to be a resistance and tumourogenic mechanism, but contrary accounts describe an anti-tumourogenic process termed autophagic cell death. EGFR signalling is counteractive to autophagy activation, largely due to the EGFR driven PI3K/AKT activation of mTOR. Therefore, we hypothesised that the reduced EGFR expression displayed by the short term treated resistant cells and treatment resistant recurrent cells increases autophagy activation **(**see **Chapter 3)**. The up-regulation of LC3 throughout the 7 day period after radiotherapy and TMZ treatment was consistent partially with our hypothesis stating the pro-resistance effect of autophagy. However, although LC3 over-expression was observed, it did not inversely correlate with EGFR expression across the time course suggesting an association between EGFR and LC3 but not a direct negative regulatory network. The latter suggestion is further supported by our #41R cells showing no detectable LC3 protein expression despite lacking EGFR gene expression.

We propose that autophagy activation - measured with LC3 levels in the present study - is not dependent on the negative regulation of EGFR activity and is a protective mechanism activated in response to radiotherapy and TMZ. This explains the lack of LC3 in the

treatment resistant recurrent cell line #41R since this cell line was not immediately exposed to radiotherapy and TMZ before assessing LC3 levels. Therefore, the lack of any immediate genotoxic stress may be the reason #41R LC3 levels were no different to #41P.

Indeed, EGFR inhibition with tyrosine kinase inhibitors can increase LC3 via reduction of AKT signalling in glioblastoma cell lines [741]. Therefore, it may appear self-evident that EGFR and LC3 are intertwined in a negative signalling network in which LC3 is the contingent protein and EGFR the causal driver; however, these findings do not contradict, and can be incorporated into, our proposal. LC3 up-regulation (and consequently autophagy activation) upon EGFR inhibition can be reconciled with the view that LC3 is not contingent upon EGFR signalling. This is because deprivation of EGFR signalling may have induced a stress response by perturbing growth signalling such that autophagy allows for cell survival and acts as a short term survival mechanism. It follows that autophagic cell death is theoretically possible if the stress response is present in the long term. However, we observed a peak in LC3 levels at Day 3 time point before levels began to reduce, the lowest LC3 expression being at the Day 7 time point. This indicates that autophagic cell death may be prevented by resistant cells by activating autophagy as an immediate survival mechanism before alternative sustainable cell survival signalling is utilised. It has been argued extensively elsewhere that short term autophagy leads to cellular senescence in the long term. Given that interleukins are also noted to sustained cellular senescence and promote tumour progression, future studies will need to further elucidate this relationship and validate our proposal [839].

Lastly, future studies require better models for the assessment of autophagy. Although LC3 may be involved in autophagy, mechanisms independent of autophagy may be involved in LC3 up-regulation; therefore, multiple autophagy-related markers are required to allow for a stronger conclusion. Furthermore, the increase in LC3 does not necessitate an increase in autophagy. This is because LC3 up-regulation may only establish autophagy induction rather than autophagic flux. For the latter to be established we are required to disrupt late-stage autophagy by inhibiting the lysosome fusing with the autophagosome followed by measuring the GFP-LC3 puncta. Therefore, in light of these considerations caution is required when establishing our conclusions pertaining to the role of autophagy in treatment resistance and recurrent glioblastoma, and more in-depth studies with improved controls are in need.

7.5 Establishing miR-221 as a therapeutic target in glioblastoma

7.5.1 Does miR-221 regulate or bind EGFR?

As evident from above, the role of miR-221 is understudied in glioblastoma compared to other drivers of glioma progression. In particular, an account on the relevance of miR-221 in treatment resistant initiated glioblastoma recurrence has so far been ignored. Furthermore, reports on miR-221:mRNA binding have largely overlooked the question of clinical relevance and validation studies using a patient cohort are commonly absent. Our project has taken these previous shortcomings into consideration in the methodologies employed in **Chapter 5**.

The regulation of EGFR via miR-221 has not been previously described in the literature. This is perhaps unsurprising given that both miR-221 and EGFR are considered oncogenic, leading to the assumption that any association between the two signalling molecules must be through positive regulation.

In the present study we have concluded that miR-221 down-regulates the EGFR expression. We demonstrated, using cell lines and human patient samples, the inverse relationship between miR-221 and EGFR suggesting a strong biological and clinical relevance for our results. However, our data has not established whether the EGFR is a direct target of miR-221 and distinguishing between the two conclusions is of crucial importance for the biology of EGFR and miR-221. Although our data demonstrated an inverse correlation between EGFR and miR-221 the relationship can be explained by mechanisms other than direct binding and mRNA degradation. Indirect down-regulation due to miR-221 targeting EGFR transcription factors and regulatory proteins were possible mechanisms for our observation. For future studies we could utilise luciferase reporter gene assays to confirm direct miR-221:EGFR binding. A luciferase gene construct consisting of the EGFR target sequence that is predicted to bind miR-221 and pre-miR-221 should be transfected into the cell – a reduction in luciferase activity will validate that the EGFR is a direct target of miR-221.

7.5.2 miR-221 regulation of EGFR is evolutionary conserved

MiR-221 was the only miRNA, out of the three short-listed miRNAs, to be over-expressed in all the EGFR- or MET-suppressed resistant cell lines tested. This both supports, though short of confirming, the tumourogenic role of miR-221 and the target validation process. A possible reason for this success perhaps lies with the evolutionary conserved target sequence pairing between miR-221 and EGFR, as indicated by TargetScan. According to TargetScan, the aggregated probability of conserved miR-221 targeting EGFR is 0.62, the fourth highest probability listed after miR-144-3p, miR-137 and miR-133. The probability of conserved targeting suggests the likelihood of the miR-221 seed sequence to bind to the EGFR mRNA is amongst the highest [840]. Phylogenetic conservation of target pairing is likely to occur for biologically relevant or significant mechanisms since paired sequences are more likely to have been selected across species due the functional interaction; therefore, conservation due to selective pressure is both reliable evidence for target prediction and biological relevance.

7.5.3 The clinical relevance of EGFR and miR-221 at recurrence

In **Chapter 4 and 5** we utilised a patient cohort to validate our pre-clinical data. We successfully demonstrated that a subset of Stupp Protocol treated patients that display lower EGFR or higher miR-221 expression trend towards poorer survival; the presence of an inverse correlation between EGFR and miR-221 within a cohort of primary and recurrent tumours; and an association between poorer survival and a high miR-221 / low EGFR gene expression signature at recurrence. Unfortunately, due to a small sample size of recurrent patients the association did not cross the threshold for significance in most of the survival analyses. Nonetheless, the survival trends that were reported warrants further investigation in elucidating the clinical significance of the EGFR and miR-221 inverse correlation.

It must be noted that this patient analysis did not control for Karnofsky score which may have introduced a bias into the final results. Future studies are required to ensure patients with severe poor performance status are excluded from the analysis. Furthermore, in addition to patient selection bias the tumour samples that were selected may also have introduced another form of selection bias. It is feasible to suggest that the human patient tumour sample we have termed recurrent did not originate from the treatment resistant

population after first line therapy. Therefore, there is a possible difference in tumour biology between our *in vitro* treatment resistant recurrent model and the recurrent tumour samples collected and utilised as part of our human patient validation studies. Taken together, although our clinical cohort studies consistent at least trended towards a confirmation of our hypothesis future validation studies need to take into account the limitations found in our project.

7.6 Establishing miR-34 as a therapeutic target for glioblastoma

The role of miR-34 in resistance to radiotherapy and TMZ in glioblastoma has not been explored at the time of writing. Although MET is a validated target of miR-34, we obtained mix results regarding its expression in our #41R, U87R and U251R cells, as well as the therapy resistant U87-V and therapy sensitive U251-V cells **(**see **Chapter 5)**. Low MET expressing #41R had lower miR-34 levels while low MET expressing cell lines U87R, U87-V and U251R showed higher levels of miR-34 compared to the respective parental controls. In several cancers, including lung, colon and prostate cancer, miR-34 is regarded as a tumour suppressor [645]. In fact, miR-34 mimic has been trialled in phase 1 clinical trial, although the study was prematurely discontinued due to adverse events [645]. Only three articles are generated on Scopus with the key terms 'miR-34' and 'glioma' and only one article is generated with key terms 'miR-34' and 'glioblastoma'. In 2013, it was reported that rescuing miR-34 inhibits proliferation in pro-neural, PDGFR-amplified glioblastoma cell lines and miR-34 targets PDGFR-a [776]. It is likely that miR-34 expression is cell line-specific and this may explain the differences in the three resistant cell lines in our study. Nonetheless, given that miR-34 is highly expressed in U87R, U87-V and U251R it is possible that its role differs compared to other malignancies though further study is required.

7.7 Concluding remarks

This thesis has demonstrated that down-regulation of RTK activity is induced by radiotherapy and TMZ, and the gene expression of two of the most studied RTKs in glioblastoma – EGFR and MET – is suppressed. Our project has questioned the rationale behind clinical trials in the past which attempted to complement the Stupp Protocol by focussing on inhibiting these two RTKs for the treatment of glioblastoma. Supported by our data, we proposed that the Stupp Protocol may select for a sub-population of resistant cells that survive independently of RTK signalling, rendering treatment with tyrosine kinase inhibitors ineffective.

To investigate mechanisms for the down-regulation of EGFR and MET, we showed that miR-221 and miR-34 were up-regulated in treatment resistance cell lines. Furthermore, miR-221 can mediate resistance to radiotherapy and TMZ by down-regulating EGFR expression, making it an ideal strategy to target this miRNA in addition to the Stupp Protocol. Given that recurrent glioblastoma patients displaying high miR-221 and low EGFR associated or trended towards poorer survival compared to their inverse counterparts supports the view that targeting EGFR is ineffective for the more aggressive cases of glioblastoma but miR-221 is a potential therapeutic target.

To answer the question of how resistant cells are capable of driving tumourigenesis and activate downstream signalling pathways, we observed that resistant cells displayed higher levels of CD44, in addition to other stem cell and EMT associated proteins. The high CD44 expression was reversed by AKT inhibition highlighting the need to target converging points of signalling pathways rather than upstream drivers.

The design of future clinical trials will need to take these reports into consideration. In the current clinical context in which the Stupp Protocol is administered as first line therapy, targeting RTKs may be ineffective. Instead inhibiting miR-221 and CD44 is an attractive strategy for clinical trials as it may pre-emptively target the intrinsically resistant sub-population of tumour cells that initiate recurrence. Lastly, this thesis emphasises the importance of adopting both radiotherapy and TMZ in pre-clinical models to better characterise glioblastoma biology and translate findings to the clinic.

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