

Circulating miRNAs as novel biomarkers and intercellular regulators in glioma

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Submitted in fulfilment of the requirements of the degree of

Doctor of Philosophy

Declaration

This is to certify that

- 1) The thesis comprises only my original work towards the Ph.D., except where indicated.
- 2) Acknowledgment has been made in the text to all other materials used.
- 3) The thesis is less than 100,000 words in length, exclusive of tables, pictures, bibliographies and appendices.

Chenkai Ma

Preface

The research presenting in this thesis are conducted in the Brain tumour laboratory, the Royal Melbourne Hospital, the University of Melbourne with the supervision of Dr. Andrew Morokoff and Dr. Rodney Luwor.

The candidate performed all cell biological experiments, and data analyses (including bioinformatic analyses). All serum sample collection and RNA extraction were performed by Lucy Paradiso. The miRNAs profile was conducted in Australia Cancer Research Faculty, the Royal Melbourne Hospital. The normalization of raw data was performed by Ramyar Molania and Professor Terry Speed. The tumour sizes from MRI were calculated by Associate Professor Frank Gaillard and Dr. Arian Lasocki. The cryo-electronic microscope was conducted by Dr. Eric Hanssen. The nanoSight analysis was performed by Dr. Hong Nguyen. The invadopodium assay was performed by Clarissa Whitehead and Dr. Stan Stylii.

The candidate is supported by Melbourne International Research Scholarship and Nick Christopher Top-Up PhD Scholarship during the whole PhD research.

The first results chapter of this thesis (chapter 3) is presented as a peer-reviewed publication and additional results are included in this chapter. Three manuscripts based on the results of chapter 4,5 and 6 are under preparation.

Acknowledgement

I would like to thank my primary supervisor Dr. Andrew Morokoff for the the mentorship and guidance over the 4 years of my PhD. It is my great privilege that I had this opportunity to study glioma biology and liquid biopsy during the course of my thesis. His insights to the scientific questions and supports to my research are valuable assets. His supportive advice to my any 'strange' idea will be the driven force to my future career of medical research. I thank my co-supervisor Dr. Rodney Luwor for the continuous suggestion and discussion on my experimental details. I learned from him that the scientific thinking is the priority thing for medical research.

I thank the head of Department of Surgery (RMH), University of Melbourne, Professor Andrew Kaye for all kindly advices during my thesis. I am also grateful to Associate Professor Kate Drummond and Professor Mark Rosenthal for their critical questions and suggestions on my presentation in fortnightly Brain Tumour Meeting.

Special thank goes to my advisory committee, Dr. Hong-Jian Zhu and Professor Chris Hovens for their continuous contribution despite their heavy research commitment.

I would like to thank research fellows in Department of Surgery. I thank Dr. Hong Nguyen for her mentoring and suggestion of my experiments and presentation when I met any questions during the four years. I also thank Dr. Stan Stylii for not only the help on the experiment setting but also the critical insight and understanding on the biological questions. I would also thank to Lucy Paradiso for her experiment preparation and sample collection. Without her work, I could not

finish my research. I also thank Dr. Andrew Gogos and Ruth Mitchell for their suggestion and guidance of cell culturing when I step in the laboratory at the beginning.

I would also like to extend my gratitude to all collaborators, Associate Professor Grank Gaillard, Dr. Arian Lasocki, Dr. Rachel Koldej, Dr. Ulrich Putz, Dr. Jason Li, Ramyar Molania and Professor Terry Speed for the contribution of their professional suggestion to my thesis.

I end with a special acknowledgement to Sandra Gou and my parents who provided a support and perspective throughout my PhD. I could not make the journey possible without their accompanying.

Publications

Publication generated from this thesis

A comprehensive meta-analysis of circulation miRNAs in glioma as potential diagnostic biomarker. C Ma, HPT Nguyen, RB Luwor, SS Stylli, A Gogos, L Paradiso, AH Kaye, AP Morokoff. PloS one 13 (2), e0189452

Exosomes derived from Glioma Stem Cells (GSCs) promote cell migration, proliferation and radiation resistance In brain cancer. C Ma, H Nguyen, L Paradiso, U Putz, R Luwor, A Kaye, A Morokoff. Neuro-Oncology 19 (Suppl 3), iii61

Preoperative serum microRNA profiles as a diagnostic tool in glioma. A Morokoff, I Bennett, L Paradiso, R Luwor, R Koldej, J Li, C Ma. Neuro-Oncology 19 (Suppl 3), iii63

Publications outside this thesis

Peripheral nerve blocks for postoperative pain after major knee surgery. J Xu, X Chen, C Ma, X Wang. Cochrane Database of Systematic Reviews, 2014

Evaluation of osteopontin as a potential biomarker for central nervous system embryonal tumours. YP Han, CK Ma, SQ Wang, A Enomoto, Y Zhao, M Takahashi, J Ma. Journal of neuro-oncology 119 (2), 343-351

The node-weighted Steiner tree approach to identify elements of cancer-related signaling pathways. Y Sun, C Ma, S Halgamuge. BMC bioinformatics 18 (16), 551

Table of contents

<i>Preface</i>	3
<i>Acknowledgement</i>	4
<i>Publications</i>	6
Publication generated from this thesis	6
Publications outside this thesis	6
<i>Table of contents</i>	7
<i>Abstract</i>	10
<i>List of Figures and Tables</i>	12
Figures	12
Tables	13
CHAPTER ONE	15
CHAPTER 1: INTRODUCTION	16
1.1. <i>Glioma and Glioblastoma Multiforme (GBM)</i>	16
1.2. <i>Emerging molecular biomarkers for glioma</i>	19
1.2.1. Isocitrate Dehydrogenase1 or 2 (IDH1/2)	19
1.2.2. Updated pathological classification	21
1.3. <i>Cancer hallmarks in glioblastoma</i>	23
1.3.1. Altered Signaling pathways.....	23
1.3.2. Subtypes of glioblastoma.....	24
1.3.3. Tumour microenvironment (TME) in glioma	26
1.3.4. Epigenetic regulation in glioma	27
1.3.5. Glioma Stem Cells (GSCs)	28
1.3.6. Subtypes of GSCs	Error! Bookmark not defined.
1.4. <i>microRNAs</i>	31
1.5. <i>Liquid biopsy</i>	33
1.5.1. ctDNA.....	36
1.5.2. Extracellular Vesicles	36
1.5.2.1. Cancer progression and metastasis.....	39
1.5.2.2. Tumour EVs in the microenvironment	40
1.5.2.3. Stromal cells EVs to microenvironment	41
1.5.2.4. Therapeutic Resistance transfer	41
1.5.2.5. Heterogeneity of glioma by EVs	42
1.5.2.6. Circulating miRNAs	43
1.6. <i>Hypothesis and Aims</i>	44
CHAPTER TWO	45
CHAPTER 2: METHODS AND MATERIALS	46
2.1. <i>Materials</i>	46
2.1.1. Antibodies.....	46
2.1.2. Chemicals.....	47
2.2. <i>Methods</i>	48
2.2.1. GSC culture	48
2.2.2. Glioma Cell Lines.....	49

2.2.3.	EVs isolation.....	49
2.2.4.	EVs size and morphology quantification	49
2.2.5.	EVs uptake assay	50
2.2.6.	Radiation treatment	51
2.2.7.	Clonogenic assay	51
2.2.8.	Cell viability assay	51
2.2.9.	Western blot	52
2.2.10.	Cell migration assay	53
2.2.11.	MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay	53
2.2.12.	Zymography and invadopodia assay	55
2.2.13.	Limiting dilution assay.....	56
2.2.14.	Immunofluorescence assay.....	56
2.2.15.	Cell cycle detection by flow cytometry	57
2.2.16.	Public datasets	57
2.2.17.	miRNA targets prediction.....	57
2.2.18.	Protein interaction analysis	58
2.2.19.	Nanostring	58
2.2.20.	Neuroimaging measurement	59
2.2.21.	EVs miRNA profiling and analysis	59
2.2.22.	Gene Set Enrichment Analysis	59
2.2.23.	Endogenous control identification.....	59
2.2.24.	Bioinformatic analysis	60
2.2.25.	Statistical analysis	60
2.2.26.	Meta analysis	61
2.2.26.1.	Search strategy	61
2.2.26.2.	Inclusion and exclusion criteria	61
2.2.26.3.	Diagnostic capacity analysis	62
CHAPTER THREE		63
CHAPTER 3: SYSTEMATIC REVIEW AND META-ANALYSIS OF CIRCULATING MIRNAS IN DIAGNOSING GLIOMAS ..		64
3.1.	Introduction	64
3.2.	Results.....	65
3.3.	Additional results	79
3.4.	Additional discussion	80
CHAPTER FOUR		85
CHAPTER 4: IDENTIFICATION OF SUBTYPES AND IDH1 MUTATION ASSOCIATED MIRNAS IN GBMS.....		86
4.1.	Introduction	86
4.2.	Results.....	89
4.2.1.	Global miRNA profile of GBM from TCGA database	89
4.2.2.	miRNAs profile of GBM vs Normal.....	90
4.2.3.	Subtype specific miRNAs.....	94
4.2.4.	IDH1 specific miRNAs.....	100
4.2.5.	The correlation of IDH1 mutation associated miRNA expression with DNA methylation	103
4.2.6.	The potential target genes and pathways of IDH1 mutation associated miRNAs.....	108
4.3.	Discussion	117
CHAPTER FIVE		126
CHAPTER 5: CIRCULATING MIRNA PROFILE IN GLIOMA		127

5.1.	<i>Introduction</i>	127
5.2.	<i>Results</i>	130
5.2.1.	miRNAs in diagnosis	132
5.2.2.	IDH1 mutation associated circulating miRNAs.....	133
5.2.3.	Circulating miRNAs in postoperative monitoring.....	134
5.2.4.	miRNAs in prognosis	135
5.2.5.	Endogenous miRNAs controls in circulation	135
5.3.	<i>Discussion</i>	136
CHAPTER SIX		139
CHAPTER 6: GLIOMA STEM CELLS DERIVED EXTRACELLULAR VESICLES PROMOTE GLIOMA PROGRESSION VIA ACTIVATION OF AKT PATHWAY		140
6.1.	<i>Introduction</i>	140
6.2.	<i>Results</i>	144
6.2.1.	Exosomes characterization and internalization	144
6.2.2.	Characterization of GSCs.....	146
6.2.3.	GSC-derived EVs enhanced tumour cell migration and spreading.....	150
6.2.4.	GSC EVs can promote radiation resistance	152
6.2.5.	Frozen storage has less effect on the function of GSC EVs	154
6.2.6.	GSCs EVs involvement in invadopodia activity	155
6.2.7.	GSCs EVs promote glioma cell proliferation by increasing cell cycle	157
6.2.8.	GSCs EVs enhance tumour progression likely via the AKT/PTEN pathway	159
6.2.9.	GSCs EVs promotes sphere formation in GSCs	165
6.2.10.	miRNAs cargo in GSCs EVs targeted PTEN/AKT pathway	166
6.2.11.	RAB27A is strongly correlated with progression in glioma	167
6.3.	<i>Discussion</i>	170
SUMMARY AND FUTURE DIRECTIONS		177
REFERENCES		179
APPENDIX		200
<i>Appendix 1</i>		200
Appendix table 1. The target genes of IDH1 associated miRNAs predicted by miRwalk		200
<i>Appendix 2</i>		227
Complimentary material: 24 h record of GSCs differentiation		227
<i>Appendix 3</i>		228
declaration forms		228

Abstract

Glioma is the most common intracranial malignant cancer despite the rarity. Glioblastoma (GBM, grade IV) has very dismal outcomes where fewer than 20% of patients survive beyond 5 years. Low Grade Glioma (LGG, grade II), on the other hand, frequently occurs in a younger population with a variable outcome that depends on their individual genetic alterations. A tissue-based biopsy requires an invasive operation for every glioma patient and it is the gold standard diagnosis. In monitoring the patients for recurrence, MRI has the evitable limitation that it could not differentiate pseudo-progression after irradiation treatment for glioma patients. Therefore, a non-invasive biomarker, in addition to tissue biopsy and MRI, is urgently needed to provide more precise cancer care and management with glioma patients. In fact, cancer cells including glioma cells, release microRNA (miRNA) into the microenvironment and peripheral circulation, which allows physicians and scientists the ability to detect the disease status by a blood test. In addition, microRNA (miRNA) displays accuracy in diagnosing and monitoring other cancer patients by analysis of miRNA abundance.

To investigate the diagnostic capacity of circulating miRNA in glioma, a meta-analysis of previous relevant studies was performed. Circulating miRNA provided an over 90% accuracy of diagnosing glioma patients from healthy controls. A bioinformatic analysis revealed seven IDH1 mutation-associated miRNAs and these miRNA signatures were down-regulated in IDH1 mutant glioblastoma due to the hypermethylation in their promoter areas. Our proof-of-concept cohort recruited 91 glioma patients and 17 healthy controls and new circulating miRNAs were identified with remarkable diagnostic capacity. However, no circulating miRNA showed an association with IDH1 mutation in our cohort. As most circulating miRNAs were packaged and delivered by extracellular vesicles (EVs), serial cell experiments were conducted to examine the function of these miRNA-enriched EVs. Glioma Stem Cells-derived EVs significantly promoted glioma

cell proliferation, cell migration and radiation resistance. This effect was largely caused by the degradation of PTEN and activation of AKT in the recipient cells through miRNAs.

Collectively, these works established circulating miRNA as a clinical biomarker where it provides a complementary tool for glioma management. These results also formed the basis of future research into the role of EVs in glioma and highlighted the potential therapeutic target of EVs to glioma.

List of Figures and Tables

Figures

Figure 1.1. IDH1 mutation leads to DNA methylation

Figure 1.2. Genetic alteration in glioblastoma occur frequently in three signaling pathways.

Figure 1.3. Glioma Stem Cells are responsible for tumour progression and recurrence.

Figure 1.4. EVs in glioma

Figure 3.1. Forest plots of miRNAs from CSF.

Figure 3.2. The summary Receiver Operation Characteristics (ROC) curve of miRNAs in CSF.

Figure 4.1. Boxplot of miRNAs expression across all samples including GBMs and normal controls.

Figure 4.2. The difference between GBMs and normal controls with respect to miRNAs expression.

Figure 4.3. Differentially expressed miRNAs between GBMs and normal controls.

Figure 4.4. All miRNA profile of GBMs samples.

Figure 4.5. The differently expressed (DE) miRNAs between subtypes.

Figure 4.6. Clustering of all GBM samples by ten subtype associated miRNAs.

Figure 4.7. The workflow of identification of IDH1 mutation associated miRNAs.

Figure 4.8. Identification of IDH1 mutation associated miRNAs.

Figure 4.9. IDH1 specific miRNAs are hypermethylated in IDH1 mutant GBM patients.

Figure 4.10. The correlation of DNA methylation in the promoter regions of IDH1 mutation associated miRNAs and their expression.

Figure 4.11. The significant pathways of putative target genes of IDH1 mutation specific miRNAs.

Figure 4.12. Interaction networks of targets of miRNAs.

Figure 4.13. Kaplan-Meier Curves of IDH1 mutation specific miRNAs in GBMs.

Figure 5.1. The normalization of raw data.

Figure 5.2. A panel of 4 miRNAs shows a differentiation capacity for glioma versus healthy controls.

Figure 5.3 The differentially expressed miRNAs between low grade glioma and GBMs.

Figure 5.4. The IDH1 mutation associated circulating miRNAs.

Figure 5.5. Circulating miRNAs is associated with the residual tumour size.

Figure 6.1. The EV production of each GSCs line.

Figure 6.2. The morphology and size distribution of EVs.

Figure 6.3. The characterization of GSCs.

Figure 6.4. Different GSCs showed different radiation resistance and TMZ resistance.

Figure 6.5. GSCs EVs promote cell migration in recipient cells.

Figure 6.6. GSCs-derived EVs (GSCs EVs) transfer radiation- and chemo- therapy resistance.

Figure 6.7. GSCs EVs induced radiation resistance in U118 and is biologically functional for recipient cells under frozen storage condition.

Figure 6.8. GSCs EVs reduced invadopodia activity of recipient cells.

Figure 6.9. GSCs EVs promote recipient cell proliferation by increasing cell cycle.

Figure 6.10. GSCs EVs activated AKT pathway in recipient cells by reducing PTEN.

Figure 6.11. The effect of AKT pathway inhibitors on glioma cells.

Figure 6.12. The effect of GSCs EVs on recipient cells are reversed by AKT inhibitor.

Figure 6.13. GSCs-derived EVs (GSCs EVs) promote intratumour heterogeneity.

Figure 6.14. RAB27A expression is correlated with progression of patients with GBM.

Figure 6.15. The schematic diagram showing the mechanism of GSCs-EVs regulating glioma cells.

Tables

Table 2.1. List of antibodies used in this study

Table 2.2. The chemicals used in this thesis

Table 2.3. The characteristics of GSCs derived from patients.

Table 3.1. Characteristics of cohorts regarding miRNAs from CSF

Table 4.1. The differentially expressed miRNAs between GBMs and normal controls

Table 4.2. The differentially expressed miRNAs between subtypes of GBMs

Table 4.3. IDH1 mutation associated miRNAs

Table 4.4. The genomic information of seven IDH1 mutation associated miRNAs

Table 4.5. Gene Set Enrichment Analysis (GSEA) of IDH1 wild type and mutant GBM

Table 4.6. Top 95 significant target genes

Table 4.7. Cox hazard proportion model of IDH1 mutation associated miRNAs

Table 5.1. The characteristics of all participants included in circulating miRNA cohort

Table 5.2. Multivariate Cox proportion hazard ration regression of circulating miRNAs

Table 5.3. The top 20 stably expressed miRNAs identified by NormFinder

Table 6.1. The top 25 highly expressed miRNAs from GSCs EVs

Table 6.2. Cox analysis of RAB27A in relation to OS and PFS of patients with GBM.

Chapter One

Chapter 1: Introduction

1.1. Glioma and Glioblastoma Multiforme (GBM)

Glioma is the most common primary malignant tumour of the central nervous system in adults and most common solid cancer in children (Behin, Hoang-Xuan, Carpentier, & Delattre, 2003; McKinney, 2004; Ricard et al., 2012). The annual age-adjusted incidence rate of brain and central nervous system cancers is 27.86 per 100,000 people according to U.S statistics while the incidence in Europe (including United Kingdom) is 3-7 per 100,000 people (Crocetti et al., 2012; McKinney, 2004; Ostrom et al., 2014). Although the most common brain and central nervous system tumours are non-malignant tumours such as meningioma, nerve sheath tumours and pituitary tumours, glioma accounts for 27.4% of all malignant brain and central nervous system tumours (Ostrom et al., 2014). There is no significant difference of incidence of all types of brain tumours between genders, where 42% of all tumours occurred in males and 58% in females. The mortality rate of all types of brain tumours is 3-7 per 100,000 in Western countries, however, the survival is still dismal for glioma patients (Ohgaki & Kleihues, 2005). From the Australia cancer registries data covering 1.7 million people, there is no improvement of five-year survival for glioma patients from 1978 to 1992, suggesting further investigation of biology of glioma is urgently required (Ohgaki & Kleihues, 2005).

Glioblastoma (GBM), the most malignant glioma has the worst prognosis of all brain tumours, which is only 15 months. The five-year survival rate for GBMs are less than 6% (Ohgaki & Kleihues, 2005; Visser et al., 2015). Current standard treatment protocol for primary GBM is maximum safe resection plus radiation and concurrent temozolomide, as demonstrated by Stupp in 2005 (Stupp et al., 2005). Targeted therapies are entering clinical practice, as showed by the application of bevacizumab, an antibody to vascular endothelial growth factor (VEGF), for the treatment of recurrence or progressive GBMs (Norden et al., 2008). As immune therapeutics including immune checkpoint blockades

and Chimeric antigen receptor (CAR)-expressing T cells (CAR-T) have displayed remarkable anti-tumour efficacy in other solid tumour and haematological neoplasms (Eggermont et al., 2018; Hellmann et al., 2019; Horn et al., 2018; Quintas-Cardama, 2018; Schmid et al., 2018; Schuster et al., 2019; Tan et al., 2016), a few clinical trials are under investigation for their application in glioma. The preliminary results from CheckMate-143 that comparing nivolumab (PD-1 inhibitor) with the anti-VEGF antibody, bevacizumab in the treatment of recurrent gliomas showed a failure of nivolumab to prolong the overall survival of patients with recurrent gliomas (Filley, Henriquez, & Dey, 2017). Despite numerous efforts to optimize GBM treatment regimen, the standard guideline treatment for GBM hasn't been modified since 2005 as there is no other treatment demonstrating a superior outcome to the Stupp protocol. More importantly, recurrence is inevitable in most cases of GBM even after the standard therapeutic treatment (Stupp et al., 2005). However, the biological mechanism of GBM recurrence remains unclear. Overall, GBM is a complex and highly heterogeneous disease with dismal outcomes; therefore further understanding for GBM tumourigenesis, therapeutic resistance and disease monitoring are urgently required (Stupp et al., 2005).

Currently the pre-diagnostic tool utilized for detection of gliomas in clinical setting is Magnetic Resonance Imaging (MRI). With the information supplied from contrasted MRI and Magnetic Resonance Spectroscopy (MRS), it clearly demonstrates the location, size and vascular supply of glioma and their nearby tissue, which help surgeons decide the suitable operational approaches for individuals. However, neuroimaging fails to provide all the biological features and biological behaviours of glioma and it doesn't demonstrate strong correlation of imaging and patients' survival. Two systematic reviews recently found that the diagnostic capacity of MRI is also limited for newly diagnosed and recurrent gliomas. Both in adults and children, the sensitivity of MRI for detecting low grade glioma and specificity for high grade glioma are 66-93% and 9-90%, respectively (Abrigo et al., 2018). For recurrent glioma, the diagnostic power is even worse. The sensitivity and specificity of MRI for diagnosing recurrence glioma are 68%

and 77%, respectively (van Dijken, van Laar, Holtman, & van der Hoorn, 2017). Pseudoprogression after glioma treatment, especially caused by irradiation treatment, might confuse surgeons and experienced radiologists as glioma necrosis induced by irradiation shows a quite similar pattern to glioma recurrence in MRI. Considering the accessibility, MRI or other neuroimaging is also not suitable for glioma screening. A novel diagnostic testing with accuracy and accessibility for glioma is needed to augment the diagnostic capacity.

The diagnosis of glioma is largely based on histopathology. Of numerous diagnostic grading systems in use, the most common is the World Health Organization (WHO) grading system for gliomas, which categorized glioma into 4 groups based on the histological features (e.g. anaplasia), under which tumours are graded from I (benign) to IV (most malignant). Specifically, the grade of glioma will increase accordingly with increased cell proliferation, nuclear atypia, mitotic activity and infiltration. This grading system has been used as the gold standard of diagnosing glioma and glioblastoma for several decades because it directly reflects the malignancy of glioma and the natural disease course. Within WHO grading system, WHO grade I tumours are slow-growing, while WHO grade IV tumours such as GBM are the most malignant and prompt to progression. Interobserver variation, especially in the context of diffusely infiltrating gliomas (Reifenberger, Wirsching, Knobbe-Thomsen, & Weller, 2017; van den Bent, 2010), might contribute to the inconsistency and bias of diagnosis, which eventually lead to the under- or over- treatment strategies for patients with gliomas. Numerous studies have revealed that molecular classification driven by genetic/epigenetic markers provides more accurate clinical stratification and outcomes than the conventional system based on histology (Cancer Genome Atlas Research et al., 2015; Suzuki et al., 2015; Weller et al., 2015; Wiestler et al., 2014). Moreover, molecular classification also provides intrinsic biological features within even one type of glioma and more insights on the intratumour heterogeneity and tumour evolution (Morokoff, Ng, Gogos, & Kaye, 2015).

1.2. Emerging molecular biomarkers for glioma

1.2.1. Isocitrate Dehydrogenase1 or 2 (IDH1/2)

The finding of isocitrate dehydrogenase1 or 2 (IDH1/2) mutation is a huge breakthrough in understanding biology of glioma as accumulating studies proved that IDH1/2 mutant status strongly predicts biological features and patients' outcomes (Balss et al., 2008; Parsons et al., 2008; H. Yan et al., 2009). IDH1 mutation is frequently observed in low grade glioma and secondary glioblastoma. IDH1 R132H mutation is the most frequent alteration in all glioma. Molecular classification based on the IDH1/2 mutation status allows clinicians to apply suitable therapeutics for patients with gliomas. Specifically, for IDH1 mutant low grade glioma, an additional survival benefit that prolonging the median survival of low grade glioma from 5.7 years to 9.4 years was observed in patients treated with procarbazine/lomustine/vincristine (PCV) chemotherapy combined with radiation. On contrast, this survival benefit of PCV plus radiation treatment was not observed in IDH1 wild type oligodendroglioma (Cairncross et al., 2014). Additionally, preliminary results from pre-clinical studies of IDH1 inhibitors also highlights that targeting IDH1 could be a promising therapeutic agent and there is a necessity of performing IDH1 testing for all glioma patients prior to treatment (Molenaar et al., 2015; Pusch et al., 2017).

Recent studies also identified IDH1/2 mutation is linked to the early stages of gliomagenesis. Along with the difference of cells DNA mutations, glioma also demonstrates distinct biological behaviours between IDH1 mutant and wild type gliomas. Absence of normal IDH1 enzyme leads to a conversion of α -ketoglutarate (α -KG) into 2-hydroxyglutarate (2-HG). The accumulation of 2-HG in IDH1 mutant glioma changes the metabolism in glioma cells, cell apoptosis and methyltransferase, therefore mutant IDH1 leads to alteration of cancer signaling pathways and tumourigenesis (B. Jiang et al., 2017) (Figure 1.1). Mechanistically, mutant IDH protein has a new catalytic activity of

converting α -ketoglutarate to 2-hydroxyglutarate, which eventually leads to DNA and histone methylation modification.

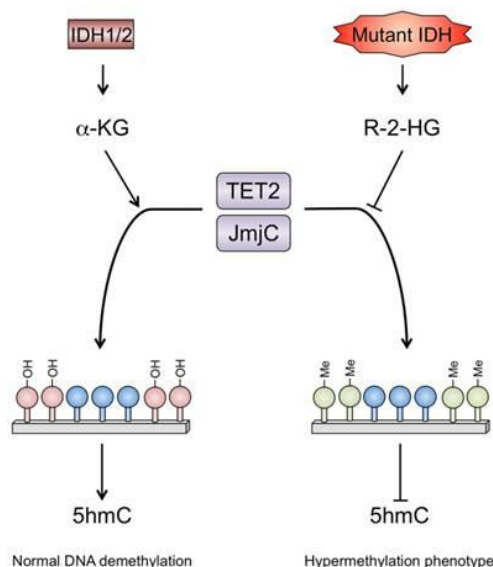


Figure 1.1. IDH1 mutation leads to DNA methylation (adapted from Int J Med Sci 2015; 12(3):201-213. doi:10.7150/ijms.11047)

Although recent studies showed conventional MRI and MRS detection of the metabolism product induced by mutant IDH1 have an excellent diagnostic capacity of glioma (Diamandis et al., 2018; Nagashima et al., 2016), immunochemical staining for IDH1 is still the gold standard for routine clinical testing. The antibody used clinically, usually targets the IDH1 R132H protein, which is the most common mutation site in glioma (accounting for 70% of IDH1 mutations), as it has already been demonstrated to produce stable results across different laboratories, regardless of whether Western blot or immunochemistry techniques are used (Brandner & Jaunmuktane, 2018; Capper, Zentgraf, Balss, Hartmann, & von Deimling, 2009). The stability and utility of this immunochemistry antibody is the mainstay for clinical testing as formalin fixed paraffin embedded tumour specimens are the tissue that are used in clinical practice. The importance of IDH1 has been widely recognized but the biological difference of IDH1 mutant and wild-type gliomas have not been fully investigated. We still do not know

whether IDH1 mutant gliomas have large percentage of DNA methylation (therefore affecting some tumour suppressor genes or microRNAs expression) compared to IDH1 wild type glioma. Further understanding of the difference between IDH1 mutant versus wild type glioma would enhance our understanding of tumourigenesis of glioma, the tumour evolution of glioma after radiation or chemotherapy treatment and ultimately benefit glioma patients.

1.2.2. Updated pathological classification

The 2016 revised version of the WHO central nervous system tumours classification has now incorporated several biomarkers including IDH1 mutation, 1p19q co-deletion based on validation studies performed in multi-centre trials (Louis et al., 2016). NCCN guideline for glioma also suggests DNA methylation test of MGMT is recommended for TMZ treatment. This new revised version of pathological handbook and guideline highlights the importance of molecular testing for glioma patients in clinical managements. As a result, the diagnosis for glioma provided by pathologists currently combines the histological and molecular information (van den Bent et al., 2017).

The molecular alteration in glioma not only redefines precise diagnosis for glioma but also improves the patient's prognostication. A few studies have uncovered the additional survival benefit for IDH1 mutant oligodendrogliomas and astrocytic gliomas with the delivery of chemotherapeutics procarbazine, lomustine and vincristine (PCV) and 1p19q-codeleted tumours should be treated with radiation and PCV (Buckner et al., 2016; van den Bent et al., 2013). In contrast, patients with IDH1 wild-type diffuse astrocytic gliomas do not benefit from this chemotherapy regimen.

In addition to genetic mutations, two other molecular markers (1p/19q chromosomal codeletion and O6-methylguanine methyltransferase (MGMT) promoter methylation status) have substantially been identified as prognostic markers for oligodendrogliomas

and predictive marker for chemotherapy, respectively. Patients diagnosed with oligodendrogliomas might have favourable outcomes if they are identified with tumours having 1p/19q co-deletion (Cairncross et al., 1998). At first, DNA methylation of MGMT promoter was not applied into clinical testing even if it was demonstrated to predict the benefit from the addition of temozolomide to radiation in the treatment of newly diagnosed GBM (Hegi et al., 2005). However in 2012, two randomized clinical trials confirmed consistently that a methylated MGMT promoter is associated with favourable outcomes of temozolomide chemotherapy (Malmstrom et al., 2012; Wick et al., 2012). Patients recruited in the German NOA-08 clinical trial, received a protocol of either standard 6-week fractionated radiotherapy or dose-dense temozolomide (week on/week off). The major conclusion of this study is that patients with methylated MGMT promoter have favourable outcomes when they are treated with temozolomide chemotherapy (Wick et al., 2012). Similarly, a Nordic trial has found that a standard temozolomide protocol produced a preferred outcomes over radiation, especially in patients with a methylated MGMT promoter (Malmstrom et al., 2012). Since then 1p/19q co-deletion and MGMT promoter status have been the prerequisite test for oligodendroglioma and changed the comprehensive treatment protocol for patients with gliomas.

This “integrated diagnosis” will not only definitely provide insights of treatment decision and more information for current glioma patients but also change the way of the designs of clinical trials. Based on the molecular testing results, physicians could select the most optimised treatment for these patients who would have effective response to the molecular targets or selecting the most suitable patients for clinical trials. Conventional randomization of clinical trials are only based on the histopathology, however, patients could be allocated according to their molecular biomarker profile. Patients with the correct molecular profiles could then be randomly allocated into new treatment or standard treatment groups, which would aid in determining whether this new treatment is “truly” effective for all biomarker-positive glioma patients. More specific biomarker-based stratification has been applied in clinical trials. Assessment for the presence of

EGFR or FGFR mutations and their expression is being considered before the most suitable patients are chosen for trials involving anti-EGFR or anti-FGFR based therapies.

Despite clinical trials have evolved and some patient could benefit from the molecular biomarkers, the postoperative monitor of glioma is largely relied on neuroimaging rather than biological biomarkers. Neuroimaging such as MRI is not accurate for the glioma recurrence during the following-up where the pseudo-progression is likely the confounder factor in the MRI for diagnosis. A new biomarker surrogating the biological behaviours in glioma and predicting the risks of recurrence in low grade gliomas is demanded especially for monitor of glioma (Tom et al., 2019).

1.3. Cancer hallmarks in glioblastoma

Cancer is a complex disease where multiple factors and interactions contribute to tumourigenesis. In glioma, alterations to signaling pathways, microenvironment adaption and (epi)genetic alteration are regarded as the main driven forces of all cancer.

1.3.1. Altered Signaling pathways

Modern techniques have enabled researchers for the exploration of genetic alteration in brain tumours (Figure 1.2). The EGFR gene encoding the receptor of the epidermal growth factor (EGF) was proposed as a primary oncogene for GBM in 1984 (Libermann et al., 1985). Subsequently, the TP53 tumour suppressor gene, playing a role in monitoring DNA damage and control cell cycle and apoptosis, was recognized as the main driver of alteration in GBMs. In 1997, the phosphatase and tensin homolog (PTEN) phosphatase was identified as a tumour suppressor and a negative regulator of the phosphoinositide 3 kinase (PI3K) pathway, which is the major activated signaling pathway to promote cell proliferation in GBMs. In the last decades, IDH1 and IDH2 were identified to be mutated in low grade glioma and small subpopulation of GBMs, in the

proneural subtype or evolved from lower grade gliomas (Parsons et al., 2008). Some studies have proposed that mutant IDH1 has an indirect oncogenic effect by activating hypoxia-inducible factor pathway that is a critical step allowing tumours to adapt to anaerobic growth microenvironment and develop new blood vessels (S. Zhao et al., 2009). This genetic alteration updates the knowledge of glioma biology which sheds a light on the biological features for diagnosis and vulnerable targets for therapeutics .

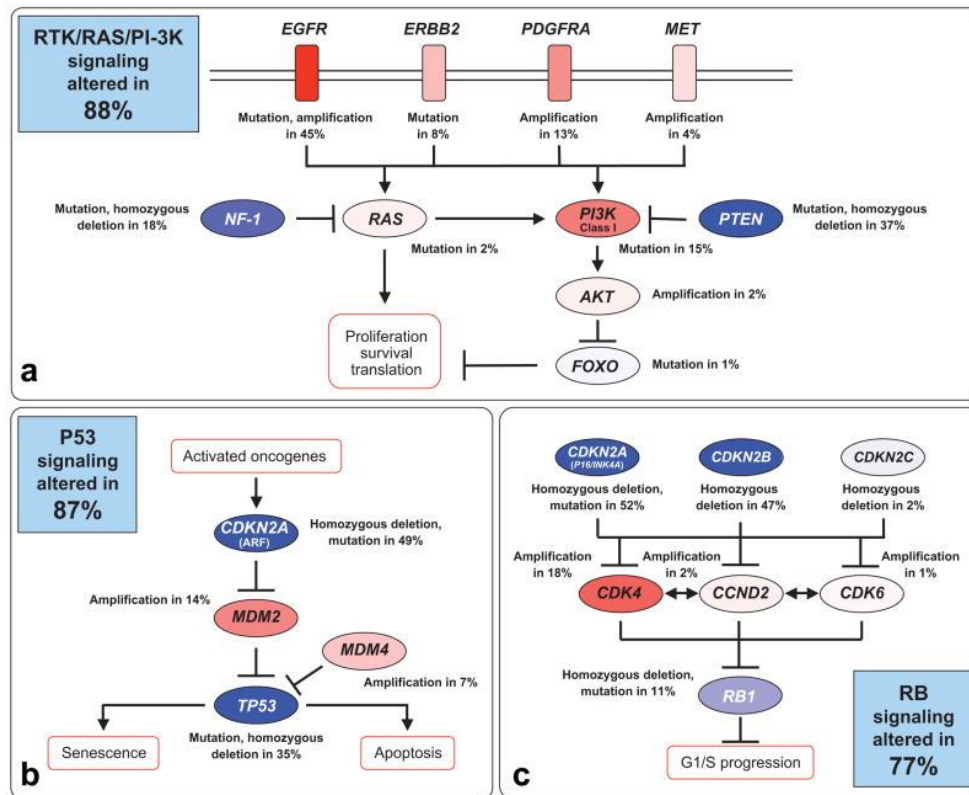


Figure 1.2. Genetic alteration in glioblastoma occur frequently in three signaling pathways. (Adapted from CA Cancer J Clin. 2010 May-Jun;60(3):166-93. doi: 10.3322/caac.20069.)

1.3.2. Subtypes of glioblastoma

Accumulating evidence shows that gliomas are heterogenetic cancers; even though they may display similar features pathologically. According to the WHO grading system, GBM is a grade IV glioma with the most malignancy and worst prognosis (Ohgaki &

Kleihues, 2005). The breakthroughs of cellular and molecular biology of GBM over the last decades have demonstrated pathological hallmarks which improved the diagnostic accuracy and treatment efficiency. However, increasing studies showed that GBM could derive from totally different precursor cells and harbors exclusively gene mutations and signaling pathways (Cancer Genome Atlas Research, 2008; Phillips et al., 2006; Verhaak et al., 2010). Current research has proved GBM is a complex disease with different featured cellular subgroups (Verhaak et al., 2010).

Due to the rapid development of biotechnology, high throughput techniques are now prevalent in cancer biology studies. Dozens of cancers have benefited from these platforms, including studies focused on establishing a greater understanding of GBM. Golub et al have showed the heterogeneity among GBM by microarrays (J. Du et al., 2009). In 2006, Phillips et al performed transcriptional microarray and concluded that there are three exclusively subgroups among GBMs, namely Proliferative, Proneural (PN) and Mesenchymal (MES) (Phillips et al., 2006). Furthermore, based on TCGA database, the worldwide database, Verhaak et al identified four subgroups (Proneural, Neural, Classical, and Mesenchymal) from GBM specimen samples by unsupervised clustering (Verhaak et al., 2010). Notably, GBMs harbor distinct DNA alteration, activated signaling pathways and response to treatment across these four subtypes (Cancer Genome Atlas Research, 2008). Although there is no consensus on the specific number of subgroups for GBM, the PN and MES classification appears to be robust and consistent. Interestingly, the featured genes of PN and MES were identified in both studies as DLL3 and OLIG2 as well as CD40 and CHI3L1/YKL-40, respectively. In addition, the glioma-CpG island methylator phenotype (G-CIMP) was uncovered on TCGA database by profiled promoter DNA methylation alterations. G-CIMP, displays in the PN subgroup mainly, are prevalent in low grade gliomas (Noushmehr et al., 2010). Integrative analysis of genomic and survival confirm MGMT DNA methylation may be a predictive biomarker for treatment response in classical subgroup (Brennan et al., 2013). Although

it remains uncertain that specific genes correlate with survivals and prognosis, subtype classification based on transcriptional level provides a novel insight into GBM biology.

Last year two studies showed the heterogeneity of gliomas is temporal as well as spatial. Patel et al found gene expression profiling and gene mutation analysis revealed a significantly regional heterogeneity by single-cell RNA sequencing. Strikingly, the gene mutation and gene expression of single cells derived from the same GBM specimen expressed different gene signatures related to cell signaling, hypoxia, complement and immune responses and oligodendrocyte function, the expression of a glioma stem-cell gene profile and the expression of GBM gene expression profiles, including Proneural, Neural, Classical, and Mesenchymal. Moreover, increased PN classification signature is associated with better survival (Patel et al., 2014). Johnson et al observed the temporal heterogeneity from comparing exome sequencing results from matched tumour samples obtained at diagnosis and at recurrence. Their data showed some gliomas did not share the identical mutations at recurrence (Johnson et al., 2014a). Considering the tumour heterogeneity of glioma, single tissue biopsy might not be suitable for glioma as tissue biopsy cannot capture the landscape of all genomic alteration in glioma.

1.3.3. Tumour microenvironment (TME) in glioma

The tumour microenvironment (TME) is emerging as key regulator in cancers, especially in glioma and has been showed to be involved in therapeutic resistance, intercellular communication and immune suppression. The lack of success of anti-cancer drugs in in vivo models, despite demonstrating high efficacy in in vitro models highlights the importance of TME in cancer management. TME contains a great variety of cells, including tumours cells as well as extracellular matrix and fibroblasts, endothelial cells and immune cells. Macrophages/microglia and astrocytes are regarded as the most common cell types in glioma TME.

Macrophages are the most common immune cells in the brain, accounting for around 30% of the tumour bulk (Graeber, Scheithauer, & Kreutzberg, 2002). Macrophage activation has been demonstrated to promote tumourigenesis and is associated with increasing tumour grade. In addition, High levels of macrophages are also strongly linked with radiation resistance and anti-angiogenesis resistance of glioma (Lu-Emerson et al., 2013). Single cell analysis also unveils the accumulation of M2 macrophage induced by depletion of NF1 associates with radiation resistance (Q. Wang et al., 2017). A survival advantage observed in other types of cancer after depletion of macrophage further indicates targeting macrophage or its secreted factors such as colony-stimulating factor-1 receptor (CSF-1) would be an effective strategy for glioma treatment (Quail & Joyce, 2017). A pre-clinical study has already provided a profound insight that inhibition of CSF-1R attenuates the growth and invasion of glioma by depletion of macrophages, which leads to a clinical trial targeting CSF-1R (Quail & Joyce, 2017).

Astrocytes are the most unique cells types in the brain and they play important roles in brain tumourigenesis and tumour spread/invasion. All cells are required to adapt or interact with astrocytes to accommodate themselves in the brain. A recent paper reported tumour suppressor PTEN was specifically downregulated in brain metastasis other than in primary and other metastasis organs. Mechanically, astrocytes reduced the PTEN expression in brain metastasis cells and then activate PI3K-Akt pathway to induce primary cells outgrowth (L. Zhang et al., 2015b). These results highlight the importance of both the microenvironment in the brain and the intercellular communication in brain tumours.

1.3.4. Epigenetic regulation in glioma

Epigenetic dysregulation is one of the typical features in tumourigenesis. Due to the advances in devices and techniques, recent studies have revealed that epigenetic alterations, especially DNA methylation and histone methylation coordinates

tumourigenesis and progression in gliomas. G-CIMP induced by IDH1 mutation leads to global DNA hypermethylation in glioma cells leading to the prevention of transcription of multiple tumour suppressor genes. Therefore, the alterations of these tumour suppressor genes contribute to overactivation of signaling pathways that drive cell proliferation. Moreover, DNA methylation has a strong effect on therapeutic resistance. MGMT promoter methylation is a strong predictive biomarker for temozolomide treatment, and thus testing for this alteration is becoming part of the routine clinical management of patients with GBM.

Considering the significant correlation of methylation with glioma, the new 2016 revised version of WHO CNS pathology guidelines has recommended DNA methylation testing for patients' stratification. Epigenetic dysregulation also interacts with cell development where some stem-like cells loss their differentiation potent in glioma or some differentiated glioma cells are reprogramed back into their unmaturred status (Bulstrode et al., 2017; Turcan et al., 2018). Ongoing studies are still looking at optimizing of DNA methylation testing, and discovery of druggable targets after methylation testing in gliomas (de Souza et al., 2018) (Cloughesy et al., 2017).

1.3.5. Glioma Stem Cells (GSCs)

For decades, there has been a hypothesis that a specific subset of cells are primarily responsible for tumourigenesis and therapeutic resistance. The definitive criteria for cancer stem cells and somatic stem cells remains obscure. However, the definition of GSC is a subpopulation of glioma cells that have the ability to self-renew, to form tumours in animal models and are capable of multipotent differentiation, which is regarded as an initiative of tumour progression and recurrence (Figure 1.3). Recent studies have demonstrated that GSCs can manipulate tumour microenvironment and parenchymal metabolism to accommodate GSCs for tumour progression and migration (P. Mao et al., 2013; van der Vos et al., 2015; L. Zhang et al., 2015a). Thus, GSC represents

an important subpopulation to further understand glioma biology, cellular phenotypes and molecular mutations.

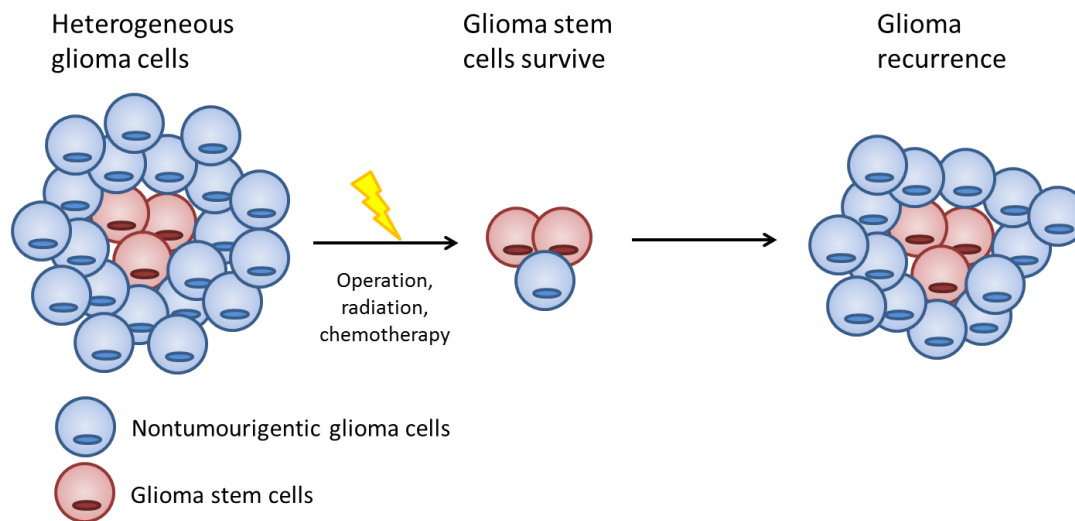


Figure 1.3. Glioma Stem Cells are responsible for tumour progression and recurrence.

Given that GSCs play a pivotal role in glioma pathogenesis and recurrence, identification and confirmation of cellular biology of GSCs is required. Although there are no universal and robust markers for sorting GSCs from bulk tumour specimen and cell lines, the neural surface marker CD133 is considered the best current marker for GSCs. Cells with CD133 positive have been shown to have the capacity for neurosphere formation, proliferation, and differentiation during culturing in the neural basal medium. In 2004, Singh et al isolated GSC by sorting brain tumour culture in terms of CD133 and proved CD133+ brain tumour cells have the capacity of tumour initiation in SCID mouse brains (Singh et al., 2004). CD133+ brain tumour cells derived from human glioma xenografts and glioblastoma specimens activate the DNA damage checkpoint and repair radiation-induced DNA damage, suggesting that GSC can promote radio-resistance and provides a therapeutic model for radio-resistant GBMs (Bao et al., 2006). Furthermore, GSC also can be isolated in the glioma cell lines, in which featured stemness signaling pathways such as hedgehog, Bmi1, Notch and PTEN/PI3K/Akt are enriched (Singh et al., 2003).

Metabolism and microenvironment are other important factors that can regulate the cellular and molecular phenotype of GSCs. Although GBM display the Warburg effect, a preferential utilization of aerobic glycolysis for energy supplies, there is no consensus on GSCs metabolism (Venneti & Thompson, 2017). The microenvironment is crucial for cancer, especially for oxygen-dependent gliomas so GSCs are intimately regulated by cluster in perivascular and hypoxic conditions (K. Yan, Yang, & Rich, 2013). Trans-differentiation of GSC into endothelial cells and pericytes has been described in a previous study to customize the suitable microenvironment and accommodate glioma. In addition, the role of TGF-beta, usually secreted by tumour-associated macrophages is highlighted during GSCs progression. TGF-beta secretion was observed to promote radioresistance of GSCs, suggesting TGF-beta could be a therapeutic marker (Hardee et al., 2012). For accommodating the progression of glioma, GSCs seek oxygen supply not only by recruiting blood vessel formation but also by maintaining the vascular niche (Gilbertson & Rich, 2007). GSCs actively interact with glioma vasculature and reshape the tumour niche by secreting factors such as VEGF.

With the advances in single molecule collection, detection and bioinformatic analysis, we can elucidate the key molecules in each single cell with high resolution. Several single cell sequencing studies confirm not only the heterogeneity of gliomas but also the hierarchy of glioma cells, where GSCs exist in high grade gliomas and IDH1 mutant low-grade gliomas (Patel et al., 2014; Tirosh et al., 2016; Venteicher et al., 2017). These results shed a light on hierarchy development in glioma as it shows the applicability of GSCs model and ex vivo culturing model of GSCs can recapture the features of gliomas. The continuous presence of GSCs in glioma development and recurrence uncovers the fact that GSCs might play a broad but critical role in tumourigenesis, microenvironment mediation, and chemoresistance. Therefore, the investigation of interaction of GSCs with the microenvironment and differentiated glioma cells would promote the diagnosis ability and provide a clue for therapeutics.

1.4. microRNAs

MicroRNAs (miRNAs), are 20-24 nucleotides in length, belongs to non-coding RNAs, which were previously considered as “junk” products in transcription. More interests in miRNAs arose in 2002 when they were discovered to play prominent regulatory roles in normal and cancer cells (Floyd & Purow, 2014). Biologically, miRNAs suppress or degrade gene expression mainly by binding to the complementary 3' UTR of target mRNA (Floyd & Purow, 2014). Through regulating the target genes transcripts, miRNAs play critical roles in human development and physiology as well as regulation of diseases processes including gliomas (J. Lu et al., 2005; Medina & Slack, 2008; Taft, Pang, Mercer, Dinger, & Mattick, 2010). MiRNAs have tissue-specific signatures, indicating that they can display the miRNA profile of the tissue of origin (Rosenfeld et al., 2008). These characteristic features allows miRNAs to be a most promising molecular biomarkers (Hua et al., 2012; Tumilson, Lea, Alder, & Shaw, 2014).

Accumulating evidence demonstrates that miRNAs play a vital role in cancer initiation, development, proliferation and metastasis. Depends on the biological features of miRNA targeted genes (oncogenes or tumour suppressors), miRNAs have double-sided effects in cancers. One miRNA could promote cancer development by inhibiting tumour suppressors in one type of cancer, and meanwhile, it could also prevent cells from malignancy transformation via suppressing oncogenes in other types of cancer (Peng & Croce, 2016). In addition, as miRNA-mRNA interaction network is complex where each miRNA having the ability of complementary binding to multiple target genes and vice versa, the role of miRNAs in cancer biology is still underestimated (Floyd & Purow, 2014).

Multiple cancer related pathways and cancer cell phenotypes are regulated by miRNAs in glioma. It has been reported that multiple miRNAs participate in the biological regulation of key signalling pathways in glioma. miR-7 is down regulated in glioma tissue samples and it inhibits the expression of EGFR and AKT, the most common altered pathway in

glioma (Kefas et al., 2008) . MiR-21, as an oncomiR in cancers, also increases gliomas invasiveness and migration by suppressing the tumour suppressor genes including PTEN (Floyd & Purow, 2014). Apoptosis is regulated by miR-148a through targeting MIG6 and BIM to activate EGFR (J. Kim et al., 2014). Down-regulated let-7 family is observed in gliomas and related with glioma invasiveness, indicating that the let-7 family acts as a tumour suppressor role in gliomas biology (X. G. Mao et al., 2013). MiRNAs such as miR-218, miR-93 and miR125b are also involved in vascular development, another crucial hallmark during glioma growth (L. Fang et al., 2011; Mathew et al., 2014).

Many miRNAs in glioma progenitor/initiating cells initiate tumour progression and development by regulating the stem cell pathways like Notch, hedgehog, Wnt, TGF-beta (Floyd & Purow, 2014). Stem cell signalling pathways or key epigenetic regulators such as polycomb repressor complex (PRC1) and Enhancer of zeste homolog 2 (EZH2) are also regulated by miRNAs to enhance glioma proliferation and self-renewal. As reported, EZH1 is critical for GSCs maintenance as depletion of EZH2 leads to a loss of the capacity to form tumours (Suva et al., 2009). MiR-128 and miR-218 reduces glioma migration, proliferation and self-renewal by binding and suppressing bmi-1, a component of the PRC1 (Godlewski et al., 2008; Tu et al., 2013), while cell cycle arrest was observed in GSCs by miR-34a and miR-125b (Guessous et al., 2010; L. Shi et al., 2010). miR-33a demonstrates its GSCs-promoting effects by inhibiting phosphodiesterase 8A (PDE8A) and UV radiation resistance-associated gene (UVRAG), the key genes in PKA and NOTCH pathways (Wang et al., 2014). Smoothened (SMO), a key regulator in the hedgehog pathway, is inhibited by miR-326 and overexpression of miR-326 significantly reduced the tumorigenicity (W. Du et al., 2015). MiR-10 enhances GSCs proliferation by regulating STAT3 and PTEN. Bian et al also found the invasiveness of GSCs is regulated by miR-20a and miR-106a targeting TIMP-2 and these two dysregulated miRNAs are activated by NF-kB. Compared to the differentiated glioma cells, miR-21 and miR-221/222 are upregulated in GSCs, suggesting these GSCs specific miRNAs are potentially the therapeutic targets (Aldaz et al., 2013). All these studies indicate miRNAs

are key player in GSCs and further investigation on how GSCs featured miRNAs mediate glioma progression and development is needed.

Due to the molecular and clinical heterogeneity of gliomas, subtypes of gliomas are developed according to transcription profiling by Phillips et al and the TCGA (Phillips et al., 2006; Verhaak et al., 2010). Different subtypes of GBM link with various clinical outcomes and feature gene expression. The comprehensive data from TCGA revealed the featured mutation pathways such as PI3K, AKT, RAS, MARK and p53. Likewise, miRNA profiling demonstrates significant differences across these exclusive subtypes. Moreover, a bioinformatics analysis of miRNA-based clusters identified five subtypes of precursor cells: oligoneural, radial glial, neural, neuro-mesenchymal and astrocytic via TCGA dataset, indicating miRNAs involve in different stages of neural differentiation during tumour progression (T. M. Kim, Huang, Park, Park, & Johnson, 2011). Although there are a few studies examining miRNAs expression pattern in different subtypes of GBMs or GSCs, we still know little about the IDH1 mutation relevant miRNAs and whether these subtype specific miRNAs could potentially be the biomarker for glioma.

1.5. Liquid biopsy

Biomarkers for patient stratification, risk definition and monitoring of treatment response have already become an integral component of clinical practice (Freidlin & Korn, 2014). The clinical importance of biomarkers based on genetics and histology is well established, but a circulating factor would be potentially useful as minimally invasive biomarker that could yield accurate diagnosis and monitoring of tumour growth and response to therapy. In several cancers including breast cancer and colorectal cancer, the “liquid biopsy” has become important in clinical practices, however a circulating biomarker for glioma has not yet been identified. (Crowley, Di Nicolantonio, Loupakis, & Bardelli, 2013; Diaz & Bardelli, 2014).

The utility of liquid biopsy is unlimited. Molecular and cellular heterogeneity is the hallmark of cancers, especially in gliomas and creates additional challenges the successful early diagnosis and therapy (Bardelli & Pantel, 2017). Liquid biopsies represent an ideal alternative, considering the difficulty of obtaining an accurate overview of the genetic landscape of solid cancers from a single tissue biopsy. Current evidence has already demonstrated Circulating Tumour Cells (CTCs) or circulating tumour DNA (ctDNA) could be from the genome of multiple metastatic tumour cells (Bardelli & Pantel, 2017). Moreover, liquid biopsy makes dynamic detection of cancer clone evolution during therapeutic treatments possible. This could be useful in brain cancer as we have observed glioma cell evolution after conventional adjuvant therapies (J. Wang et al., 2016; Q. Wang et al., 2017). Early detection of cancer is always attractive for physicians, researchers and patients because the results from early screening detection enables physicians to take feasible therapeutic actions and prolong patients' survival before they become late-stage incurable cancers. The sensitivity and specificity of this early cancer screening would be the most challenging issue before it is applied into clinical usage. Some cancer-associated mutation could be discovered in populations with increasing ages, which do not drive cancer progression in particular individuals during their lifetime (Bardelli & Pantel, 2017). TP53 mutation could be detected in 11.4% of non-cancer healthy populations (Fernandez-Cuesta et al., 2016). A combinational panel of current protein biomarkers and ctDNA is probably a solution to this issue with high sensitivity and specificity. A multi-analyte blood based test recruiting 1005 surgically resectable cancers and 821 healthy controls demonstrated an appealing outcome for early detection and localization of cancers with 70% sensitivity and >99% specificity (Cohen et al., 2018b). In this tool, Cohen et al combined 8 protein markers and 61 amplicons from 16 most mutant genes in cancer-associated pathways to optimize the panel for the early detection of cancers. Monitoring the minimal residual disease and prediction of target therapy responses are other aspects for clinical application of liquid biopsy. The continuous presence of CTCs or ctDNA in blood samples taken from cancer patients has already been found to indicate the unfavourable outcomes after adjuvant therapies (Rack

et al., 2014). Monitoring stratification may apply to the ‘high-risk’ cancer patients identified by liquid biopsy as these patients might require additional adjuvant therapies. More importantly, the approval of blood based EGFR testing for lung cancer patients during anti-EGFR agents unveils this proof-of concept is clinically applicable (Bardelli & Pantel, 2017). Additionally, liquid biopsy could be an effective tool in the era of immunotherapy because we could detect not only tumour alterations and tumour burden but also T cells activation and response simultaneously (Schumacher & Scheper, 2016). Using the techniques developed from liquid biopsy, isolating the neoantigen specific T cells is feasible in the future. Collectively, liquid biopsy is an appealing alternative for cancer patients, though tissue-based biopsy is still the gold standard for cancer diagnosis and monitoring.

A perfect circulating biomarker should represent the whole tumour or demonstrate the biological relevance and reliability of tumours to a large extent (Westphal & Lamszus, 2015). Thus, CTCs may not be the most suitable biomarkers for gliomas or GBMs. As gliomas are heterogeneous tumours, CTCs may only contain a small proportion of the tumour, which might not reflect the complete biological landscape of all glioma cells within the overall population. Although CTCs are not ideal circulating biomarkers for cancer screening, CTCs are great tools for drug screening. Based on a novel microfluidic tool, CTCs can be collected for assessing drug response without any enrichment before culturing (Hampton, 2016). This new tool could maintain cells status more effectively and vivid ex vivo. In contrast to CTCs, circulating tumour DNA (ctDNA) has drawn significant attention in recent years. However, the sensitivity of ctDNA for cancer detection should be considered or solved before large cohorts of patients are utilised commercially. Extracellular vesicles (EVs) that are subjectively secreted into the circulation are considered as the representation of the whole cell status, heterogeneity of gliomas and an ideal source of biomarkers (Minciacchi, Freeman, & Di Vizio, 2015).

1.5.1. ctDNA

Although ctDNA testing has demonstrated remarkable accuracy in lung cancer, breast cancer, colorectal cancer and prostate cancer (Dawson et al., 2013; Tie et al., 2016; Wyatt et al., 2017), the utility of ctDNA in glioma remains challenging. A pooled analyses of ctDNA from the most common 15 types of cancers show the abundance and the detectable rate of ctDNA in glioma patients is the lowest across all types of cancers (Bettegowda et al., 2014). This might be due to the blood-brain barrier. Detection of ctDNA from cerebrospinal fluid increases the accuracy to 74% but it still requires a specialist to perform an invasive procedure for collecting cerebrospinal fluid (lumbar puncture), which is not an ideal way for monitor of glioma progression (De Mattos-Arruda et al., 2015).

1.5.2. Extracellular Vesicles

Increasing attention has been raised regarding extracellular vesicles (EVs) as the main component of intercellular communication of neighboring cells and distant cells (Tkach & Thery, 2016). EVs are composed of the same lipid bilayer that is present in cells. EVs are a mixed concept, which includes two ways of forming small circulating vesicles. The first is formed from the cells' plasma membrane directly, which are generally known as microvesicles, ectosomes, or microparticles (Tkach & Thery, 2016). The distribution of their sizes are varied from 100 – 1000 nm in diameter. The other EVs, known as exosomes, are generated by multivesicular endosomes or multivesicular bodies (MVBs) and fused with cell plasma membrane before being secreted by cells (Tkach & Thery, 2016).

Once EVs have attached to the target cells, the signaling of recipient cells are activated either by receptor-ligand interaction or internalization by endocytosis and phagocytosis which leads to a changd biological status of the recipient cells. Cell communication via

EVs is widely investigated in fields including cardiac diseases, neurological diseases as well as cancers. In cancer, tumour cells or stromal cells in the microenvironment are shown to take part in variety of biological activities to promote cancer progression and metastasis.

The cargo of EVs varies but usually contains proteins (including transmembrane protein and enclosing cytosolic protein) and RNA. In the tumour setting, the cargo of EVs plays an important role in promoting cancer progression, transferring radiation or chemotherapeutic resistance, adaption to the microenvironment, regulation of target signaling pathways and formation of cancer heterogeneity.

EVs are nanometer in size and contain exosomes and microvesicles (Godlewski, Krichevsky, Johnson, Chiocca, & Bronisz, 2015). All types of cells can produce EVs as part of their secretome. In the case of cancer cells, EVs could be the most effective way of response to environment change (Kucharzewska & Belting, 2013). EVs are secreted either directly from the plasma membrane, or by section where the tumour cell membrane fuses with microvesicular bodies. A diverse spectrum of molecules such as genomic DNA, various RNAs and proteins can be carried by EVs (Godlewski et al., 2015), which are all packaged selectively rather than randomly. This feature allows the potential for EVs to be promising biomarkers. Although we still know little about the dynamics of EVs secretion, one study found the half-life of EVs is quite short and it could change rapidly to reflect the donor cells status, making them suitable for cancer biomarkers.

Beyond biomarkers, EVs have the potential to be the reservoirs of immunological effector molecules (Figure 1.4). CD163 expression on normal blood monocytes is up-regulated by exosome-enriched serum, which indicate exosomes could modulate the immune system by T-helper cells. Glioma cells secrete cytokines, growth factors and chemokines, which is essential to support tumour initiation, angiogenesis and invasion. EVs can pack and transport as cargo a diverse spectrum of biomolecules including genomic DNA, RNA (mRNA and non-coding RNA) and proteins (Godlewski et al.,

2015). The recipient cells including normal cells within the tumour microenvironment and other tumour cells uptake EVs secreted from tumours (donor cells), establishing intracellular communication between cells. Although the regulation of a single EV may be weak, their combinatory regulatory effect by binding multiple target gene sets may generate substantial and significant cascade effects on microenvironment regulation (Godlewski et al., 2015). Until now the mechanisms of EVs cargo uptake remains obscure but it provides a promising platform for therapeutic and diagnosis of gliomas. EVs networks can modify the microenvironment by direct or indirect reprogramming of the recipient cells, which means they could be an ideal platform for targeted therapy. In fact, the recipient cells can be regulated in translational, transcriptional, and proteome levels through the delivery of EVs, which is observed in variant tumours (Godlewski et al., 2015). Furthermore, EVs carrying oncogenic and tumour suppressive proteins like EGFR, PDGFRA and PTEN can be uptaken in several models of gliomas.

More recently exosomes were observed to induce Proneural-Mesenchymal Transit and promote progression in glioma. Interestingly, proneural glioma is associated with worse survival if it obtains mesenchymal signatures and vice versa (Ricklefs et al., 2016b).

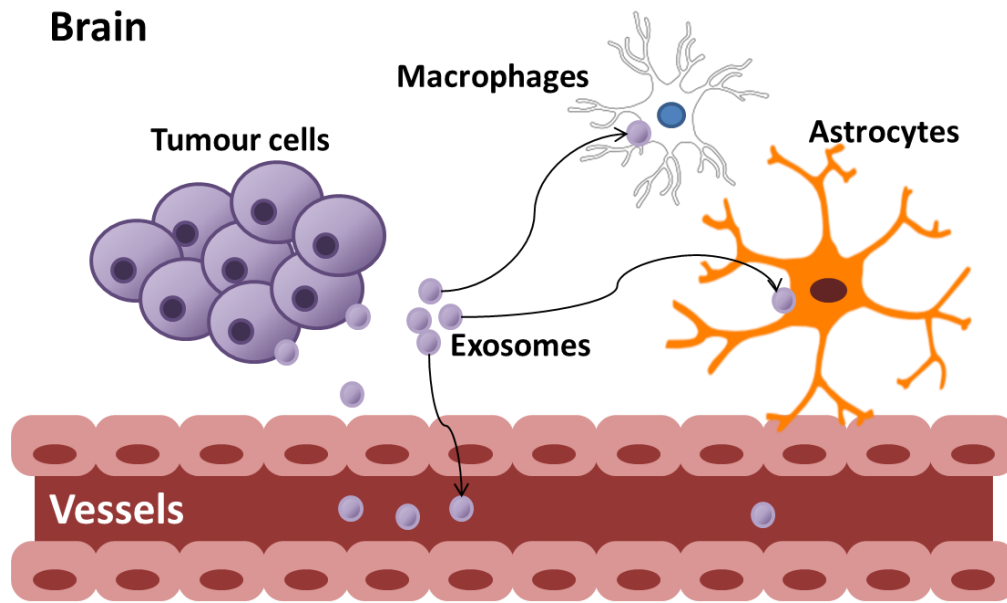


Figure 1.4. EVs in glioma

1.5.2.1. Cancer progression and metastasis

Accumulating studies showed that EVs take part in multiple steps of cancer metastasis. EVs derived from tumour cells directly mediate tumour cells intrinsic invasiveness. Tumour cells motility is activated by EVs through ECM components (Sung, Ketova, Hoshino, Zijlstra, & Weaver, 2015). EVs were also reported to be tightly associated with invadopodia. Invadopodia provided critical docking and secretion sites for EVs and thus EVs secretion was attenuated by inhibition of invadopodia formation. On the other hand, invadopodia formation and stabilization themselves were regulated by EVs, indicating the cargos of EVs would be critical to promote invasive activity in tumour cells (D. Hoshino et al., 2013). In addition, tumour EVs promote cancer progression via inducing vascular permeability to set up premetastasis status (W. Zhou et al., 2014). In particular, miR-105 present in the cargo of cancer EVs, was secreted by tumour cells to destroy the intercellular junction through binding to the tight junction protein ZO-1 in endothelial cells.

EVs do not only predict tumour progression, but also indicate the potential metastasis sites. ITGs expression profiling from breast or pancreatic cancer patients' plasma is strongly linked with sites of metastasis. Exosomal ITGs as organotropism biomarkers (A. Hoshino et al., 2015).

1.5.2.2. Tumour EVs in the microenvironment

Considering the broad communication between tumour cells and stromal cells, many studies have demonstrated tumour cells selectively package and release EVs into the microenvironment in order to manipulate and reshape the stromal cells, which are modified to form a more suitable microenvironment to accommodate the metastasis tumour cells. Notably, EVs-induced metastasis is organotropic. Lung fibroblasts, brain endothelial cells and liver Kupffer cells preferentially take up tumour derived exosomes, which provides a possible explanation of organ preference of cancer metastasis, namely lung, brain and liver are clinical common metastasis site from a variety of primary cancers. In other words, tumour derived EVs are delivered into circulation or tumour microenvironment to address tumour cells to specific organs, where tumour cells EVs have already set up the pre-metastasis niche. EVs from tumour cells of different metastatic sites have remarkably showed preference to organ tropism. Although EVs from breast cancer origin are similar in size and morphology, their biodistribution is quite different from others. Analysis of the proteome of metastatic organotropic EVs identified that members of the integrin family are significantly highly expressed molecules, suggesting integrin expression profiles determine the organotropism. Besides, ITGs regulate tumour metastasis relevant genes expression by activating the S100 gene to facilitate tumour metastatic cells (A. Hoshino et al., 2015).

1.5.2.3. Transfer of EVs from Stromal cells to the microenvironment

Recently, increasing studies showed stromal cells actively participate in tumour metastasis other than are regulated by EVs of tumour cells passively. Communication between stromal cells and tumour cells by EVs in brain has been reported to examine the role of stromal derived exosomes in metastasis. Specifically, reactivated astrocytes secrete exosomes that contain miRNAs that regulate the expression of PTEN in tumour cells and subsequently promoting metastatic outgrowth in breast cancer. This work confirmed Stephen Paget's "seed and soil" hypothesis on cancer metastasis, that is cancer cells tailor the microenvironment to a suitable environment for metastasis by EVs and exosomes secreted by stromal cells develop cancer evolution in vitro and in vivo. More importantly, this work also highlights that stromal exosomes have the ability to reversibly regulate tumour cells mRNAs expression (L. Zhang et al., 2015a).

1.5.2.4. Therapeutic Resistance transfer

Therapeutic resistance is common and inevitable during cancer treatment, where it usually occurs within one year of treatment. Although some evidence showed resistance might be associated with activation of genes that drive the expression of drug transport pumps, cancer therapeutic resistance can also be induced by new mutations in genes involved in tumour evolution, epigenetic modification such as histone methylation, DNA methylation and histone acetylation induced by chemo-agents. Accumulating evidence has demonstrated that there are pre-existing subpopulations of tumour cells that are resistant, and they can transfer their resistance mechanism to surrounding cells by cellular communication (Andre Mdo, Pedro, & Lyden, 2016; Ayob & Ramasamy, 2018; Giallombardo, Taverna, Alessandro, Hong, & Rolfo, 2016).

1.5.2.5. Heterogeneity of glioma by EVs

The concept that there is a complex intratumour heterogeneity in cancer is now well recognized. Specifically, a tumour is composed of several cellular sub-populations that vary in their proliferative rate and therapeutic response. In some cases, one subpopulation has adapted to the local microenvironment and dominates other subpopulations and triggers clonal cooperation by direct cell-to-cell crosstalk or indirect paracrine secretion to drive intratumour heterogeneity. However, one population of tumour cells cannot completely drive the complicated but delicate properties of tumour growth and metastasis simultaneously. Instead, collaborative crosstalk of multiple subpopulation enable tumour outgrowth triggered by one subclone secreting proteinase to degrade the extracellular matrix while another subclone benefits from this and actively proliferates (Tabassum & Polyak, 2015). Thus, further understanding of the role of the crosstalk between subpopulations in tumour progression and therapeutic resistance would allow for improved patient treatment regimens.

Exchange of GSCs EVs between subpopulation of GSCs is currently under investigation as EVs have been shown to be broadly involved in heterogeneity of solid tumours and adaptation to microenvironment in glioma. Although EVs of proneural or mesenchymal GSCs origin display distinct protein expression signatures, they still mirror their donor cells protein composition. Moreover, tumour growth benefit from the exchange of diverse EVs signatures between two GSCs subtypes, suggests that GBM is a highly heterogeneous disease. More importantly, the heterogeneity within tumour propagated by EVs is associated with decreased survival (Ricklefs et al., 2016b). Despite the crosstalk between EVs of GSCs subtypes having been demonstrated to contribute to the progression of glioma, the exact mechanism of this is still unclear.

Despite no single or panel of subtype specific miRNAs have been identified in four transcriptome subtypes of GBM tumours nor in GSCs EVs, unsupervised clustering of GBM miRNA expression profile identified two major groups of GBM, known as PN and

MES, suggesting miRNAs in GBM mainly participate in the regulation of two subpopulation of GBM. The diverse miRNAs expression in subtypes of GBM also partially confirm the heterogeneity of transcriptome in GBM might be induced by miRNAs. In other words, if miRNAs from EVs of MES subtype of GSCs were transferred into PN subtype of GSCs, GSCs would be more diverse than before and the transcriptome of recipient GSCs would be changed accordingly, which was experimentally confirmed by Godlewski and colleagues (Godlewski et al., 2017b). In addition, exchange of EVs miRNAs between GSCs subtypes help GSCs adapt to the microenvironment. Mesenchymal-like GSCs always exist in the core but infiltrate into the surrounding areas in the presence of proneural-like GSCs due to the EV subtype specific miRNA transfer.

1.5.2.6. Circulating miRNAs

Besides the regulatory function in glioma, miRNAs are also released into peripheral circulation by glioma cells. Several studies have identified circulating miRNAs from blood or cerebro-spinal fluid suggesting that circulating miRNA could be a viable diagnostic tool. Circulating miRNAs can be either packaged in EVs or vesicles free. miR-185 is significantly upregulated in glioma patients' blood sample, comparing to normal healthy controls. More interestingly, the level of miR-185 was significantly reduced after surgical operation, which indicates that miR-185 is associated with glioma and is a potential biomarker (Zachariah, Oliveira-Costa, Carter, Stott, & Nahed, 2018). However, there is no consensus on which miRNAs could be an ideal diagnostic biomarker which monitors the tumour burden of glioma. Furthermore, the correlation of miRNAs and molecular alteration such as IDH1 mutation has not been fully elucidated.

1.6. Hypothesis and Aims

The hypothesis for this thesis is “Circulating miRNAs regulate glioma pathogenesis through activation of signalling and cell cycle induction cascades, and can be detected through serum biomarker evaluation”. This hypothesis is investigated in the following aims.

- To investigate the diagnostic and prognostic capacity of circulating miRNAs for gliomas (Chapter 3)
- To investigate the IDH1 mutation associated miRNAs by bioinformatic analyses (Chapter 4)
- To investigate the monitoring capacity of the tumour burden of glioma by serum miRNAs (Chapter 5)
- To investigate the mechanism of regulation by GSCs EVs in gliomas (Chapter 6)

Chapter Two

Chapter 2: Methods and Materials

2.1. Materials

2.1.1. Antibodies

All antibodies used in this thesis are listed in table 2.1.

Table 2.1: List of antibodies used in this study

Antibody	Company	Host / Species	Dilution	Catalogue number	Isotype
PTEN	CST	Mouse	1:1000	9556	IgG
N-cadherin	BD	Mouse	1:1000	610921	IgG ₁
pAKT (Ser473)	CST	Rabbit	1:1000	9271	IgG
GSK3ab	CST	Rabbit	1:1000	5676	IgG
CD44	CST	Mouse	1:1000	5640	IgG
EGFR	CST	Rabbit	1:1000	4267	IgG
AKT	CST	Rabbit	1:1000	9272	IgG
Oct3/4	SC	Rabbit	1:500	Sc-9081	IgG
E-cadherin	BD	Mouse	1:500	610182	IgG _{2a}
Nestin	Millipore	Rabbit	1:1000	ABD69	n/a*
Olig1	Chemicon	Mouse	1:1000	MAB5540	IgG _{2b}
PARP	CST	Rabbit	1:1000	9542	IgG
GAPDH	CST	Rabbit	1:1000	2118	IgG
Alix	CST	Mouse	1:1000	2171	IgG ₁
CD9	Invitrogen	Mouse	1:250	106 26D	IgG
CD81	Invitrogen	Mouse	1:250	106 30D	IgG
TSG101	BD	Mouse	1:500	612 696	IgG ₁

2.1.2. Chemicals

All chemicals used in this thesis are listed in table 2.2.

Table 2.2. The chemicals used in this thesis

Items	Company
AKT-X	Calbiochem-Merk-Millipore
BKM120	selleckchem
B27	Thermo Fisher Scientific
FGF	bdBiosciences
EGF	bdBiosciences
Dimethyl sulfoxide (DMSO)	Thermo Fisher Scientific
Foetal calf serum (FCS)	Life Technologies
Ethanol	Chem-Supply Pty Ltd
Propidium iodide (PI)	Sigma-Aldrich
Tri-sodium citrate	VWR International
Trizol	Life Technologies
Trypan blue solution	Thermo Fisher Scientific
Tween-20	Atlas Chemical Industries
Polyvinylidene fluoride membranes	GE
Amersham ECL Western Blotting Detection Reagent	GE
Glutaraldehyde	Sigma-Aldrich
Crystal violet	Sigma-Aldrich
Phosphate buffered saline (PBS) powder, without calcium and magnesium 10X for 1L	Thermo Fisher Scientific

2.2. Methods

2.2.1. GSC culture

For this study, a written consent form was signed by each patient participant. Ethical approval was obtained from the Royal Melbourne Hospital Human Research and Ethics Committee (HREC number 2009.016). Glioma stem cells (GSCs) were derived from patient tumour samples (Table 2.3) obtained intraoperatively at the Royal Melbourne and Melbourne Private Hospitals and stored in the local tissue bank. GSCs are grown as gliomaspheres in ultralow adherent plates/flasks were cultured in serum-free media (SFM) containing DMEM-F12 (Gibco) supplemented with 1x vitamin A-free B27 (Gibco), 10 ng/ml EGF and 10ng/ml FGF (both BD Biosciences) and 1x or 1% penicillin/streptomycin. Briefly, GSC cells are allowed to culture in for 7-10 days in a humidified incubator of incubated at 10% CO₂ at 37°C.

For cellular passage, GSC gliomaspheres in DMEM-F12 (Gibco) supplemented with 1x vitamin A-free B27 (Gibco), EGF 10ng/μl (bdBiosciences), FGF 10ng/μl (bdBiosciences) and penicillin/streptomycin 1:200. Cells are dissociated every 7-10 days using 0.05% trypsin, followed by inactivation with DMEM-F12 with 2% foetal calf serum (FCS), washing and re-suspension with SFM in ultralow adherence plates or flasks.

Table 2.3. The characteristics of GSCs derived from patients.

GSC ID	MU004	MU020	MU035	MU039	MU041
AGE	58	39	61	78	82
GENDER	Male	Male	Female	Female	Male
DIAGNOSIS	GBM	GBM	GBM	GBM	GBM
PRIMARY OR RECURRENT	Primary	Primary	Recurrent	Primary	Primary
SURVIVAL TIME (MONTHS)	4	14	3	7	4

2.2.2. Glioma Cell Lines

The human glioma cell line LN229, U118 and U87MG were available within the Department of Surgery, Royal Melbourne Hospital. These cell lines are immortalized line and obtained commercially from ATCC. Cells were cultured in monolayer in using previously described protocols (Ng, 2016). Briefly, cells were incubated in 10% CO₂ at 37°C using DMEM media supplemented with 10% foetal calf-serum (FCS) and penicillin/streptomycin 1:200. The cells were passaged using 0.25% trypsin, inactivated with media, spun at 500g for 5 min to remove the supernatant and re-plated in 75 cm² flasks (Corning) at a 1:6 dilution.

2.2.3. EVs isolation

GSCs-EVs isolation and characterisation were performed according to a well-established protocol (Thery, Amigorena, Raposo, & Clayton, 2006). Briefly, conditioned medium (CM) were collected from glioma spheres. CM were centrifuged at 300 g for 5 min to remove dead cells and then they were subjected to centrifuge for another 10 min to remove debris at 2,000 g. CM were further centrifuged at 10,000 g for 30 mins to remove apoptotic bodies. Supernatant was then collected and ultracentrifuged at 100,000 g for 70 mins. EVs pellet was concentrated and washed with cold phosphate buffered saline (PBS) at 100, 000 g for 70 mins and stored at -80 °C for subsequent functional studies.

2.2.4. EVs size and morphology quantification

Cryo-Electron Microscopy (CryoEM) was used to record GSCs-EVs size and morphology. Briefly, 3 µl of freshly prepared GSCs EVs was applied to a glow-

discharged and vitrified carbon grid. After excess liquid was removed, the grid was mounted into a Gatan cryoholder (Gatan, Inc., Pleasanton, CA, USA) and stored in liquid nitrogen for future analysis. Typical images of GSCs-EVs were acquired using a 300 kV Tecnai G2 F30 (FEI, Netherlands). A minimum of 15 fields of view were recorded per sample.

Nanoparticle tracking analysis (NTA, Nanosight NS300 instrument, Malvern, UK) was used to record the size and particle concentration of GSC-EVs using previously described protocol (Filipe et al 2010). Tracking analyses were performed with the assistance of Dr. Hong Nguyen. Briefly, NS300 instrument was turned on and equilibrated to room temperature prior to analysis. A mixture of sample (100 μ l of GSCs EVs: 900 μ l of distilled water) was injected into the sample chamber using a 1ml sterile syringe (BD, New Jersey, USA) until the first droplet of liquid formed at the tip of the nozzle. Using the NTA software to optimize for the field of view (50-100 particle/view) and set recording for triplicates/sample. Data including size and particle concentration/ml were recorded and exported as .csv or .pdf format.

2.2.5. EVs uptake assay

To assess whether GSCs-derived EVs (GSCs EVs) can be taken up by glioma cells, the EVs uptake assay was performed according to previous method with modification (Escrevente, Keller, Altevogt, & Costa, 2011). Concisely, GSCs EVs pellets collected after 100,000 $\times$ g centrifugation was incubated with 0.6 μ l carboxyfluoresceine diacetate succinimidyl-ester (CFSE) in final volume of 400 μ l PBS at 37°C for 30 min. Labelled GSCs EVs were resuspended in PBS and excess CFSE dye removed by centrifugation at 100,000 $\times$ g for 70 min and stored at -80 C for future uptake assay. LN229 cells were

seeded in 6-well plates at concentration of 2×10^5 cells/well 24 h prior to GSCs EVs harvesting.

2.2.6. Radiation treatment

Cells were irradiated at indicated dose (2 and 5Gy for adherent cells; 2Gy, 4Gy, 6Gy and 8Gy for GSCs) in house at the WEHI. Briefly, adherent cells were seeded in 6-well plates at the concentration of 1×10^3 cells per well at least 12 h before irradiation to allow adherent on the plate while GSCs were disassociated by trypsin into single cells and seeded in ultra-low attachment plates at least 2 h before irradiation.

2.2.7. Clonogenic assay

Clonogenic assay was performed according to the previous protocol with minor modification (Franken, Rodermond, Stap, Haveman, & van Bree, 2006). Briefly, recipient cells (LN229 and U118) were seeded in 6-well plates at 1×10^3 cells/well in triplicate for 7 days to allow for colony formation. Cells were treated with radiation (2 or 5 Gy) with/without GSCs EVs treatment after 48 hours. After colony formation was observed at day 7, cells were washed by PBS and then stained with crystal violet mixture (6.0% glutaraldehyde and 0.5% crystal violet) at room temperature for 30 min. Images of colony formation were taken after cells were washed in tap water.

2.2.8. Cell viability assay

To determine difference in cell proliferation and cell viability, a luminescent readout assay was performed according to manufacturer's instructions. In brief, glioma cell lines (LN229 and U118) were washed twice with PBS and incubated with $1 \times$ lysis buffer

(Luminescent Cell Viability Assay, Promega) at room temperature for 15 min. The volume of 1× buffer was 40µl per well of 96-well plate or 500 µl per well of 6-well plate. After incubation, 30 µl of cell-buffer mixture was transferred to a new clear white 96-well plate and read using a luminometer. All read outs of each group from the luminometer were normalized to the non-treatment group, and the change ratio of each group was reported.

Recipient cells (LN229 and U118) were seeded for 24 h and treated with 2 mM TMZ for 24 h. On day 3, 20 µg/ml GSCs-EVs were added to the TMZ-treated recipient cells and incubated for 48 h. Cell viability was studied as previously described.

2.2.9. Western blot

GSCs EVs or recipient cells with or without GSCs EVs treatment were lysed and protein levels were quantified using the Pierce BCA Protein Assay kit (Life Technologies) according to manufacturer's instructions. Total protein (10 - 50 µg) was mixed with sample reducing buffer and sample buffer (Life Technology) before heating at 95 °C for 5 mins. Afterwards, a total of 15 µg protein were run on 4-12% Bis-Tris gels (Life Technology), and proteins were transferred on to 0.45 µm polyvinylidene fluoride membranes (GE). The membrane was then blocked by blocking buffer (5% skim milk in TBST) for one h on the vertical shaker. The membranes were incubated with primary antibodies at final dilutions of 1:1000 overnight in 4°C fridge. All antibodies used are included in Table 2.1. Membranes were washed 3 times for 15 min by TBST. Secondary antibodies were applied for one h and then the wash once more for 15 min, 3 times. The signal of each band was visualized by the Amersham ECL Western Blotting Detection Reagent (GE Health) and exposed to X-ray film (Fujifilm Australia, Australia). The band intensity was analysed by ImageJ.

Three days after glioma sphere culturing, GSCs were transferred into Dulbecco's modified Eagle's medium containing 10% fetal calf serum to stimulate GSCs differentiation. Typical images were taken under the microcopy every 2-3 days, lasting for 28 days. For stemness marker validation, differentiated GSCs were harvested in protein lysis buffer and subjected to -20 °C freezer for western blotting.

2.2.10. Cell migration assay

To examine the effect of GSCs EVs on glioma cell migration and invasion, recipient cells (LN229 and U118) were seeded (1×10^4 cells/well) into 48-well plate and allowed to reach 100% confluency. A scratch was made using a 200 μ l sterile tip following the addition of 10 μ g GSCs EVs were added and monitored for 48 h. The amount of cell migration was captured at time 0, 24 h and 48 h. Percentage of cell migration was calculated as the distance of cell migrated overtime to the distance of the initial scratch x 100%. All experiments were performed at least 3 times in triplicates.

2.2.11. MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

To test the cell viability of GSCs and recipient cells after AKT inhibitor treatment, MTT assay was employed to detect the cell proliferation and cell number after the given treatments. Cells were seeded in 96 well plate at 1000 cells/well 1 day prior to the treatment. A total of 20 μ l of MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution was added in the media of cell culturing directly. After 4 hours incubation in the 37 °C incubation, all media was removed and then 150 μ l of DMSO

was added in each well of cells. After 3 min shaking in colorimetric plate reader, the absorbance of each well was recorded under the wavelength of 570 nm.

Single cell suspensions of GSCs (2×10^3 cell/well) were seeded into 96-well plate in triplicates for 24 hr. Temozolomide (500 μ M TMZ) was added and incubated for 5-7 days for sign of proliferation and sphere formation. For proliferation, MTT assay was used according to manufacturer's instructions. For sphere formation, sphere number was counted by phase contrast microscope.

Akt inhibitor X (Calbiochem-Merk-Millipore, Burlington, Massachusetts, USA) was utilized to block the phosphorylation of Akt induced by GSC-EVs. Recipient cells (LN229, U118) were grown as monolayer (1×10^3 cells/well) in 12-well plate for 24 h followed by incubation with GSC-EVs for 24 h. Akt inhibitor X (10 μ M) was added to cells with or without GSC-EVs treatment group and incubated for a further 24 h. Cell viability of all treatment groups was studied.

BKM120 was kindly donated by Dr. Theo Mantamadiotis. Titration assay of BKM120 (0.5 μ M, 1 μ M, 2 μ M, 4 μ M) was applied to LN229 and U118 to determine the concentration of BKM120 in Akt inhibition experiments. Time course experiments were also employed. A total of 5×10^4 cells for each concentration of BKM120 were seeded in 24 well plates one day before BKM120 treatment. Six and 24 hours after BKM120 treatment, all cells were washed by PBS and digested in protein lysis buffer on ice and stored in -20 °C freezer for Akt activation detection by western blotting. For GSCs EVs treatment experiment, a total of 5×10^4 cells was seeded in 24 well plate 24 hour before GSCs EVs treatment (40 μ g/mL). After another 24 hours, BKM120 (2 μ M) was applied in GSCs EVs treated cells.

2.2.12. Zymography and invadopodia assay

With help and support from Clarissa Whitehead and Dr. Stanly Stylli, zymography and invadopodia assays were performed to detect the levels of MMP expression and invasion, respectively. For gelatinase zymographic analyses, 10 µg of GSCs EVs (MU004EV, MU020EV and MU039EV) or conditional media (100 µl/sample of serum free media from GSCs EVs treated U118 and LN229 glioma cells) were diluted 1:1 with Novex Tris-Glycine SDS sample buffer and separated by electrophoresis on Novex 10% Zymogram (gelatin) gels in Novex tris-glycine sodium dodecyl sulfate running buffer (Life Technologies). Sample loading was normalized against the concentration of the corresponding cell protein lysates, which were determined by the BCA protein assay (Pierce, ThermoFisher Scientific). Subsequently the gels were incubated in a Novex zymogram renaturing buffer for 30 min (ThermoFisher Scientific), followed by a 30 min incubation in a Novex zymogram developing buffer (ThermoFisher Scientific) that was replaced with new developing buffer and incubated overnight at 37°C. The gels were then washed in distilled water before being stained for 1 h in SimplyBlue Stain (Life Technologies). The molecular weight was identified against the loaded Precision Blue (BioRad) protein markers. The densitometric analysis of gels were then scanned on a flatbed scanner using ImageJ (v1.51s, NIH).

For the invadopodia experiments, LN229 and U118 recipient cells were prepared by incubating 20 µg of GSC-EVs (MV004EVs, MV020EVs, MV039EVs or untreated) for 48 hours, trypsinised and seeded (7.5×10^4 cells) per FITC-gelatin coated coverslip and incubated for another 24 hours as described (L. Mao et al., 2017; Stylli et al., 2009). Coverslip was stained with Rhodamine-phalloidin (dilution 1:75, Cytoskeleton Inc) and DAPI nuclear staining (5 µg/ml, Sigma-Aldrich) as per manufacturer's instructions. Images of actin puncta of the invadopodium core were acquired and analyzed using a Nikon A1+ confocal microscope system equipped with a Plan Apo VC 60x DIC N2 oil immersion objective. A total of 10 random image per field of view were captured by this

microscope and degraded FITC-labelled gelatin was defined as a black area depleted of fluorescent gelatin within each acquired image. The area of degradation was computed using the particle counter function and normalized with respect to the number of DAPI-positive nuclei.

2.2.13. Limiting dilution assay

For limiting dilution assay, GSCs were plated at one, 10, 20, 40, 60, 80, 100 cells per well into a 96-well ultra-low attach plate before radiation with the indicated dosage. Seven days after plating, the number of gliomaspheres in each well were quantified by manual counting. Glioma spheres were defined as the diameter of spheres were larger than 100 μm . The statistics and plots were performed by Extreme limiting dilution assay analyses (ELDAs) as previously described (Y. Hu & Smyth, 2009).

2.2.14. Immunofluorescence assay

Immunofluorescence assay was applied to detect Ki-67 expression in recipient cells after GSCs EVs treatment. After GSCs EVs treatment, recipient cells (LN229 and U118) were washed by PBS once, followed by fixation using 2.7% formamide for 5 min. After washing by PBS, Triton-X 100 was applied for 5 min. 1% BSA was then added at 4°C overnight to block the unspecific binding. After washing in PBS twice, cells were probed with primary antibody at room temperature for 1 h before being probed with secondary antibody. Hoechst was applied for another five min for nucleus staining after 2 PBS washes. After washed in double distilled water, cells were subject to fluorescence microscope for taking images.

2.2.15. Cell cycle detection by flow cytometry

Flow cytometry was utilized to assess the cell cycle after GSCs EVs treatment with the support of Xin Du (Peter Mac Cancer Center). All cells were trypsinised and washed by PBS once to remove media after a variety of treatments. After removed of PBS, cells were fixed in cold 70% ethanol. Fixed cells can be stored at 4 °C fridge for one week. After cells were washed with PBS twice, cells were stained with propidium Iodide (in 2% Fetal bovine serum of PBS) and then subject to flow cytometry (BD LSR II) for cell cycle detection. After gating the complete cells out by plotting forward scatter versus side scatter, all analyses were performed in the software flowjo 7.6.1. Dean-Jett-Fox model was applied for cell cycle analyses. Cell cycle of all detected cells were divided into three groups: G0/G1, S and G2/M phase, and the proportion of each phase between different treatment group was demonstrated and compared using Graphpad Prism 7.0.

2.2.16. Public datasets

MiRNA expression profiling was performed in Agilent 15K Human microRNA microarrays. The processed (level 3) data of miRNAs expression matrix were retrieved from The Cancer Genome Atlas (TCGA) Data Portal at <http://tcga-data.nci.nih.gov/tcga/>. Clinical information annotation including subtypes classification was also downloaded from TCGA Data Portal. IDH1 mutation status was inquired on TCGA website and then added into clinical annotation table manually. Similarly, level 3 data of DNA methylation dataset (Illumina Human Methylation 450K) from 5 IDH1 mutant and 6 IDH1 wild type GBM patients was also downloaded from TCGA.

2.2.17. miRNA targets prediction

The potential targets of miRNAs were predicted by miRWalk 2.0 (<http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/>). Three mutually exclusive algorithms (TargetScan,

MiRanda, miRWalk) were utilized to predict the targets of miRNA candidates. The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) involved by the potential targets were listed by mirPath V.3 (<http://snf-515788.vm.okeanos.grnet.gr/>). In GO analysis, the biological process (BP) was analyzed and a p value <0.05 was considered as statistically significance. In KEGG analysis, enriched pathways were identified as the hypergeometric distribution with a p value < 0.05 (X. H. Shi et al., 2018).

2.2.18. Protein interaction analysis

All the miRNAs target genes were transferred into the Entrez ID and then subject to the STRING online tool to predict the potential protein interaction among them among them. The Entrez ID of target genes of each miRNAs were uploaded into STRING separately. Moderate confidence score of <0.75 was utilized as the threshold value. The associations of one target genes with the other target genes indicate the importance of these genes in the protein interaction network.

2.2.19. Nanostring

A total of 47 LGG and 44 GBM patients peripheral blood was collected. All miRNAs from health controls are kindly provided by the ACRF Translational Research Laboratory. The total miRNAs of each serum were extracted by Exiqon miRCURY RNA isolation kit and detected in nCounter platform (Nanostring Inc.), following the manufacturer's guide. The Nanostring miRNA panel (Human v3) contains 827 miRNAs, which can be found here: <https://www.nanostring.com/products/mirna-assays/mirna-panels>. Quality control and normalization of raw data were conducted in nSolver software (Nanostring Technologies, Seattle WA). As Nanostring miRNA panel provides the absolute copies of each miRNA given samples, we utilized the copy counts for the statistical analysis.

2.2.20. Neuroimaging measurement

All neuroimaging measurements were kindly provided by Royal Melbourne Hospital neuroradiologists Dr. Lasocki Arian and Dr. Frank Gaillard. In brief, contrasted MRI imaging was utilized for estimating the area in MRI image of each tumour. The tumour size was calculated by an $A \times B / 2$ on the largest “slice”. Line charts of sum of the tumour area in MRI image were plotted over all time points of follow-up.

2.2.21. EVs miRNA profiling and analysis

miRNA profiling was performed on GSCs EVs using Nanostring (Nanostring Technologies, Seattle, USA). Briefly, total RNA was extracted from GSCs EVs using Exiqon miRNA extraction kit according to manufacturer’s instructions and profiled with Nanostring miRNA assay profiling. Data was analysed using nSolver Analysis Software (v3.0, Nanostring Technologies) and R statistical package.

2.2.22. Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) was employed to validate the different expression of miRNAs target genes between IDH1 mutant and IDH1 wild type glioma. All GSEA were carried out in the Java based tool developed by Broad Institute (www.broad.mit.edu/gsea/).

2.2.23. Endogenous control identification

Endogenous control for serum miRNAs study was identified by NormFinder (Andersen, Jensen, & Orntoft, 2004; Vandesompele et al., 2002; Xie, Xiao, Chen, Xu, & Zhang,

2012). All spike-in and ligation normalized data generated from nSolver were subject to NormFinder to discover the endogenous controls.

2.2.24. Bioinformatic analysis

All bioinformatic analysis was performed in R environment (R version 3.4.0). LIMMA package was utilized to identify differentiated expression miRNAs. Clustering was conducted by k-means. Data visualization was conducted with the support of the following packages: ggplot2, gplots, ggfortify. All programming codes are available here: <https://github.com/mackaay>

2.2.25. Statistical analysis

For differentially expressed miRNAs, LIMMA package was utilized in R 3.4.2 based on the following criteria: log fold change larger than 1 and – log adjusted p value larger than 2. The principal components analysis (PCA) and t-SNE was also conducted in R software for demonstrating the distribution and distance of samples by reducing the dimensions. CpG island methylation level, correlation of methylation and miRNAs expression and the Kaplan-Meier Curve of survivals were performed in Graphpad Prism 7. Multivariate Cox proportion hazard regression were performed to identify the independent prognostic factor. For survival analysis, log-rank analysis was utilized to determine the difference between two groups. The receiver operating characteristic (ROC) curve was generated to predict the diagnostic accuracy of one miRNA or miRNA panel for glioma by calculating the area under the curve (AUC). All cell experiments in Chapter 6 were repeated at least three time in triplicates. A P value of <0.05 was regarded as statistically significant.

2.2.26. Meta analysis

2.2.26.1. Search strategy

We performed a comprehensive search for original papers demonstrating diagnostic power of circulating miRNAs in patients with glioma. Databases including Pubmed, Embase, the Cochrane Library Database and Google Scholar were used to obtain all relevant studies up to February 2017. Key words including “glioma” or “glioblastoma”, “microRNA” or “miRNA” or “miR” and “plasma” or “serum” or “circulating” or “blood”, which worked as Medical Subject Headings (MeSH) and free-style words in searching retrieval. No publication date was restricted in our search. All publications containing the relevant keywords were included in this meta-analysis.

2.2.26.2. Inclusion and exclusion criteria

Selection criteria have been set up for literature selection before performing meta-analysis. The inclusion criteria: (1) studies aim to identify the diagnostic capacity of miRNAs for glioma, (2) all patients must be diagnosed as glioma by using the gold standard test (namely by histopathology examinations), (3) sample sizes, sensitivity, specificity or sufficient data were reported in the original studies to analyze the diagnostic ability and (4) studies that used plasma or serum as the source of study materials. Exclusion criteria: (1) duplicate publications, (2) insufficient data provided, (3) review, letters, editorials and commentaries and (4) studies on miRNAs in cerebrospinal fluid (CSF).

The studies considered satisfactory and included in this meta-analysis all have glioma indication as patient cohort and compared with non-glioma controls. Characteristics of included studies were collected as following: first author’s name, year of publication, numbers of cases and controls, types of glioma, methods used to detect miRNAs, miRNA names, sources of samples, the sensitivity and specificity, the test for true positive (TP),

false positive (FP), false negative (FN) and true negative (TN) and any other additional information required for quality evaluation. The methodology quality (risk of bias and applicability of primary diagnostic accuracy) of each trial was evaluated by the revised Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) tool (Whiting et al., 2011).

2.2.26.3. Diagnostic capacity analysis

All statistical analysis was conducted using Stata software version 13 (StataCorp LCC, Texas USA). The number of participants with TP, FP, FN and TN were extracted in order to calculate the pooled sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR), diagnostic odds ratio (DOR) and corresponding 95% confidence intervals (CIs). The summary receiver operator characteristic (SROC) curve and area under the SROC curve (AUC) were evaluated to test the pooled diagnostic performance of circulating miRNAs reported from original studies. Deek's funnel plot was conducted to assess the publication bias in our meta-analysis. A p value < 0.05 was considered statistically significant.

Chapter Three

Chapter 3: Systematic review and meta-analysis of circulating miRNAs in diagnosing gliomas

3.1. Introduction

Considering that miRNAs are extensively involved in the regulation of expression of many target genes of neurological disease and cancers, miRNAs are considered as a potential biomarker for disease diagnosis and monitoring. In glioma, several clinical studies have already identified circulating miRNAs (derived from blood or CSF) are capable of diagnosing glioma from the normal controls. However, the diagnostic accuracy of these circulating miRNAs is unparalleled in these clinical studies. Ivo D'Urso et al found miR-15 is up-regulated in glioma patients and achieved 90% sensitivity and 100% specificity (Ivo D'Urso et al., 2015). Similarly, another cohort including 70 glioma patients found miR-454-3p was promising diagnostic biomarker with 99.05% sensitivity and 82.86% specificity (Shao, Wang, Xue, Wang, & Lan, 2015). In contrast, Xiao et al reported the sensitivity of miR-182 in circulation for glioma diagnosis was less than 60% and circulating miR-125b had 66.7% diagnostic specificity in another glioma study (Regazzo et al., 2016; Xiao et al., 2016). These inconsistent results within miRNA clinical studies will undermine the utility of circulating miRNAs. Therefore, there is a necessity of performing a meta-analysis to confirm the accuracy of circulating miRNAs in glioma.

3.2. Results

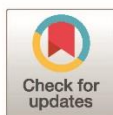
RESEARCH ARTICLE

A comprehensive meta-analysis of circulation miRNAs in glioma as potential diagnostic biomarker

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OPEN ACCESS

Citation: Ma C, Nguyen HPT, Luwor RB, Stylli SS, Gogos A, Paradiso L, et al. (2018) A comprehensive meta-analysis of circulation miRNAs in glioma as potential diagnostic biomarker. PLoS ONE 13(2): e0189452. <https://doi.org/10.1371/journal.pone.0189452>

Editor: Bing-Hua Jiang, Thomas Jefferson University, UNITED STATES

Received: August 7, 2017

Accepted: November 24, 2017

Published: February 14, 2018

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Data Availability Statement: All figures files are available from the figshare database: <https://figshare.com/s/8f9716cc23d0ac211041>.

Funding: We would like to acknowledge the Cure Brain Cancer Foundation, the Royal Melbourne Hospital Neuroscience Foundation for their funding support to Dr A.Morokoff and Melbourne International Research Scholarship to Mr C.Ma, and the Victorian Government for Infrastructure support.

Abstract

Glioma is the most common malignant intracranial tumour. Recently, several publications have suggested that miRNAs can be used as potential diagnostic biomarkers of glioma. Here we performed a meta-analysis to identify the diagnostic accuracy of differentially expressed circulating miRNAs in gliomas. Using PubMed, Medline and Cochrane databases, we searched for studies which evaluated a single or panel of miRNAs from circulating blood as potential biomarkers of glioma. Sixteen publications involving 23 studies of miRNAs from serum or plasma met our criteria and were included in this meta-analysis. The pooled diagnostic parameters were calculated by random effect models and overall diagnostic performance of altered miRNAs was illustrated by the summary receiver operator characteristic (SROC) curves. The pooled sensitivity, specificity, positive likelihood ratio (PLR) and negative likelihood ratio (NLR) from each study were calculated. The pooled PLR, NLR and Diagnostic Odds Ratio were 6.39 (95% CI, 4.61–8.87), 0.15 (95% CI, 0.11–0.21) and 41.91 (95% CI, 23.15–75.88), respectively. The pooled sensitivity, specificity and area under the curve (AUC) were 0.87 (95% CI, 0.82–0.91), 0.86 (95% CI, 0.82–0.90) and 0.93 (95% CI, 0.91–0.95), respectively. This meta-analysis demonstrated that circulating miRNAs are capable of distinguishing glioma from healthy controls. Circulating miRNAs are promising diagnostic biomarkers for glioma and can potentially be used as a non-invasive early detection.

Introduction

Glioma, the most prevalent malignant cancer in the central nervous system, has a content of latent progression before histopathological diagnosis [1]. The incidence of glioma in adults varies from 1.32 to 5.73 per 100,000 person, depending on geographic location [2]. Glioma is categorized according to the World Health Organization (WHO) grading system, grade I and II are classified as benign and low grade glioma while grade III and IV are high grade glioma. The current protocolized treatment for high grade glioma is surgery followed by concurrent radiotherapy and temozolomide chemotherapy [3]; despite this, glioma remains a highly

Competing interests: The authors have declared that no competing interests exist.

aggressive cancer with extremely poor prognosis, resulting in median survival of less than 15 months. Glioma recurrence is almost always seen and is usually accompanied with higher malignancy and infiltrative growth, causing rapid progression of disease and larger community-family burden [4] with 5-year survival rate remaining less than 3% [5].

Currently, contrast-enhanced magnetic response imaging (MRI) is a useful guide for pre-surgical diagnosis and post-surgical monitoring of glioma. It cannot detect micro lesions nor provide all of the biological information regarding the tumour [6, 7]. Furthermore, it is expensive and inconvenient for many patients. Histology is the basis of glioma diagnosis and prognosis, but requires invasive neurosurgery to obtain tumour tissue. In light of this, there is urgency to discover an improved minimally invasive and rapid approach for diagnosis and monitoring of glioma.

Biomarkers from bio-fluid have been widely studied in clinical diagnosis and disease monitoring in cancer and some are in clinical use. MicroRNAs (miRNAs), a family of small non-coding RNAs (19–22 nucleotides), have been shown to take part in multiple biological activities such as immunology regulation, embryo development and cancer progression through post transcriptional modifications. Its mechanism of action is to bind the complementary 3' UTR of target mRNAs and degrading the mRNAs [8, 9]. Deregulated miRNAs can be detected in the surgical specimens and the bio-fluid like serum, plasma, urine and cerebrospinal fluid (CSF) from patients, suggesting altered miRNAs have the potential to become a non-invasive biomarker for cancer detection [10, 11]. Recently, several studies have shown that a single, or panel of, circulating miRNAs can be used as diagnostic tools for several cancers including bladder cancer, hepatocellular carcinoma, lung cancer, cervical cancer, ovarian cancer, gastric cancer and colorectal cancer with high sensitivity and specificity [12–18]. The expression of circulating miRNA has also been correlated with patient outcome [19]. The diagnostic and prognostic utility of miRNAs in bio-fluid for glioma, however, remains ill-defined. We performed a meta-analysis to assess the published clinical studies about circulating miRNAs in glioma patients in order to better evaluate this biomarker.

Methods

Search strategy

We performed a comprehensive search for original papers demonstrating diagnostic power of circulating miRNAs in patients with glioma. Databases including Pubmed, Embase, the Cochrane Library Database and Google Scholar were used to obtain all relevant studies up to February 2017. Key words including “glioma” or “glioblastoma”, “microRNA” or “miRNA” or “miR” and “plasma” or “serum” or “circulating” or “blood”, which worked as Medical Subject Headings (MeSH) and free-style words in searching retrieval. No publication date was restricted in our search. All publications containing the relevant keywords were included in this meta-analysis.

Inclusion and exclusion criteria

Selection criteria have been set up for literature selection before performing meta-analysis. The inclusion criteria: (i) studies aim to identify the diagnostic capacity of miRNAs for glioma, (ii) all patients must be diagnosed as glioma by using the gold standard test (namely by histopathology examinations), (iii) sample sizes, sensitivity, specificity or sufficient data were reported in the original studies to analyze the diagnostic ability and (iv) studies that used plasma or serum as the source of study materials. Exclusion criteria: duplicate publications, insufficient data provided, reviews, letters, editorials and commentaries and studies on miRNAs in cerebrospinal fluid (CSF).

The studies considered satisfactory and included in this meta-analysis all have glioma indication as patient cohort and compared with non-glioma controls. Characteristics of included studies were collected as following: first author's name, year of publication, numbers of cases and controls, types of glioma, methods used to detect miRNAs, miRNA names, sources of samples, the sensitivity and specificity, the test for true positive (TP), false positive (FP), false negative (FN) and true negative (TN) and any other additional information required for quality evaluation. Values obtained for these parameters were additionally calculated using Revman 5 software (Review Manager, version 5.3. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014). The methodology quality (risk of bias and applicability of primary diagnostic accuracy) of each trial was evaluated by the revised Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) tool [20].

Diagnostic capacity analysis

All statistical analysis was conducted using Stata software version 13 (StataCorp LCC, Texas USA). The number of participants with TP, FP, FN and TN were extracted in order to calculate the pooled sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR), diagnostic odds ratio (DOR) and corresponding 95% confidence intervals (CIs). The summary receiver operator characteristic (SROC) curve and area under the SROC curve (AUC) were evaluated to test the pooled diagnostic performance of circulating miRNAs reported from original studies. Deek's funnel plot was conducted to assess the publication bias in our meta-analysis. A *p* value less than 0.05 was considered statistically significant.

Results

Studies characteristics

Using our retrieval strategy, a total of 138 publications were likely identified as relevant studies about miRNAs as potential biomarkers of glioma (Fig 1A). By reviewing the abstracts, 118 were excluded as they are reviews and/or studies unrelated to glioma diagnostic analysis topic. Of the remaining 20 publications, three studies were also removed as they failed to provide diagnostic information. One other article was also excluded as it studied diagnostic accuracy of circulating miRNA between glioblastoma and low grade glioma rather than miRNA biomarker in tumour and normal control. Consequently, 16 publications met our inclusion criteria and were included in this meta-analysis for [21–33] [29, 34, 35] (Fig 1A).

The 16 publications (ranging from year 2011 to 2017) included in this meta-analysis reported 23 cohorts, totaling 2325 participants. Seven publications reported a panel of several miRNAs as a diagnostic biomarker, while nine studies discussed a single miRNA candidate (Table 1). D'Urso et al, Manterola et al, Zhang et al and Zhi et al reported two study cohorts in each of their articles [22, 32–34]. Articles by Wang et al and Huang et al included a subset of three studies [26, 35]. The remaining articles reported one study each. In all studies, the level of miRNA expression in plasma (*n* = 5), serum (*n* = 10) or blood (*n* = 1) was detected by quantitative real-time polymerase chain reaction (qRT-PCR).

Of the 16 studies analyzed (Table 1), 5 publications [22, 23, 30, 33, 34] tested global miRNA expression using a panel of miRNA available commercially including those using the Taqman Low Density Array (TLDA) based on different versions of miRNA databases from mirBase [22, 33]. Nine publications only focused on a targeted miRNA that has previously been assessed as potential biomarker or diagnostic marker in glioma or other cancer types (Table 1). Some studies used healthy volunteers as their controls, while one study included patients with other neurological conditions as controls [34]. All studies collected biofluid at the time of

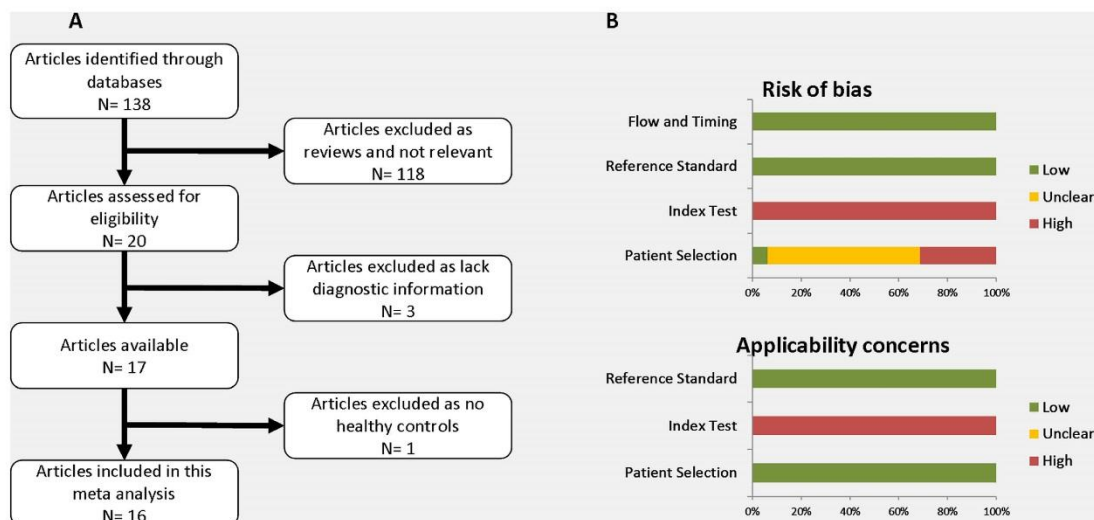


Fig 1. Flow chart of studies selection and quality assessment of studies. A) A total of 16 published articles were included in this meta-analysis after filtering through the inclusion criteria. B) QUADAS-2 assessment. Bar chart showing the summary of risk of bias and applicability concerns, expressed as percentage. Each study occupied the bar equally (1/16, 6.25%). Red: high risk; Yellow: unclear risk; Green: low risk.

<https://doi.org/10.1371/journal.pone.0189452.g001>

glioma diagnosis or prior to operation, however some studies also included blood collected post-surgery.

Potential miRNA biomarkers

There were several miRNAs identified more than once among the studies in this meta-analysis (Table 1). The most common differentially expressed miRNAs between glioma and healthy controls were miR-15b [30, 34] and miR-125b [27, 36] (Table 1). The direction of differential expression was contradictory between some reports; notably, miR-128 was reported as significantly down-regulated in two studies [25, 26], but up-regulated in a third publication [23].

The QUADAS-2 tool was used to review the risk of bias and applicability for each included article, where all included records displayed a moderate quality (Fig 1B). The highest risk after assessment was from the index test because there was a large variance between the technical methods, or the thresholds that the original studies used. Since it is not clinically warranted to perform 'screening' miRNA testing without surgical biopsy, blinding of miRNA results to histopathological diagnosis may not have been reliable, which eventually raises the risks of testing assessment.

The reference genes chosen to normalize in the studies also contributed to the risks. Noticeably, all studies were assessed as high risk in index test sections as they used different endogenous genes or miRNAs as control for normalization (Table 1). The difference in the report of miRNA-128 expression between studies may be caused by the use of different reference genes (i.e. RNU6 in Roth's study and spiked-in miRNA in Wang and Sun's studies). Overall, RNU6 (including RNU6B) was the most common reference gene used for normalization, followed by miR-16. Interestingly, RNU6-1 is reported to be an up-regulated biomarker in another study [22] that compared glioma to control serum. This variability in normalization due to lack of

Table 1. Characteristics of included circulating miRNA studies in this meta analysis.

Study	Year	Case size	Control size	Cohort Source	Cancer type	Methodology	miRNA Signatures (up- or down-regulated in glioma)	Source	Normalization
D'Urso et al	2015	30	30	Training Set and Validation Set	Glioma (grade I to IV)	qRT-PCR	miR-15b (up)	Plasma	miR-24
Tuang et al	2017	100	50	Training Set	Glioma (grade I to IV)	qRT-PCR	miR-376a, miR-376b, miR-376c (down)	Serum	RNU6
Lai et al	2015	126	40	Validation Set	Glioma (grade I to IV)	qRT-PCR	miR-210 (up)	Serum	miR-16-1
Manterola et al	2014	75	55	Training Set and Validation Set	Glioblastoma (grade IV)	qRT-PCR	RNU6-1, miR-320, miR-574-3p (up)	Serum	RNU48
Regazzo et al	2016	15	10	Training Set	Glioblastoma (grade IV)	qRT-PCR	miR-497, miR-125b (down)	Serum	UniSP2
Roth et al	2011	20	20	Validation Set	Glioblastoma (grade IV)	qRT-PCR	miR-128 (up), miR-342-3p (down)	Blood	RNU48
Shao et al	2015	70	70	Training Set	Glioma (grade I to IV)	qRT-PCR	miR-454-3p (up)	Plasma	cel-miR-39
Sun et al	2015	151	53	Training Set	Glioma (grade I to IV)	qRT-PCR	miR-128 (down)	Serum	cel-miR-39
Wang et al	2012	10	10	Training Set	Glioblastoma (grade IV)	qRT-PCR	miR-21 (up) miR-128, miR-343-3p (down)	Plasma	mmu-miR-295
Wei et al	2014	33	33	Training Set	Glioma (grade I to IV)	qRT-PCR	miR-125b (down)	Serum	miR-24
Wu et al	2014	83	69	Training Set	Glioma (grade I to IV)	qRT-PCR	miR-29 (down)	Serum	miR-24
Xiao et al	2016	112	54	Training Set	Glioma (grade I to IV)	qRT-PCR	miR-182 (up)	Plasma	RNU6B
Yang et al	2012	133	80	Training Set and Validation Set	Astrocytoma (grade II to IV)	qRT-PCR	miR-15b ¹ , miR-23a, miR-133a, miR-150 ⁴ , miR-197, miR-497, miR-548b-5p (down)	Serum	Serum Volume
Yue et al	2016	64	45	Training Set	Glioma (grade I to IV)	qRT-PCR	miR-205 (down)	Serum	miR-16
Zhang et al	2015	50	51	Training Set	Glioma (grade I to IV)	qRT-PCR	miR-221/222 (up)	Plasma	miR-16
Zhi et al	2015	140	160	Training Set and Validation Set	Astrocytoma (grade II to IV)	qRT-PCR	miR-15b-5p, miR-16-5p, miR-19a-3p, miR-19b-3p, miR-20a-5p, miR-106a-5p, miR-130-3p, miR-181b-5p, miR-208a-3p (up)	Serum	Serum Volume

<https://doi.org/10.1371/journal.pone.0189452.t001>

well-established guidelines for assessing circulating miRNA, contributed to an increase in risk of bias and heterogeneity of studies.

Diagnostic accuracy

Diagnostic accuracy, sensitivity and specificity of each dysregulated single miRNA or miRNAs panel are summarized in [Table 2](#). Forest plots of pooled data (23 studies from 16 articles) demonstrated a large heterogeneity of included studies ([Fig 2](#)). Thus, the mixed effect model was applied to yield the sensitivity (0.87 (95% CI, 0.82–0.90) ([Fig 2A](#)), and specificity 0.86 (95% CI, 0.82–0.90) ([Fig 2B](#)) of this meta-analysis. The Diagnostic Odds Ratio (DOR) was identified as 41.91 (95% CI, 23.15–75.88) and the pooled PLR and NLR were 6.39 (95% CI, 4.61–8.87), 0.15 (95% CI, 0.11–0.21), respectively ([Fig 3A](#)). In addition to diagnostic accuracy, the summary receiver operator characteristic (SROC) curve was plotted and the Area Under the Curve (AUC) was 0.93 (95% CI, 0.91–0.95) ([Fig 3B](#)), suggesting that circulating miRNAs may be valuable as diagnostic tool. Subtype analysis was also performed according to the type of sample used (serum and plasma) ([S1 Fig](#)).

Table 2. Diagnostic characteristics of each single miRNA and miRNA panel.

miRNA(s)	Expression	Sensitivity	Specificity	AUC	Study
Single miRNA					
miR-15b	Upregulated	98%	98%	0.98	D'Urso et al, 2015
miR-376a	Downregulated	81%	82%	0.872	TUang et al, 2017
miR-376b	Downregulated	82%	78%	0.890	Huang et al, 2017
miR-376c	Downregulated	90%	70%	0.837	Huang et al, 2017
miR-182	Upregulated	58.5%	85.2%	0.778	Xiao et al, 2016
miR-128	Downregulated	86.75%	88.68%	0.9095	Sun et al, 2015
		90%	100%	1	Wang et al, 2012
miR-29	Downregulated	68.5%	77.3%	0.74	Wu et al, 2014
miR-125b	Downregulated	78.79%	75.76%	0.839	Wei et al, 2014
miR-210	Upregulated	91.27%	72.50%	0.927	Lai et al, 2015
miR-454-3p	Upregulated	99.05%	82.86%	0.9063	Shao et al, 2015
miR-21	Upregulated	90%	100%	0.93	Wang et al, 2012
miR-342-3p	Downregulated	90%	100%	1	Wang et al, 2012
miR-205	Downregulated	86.3%	92.2%	0.935	Yue et al, 2015
miR-221	Upregulated	73.5%	80%	0.83	Zhang et al, 2015
miR-222	Upregulated	85.7%	87.5%	0.88	Zhang et al, 2015
MiRNA panel					
miR-497, miR-125b	Downregulated	88.9%	66.7%	0.861	Regazzo et al, 2016
miR-15b*, miR-23a, miR-133a, miR-150*, miR-197, miR-497 and miR-548b-5p	Downregulated	88%	97.87%	0.972	Yang et al, 2013
180 miRNA panel					
RNU6, miR-320, miR-574-3p	Upregulated and downregulated	83%	79%	0.81	Roth et al, 2011
		87%	86%	0.926	Manterola et al, 2014
miR-15b-5p, miR-16-5p, miR-19a-3p, miR-19b-3p, miR-20a-5p, miR-106a-5p, miR-130a-3p, miR-181b-5p, miR-208a-3p	Upregulated	70%	71%	0.722	Manterola et al, 2014
		93.3%	94.5%	0.9722	Zhi et al, 2015
		94.0%	92.0%	0.9576	Zhi et al, 2015

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Sensitivity analysis

The goodness of fit included in our analysis shows a clear representation of the population. Q-Q plot (Fig 4) demonstrated the included studies were normally distributed despite a few outliers. Influence analysis showed that this meta-analysis was mainly contributed by the following studies D'Urso et al, Shao et al, Xiao et al and Yang et al in weight (Fig 4). Detection analysis identified D'Urso and Xiao (Fig 4), indicating these two papers might be the source of heterogeneity in this meta-analysis. In addition, there is no potential publication bias detected by Deeks' funnel plot asymmetry test ($p = 0.53$), as shown in the funnel plot (S2 Fig).

Discussion

In this meta-analysis study, we analyzed 16 publications relating to miRNAs as potential diagnostic markers of glioma. In our meta-analysis, the overall AUC was 0.93, suggesting that circulating miRNAs could be useful in detecting glioma. The overall specificity and sensitivity of circulating miRNAs was 0.87 and 0.86, respectively. This level of diagnostic accuracy is similar to that reported for proton magnetic resonance spectroscopy (specificity 0.87 and sensitivity 0.85) [37]. The pooled positive likelihood ratio of 6.39 indicated that the probability of glioma is 6-fold increased when the studied miRNAs was positive while the pooled negative likelihood

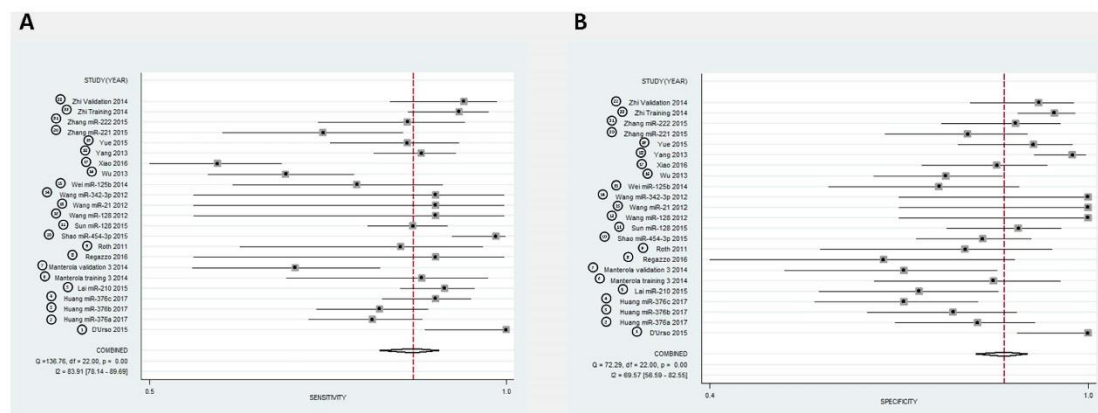


Fig 2. Forest plots of all 16 studies included in this meta-analysis. The pooled A) sensitivity and B) specificity is 0.87 and 0.86, respectively of miRNAs in diagnosis of glioma.

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ratio of 0.15 suggested that the probability of glioma decreased by 85% with negative miRNA testing. The diagnostic odds ratio of circulating miRNAs of 41.91 also showed an outstanding test performance. The validity of circulating miRNA for glioma diagnosis is the key point we need to address prior to its introduction into clinical practice. All studies included in this

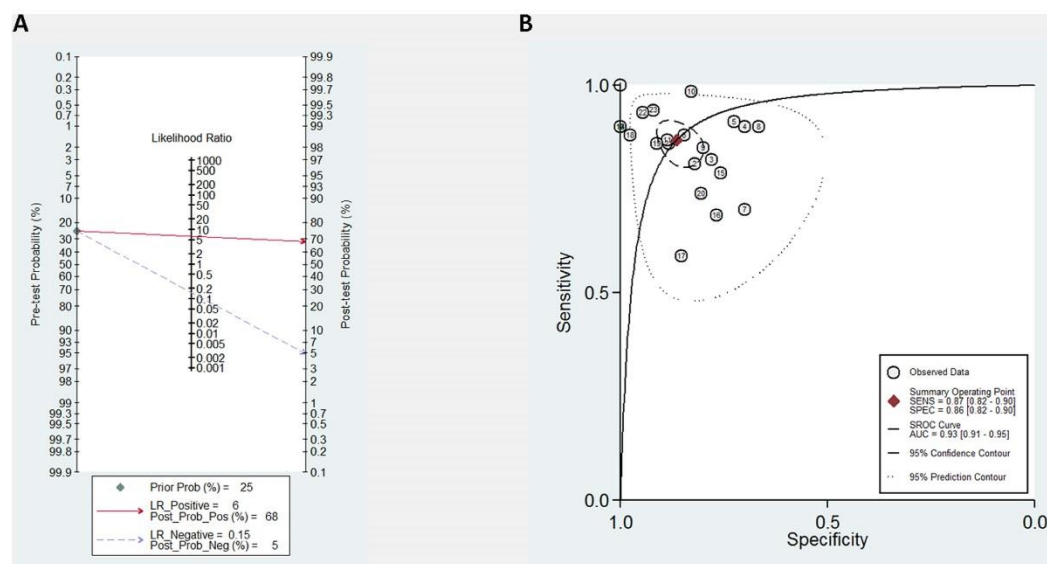


Fig 3. Diagram of SROC curves describing the diagnostic performance of miRNAs. A) The PLR and NLR is 6.39 and 0.15 respectively, showing the pre-test probability set as 25%, the positive and negative post-test probability of 68% and 5%, respectively. B) The AUC is 0.93 (95%CI, 0.91–0.95). Each number within a circle represents the order of study identifier in Fig 3.

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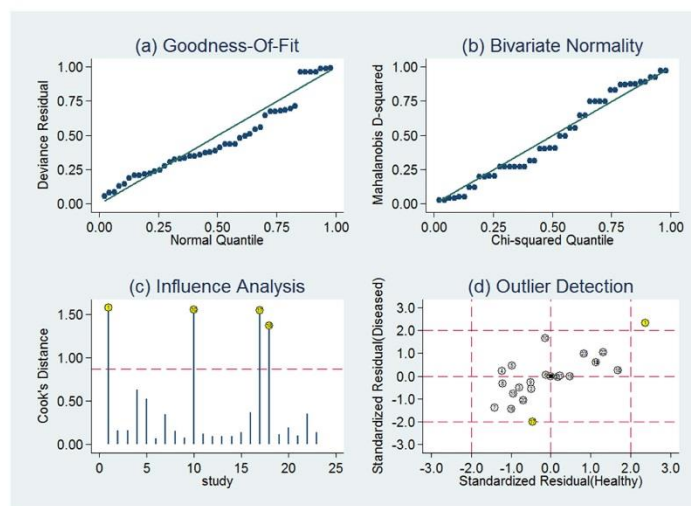


Fig 4. The diagram shows the (a) goodness-of-fit, (b) bivariate normality, (c) influence and (d) outlier detection analyses. Goodness-of-fit and bivariate normality showed this analysis fitted the model well. Influence analysis identified the most dominant studies in this meta-analysis. Outlier detection demonstrated one study (D'Urso et al and Xiao et al) is outside the standard residual square. Each number within circle represents the order of study identifier in Fig 3.

<https://doi.org/10.1371/journal.pone.0189452.g004>

meta-analysis utilized qRT-PCR to detect circulating miRNA; this could be the best choice of technique for future application as it is highly sensitive for detecting low copies of miRNAs in serum samples, which is critical for routine test in clinical setting [38]. However, this meta-analysis identified a large heterogeneity of circulating miRNAs within all studies included ($I^2 = 87.89$), which was likely due to data normalization.

Three miRNAs (miR-15b, miR-125b and miR-128) were identified by several studies [25, 26, 30, 33, 34] to be dysregulated in blood of glioma patients. In two of these studies [33, 34], miR-15b was found upregulated in glioma samples when compared to healthy controls. High levels of miR-15b in glioma tissue are strongly associated with poor prognosis [39] and biologically, miR-15b can induce cell cycle arrest, promoting cell proliferation and apoptosis by targeting cyclins [39, 40], suggesting its role in glioma development.

Serum miR-128 was also reported significantly dysregulated in glioma [23, 25, 26] and upregulated in patients post-surgery [23, 25]. In line with this finding, miR-128 has been reported to be downregulated in glioma tissues compared to normal brain tissue [41]; it is regarded as a critical regulator in glioma as it manipulates multiple biological phenotypes such as apoptosis, proliferation, angiogenesis, self-renewal, cell adhesion and chemotherapy resistance [41–45].

Serum miR-125b, was also commonly found at lower levels in high grade glioma patients compared to controls [27, 36]. However, in contrast, high levels of miR-125b expression are linked to malignancy in glioma cells [46], and it functions to regulate apoptosis via p53 and p38-MAPK and NF- κ B pathways, common in glioma development. These opposite findings may be related to different roles or expression mechanisms for this miRNA in tissue vs serum and supports the need for careful selection of miRNA for glioma diagnosis prior to translating to clinical practice.

In the studies we retrieved here, some miRNAs were compared between pre-operative and post-operative patients. miRNAs including miR-205, miR-21, miR-128, miR-454, changed dramatically post-surgery [23, 24, 31], suggesting them as possible candidates for monitoring glioma progression. Our group recently published a study showing significant changes in circulating level of miR-21 and miR-10b in glioma patients undergoing treatment with an anti-angiogenic reagent, bevacizumab, indicating that these circulating miRNAs are potentially monitoring biomarkers for glioblastoma [47]. Another study showed that a panel of miR-21, miR-128 and miR-342 can be used as potential predictors of therapy response, as the levels of these circulating miRNAs were altered after chemo-radiation treatment [26]. However, post-operatively, serum miRNA levels are likely to reflect drug treatments and host factors as well as directly related to glioma progression, thus caution is required in interpreting this early evidence.

Several different endogenous controls were used in the studies analyzed, which possibly contributed to the heterogeneity of the data. There is no universal housekeeping control for miRNA and the process of choosing a suitable reference is still controversial [48]. It is known that RNU6 is widely used as an endogenous control in some cancer tissues or cellular samples, but it does not seem to work in bio-fluid samples [49][49][49][49], yet through this analysis, we have identified RNU6 as a reference control in several studies, which could likely cause misinterpretation in the dataset.

There are other confounding factors contributing to the heterogeneity within and between studies including the patients' ethnic group, geographic location of the studies, and the statistical package used in data filtering and analysis. Another important factor affecting the future application of circulating miRNA in the clinic is the current lack of a standardized protocol for processing blood samples as well as RNA/miRNA isolation prior to qRT-PCR analysis, which is likely the main reason for the 'high risk' seen in applicability concerns of QUADAS-2 analysis.

A larger sample size may have improved the reliability of the conclusions in our meta-analysis, however, we are limited by the numbers of current published studies in this novel field of circulating miRNA in glioma. The sample size of 16 publications in our study is within the average range compared to meta-analyses of miRNA expression in various other cancers.

Based on our meta-analysis, circulating miRNAs can potentially be used as diagnostic tool for glioma with high sensitivity and specificity. The clinical role of serum miRNA analysis is likely to be in conjunction with MRI and histopathology at the time of diagnosis, as well as for monitoring for glioma recurrence post operatively. Towards this aim, we have recently performed a global miRNA profile analysis of serum from a large cohort of glioma patients in our local institution that has identified a set of miRNAs, some of which overlap with those in this meta-analysis. These biomarkers need to be validated in future clinical trials. Furthermore, a consensus needs to be established for a standardized sample processing and miRNA detection methods before introduction into clinical practice.

Supporting information

S1 File. Prisma Checklist.
(DOC)

S1 Fig. SROC curves describe the diagnostic performance of miRNAs in discriminating glioma from non-cancer subjects from plasma or serum sample. A) AUC is 0.96 (95%CI, 0.94–0.97) for plasma and B) AUC is 0.92 (95%CI, 0.89–0.94) for serum.
(TIF)

S2 Fig. The Deek's funnel plot of all included studies. This Deek's funnel plot demonstrated that there is symmetry in all included studies, indicating low risk of publication bias in this meta-analysis, indicating ($p = 0.53$). Each number within circle represents the order of study identifier in Fig 3. (TIF)

Acknowledgments

We would like to acknowledge the support of the Cure Brain Cancer Foundation, the Royal Melbourne Hospital Neuroscience Foundation (Dr A Morokoff) and The University of Melbourne International Research Scholarship (Dr C Ma).

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3.3. Additional results

To further explore the diagnostic capacity of miRNA in CSF for glioma, a meta-analysis was also performed. Through the literature search, a total of four publications with five cohorts regarding diagnostic miRNAs in CSF for glioma was identified after removing the relevant reviews and studies that failed to report diagnostic accuracy (Akers et al., 2013; Baraniskin et al., 2012; R. Shi et al., 2015; Teplyuk et al., 2012). The characteristics of these papers are listed in table 3.1. miR-21 was the most commonly identified miRNAs across all four studies, suggesting its high specificity in circulating miRNAs for diagnosing glioma.

Table 3.1. Characteristics of cohorts regarding miRNAs from CSF

Study	Case number	Control number	miRNA	Cancer type	Normalization
Akers et al. (training set)	13	14	miR-21	Glioblastoma	Input EV number
Akers et al. (validation set)	15	16	miR-21	Glioblastoma	Input EV number
Baraniskin et al.	10	10	miR-15b	Glioma	miR-24
Shi et al.	70	25	miR-21	Glioma	GAPDH
Teplyuk et al.	19	15	miR-10b, miR-21, miR-125b, miR-141, miR-200a, miR-200b, and miR-200c	Glioblastoma	miR-24

The forest plots demonstrated the pooled sensitivity and specificity of miRNAs in CSF for diagnosing glioma (Figure 3.1). The sensitivity and specificity of these miRNAs were 0.89 (95% CI, 0.83-0.93) and 0.95 (95% CI, 0.88-0.98), respectively. The summary ROC curve showed the summary area under the curve (AUC), where the AUC for miRNAs in

CSF was 0.97 (95% CI, 0.95-0.98) (Figure 3.2). Notably, there was no study investigating the IDH1 or MGMT promoter methylation relevant miRNAs in biofluid, which could be helpful in glioma management.

Figure 3.1. Forest plots of miRNAs from CSF. Pooled sensitivity (A) and specificity (B) of miRNAs in CSF. Red line, the overall sensitivity or specificity. Diamond, the 95% CI of sensitivity of specificity.

3.4. Additional discussion

Our results have demonstrated that circulating miRNAs (derived from either CSF or blood) have diagnostic capacity for glioma. The accuracy of circulating miRNAs in CSF is slightly better than that in plasma or serum with a better sensitivity and specificity. Due to the blood-brain barrier, cell free miRNAs in CSF miRNAs might be prevented going through and miRNAs in CSF might be a more effective biomarker for diagnosing glioma. However, the clinical utility of CSF miRNAs is still limited. Although our results show the accuracy of CSF miRNAs are slightly better than blood miRNAs regarding predicting

diagnosis, collection of CSF still requires a specialist to perform lumbar puncture and this procedure leads to injuries and inconvenience for patients. Therefore, detection of miRNAs from CSF could be considered complementary to the conventional neuroimaging but not an ideal way for early stage cancer screening and a non-invasive tool.

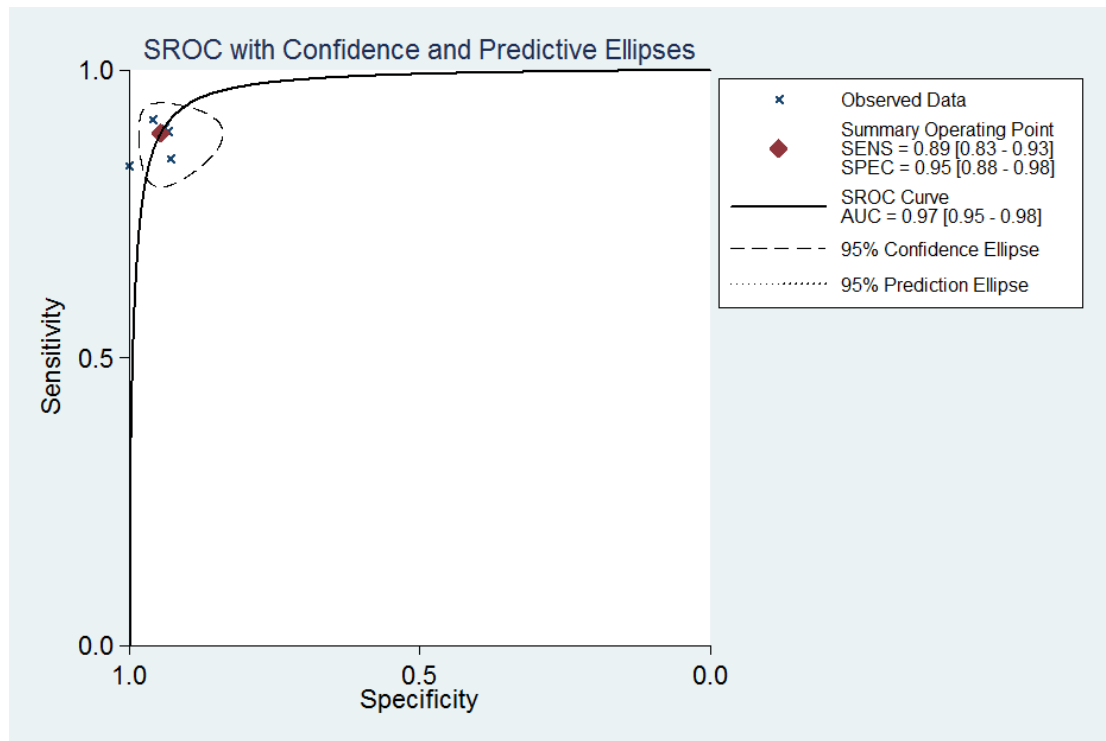


Figure 3.2. The summary Receiver Operation Characteristics (ROC) curve of miRNAs in CSF. Cross, the observed data. Red square, the summary point of all observed data. Solid line, the summary ROC curve. Dash line, the 95% CI.

In contrast, miRNAs in blood are promising biomarkers for glioma in the ‘liquid biopsy’ era. Despite the slight inferior accuracy of miRNAs in blood to that in CSF, miRNAs in blood can still provide more than 90% accuracy of diagnosis. In addition to the accuracy, the convenience of collection of blood from patients should also be considered in the development and management of biomarkers.

Slightly different from miRNAs from blood, miR-21 is the most commonly identified miRNAs in CSF. This result is consistent with previous studies where miR-21 is the key

regulator in variety of cancers and associated with the grade of cancer (Z. Shi et al., 2013). miR-21 plays an important role in tumourigenesis and progression of glioma through targeting the tumour suppressors such as PTEN (Papagiannakopoulos, Shapiro, & Kosik, 2008). A few meta analyses have found miR-21 is a promising diagnostic biomarker in multiple types of cancers (Y. Gao et al., 2016; S. R. Yan, Liu, Yu, & Bao, 2015; Yin, Zhou, Dang, Yan, & Zhang, 2015), suggesting miR-21 is the most promising biomarker of all miRNAs for diagnosis of cancer.

Another consideration that we need to highlight is that there are few studies exploring the grade or molecular markers such as IDH1 mutation, 1p/19q co-deletion or MGMT promoter methylation related miRNAs, which might have more practical information in therapy and prognosis management. Clinically, it might not be difficult to distinguish glioma from normal brain. Rather, it would be more useful if we could further understand the biological and prognostic information simply by the collection of patients' blood and detection of miRNAs expression before we apply the initial diagnosis and therapies to patients. For low grade glioma, miRNAs testing that elucidate which patients are highly likely to develop to high grade glioma or have high risk of recurrence is the ultimate aim for developing an identifying miRNA biomarkers in glioma. Once the molecular markers relevant miRNAs are identified, patients with a low risk of recurrence identified by circulating miRNA testing may not need to receive further chemotherapy after the standard treatment. Before establishing the association of miRNAs and IDH1 mutation in circulation, there still is little known about IDH1 mutation relevant miRNAs in glioma. One study has already revealed miR-148a is silenced in IDH1 mutant glioma and the methylation of miR-148a is complementary to the prognostication of glioma patients survival (S. Li et al., 2014). However, the other miRNAs relevance to IDH1 mutation and methylation remain unclear. To address this question, identification of IDH1 relevant miRNAs from tissue samples were performed in the next chapter of this thesis (Chapter 4).

As we noted, there is some limitations of previous study that qRT-PCR is the main method they used for circulating miRNAs signature determination and relative quantification detection. The qRT-PCR could not provide a comprehensive overview of circulating miRNA patterns in cancer patients. The qRT-PCR is widely used for nucleic acid detection and it is significantly sensitive for detection with limited inputs. However, the sequences of targeted genes / loci and the primers designed for PCR reactions are the prerequisites for all PCR based detections. In the biomarker discovery, a genome-wide biomarker screen should be performed to shortlist and optimize the best combination of biomarker candidates. Since the circulating miRNA profiles of glioma has not established yet, it is worthwhile that performing a comprehensive discovery for circulating miRNAs. In addition, qRT-PCR only yield the relative expression of certain miRNAs rather than the absolute values. The most widely used quantification for qRT-PCR results is $\Delta\Delta C_t$ value, which requires a housekeeping gene or a invariable gene across all tested samples. Unlike the housekeeping genes in tissues are well-established, the reference genes for circulating miRNAs are still controversial. The results variable between studies likely depend on the reference gene chosen by each study. Therefore, for biomarker validation and clinical practice, digital droplet PCR (ddPCR) could be considered to replace the conventional qRT-PCR.

The material source of circulating miRNAs (serum or plasma) is another bias for circulating miRNA biomarker discovery. Foye et al directly compared circulating miRNA expression patterns between serum and plasma of 28 patients (Foye et al., 2017). There is a significant difference in both the numbers of miRNAs and the abundance of miRNAs between plasma and serum, which suggests the results from plasma and serum might not be directly comparable. Further investigations are needed to explore the mechanism of the difference of miRNA patterns between plasma and serum. It is also notable that the reference genes used in plasma could not be suitable for studies in serum and vice versa.

We also noticed the disease condition might be another bias for performing and interpreting the meta-analysis. Healthy population samples are used as control in most studies but only one cohort involved various neurological disorders (Ivo D'Urso et al., 2015). Although this did not have a large effect on this meta-analysis, neurological diseases specific miRNAs have been identified from variety of studies (Y. Ma, 2018; Martinez & Peplow, 2020). To optimize the miRNA biomarker panel, samples from healthy population should be utilized for controls.

As a proof-of-concept study, further investigation on the clinical utility of circulating miRNAs is required to test whether it works as a companion diagnosis in glioma. Although these meta-analyses demonstrate high accurate circulating miRNAs in glioma patients, these patients have been pathologically diagnosed with suspicious lesions in CT or MRI scans. A prospective clinical trial is needed to test the clinical utility of circulating miRNAs as a companion diagnosis tool in glioma.

From this meta-analysis, we observe more down-regulated miRNAs than up-regulated ones. These down-regulated miRNAs mediate the gliomagenesis by targeting oncogenes. However, from the point view of biomarker, up-regulated miRNAs in cancer patients are likely to be more suitable biomarkers than down-regulated ones in the clinical practice. Even with the reference genes, the down-regulated miRNAs have more possibility of false negative than up-regulated miRNAs, where we could not speculate whether one miRNA that not expressed in one sample is due to the failure of reaction or the true downregulation.

Chapter Four

Chapter 4: Identification of subtypes and IDH1 mutation associated miRNAs in GBMs

4.1. Introduction

Gliomas are well recognized as a heterogeneous disease which is characterized by EGFR and/or PDGFRA mutation, TERT promoter mutation, DNA methylation and IDH1 mutation (Brennan et al., 2013). IDH1 mutation is frequently found in secondary GBMs and grade II glioma such as oligodendrogliomas and astrocytomas while only 6% of primary GBMs are IDH1 mutant. Of them, IDH1 R132H is the most frequent alteration in IDH1, followed by R132G, G300C and Y319F. Furthermore, mutations in IDH1 are early events in gliomagenesis, which leads to the distinct biological phenotypes and response to treatment between IDH1 mutant and wild type GBMs. Therefore, GBMs can be categorized into two subgroups: those that harbor IDH1 mutations and those that are IDH1 wild type. Due to the relatively favourable outcomes for IDH1 mutant GBMs, they are assigned to a separate subgroup in the newly published WHO central nervous system classification (WHO 2016) where patients are administered with different treatment strategies compared to patients with tumours that express wild-type IDH1 (Paul, Mondal, Patil, & Somasundaram, 2017; van den Bent et al., 2017). This updated WHO classification highlights the importance of evaluating IDH1 mutation status as it provides further insights to glioma behaviour and response to treatment. In low grade glioma, IDH1 mutant is a prognostic marker, where its expression dictates the clinical management strategy in anaplastic gliomas. A randomized clinical trial found an additional survival benefit of procarbazine/lomustine/vincristine (PCV) chemotherapy combined with radiation in IDH1 mutant anaplastic glioma, compared to patients treated with radiation alone (Cairncross et al., 2014). Despite the presence of IDH mutations in

only a minority of GBMs, its presence indicates distinct biological and clinical behaviours with IDH1 wild type GBM. Therefore, further investigation of IDH1 mutation in GBMs is required to understand the biological difference caused by IDH1 mutation in GBMs.

IDH1 mutation is a frequent event in low grade glioma and leukemia. Mutant IDH1 protein catalyses α -ketoglutarate (α -KG) to D-2-hydroxyglutarate (D-2-HG). As a consequence, the α -KG dependent dioxygenases such as ten-eleven translocation (TET) family 5-methylcytosine hydroxylases and the Jumonji-C-domain-containing histone-lysine demethylases are accordingly inhibited (C. Lu et al., 2012). Once dioxygenases are reduced due to mutant IDH1, methylation of DNA is accordingly increased and then global hypermethylation of CpG island will be formed. Mutant IDH1 re-defines the chromatin status, DNA methylation level and maintains the stem cell like status (Turcan et al., 2018), which eventually enable hematopoietic progenitors to lose their differentiation ability (Sasaki et al., 2012). This DNA hypermethylation status is not only observed in leukemia but is also seen in glioma, and is called the ‘glioma CpG-island methylator phenotype’ (G-CIMP) (Noushmehr et al., 2010). In cancer, the aberrant DNA methylation level is characterized by hypermethylation of the promoter region of genes, gene bodies and global hypomethylation of the non-promoter elements (Malta et al., 2018). This remodeling of the DNA methylome leads to the genes transcription suppression by misrecognition of transcription site of DNA-binding transcription factors, which regulates genes transcription. Hypermethylation of CpG sites in cancer is strongly associated with prolonged patient survival. A better survival was observed in GBMs patients carrying G-CIMP, as compared with those not carrying G-CIMP (Malta et al., 2018). G-CIMP is distinct from other subtypes of glioma in terms of gene alteration, which suggests G-CIMP tumours are biologically and clinically different from other types of glioma. A recent machine-learning-based classifier also demonstrated DNA methylation classification increased diagnostic power along with conventional pathological diagnosis (Capper et al., 2018; Hegi, Kleihues, Wen, & Suva, 2018).

Along with DNA methylation, microRNA (miRNA) is another epigenetic regulation mechanism that plays a vital role in gliomagenesis and progression. MiRNAs are small non-coding RNA, found in all cell types. They are approximately 22 nucleic acid sequences in length and complimentary to 3' UTR of their potential target mRNAs, where they could regulate target genes expression by preventing mRNA translation and/or inducing degradation of target mRNAs. Accumulating studies reveal the regulatory power of miRNAs in gliomagenesis and they are differentially expressed in glioma compared to normal tissues. For example, miRNA-222 induces tumourigenesis by inhibiting DKK2 and activation of Wnt pathways in glioma. In addition, miRNAs are secreted by cells into the microenvironment and have been demonstrated as potent, novel diagnostic tools in glioma, (see chapter 3 of this thesis) (C. Ma et al., 2018). Clustering based on miRNA expression demonstrates the differentiation capacity of the temporal and spatial heterogeneity. Unsupervised clustering of 121 miRNA expression and subtypes classified by these miRNAs are linked with neural differentiation stages. Moreover, miRNA expression also correlates with the distinct subtypes of glioma. An integrative analysis of RNA, DNA copy number, DNA methylation and miRNAs expression patterns from diffuse lower-grade glioma uncover unsupervised clustering of miRNA expression has consensus with IDH1 mutation clustering (Cancer Genome Atlas Research et al., 2015; C. Ma et al., 2018). In this study, four consensus groups were identified by means of miRNAs expressions, which was consistent with IDH1 mutation status. A total of 15 up-regulated miRNAs including miR-196b-5p, miR-10a-5p, miR-204-5p, miR-21-3p, miR-148a-3p, miR-455-3p, miR-155-5p, miR-21-3p, miR-193a-5p, miR-146b-5p, miR-27a-3p, miR-99b-3p, miR-23a-3p, miR-221-3p and miR-24-3p were enriched in IDH1 wild types while only five miRNAs (miR-181a-2-3p, miR-9-5p, miR-105-5p, miR182-5p, miR-183-5p, miR-891a-5p) were associated with IDH1 mutation under a threshold of p value less than 0.05. Recent studies have already revealed GBM is a highly heterogeneous disease with respect to both intertumour and intratumour differences, and these are also associated with therapeutic treatment response and

survival (Soeda et al., 2015). However, the miRNA expression pattern in GBMs, especially for IDH1 mutant GBMs remains unclear.

Here, we utilized a miRNA array dataset, a clinical information dataset and DNA methylation datasets downloaded from TCGA to explore the IDH1 mutation associated miRNAs in GBMs. We also explored the correlation of DNA methylation induced by IDH mutation with miRNA expression in GBMs. We further investigate methylation of these differentially expressed miRNAs and the pathways of the targets regulated by these miRNAs to explore the potential therapeutic targets for GBMs.

4.2. Results

4.2.1. Global miRNA profile of GBM from TCGA database

All miRNA expression data in GBM was downloaded from TCGA database. A total of 615 cases were obtained from public data base and 566 of them attached with clinical information. Therefore, 566 cases of GBM with miRNA expression and clinical information were retrieved and included in this following analysis. All gene expression data of these cases were also downloaded. All data pre-processing is included in filtering, normalization. Boxplot graphing was employed to get the distribution of all miRNAs or genes across all samples after normalization. Principal component analysis (PCA) and supervised clustering showed by heatmap were conducted to explore the clustering status based on miRNAs expression over all eligible samples. All significant differential expressed miRNAs were highlighted by volcano plot and exported.

4.2.2. miRNAs profile of GBM vs Normal

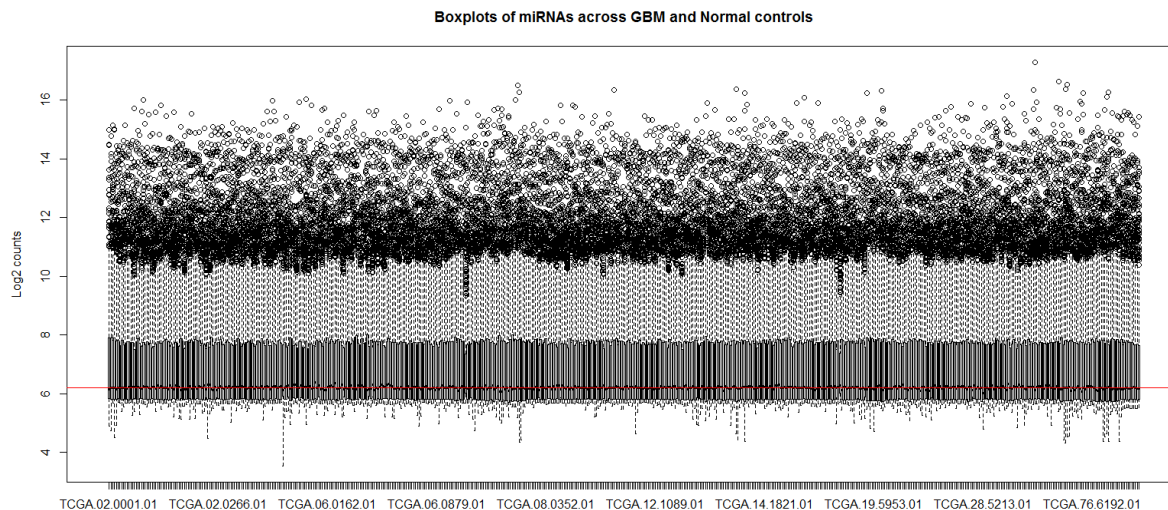


Figure 4.1. Boxplot of miRNAs expression across all samples including GBMs and normal controls. Red line indicates the median of all samples.

A comparison of all GBMs and normal *tissues* was first performed to identify the GBM specific miRNAs (note this is not based on circulating miRNA). A total of ten normal brain tissue samples are available in the TCGA database. Boxplot analysis demonstrated that there was no significant variation of miRNAs expression across all samples, indicating all level 3 data are reliable and suitable for downstream analysis (Figure 4.1). PCA showed all samples including normal controls are clearly clustered into two main subsets, indicating GBM had distinct miRNA expression pattern with normal controls (Figure 4.2A). The first and second principal component indicated 17.36% and 12.81% variation between GBMs and normal controls, respectively. Supervised clustering of miRNAs expression showed the top 100 variable miRNAs between GBMs and normal controls by heatmap (Figure 4.2B). Volcano plot demonstrated the shortlisted differentiated miRNAs when the threshold was set as log fold change larger than 2 and adjusted p value smaller than 0.01 (Figure 4.3). In this analysis, fourteen miRNAs (miR-129, miR-139, miR-491, miR-218, miR-137, miR-128a, miR-128b, miR-132, miR-138, miR-124a, miR-7, miR-410, miR-338, miR-219) are highly expressed in normal controls

while four miRNAs (miR-27a, miR-23a, miR-21, miR-210) are GBMs associated miRNAs. A heatmap of these 18 differentially expressed miRNAs demonstrated GBM and normal controls were well clustered (Figure 4.3). All shortlisted miRNAs to differentiate GBM and normal brain tissue are listed in Table 4.1.

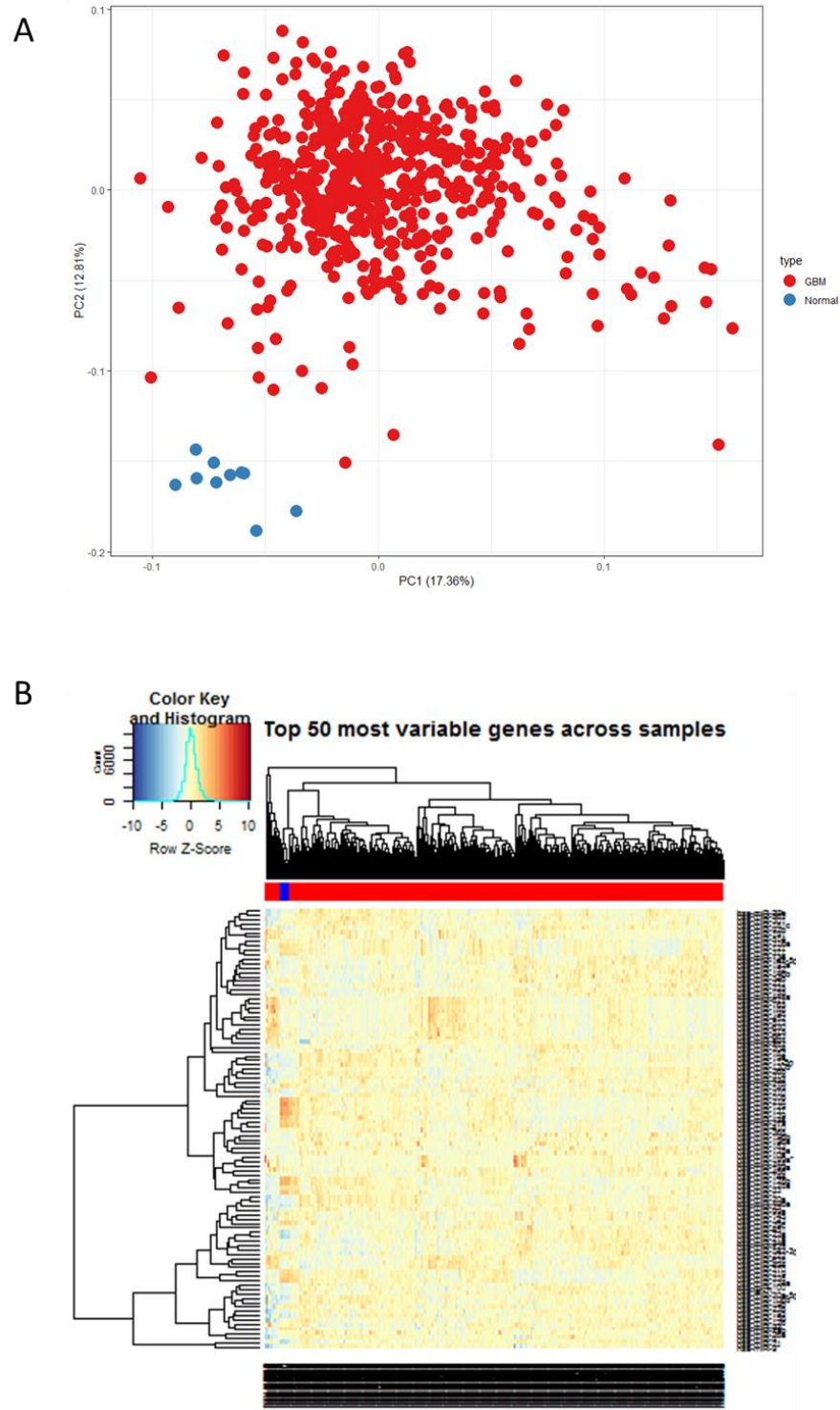
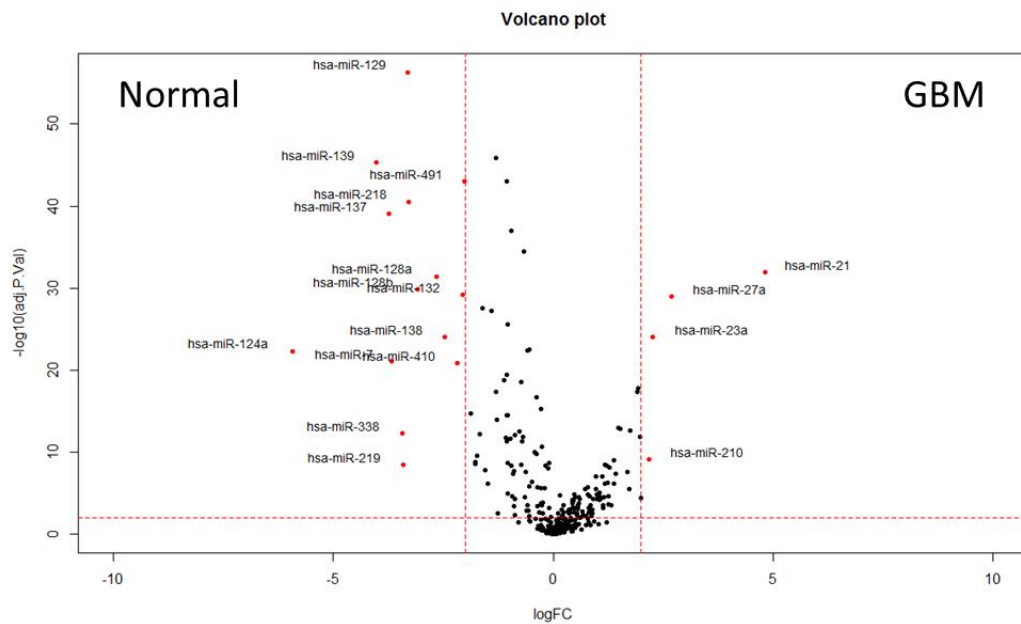


Figure 4.2. The difference between GBMs and normal controls with respect to miRNAs expression. **A**, PCA plot of all samples. Red dots= GBM samples; blue dots= normal controls. **B**, the top 100 most variable miRNAs between GBMs and normal controls. Red bar, GBM samples; blue bar, normal controls.

A



B

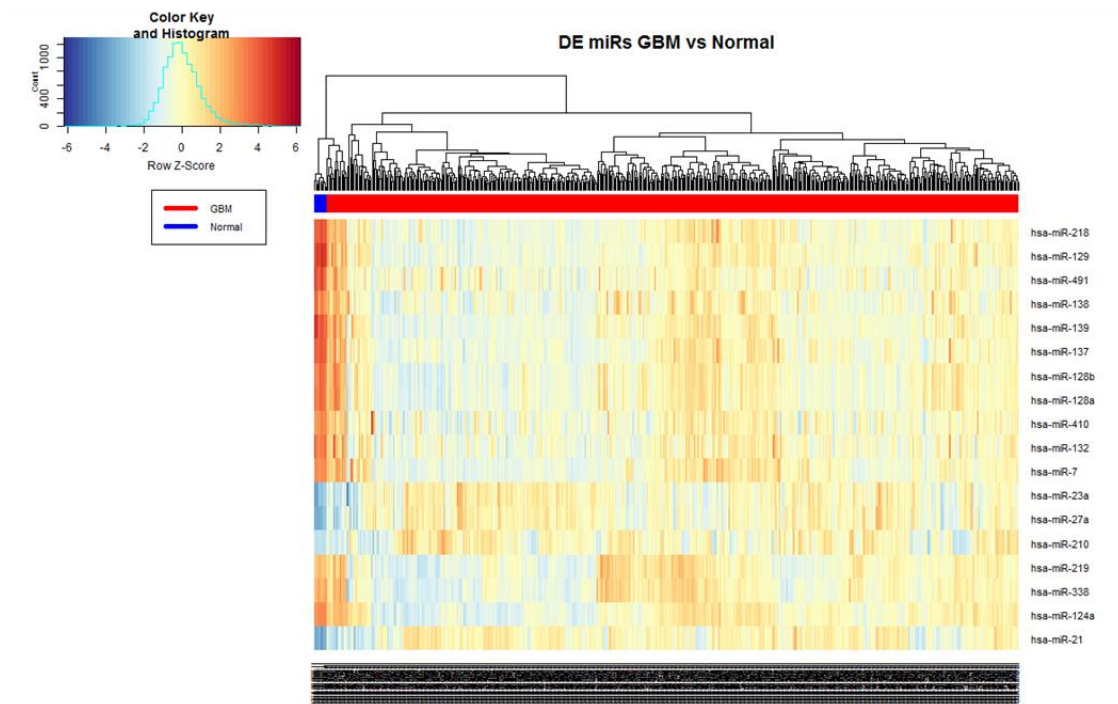


Figure 4.3. Differentially expressed miRNAs between GBMs and normal controls. A, Volcano plot showing 18 differentially expressed miRNAs. Dots = miRNAs; red dots = significant miRNAs. Red lines indicates the threshold. Left = highly expressed miRNAs in normal controls; right = highly expressed miRNAs in GBMs. **B,** Heatmap showing the clustering of all samples with respect to 18 differentially expressed miRNAs. Red bar= GBM samples; blue bar = normal controls.

Table 4.1. The differentially expressed miRNAs between GBMs and normal controls

miRNA names	Highly expressed in
miR-129	Normal controls
miR-139	Normal controls
miR-491	Normal controls
miR-218	Normal controls
miR-137	Normal controls
miR-128a	Normal controls
miR-128b	Normal controls
miR-132	Normal controls
miR-138	Normal controls
miR-124a	Normal controls
miR-7	Normal controls
miR-410	Normal controls
miR-338	Normal controls
miR-219	Normal controls
miR-27a	GBM
miR-23a	GBM
miR-21	GBM
miR-210	GBM

4.2.3. Subtype specific miRNAs

We next explored the transcriptome subtype specific miRNAs of GBM using all subtype labelled data retrieved from TCGA database. Based on the information from TCGA, there are 144 cases from the classical subtype of GBM, 137 cases from the proneural subtype, 85 cases from the neural subtype and 157 cases from the mesenchymal subtypes respectively. Boxplot showed that all included samples were comparable (Figure 4.4A). PCA expression demonstrated 15.87% and 10.39% of variance between GBM subtypes

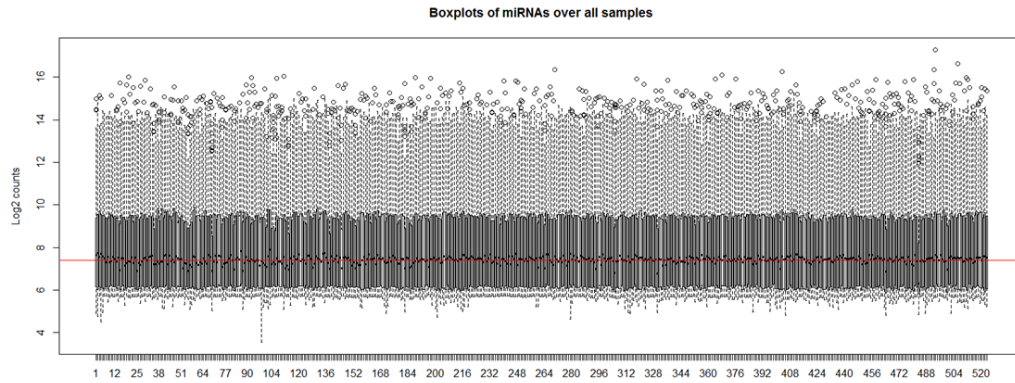
could be explained by principal component (PC) 1 and 2 of all miRNA expression, respectively, which were also confirmed by scree plot. Most mesenchymal GBMs were located in the right part of PC1 while proneural GBMs were located to the left of PC1, suggesting there was distinct miRNA expression pattern between mesenchymal GBM and proneural GBM. Classical and neural GBMs were located between proneural and mesenchymal GBM with big overlap of proneural GBM and mesenchymal GBM, suggesting there are only slight differences between these groups with respect to miRNA expression profiles (Figure 4.4).

Table 4.2. The significantly differentially expressed miRNAs between subtypes of GBMs

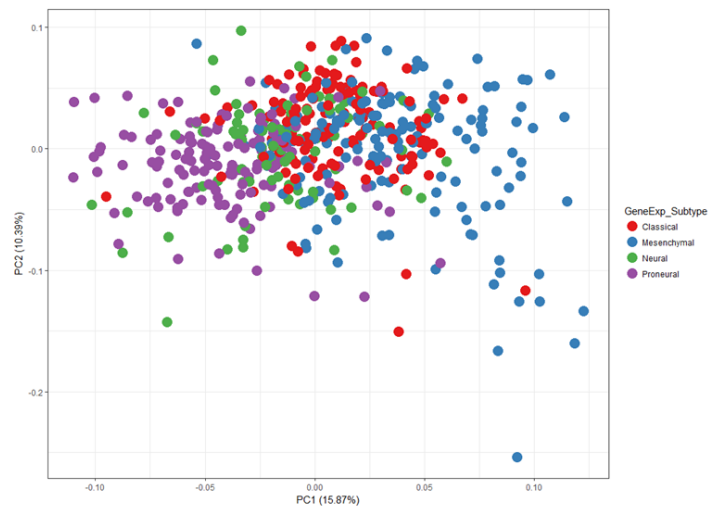
Comparisons	miRNAs
Classical versus Neural	miR-219, miR-338, miR-124a
Classical versus Mesenchymal	n/a
Classical versus Proneural	miR-219, miR-338, miR-124a, miR-10b, miR-204, miR-222 and miR-34a
Neural versus Proneural	miR-204
Mesenchymal versus Proneural	miR-21
Mesenchymal versus Neural	miR-124a

n/a: not available

A



B



C

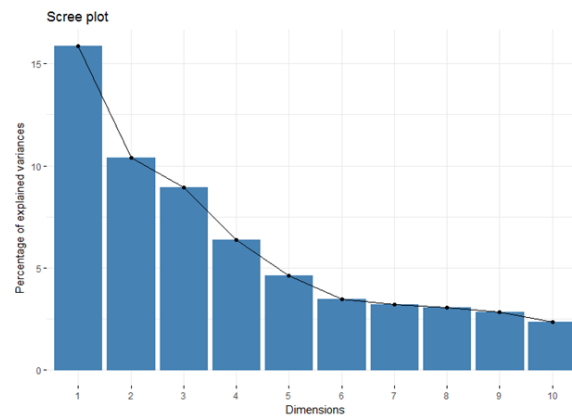


Figure 4.4. All miRNA profile of GBMs samples. **A**, boxplot showing the miRNA expression across all 523 GBM samples. **B**, PCA of all GBM samples with respect to miRNA expression. Red spots = Classical GBM; blue spots= mesenchymal GBM; green spots=Neural GBM; Purple spots=Proneural GBM. **C**, Scree plot show the variance explained by all miRNAs expression.

To further explore the subtype associated miRNAs, the differentially expressed miRNAs between subtypes were calculated by LIMMA package in R. Based on the selection criteria that log fold change larger than 1 and adjusted p value smaller than 0.01, a few featured miRNAs were identified. Then miRNAs in each subtype were tested against all other three subtypes. Comparing classical GBM with neural GBM, miR-219, miR-338, miR-124a were lower expressed in classical GBM without any higher expressed miRNAs. There was no significant differentially expressed miRNA identified between classical and mesenchymal GBMs, suggesting it was difficult to differentiate classical GBM from mesenchymal GBM on the miRNA expression pattern. MiR-219, miR-338, miR-124a along with miR-10b were under-expressed while miR-204, miR-222 and miR-34a were over-expressed in classical GBM, as compared with proneural GBM. There was only one miRNA (miR-204) identified that was differentially expressed between neural and proneural GBM. One up regulated miRNA (miR-21) and one down regulated miRNA (miR-124a) were observed in mesenchymal GBM compared with neural GBM. Consistent with the PCA plots, a total of ten differentially expressed miRNAs were identified between proneural and mesenchymal GBMs, where miR-222, miR-221, miR-34a, miR-223, miR-21, miR-204 were highly expressed in mesenchymal GBMs while miR-124a, miR-10b, miR-338 and miR-219 were up regulated in proneural GBMs. Heatmaps of these differentially expressed miRNAs revealed different subtypes could be clearly clustered based on these miRNAs expression (Figure 4.5) Table 4.2.2 highlighted the significantly differentially expressed miRNAs between subtypes of GBMs.

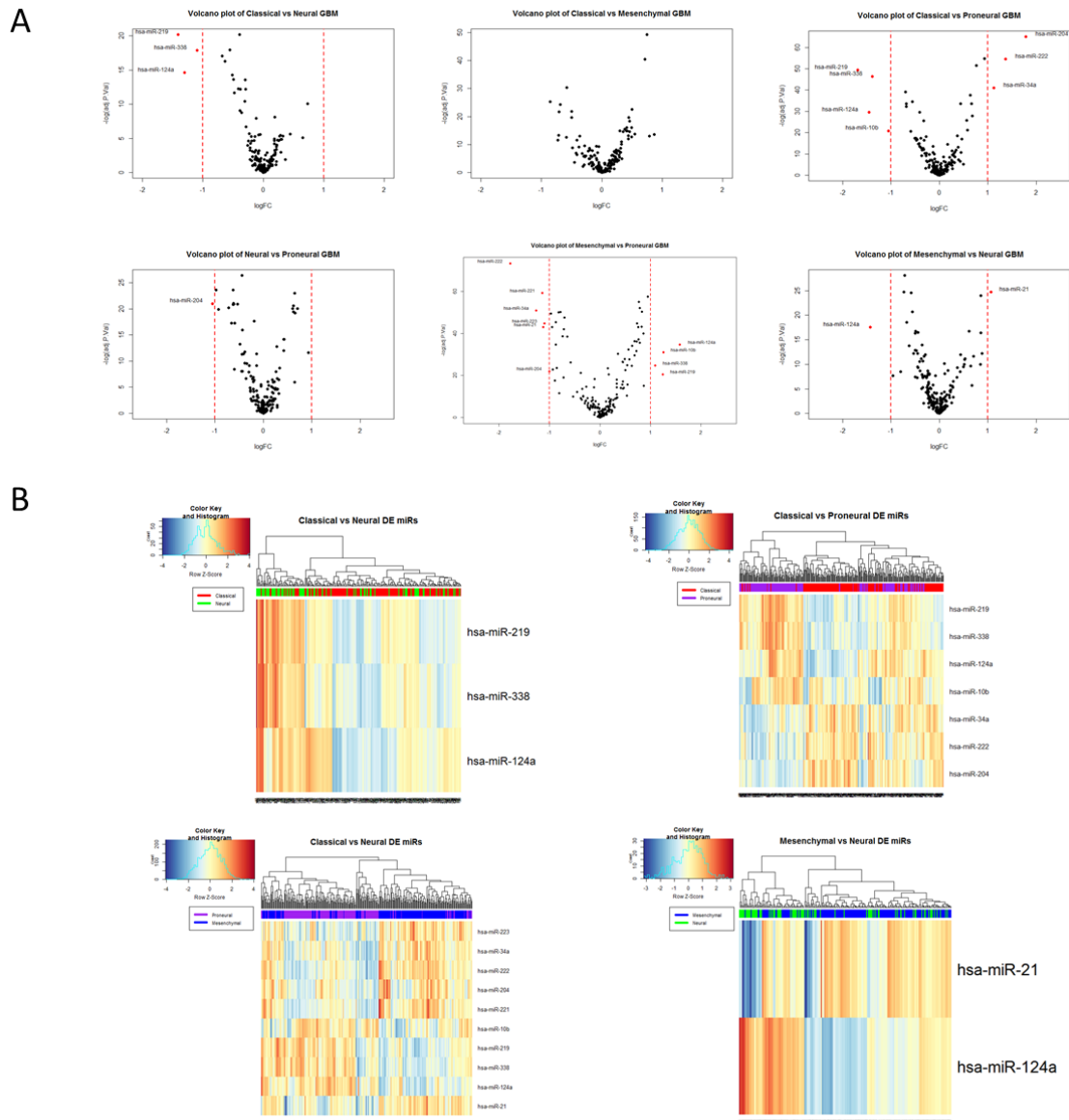


Figure 4.5. The differentially expressed (DE) miRNAs between subtypes. A, volcano plot highlighting the DE miRNAs between the given two subtypes. **B,** the heatmaps showing the clustering of the given subtypes based on the DE miRNAs.

To further test whether these 10 differentially expressed miRNAs clustered GBMs into subtypes, clustering, PCA and t-Distributed Stochastic Neighbor Embedding (t-SNE) were conducted. As expected, there was a remarkable difference between proneural and mesenchymal GBM and a great overlap between neural GBM and proneural GBM. It was also difficult to separate classical GBM from mesenchymal GBM, suggesting there was

no significant difference in these 10 miRNAs. We could use these 10 miRNAs expression to interpret 42.56% and 18.69% of GBMs variance in PC1 and PC2, respectively (Figure 4.6). Taken together, a total of 10 subtype-associated miRNAs were sorted out and proneural GBMs had a large difference miRNA expression pattern with mesenchymal GBMs.

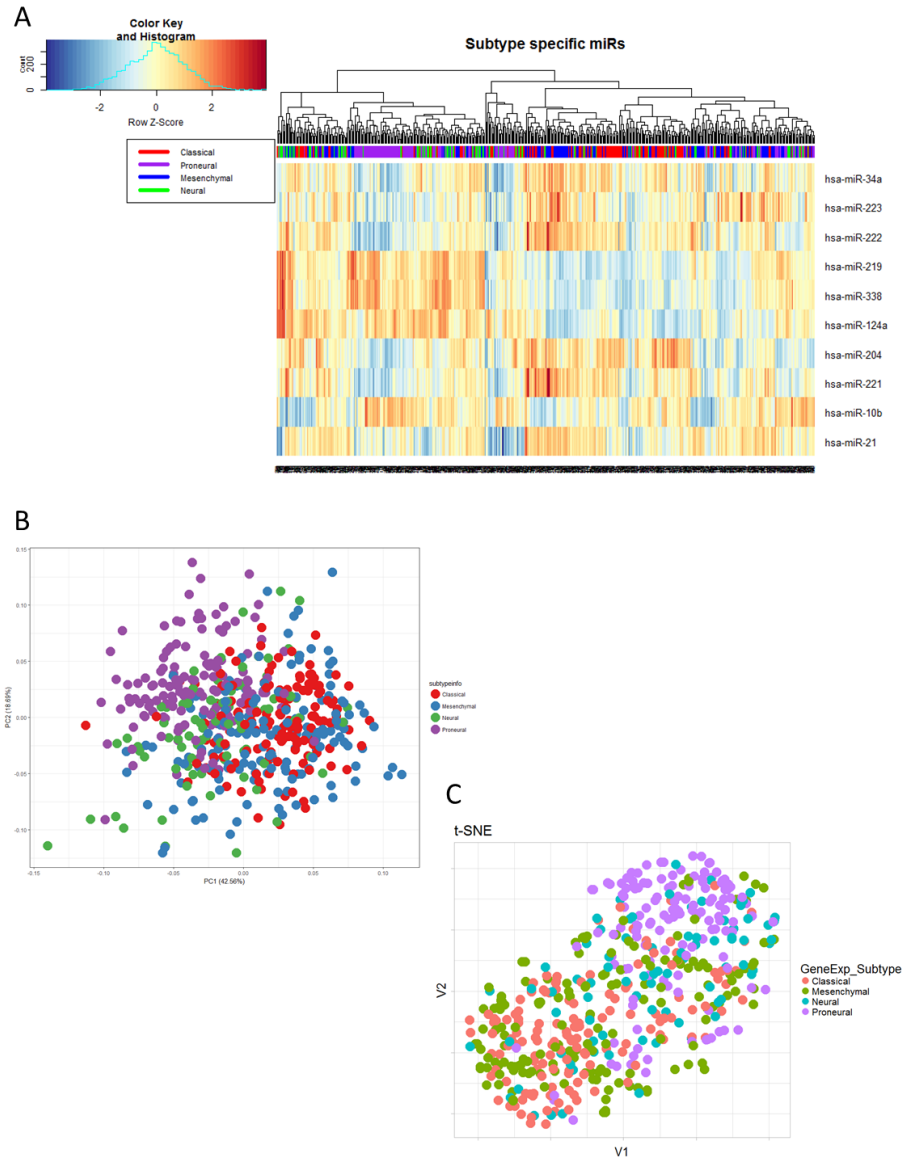


Figure 4.6. Clustering of all GBM samples by ten subtype associated miRNAs. A, heatmap showing the clustering of all GBM samples by ten subtype associated miRNAs. **B,** PCA of all GBM samples by ten subtype associated miRNAs. **C,** t-SNE showing the distance of all GBM samples by ten subtype associated miRNAs.

4.2.4. IDH1 specific miRNAs

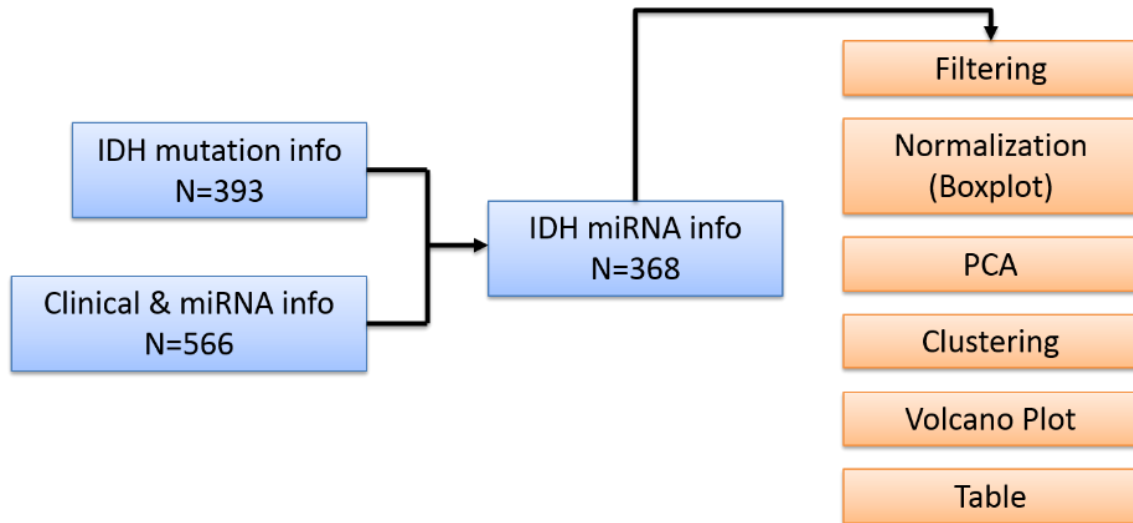


Figure 4.7. The workflow of identification of IDH1 mutation associated miRNAs.

We next performed bioinformatic analysis to determine IDH1 mutation specific miRNAs. A workflow of identification of IDH1 mutation associated miRNAs is demonstrated in Figure 4.7. A total of 572 GBMs miRNAs expression matrix were downloaded from the TCGA database. Of these, 566 data points contained patients clinical information annotations. A total of 393 cases were provided with IDH1 mutation information in TCGA database. After comparing 566 cases with clinical and miRNA information, 368 cases remained for analysis. Of these, 22 were IDH1 mutant cases, with 19 cases of IDH1 R132H, one case with IDH1 R132H mutation, one case of IDH1 R132G, one case of IDH1 Y319H and one case harbouring dual mutation (R132H and G300C). Based on all miRNAs expression of both IDH1 mutant and wild types, PCA identified that all cases with IDH1 mutation were clustered in lower PC1 direction and lower PC2 direction without significant distinction with IDH1 wild type cases. Subsequently, differentially expressed miRNAs were identified by LIMMA package and volcano plot revealing the

differentially expressed miRNAs comparing GBMs with IDH1 mutations versus IDH1 wild type. All differential expressed miRNAs were highlighted and the threshold was set as log fold change larger than one. Notably, all differential expressed miRNAs (miR-148a, miR-155, miR-204, miR-221, miR-222, miR-34a, miR-34b) were upregulated in IDH1 wild type samples and no IDH1 mutation specific miRNA was identified, indicating IDH1 mutation might lead to miRNAs down regulation in GBM. This might be caused by global genomic methylation, known as a glioma CpG island methylator phenotype (GCIMP), induced by mutant IDH1 and therefore closed miRNAs transcription. A Heatmap and table also highlights the IDH1 wild type specific miRNAs (Figure 4.8 and Table 4.2).

Table 4.3 IDH1 mutation associated miRNAs

miRNA names	Highly expression
miR-148a	IDH1 wild type GBM
miR-155	IDH1 wild type GBM
miR-204	IDH1 wild type GBM
miR-221	IDH1 wild type GBM
miR-222	IDH1 wild type GBM
miR-34a	IDH1 wild type GBM
miR-34b	IDH1 wild type GBM

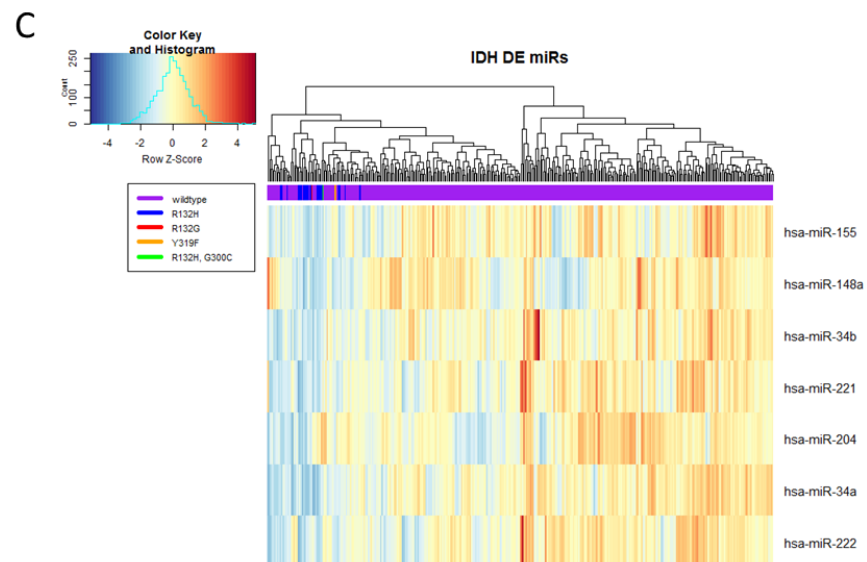
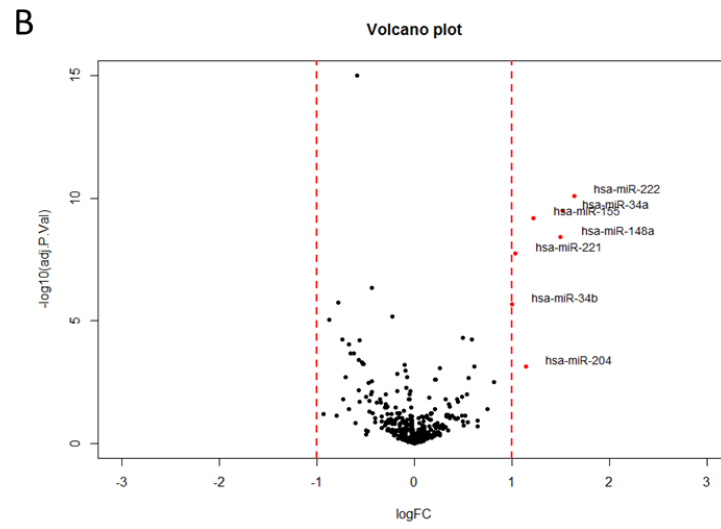
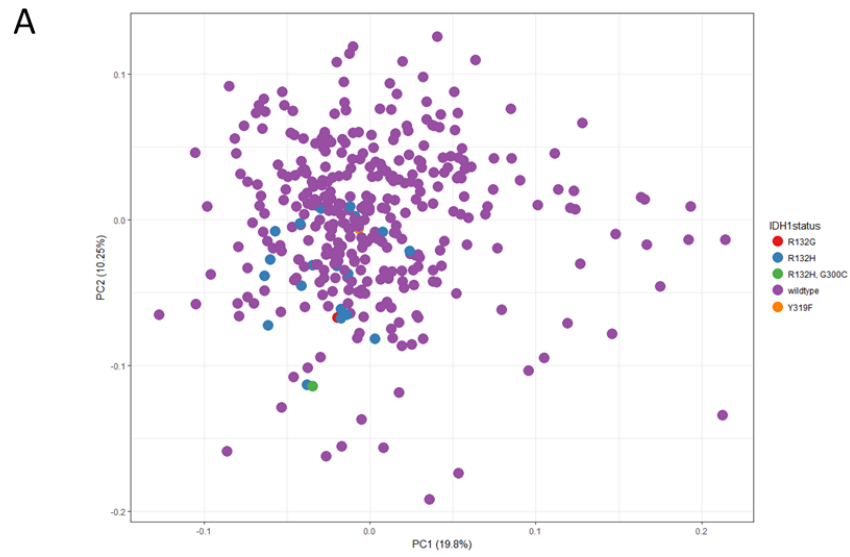


Figure 4.8. Identification of IDH1 mutation associated miRNAs. **A, PCA plot of miRNA expression across 368 samples.** PC1 and PC2 represents 19.8% and 10.25% of variation of miRNA expression. Red= IDH1 R132G mutation; Blue= IDH1 R132H mutation; Green= IDH1 R132H and G300C mutations; Purple= IDH1 wild type; Orange= IDH1 Y319F. **B, Volcano plot showing the differential expressed miRNAs.** Each dot represents a single miRNA and differential expressed miRNAs are highlighted in red (log fold change larger than 1 or smaller than -1). **C, Heatmap of clustering by differential expressed miRNAs.** IDH1 status of each sample is labelled on the top of heatmap. Red = IDH1 R132G mutation; Blue = IDH1 R132H mutation; Green = IDH1 R132H and G300C mutations; Purple = IDH1 wild type; Orange = IDH1 Y319F.

4.2.5. The correlation of IDH1 mutation associated miRNA expression with DNA methylation

Considering the totally different genomic location of these miRNAs and their relatively low expression level, we hypothesized that down regulation of these miRNAs might be induced by DNA methylation in glioma. To explore the DNA methylation status of these miRNAs, we compared the DNA methylation dataset between five IDH1 mutants and six IDH1 wild types. Of all seven differentially expressed miRNAs we identified, miR-155, miR-221/222 and miR-34a had their own target genes for transcription while miR-148a and miR-34b initiated transcription from their precursors directly. As miR-204 was located between exon 6 and exon 7 of TRPM3 in the genomic location, it shared the promoters' region with the transcripts of TRPM3 (Table 4.3).

Table 4.4. The genomic information of seven IDH1 mutation associated miRNAs

name	Host target Gene	type	Exon	Genome coordinates (hg19)	size (bases)	orientation
miR-148a	N/A	intergenic	1	chr7:25,949,903-25,950,013	111	minus strand
miR-155	MIR155HG	intragenic	4	chr21:25,561,909-25,575,168	13,260	plus strand
miR-204	TRPM3	intergenic	1	chr9:70,809,957-70,810,087	131	minus strand
miR-221/222	MIR221HG	intragenic	2	chrX:45,745,211-45,770,274	25,064	minus strand
miR-34a	MIR34AHG	intergenic	2	chr1:9,148,011-9,182,392	34,382	minus strand
miR-34b	N/A	intergenic	1	chr11:111,512,931-111,513,040	110	plus strand

N/A: not available

Notably, IDH1 mutants had higher methylation level in the promoter area in which these IDH1 mutant associated miRNAs located than those of IDH1 wild types, except for miR-221/222 and miR-34a (Figure 4.9). For miR-221/222 there was no methylation data available in the miR-221/222 promoter area from TCGA dataset. Although no promoter was identified in up-stream 3 kb of transcription start site (TSS) of miR-34a host gene, a high level of methylation was observed up-stream of IDH1 mutant GBMs, suggesting a high level of methylation in front of TSS might be associated with down regulation of miR-34a by preventing transcription (Figure 4.9).

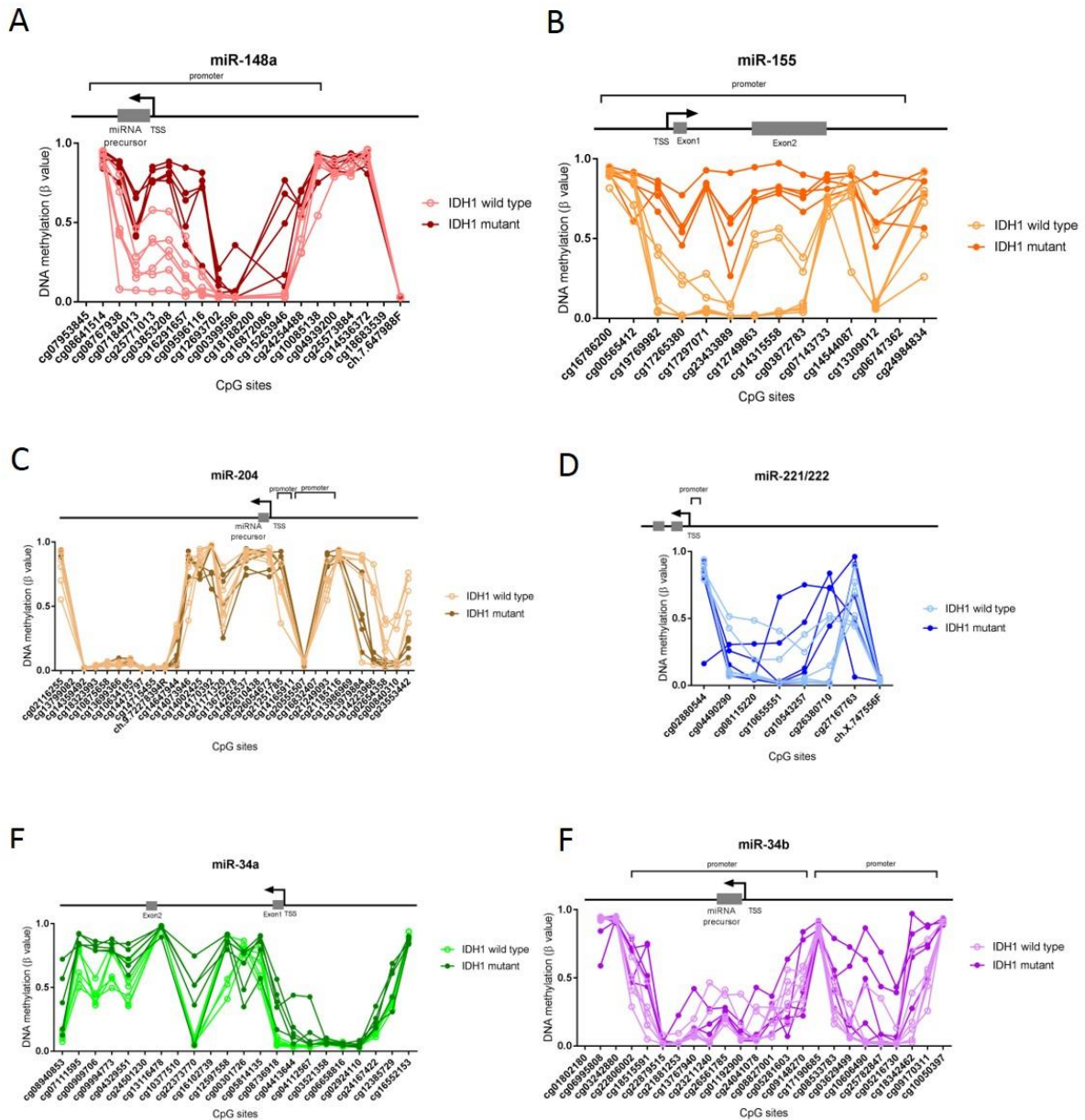


Figure 4.9. IDH1 specific miRNAs are hypermethylated in IDH1 mutant GBM patients. The DNA methylation status of miR-148a (A), miR-155 (B), miR-221/222 (C), miR-204 (D), miR-34a (E) or miR-34b (F) located CpG sites in IDH1 mutant and IDH1 wild type GBMs.

We further investigated the correlation of these miRNAs expression and DNA methylation to explore whether DNA methylation in GBM was linked with low miRNAs expression. Interestingly, there was a significantly inverse correlation between five of

these miRNAs expression (miR-148a, miR-155 and miR-34a) and their promoter DNA methylation level. At least one promoter DNA methylation was inversely correlated with miR-204 and miR-34b expression, suggesting that the expression of these miRNAs may be regulated by DNA methylation (Figure 4.10). For miR-221/222, no correlation could be analyzed as there was no DNA methylation available. Taken together, our results suggest that the global DNA methylation induced by IDH1 mutation likely led to the reduction of these differentially expressed miRNAs in GBM.

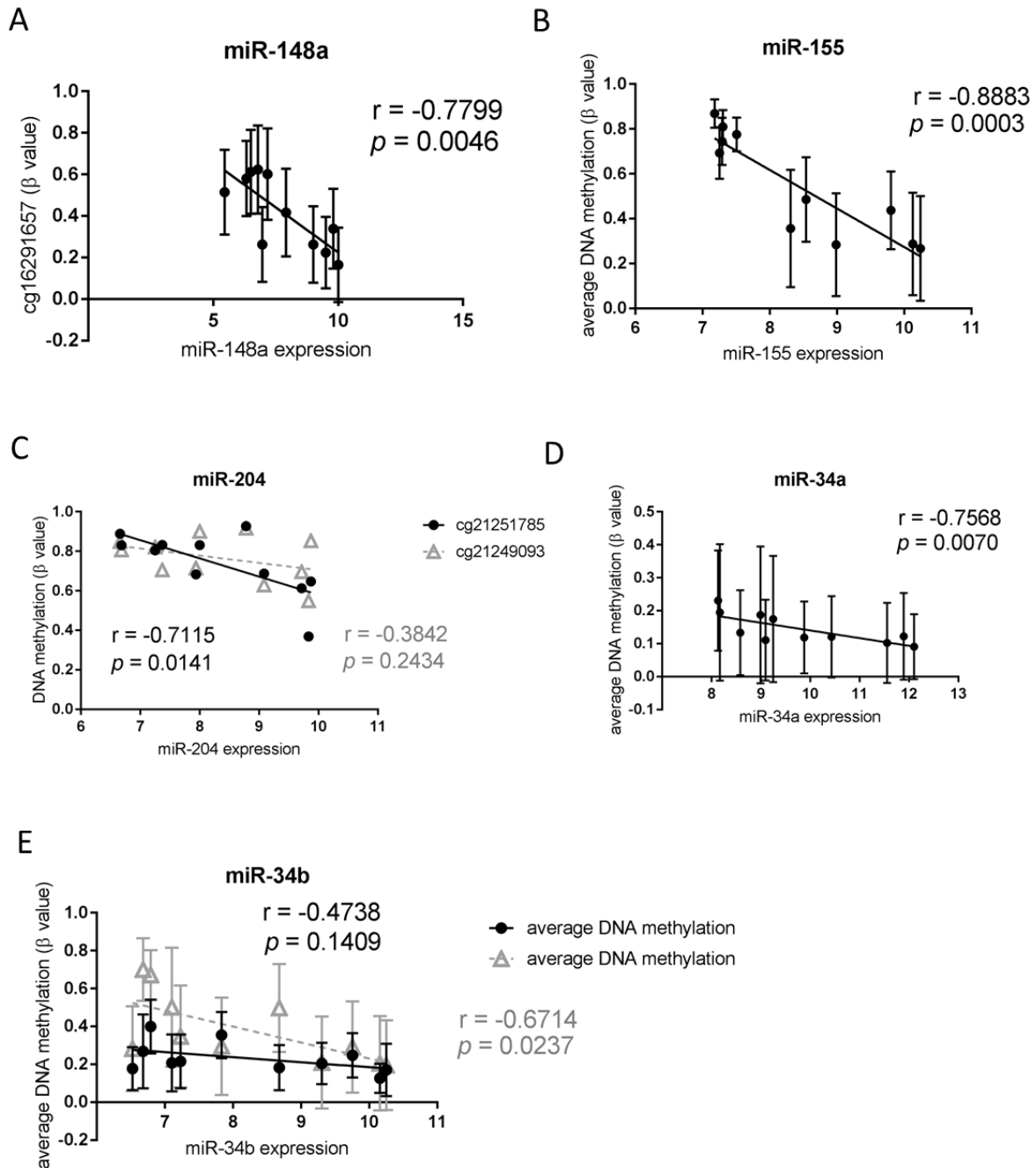


Figure 4.10. The correlation of DNA methylation in the promoter regions of IDH1 mutation associated miRNAs (A, miR-148a; B, miR-155; C, miR-204; D, miR-34a; E, miR-34b) and their expression. Whisker, 95% confident interval. r , correlation (Pearson correlation test).

4.2.6. The potential target genes and pathways of IDH1 mutation associated miRNAs

Next, we explored the pathways that these IDH1 mutation associated miRNAs targeted. To investigate which transcripts these miRNAs targets, we then predicted the target genes of these miRNAs via miRWalk 2.0. We choose these targets as they were predicted from all three algorithms to ascertain the validation. Based on three different algorithms (miRwalk, miRanda, Targetscan), a total of 5,905 targets of these miRNAs were generated after removal of overlapping (Appendix table 1). Pathway enrichment analysis was utilized to explore which pathway these targets were enriched in. Prion diseases, glycosphingolipid biosynthesis- lacto and neolacto series, mucin type O-glycan biosynthesis, microRNAs in cancer, glycosphingolipid biosynthesis - globo series, folate biosynthesis, ECM-receptor interaction, PI3K-Akt signalling pathway, hematopoietic cell lineage and axon guidance were identified out in all signalling pathways with significance (Figure 4.11). The targets genes significantly enriched biological processes including the cellular protein modification process, cellular nitrogen compound metabolic process, L-fucose catabolic process, axon guidance, post-translational protein modification, cell motility, neurotrophin TRK receptor signaling pathway, cell adhesion and the cellular protein metabolic process (Figure 4.11). To further investigate the differently expressed pathways between IDH1 mutation and IDH1 wild type GBMs, Gene Set Enrichment Analysis (GSEA) was then conducted. Apart from multiple pathways of cell metabolism including glycolysis that was induced by IDH1 mutation, ECM receptor interaction, axon guidance, PI3K-AKT signaling and adhesion were also identified for enrichment in IDH1 mutant or wild type GBMs (Table 4.4 and Figure 4.11). Taken together, these results indicated the potential therapeutic targets in IDH1 mutant GBMs by manipulation of miRNAs.

Table 4.5. Gene Set Enrichment Analysis (GSEA) of IDH1 wild type and mutant GBM

GS follow link to MSigDB	SIZE	ES	NES	NOM p-val	FDR q-val
KEGG_STARCH_AND_SUCROSE_METABOLISM	31	-0.66	-2.16	0	0.017
GO_PROTEIN_TRIMERIZATION	29	-0.72	-2.02	0	0.141
GO_CARBOHYDRATE_BIOSYNTHETIC_PROCESS	102	-0.56	-1.93	0	0.265
GO_ORGANONITROGEN_COMPOUND_CATABOLIC_PROCESS	273	-0.54	-1.88	0	0.328
GO_GLUCAN_BIOSYNTHETIC_PROCESS	22	-0.67	-1.87	0.002	0.295
GO_REGULATION_OF_CELLULAR_AMINE_METABOLIC_PROCESS	80	-0.48	-1.86	0.004	0.268
KEGG_ARGININE_AND_PROLINE_METABOLISM	45	-0.61	-1.85	0.002	0.106
GO_PYRIMIDINE_NUCLEOSIDE_METABOLIC_PROCESS	36	-0.63	-1.83	0	0.197
GO_NUCLEOTIDE_PHOSPHORYLATION	48	-0.6	-1.83	0.002	0.21
GO_MONOSACCHARIDE_BIOSYNTHETIC_PROCESS	50	-0.59	-1.83	0.002	0.234
GO_PHOSPHATIDYLGLYCEROL_METABOLIC_PROCESS	20	-0.64	-1.83	0.002	0.267
GO_PYRIMIDINE_CONTAINING_COMPOUND_CATABOLIC_PROCESS	25	-0.64	-1.83	0	0.304
GO_LONG_CHAIN_FATTY_ACID_TRANSPORT	33	-0.66	-1.82	0	0.168
GO_GLUCOSE_6_PHOSPHATE_METABOLIC_PROCESS	19	-0.64	-1.82	0	0.172
GO_NIK_NF_KAPPA_B_SIGNALING	76	-0.53	-1.82	0.006	0.183
HALLMARK_HYPOXIA	187	-0.68	-1.81	0	0.037
GO_ANTIGEN_PROCESSING_AND_PRESENTATION_OF_PEPTIDE_ANTIGEN_VIA_MHC_CLASS_II	84	-0.58	-1.8	0.008	0.203
GO_CELLULAR_MODIFIED_AMINO_ACID_BIOSYNTHETIC_PROCESS	40	-0.56	-1.8	0.004	0.207
GO_BICARBONATE_TRANSPORT	32	-0.7	-1.79	0	0.201
GO_VASODILATION	24	-0.71	-1.79	0.004	0.206
GO_CARBOHYDRATE_DERIVATIVE_CATABOLIC_PROCESS	145	-0.55	-1.79	0	0.211
KEGG_GLYCOLYSIS_GLUONEOGENESIS	54	-0.51	-1.78	0.01	0.135
GO_RESPONSE_TO_COLD	37	-0.65	-1.78	0.004	0.194
KEGG_PEROXISOME	65	-0.57	-1.77	0.006	0.118
GO_CARBOHYDRATE_TRANSPORT	68	-0.51	-1.77	0.004	0.215
HALLMARK_GLYCOLYSIS	182	-0.58	-1.76	0.002	0.046
KEGG_LYSOSOME	108	-0.56	-1.75	0.017	0.125
KEGG_GLYCOSAMINOGLYCAN_DEGRADATION	21	-0.67	-1.73	0.006	0.121
HALLMARK_UV_RESPONSE_UP	152	-0.53	-1.72	0	0.056
HALLMARK_COMPLEMENT	183	-0.62	-1.71	0.002	0.033
HALLMARK_XENOBIOTIC_METABOLISM	182	-0.53	-1.71	0.004	0.039
HALLMARK_BILE_ACID_METABOLISM	99	-0.55	-1.71	0	0.046
KEGG_ALPHA_LINOLENIC_ACID_METABOLISM	15	-0.67	-1.7	0.012	0.143
HALLMARK_CHOLESTEROL_HOMEOSTASIS	60	-0.59	-1.69	0	0.03
HALLMARK_COAGULATION	133	-0.62	-1.69	0.004	0.033
KEGG_TRYPTOPHAN_METABOLISM	30	-0.63	-1.69	0.006	0.127
KEGG_ECM_RECEPTOR_INTERACTION	78	-0.7	-1.69	0	0.139
HALLMARK_APOPTOSIS	156	-0.6	-1.68	0	0.029
HALLMARK_MTORC1_SIGNALING	193	-0.54	-1.67	0.008	0.03
HALLMARK_ADIPOGENESIS	168	-0.49	-1.66	0.006	0.026
HALLMARK_FATTY_ACID_METABOLISM	141	-0.47	-1.66	0.015	0.028
KEGG_PENTOSE_PHOSPHATE_PATHWAY	24	-0.58	-1.66	0.014	0.138
KEGG_BETA_ALANINE_METABOLISM	21	-0.56	-1.66	0.01	0.148
KEGG_GALACTOSE_METABOLISM	24	-0.52	-1.65	0.025	0.14
HALLMARK_REACTIVE_OXYGEN_SPECIES_PATHWAY	44	-0.57	-1.64	0.032	0.03
HALLMARK_ANGIOGENESIS	36	-0.76	-1.63	0.002	0.028
HALLMARK_P53_PATHWAY	186	-0.53	-1.63	0.002	0.029
KEGG_FOCAL_ADHESION	182	-0.55	-1.63	0.01	0.156
HALLMARK_TNFA_SIGNALING_VIA_NFKB	189	-0.69	-1.62	0.009	0.031
KEGG_SNARE_INTERACTIONS_IN_VESICULAR_TRANSPORT	31	-0.57	-1.62	0.029	0.162
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	191	-0.73	-1.6	0.011	0.036
KEGG_AMINO_SUGAR_AND_NUCLEOTIDE_SUGAR_METABOLISM	39	-0.59	-1.6	0.022	0.18
HALLMARK_MYOGENESIS	196	-0.5	-1.59	0.005	0.036
KEGG_PYRUVATE_METABOLISM	34	-0.49	-1.59	0.024	0.179
HALLMARK_PI3K_AKT_MTOR_SIGNALING	98	-0.44	-1.58	0.012	0.038
KEGG_ABC_TRANSPORTERS	36	-0.52	-1.58	0.019	0.176
KEGG_LINOLEIC_ACID_METABOLISM	24	-0.59	-1.57	0.028	0.184
KEGG_GLYCINE_SERINE_AND_THREONINE_METABOLISM	27	-0.65	-1.55	0.026	0.192
KEGG_HISTIDINE_METABOLISM	23	-0.54	-1.55	0.023	0.199
HALLMARK_INFLAMMATORY_RESPONSE	188	-0.62	-1.53	0.028	0.058

HALLMARK_E2F_TARGETS	166	0.17	0.39	0.973	0.994
HALLMARK_G2M_CHECKPOINT	184	0.29	0.68	0.76	1
KEGG_CARDIAC_MUSCLE_CONTRACTION	60	0.26	0.86	0.583	0.862
KEGG_CHRONIC_MYELOID_LEUKEMIA	70	0.27	0.86	0.625	0.906
KEGG_NEUROACTIVE_LIGAND_RECEPTOR_INTERACTION	227	0.24	0.87	0.675	0.956
KEGG_COLORECTAL_CANCER	61	0.29	0.9	0.591	0.939
KEGG_LONG_TERM_POTENTIATION	65	0.27	0.91	0.568	0.972
HALLMARK_PANCREAS_BETA_CELLS	40	0.36	0.91	0.585	1
KEGG_PROGESTERONE_MEDIATED_OOCYTE_MATURATION	73	0.28	0.93	0.546	0.994
KEGG_AXON_GUIDANCE	110	0.28	0.94	0.554	1
KEGG_MATURITY_ONSET_DIABETES_OF_THE_YOUNG	22	0.35	0.99	0.489	0.972
KEGG_NOTCH_SIGNALING_PATHWAY	36	0.34	1.01	0.478	0.991
KEGG_MELANOGENESIS	88	0.31	1.07	0.36	0.91
KEGG_TGF_BETA_SIGNALING_PATHWAY	78	0.34	1.12	0.307	0.862
KEGG_THYROID_CANCER	29	0.39	1.13	0.33	0.908
KEGG_GLYCOSPHINGOLIPID_BIOSYNTHESIS_LACTO_AND_NEOLACTO_SERIES	24	0.39	1.14	0.289	0.991
KEGG_BASAL_CELL_CARCINOMA	46	0.38	1.15	0.277	1
KEGG_BASAL_TRANSCRIPTION_FACTORS	30	0.39	1.16	0.311	1
KEGG_WNT_SIGNALING_PATHWAY	132	0.34	1.18	0.228	1
KEGG_ACUTE_MYELOID_LEUKEMIA	55	0.4	1.24	0.218	1
HALLMARK_WNT_BETA_CATENIN_SIGNALING	38	0.43	1.29	0.154	0.563
KEGG_RIBOSOME	73	0.49	1.39	0.156	0.864
KEGG_TASTE_TRANSDUCTION	32	0.5	1.47	0.062	0.831
GO_NEGATIVE_REGULATION_OF_EPITHELIAL_TO_MESENCHYMAL_TRANSITION	16	0.6	1.47	0.072	1
GO_CEREBELLAR_CORTEX_FORMATION	19	0.63	1.47	0.074	1
GO_MUSCLE_HYPERTROPHY	25	0.53	1.5	0.042	1
GO_RETINAL_GANGLION_CELL_AXON_GUIDANCE	16	0.62	1.5	0.045	1
KEGG_HEDGEHOG_SIGNALING_PATHWAY	45	0.44	1.51	0.044	1
GO_CEREBELLAR_PURKINJE_CELL_LAYER_DEVELOPMENT	22	0.59	1.51	0.062	1
GO_ADRENERGIC_RECEPTOR_SIGNALING_PATHWAY	17	0.56	1.51	0.075	1
GO_POSITIVE_REGULATION_OF_HEART_GROWTH	23	0.57	1.52	0.013	1
GO_CHROMATIN_DISASSEMBLY	15	0.62	1.52	0.058	1
GO_POSITIVE_REGULATION_OF_CARDIAC_MUSCLE_CELL_PROLIFERATION	17	0.59	1.53	0.02	1
GO_SEX_DETERMINATION	20	0.57	1.55	0.029	1
GO_POSITIVE_REGULATION_OF_MULTICELLULAR_ORGANISM_GROWTH	26	0.47	1.55	0.043	1
GO_ANTERIOR_POSTERIOR_AXIS_SPECIFICATION	37	0.53	1.56	0.017	1
GO_CARDIAC_MUSCLE_TISSUE_MORPHOGENESIS	47	0.53	1.56	0.018	1
GO_POSITIVE_REGULATION_OF_EMBRYONIC_DEVELOPMENT	25	0.5	1.56	0.026	1
GO_CHROMATIN_SILENCING_AT_RDNA	24	0.51	1.56	0.085	1
GO_CHROMATIN_SILENCING	58	0.47	1.59	0.072	1
GO_PITUITARY_GLAND_DEVELOPMENT	37	0.53	1.61	0.006	1
GO_CELL_DIFFERENTIATION_IN_HINDBRAIN	18	0.71	1.63	0.026	1
GO_NEGATIVE_REGULATION_OF_GENE_EXPRESSION_EPIGENETIC	72	0.48	1.64	0.065	1
GO_GASTRULATION_WITH_MOUTH_FORMING_SECOND	26	0.57	1.71	0.008	1

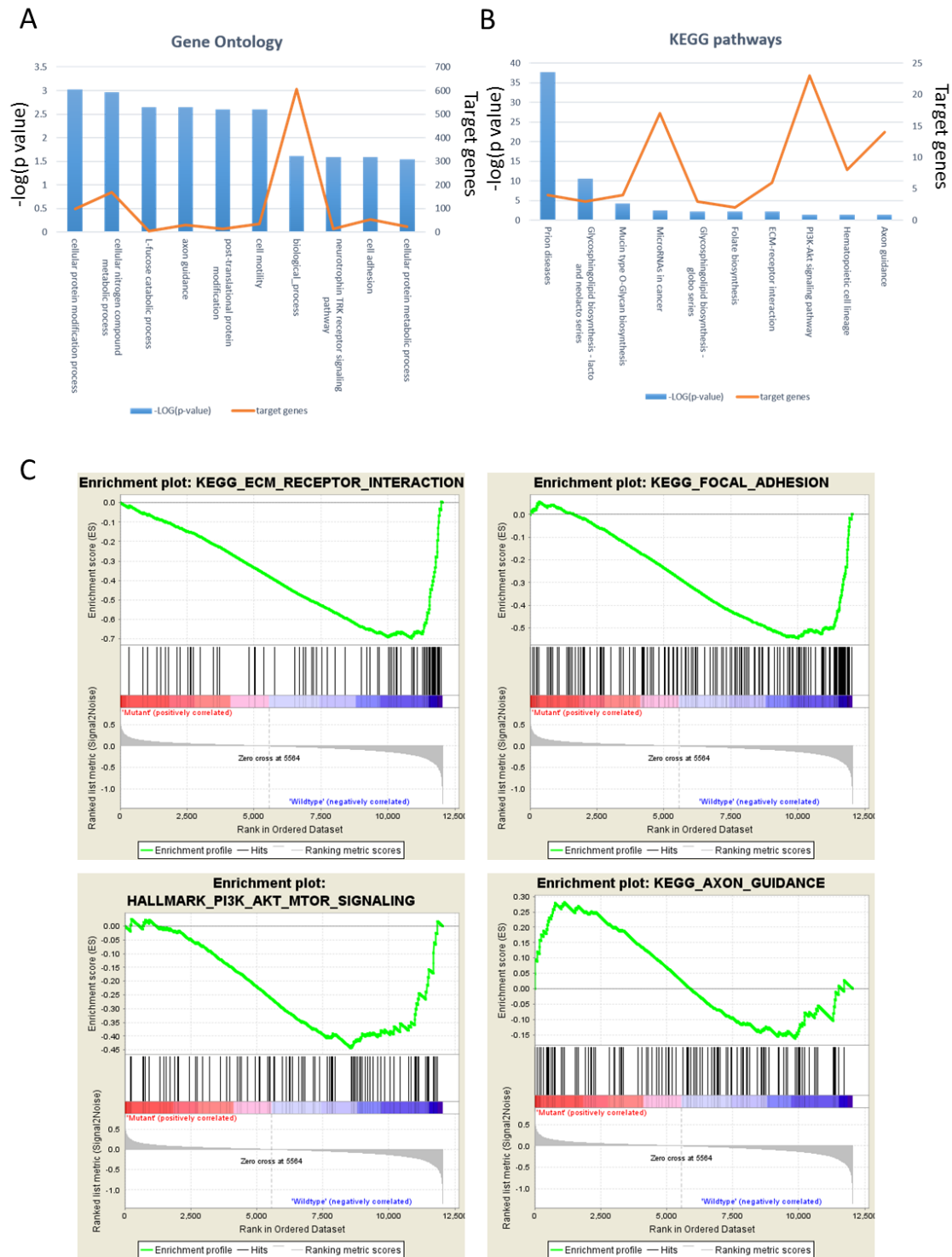


Figure 4.11. The significant pathways of putative target genes of IDH1 mutation specific miRNAs. **A**, enriched Gene Ontology of target genes. **B**, enriched KEGG pathways of target genes. Blue bar= $-\log(p \text{ value})$; orange line= the number of target genes. **C**, Gene Set Enrichment Analysis (GSEA) comparing the pathways between IDH1 mutant and wild type GBMs.

To further explore the protein-protein interaction (PPI) of these target genes, we utilized the STRING database to predict the protein interaction networks. The STRING score was set to >0.7 for acquirement of the interaction with high confidence. The target genes of each miRNAs were mapped into STRING separately. These results were demonstrated in Figure 4.12. For miR-148a, a total of 1,336 predicted target genes were mapped into STRING and 691 PPIs were identified with the p value of less than 1×10^{-16} . These proteins were enriched in neurological development and cGMP-PKG signalling pathway, Ras signalling pathway, glioma pathway and PI3K-AKT signalling pathway (Figure 4.12). In the PPI of target genes of miR-155, 1624 interactions were identified from STRING ($p = 1.52 \times 10^{-8}$). The pathways that these target genes involved were metabolic regulation, ion binding and TGF-beta signalling pathway. A total of 5452 interactions were mapped in miR-204 target genes by STRING ($p = 3.22 \times 10^{-14}$). These target genes participated in positive regulation of biological process, cellular process, cell communication, protein or ion binding and MAPK signalling pathway. Similar to miR-204, the pathways involved in the target genes of miR-221/222 were positive regulation of biological process, cellular process and ion binding with 1540 interactions ($p = 6.23 \times 10^{-4}$). Notably, MiR-221/222 target genes were also involved in FoxO signalling pathway. Development process and ion binding were involved in target genes of miR-34a and miR-34b, with 3226 ($p < 1 \times 10^{-16}$) and 1461 interactions ($p = 5.37 \times 10^{-6}$), respectively. Adrenergic signalling and FoxO signalling pathway were the most significant pathways in which the target genes of miR-34a and miR-34b involved in, respectively.

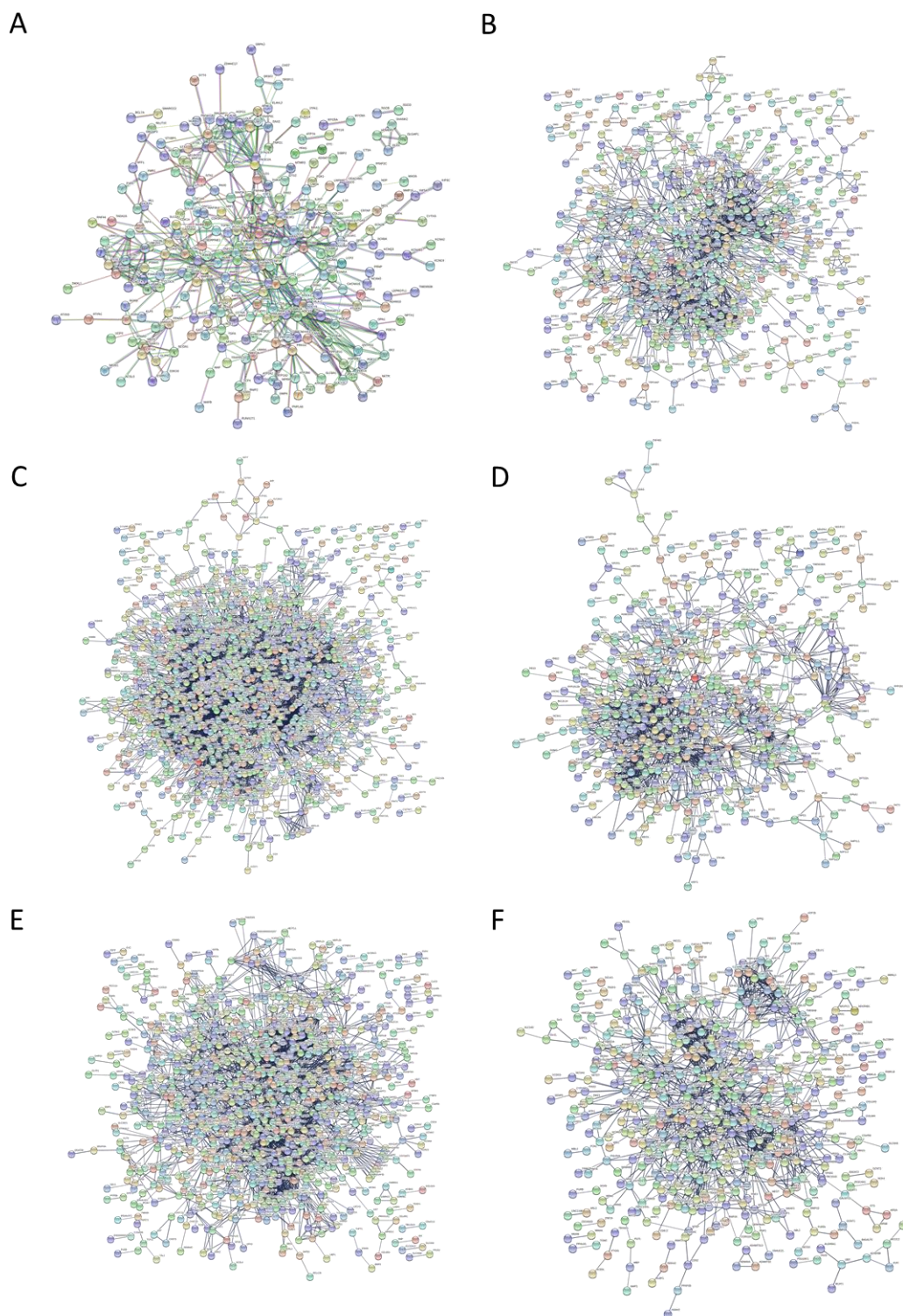


Figure 4.12. Interaction networks of targets of miRNAs. A, miR-148a; B, miR-155; C, miR-204; D, miR-221/222; E, miR-34a; F, miR-34b.

To further investigate the most significant target genes of these IDH1 associated miRNAs and PPIs, we employed the Streiner algorithm with minor modification. The top 95 significant target genes were listed in Table 4.5. CDK6, CREB1, FLT1, MEF2A and NRAS were the most critical target genes while CDK6 with CDKN1A, CREB1 with TP53 and CREB1 with FOS were the most critical PPIs in these targets of IDH1 associated miRNAs, indicating these target genes would be the driven force to the difference between IDH1 mutation and IDH1 wild type GBMs and the druggable therapeutic targets.

Table 4.6. Top 95 significant target genes

CDK6	NOTCH1	SP1	PRDM10	MAPK1
CREB1	OAS2	YWHAЕ	CDC42	SYK
FLT1	OAS3	YWHAZ	DNM2	IRS1
MEF2A	MAPK9	HDAC4	EGR1	FGFR1
NRAS	RAD51	EGFR	EPHB2	KIT
CDK14	RET	HDAC2	MX1	PDGFRA
MAP2K1	RNASEL	HIF1A	PDGFRB	RRAS
PTEN	TBP	NOS3	MAPK11	SRC
RPS6KB1	YWHAG	PTPN11	MAPK13	FURIN
SNCA	ACTA1	RAC1	RAF1	STAT3
SRF	FOXO3	YES1	RPS6KA2	HACE1
STK11	FOS	HDAC9	UMPS	GLI3
TEK	IGF1R	AR	CSNK2A1	GSK3B
TP53	JAK2	BCL2	ESR1	RAP1B
YWHAB	KRAS	ATF2	KDR	YWHAQ
AKT2	NT5E	DECR1	RALA	
CDK1	PAK2	SMAD4	RND2	
CDKN1A	PIK3CA	MEF2C	AKT3	
ERBB3	PPP2CA	PRKCA	MYOD1	
INSR	RPS6KA3	SHC1	PRKN	

Lastly, we investigated whether these IDH1 associated miRNAs were linked with patients' survival. Univariable Cox hazard proportion regression revealed higher expression of all these IDH1 associated miRNAs linked with unfavourable outcomes in all GBM patients with statistical significance regardless of IDH1 mutation status (Table

4.6). Kaplan-Meier survival curves of 6 IDH1 associated miRNAs (miR-148a, miR-204, miR-221, miR-222, miR-34a and miR-34b) also demonstrated patients with low expression of miRNAs had better survival advantages, as compared with patients with high expression of miRNAs (Figure 4.13).

Table 4.7. Cox hazard proportion model of IDH1 mutation associated miRNAs

	univariate OS of IDH1 associated miRNAs		
	HR	95% CI	p value
miR-148a	1.202	1.105-1.306	< 0.001
miR-155	1.153	1.026-1.294	0.016
miR-204	1.107	1.034-1.184	0.003
miR-221	1.300	1.169-1.446	< 0.001
miR-222	1.260	1.166-1.362	< 0.001
miR-34a	1.188	1.091-1.294	< 0.001
miR-34b	1.153	1.040-1.279	0.007

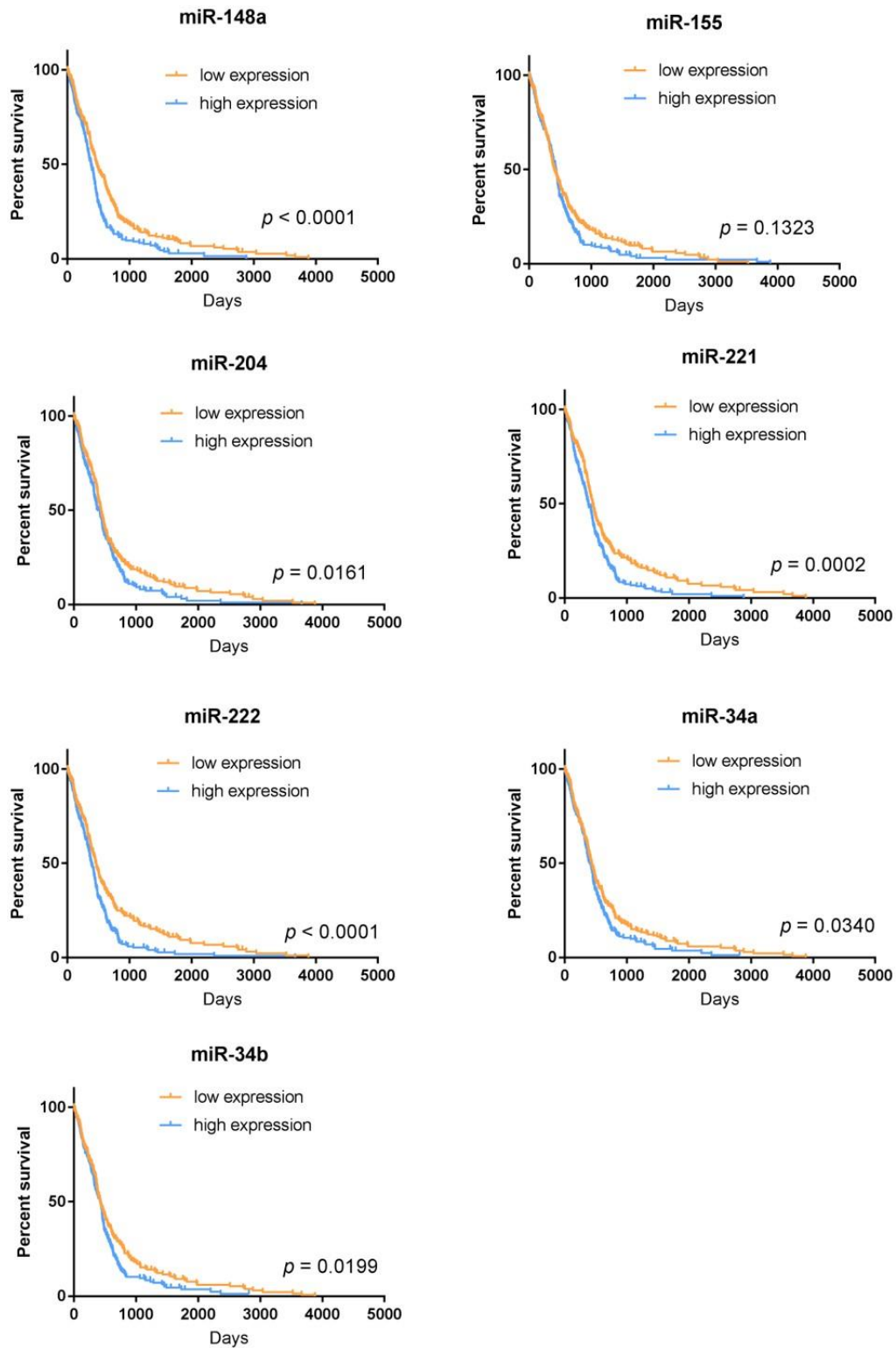


Figure 4.13. Kaplan-Meier Curves of IDH1 mutation specific miRNAs in GBMs. Significance was tested by Log-rank test.

4.3. Discussion

Classification systems of brain tumours based on their histology are troubled by high inter-observer variation and are not perfectly prognostic or predictive. Since 2009, molecular markers have sparked researchers' attention where IDH1 mutation was identified as a key marker in glioma (H. Yan et al., 2009). Along with other molecular markers such as 1p/19q codeletion, TERT mutation and MGMT promoter methylation uncovered to be strongly linked with glioma prognosis and therapeutic response, these molecular markers have been incorporated into the WHO classification of central nervous system tumours from 2016 (Paul et al., 2017; van den Bent et al., 2017). Other comprehensive bioinformatic analyses of glioma datasets in TCGA also identified high risk gene signatures that have prognostic capacity and potential therapeutic targets in low grade glioma and glioblastoma (Cancer Genome Atlas Research et al., 2015). Besides the genomic alteration in glioma, epigenetic variance has also played a critical role in glioma. A DNA methylation based classification based on 2801 mixed samples has shown a more precise diagnostic capability in brain tumours than the standard diagnosis, suggesting it could be a complimentary diagnostic method (Capper et al., 2018). As an important part of the epigenome, miRNA expression in glioma also demonstrate a strong correlation with patient survival and classification. A panel of differentially expressed miRNAs has been uncovered between IDH1 mutant low grade glioma and IDH1 wild type low grade glioma (Cancer Genome Atlas Research et al., 2015). More importantly, the targets of these differentially expressed miRNAs in low grade glioma also have distinct expression between IDH1 mutant and wild type low grade glioma, indicating that the miRNAs regulation may be involved in gliomagenesis.

Our results demonstrated that distinct miRNA expression patterns exist between GBM and normal controls, suggesting differentially expressed miRNAs could be utilized as

potential biomarkers in glioma. We also delineated subtype-associated miRNAs where 10 miRNAs were differentially expressed between subtypes. In addition, distinct miRNA expression patterns between IDH1 mutant and IDH1 wild type GBM was identified and their target pathways were accordingly identified.

A total of 14 miRNAs were down regulated in GBM, compared with normal controls, suggesting that these glioma specific miRNAs play a critical role in tumourigenesis and glioma development. A few studies revealed miR-129 acts as a tumour suppresser gene in glioma and upregulation of miR-129 is associated with the FoxP1, IGF2BP3 and MAPK1 that are key regulators of cell cycle progression and cell proliferation (D. Z. Fang et al., 2018; Haapa-Paananen et al., 2013; Kouhkan et al., 2016; Tian, Zhang, Sun, & Li, 2015; Y. Yang, Huang, Zhang, & Shen, 2015). miR-139 has demonstrated its anti-tumour function in glioma by inhibition of the cell cycle, migration and invasion (Dai, Wang, Li, & Cao, 2015; H. Wang et al., 2017; S. Yue et al., 2015). In vitro and in vivo studies showed miR-491 reduces cell proliferation, invasion and stem cell propagation (X. Li et al., 2015; Qi et al., 2016; W. Yan et al., 2011). miR-218 could inhibit stem cell renewal, cell proliferation, cancer cell metabolism, cell migration and invasion by targeting multiple key genes in pathways (Z. Feng et al., 2017; X. Gao & Jin, 2014; J. J. Gu, Gao, & Zhang, 2015, 2016; Jun, Zhong, & Ming, 2015; Mathew et al., 2014; Tu et al., 2013; Z. Wu et al., 2016; J. Xu, Xu, & Zhu, 2015; Y. Zhang et al., 2015). Increasing miR-137 could attenuate cell proliferation, migration and invasion by regulating of PI3K-Akt pathway (Chakrabarti & Ray, 2015; Liang et al., 2016; J. Sun, Zheng, Gu, & Guo, 2015; L. Wang et al., 2016). Moreover, miR-137 is significantly down regulated in glioma patients' serum, as compared with healthy controls, suggesting circulating miR-137 would be a diagnostic marker for glioma (H. Y. Li, Li, Li, Shi, & Chen, 2016). miR-128a is involved in chemoresistance and MGMT promoter methylation status (Lakomy et al., 2011; Slaby et al., 2010). By targeting multiple genes such as Gli1, TUSC3, MMP16 and SMAD7, miR-132 induces cell apoptosis, cell progression and invasion in glioma cell lines (Z. X. Cheng, Yin, & Wang, 2017; F. Geng et al., 2016; H. Wang, X. T. Li, et al.,

2015; Y. Z. Wang et al., 2018; Z. H. Wang et al., 2015). Although miR-132 is down regulated in GBM, the high expression of miR-132 is associated with worse outcomes for patients after treatment with the Stupp protocol (Parker et al., 2013). As miR-138 targets CTLA-4 and PD-1, it showed potential antitumour efficacy in glioma (J. Wei et al., 2016). Endogenous delivery of miR-124 into glioma cells significantly reduced cell proliferation and migration and increased chemo-sensitivity (Sharif, Ghahremani, & Soleimani, 2018). A further study found that expression of exosomal miR-124 is elevated in high grade glioma, as compared with low grade glioma (Santangelo et al., 2018). A few studies have confirmed that miR-7 is strongly associated with cell apoptosis and autophagy in glioma cells by inhibiting XIAP and PKC α (Chakrabarti & Ray, 2016; X. Zhang et al., 2017) and its reduced expression in serum increases the potential of blood screening for glioma (Dong et al., 2014). Through targeting MET, miR-410 reduces proliferation and invasion in glioma (L. Chen et al., 2012). miR-338 has also demonstrated anti-tumour efficacy of suppressing proliferation, migration, invasion and promoting apoptosis in glioma (Howe et al., 2017; Lei et al., 2017; D. Z. Liu, Zhao, Zou, & Ma, 2017). It is reported that miR-219 inhibits cell proliferation, migration and invasion of brain tumours by targeting CD164 and EGFR, indicating miR-219 could sensitize the efficacy of EGFR inhibitors for glioma treatment (Jiang, Yin, Jing, & Zhang, 2015; J. A. Shi, Lu, Huang, & Tan, 2014). In contrast, inhibition of miR-27a significantly reduces cell proliferation and invasion in vitro and in vivo (S. Y. Feng et al., 2012; W. Xu et al., 2013).

As an “oncomiRNA”, miR-21 has been found to be elevated in a variety of cancers and associated with unfavourable outcomes. In glioma, miR-21 is highly expressed in numerous studies. In addition, the expression of miR-21 is highly increased in blood and CSF from patients with glioma, suggesting circulating miR-21 as a potential diagnostic biomarker in glioma – this will be explored in the next chapter (Santangelo et al., 2018; Ye et al., 2017; Q. Zhou et al., 2018). miR-21 promote cell proliferation, invasion and migration by preventing PTEN, a key tumour suppressor in glioma, from transcription and thereby increase glioma cell progression by removing the regulation of the PI3K-Akt

pathway (Chai, Song, Han, Li, & Li, 2018; Guo et al., 2018a). Likewise, miR-210 is also up regulated in glioma patients' blood and demonstrates its potent of diagnosis in glioma (Lai et al., 2015). Our previous study also reveals the expression of miR-210 is a response indicator of bevacizumab in high grade glioma (Siegal et al., 2016).

We observed a large overlap of differentially expressed miRNAs between subtypes associated miRNAs. In fact, most subtypes associated miRNAs are derived from the differentially expressed miRNAs between proneural and mesenchymal GBM, which are very similar in profile to IDH1 mutation associated miRNAs. Interestingly, all differentially expressed miRNAs were associated with IDH1 mutation, indicating there is a distinct epigenome between IDH1 mutant and wild type GBM. A previous study identified four miRNAs could be utilized for predicting IDH1 mutation status (W. Cheng, Ren, Zhang, Han, & Wu, 2017). Because of the distinct analytic method used, there is no overlap of differentially expressed miRNAs between previous one and this one. In this study, we also further explored the IDH1 mutation associated miRNAs are probably mediated by DNA methylation. Several studies have revealed miR-148a induces cell proliferation and migration in glioma (Q. Cai, Zhu, & Gong, 2018; Cui et al., 2017; H. Wang, J. Q. Pan, et al., 2015). Cui et al found exogenous miR-148a targeted GADD45A to promotes glioma migration and invasion of IDH1 mutant glioma. In addition, miR-148a is increased in exosomes from glioma patients and binds directly to the tumour suppressor CADM1 (Q. Cai et al., 2018). Hence, it is likely that miR-148a increases IDH1 mutant glioma progression. Yang et al found the presence of miR-155 promotes glioma proliferation by targeting CDX1. Moreover, knockdown of miR-155 sensitize chemotherapy of temozolomide and overexpression of miR-155 is linked with worse outcome in glioma (J. Sun et al., 2014). Kim et al also found miR-148a is highly expressed in IDH1 mutant GBM and it regulates EGFR and apoptosis by targeting MIG6 and BIM (J. Kim et al., 2014). On the other hand, Gu et al uncovered suppression of miR-155 inhibit glioma proliferation and invasion and Wu et al also found inhibition of MIR155HG, the precursor miRNA of miR-155 attenuates glioma invasion, migration and

glioma growth in vivo (J. Gu et al., 2017; X. Wu et al., 2017). Ying et al found reduction of miR-204 expression enhanced glioma migration and the stem cell-like phenotype and Xia et al also found that the loss of miR-204 expression promoted glioma growth and invasion (Xia, Liu, Zhang, & Liu, 2015; Ying et al., 2013). However, Mao et al found miR-204 inhibits glioma migration and invasion by targeting ezrin (J. Mao, Zhang, Zhong, Zhang, & Lv, 2014). Song et al also showed ectopic expression of miR-204 reduces glioma proliferation, migration and invasion by targeting ATF2 (Song, Fajol, Tu, Ren, & Shi, 2016). These results suggest the complicated biological function of miR-204 in glioma. miR-221/222 is widely involved in the development of glioma and their expression are significant diagnostic and prognostic markers in glioma. Yang et al found exosomal miR-221 targets DNMT3 to promote glioma progression and induce temozolomide resistance, suggesting exosomes could promote glioma resistance by transferring miRNAs into recipient cells (J. K. Yang et al., 2017). Cai et al also found reduction of miRNA-221 decrease cell proliferation and invasion (G. Cai, Qiao, & Chen, 2015). Quintavalle et al suggests the expression of miR-221 has lower abundance in IDH1 mutant glioma than that in IDH1 wild type glioma (Quintavalle et al., 2013). In addition, higher expression of miRNA-221/222 is associated with glioma, as compared with normal controls and distinct expression patterns are also confirmed in glioma patients' plasma samples, suggesting miR-221/222 are potentially diagnostic markers (X. Li, Zheng, Chen, Diao, & Liu, 2016; Zhang et al., 2016). Two studies reveal suppression of miR-23a inhibits glioma cell invasion and growth (X. Hu, Chen, Cui, Li, & Huang, 2013; Lian et al., 2013). Additionally, serum miR-23a expression in glioma patients is remarkably higher than that in normal controls. In terms of miR-23b, its molecular function is still controversial in glioma. Chen et al found knockdown of miR-23b by lentivirus diminished cell invasion, migration and in vivo cell growth and Agrawal et al identified miR-23b is strongly associated with hypoxia in glioma (Chen et al., 2014). However, Geng et al found miR-23b enhances the sensitivity of temozolomide treatment in glioma cell lines and ectopic miR-23b promote cell cycle arrest and proliferation inhibition (J. Geng et al., 2012).

Our results indicate that these differently expressed miRNAs are potentially silenced by DNA methylation induced by mutant IDH1. An unsupervised k-means consensus clustering was performed in pan-glioma samples in a previous study (Ceccarelli et al., 2016). IDH1 mutation status is the main driving force of clustering glioma methylome and transcriptome. A high methylation level of tumour specific CpG sites was segregated in IDH1 mutant glioma, suggesting the correlation of IDH1 mutation and increased methylation in glioma. In addition, a distinct gene expression was showed to be significantly associated with IDH1 mutation status and DNA methylation. Loss of methylation in CpG sites promoted gene expression in glioma. Further analyses of DNA motif signatures and transcription factor binding sites identified hypomethylation in CpG sites is linked with the activation of cell cycle progression and progression of glioma. Moreover, patients with hypermethylation have worse survival outcomes, as compared with those with hypomethylation. Hence, these results indicated methylation induced by mutant IDH1 could be a contributing factor of the observed distinct gene expression within GBM and a driving force of the progression of glioma. From our analyses, all significant IDH1 associated miRNAs are highly expressed in IDH1 wild type GBM. In contrast, there is no miRNA identified as highly expressed in IDH1 mutant GBM. Considering the notion that the hypermethylation of CpG sites induced by IDH1 mutation prevent genes expression, we compiled DNA methylation analyses of these seven differentially expressed miRNAs. We observed there is hypermethylation in the CpG sites of the host genes of IDH1 mutation associated miRNAs, except for miR-221/222. The correlation of DNA methylation and these miRNAs expression also confirms mutant IDH1 reduces these miRNAs expression by inducing DNA methylation in the CpG sites. These results also are confirmed by other studies. Huang et al also confirmed MIR155HG, the precursor miRNA of miR-155 is down regulation in IDH1 mutant glioma by DNA methylation induced by mutant IDH1 (G. H. Huang et al., 2018). miR-23a is also methylated in GSC rather than in cell lines, indicating these IDH1 associated miRNAs are regulated by methylation (J. Geng et al., 2012). For miR-221/222, the transcription is

regulated by other factors such as SF2/ASF silencing regulation, therefore, we failed to establish the correlation of DNA methylation and their expression (H. Wu et al., 2010).

Our results of the differentially expressed miRNAs have a great overlap with those in low grade IDH1 mutant glioma (Cancer Genome Atlas Research et al., 2015), suggesting these miRNAs are the key factors in glioma progression. As a key regulator in tumorigenesis and cancer progression, miRNA mediate multiple signaling pathways by targeting 3' UTR of genes and then preventing their translation or degrading them directly. Our results found ECM receptor interaction, axon guidance, PI3K-AKT signaling and adhesion are regulated by these differentially expressed miRNAs, also confirmed by other studies (Bai et al., 2016; S. Liu et al., 2014). ECM receptor interaction plays an important role in glioma invasion (Goldbrunner, Bernstein, & Tonn, 1999). Glioma is prone to invade into the surrounding brain, making it difficult to cure surgically. Glioma cells penetrate into normal brain by secreting multiple proteases to degrade the extracellular matrix. Therefore, the invasion of glioma is largely reliant on the interaction of glioma cells and extracellular matrix. Over-expressed and uncontrolled activation of receptors enables glioma cells to degrade the extracellular matrix and spread into surrounding brain. Knockdown of integrin $\alpha 7$ contributes to a remarkable reduction of glioma growth and invasion in vitro and in vivo. A few compounds have already been tested for preventing glioma invasion by inhibiting integrin receptors and matrix-metalloproteinases (Groves et al., 2002; Scaringi, Minniti, Caporello, & Enrici, 2012) where the promising data show the potential for treating glioma progression. PI3K-Akt signaling pathway is the most altered signaling in glioma and hundreds of inhibitors targeting this pathway have been attempted (Van Meir et al., 2010). An inverse correlation between PI3K-Akt pathway and IDH1 mutation was found in glioma patients tissue samples and we validated this correlation to show that this is likely due to the aberrant miRNAs profiles in IDH1 mutant glioma (Birner et al., 2014). As a key pathway in glioma, the activation of PI3K-Akt pathway enhances multiple downstream pathways. Overactivation of mTOR, the key transcription factor in PI3K-Akt pathway promotes

cancer cell growth by loss of control of cell cycle regulation. Previous studies have displayed that IDH1 mutation leads to an abnormal expression of cyclin-dependent kinase 4 (CDK4) and cyclin-dependent kinase inhibitor 2A (CDKN2A), which promotes glioma cell proliferation (Malta et al., 2018). CDK6, the key factor in cell cycle regulation is also regarded as the most critical molecule in all targets of IDH1 mutation associated miRNAs by our analysis.

The survival analyses of IDH1 specific miRNAs demonstrated their prognostic capacity as a biomarker. The most common test for IDH1 mutation in clinical practice is anti-IDH1 mutation antibody-based immunohistochemistry assay, which is easy access and inexpensive to perform. However, there is inter-laboratory bias on immunohistochemistry assay. Protein based assay does not surrogate the mutation status as protein product is likely to be post-transcription regulated, therefore, immunohistochemistry assay is still not ideal for IDH1 mutation test (Nassiri, Zadeh, & Aldape, 2017). This bioinformatic analyses highlighted the potential capacity of biomarker using IDH1 specific miRNAs.

Some limitations of this study are needed to be highlighted here. As there is a bioinformatical analysis (*in silico*) of public datasets, the results here demand a validation in cell lines or primary tumour tissues prior to the clinical application. An external validation of the association of miRNAs expression and their promoter areas methylation would be another advantage to these results. The numerous putative targets and pathways of these IDH1 mutation associated miRNAs are also needed to be refined in the “wet laboratory”. Due to the computer capacity of RAM and cores, only eleven gliomas (5 IDH1 mutant and 6 IDH1 wildtype) were examined in this study. The correlation of DNA methylation and miRNA expression will be validated in a large cohort. We also noticed that some expression of IDH1 specific miRNA were not inversely correlated with their DNA methylation status, suggesting other regulation mechanisms beyond methylation are likely involved in miRNA expression. This will also be tested in the large cohort in the future.

In summary, we identified that seven miRNAs are down regulated in IDH1 mutant GBM and they have effects on cell proliferation and PI3K-Akt pathway by targeting their putative genes. The expression of these differentially expressed miRNA are linked with patients' survival, suggesting their prognostic capacity in glioma management. Further investigation to determine whether these IDH1 mutation associated miRNAs are released into peripheral blood and have clinical utility are conducted in the next chapter of this thesis (chapter 5).

Chapter Five

Chapter 5: Circulating miRNA profile in glioma

5.1. Introduction

Early diagnosis and monitoring patients' response to the therapeutic treatment are still challenging for most cancers. In the brain tumour setting, particularly in gliomas, the standard of care of diagnosis before surgical biopsy, and monitoring patients in follow-up after resection, operation largely relies on Magnetic Resonance Imaging (MRI). However, MRI does not give complete information and it can be difficult to distinguish glioma progression from radiation necrosis (Zachariah et al., 2018). Hence, there is an urgent need for a minimal invasive tool for molecular diagnosis and monitoring of glioma patients with high sensitivity and specificity. "Liquid biopsy" has emerged as a non-invasive alternative to conventional tissues biopsy and neuroimaging with exquisite specificity (Pantel, Diaz, & Polyak, 2013).

Another advantage of liquid biopsy is to gain access to the complete genetic information from patient biofluid to avoid the problem of spatial heterogeneity of cancers. Due to a marked spatial and temporal heterogeneity in gliomas, serial liquid biopsy could allow us to get more comprehensive mutational landscapes of glioma patients than a single tissue biopsy has in the past. As such, physicians would not miss the critical driver mutations which often could not be reached in a single tissue biopsy and then deliver suitable therapy (standard treatment or additional adjuvant therapies) for individual patients. In general, biomarker driven therapeutic strategy eventually benefits both high-risk and low-risk gliomas. For glioma patients with high risk, adjuvant therapy is applied and significantly increases the progression free survivals and reduces the mortality rate (Buckner et al., 2016; Shaw et al., 2012). On the other hand, further radiotherapy increased a long term risk for cognitive impairs in low-risk gliomas (Shaw et al., 2012).

In addition, a longitudinal genome and transcriptome study of 114 GBMs patients identified clonal evolution after standard treatments, indicating a single molecular diagnosis might be not enough for long-term glioma patient management (J. Wang et al., 2016). Considering the clinical difficulty in performing tissue biopsy for recurrent GBMs and tissue scarcity of brain tumours, blood-based testing for molecular alteration in cancer patients warrants its clinical utility and reproducibility. Therefore, liquid biopsy is an ideal alternative for detecting the molecular alternations in glioma precisely and monitoring the cancer evolution after adjuvant therapeutics with minimal invasion (Aizenberg, 2016; Johnson et al., 2014b; J. Wang et al., 2016).

The term “liquid biopsy” is regarded as all analysis of circulating biomarkers (typically involving circulating tumour cells (CTCs), circulating tumour DNA (ctDNA) and extracellular vesicles (EVs)) from body fluids such as blood and cerebrospinal fluid (CSF) in brain tumour patients. CTCs are identified in a variety of cancers including gliomas and as a subpopulation of primary cancer cells with metastatic properties. More importantly, CTCs represent their primary cancer cells and retain the signature profiles, which is a helpful tool for investigation of primary cells and their metastasis. The detectable sensitivity of CTCs in glioma varies from 20.6% to 71% according to the markers for positive selection. By CTC-iChip along with microfluidic technology, CTCs were found in 39.3% (13/33) of patients with GBMs and these detected CTCs were enriched for mesenchymal expression such as TGFB1, TGFBR2 and VIM (Sullivan et al., 2014). CTCs were found in 20.6% (29/141) of patients’ samples when glial fibrillary acidic protein (GFAP) was utilized as the CTCs markers in glioma (Muller et al., 2014). In contrast, the detection rate of CTCs increased to 71% of patients if nestin was used for the selection markers (Macarthur et al., 2014). Although CTCs have potential, no consensus on the selection markers for glioma CTCs and no solid evidence of association with CTCs and clinical survival will impair their applicability in glioma.

Since EVs from GBMs cells were first reported to carry EGFRvIII, the most frequent mutation in GBMs, and miRNAs ten years ago (Skog et al., 2008), several other studies

focused on the biology of these EVs, aiming to identify the molecular regulation of these EVs into glioma microenvironment. A study of EVs from 71 GBMs CSF revealed the sensitivity and specificity of detection of EGFRvIII status were 61% and 98%, respectively (Figueroa et al., 2017). Due to the low sensitivity of EVs in glioma and few study on the association between EVs and clinical implications, further work should be carried out prior to clinical use. ctDNA has demonstrated its promisingly clinical utility in a variety of cancers. Detection of the somatic mutation from ctDNA could allow us to diagnose cancer in early stage, monitor the minimal tumour residual before presenting in imaging and detect the emergence of therapeutic resistance and cancer evolution during adjuvant therapies (Bettegowda et al., 2014; Dawson et al., 2013; Tie et al., 2016). In recurrent or metastatic lung cancer, detection of ctDNA is one of the alternative option to determine the choice of personalized cancer therapy (Chaudhuri et al., 2017; Comino-Mendez & Turner, 2017). Combination of ctDNA and protein markers significantly overcame the mutual drawbacks: low specificity of ctDNA and low sensitivity of circulating proteins (Cohen et al., 2017). As a result, a panel of 61 amplicon ctDNA and 8 circulating proteins could achieve a median sensitivity of 70% and specificity of >99% in eight most common types of cancers (Cohen et al., 2018a). Due to the low abundance of ctDNA in blood samples of patients with glioma, the detectable rate of ctDNA is the lowest of all 15 types of cancers, with less than 10%, which restricted the application of ctDNA in blood based detection for glioma patients (Bettegowda et al., 2014). On the other hand, detection of ctDNA from CSF samples have showed a higher sensitivity than that from blood samples (T. Y. Huang et al., 2017; Y. Wang et al., 2015) but it requires a physician to perform lumbar puncture and collect CSF, which might be impractical for some patients living in rural areas where there are not neurosurgeons available and too invasive for multiple sampling.

In Chapter 4, circulating miRNAs have demonstrated a remarkable capacity of diagnosing glioma but we are still unclear whether circulating miRNAs are associated with IDH1 mutation status, which is more useful in clinical practice. In addition, the

correlation of circulating miRNAs expression with tumour area in MRI image after surgical resection are not fully investigated. Here, we recruited 92 glioma patients and collected their serum samples pre and post operatively to investigate circulating miRNAs profile in glioma patients. We also explored the endogenous controls for circulating miRNAs.

5.2. Results

Our research group has been prospectively collecting blood, plasma and serum samples from glioma patients (n=250) since 2009. For this study we selected 91 patients who were diagnosed with either WHO II or WHO IV gliomas and had preoperative and at least one postoperative serum sample. All pathological diagnosis and IDH1 mutation testing were based on the histopathological examination performed by pathologists at the Royal Melbourne Hospital. The summarized characteristics of all included are listed in table 5.1. Serum sample from 47 low grade glioma patients, 44 high grade glioma patients and 17 healthy controls were included in this cohort. The mean age of patients with low and high grade glioma were 49.8 and 49.9, respectively. The gender ratio between these two groups were also similar. The overall survival of low and high grade glioma were 41 and 16 months, which was similar to the previous international studies (Cancer Genome Atlas Research, 2008). It is notable that there was a higher percentage of IDH1 mutation (52.3%) in high grade glioma of our population than that reported in previous studies (approximately 10%), suggesting there were a high proportion of secondary GBMs in our cohort.

Before we performed analyses, we observed a large batch effect of the raw counts within our samples (Figure 5.1A). Regardless of the normalization methods recommended by the nSolver (the official software for analyzing nanoString data), we still found the expression of miRNAs in batch 3 and 4 were higher than the other batches. After

consulting with bioinformaticians in WEHI, the algorithm Remove Unwanted Variant version III (RUVIII) was performed to the raw counts. After calibrated by RUVIII, the batch effect was remarkably reduced and then the normalized data was subjected to downstream analyses (Figure 5.1B).

Table 5.1 The characteristics of all participants included in circulating miRNA cohort

	GLIOMA		HEALTHY CONTROLS
	Low Grade (n = 47)	High Grade (n = 44)	VBDR (n = 17)
AGES (YEARS, MEAN±SD)	49.8 ± 15.3	49.9 ± 15.4	37.6 ± 10.5
GENDER (MALE, %)	53.2 %	63.6 %	41.2 %
MEDIAN SURVIVAL (MONTHS)	41	16	n/a
IDH1 MUTATION (POSITIVE, %)	68.1 %	52.3 %	n/a

VBDR: Victorian Blood Donor Registry, kindly provided by Dr. Rachel Koldej and ACRF.

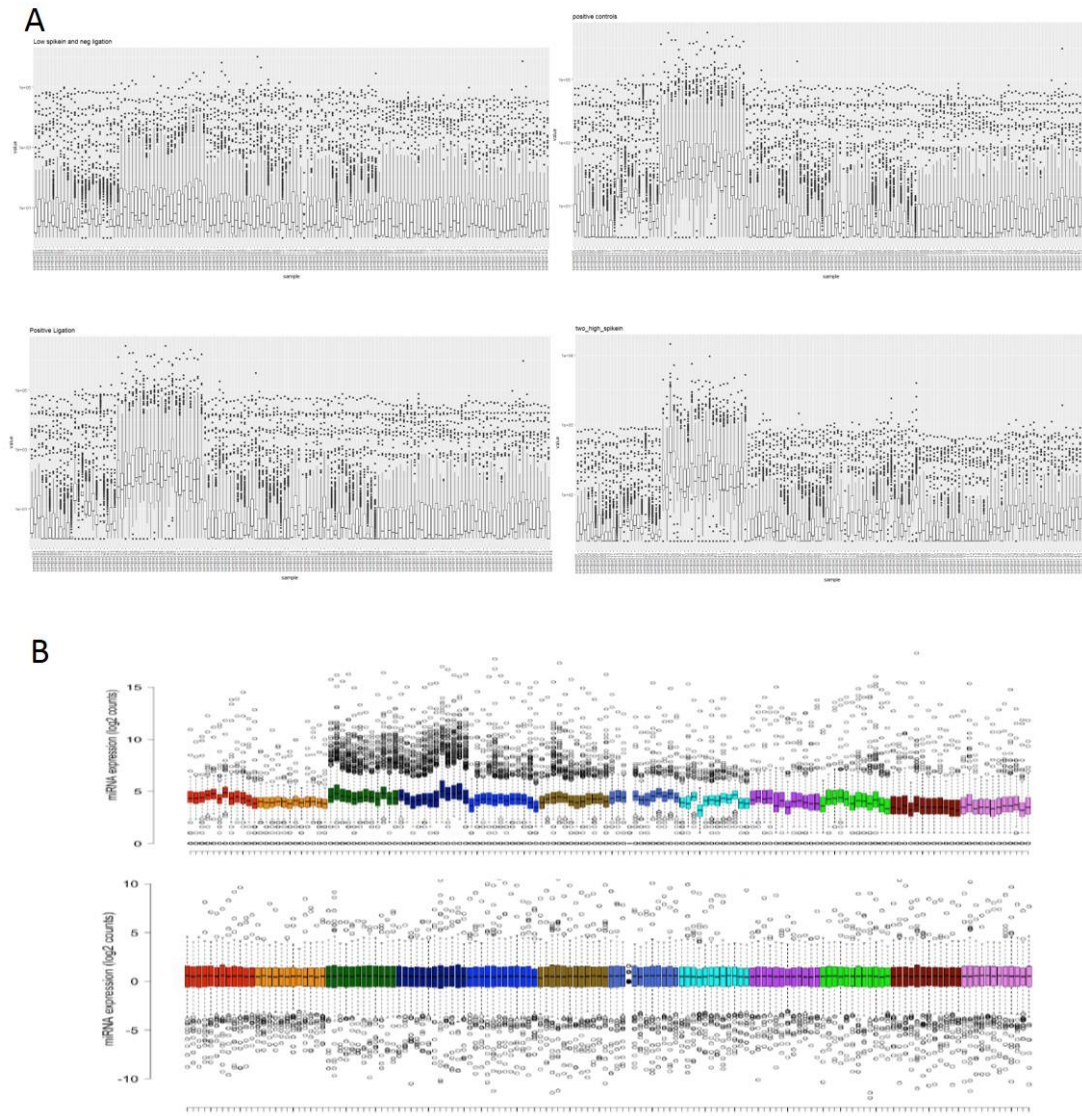


Figure 5.1. The normalization of raw data. A, boxplot showing the global miRNAs distribution after normalization by Spike-in and positive controls. B, boxplot showing the global miRNAs distribution before (upper) and after (lower) RUVIII applied. Each box represents one sample. Samples coming from one batch are labeled in one color. Kindly generated by Ramyar Molania and Professor Terry Speed.

5.2.1. miRNAs in diagnosis

This part is now not public due to confidential and sensitive materials.

5.2.2. IDH1 mutation associated circulating miRNAs

As there were few studies investigating the association of circulating miRNAs with IDH1 mutation, we then performed differential analysis and validated the IDH1 mutation associated miRNAs (that we found in Chapter 4) in our serum cohort. PCA demonstrated there was a substantial overlap of IDH1 mutant glioma and IDH1 wild type glioma in regard to circulating miRNAs (Figure 5.4A), suggesting circulating miRNAs might not be a biomarker for IDH1 mutation. Similarly, t-SNE plot also showed an overlap of IDH1 mutant and wild type glioma in content of miRNAs expression (Figure 5.4B). Differentiated expression analysis failed to identify any significant miRNA as shown in the volcano plot (Figure 5.4C). Shortlisted IDH1 mutation associated miRNAs (miR-148a, miR-155, miR-204, miR-221, miR-222, miR-34a and miR-34b) generated from TCGA were also utilized to determine whether tissue IDH1 mutation associated miRNAs differentiate IDH1 mutation. Not surprisingly, these 7 miRNAs expression in serum from our cohort showed no association with IDH1 mutation, suggesting no circulating miRNAs were associated with IDH1 mutation (Figure 5.4D).

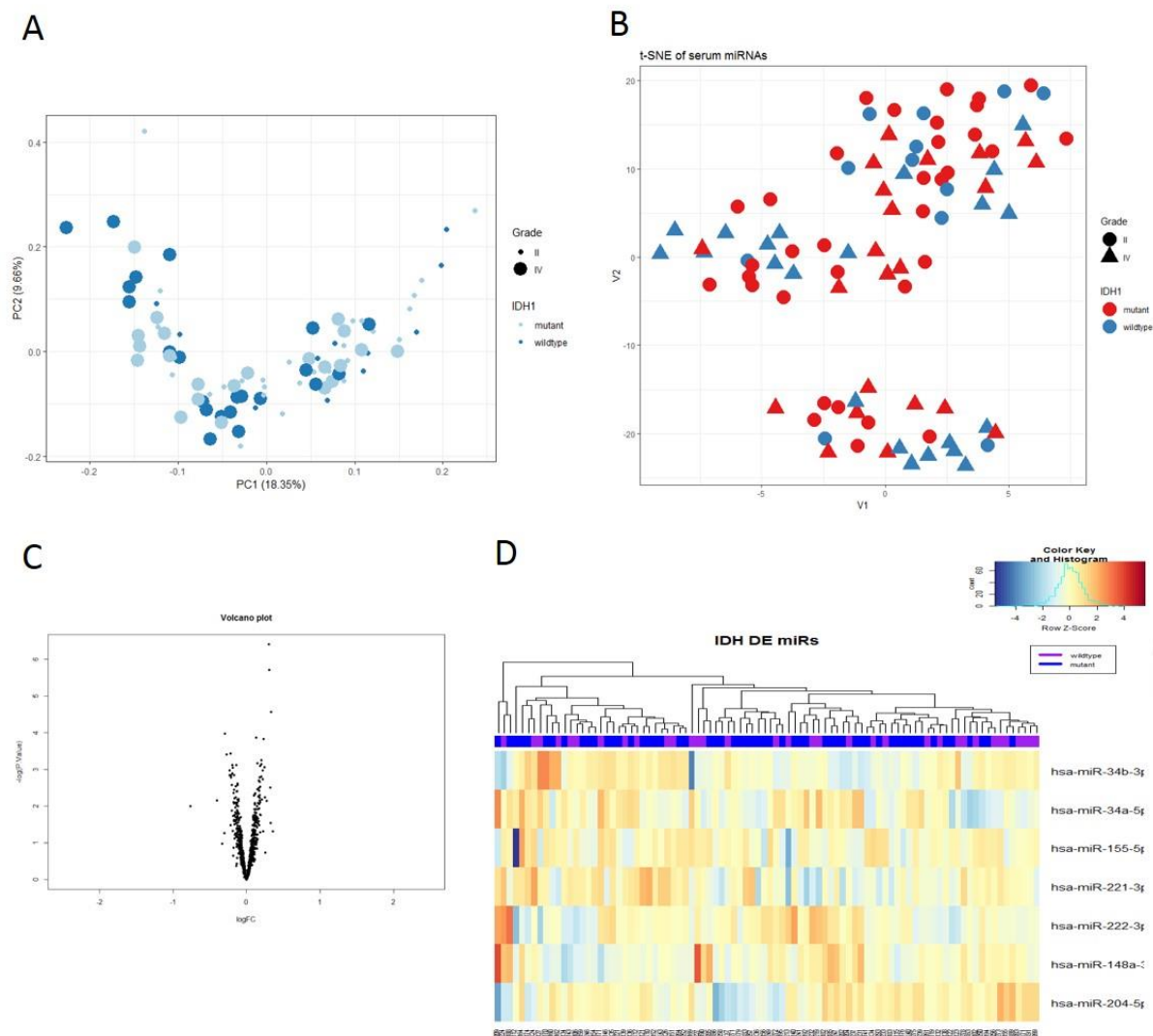


Figure 5.4. The IDH1 mutation associated circulating miRNAs. A, PCA plot of miRNA expression over all samples. PC1 and PC2 represents 18.44% and 6.79% of variation of miRNA expression. Light blue, IDH1 mutants; Dark blue, IDH1 wild types. Large dots, GBMs; small dots, low grade gliomas. B, t-SNE plot showing the sample clustering. Round, low grade glioma; triangle, GBMs. Red, IDH1 mutants; Blue, IDH1 wild types. C, Volcano plot showing the differential expressed miRNAs of 91 serum samples. Each dot represents single miRNA and no significant miRNA was identified (log fold change larger than 1 or smaller than -1). D, Heatmap of clustering by differential expressed miRNAs generated from TCGA. Grading of each sample are labelled on the top of heatmap. Purple, IDH1 wild type; blue, IDH1 mutation.

5.2.3. Circulating miRNAs in postoperative monitoring

This part is now not public due to confidential and sensitive materials.

5.2.4. miRNAs in prognosis

This part is now not public due to confidential and sensitive materials.

5.2.5. Endogenous miRNAs controls in circulation

Unlike the RNU6 that was extensively utilized as the endogenous control for miRNAs study in tissue and cell lines (Das, Andreassen, Haugen, & Furu, 2016), the endogenous controls for circulating miRNAs have yet to be established (C. Ma et al., 2018). To explore the endogenous controls for circulating miRNAs for glioma study, we performed endogenous single miRNAs controls identification by NormFinder in our cohort (91 gliomas and 17 healthy controls) (Andersen et al., 2004). A total of 589 miRNAs remain for NormFinder analysis after removed these miRNAs if their expression of more than 25% samples below the threshold in nSolver. Let-7b was identified as the best endogenous controls for circulating miRNA study of glioma mixed with healthy controls with 11.088 of the stability value, followed by miR-155 and let-7d (table 5.4).

Table 5.3. The top 20 stably expressed miRNAs identified by NormFinder

miRNA name	Stability value
hsa-let-7b-5p	11.08795
hsa-miR-155-5p	13.25654
hsa-let-7d-5p	13.57218
hsa-miR-376a-3p	15.16084
hsa-miR-591	15.38618
hsa-miR-337-3p	16.39371
hsa-miR-499b-3p	16.83099
hsa-miR-519b-3p	16.8463
hsa-miR-1245b-5p	17.07942
hsa-miR-520c-3p	17.54847
hsa-miR-363-3p	17.60588
hsa-miR-1257	18.79376
hsa-miR-522-3p	20.25504
hsa-miR-574-5p	20.31111
hsa-miR-549a	20.74811
hsa-miR-320d	21.86925
hsa-let-7i-5p	22.22213

hsa-miR-873-3p	22.32354
hsa-miR-612	22.66448
hsa-miR-93-5p	23.72742

5.3. Discussion

A few paragraphs are restricted due to confidential and sensitive materials.

One issue undermining the application of circulating miRNAs into clinical practice is that there is no consensus on the endogenous controls. In addition, few studies investigated the endogenous controls for circulating miRNAs (J. Hu et al., 2014). RNU6, the widely used endogenous control in tissue, is not stable for circulating miRNAs after multiple cycle of freezing and thawing (Xiang et al., 2014). miR-16 was suggested to be another endogenous control in one study but Vigneron et al who found exogenous controls offered less inter-sample variability than miR-16 did in normalization of circulating miRNAs (Alegre et al., 2014; Vigneron et al., 2016; Xiang et al., 2014). In our study, we identified out hsa-let-7b-5p, hsa-miR-155-5p and hsa-let-7d-5p are the most stable miRNAs across all samples. The previously identified endogenous miRNAs such as miR-16 and miR-1228 are not included in the top 25 stable miRNA controls in our study. The let-7 family has been reported to be associated with cell development, indicating it is key regulator in cells (Morgado, Rodrigues, & Sola, 2016; H. Song et al., 2016; Y. Tian et al., 2015; Z. Zhang et al., 2017).

The progression signature of miRNAs is significantly associated with tumour recurrence and tumour evolution in breast cancer (Dadiani et al., 2016). Our analysis found that the expression profiles of five circulating miRNAs revealed the tumour progression pattern. Although no miRNA demonstrates progression pattern in all four recurrence patients

(NS69, NS122, NS123, NS152) we profiled, at least three patients (NS69, NS122, NS152) have revealed these miRNAs indicated the progression. In addition, the PS based on the average of these five miRNAs strongly suggest the risk of glioma recurrence (Table 5.2). These five miRNAs composed of PS broadly participate in hypoxia regulation pathway and glioma stem cells development in glioma (Y. Jiang et al., 2017; Xi et al., 2017; D. Zhao et al., 2014). As glioma stem cells are regarded as being responsible for tumourigenesis and recurrence and glioma stem cells induce hypoxia in tumour microenvironment, the level of progression signature miRNAs might indicate the risk of glioma recurrence in clinical practice. Further study will be conducted to validate the accuracy of the 6 miRNA-based PS in a large independent population and the biological function of these 6 miRNAs should also be further investigated.

One patient selection bias should be addressed here. As a comprehensive cancer centre, most patients with gliomas here are likely treated before and referred by other hospitals. That would be the reason why more secondary GBMs are included in this study.

In the future, a clinical trial is going to be conducted to validate these five miRNAs for their diagnostic/prognostic capacity. Patients diagnosed with low grade glioma will be included in this prospective trial. The serum samples of each patients will be taken to extract these six miRNAs expression during the follow-up. The included patients will randomly be allocated into two groups: standard care group or circulating miRNA monitor group. In standard care group, patients will be monitored by the CT scan and the aggressive treatment will only be delivered when the CT image indicates a recurrence. For circulating miRNA monitoring group, once the expression of six miRNAs are elevated in one follow-up time point, the adjuvant therapeutics will be delivered to this particular patient. After 5 years follow-up, the recurrent rates of each group will be tested and cost effectiveness analysis will be also calculated in order to evaluate the best care for low grade glioma patients.

Although liquid biopsy is not a replacement for tissue biopsy, it provides an option for those who are not suitable for tissue biopsy or those whose reliable molecular test of tissue is not accessible. Our discovery phase studies showed circulating miRNAs can differentiate glioma from healthy controls but we did not demonstrate glioma grade or IDH1 mutation associated serum miRNAs. Given that circulating miRNAs can be packaged and released by extracellular vesicles, the molecular function of extracellular vesicles in glioma is investigated in next chapter (chapter 6).

Chapter Six

Chapter 6: Glioma Stem Cells derived extracellular vesicles promote glioma progression via activation of AKT pathway

6.1. Introduction

The most common brain cancer in adults is glioma and the most aggressive subtype, glioblastoma multiforme (GBM), remains a devastating disease with median survival less than one year (Kaye & Morokoff, 2014; Stupp et al.). Despite surgery, chemotherapy and radiotherapy, GBM has an almost inevitable recurrence with tumour cells continuing to invade throughout the brain. A major issue in designing therapies for GBM is its complex heterogeneity at a phenotypic and molecular level (Sottoriva et al., 2013).

A small subpopulation of stem-like cancer cells (glioma stem cells, GSC) are associated with resistance and subsequent repopulation of glioma cells after treatment (Bao et al., 2006; J. Chen et al., 2012). Moreover, accumulating studies have already demonstrated GSCs exist in low grade glioma, suggesting GSCs are responsible for gliomagenesis (Tirosh et al., 2016). Like neural stem cells in brain, GSCs have also the ability of self-renewal and differentiation into distinct lineages. This plasticity of differentiation or trans-differentiation capacity of GSCs is regarded as the driven force behind glioma recurrences and resistance to therapeutic treatment (B. Hu et al., 2016; Zheng et al., 2008). However, the mechanisms of how GSCs facilitate their differentiated glioma cell counterparts to progress are not fully elucidated (Brown et al., 2017).

Inter-cellular communication is emerging as an important feature of cancer, allowing the cancer cell to have effects on other neighbouring tumour cells and its surrounding microenvironment, which in the brain includes astrocytes, neurons, extracellular matrix,

endothelial cells and immune cells. This communication is mediated by extracellular vesicles (EVs): nanometer size spherical bodies composed of organelle-free cytosol with a lipid bilayer. They include exosomes and microvesicles, and are part of the so-called Secretome (Godlewski et al., 2015; Luga & Wrana). In normal brain, hypothalamic neural stem cells regulate ageing by releasing miRNAs in EVs (Y. Zhang et al., 2017), which suggested that stem cell-derived EVs are prone to mediate the brain microenvironment. Glioma-derived exosomes contain a specific profile of DNA, RNA, miRNA and proteins that can cause gene expression changes in recipient tissues (Akers et al., 2013; Putz et al.). It has been shown that GBM-derived EVs containing miRNAs can be taken up by surrounding microglia, monocytes and macrophages and other glioblastoma cells (Skog et al., 2008; van der Vos et al.). Recently, EVs have been shown to play a role in mediating a switch to a mesenchymal glioma stem cell phenotype throughout heterogeneous tumours (Ricklefs et al.).

Cancer derived EVs are reported to be associated with tumour progression and metastasis by setting up the pre-metastasis niche for accommodation of cancer cells and regulating invadopodia formation (D. Hoshino et al., 2013). On the other hand, cancer cells derived EVs also play a critical role in therapeutic resistance transfer and this resistance transfer is most likely induced by EVs cargos, specifically miRNAs or lncRNAs (Au Yeung et al., 2016; Boelens et al., 2014; Qu et al., 2016a; J. K. Yang et al., 2016). Resistance cancer cells photocopy their radiation or chemotherapy resistance to the sensitive cancer cells where EVs from resistance cells transport the critical molecules in resistance to the recipient cells. Afterwards, the emergence of acquired resistance of cancer cells is inevitable in clinical setting. However, we still know little about whether GSCs induced resistance to the differentiated glioma cells and the signalling pathway change that occur after GSCs EVs exposure. Further understanding about the process of acquired resistance in glioma and exploration of inhibitory drugs on this process would most likely reduce the emergence of resistance and improve patients' outcomes.

The concept that there is a complex intratumour heterogeneity in cancers is well recognized in the last decade. Specifically, a tumour is most likely composed of several cellular subpopulations that vary in the proliferative rate, therapeutic response. Moreover, subpopulation interaction almost exists in all tumour cells as the main feature of the tumour ecological system. Although, in some cases, one subpopulation that adapts to the microenvironment better might dominate another subpopulation occasionally, clonal cooperation achieved by direct cell-to-cell crosstalk or indirect paracrine activation is observed and thought to be the driving force of intratumour heterogeneity. One tumour cell cannot progress growth and metastasis simultaneously, instead, collaborative crosstalk of multiple subpopulation enable tumours to grow by for example, one subclone secreting proteinase to degrade the extracellular matrix while another subclone utilises this advantage to proliferate (Tabassum & Polyak, 2015). Thus, further understanding of the role of the crosstalk between subpopulations in tumour progression and therapeutic resistance is necessary.

Extracellular vesicles are regarded as a common mechanism of heterogenetic transfer the heterogeneity in glioma cells. EGFRvIII was identified to be highly expressed in glioblastoma cells, which highlighted the clinical utility for glioma diagnosis (Skog et al., 2008). Further experiments showed EVs carrying EGFRvIII significantly changed the transcript and proteomic expression profiles. Proteomic analysis showed EVs with EGFRvIII had a large expression of pro-invasion markers such as CD44, ITGA6, and ITGB4 (Choi et al., 2018). GO analysis also demonstrated the protein enriched in EGFRvIII EVs are highly relevant to cell adhesion. However, whether the pro-invasive EVs promote glioma cells progression is still unclear. In addition, GSCs EVs were responsible for the intercellular communication within GSCs and miRNAs packaged in EVs propagated glioma heterogeneity (Godlewski et al., 2017a; Rooj et al., 2017). Distinct subtypes of GSCs had different miRNAs profile in EVs secreted by GSCs and miRNAs in EVs proxied their donor glioma cells miRNAs signatures. As a result, transfer of the EVs from different subtypes GSCs shifted the miRNA expression

signature and the expression of the miRNA targets in the recipient cells accordingly (Godlewski et al., 2017a; Rooj et al., 2017). Afterwards, a remarkably cell phenotype shift was observed in the recipient cells, indicating miRNAs in EVs promote glioma cell progression.

As cancer cells actively secrete EVs into microenvironment to re-shape the niche for cancer progression and metastasis, the key molecules involving in EVs biogenesis and secretion will be potentially the diseases biomarkers and therapeutic targets. Rab27a and Rab27b are two proteins in Rab family participating the docking of vesicles into multivesicular endosomes. Silencing of Rab27a and Rab27b significantly reduced the exosomes secretion into the cell supernatant (Ostrowski et al., 2010). Further investigation on Rab27a in lung cancer reveals blockage of Rab27a significantly reduced the dissemination of metastasis lung cancer (Bobrie et al., 2012). However, the relationship of Rab27a and the progression free survival of cancer patients as well as whether Rab27a is a predictive biomarker in glioma progression have yet been fully elucidated.

In summary, glioblastoma is the most aggressive brain tumour with short survival, partly due to resistance to conventional therapy. It is likely that glioma stem cells play a role in treatment resistance, by releasing extracellular vesicles that contain specific molecular cargo that can modulate the tumour microenvironment. Here, we studied the extracellular vesicles secreted by glioma stem cells (GSC-EVs) and their effects on tumour growth and progression. Here we show that GSC-derived exosomes can transfer increased migration, radio- and chemoresistance signals and confer stem-like characteristics to non-stem glioma cell lines and that this effect is likely mediated by miRNAs through inhibition of PTEN function in the recipient cells, leading to upregulation of the AKT pathway.

6.2. Results

6.2.1. Exosomes characterization and internalization

To verify whether EVs (30-150 nm) were secreted by glioma stem cells, we isolated culture media and examined EVs morphology and marker expression after differential ultra-centrifugation (Figure 6.1A). The expression of EVs markers were variable between three GSCs. It should be noted that level of TSG101 and Alix expression were variable among each GSC line examined MU004 showed very low TSG101 and Alix expression in their EVs. The expression of CD9 was similar across all three GSCs, indicating CD9 would be a suitable marker for GSCs EVs (Figure 6.1B).

The correlation of GSCs number and GSCs EVs total protein were explored to investigate EVs production efficacy of GSCs. The EVs production efficacy in MU020 and MU039 were significantly higher than that in MU004. The average total yield EVs protein amount of 100,000 GSCs were 0.09 µg, 0.38 µg and 0.33 µg in MU004, MU020 and MU039, respectively (Figure 6.1C). Mesenchymal-like GSC (MU020) has the highest exosome producing efficacy than the other two lines, indicating mesenchymal-like GSCs prompt to secrete EVs in order to influence surrounding cells.

Western blot further confirmed GSCs EVs surrogate their parental cells signatures. Comparing the expression of CD44, known as a marker of mesenchymal cells, in GSCs EVs and their parental cells, we found there was a distinct expression between GSCs EVs but a similar expression pattern between EVs and their donor cells (Figure 6.1D), indicating the heterogeneity of GSCs EVs and EVs could surrogate the proportion of mesenchymal glioma cell in whole tumour. Re-plotting the mass spectrum of a previous study (PMID: 27770278) also demonstrated that the proteomic profiles of six glioma cell lines were different (Figure 6.1E).

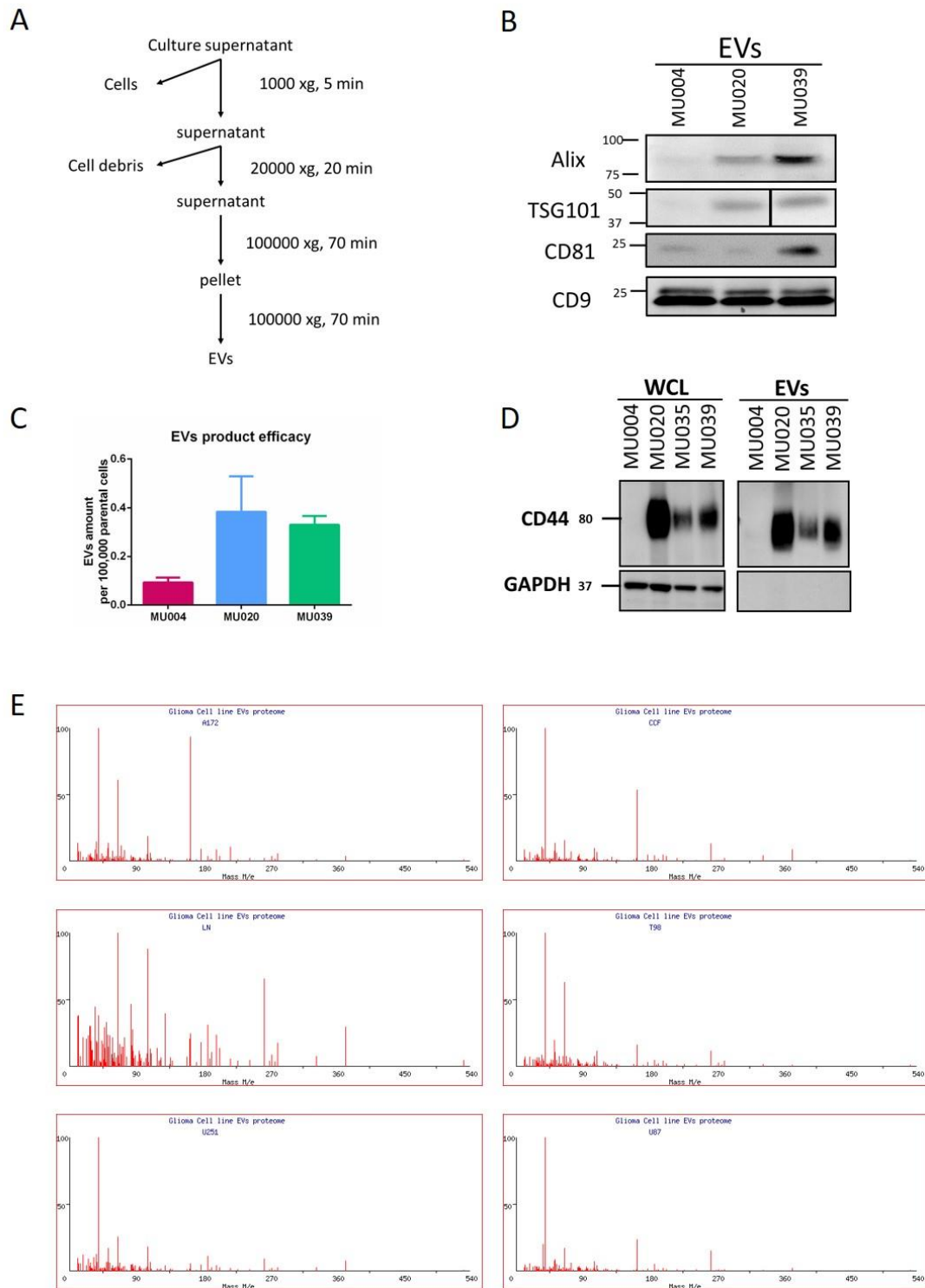


Figure 6.1. The EV production of each GSCs line. A, the brief flow chart showing the procedure of EVs isolation. B, the EVs markers expression in each GSCs EVs. C, the total protein amount of each 100,000 GSCs. D, CD44 and GAPDH expression of GSCs EVs and their parental cells. WCL, whole cell lysate. E, the mass spectrum showing the proteomic profiles of six glioma cell lines.

Exosomes/EVs (30-150 nm, mode size 106 ± 4.7 nm) with round morphology and membranous cup-like shape were secreted by glioma stem cells, as observed in cryo-transmission electron microscope and Nanosight distribution (Figure 6.2A). GSC-EVs concentration from 1.6×10^8 particles/ml to 4.85×10^8 particles/ml (Figure 6.2B).

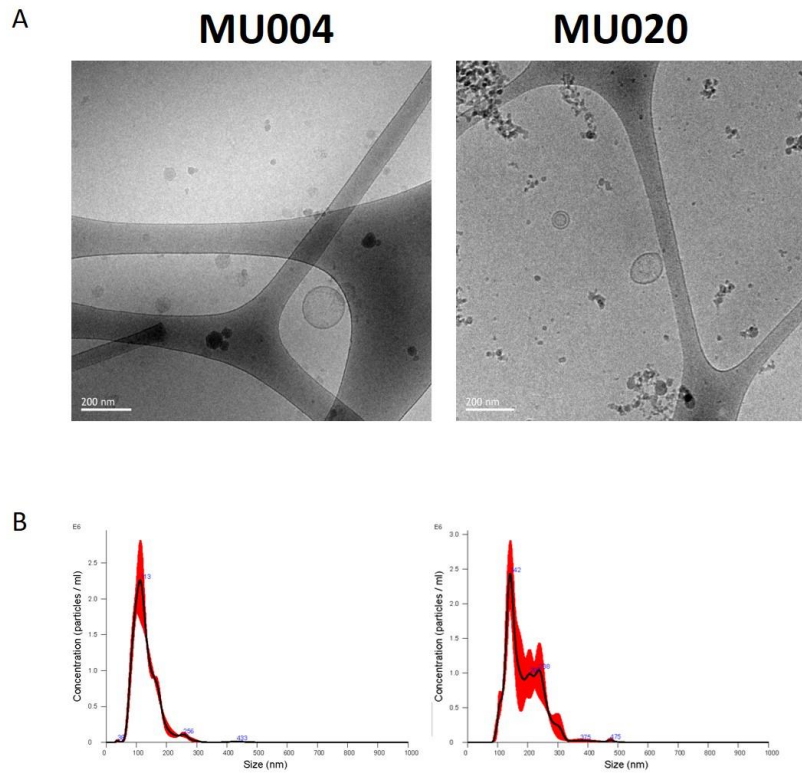


Figure 6.2. The morphology and size distribution of EVs. A, the cryo-Electronic Microscope scanning results for the product from ultra-centrifugation isolation. Scale bar, 200 nm. B, the size distribution and concentration of the isolated product by Nanosight.

6.2.2. Characterization of GSCs

Neurospheres were firstly confirmed as GSC by the presence stem cell markers (Sox2, Oct4, Nestin and pSTAT3) prior to exosomes isolation (Figure 6.3A&B). All 3 GSCs cell lines (MU004, MU020, MU039) expressed stem cells markers but not in differentiated cell lines (LN229 and U118) (Figure 6.3C). Notably, GSCs also expressed HDAC6, indicative of their primitive status compared to the differentiated cells. MU020 also

expressed much higher level of cleaved PARP (cPARP) than MU004 and MU039, suggesting that MU020 might be associated with therapeutic resistance (Figure 6.3B). When GSCs neurospheres were cultured in serum-stimulating differentiation medium, they loss their stemness markers (Sox2, Oct4 and Nestin) but still retained astrocytic marker GFAP (glial fibrillary acidic protein) (Figure 6.3C). A 24 h live cell record documented the differentiation of MU039 (Appendix 2). Differentiation assay also revealed three GSCs differentiated into specific lineages and had different morphology after 17 days serum stimulation culturing (Figure 6.3D). More interestingly, flow cytometry showed there were more cycling cells (S phase and G2/M phase) in GSCs than that in differentiated glioma cells, suggesting a higher proliferating rate in GSCs (Figure 6.3E), as being consistent with the results of single cell sequencing (Tirosh et al., 2016).

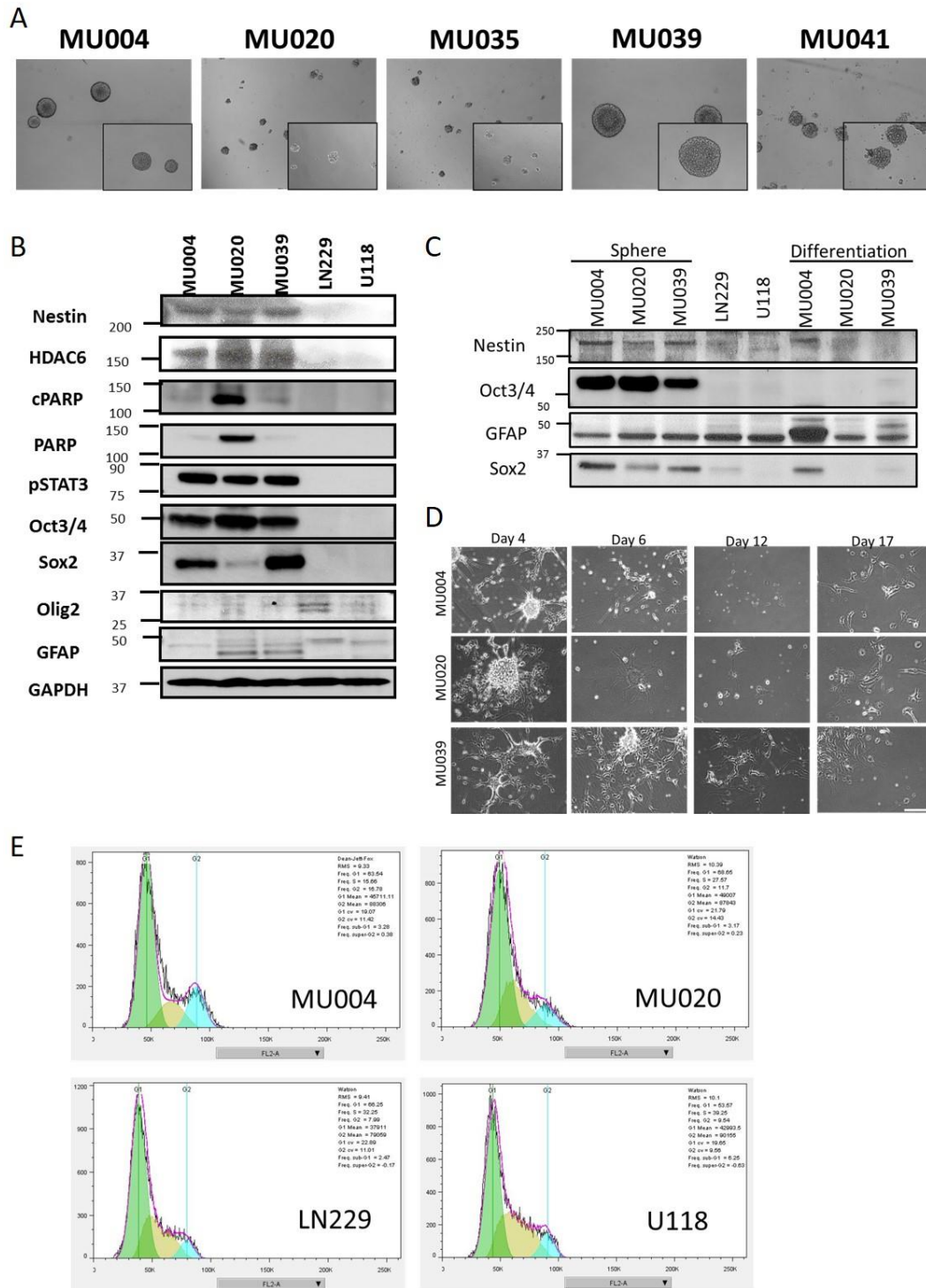


Figure 6.3. The characterization of GSCs. A, typical images under bright white field view of GSCs spheroid formation 7 days after association. Whole image, magnification X4. lower right, magnification X10. B, the expression of stemness markers in GSCs and differentiated glioma cell lines. C, the change of stemness and glial cells markers in GSCs after serum-stimulating differentiation. D, phase contrast view of GSCs during differentiation. Scale bar, 100µm. E, histogram of flowcytometry results for MU004, MU020, LN229 and U118. green, G0/G1; yellow, S phase; blue, G2/M.

Given the heterogeneity of GBM and GSCs were reported recently, we detected the response of radiation and chemotherapy from our GSCs. Both limited dilution assay and spheres formation assay demonstrated that MU020 cells had greater radiation resistance than the other two GSCs (Figure 6.4A, B). To identify the chemoresistance of GSCs, we then performed cell proliferation assay in three GSCs after TMZ treatment. Similarly, proliferation assay demonstrated there was no different between non-treatment group and TMZ treatment group in MU020 lines (Figure 6.4C).

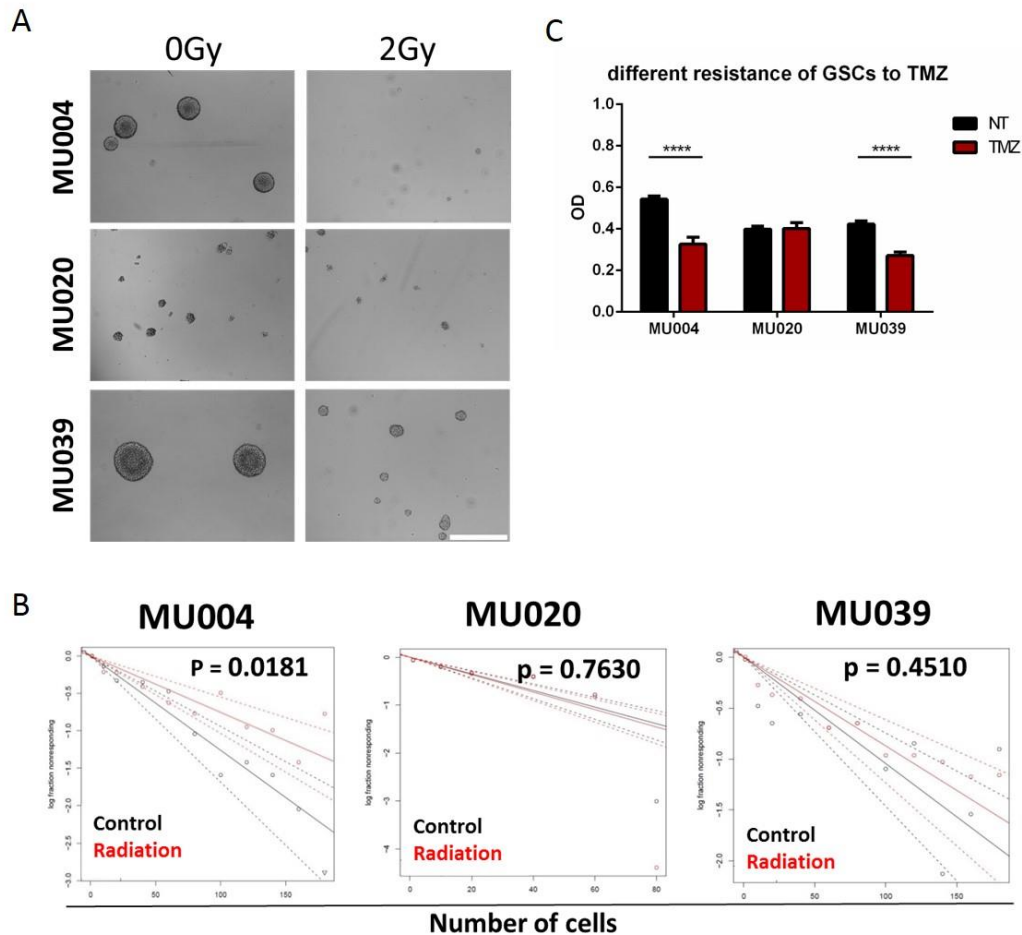


Figure 6.4. Different GSCs showed different radiation resistance and TMZ resistance. A, Typical images of GSCs spheres on 7 days after radiation treatment (0Gy or 2Gy). Scale bar, 100 μ m. B, Limited dilution assay showing sphere formation of MU004 largely is affected by radiation but not in MU020 and MU039. Black: non-radiation treatment; Red, 8Gy radiation treatment. Solid line: mean of linear regression of each group; dash line: standard deviation of linear regression of each group. C, MTT assay showing TMZ affects on proliferation of MU004 and MU039 but not on MU020 7 days after TMZ treatment. TMZ concentration: 500 μ M. NT, non-treatment group.

6.2.3. GSC-derived EVs enhanced tumour cell migration and spreading

To investigate whether GSCs EVs promote cell migration ability, we first utilized the spheroid migration assay and wound healing assay. After seeding the glioma spheroid in the plate, GSCs EVs were added into the culturing media and incubated for 3 days. A significant increase of migrated distance was observed in GSCs EVs treatment group compare to that in non-treatment control group (Figure 6.5A).

Both LN229 and U118 cells migrated faster in the presence of GSCs EVs (Figure 6.5B). Significant rate of migration was observed in 48h but not 24 h and were most prominent in response to MU004EVs (Figure 6.5B). GSCs EVs did not increase LN229 migration in 24 h but stimulated LN229 migration at 48 h after MU004 EVs exposure (Figure 6.5B). The percentage of migration distances after NT, MU004EVs, MU020EVs and MU039EVs addition to LN229 were 43.97%, 44.64%, 39.26% and 40.39% at 24 h, respectively. Forty-eight hours after GSCs EVs exposure, MU004 EVs increased to 77.08% of migration distance in LN229, compared to 64.37% of that in NT group ($p = 0.049$). In contrast, wound healing assay showed all sources of GSCs EVs promote U118 migration after 24 h and maintained the significance of high migration after 48 h (Figure 6.5B). With regards to U118, 75.09%, 70.16% and 75.41% of the distances had been migrated in MU004EVs, MU020EVs and MU039EVs in 24 hours after GSCs EVs exposure, compare to 42.76% of distance in NT group, with the statistical significance of $p = 0.003$, 0.001 and 0.004 , respectively. The significance of these three groups is also observed in 48 h after GSCs EVs exposure, with p value of 0.003 in all groups. Specifically, MU004EVs, MU020EVs and MU039EVs promoted U118 migrated 93.11%, 93.18% and 95.93% of wound, compared to 67.82% of that in NT group. The quantified cell-migrated distance was also shown in the right panel.

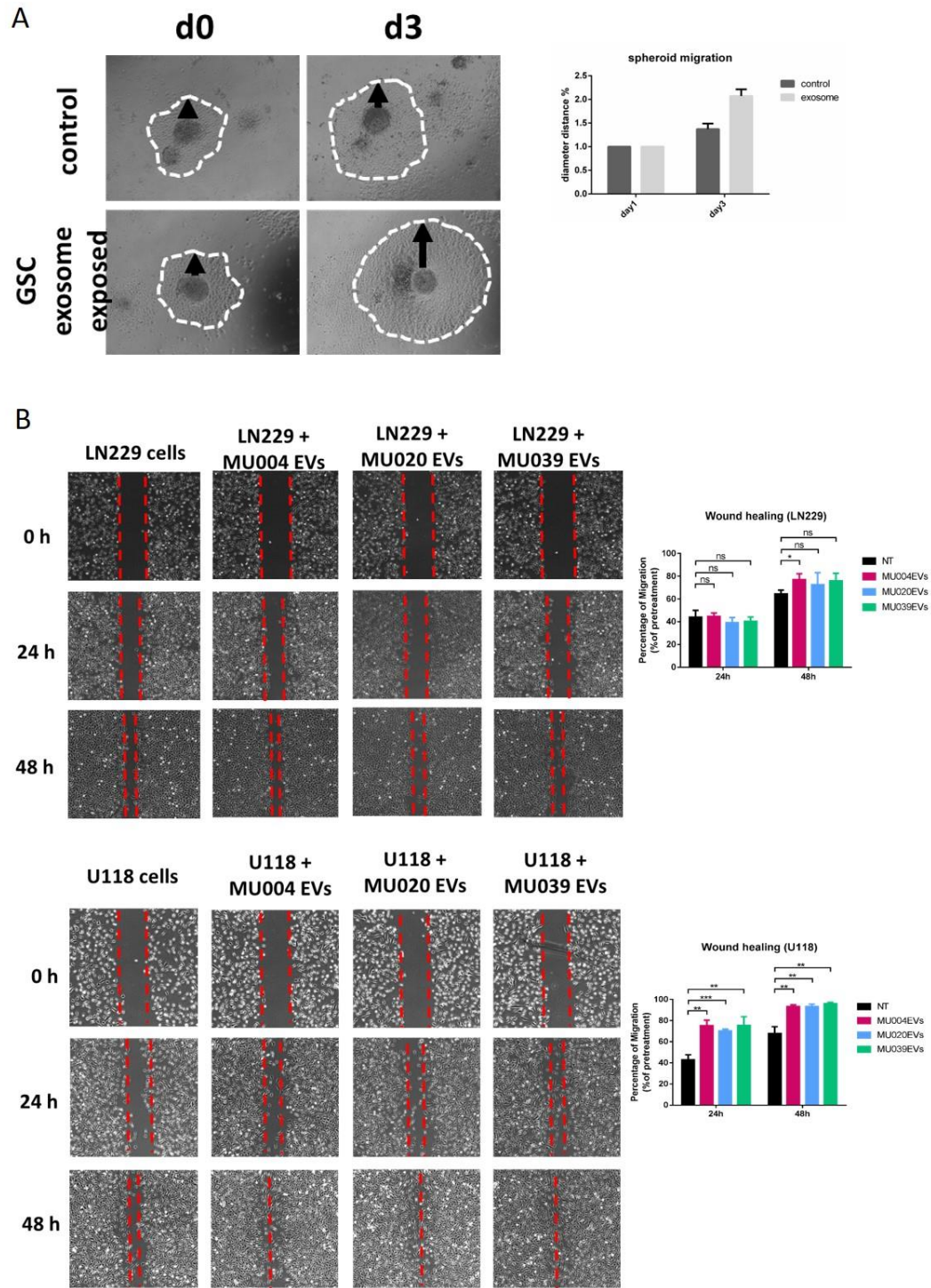


Figure 6.5. GSCs EVs promote cell migration in recipient cells. A, white field images of spheroid migration before and 72 hours after GSCs EVs treatment. Black arrow, the migrated distance; white circle, the migrated area. B, Phase contrast images of cells migration after exposure to EVs from MU004, MU20, MU39-GSCs in 24h and 48h in U118 Cells and LN229 cells. Bar graph showing percentage of migration of U118 cells and LN229 cells in presence GSC-derived EVs. One-way ANOVA, Significance: ns, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$

6.2.4. GSC EVs can promote radiation resistance

To test whether GSCs EVs could enhance radiation resistance of recipient cells, we performed irradiation to recipient cells 6 hours prior to GSC-EVs addition, followed by a colony formation assay. All EVs derived from 3 GSC lines can transfer radiation resistance to LN229 and U118 recipient cells (Figure 6.6A). More visible colonies were seen after 2 Gy treatment compared to 5 Gy treatment. Notably, recipient cells survived 5 Gy exposures and formed colonies in the presence of MU020EVs (Figure 6.6A). These results were also supported by cell viability assay (Figure 6.6A). More interestingly, the most formed colonies and cell amount of recipient cells were identified in CD44 highly expressed MU020 EVs treatment. Considering CD44 is highly relevant to mesenchymal subtype and radiation resistance, these CD44 enriched EVs delivered therapeutic resistance into the recipient cells likely through their cargo.

To determine whether GSC-EVs promote cells survival after temozolomide treatment, we performed cell viability assay after temozolomide plus GSCs EVs treatment to differentiated cells. Interestingly, chemotherapy resistance transfer is more significant in MGMT promoter methylation cell lines (U118) than non-MGMT-methylated cells (LN229), indicating chemotherapy resistance might be due to a DNA methylation dependent mechanism (Figure 6.6B). Taken together, GSCs likely transfer the therapeutic resistance to the surrounding recipient cells by EVs.

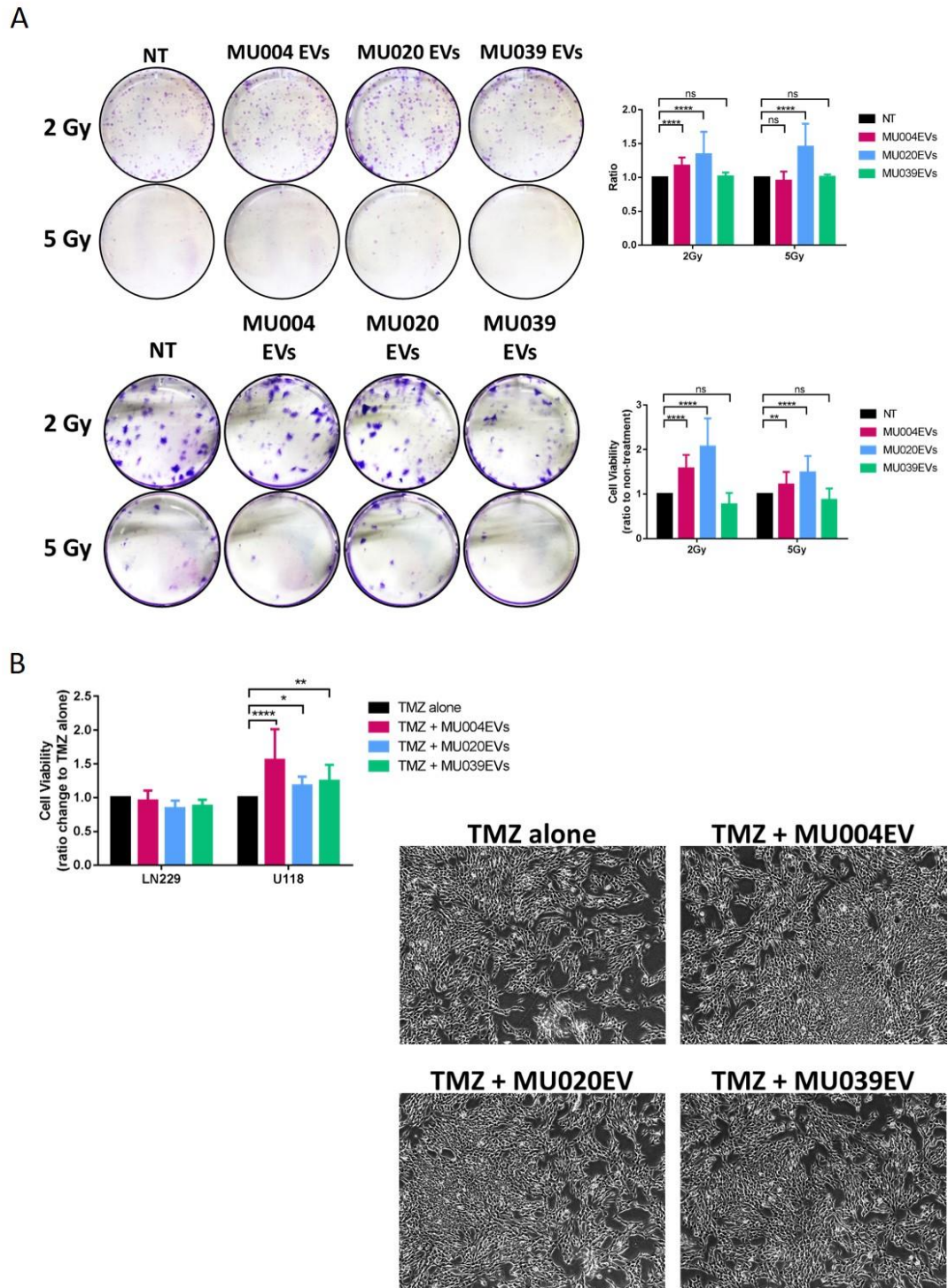


Figure 6.6. GSCs-derived EVs (GSCs EVs) transfer radiation- and chemo- therapy resistance. A, Representative images of colony formation of recipient cells with or without GSC-EVs after exposure to 2Gy and 5Gy radiation dosage. Upper, LN229; Lower, U118. Right panel, the cell viability of LN229 and U118 after exposure to 2Gy and 5Gy radiation and GSCs EVs response. B, Response to temozolomide of recipient cells after GSCs EVs treatment. Right panel, contrast field images of U118 cell 48 hours after temozolomide and GSCs EVs treatment. Significance: ns, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$

6.2.5. Frozen storage has less effect on the function of GSC EVs

As EVs have demonstrated their clinical utility of targeting delivery of drugs and non-invasive circulating biomarkers, we explored whether storage in -20°C of EVs has an effect on the structure and function of EVs. To mimic the clinical transport and storage, the EVs isolated from GSCs were stored at -20°C freezer for two weeks. We performed cryo-TEM and wound healing assay to examine the frozen EVs structure and biological function. As we expected, cryo-TEM showed GSCs EVs retained their membrane structure and morphology regardless of culture media storage conditions (-20°C for two weeks) (Figure 6.7A). In addition, we observed a significant increase of colonies after frozen GSCs EVs treatment in colony formation assay and there was no statistical difference between fresh and frozen GSCs EVs treatment (Figure 6.7B). Similarly, frozen GSCs EVs promoted recipient cells migration and no difference was observed between fresh and frozen GSCs EVs treatment groups (Figure 6.7C).

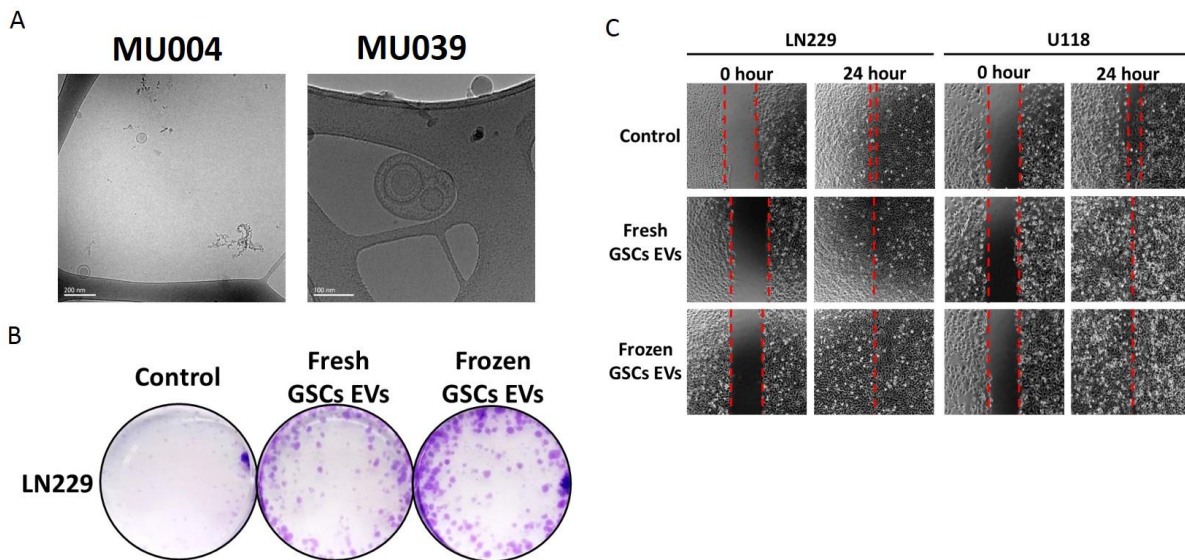


Figure 6.7. GSCs EVs induced radiation resistance in U118 and is biologically functional for recipient cells under frozen storage condition. A, the cryo-TEM showing the structure of GSCs EVs after 2 weeks storage in -20°C . Scale bar is showed as indicated units. B, clonogenic assay showing the formed colonies of LN229 and U118 after fresh GSCs EVs or frozen GSCs EVs exposure. C, Wound healing assay showing the cell migration at 24 hours after fresh

or frozen GSCs EVs exposure. Fresh GSCs EVs, isolated the day for GSCs EVs exposure; frozen GSCs EVs, stored in -20°C for 2 weeks.

6.2.6. GSCs EVs involvement in invadopodia activity

To determine the potential role of GSC-EVs (MU004EVs, MU020EVs and MU039EVs) in extracellular matrix degradation and invasion, gelatin-based zymographic analysis was studied. GSC-EVs contained MMP cargo and both MMP2 and MMP9 bands were detected (Figure 6.8A), with the predominant MMPs being MMP2 and MMP9 in MU020EVs and MU039EVs respectively as compared to MU004EVs (Figure 6.8A). However, incubation of LN229 and U118 recipient cells with GSCs EVs, did not result in a significant increase of MMP2 or MMP9 secretion from these recipient cells (Figure 6.8A). To further determine if MMP2 from GSCs EVs was loaded into the recipient cells, a western blot was performed and an increase of MMP2 was identified in GSCs EVs treated recipient cells (Figure 6.8B).

To further investigate the effect of GSCs EVs on cell invasion, we wanted to ascertain if GSCs EVs uptake could influence invadopodia activity in GBM cells. Surprisingly, pre-incubation of the LN229 and U118 cells with GSC-derived EVs (MU004EVs, MU020EVs or MU039EVs) resulted in a decrease in the level of invadopodia-mediated FITC-gelatin degradation relative to the control cells which were not pre-incubated with GSC-derived EVs (Figure 6.8C). Taken together, these results suggested recipient cells maintained MMPs in cells and slightly reduced the invasion after taking up MMP family from GSCs EVs. Along with previous studies that demonstrated MMPs enriched EVs from cancer cells could degrade extracellular matrix directly and accommodating for cancer cells invasion and migration, we thought GSCs EVs coordinate cancer cells themselves to be less invasive. While there was a dichotomy invasion / proliferation were regarded as two mutually exclusive phenotypes in glioma cells that (Berens & Giese,

1999), we then hypothesized that recipient cells were more proliferative but less invasive after the addition of GSCs EVs.

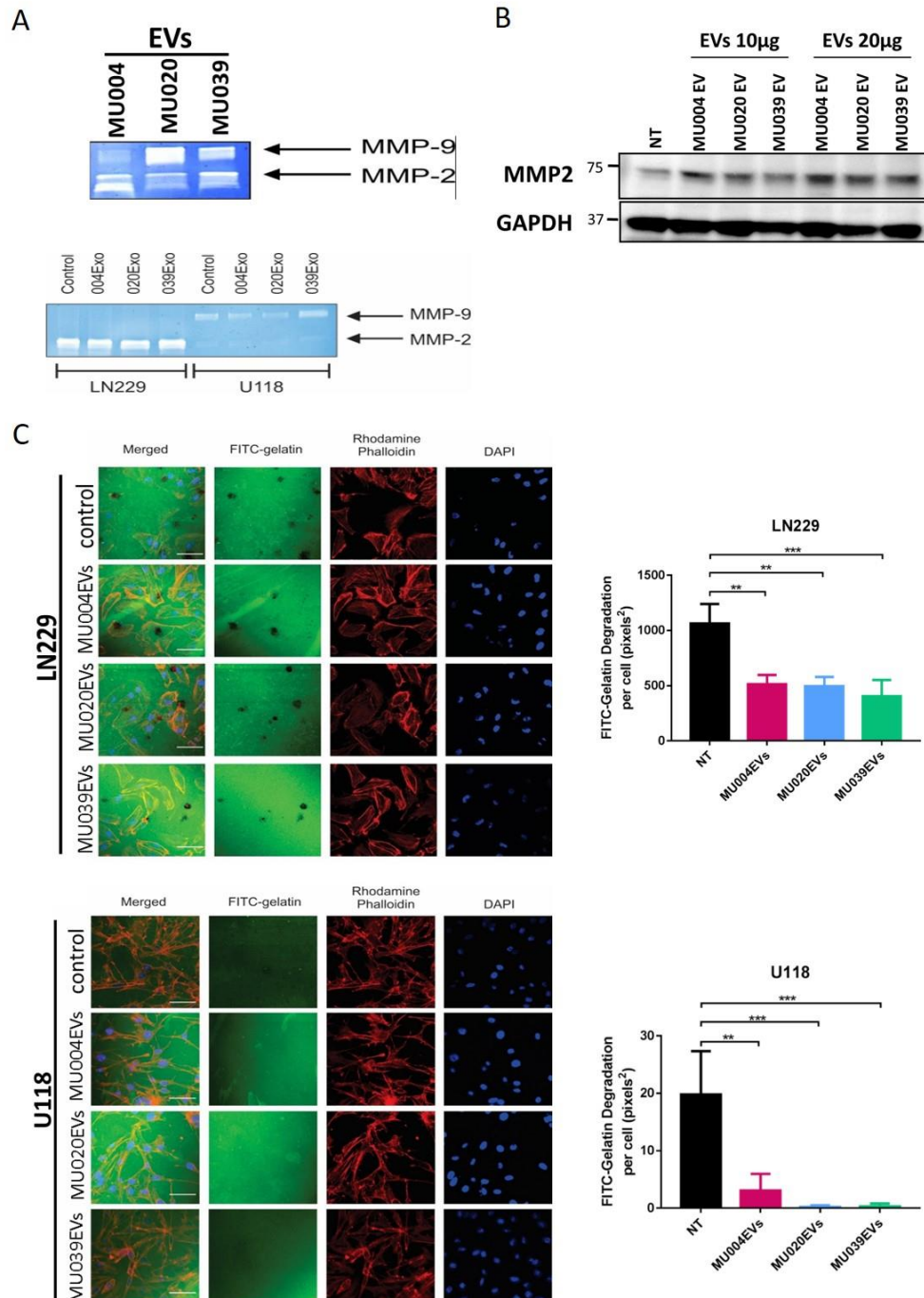


Figure 6.8. GSCs EVs reduced invadopodia activity of recipient cells. A, zymogram assay showing MMP2 and MMP9 expression in GSCs EVs (upper) and GSCs EVs treated recipient cells (lower). B, western blot showing GSCs EVs contained MMP2. C, FITC-gelatin coated degradation of LN229 and U118 cells after GSCs EVs exposure. Significance: ns, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$

6.2.7. GSCs EVs promote glioma cell proliferation by increasing cell cycle

We next investigated the proliferation of recipient cells after GSCs EVs treatment. As we expected, trypan blue assay revealed all GSCs EVs significantly increased the recipient cells numbers at 48 h after treatment as compared with the non-treatment group (Figure 6.9A). Moreover, GSCs EVs also appeared to promote a proliferative phenotype in the recipient cells as confirmed by a significant increase in Ki-67 expression in the GSCs EVs treated groups (Figure 6.9B).

As Ki-67 was expressed in all cell cycle phases in cycling cells, we were not clear which cell cycle phase of recipient cells was increased after GSCs EVs treatment. To further determine which cell cycle phase has been affected by GSCs EVs, we performed cell cycle analyses by flowcytometry. A remarkable increase of G2/M phase was observed in U118 cells 48 h after GSCs EVs treatment and all source of three GSCs EVs promoted significantly G2/M in recipient cells (Figure 6.9C). Taken together, these results indicated that GSCs EVs may facilitate recipient glioma cells to be more proliferative, by increasing the number of cells in G2/M phase and GSCs promoting glioma progression by coordinating differentiated glioma cells to proliferate faster.

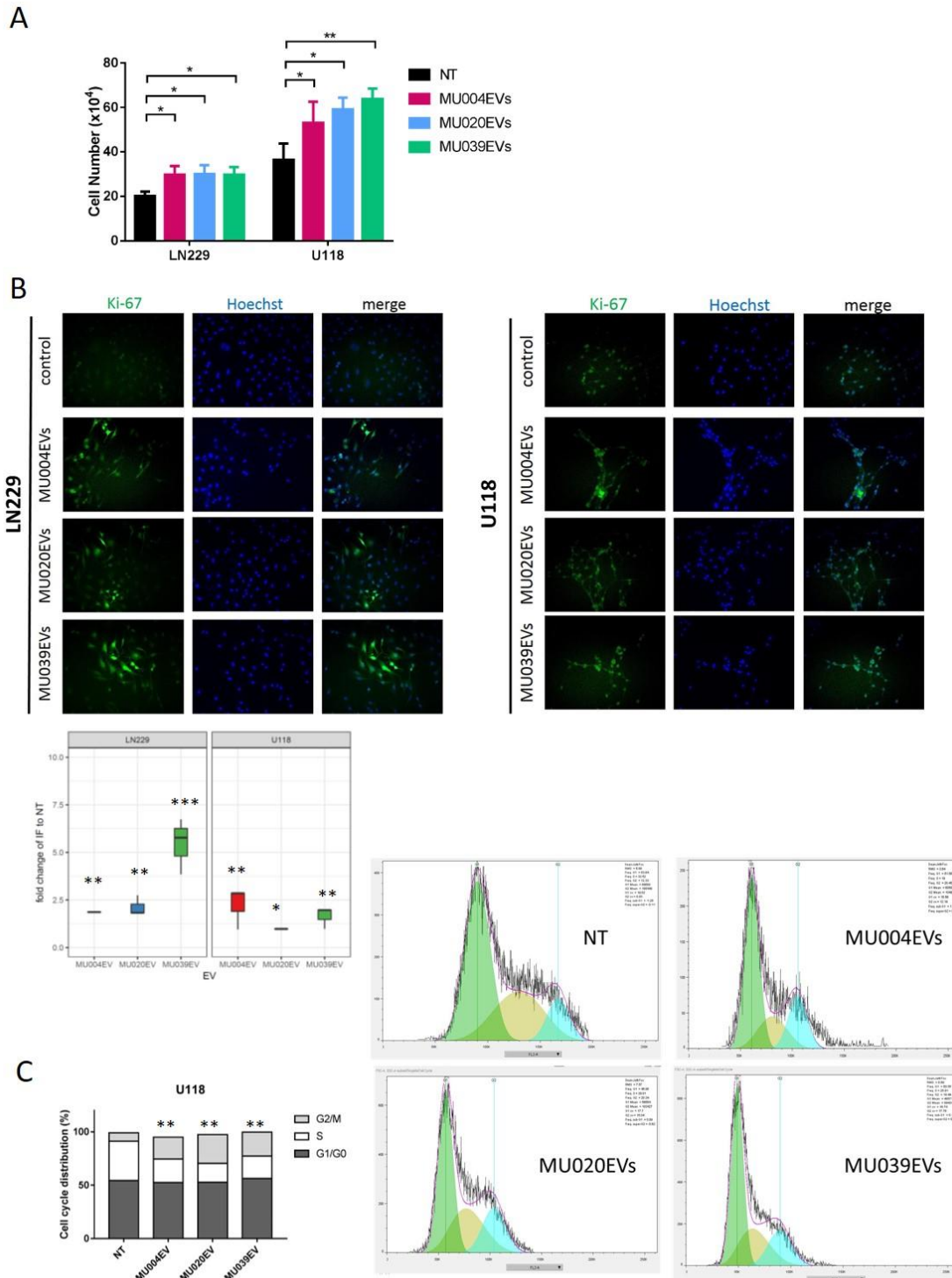


Figure 6.9. GSCs EVs promote recipient cell proliferation by increasing cell cycle. A, Trypan blue assay of increasing cell number of LN229 and U118 in the presence of GSCs EVs. (Mean±SEM) B, Representative images of LN229 and U118 cell proliferation (Ki-67, green; Hoechst, blue) in the presence of GSCs EVs. The quantification of the relative immunofluorescence to NT was calculated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. C, flowcytometry showing the cell cycle change after GSCs EVs treatment (N=2). **, $p < 0.01$.

6.2.8. GSCs EVs enhance tumour progression likely via the AKT/PTEN pathway

We have already demonstrated that GSCs EVs promoted recipient cells proliferation and resistance to therapeutics. These in vitro experiments have confirmed GSCs EVs were functional with regard to regulating tumour microenvironment and accommodating glioma cells invasion and progression. However, the detailed regulatory mechanism remains unclear. Given that the AKT/PTEN pathway is the commonly altered in GBM and another study demonstrated GSCs could activate AKT pathway in differentiated glioma cells (X. Wang et al., 2018), we next investigated this pathway in relation to GSCs EVs exposure. Interestingly, we found PTEN, a tumour suppressor, was significantly down-regulated and subsequent phosphorylated AKT was increased after serial concentration of GSCs EVs exposure (Figure 6.10A&B). The effect of GSCs EVs on recipient cells was in a dose dependent manner (Figure 6.10B&C). Next, we investigated the PTEN expression in primary and recurrent glioblastoma to validate that GSCs increased the malignancy by reduction of PTEN in recurrent GBMs. To address this question, we utilized a public dataset (GSE62153) from Gene Expression Omnibus (GEO) investigating the genes expression from 25 primary and 18 recurrent GBMs. Surprisingly, there was no significant difference of PTEN expression between primary and recurrent GBMs (Figure 6.10D). After further investigation of the clinical information of these 43 GBMs, we found only 5 recurrent GBMs had primary matched samples. Using all unmatched GBMs to investigate the change of PTEN expression would increase the bias and the true significance would likely be concealed by the majority of the whole population. Therefore, we then explored the PTEN expression in five matched samples. As expected, the PTEN expression were significantly reduced in recurrent GBMs as compared with primary GBMs ($p = 0.0313$) (Figure 6.10D), suggesting reduced PTEN expression relative to presence of GSCs EVs is a feature of recurrent GBM. Taken together, our preliminary results showed GSCs EVs activate AKT pathway by reducing PTEN expression, which contributes to glioma progression.

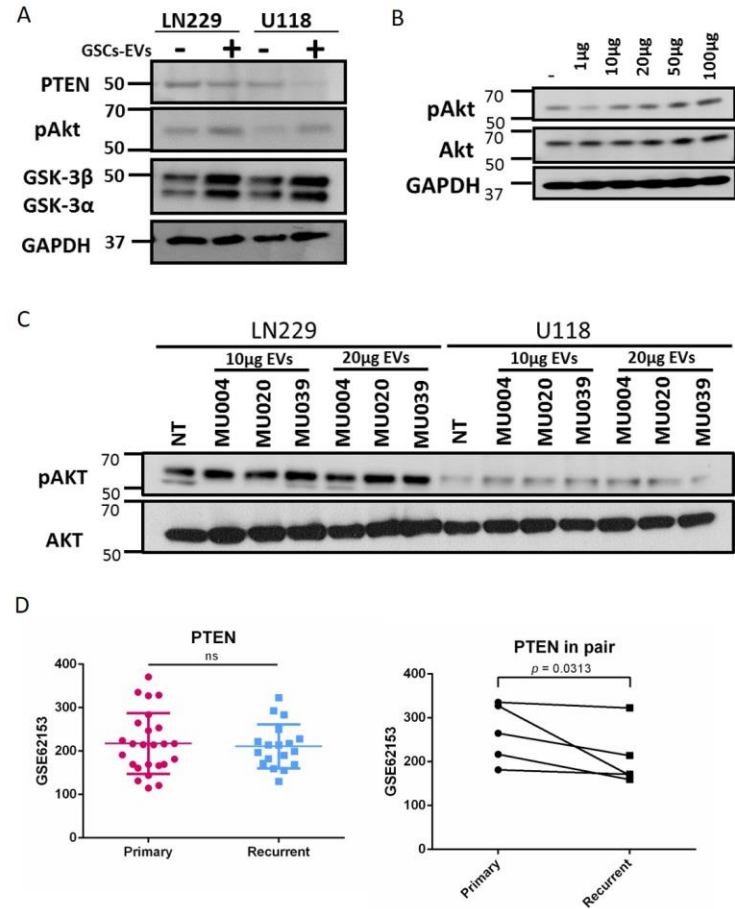


Figure 6.10. GSCs EVs activated AKT pathway in recipient cells by reducing PTEN. A, Western blot showing the PTEN and pAKT expression after GSCs EVs exposure. B & C, Western blot showing the pAKT expression after a exposure of serial concentration of GSCs EVs. D, a public dataset (GSE62153) showing the PTEN expression between 43 primary and recurrent GBMs (left) and 5 matched GBMs (right).

Before further investigating that the AKT pathway activation is required for glioma progression, we tested the different concentrations of AKT pathway inhibitors in vitro experiments. The western blot results of titration of AKT inhibitor X in LN229 and U118 suggested 10 μ M of AKT inhibitor X reduced pAKT expression slightly and 20 μ M of it decreased pAKT expression significantly (Figure 6.11A). Next, we performed cell viability assay to examine whether the AKT inhibitor X has an effect on the glioma cells and which concentration was suitable for reducing the pAKT activation induced by GSCs EVs. There was no effect on glioma cells under 0.1 μ M, 2.5 μ M or 5 μ M of AKT

inhibitor X as compared with non-treatment control group in 6 h of treatment (Figure 6.11B). The application of 10 μ M of AKT inhibitor X showed a nearly 50% reduction of cell viability and 20 μ M of AKT inhibitor X decreased 75% cell viability. To further validate the applicable concentration of AKT inhibitor X, we determined the IC₅₀ of AKT inhibitor X by performing IC₅₀ curve. The result of IC₅₀ also suggested 10 μ M of AKT inhibitor X would be applicable concentration because under this point AKT inhibitor X inhibited 50% of glioma cells (Figure 6.11C). The concentration of AKT inhibitor X was determined at 10 μ M for the following experiments because the restoration of AKT phosphorylation via exosomal miRNAs unlikely happen in the recipient cells if AKT inhibitor X induced more than 75% cell death. In addition, we compared the inhibition effect of BKM120 on glioma cells with that of AKT inhibitor X. Interestingly, western blots demonstrated there was no remarkable inhibitory effects on glioma cells at 6 hours after treatment while we found a significant reduction of AKT in 2 μ M and 4 μ M of BKM120 in both glioma cell lines (Figure 6.11D). Taken together, these results indicated 10 μ M of AKT inhibitor X and 2 μ M of BKM120 were applicable concentration for reversing the effect of GSCs EVs on recipient cells.

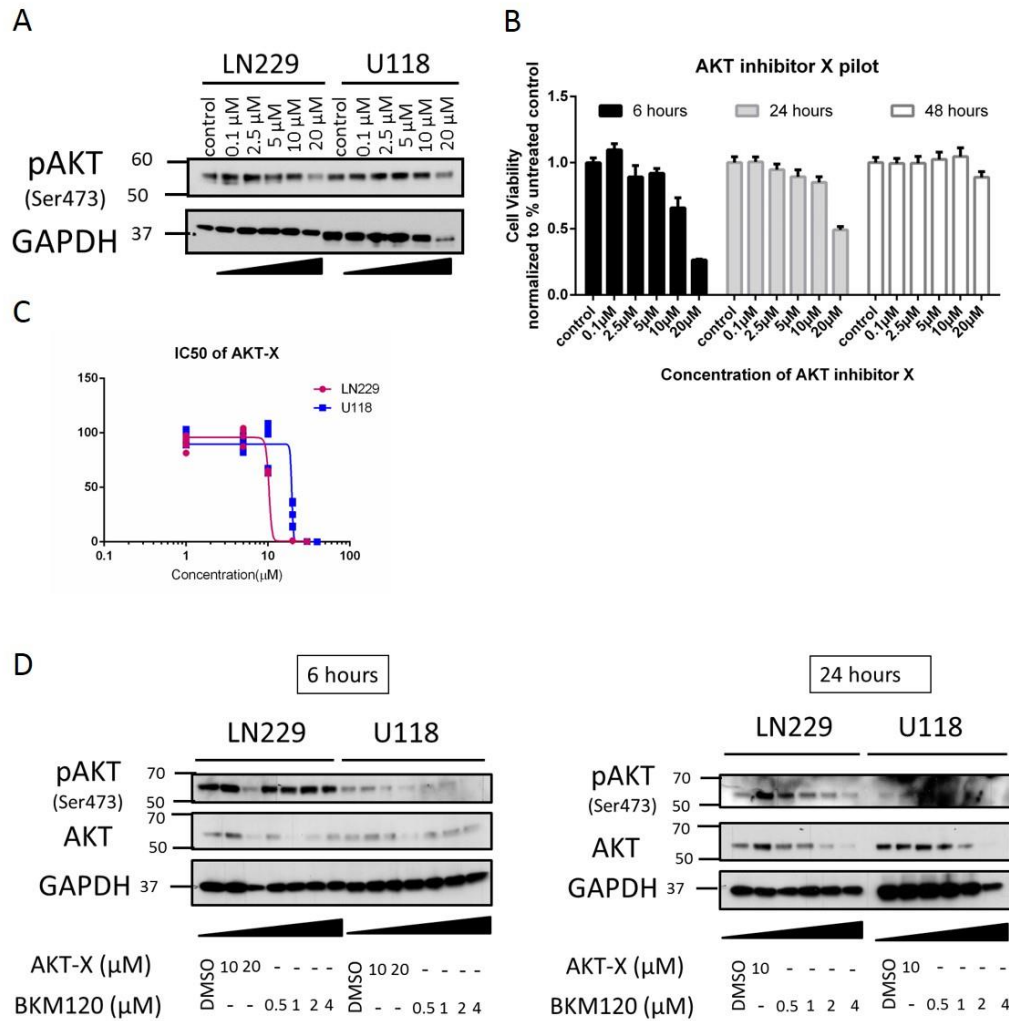


Figure 6.11. The effect of AKT pathway inhibitors on glioma cells. A, Western blot showing the pAKT expression under the titration of AKT inhibitor X. B, the cell viability of U118 under the titration of AKT inhibitor X over 48 hours. C, the IC₅₀ curves of LN229 (red) and U118 (blue). D, Western blot showing the pAKT expression of LN229 and U118 6 hours (left) or 24 hours (right) after the titration of AKT inhibitor X or BKM120.

Once we had determined the suitable concentration of AKT inhibitors (AKT-X), we combined GSCs EVs and AKT inhibitors into LN229 and U118 to investigate the hypothesis that inhibition of AKT attenuated the effect of GSCs EVs on recipient cells. We first tested the pAKT level of recipient cells after GSCs EVs and AKT inhibitor X treatment and found AKT inhibitor X slightly reduced the pAKT expression (Figure 6.12A). Cell viability assay also showed that AKT inhibitor X could partially attenuate

the activation of AKT pathway by GSCs EVs in both LN229 and U118 cell lines, indicating GSCs EVs promote recipient cells progression via activation of AKT pathway (Figure 6.12B). For LN229, AKT inhibitor X significantly reduce the ratio of cell viability from 1.034 to 0.879 ($p < 0.0001$), from 0.993 to 0.922 ($p = 0.0003$), from 1.014 to 0.956 ($p = 0.0082$) in MU004EVs, MU020EVs and MU039EVs treatment groups, respectively. In terms of U118, a significant reduction of cell viability ratio after AKT inhibitor X treatment from 1.129 to 0.978 was observed in MU039EVs group, with the p value of 0.031. There was no statistical significance within MU004EVs ($p = 0.3574$) and MU020EVs ($p = 0.0719$) group, though decreases of cell viability of MU004EVs and MU020EVs group were observed from 1.091 to 1.057 and from 1.126 to 1.036, respectively. We further tested whether the PI3K inhibitor, BKM120 impaired the pAKT level in the presence of GSCs EVs. As expected, the effects of GSCs EVs on LN229 and U118 were significantly decreased by BKM120, suggesting that the inhibition of the AKT pathway was likely an alternative to block the glioma progression induced by GSCs EVs (Figure 6.12C). To further confirm our hypothesis that blockage of the AKT pathway could reduce cell progression after GSCs EVs, we increased the concentration of AKT inhibitor X to 15 μ M and found the cell viability of LN229 and U118 were significantly inhibited (Figure 6.12D). Taken together, these results indicated inhibition of AKT pathway could effectively inhibit glioma cell progression by decreasing the GSCs EVs-induced activation of AKT pathway.

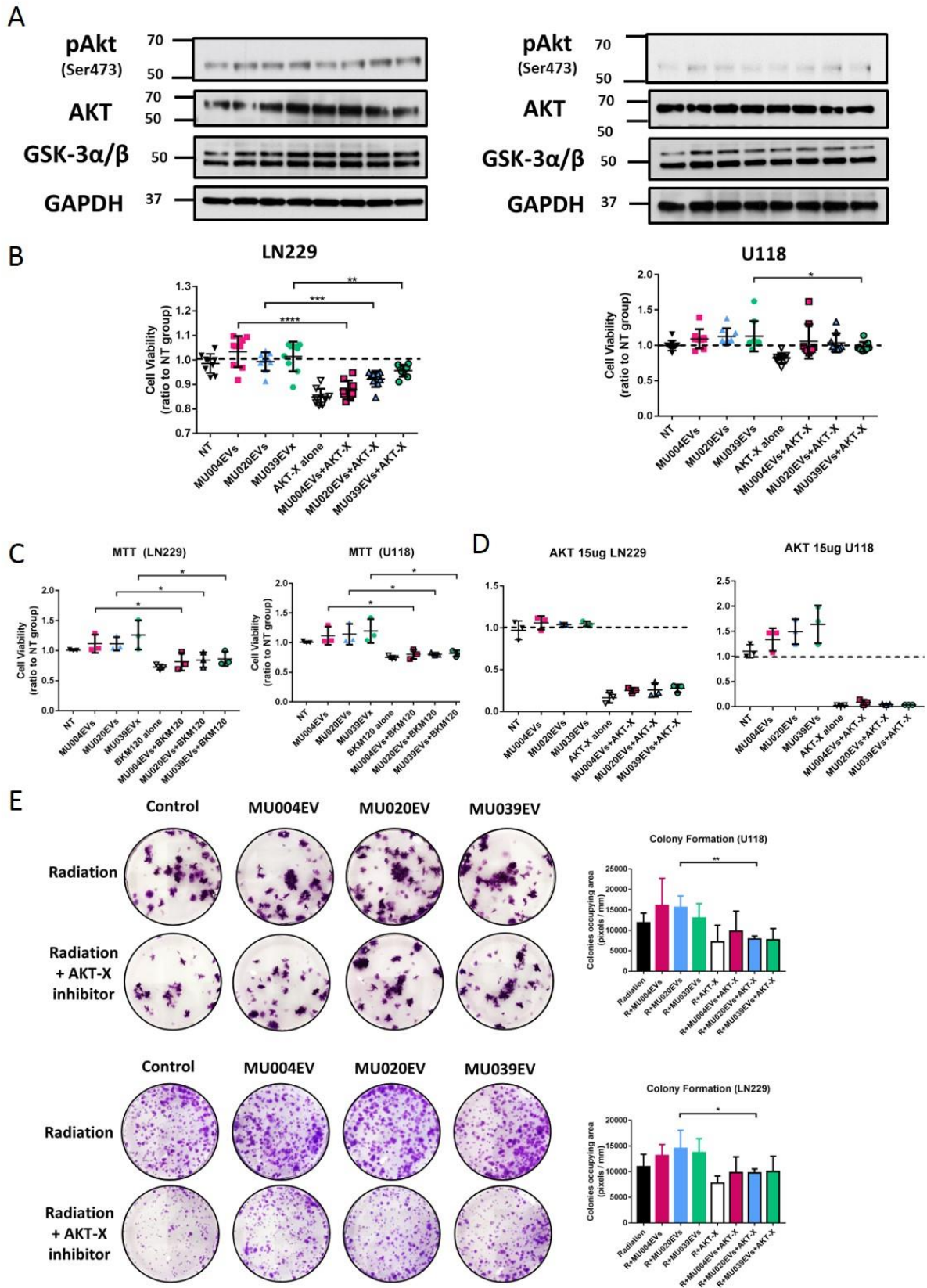


Figure 6.12. The effect of GSCs EVs on recipient cells are reversed by AKT inhibitor. A, western blot showing the pAKT level after GSCs EVs with or without AKT inhibitor X (AKT-X) treatment. Left, LN229; Right, U118. B, Cell viability assay showing LN229 (left) and U118 (right) after GSCs EVs exposure with or without AKT inhibitor X. GSCs EVs concentration: 20µg/ml; AKT-X concentration: 10µM. C, MTT assay showing LN229 (left) and U118 (right) after GSCs EVs exposure with or without BKM120 treatment. GSCs EVs concentration: 20µg/ml; BKM120 concentration: 2 µM. D, MTT assay showing the cell viability after GSCs EVs with/without 15 µM of AKT inhibitor X treatment. E, Colony formation assay showing the colony number and area of LN229 (upper) and U118 (lower) after GSCs EVs with or without AKT inhibitor X in presence of 2 Gy irradiation. Student's t test was applied. Significance: ns, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$

Then, to further investigate whether AKT inhibition reduce the therapeutic resistance transfer by GSCs EVs, we performed the colony formation assay again. After 2 Gy irradiation along with GSCs EVs treatment to LN229 and U118, AKT inhibitor X reduced the colonies numbers and the covering area of colonies as compared with irradiation treatment alone (Figure 6.12E). Considering the MU020 were strongly associated with radiation resistance, we then investigated whether AKT inhibitor X reduced the colonies after irradiation and MU020EVs treatment. Interestingly, AKT inhibitor X significantly decreased the radiation resistance induced by MU020EVs in both recipient cell lines, suggesting the resistance transfer by MU020EVs required the activation of AKT pathway (Figure 6.12E). In summary, the effect of cell progression and radiation resistance transfer induced by GSCs EVs likely required activation of AKT pathway. Further experiments that applying GSCs EVs to AKT knockout or knock-down glioma cells are also needed to validate our hypothesis in the future.

6.2.9. GSCs EVs promotes sphere formation in GSCs

To test the transfer of GSCs EVs between GSCs, sphere formation assay was employed after GSCs EVs exposure to MU035 and co-culture of MU035. MU020 and MU039 EVs significantly promoted MU035 spheres formation (Figure 6.13A). To investigate the mutual interaction and communication of GSCs, co-culture assay was also performed. As our laboratory has identified MU035 is proneural GSCs and the change of cell

phenotypes of proneural GSCs with GSCs EVs remains unclear, we chose MU035 as recipient cell here. Similarly, our co-culturing assay revealed all GSCs increased the spheres numbers after 3 days co-culture, indicating the transfer of EVs between GSCs would enhance GSCs self-renewal (Figure 6.13B).

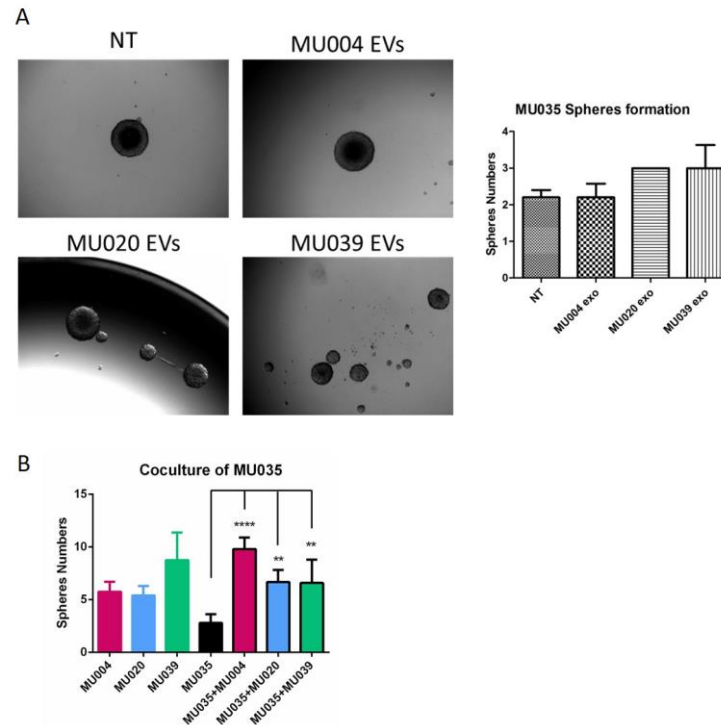


Figure 6.13. GSCs-derived EVs (GSCs EVs) promote intratumour heterogeneity. A, Spheres formation assay showing the sphere sizes of MU035 after GSCs EVs exposure. Typical images were represented (magnification: X100). B, Co-culture assay showing the sphere numbers after 3 days of co-culture. A total of 2000 GSCs was seeded in 96 well plates. Co-culture ratio is 1:1, namely 1000 MU035 cells and 1000 other GSCs.

6.2.10. miRNAs cargo in GSCs EVs targeted PTEN/AKT pathway

As miRNAs were important cargo in neurological disease and the transfer of miR-21 targeting PTEN in astrocytes from breast cancer accommodated breast cancer metastasis (Becker et al., 2016; L. Zhang et al., 2015b), we then examined the profile of miRNAs expression from our four GSCs EVs (MU004, MU020, MU035 and MU039). GSCs EVs were profiled using Nanostring miRNA assay and analysed using nSolver analysis

software (Nanostring version 3) and R statistical program package. A list of 25 miRNAs were found commonly expressed in EVs derived from 4 GSC cell lines investigated (Table 6.1). Of this, 6 miRNAs can target the PTEN pathway when analysed and compared using TargetScan, suggesting the decreased PTEN expression were likely caused by loading and release of miRNAs after internalization of GSCs EVs.

Table 6.1. The top 25 highly expressed miRNAs from GSCs EVs

This table is now not public due to confidential and sensitive materials.

6.2.11. RAB27A is strongly correlated with progression in glioma

Considering Rab27a controls the secretion of EVs in cancer cells and higher expression of Rab27a is associated with higher production of EVs (Bobrie et al., 2012; Ostensfeld et al., 2014), we then explore the RAB27A expression in glioma using public database. RAB27A expression in grade IV glioma were significantly higher than those in grade III glioma and grade II glioma (Figure 6.14A), indicating RAB27A is strongly associated with the malignancy of glioma. Moreover, Kaplan-Meier curves of overall survival and progression free survival revealed patients with higher expression of RAB27A would have an unfavourable survival in three independent cohorts (Figure 6.14B). Cox proportion hazard regression analyses also demonstrated RAB27A and IDH1 mutation status were independent prognostic markers in GBMs (Table 6.2). Due to mesenchymal subtype of GBM is linked with therapeutic resistance, we then investigated RAB27A expression in subtypes of GBM. Expectedly, RAB27A is markedly higher in mesenchymal GBM than the other three subtypes (Figure 6.14C). To further explore the association of RAB27A and stemness markers, we then calculated the Pearson correlation of these genes expression in GBMs. Surprisingly, RAB27A was inversely correlated with all four glioma stemness markers (Figure 6.14D), indicating GBMs containing less GSCs

properties likely released more EVs into microenvironment. Further experiments are required to investigate the relationship and underlying mechanism of GSCs and RAB27A.

Table 6.2. Cox analysis of RAB27A in relation to OS and PFS of patients with GBM.

Overall Survival						
Variable	Univariate			Multivariate		
	HR	95% CI	p Value	HR	95% CI	p Value
Age	1.004	0.997-1.011	0.214			
Karnofsky Score	0.998	0.990-1.006	0.613			
IDH1 status	0.377	0.216-0.659	0.001	0.376	0.215-0.656	0.001
RAB27A	1.097	0.998-1.207	0.055	1.101	1.000-1.212	0.045
Progression Free Survival						
Variable	Univariate			Multivariate		
	HR	95% CI	p Value	HR	95% CI	p Value
Age	1.003	0.995-1.010	0.456			
Karnofsky Score	0.997	0.989-1.006	0.524			
IDH1 status	0.582	0.350-0.966	0.036	0.599	0.361-0.996	0.048
RAB27A	1.129	1.021-1.248	0.018	1.121	1.013-1.240	0.027

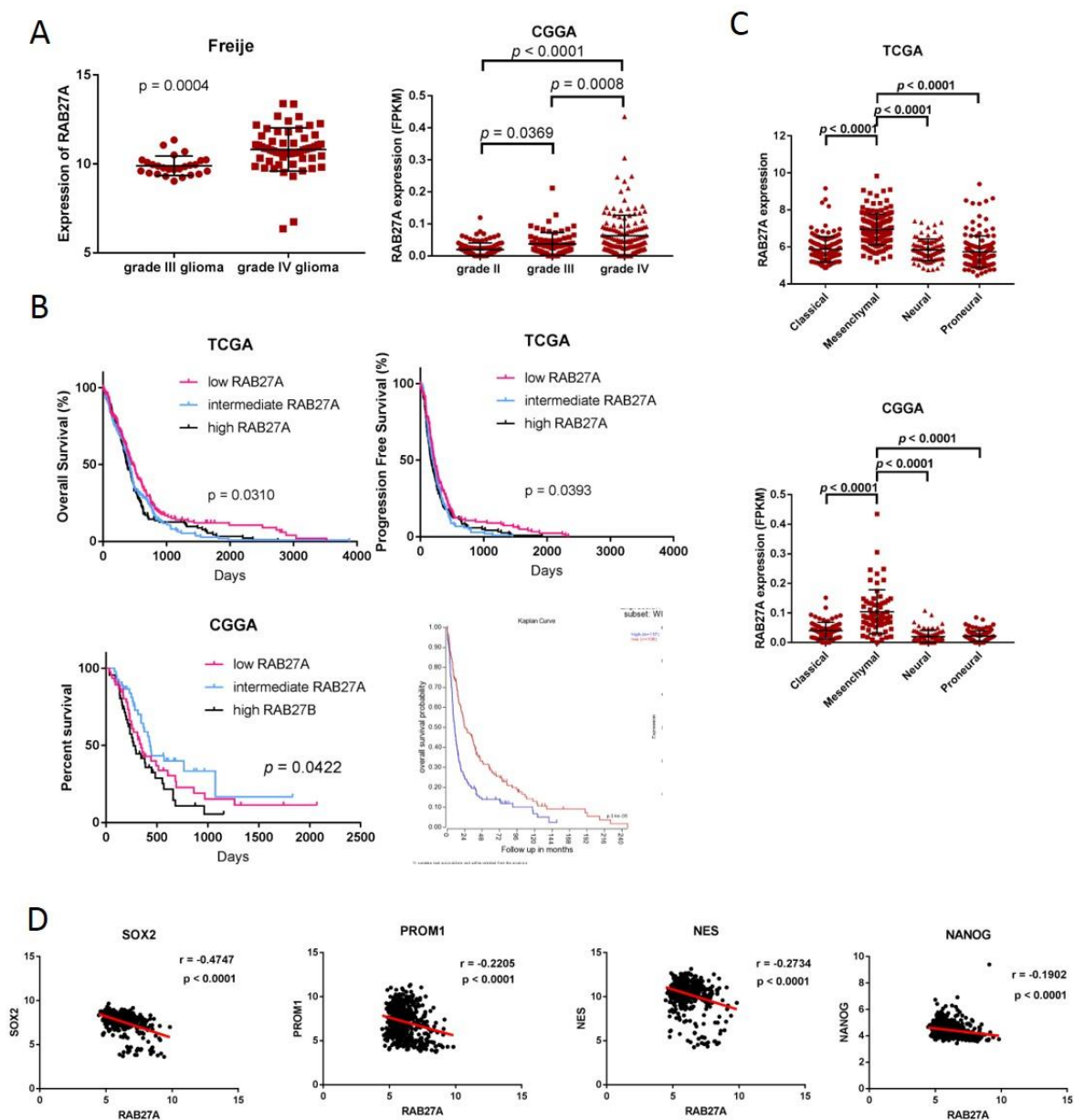


Figure 6.14. RAB27A expression is correlated with progression of patients with GBM. A, the expression of RAB27A in grade III glioma and grade IV glioma. B, Kaplan Meier Curve for overall survival and PFS of GBMs High RAB27A versus low RAB27A. C, the expression of RAB27A between subtypes of GBMs. D, the Pearson correlation of RAB27A and stemness markers. Red line, linear regression line.

In conclusion, EVs released by GSCs could be taken up by recipient glioma cell in the microenvironments. Once glioma cells took up GSCs EVs, they would become more proliferative and promote glioma progression by activating AKT pathway (Figure 6.15).

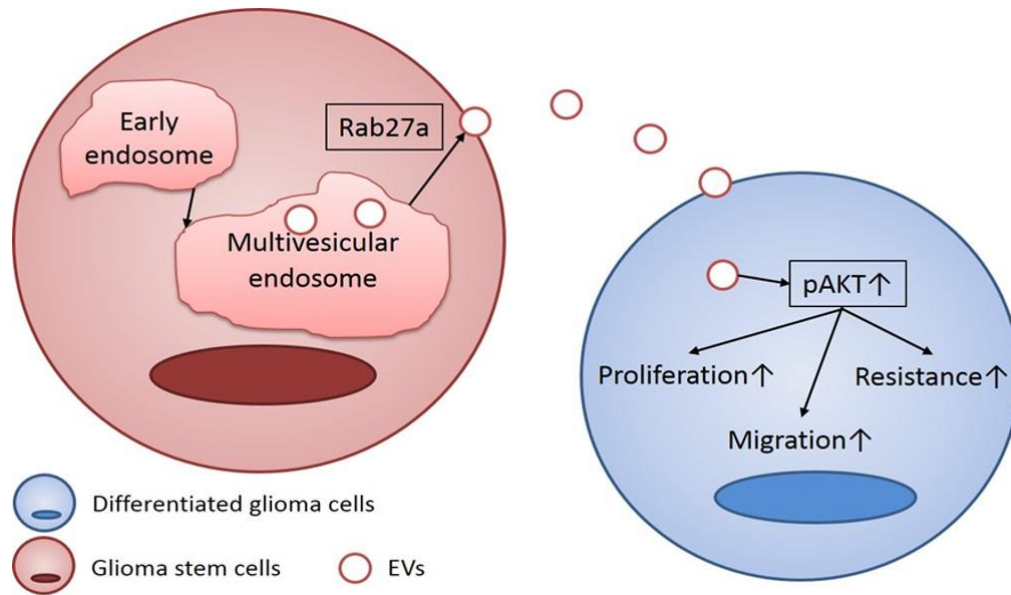


Figure 6.15. The schematic diagram showing the mechanism of GSCs-EVs regulating glioma cells.

6.3. Discussion

Accumulating evidence demonstrates that EVs derived from cancer cells promote cancer progression by setting up the pre-metastasis niche and confer enhanced therapeutic resistance to sensitive cancer cells to resistance to them (Becker et al., 2016; Costa-Silva et al., 2015; A. Hoshino et al., 2015; Y. Liu et al., 2016; Matei, Kim, & Lyden, 2017; Pavlyukov et al., 2018; Peinado et al., 2017; Qu et al., 2016b; L. Zhang et al., 2015b). The cargos in EVs, mainly miRNAs and proteins, packaged and transported by EVs, are taken up by the recipient cells by reprogramming or modulating the signaling pathways or specific genes expression of recipient cells (L. Zhang et al., 2015b). Cancer cell

derived EVs usually mediate cells in microenvironment such as fibroblast, macrophage and microglia in brain to accommodate their metastasis in turn (A. Hoshino et al., 2015; Kamerkar et al., 2017; L. Zhang et al., 2015b). Moreover, further understanding of neural stem cells derived EVs shed a light on the intercellular regulatory mechanism by stem cells derived EVs in brain (Y. Zhang et al., 2017). Our results revealed that GSCs derived EVs promote glioma cells migration and induce cell survival after radiation and chemotherapy treatment. We also found GSCs EVs are likely to promote recipient cancer cell progression by activating AKT signaling pathway.

With the advances in high throughput sequencing, the heterogeneous profile of glioma was recognized and moreover, cellular hierarchy was revealed in glioma progression and therapeutic resistance. We observed a large variance of cell lines and GSCs in all experiments due to the heterogeneity of gliomas. Both cell lines and GSCs only represent a small subpopulation of glioma. LN229 is a p53 mutated and PTEN wild-type glioma cell line while both p53 and PTEN mutation are identified in U118, which leads to the variance of cell phenotypes and biological behaviors between these two cell lines. Furthermore, GSCs were found in low grade glioma, the early stage of glioma development as well as present in grade IV glioma and recurrent gliomas. A few studies have recaptured these features of GSCs EVs cargoes like RNA and protein profiles by next generation sequencing and mass spectrometry. The features from previous studies showed the cargoes in EVs derived from glioma cells contained RNAs and proteins that were associated with cell migration and invasion. GBM-derived EV increased angiogenic growth in human microvascular endothelial cells (HBMVEC), migration and neurosphere formation in recipient cells (Bronisz et al., 2014). However, whether GSCs EVs are taken up by the recipient glioma cells and what their function is remains unclear. The feature that cancer cells can invade and migrate to distant sites is regarded as the driving force to cancer progression and lethality. Our results demonstrated that GSC-EVs promote differentiated glioma cells migration, indicating that the distance dissemination is likely to occur in presence of GSCs EVs. To further explore whether this enhanced mobility of

recipient cells is accompanied with the degradation of extracellular matrix to penetrate and invade the microenvironment, we employed invadopodia activity assay. Notably, invasion of recipient cells is not improved, instead, it is reduced. Although both proliferation and invasion are cancer cell characteristics, these two features are likely to be mutually exclusively (Kohrman & Matus, 2017). Compared with normal cells, tumour cells are more proliferative and invasive. For a tumour cell, it needs to invade by stopping dividing. We chose commercial glioma cell lines as recipients because differentiation induced GSCs could be cultured as glioma spheres when they were transferred into neural basal media (data not shown). Therefore, commercial glioma cell lines are more suitable for the recipient cells than differentiated GSCs. Meanwhile, the cargoes of GSCs EVs are subtype specific, where it coordinates the equilibrium of GSCs hierarchy. Mesenchymal GSCs derived EVs transfer their subtype specific molecules into proneural GSCs and affect proneural GSCs phenotypes but could not affect differentiated mesenchymal GSCs, suggesting GSCs EVs might not affect differentiated GSCs counterparts (Garnier et al., 2018; Nakano, Garnier, Minata, & Rak, 2015). Therefore, to explore the resistance transfer of GSCs EVs, LN229 and U118 were utilized as recipient cells.

Before the investigation of the biological features of EVs in glioma, we also tested the stability of EVs isolated from GSCs to confirm the storage conditions does not affect the structure and biological functions. The reason why we should test it is because EVs samples from patients biofluids might be transported and stored in freezer before testing. Our results supported EVs are stable in frozen status.

It is widely accepted that therapeutic resistant cancer cells have the ability to transfer resistance to sensitive cancer cells by EVs in renal cancer, breast cancer and colorectal cancer (Boelens et al., 2014; Y. Hu et al., 2015; Qu et al., 2016a). There are two ways to enhance therapeutic resistance in the ecosystem. Cancer cells secrete EVs containing lncRNA and proteins to educate drug or radiation sensitive cells in the microenvironment. On the other hand, stromal cells in turn help cancer cells survive after treatment.

However, the drug resistance in glioma transferred by EVs has not been fully elucidated yet. High throughput tool has already screened out glioma diagnostic markers such as EGFR, EPHA2, are prone to be found in EVs. More importantly, drug resistance markers like MGMT, APNG, ABCC3 and IGFBP2 were also identified in GBM derived EVs but the issue whether the EVs containing resistance markers can be taken up by the recipient cell and then help glioma cells survive after drug or radiation remain unclear (H. Shao et al., 2015). Our results revealed glioma cells treated with GSCs EVs formed more colonies after radiation and survive after TMZ treatment, indicating GSCs not only resist the therapeutic treatment themselves but also have effect on the neighboring cells to coordinate the tumour evolution after treatment where resistance glioma cells are the dominant population in the treated glioma and recurrent glioma (J. Wang et al., 2016). Noticeably, CD44 enriched GSCs EVs showed the most significant effect on rescuing the radiation effects on recipient cell after GSCs EVs exposure. Our previous study has demonstrated CD44 is a reliable surface marker for mesenchymal GSCs (Brown et al., 2017) and mesenchymal GSCs are strongly linked with worse outcomes in patients' survival and treatment response (Shahar et al., 2017). Moreover, incubation of cells with mesenchymal-derived exosomes can facilitate tumour progression. The mesenchymal-derived exosomes were not only observed to have an effect on the normal support cells (astrocytes), but they also produced a phenotypic change on the other molecular subtypes of GBM that were present.(de Vrij et al., 2015) Therefore, inhibition of EVs releasing or neutralizing in glioma will be the potential therapeutic target in the future.

Invasion related proteins were identified from glioma cells EVs in previous study, which suggested they might be responsible for promoting glioma invasion after these EVs were neutralized in recipient cells. However, our results demonstrated the proliferation, not invasion of recipient cells is enhanced after GSCs EVs treatment, which are consistent with 'Go or Grow' theory (Bronisz, Godlewski, & Chiocca, 2016). This invasion or proliferation dichotomy was described as invasive glioma cells are usually low proliferative cells while less invasive glioma cells are likely to be associated with high

proliferation (Dalrymple et al., 1994; Schiffer, Cavalla, Dutto, & Borsotti, 1997). This inverse correlation of invasion and proliferation of glioma cells warrants the dynamic balance of cells survival when confronted with therapeutic stress (Giese, Bjerkvig, Berens, & Westphal, 2003). Considering GSCs are relatively slow cycling cells, we believe GSCs must maintain glioma outgrowth by coordinating differentiated cells into fast cycle of cell growth, where GSCs achieve this goal by EVs. The extracellular matrix is probably degraded by EVs directly through releasing invasion related proteins into microenvironment where GSCs EVs pave the way to glioma cell outgrowth (D. Hoshino et al., 2013). It also explains why invasion related proteins were identified in glioma EVs but they failed to promote recipient cells invasion instead.

Given GSCs EVs promote differentiated glioma cells proliferative rather than invasive phenotype, we explored the proliferation-related signal pathways. In glioma, PI3K/AKT pathway is one of the most mutated and it promotes cell growth when activated (Cancer Genome Atlas Research, 2008). Our results confirmed after GSCs EVs loading the AKT pathway of recipient cells were activated by phosphorylation of AKT. Due to the key role of signal transduction process in cell proliferation and cell growth, the activation of AKT results a significant increase in cell number after EVs treatment to support glioma outgrowth. Considering GSCs EVs could accommodate cell grow and progression by degrading extracellular matrix directly using EVs cargoes, glioma cells have the suitable environment to expand and therefore it leads to glioma recurrence. This coordinated biological system regulated by GSCs EVs elaborately provide a novel target to treat glioma in the future.

Besides the AKT pathway, a few oncogenic signaling pathways are activated by EV transfer of miRNAs. Yue et al found through targeting TCEAL7, hypoxic glioma cells derived exosomal miR-301a mediates Wnt/ β -catenin signaling and promotes radiation resistance (X. Yue, Lan, & Xia, 2019). Hypoxic glioma cells also suppressed immune cells response via exosomal transfer of miR-10a and miR-21 targeting Rora and PTEN pathway, respectively (Guo et al., 2018b). Glioma derived exosomal miR-21 also

participates in the angiogenesis by targeting VEGF signaling (X. Sun et al., 2017). The networks mediated by exosomal miRNAs highlight the complexity of microenvironment in glioma.

Although Rab27 family expression is strongly associated with patient survival in prostate cancer (Ostenfeld et al., 2014), few studies have investigated the relationship between RAB27A expression and GSCs. We found consistent with a previous small cohort, RAB27A is an independent prognostic marker and their expression indicate the worse overall survival. Notably, GSCs have more expression of Rab27a than their differentiated counterparts, suggesting GSCs likely prompt to mediate neighboring cells into GSCs-like feature by transporting cargoes in EVs. This dynamic regulation by GSCs is also described as the phenotype copying in cancer cells that some phenotypic traits are likely to be transferred into the secondary cells by EVs (O'Brien et al., 2013). On the other hand, we have already demonstrated that the effect of EVs transfer is a dose dependent manner, indicating that higher proportion of GSCs in glioma is probably responsible for the recurrence and therapeutic resistance.

There are also some limitations in this Chapter. Although we characterized the top expressed miRNAs in GSCs EVs and their putative target, PTEN, it still needs to be validated that these GSCs EVs featured miRNAs can bind to the 3' UTR of PTEN by luciferase assay. Secondly, we found glioma cells have required the radiation resistance by GSCs EVs but we did not test whether stemness could be induced by GSCs EVs in differentiated glioma cells through the activation of AKT pathway. Lastly, we investigated the phenotype changing of recipient cells when GSCs acted as donor cells but we did not test whether differentiated glioma cells could promote radiation resistance and cell proliferation. Other group has reported the gene expression pattern of normal endothelial cells is largely altered by glioma cells EVs but transfer of glioma cells EVs to glioma cells and its consequences have not been reported yet (C. C. Li et al., 2013).

The utility of EVs are unlimited in the future as EVs are easily captured in patient blood and an ideal biofactory for drug delivery. EVs in the circulation was a promising biomarker in cancer. EGFRvIII, the most common variant in GBM, are detectable in glioma cell derived EVs (Skog et al., 2008), which allows EVs becoming a non-invasion biomarker for diagnosis and dynamic monitoring GBM patients. Considering EVs is a biorepository enriched with non-coding RNAs (Z. Wei et al., 2017) along with the fact that miRNAs are promising biomarker for glioma (C. Ma et al., 2018), the potential role of miRNAs in EVs should be further investigated in the future.

Summary and future directions

Glioma is a heterogeneous disease and results from simple tissue biopsy sometimes are not the best proxy for the molecular profiles of glioma. Considering the invasiveness of tissue biopsy, liquid biopsy is a promising complementary test to the current standard of management. To identify the clinical utility of circulating miRNAs in glioma, we performed a meta analyses of existing literature, which demonstrated that circulating miRNAs could be very good diagnostic biomarkers in glioma with high sensitivity and specificity. Interestingly, miRNAs from CSF are more accurate in diagnosing glioma than miRNAs from blood. However, CSF is much more difficult to collect than blood samples in clinical practice due to its technique requirements, whereas detection of miRNAs from blood sample is an ideal method for liquid biopsy in glioma.

Through bioinformatic analyses, we found IDH1 mutation associated miRNAs in the TCGA dataset. All these miRNAs are down-regulated in IDH1 mutation glioblastoma and their promoter areas are hypermethylated. These results suggest IDH1 mutation introduced global methylation not only leads to the transcripts alteration but also the change of expression profile of miRNAs in glioma. In addition, these IDH1 mutation associated miRNAs are potentially the biomarkers for detection of IDH1 mutation in glioma. Further studies are needed to explore the correlation between these miRNAs and IDH1 mutation status and their diagnostic capacity in glioma.

Although IDH1 mutation associated miRNAs are identified from tissue samples, our results found none of these miRNAs in serum has a direct relation with IDH1 mutations. Considering the results from our proof-of-concept study that circulating miRNAs are remarkable indicators of tumour status and potential indicators of recurrence, further serum biomarker discovery study need to be conducted to investigate the concordance of circulating miRNAs and outcomes based on MRI and survival.

EVs, the intercellular communicator, play important roles in cancer metastasis and tumour microenvironment formation. MiRNAs are packaged and delivered into distinct cells by EVs in variety of cancer types. Our results found GSCs derived EVs promote cell progression by activation of AKT pathway. MiRNAs in GSCs EVs are thought to be the driving force promoting glioma progression via EVs, by targeting and downregulating PTEN. Future studies including in an animal model will be conducted to further explore the effects of inhibition of EVs releasing or uptaking and GSCs featured circulating miRNAs on glioma progression. In this animal experiment, RAB27A inhibition will be delivered into SCID mice with GSCs to prohibit the release of EVs containing miRNAs from GSCs. The response to the radiation and chemotherapy as well as the survival of these mice will be compared between the groups with or without RAB27A inhibition. The blood samples of mice will be taken to evaluate the inhibition effectiveness of RAB27A inhibition via the abundance of GSCs derived EVs and EVs miRNAs. This pre-clinical study will be a proof-of-concept that inhibition of GSCs EVs sensitize the radiation and chemotherapy in glioma.

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Appendix

Appendix 1

Appendix table 1. The target genes of IDH1 associated miRNAs predicted by miRwalk.

ABCB7	BMPR2	DRG2	GRM6	LGALS8	MPP2
ABL2	BNIP2	DSC2	GTF2H1	ABLIM1	MPZ
ACHE	DST	DYRK1A	GUCY2C	LIPA	MTF1
ACVR1	KLF5	S1PR1	HAS3	LMAN1	MTR
ADAM10	LDLRAD4	EDNRA	HLA-C	LRP2	MYBL1
ADCY2	TMEM50B	MEGF9	HLA-DQB1	MXD1	MYH11
ADCY7	CACNA1E	ELAVL2	HLA-DQB2	MAF	MYO1D
ADCY9	CAMK2A	ELAVL4	HOXC8	MAFG	MYO5A
AK2	CANX	EMP1	AGFG1	MAOA	MYO9A
ALCAM	CAST	EPAS1	IKBKB	MAP1B	PPP1R12A
ANGPT2	RUNX1T1	EPHA4	IL6ST	MCC	NAB1
ARF4	CCKBR	EPHA8	IL15	MDM4	NAGA
ARSB	CCT6A	ESRRG	INHBB	MEF2A	NAP1L1
ASPH	ENTPD1	ETF1	ITGA3	MAP3K4	NCAM1
ASTN1	CDK6	ETS1	ITGA5	MEOX2	NCAM2
ZFHX3	CFL2	EYA3	ITGB8	MET	NDP
ATP1A2	CHD4	ACSL3	ITPK1	MGAT5	NDUFA10
ATP2A2	CHRNA2	FGF2	JARID2	CIITA	NEUROD1
ATP2B2	COL2A1	FGF9	KCNA2	MITF	NFATC2
ATP2B3	COL4A1	FLT1	KCNC4	ATXN3	NFIA
ATP2B4	CPT1A	FMR1	KCNN3	MAP3K9	NFIX
ATP7A	CREB1	GJA3	KCNQ3	KMT2A	NFYA
BAI3	CSF1	GLG1	KIF5A	AFF1	NHS
BCL7A	GADD45A	GNAO1	KIF5C	MLLT6	NID1
CEACAM1	DMXL1	GNS	TNPO1	MMP13	NKTR
BNC1	DLG2	GPM6A	LBR	MMP15	CNOT4
BMP3	DMPK	GRID2	LCP2	MNT	NPTX1
BMPR1B	DNMT1	GRIK3	LDLR	MOBP	NRAS

NUMA1	PPP2R2C	SGCB	TGFB2	CUL3	KIAA0226
OPA1	PRKAA1	SGCD	TGFBR1	PIK3R3	SART3
OTC	PRKAR2A	SIM1	NR2E1	LMO4	PHACTR2
OTX1	PRKCB	SKP1	TRAPPC10	CDC14A	TBKBP1
OTX2	PRKG2	SLC1A4	TNFRSF1B	PPAP2C	KIAA0232
PAFAH1B1	MAPK10	SLC2A1	TP53	NCOA1	NUPL1
PAFAH1B2	MAP2K1	SLC2A3	HSP90B1	PDE5A	TOMM70A
SERPINE1	PRNP	SLC5A3	TRAF3	VAMP4	ZBED4
PAM	PRRG1	SLC7A2	TRPS1	NOL4	SNAP91
PAX6	MASP1	SLC8A1	TSPYL1	ADAM23	SRGAP3
PBX3	PSMA5	SLC22A5	UBE2D1	NRP1	KIF14
PCCB	PSMD1	SMARCC2	UBE2D3	CDK5R1	MAFB
PDE1A	PSMD9	SMS	UCP3	SYNJ1	OXSRI
PDE1C	PTEN	SNCA	UGCG	MTMR3	TNFSF15
PDE4B	PTGER2	SNX2	USP4	BSN	SH2B3
PDE4D	PTGER3	SOS1	USF1	BTRC	RASGRP1
PDE7A	PTGS1	SOS2	VAV2	LIMD1	LRPPRC
PDK1	PTGS2	SOX11	YWHAB	SLC13A2	ATP6AP2
PDK4	QSOX1	SPTB	ZNF74	USP6	STX6
SLC26A4	PTPN14	SRF	SLC30A4	SLC16A6	IKZF1
PFAS	PTPRA	SRPK2	FZD5	DYRK1B	PGRMC2
CDK14	PTPRD	SSR1	BRPF1	DLGAP1	LRRC41
PHEX	PVRL1	STAR	USP7	CYTH3	VTI1B
PIGA	ABCD3	STK11	KCNAB1	SRSF11	CRTAP
PIK3C2A	PXMP2	STX3	TFEB	B4GALT5	NEBL
PI4KA	RAG1	STXBP1	ARHGEF5	PLAA	TGOLN2
PLN	KDM5A	SYT1	TUSC3	ZNF264	TXNIP
PML	ROBO2	SYT4	KAT6A	NTN1	GNB5
PODXL	ROCK1	TADA2A	MLLT10	HOMER1	SLC12A7
POU3F2	RORA	TAF4	CCDC6	PCYT1B	NFAT5
POU4F2	RORB	TAF4B	CUL5	ONECUT2	MAP3K2
PPARA	RPS6KB1	TCF4	FXR1	SLC4A8	ZNF274
PPARD	ATXN1	TEAD1	SLC7A5	STXBP5L	FRS2
MED1	SCN2B	TEK	PICALM	CLOCK	PNPLA6
CTSA	SCN9A	TERF2	SNN	FEZ2	GLIPR1
PPP1CB	CXCL12	TFDP2	RASAL1	SH3PXD2A	ADAMTS5
PPP1R10	SRSF2	TGFA	DYRK2	SLC25A44	KAT7

BAZ2A	MMD	KRT76	HIF1AN	KIAA1430	TBL1XR1
AKAP11	DDAH1	PRKAG2	FAM178A	KIAA1456	VPS37B
RASSF8	SSBP2	SFMBT1	TXLNG	PITPNM2	SLTM
CA5B	SLC7A11	C9orf114	PAG1	UVSSA	PEAK1
ITGA11	CADM1	RAB14	CAND1	EPB41L5	ADM2
MRAS	C22orf24	NT5DC3	CTTNBP2NL	KIAA1549	ERMP1
RNF44	DSTYK	ASB4	MOSPD1	TNRC6C	PHC3
CPEB3	GLCE	ACSL5	CYP26B1	WDFY4	LRRTM4
PPP6R1	ABHD12	ATP8A2	BCCIP	MIER1	BICC1
BTBD3	GAPVD1	SIX4	TMEM9B	SLC7A14	CPEB4
EPN2	SERBP1	PHAX	DPYSL5	EPG5	DNAJC5
TRAK1	STAP1	ADAM22	SPIRE1	RXFP1	CEP44
SHANK2	SACS	SETD4	NCLN	EXOC4	TUBB1
RAB21	AGO1	NLE1	DUSP22	TGIF2	VMP1
STK38L	FOXP1	FAM105A	RGMA	BACH2	VANGL1
ZHX3	EIF2AK1	MTMR12	STOX2	PKNOX2	SBF2
CMTR1	CYTH4	FBXL19	BBX	XPNPEP3	FCRL5
CDK19	PALD1	VSIG10	TULP4	PRDM16	SESN2
N4BP3	DISC1	OTUD4	C12orf4	XPO4	CD99L2
GRAMD4	ARFIP1	EPDR1	NAT14	ZMAT3	RAB34
MPRIIP	AHDC1	IL17RD	PLXDC1	MTMR14	ZNRF3
ARL6IP1	TNRC6A	DNAJB12	LYRM2	SUDS3	FAM161A
DTX4	TMEM97	TET2	SLC4A10	MRPS25	USP48
ATP11A	CECR6	FAM46C	PBXIP1	NUCKS1	MAF1
MCF2L	CNIH4	SNRK	SLC24A3	SMURF2	TMEM246
KLHL18	SAP30BP	INO80D	GALNT16	YTHDC2	NEURL4
TRIM2	CDON	ELOVL2	TMCC3	CDCP1	MEGF10
RPGRIP1L	PDE11A	PARBPB	GATAD2B	TRAK2	GHDC
TTC28	TMED7	PTCD3	CNOT6	MTMR9	SRRM4
ZNF629	ST8SIA3	MRPS10	GRAMD1B	C1orf116	KIAA1841
ARHGEF12	SAR1B	LGI2	USP31	BHLHE41	MFSD9
ZDHHC17	RLIM	GOLPH3L	KIAA1210	FYCO1	FAM73B
DICER1	RAB9B	UBA6	ESYT2	RNF219	TBRG1
NCS1	GLRX5	UBE2W	HECW2	PPP1R3B	FAM104A
AP4E1	FAM13B	SPTLC3	KCTD16	STEAP4	ZNRF1
NPTXR	SLC25A37	FOXRED1	ARRDC3	ZNF322	KIAA1644
LEPROTL1	ZBTB7A	ZDHHC7	UNC79	COLGALT1	TANC1

ANKRD13A	TOM1L2	ZDHC22	APBB2	HTT	NOS1
FCHSD1	SLFN13	PRTG	ARG2	HLA-A	NOTCH1
C5orf30	RC3H1	C16orf91	ARRB1	HLA-B	NPC1
BMF	SIK1	ZADH2	ARSD	HLA-G	NPTX2
STARD13	PPM1L	DUSP28	RERE	PRMT2	NPY1R
FAM104B	CNTN4	EOGT	FXD2	FOXN2	NPY2R
PYGO2	C7orf60	GPRIN3	ATP2A3	HTR5A	NTRK2
SESTD1	VKORC1L1	C7orf71	ATP4B	IDH3G	NTSR1
PIGS	RDH10	YIPF6	KIF1A	IGF1	GPR143
SMIM12	USP51	TRIM59	BAG1	IGF2	OAS2
PIK3IP1	GPR180	EIF4E3	C8A	ILF3	OAS3
TMEM132B	CABP7	FAM19A2	MPPED2	ING2	OPHN1
ESCO1	PRICKLE2	NAT8L	CD1D	INSR	OPRM1
OSBPL11	KBTBD12	ZC3H12B	CD72	KCNA3	OVOL1
Mar-03	SGMS2	ATXN1L	CDK1	KCNH1	P2RY2
ZNF689	DCP2	C2orf71	CDKN1A	KCNQ2	PCSK6
RAB3C	ADAMTS15	PLEKHM3	CHRN3	KLC1	PAK3
LYSMD3	AGO4	BEND4	CHUK	KRT3	PAX1
MED12L	ANKS6	LIN28B	CLCN6	KRT85	PCSK1
GALNT15	C3orf58	GTF2H5	CLTA	LAIR1	PDE2A
TMEM52B	LRRC55	TMEM150C	CMKLR1	LALBA	PDE6A
SLC15A4	FAM124A	NRARP	CLDN7	LAMB2	PEX14
IKBIP	RNF152	RNF165	CSNK1D	LAMP2	PHB
SLC24A4	GPR116	ZNF704	NKX2-5	MGP	PHKG2
C15orf27	ZSCAN25	WIPF3	CTSL	MLH1	SERPINB8
TMEM170A	KIAA1324L	OC90	DHX8	MLLT1	PKD2
SYT2	ATXN7L1	ZNF605	DHODH	MMP10	PKLR
UHK1	RICTOR	ASIC1	DPP4	MMP19	PKHD1
FAM168B	CERS6	ACOX1	EGR3	MPL	PLEK
ZPLD1	UBN2	ACVR2B	ELF5	MTHFR	UBL3
JMY	BRWD3	ADD2	EMR1	MYO5B	POLH
DPCR1	C12orf79	ADPRH	ERBB3	MYO9B	POLR2D
VPS37A	LYSMD2	ADRBK2	FCAR	NCK1	POU2F1
C20orf112	CXorf23	CRISP1	FXN	NDUFB9	PPIB
ISM1	PGM2L1	AHR	FUT2	Sep-02	PPP1R1A
ISM2	ANKRD52	AK4	GALNS	NIT1	PPP2R1B
TTBK2	GPR137C	AKT2	GPD1	NMT1	PRKCZ

MAPK9	SSTR5	ZNF79	S1PR2	LILRB4	CNTNAP2
PRKX	STAU1	ZNF175	TRIP12	TPPP	ERC2
KLK10	CDKL5	RNF112	B4GALT6	KRR1	C20orf26
PSAP	STX4	ZNF217	SNAP29	LDB3	ZBTB20
PSMC2	SULT1C2	ZNF223	NRXN3	ABCB8	TRAF3IP1
RAD51	SYPL1	PTP4A1	CIAO1	HPS5	NAT9
RANGAP1	SYT5	LRP8	FAM189A2	MGLL	FBXW2
RASGRF1	TBP	ST8SIA4	QKI	TFEC	LRRC29
RBBP5	GCFC2	EPM2A	SPTLC2	RASA3	CKAP2
RBM3	TG	SF3A2	GTPBP1	ZBTB1	NPTN
RBMS2	TGM1	TMEM187	NFE2L3	SCAP	B9D1
RET	TH	HIST1H3E	ABCG1	KIF21B	BBC3
RFX3	TMF1	TAGLN2	CELSR1	KIF1B	INVS
RHCE	TMPO	STX7	PPM1F	GPATCH8	SIGLEC8
RHD	TMSB4X	ZNF282	VGLL4	PHF8	ZNF544
RNASEL	TNNI1	STK24	ST18	EPB41L3	POLM
RPL13	TP73	DGKE	KIAA0513	TTLL12	PCLO
RPL27A	TPM2	SNX3	SCRN1	CEP68	HIPK2
RPL36A	TPM3	RNMT	TRIM14	POFUT2	CD274
RSU1	TSC2	TNFRSF11A	UBAP2L	DOCK9	TRPM5
RXRA	UBA52	CFLAR	HELZ	KIAA1045	HCFC2
SCN8A	UBE2G2	NR1I2	DGCR2	NUDCD3	GPR132
SELL	UCK2	TAX1BP1	BCL2L11	SIRT4	PADI1
SFPQ	UQCRB	CACNA1H	DSCR3	ZFYVE26	EMR2
ST6GAL1	EZR	TAF1A	SMYD5	KCTD2	SOCS7
SLC1A2	CORO2A	SEMA5A	SPON2	R3HDM1	ITSN2
SLC6A12	WHSC1	PAPSS2	EMG1	SEZ6L	IRX4
SLC8A3	WNT1	NMI	COG5	CLEC5A	ZNF691
SLC19A1	WNT10B	CBFA2T2	SEMA4F	MAFF	A4GNT
SLCO1A2	WNT9B	SCAF11	MRPL28	PANX1	TUBE1
SLIT3	XPNPEP2	IL1RL1	ST6GALNAC2	SLC24A2	ABI3
SMN1	YWHAG	DEDD	FUT9	PARM1	MRPL37
SMN2	ZFP36	MAGI1	HBS1L	USP49	DNAJC27
FSCN1	ZNF3	PNMA1	ARPP19	SUMF2	ZDHHC3
SNRPD1	ZNF33A	UBE2L6	ZNF268	TPGS2	UCHL5
SPIB	ZNF37A	RPS6KA5	CPLX2	PTCD1	AMOTL2
SSFA2	ZNF75A	BCL7C	RAB40B	CHD5	UBE2J1

NCKIPSD	SLC39A9	ALX4	PQLC1	TRIM4	AGPAT6
CHMP3	HES6	SMOC1	ORAI2	UBE3B	TLDC2
RAB23	MAML3	PDLIM2	TRIM45	SNX21	STK35
TM7SF3	ZNF821	AKTIP	ULBP1	PPP1R3E	DYDC1
BCL11A	NAGK	S100PBP	SRCIN1	SLC25A46	ZNF548
SHC3	PEX26	TBC1D15	SETD7	PLXNA4	ZNF681
TPCN1	MREG	RABL5	SYNC	CABLES1	IL23R
STX18	CARKD	GNPNAT1	SLC38A1	NXPE3	TEKT4
S1PR5	NUP133	VWA1	VOPP1	MYLK3	C2orf15
RAB24	CENPN	FUNDC2	NETO1	WDR20	PROM2
BRWD1	TDRD1	LYNX1	KRTAP2-1	MYOZ3	DTX3L
RIPK4	AGPAT3	C7orf49	DNAL1	DPH7	XXYLT1
ERRFI1	C1GALT1	PRRG4	NUDT12	TMEM44	AMZ1
ARMCX6	C11orf30	TAF1D	YPEL3	PHF21B	MCMDCC2
SDK2	PRDM11	TNIP2	ITFG3	GLB1L3	LONRF2
UGT1A10	CTNNBIP1	CENPO	NCALD	SCAMP4	ZNF778
UGT1A8	CABP4	ULBP3	SYDE2	TMEM54	STRA13
UGT1A7	C12orf5	HMBOX1	NLRC5	ABHD15	FAM153B
UGT1A6	PNPLA2	ROGDI	ZDHHHC18	AGAP1	TSNARE1
UGT1A5	ZNF695	EFCAB1	DOT1L	MRGPRX3	SLC25A43
GNB1L	CD177	C22orf29	SLX4	TRIM6	ATAD3C
LZTFL1	LYRM4	MLF1IP	HOPX	ZNF488	SLC37A2
UGT1A9	KIAA1147	NPEPL1	ZNF594	DEPDC4	TBCEL
INO80	ZNF248	CLIP4	USP32	NIPA1	DLEU7
UGT1A4	SLC45A4	CCDC82	PPP1R9B	TBC1D16	Mar-08
UGT1A1	ISY1	KLHL36	UNC119B	INO80C	SPATA13
UGT1A3	ZNF490	ALG9	HAVCR2	CALR3	RBM24
RBFOX1	AHRR	AGBL2	RSPO3	TNFAIP8L1	PHACTR1
FAM20A	MTA3	RBFA	ZNF587	NR2C2AP	REXO1L1
BNC2	KIAA1328	MAP9	RAB2B	SHE	TAC4
ZCCHC2	DOCK6	CNTNAP3	MICALCL	FAM213B	PLA2G4F
CHCHD3	CRAMP1L	NOL10	FBXL20	GOLT1A	MYADML2
PARP16	FBRSL1	PHF17	AJUBA	ZNF831	NEGR1
ATP5SL	CTDSP1	DENND2D	TMEM128	TYW5	BCL9L
MAGOHB	ENOPH1	NAA25	STON2	UBE2QL1	B4GALNT3
PNPO	TRPV4	TRPM3	KIAA1751	STXBP5	SMIM21
MOB1A	SLC5A7	CCDC170	TSLP	B3GAT2	EPHA10

RIMKLA	ZBTB42	BACH1	FANCF	SMAD5	PRKAB2
FAM153A	LOC100132874	BARD1	FECH	SMAD9	PKIA
PRR18	C17orf99	BCAT1	FGF5	MECP2	PRKAR1A
TREML4	LOC100289561	ZFP36L2	FGF7	MEIS1	PKN2
C9orf47	TPBGL	CACNA2D1	FOXE1	MEST	PRKG1
P2RY8	TMEM178B	CACNB4	FOXO3	MFAP3	PROX1
S100A7A	LOC100507747	CAMK4	FOS	MKLN1	PSKH1
CLEC4D	ATP5J2-PTCD1	CCNT2	FUS	MAP3K10	TWF1
LUZP2	RNF103-	CD28	GABRA1	MMP16	PTPN4
SLC6A19	CHMP3	CD36	GABRB2	MOCS2	PTPRG
MYLK4	LOC100653133	CD47	GATM	MRC1	PURB
VSIG1	LOC100653247	CDH6	GCNT1	MYO1B	RAB1A
ZNF445	CYP3A7-	CDS1	GFPT1	MUT	RAB3B
BMP8A	CYP3AP1	CDX1	GFRA1	MYB	RAB6A
ZBTB41	LOC101060212	CEBPB	GJA5	MYO10	RAP2A
YJEFN3	LOC101060835	RCBTB2	GOLGA3	PPP1R12B	ARID4A
CEP85L	LOC101927903	CLCN3	GPM6B	NARS	RBP2
LDLRAD1	LOC101928210	CLCN4	GRIN2A	NEO1	RCN2
C2orf80	LOC101928454	CLCN5	GRSF1	NOVA1	RELA
IYD	LOC101928498	COL4A4	H3F3A	SLC11A2	RPE
SPRED3	LOC101928558	COL8A1	HIVEP2	NRCAM	RPL17
IL17REL	LOC101928970	CSF1R	HMGB3	NT5E	RPS3
C2orf91	LOC101930424	CSNK1A1	HMGCS1	OMD	RPS6KA3
C7orf65	ABAT	CSNK1G2	HNRNPU	OGN	RREB1
SNX30	ACADSB	CSRP2	DNAJB1	PAK2	SORT1
GOLGA7B	ACTA1	DYNC1I1	IAPP	CNTN3	SATB1
IQSEC3	ACVR2A	DOCK1	IGF1R	PCDH9	SCN1A
COA5	ADD3	DRP2	IL6R	PDE3A	SCN2A
LURAP1	ABCD2	DSG2	INPP4A	PEG3	SRSF1
FBXO48	AMBN	EPYC	INPP5D	PFKFB2	SHOX
POTEM	APAF1	E2F2	JAK2	PGR	SKI
TEX22	XIAP	PHC2	KCNA1	PIK3CA	SLA
CNTNAP3B	TRIM23	EN2	KPNA1	PIK3R1	SLC1A3
FLJ45513	RND3	EPHA5	KRAS	PLS1	SLC20A2
TMEM242	ARHGAP5	ERBB4	LIFR	PPP2CA	HLTF
SHISA9	ATF3	EYA1	LMX1A	PPP3R2	SMARCA4
RFX8	ATP2B1	F3	LPP	PRKAA2	SNX1

SNTB2	ZNF236	TOMM20	ATF6	ATRN1	PELO
SOX1	SCG2	RGP1	SEPHS1	ZNF451	POLE3
SP1	FZD3	ZBTB39	ELL2	LRRTM2	GPR85
SP3	GLRA3	G3BP2	TRIM32	LSM14A	ATAD2B
SPARC	SSPN	LPGAT1	KDM2A	HERC4	CCSER2
SPAST	ST8SIA2	DMTF1	HECW1	ARL5A	XRN1
SPOCK1	USP9X	ACTR2	TAB2	FBXO22	RBM47
STAC	MAP4K3	TSPAN5	GPD1L	CNNM1	RC3H2
STYX	UNC5C	FEM1B	LPIN1	INTS6	EGLN1
SVIL	SOCS1	MBNL2	LARP4B	NUFIP1	CNGB3
TAF7	DDX3Y	ABI2	RCOR1	HBP1	HAUS6
TAF12	PEA15	AASS	GANAB	CYFIP2	WBP1L
MAP3K7	CD84	LPAR6	ATP11B	ATP2C1	IMPAD1
TCEB1	KSR1	INADL	FAM168A	RBMS3	ACSM5
TCF7L2	BCL10	CTDSPL	ANKRD12	RAB30	Mar-01
TCF12	KALRN	SPRY3	WWC1	ANGPTL3	C14orf119
TERF1	TBX18	Mar-06	ACSL6	N6AMT1	C17orf80
TGFBR2	USP8	TLR6	TMEM194A	TMOD2	PDPR
TIA1	SLC16A7	SEMA3A	SATB2	STRN3	UACA
TLE4	ZMYM4	ZBTB18	DPY19L1	UBQLN1	GPATCH2
TLL1	ZMYM5	EXOC5	MED13L	TMED5	SLC25A36
UBE2G1	VAPA	KDM5B	SIRT1	RRP15	LRRC59
WEE1	LRAT	FGL2	CBX5	VPS36	YOD1
WNT5A	AIMP1	ACTL7A	LRRC8B	KLHL5	ELMOD1
YWHAE	TRIP13	PAPOLA	MORC3	KCTD3	PPP1R9A
YWHAZ	PPIG	SUB1	SMUG1	INSIG2	G2E3
ZIC3	MED21	ABHD2	CARHSP1	C3orf18	RUFY2
ZNF12	FHL5	DUSP14	ZKSCAN5	ZDHHC2	TENM3
ZNF33B	RASAL2	AKAP10	SGK3	HOOK1	Sep-11
ZKSCAN1	BAG5	SYNRG	GABARAPL1	CDC40	FGD6
ZSCAN21	SOCS5	ZNF652	TMEM245	BIN2	MBNL3
ZNF91	PHF14	COBLL1	MOB4	ANKFY1	FAM212B
VEZF1	CLINT1	RAB11FIP2	RNF19A	LGSN	BAIAP2L1
ZKSCAN8	RAPGEF2	NLGN1	RCHY1	HECA	PCDHA6
ZMYM2	RIMS2	INPP5F	KBTBD2	CAB39	ANKH
ZFAND5	SEC14L5	ANKRD6	TIPARP	LRP1B	EIF5A2
ZNF230	HDAC4	ZHX2	UPF2	PANK1	RAD18

TM9SF3	ZNF385D	SERAC1	UPP2	CYB561D1	ARVCF
FEM1C	MCTP1	SSH2	IGSF11	UNC80	BAAT
CDC42SE2	TTC21B	NAV3	PAQR3	ZNF621	BDKRB2
SLC39A10	TRAPPC13	MVB12B	CREBRF	TCAIM	CA6
THOC2	PGAP1	MIDN	SLC25A48	TPRG1	CBL
ADAMTSL3	DCAF17	CCDC126	RNF217	C5orf64	CCNH
GPR126	VCPIP1	SLFN11	FAM91A1	SREK1IP1	CD33
AICDA	FBXO11	CHURC1	FREM1	FAM133A	CD59
NUFIP2	CHD9	MARVELD3	ZXDB	FAM149B1	CHRM3
KLHL42	KCNIP4	UBTD2	ZDHHHC15	TMEM202	CCR1
PDP2	THSD7B	RBM18	CCDC67	ZNF260	COL19A1
ARHGAP20	NDFIP1	TADA2B	TMTC2	KIAA2022	COX11
FAM135A	JHDM1D	TBCK	SPRED1	FREM2	CUX1
WDFY1	KIAA1715	ARHGAP18	IFNLR1	ECT2L	AKR1C2
KIAA1462	C1orf21	CEP41	MMAA	IRF2BP2	DRD1
RANBP10	CSRNP2	GLCCI1	LCA5	NHLRC2	DTNA
TSHZ3	COL21A1	CSMD3	SPTSSA	PTPRQ	EGFR
VPS18	TSPAN14	ZNF618	ARID2	PTAR1	EPHA3
STIM2	PUS7L	FAM210B	PLD5	CA13	ERCC6
RNF213	TMEM47	RAB3IP	TAPT1	FAM69A	FGF14
ZSWIM6	FSD1L	ANTXR2	ANO5	TSPYL6	FKBP5
USP37	TSSK1B	PSTK	KIAA2018	C2orf68	FMO5
IGDCC4	B3GNT5	FGD4	RTKN2	GDF6	GABRA4
RBAK	FBXO30	USP43	MPEG1	C11orf87	GALC
NEUROG2	USP44	ZFP3	HNRNPA3	KLHL31	GALT
PAPD5	SLC41A2	KRT222	ARL5B	SAMD12	GLUL
HHIP	ANTXR1	CLHC1	TDRD6	SLC25A53	GNAS
ACBD3	FAR1	ACVR1C	SMIM13	PTCHD4	GUCY1A2
PAPOLG	ANKRD32	PPARGC1B	C7orf41	TMEM200C	GYG1
RAPH1	SGIP1	UBXN2B	ZBTB38	FAM227A	GYPA
WNK1	DDI2	FAM199X	MMS22L	SLC35E2B	HAL
OTUB2	LCOR	CACUL1	FBXO33	TMEM194B	HDAC2
TRPM8	C15orf41	LDLRAD3	TCP11L2	ACRV1	HIF1A
CDC73	MYPN	ZNF664	FAM133B	ACYP1	CYR61
DHX40	CBR4	SLC30A7	C12orf40	ADRA1A	IGJ
SAP30L	NFATC2IP	KANSL1L	FAM177A1	ANXA2	INHBC
ZMAT4	SLC35B4	CCNYL1	FAM102B	STS	IRF6

STT3A	RBBP6	MALL	ARL6IP5	NSL1	BSDC1
LIG4	RBP1	UBXN8	GNA13	IPCEF1	PBRM1
SMAD1	REST	SMARCA5	TBR1	LDLRAP1	DCUN1D2
SMAD2	RGS13	GPR65	YME1L1	TRPC4AP	CPPED1
MGAT2	RLN2	PRSS12	CCR9	TAF5L	AGPAT5
MLLT4	RPS14	PPM1D	ZNF275	PDCD4	C17orf85
MPI	S100B	DDO	PPP1R17	TINAG	TRIM36
MUSK	SCN7A	PIAS1	DBF4	MRPL18	IL17RB
MYLK	SDCBP	ASAP2	MSL3	CARD10	BMP2K
NKX3-1	SH3GL2	AP1S2	SERINC3	CNOT7	FAM46A
NOS3	ST8SIA1	CDKL2	LILRA1	TPRKB	ZNF701
NPHS1	SLC10A2	MAP3K14	STON1	GOLT1B	ZNF83
OPRL1	SLC15A2	SLC33A1	LECT1	RMDN1	TBC1D23
ORC4	SLC22A2	TRIP11	WDR45	CCDC41	LRIF1
CLDN11	SPI1	ITGBL1	NUDT4	SERPINA10	DCP1A
PAWR	SSX2	NMT2	PSIP1	KRCC1	MYNN
PCCA	TACR1	AKAP5	MST1L	HMP19	KLHL4
PCSK5	TCEB3	ADAMTS1	SEC63	TMEM66	PCDHB10
PDK3	TCOF1	SCAMP1	ZNF507	NAA38	PCDHAC1
ENPP1	TDO2	ZNF254	AAK1	MYOZ2	PCDHA13
PF4V1	TFAP2B	ENTPD4	CLUAP1	BTBD1	PCDHA12
POLR2C	TNFAIP2	KIAA0430	ERP44	TM6SF1	PCDHA11
PPEF1	TSC1	EDEM1	PEG10	SPIN2A	PCDHA10
PPM1A	TTF1	HDAC9	MRPS27	GNL3L	PCDHA8
PRKAR1B	TYRP1	PCDHA9	PDS5A	CRLS1	PCDHA7
PRKDC	UFD1L	ZSCAN12	RPRD2	SLC35F2	PCDHA5
DNAJC3	USH2A	RIMS3	TBC1D30	XAF1	PCDHA4
PTGIS	VIPR1	KIAA0141	SRGAP2	TRIM44	PCDHA3
PTN	LAT2	SLC35E2	EXOSC2	MBTD1	PCDHA2
PTPN2	YES1	KBTBD11	CDC42EP4	ZNF562	PCDHA1
PTPN11	ZNF28	NR1I3	RABGAP1	SYTL2	ZNF253
RAD1	ZNF43	DOPEY2	NPAP1	DCAF16	IL36G
RAB27B	ZNF85	SLC12A6	BHMT2	WHSC1L1	CELF4
RAB5C	ZNF138	ARPC3	TNFAIP8	RHBDL2	ENY2
RAC1	ZBTB16	PLXNC1	BACE2	PHIP	LHX9
RAD21	ZFP112	BTN2A2	FBXL2	PLEKHB2	OTUD7B
RAP1GDS1	RNF103	DDX17	THUMPD3	DET1	CIAPIN1

C16orf62	STARD5	EPSTI1	CNST	C6orf120	ATP6V1C1
TWSG1	EEPD1	TP53INP1	PARP15	POTEC	ATRX
AGTRAP	APOL6	CYP2U1	ZNF92	ZNF808	BCL2
PAK7	GFOD2	FMNL2	SPIN3	ZNF761	BCL2L2
RAB22A	SLCO5A1	ELFN2	ZNF431	ZNF470	BCL9
SERINC1	ZNF611	SORCS1	ZNF721	VGLL3	BDNF
TBC1D14	CHST9	TMEM123	SYNPO2	SAMD5	BICD1
SYT13	CALN1	TRMT61A	FAM9C	SERTM1	PRDM1
FNIP2	SYT16	WDFY2	ZNF626	LOC400682	BMPR1A
SLAIN2	SPRTN	FOXP4	C3orf67	ZNF880	FOXL2
ZNF492	ARMC2	CYYR1	PDE12	BCL2L15	BPGM
HCN3	TXNDC2	RAB39B	VMA21	PIWIL3	BRAF
SCUBE2	DCUN1D5	WDR17	ORAOV1	OOEP	KLF9
EPS15L1	UTP23	SSX2IP	CCDC89	SPIN2B	BTN1A1
ENPP5	ZNF528	PARD3B	NAPEPLD	LOC729020	MYRF
PLEKHA1	ZNF333	C10orf90	LNK2	SLC15A5	CA11
RNF123	MCM8	C10orf32	ZNF396	ZNF812	CA12
PRDM15	GPR174	ANAPC16	ZDHHC20	RELL1	CAMK2D
ARHGEF28	MASTL	TDRD9	LCORL	ZNF737	RUNX2
MPP5	USP45	TYW3	WDR72	MTRNR2L1	CBFB
FBXL17	ZIC5	UBXN10	C10orf67	HSPE1-MOB4	CCND2
NABP1	EPT1	UGT3A1	HS6ST3	C8orf44-SGK3	CCNF
ZBTB10	DPH6	ACOT12	KSR2	LOC101060179	CCNT1
SECISBP2	FAM105B	CTCFL	NUDT7	LOC101060405	CD5
RNF26	ZNF468	HECTD2	GDPD1	LOC101927515	TNFRSF8
CARS2	ZNF160	ZNF578	RNF149	LOC101928218	TNFSF8
CCNJL	MCFD2	ZNF98	CPLX4	LOC101928793	CHN2
SH3TC2	L3MBTL4	ZNF714	ZNF678	ACADL	AP1S1
MANEA	DDX60L	GCSAML	GADL1	ADCY1	CCR5
OGFOD3	BOC	ENTHD1	ZNF680	ADCY5	CNP
ZFAND1	ZNF765	PPP4R2	GLDN	ADCY6	COX10
ZNF665	ZNF845	ZC2HC1B	ZNF860	AP2A2	ATF2
METTL8	FAM114A1	SLC2A12	LRIT3	ALPL	CRKL
SPEF2	ZNF561	KIAA1958	OR2A1	ANGPT1	HAPLN1
L2HGDH	PRDM6	TMEM229B	GATS	APBA1	CYP1B1
PDZD7	ACTR8	ZNF320	ZNF829	AR	DACH1
LRRC27	PAXBP1	DENND1B	C1orf95	ARCN1	DGKG

DBH	NR3C1	MCL1	PLXNA2	SDHD	TUB
DECR1	GRM1	ADAM11	PRRX1	SELE	TXNRD1
DHCR24	GRPR	MEF2C	POLA1	SEMA3F	UBE2H
DLG3	GSPT1	MAP3K3	POLR2F	MAP2K4	UBP1
DLX6	GSTM3	MGAT3	PPP1CC	SFRP1	VHL
DNA2	GUCA1B	KITLG	PPP3R1	SHC1	WNT2
DPYSL3	HAS2	MLLT3	PPP6C	FBXW4	ZNF22
DSG3	HHEX	NR3C2	PPT1	SIX1	ZNF24
DUSP3	HMGCR	TRPM1	PRELP	SKIL	ZNF75D
DUSP4	HMX1	ALDH6A1	PRKACB	SLC1A1	ZNF135
DVL3	FOXA1	MSR1	PRKCA	SLC5A1	ZNF148
EFNA5	HNRNPA2B1	MTTP	PRKCI	SLC22A3	ZNF180
EFNB3	TLX2	MUC4	PROC	SNAI2	LUZP1
CELSR3	HSD17B2	MXI1	PSMD5	SNAI1	PRDM2
ELAVL3	IDS	NEDD4	PSME1	SOX4	EVI5
ELF2	IGF2R	NEUROG1	PTCH1	SSR3	CXCR4
ELK4	IGFBP5	NFATC3	PTGDR	SSRP1	ALDH5A1
EPB41L1	IL1B	NFIC	PTPRR	SS18	NR4A3
EPHB1	IL1R1	NOTCH2	RAB3IL1	STAT5B	ADAM12
EPHB6	IL16	NPR3	RAB5B	HSPA13	KMT2D
EPS15	FOXK2	ROR1	RAD23B	ELOVL4	HMGA2
EVX1	ITGB3	NUCB2	RAD52	TAF5	GPR68
ACSL4	ITPR1	NR4A2	RANBP2	TAL1	GAN
FBN2	KCNA6	OCM2	RARG	TBX3	AKAP1
GPC4	KCNA7	OPCML	RASA2	ZEB1	C21orf33
FOXC1	KCNJ1	OPRK1	RASGRF2	TFAM	CDC7
FOSB	KCNJ14	P2RX7	RGS16	TGFB3	EOMES
GABRG2	KCNMA1	P4HB	RLF	TGM2	FZD4
NR6A1	IPO5	PAFAH2	RP2	THBS1	PIP4K2B
GCNT2	LAMP1	PDE4A	RRM2	TCHH	SPOP
GPC3	FADS1	ATP8B1	CLIP1	THRB	CUL4B
GLRB	LRP6	PGGT1B	ATXN7	TIE1	ATRN
GNAL	M6PR	PIK3C2B	SCN4A	TMPRSS2	OGT
GNAQ	NBR1	PITX2	SCN4B	TNFAIP3	SLC43A1
GP2	SMAD4	PLAG1	SCN5A	TRPC5	ENC1
GPR26	MAPT	PLCG1	SCTR	DNAJC7	GAS7
GRIN2B	MBNL1	SERPINF2	CX3CL1	TPPA	AGPS

TCAP	CCPG1	ABCB6	METAP2	SIRT3	AFF4
DENR	SOCS6	HHLA1	PTPRT	SLC44A1	BRPF3
KMO	COX5A	ARL4C	KIF3A	ANGPTL2	SERP1
YARS	GRAP2	FRY	IRAK3	CBX6	GPKOW
PRKRA	EIF2AK3	FARP1	PADI2	BRD4	BHLHE22
STK16	EIF4E2	DHRS9	NXPH4	RIMBP2	TOX3
KLF7	PIGL	CNIH1	GPR45	RBFOX2	TRIB2
STC2	GABBR2	TENM1	CHP1	HYAL4	C16orf72
CDK13	CREB5	TRIM13	KLF12	CORO1C	ZCCHC4
TP63	GDA	LANCL1	GABARAPL2	ZMYND8	RBM15B
JRKL	HS2ST1	CRISP3	IKZF2	LSM5	POMT2
CDC23	LPIN2	PCGF3	HSPA4L	STX12	SLC25A24
RNGTT	CCDC144A	HMG20A	ADNP2	FLRT2	NRBF2
RIPK1	NOS1AP	CACNG2	CNKSR2	ATXN10	ZBTB21
NAPG	SEMA3E	SCML2	MLXIP	ARMC8	COL5A3
IL18R1	CCP110	ATP8A1	FAN1	ZNF521	DCAF8
DCAF5	FAM115A	SYNCRIP	DIP2C	C20orf194	DHH
FGF18	SLK	IPO8	PLCH1	NUDT13	SPOCK3
NRP2	TOX	ATG7	GGA2	C2CD2	STMN3
GMPS	RASSF2	SLC19A2	ZNF423	IBTK	RDH11
WISP1	TM9SF4	DRAP1	ZC3H13	NSMF	MRPL35
HCAR3	DHX38	ARID3B	ARHGAP26	PLEKHG3	FAM8A1
DDX18	IST1	IGF2BP2	RAP1GAP2	SUSD5	CTDSPL2
ENDOU	RB1CC1	CELF2	TNRC6B	SETBP1	UFM1
PHOX2B	GAB2	CD226	ATP10B	FAM169A	ASB1
STBD1	C2CD5	GMEB1	COLGALT2	ARHGEF26	SELT
MPZL1	MFAP3L	AHCYL1	Sep-06	SZRD1	FBXO40
USP14	SEC24D	POP4	SNX13	TANC2	CDK12
MTMR6	TBC1D4	HSPH1	RTF1	PHF19	RAB8B
MTMR7	RHOBTB1	GPR83	FAM175B	GREM1	RSF1
LATS1	LPPR4	RAB10	ZCCHC14	RPS6KC1	DCUN1D1
SEC22C	NUAK1	PPARGC1A	GSE1	SEC22A	FBXO42
PRPF3	SV2B	MAN1A2	ZC3H4	AP3M1	FAM134B
MAP3K13	DENND4B	SPIN1	RHOBTB2	MLH3	CCDC93
SLC24A1	RABGAP1L	AP3M2	FBXW11	NSG1	SGTB
LARGE	FAM20B	CBX1	DNAJC13	FAM155B	SPATA6
VAPB	ZBTB5	MAPRE2	SZT2	PDE7B	CCNJ

SLC6A20	LMO3	FAM204A	DNAJB14	RELT	SOCS4
ZCCHC10	ZC4H2	FAM217B	GREB1L	UBASH3B	ACSM2A
NDE1	LANCL2	TMBIM1	TMEM156	FRMD5	DCUN1D3
MFSD6	TEX15	RRAGC	WWC2	CEP19	CYB5D1
PGPEP1	LMOD3	ELTD1	FRAS1	PAQR8	ZNF792
LAX1	AGPAT4	XYLT1	NAA15	EAF1	AADACL3
RNF125	C14orf132	ZFYVE20	MYCT1	DCLK3	TPRG1L
PID1	ATXN7L3	IKZF5	PROSER1	HPS4	TEDDM1
TMEM255A	PRDM10	EBF2	WDR26	PAPLN	TBC1D20
CCDC40	SLC17A7	CREB3L2	SP6	CCDC120	AP1S3
LRRC8D	ANKMY2	USP46	WDR82	C12orf23	NUDT16
SEC61A2	ENTPD7	BCL11B	ASXL3	SHF	AFAP1L1
VPS13D	DOLPP1	TTC23	ANKRD13C	CEP95	NCOA7
UQCC1	MCOLN1	REEP1	CDADC1	TRIM41	HGSNAT
TMEM39A	KIAA1244	TMEM237	DOCK8	FMNL3	SLITRK4
VPS53	ERGIC1	SOWAHC	RNF170	RNF185	AMER1
ZNF654	JPH3	UBE2Z	APH1B	ISX	PTCHD1
RHOT1	TTYH1	WNK3	RASSF5	CHRD1	TRPM6
ACER3	CYP20A1	SCNM1	AMMECR1L	CALML4	DUSP19
STRBP	NDRG3	KXD1	C10orf11	STRADA	XRRA1
TCP11L1	MTUS1	ELOVL6	KREMEN1	MMGT1	TMEM86A
STYK1	XPO5	PRR15L	HDHD2	MYOCD	CPNE8
NOP10	GPR158	C22orf46	TMEM164	DTD2	LACC1
GALNT10	TAOK1	SRD5A3	RHBDD1	CASC4	CD300LF
VEZT	SEMA6A	AKIRIN1	YIPF4	FAM83F	SYT6
LRRC40	TMEM181	ARHGAP10	C2orf88	PALM2	SAMD13
FLVCR2	NYAP2	DYNC2H1	COL25A1	SLITRK1	PHF13
TMEM127	USP28	ZDHHC14	ST6GAL2	MYSM1	MANEAL
FRMD4A	SFMBT2	LIN28A	FAM126A	RNF157	EXOC8
IPO9	CYP4F11	CLMP	SLC9A7	KLHL29	CCDC117
BTBD7	ZNF462	NAA40	GPT2	OSBPL6	FBXO41
FAM222B	CACNG8	THSD4	FUT10	PCMTD1	FAM84A
ENAH	ELOVL5	CCDC134	EFCAB4B	ARAP2	FGD5
AGK	TRMT11	CYBRD1	ZNF496	SFXN2	AMOT
TMEM30A	GUF1	NAA60	PLXDC2	FAT3	TMEM213
PRR11	SPCS3	SYNPO2L	ARHGEF39	TMEM132D	TTC39B
WWC3	MRPL17	WDR76	TMEM87B	AEBP2	PRUNE2

EML5	MTX3	BIN1	F2R	IL12RB2	PHKB
Mar-10	ACSM2B	BIRC2	F11	JAK3	PIGF
DEDD2	HCN1	AQP4	FBN1	KCNJ5	PIN4
SASS6	TICAM2	ARL3	MS4A2	KRT82	PLCB2
BMPER	LHFPL4	ATP5G3	FCN1	LEPR	EXOSC9
TMEM64	ILDR2	AUH	FGF1	LIG3	POLR2E
GLIS3	SETD8	AUP1	FSHB	LIMS1	POLR2K
ARX	C15orf52	BBS4	GABRB1	VWA5A	POU2F2
ZNF384	TMEM220	BCL2L1	GABRG1	LSAMP	PPIA
MPZL3	ARHGEF37	OSGIN2	GALR1	MAGEB4	PPIC
ZNF48	C8orf86	C21orf2	GAS1	CHST6	PPOX
SLC39A11	XKR9	CACNA1C	GAS8	MDM2	PPP1R3A
ZBTB7C	IER5L	CD8B	GBP3	MEF2D	PRF1
C9orf72	KBTBD13	CD80	GFAP	MEFV	PRKACA
FAM219A	ZNF793	CDC42	GGT7	MFGE8	PRKCH
NRK	METTTL10	CDH4	GLP1R	MFI2	MAPK11
SLC36A1	PAIP2B	CHML	GNA15	MICB	MAPK13
AMER2	PLEKHG7	AP3S1	GNAT1	CXCL9	PRLR
YPEL4	CAPN14	CNR2	GNB3	MME	TMPRSS15
TMEM26	TMEM151B	COL4A5	GNRHR	MMP9	PSMB9
ZCCHC24	C18orf32	COX15	GPD2	MN1	PSMC1
UNC5B	SDHAF1	CSF2RB	GPLD1	CD200	PSMC4
CCNY	SMIM10	VCAN	ARHGAP35	MX1	PSMC6
C11orf45	GXYLT2	CYBB	GTF3C1	MYF6	PTGER4
KCTD20	ZBTB8B	DBT	GUCY1A3	MYT1	PTPN1
LCLAT1	ARHGEF33	COCH	HIP1	CEACAM6	PTPN3
GCSAM	TMED7-	DMD	HLA-DQA1	NBL1	PTPRC
TAB3	TICAM2	DNM2	MR1	NFATC1	PTPRJ
MAGI3	POC1B-	DR1	HLCS	NRL	PTPRO
AQP11	GALNT4	RCAN1	HNRNPA1	DDR2	PTX3
BOD1L2	ACACA	EBF1	HPCA	P2RX1	PVR
ANKRD34A	ACAT1	EGR1	HTR1F	P2RX5	PEX19
LSMEM1	ADARB2	EIF4B	HYAL1	PBX1	RAB2A
TRIQK	ADH5	EPHB2	IL1RAP	PCDH7	RGL2
HCAR2	ADORA2A	ERV3-1	IL7R	PDGFRB	RAB13
RSPO4	AFM	EVC	IL10RA	PDK2	RAD51B
PLCXD3	AIF1	EXTL3	IL11	ENPP3	RAD51D

RAF1	STX5	ZNF213	ATG12	BET1	HABP4
RAP2B	MED22	ZNF224	FAIM3	LILRB2	SLC4A1AP
RBL1	VAMP7	SLC30A2	ZBTB22	SIRPB1	NT5C2
RBM4	TAF2	PAX8	GPR52	EMC8	SORCS3
PRPH2	TBL1X	ST7	SNRNP40	RAPGEF3	CNOT1
RELB	TCEB2	PDHX	KCNK6	CD2BP2	MON2
RFX2	HNF1A	PTP4A2	MAP4K4	RBM14	VWA8
RFXAP	TFE3	DPF3	ARHGEF6	OLFM1	SARM1
RGS2	THPO	DPF1	AKAP7	TAB1	ZBTB43
RHAG	TIAL1	HDHD1	EEF1E1	TRIM38	DCUN1D4
RNF4	TKT	KDM5C	TP53I11	SEMA4D	LRCH1
RPS6KA2	TNS1	UBL4A	GOSR2	MYBBP1A	EXOC6B
RPS23	TP53BP1	USP9Y	CLCA2	SMC2	CAMTA1
SALL2	TPD52	FZD1	ZNF592	RASL10A	DNMBP
SCD	TRAF1	HIST1H2AC	IPO13	IGF2BP1	NEDD4L
SEL1L	TRHR	SOX14	GREB1	KHDRBS3	UFL1
SELPLG	TRPC1	KLF11	RAB11FIP3	NES	KIAA0947
SEMG2	TTC4	IRS4	TBC1D5	SRSF8	GPR161
TRA2B	TTN	RGS5	PTDSS1	ASCC3	TARDBP
SFTPBP	UBE3A	PPFIBP2	MAML1	ATF7	ATP1B4
SGSH	SLC35A2	CAMK1	MRPL19	RBPM5	HEY2
SHB	UGDH	BCAS1	ZBTB24	PIM2	SLC39A14
SHOX2	UGT2B10	DEGS1	ELMO1	C10orf10	TTC33
ST3GAL3	UMPS	MADD	EPM2AIP1	HNRNPUL1	CCNDBP1
SLAMF1	UNG	CASK	TRIM66	WDHD1	ARFIP2
SLC6A2	KDM6A	RGS20	POM121	FAM107A	PLA2G15
SLIT1	VASP	ABCB11	TECPR2	AKAP13	TMEFF2
SUMO2	VCAM1	GALNT4	IQSEC1	PTGDR2	KIAA1549L
SNRNPB2	VCP	ADAM19	MED12	GPN1	MTHFD1L
SP100	VIPR2	TNFRSF10D	PDCD6	IKZF3	AHCTF1
SPTBN1	VSNL1	VNN1	GJC1	RHOBTB3	C1orf43
SRP19	WFS1	AP1G2	UBA2	PPM1E	PVRL3
SSTR1	WNT3	GYG2	SAE1	VASH1	KANK2
SSTR2	WNT8A	CDK5R2	ABCC9	LPHN1	TMEM87A
STAT2	XK	NOL3	TSPAN2	UBOX5	SPATS2L
STIM1	ZFX	ANGPTL1	RASA4	DIS3	GBGT1
STRN	ZBTB25	BPY2	STAM2	CD93	PTPN18

SLC17A5	CPSF2	H2AFJ	HS1BP3	STMN4	NAV1
TIMM10B	FAM3B	TRERF1	GREM2	FAM167A	SEC11C
OR1J2	WNT4	RNF20	ZNF106	FAM57B	AP5B1
RANBP6	RNF216	ZC3HAV1	GZF1	RIOK1	ANKRD44
DIEXF	UGT2B28	MDM1	GINS3	RBP5	CENPL
B3GAT1	TMX3	PRDM8	LPIN3	ESYT3	SNX29
RPS6KA6	TMCO1	CHRNA10	MRPS34	SETDB2	OXNAD1
MRPL42	WDR5B	PITHD1	C11orf95	RASSF4	MEX3A
MCTS1	MAP10	JPH2	TMEM108	IMMP2L	C17orf72
TMEM230	GTPBP2	FAM219B	GID4	FAM160A2	MOB1B
ZBTB44	CROT	VN1R1	AHNAK	SLC10A7	PKHD1L1
MRPL22	LEPROT	SPRYD7	METTL16	FAM175A	OPALIN
SEC61A1	KLHL24	ZNF286A	SMIM7	SLA2	MAPK1IP1L
PNMA3	SAMD9	ODF2L	TRIM48	ZCCHC9	TRMT10A
A1CF	RETSAT	RIMKLB	TMEM185B	LDOC1L	SFXN1
VSX1	CDKAL1	MAVS	BCL2L14	SLC37A3	SFXN5
SLC35B3	KANSL2	INTS2	LRRC2	PRADC1	RWDD2A
COQ6	CXorf48	KIAA1324	CHMP6	GINS4	PWWP2A
PI15	SLC35A5	SORCS2	NARG2	HOOK3	C1QTNF3
MRPL4	ATG2B	MICAL3	PPCS	MAML2	C1QTNF7
SEPSECS	C11orf57	STAMBPL1	CXorf36	FAXC	DDIT4L
SUV420H1	CCDC25	IFT80	ASB13	PLA2G12B	ARL11
PLCE1	WDR41	TRMT5	C1orf115	EMR3	SNAP47
NIN	CWF19L1	POGK	C12orf49	HINT2	FTSJ3
BET1L	UEVLD	GATAD1	BORA	FAM71F1	OLFM3
GOLM1	RNLS	ZP4	RIN3	CAPS2	CPXM2
ARL17A	SYNJ2BP	CREBZF	DNAJC22	PIGO	ZNF641
MBTPS2	ENOSF1	PLEKHA2	FRMD1	PRRC2B	SPPL3
MRPS17	CDV3	HRH4	FAM192A	AGBL4	TEX26
POLK	MED29	AFAP1	SLC8B1	RNFT2	TRAPPC6B
PTPLAD1	ANKRD10	AASDHPPT	PIF1	ABHD13	C17orf64
PEX5L	FERMT1	SH2D4A	TTI2	RGS8	CNTD1
UBE2D4	CHD7	BLOC1S5	ADAM33	KIAA1755	B4GALNT2
RBMX2	SLC48A1	PERP	DCAF11	DIXDC1	ZNF441
YPEL5	SLC30A6	EPB41L4A	ITIH5	MBD3L1	IRGQ
PTRH2	KANSL3	PJA1	CYB5B	HSFY1	MYOM3
LUC7L3	VAC14	MS4A6A	DUSP16	GAL3ST3	ZNF648

C1orf216	AAED1	SCAI	CTXN2	FAM47E-	AFF2
KLHDC7A	ZFC3H1	ATP11C	DTHD1	STBD1	KDSR
PLEKHH2	ANO6	TUSC5	CCSER1	LOC100653071	GAB1
C2orf76	NLRC3	CCDC85C	RGS7BP	LOC100653194	GNAI2
KLHL40	PLD6	RAB7B	SRRD	LOC100996273	GNAI3
EVC2	C4orf46	C17orf51	FNDC9	LOC100996571	GOLGA1
CMBL	TMEM192	ZBTB8OS	ACBD7	SRGAP2D	GRB10
DNAJC21	SLC44A5	PRSS38	MXRA7	LOC100996779	GRIA4
GRPEL2	SENP5	KY	TTLL13	LOC101927402	HOXC10
DCAF4L2	TMEM218	ZDHHC21	PRAMEF6	LOC101927989	HOXD1
NACC2	BEND6	RGAG4	NUTM2G	LOC101928728	INSIG1
ZFP92	C6orf89	NANOS1	BPY2B	LOC101928805	KCNK2
ZFP28	FO XK1	LRIT2	BPY2C	LOC101929728	KDR
SMCR8	THSD7A	ZKSCAN2	C18orf42	LOC101930064	KPNA4
SRXN1	PELI3	CD300E	SMIM15	ALDH1A1	MARK1
ZSWIM3	YTHDF3	ZSCAN22	LOC643355	ANXA1	MAT2A
FAM65C	SNX32	PAQR9	RASSF10	ARNT	MYO1C
ABHD12B	FAM26E	EYS	CCNI2	ASPA	NAP1L2
BEAN1	BPIFC	ZNF391	PRAMEF19	BMI1	NEFH
WIPF2	MPV17L	IGFBPL1	SRGAP2B	BNIP3L	NFYB
CCBE1	TMCO4	CLEC18A	ANKRD33B	CALM1	NTF3
LYPD4	SYT14	NIPAL4	SRGAP2C	CASP10	PAK1
KNCN	SCML4	FOXD4L4	TMEM236	CASR	PHF2
ZNF362	ARHGAP30	GALNT18	FAM155A	KRIT1	SERPINB5
CLDN19	RBM20	NCR3LG1	LOC728392	MS4A1	PPP2R5E
COMMD7	GATC	ATP9B	CCR2	CDKN1C	PRPS1
PPP1R1C	PGP	ZNF773	ZNF286B	CNR1	PTGFR
PPM1K	CLEC18C	C3orf62	FOXD4L2	CSNK2A1	PTPRM
PLEKHG4B	C17orf58	SUMO4	LOC100129361	CYLD	PURA
SNRNP48	TMEM105	SOGA3	LOC100130705	CYP7A1	RALA
KCTD7	ZNF780A	C10orf113	KLLN	TRDMT1	RIT2
ESCO2	SLC25A34	SHISA6	LOC100289097	DNMT3B	CXCL11
C8orf37	C3orf70	CCDC103	SPATA1	TSC22D3	SRSF7
ZNF483	DCAF4L1	USP27X	RPL17-C18orf32	EIF1AX	SH3BGRL
OTUD7A	C5orf51	PRAMEF12	MINOS1-NBL1	EIF2S3	SIM2
ITPRIPL2	MICU3	PRAMEF18	TNFAIP8L2-	ERCC4	SNCB
TXLNB	C8orf31	SLC25A35	SCNM1	ESR1	SNTB1

SOX10	CTCF	FNBP1L	FAM110B	EML6	KIR3DL1
T	CAPN9	LPPR1	SLC2A13	AADAC	KPNA2
TIAM1	ZNF460	CASZ1	OSBPL8	ACADM	AFF3
TIMP3	SF3A3	FIGN	TTC8	ACTC1	STMN1
TLE3	NUDT21	TBC1D19	TTC39C	AQP3	MAP6
SEC62	ADAMTS6	C5orf22	EGFLAM	ARF1	MMP1
TP53BP2	PDCD10	ZNF407	C5orf24	ARL1	MSN
TPD52L1	FOXJ3	SYBU	CLVS2	ATP1A1	MYOD1
RND2	RAB18	BRK1	ZNF572	BCHE	NDUFA1
SMC1A	POGZ	GNG12	ASB7	BLVRA	NDUFC2
RECK	FBXO28	SLC17A6	RIMS4	BST1	NFATC4
CDS2	PACS2	SENP7	SESN3	C6	SERPINB2
CD164	DDHD2	PCDH10	ARHGAP42	CAV2	PARK2
PEX11A	CUX2	KIAA1598	ZFP90	SCARB2	PDHA1
INPP4B	U2SURP	PRUNE	DPY19L3	SLC31A1	PEX13
MBD2	FRAT2	DMRT3	CCDC50	CRX	PIK3CD
SOCS3	ZFPM2	MESDC1	USP54	CTSE	PKP1
CLDN12	WWTR1	MRPS14	C15orf26	DVL2	PLAUR
INA	ZNF385A	LMBR1	RASSF6	EFNB2	PPP1R2
NDST3	L3MBTL1	IKZF4	MIER3	EIF5	MAPK1
SLC4A7	OSBPL3	ZSWIM4	SPRED2	CLN8	PSMD11
KIAA0040	DNM3	WDR77	STK32A	FABP2	PWP2
USP6NL	PITPNC1	PANK3	KHDRBS2	FKTN	PEX2
ZFYVE16	CABYR	CARF	ZNF25	FGF12	PYGB
SPCS2	EHF	BAALC	RSBN1L	GFER	RDX
EFCAB14	TSPAN13	SCD5	PRR14L	GGCX	RPL37
SPATA2	PCDH11X	SEMA6D	CADM2	GLS	RS1
JAKMIP2	OLA1	SLC35F5	RALGAPA1	GPI	SC5D
KIAA0319	PDZRN4	PDGFD	RNF169	GPR12	SH3BP2
MFN2	NXPH1	APOLD1	RNF144B	H1F0	SLC6A4
AKT3	MYEF2	ST6GALNAC5	NAP1L5	HLA-DPB1	SNRPD3
DNAJB6	KLF3	PCDH11Y	KANSL1	HLA-F	SOD2
EIF1	NLK	SLITRK6	FMN1	HNF4G	SORL1
TFG	ANKIB1	PLEKHA8	CCDC18	IFRD1	SPTBN2
SEC24B	MRPL50	TMEM25	MIA3	IRF4	SYK
CHERP	DIRAS2	PPP1R15B	ZC3H6	ITPR2	ADAM17
HEXIM1	ARHGEF38	KLHL6	ANKRD34C	KIR2DS4	TAF11

TAPBP	ACTR3	CHORDC1	ZSCAN31	LDHAL6B	RNF180
TCEA1	TSFM	WDR91	AZI2	KCNG4	FAM115C
TFRC	RNF41	PURG	NXN	ZNF670	DPY19L4
THBS2	NUTF2	PNPLA8	IQCH	SLC35A4	TMSB15B
GPR137B	B3GALT5	TFB1M	LRRC19	SLAMF6	CYP27C1
TNF	PPIE	PPHLN1	PCDH15	LRRC58	SH3RF3
TRAF2	SEMA3C	TAF9B	DSCC1	DNAJC24	LNP1
TRPC3	RBCK1	PPIL1	ZNF557	JDP2	TBC1D26
TXK	CELF1	PRKAG3	ZXDC	ZNF543	USP41
UBE2N	SRSF10	CLDN22	PAGR1	ALDH16A1	KRT77
UGT2B15	WDR4	TMEM106B	NOL9	CCSAP	NSUN4
CLRN1	GJB6	NSMCE4A	OBFC1	PQLC3	TMEM179
VDR	NEU3	KLHL28	OPA3	CCDC148	TEX19
TUBA1A	GLMN	ZNF280D	TNKS2	C4orf33	NBPF9
SEMA3B	ZHX1	CEP55	LAS1L	SAMD8	FLJ25363
AP3B2	KLF8	LINS	FERMT3	TRUB1	FIGNL2
ARID1A	DDX42	DHTKD1	KCTD10	ZNF555	TNFSF12-
GEMIN2	ZFP30	CDCA7L	SLC7A6OS	NBPF12	TNFSF13
PEX3	KLHDC10	DEPDC1	ZNF541	TTL	HIST2H2BF
ITGA10	SPG20	YEATS2	NBPF3	TMEM167A	CT45A5
SNX4	RGL1	CNDP2	LRRC8C	CXorf38	C8orf22
URI1	TBC1D12	NXT2	FYTDD1	C18orf54	IQCJ
TNFSF13	FAM208A	TMPRSS4	PRAC1	SPATA5	NBPF8
TNFSF10	ZCCHC11	C10orf2	DCTN5	NBPF11	NBPF24
SNAP23	NCAPH	PLSCR2	TMEM185A	JMJD1C	NBPF16
FUBP1	TTC9	SMURF1	KRTAP4-4	AK9	ZNF814
SKAP2	POFUT1	TTC7A	TTBK1	TWISTNB	LOC730159
PGLYRP1	RAB38	SMEK2	SFT2D3	FAM185A	C11orf83
PIAS2	BRD1	TBC1D24	ZNF341	CCDC129	TMEM170B
CHST3	LMOD1	ZNF624	TMEM209	SLC17A8	LOC100288142
GOSR1	NBPF14	KIAA1377	SLC45A3	ELMOD2	LOC100289187
SEC24C	NIPBL	GPAM	ZCRB1	COL6A5	ZNF587B
KIAA0586	ABTB2	SIGIRR	LRRCC1	SVIP	SMLR1
TRIL	MOXD1	MRPS35	LRSAM1	CYP4X1	COMMD3-
DCLRE1A	LRP10	SCOC	PKDCC	ZNF615	BMI1
USP3	ACOT11	PIEZO2	ARRDC4	NBPF15	LOC100996646
PIGK	GGA1	C17orf75	MTDH	C20orf203	LOC101060226

LOC101060318	BTN3A2	BMP8B	GRK6	TBC1D25	SLC6A1
LOC101060684	SCN11A	DAGLA	GPX3	OGDH	SLC16A2
LOC101928686	MGAT4A	CA7	GRID1	PAPPA	SLCO2A1
LOC101929020	FILIP1	CACNB3	GRM7	PAX5	SOX9
CDKN1B	IMPG2	CALB1	HK1	PDGFRA	SP2
CXADR	MRPS7	CALCA	HLF	PFKM	SPG7
DYNC1LI2	C10orf118	CALCR	HNF4A	PGM1	SRC
GALNT3	CDK15	CAPN6	TLX1	PIK3CB	SRPR
AXIN2	FBXO31	CASP2	HOXA13	PI4KB	STC1
ZEB2	GRIP2	CAV3	PRMT1	PKNOX1	SUPT6H
APPBP2	JAM3	CBFA2T3	HSBP1	PLAGL1	SURF4
IPO7	ZFHX2	CFTR	HSPA1B	PLAGL2	VAMP2
RPH3A	ZNF616	CHD3	IGSF3	PLIN1	CNTN2
OSTM1	C22orf39	CPD	NDST1	PLOD1	TBCD
CCDC88A	TMEM56	CR2	HTR2C	PNOC	TCF7
PHTF2	TMEM74	CRHR1	CXCL10	POU6F1	PPP1R11
FAM160B1	ZNF100	CRY2	IRF1	PPP1R8	TGFB1
ARHGAP28	FUK	DAG1	KCNK3	PPP2R3A	TGFBR3
PYROXD1	RFESD	BRINP1	KIT	PPP2R5A	TLR4
PARP9	CCDC144NL	DCX	LASP1	PPP2R5D	TSN
ZIM3	ZNF181	DMWD	LDHA	PRKAG1	TULP1
NOP9	IRG1	DOCK3	LMNA	PRKCE	VCL
ZNF367	ABR	E2F3	LPO	PTPRN	BEST1
HIPK1	ASIC2	E2F5	MFAP2	RAB4A	WNT2B
FCGR2A	ADK	EFNB1	MGAT1	RAP1GAP	XBP1
FOXL1	ADRA1D	EIF4EBP2	MMP2	DPF2	XPC
HDLBP	AP1B1	F2RL2	MOG	RFX1	ZNF16
HNMT	AGTR1	F8	MSI1	RGS3	ZNF207
HSPA8	ABCD1	FGFR1	MYCN	RRAS	SLC30A3
IRS1	ALDOA	FLOT2	MYH9	SCNN1D	FOSL1
DDX21	ANK3	FUT1	NEDD8	CCL22	FGF23
NRXN1	AREG	FUT8	NEU1	SDHC	HIST1H4H
GPRIN2	ARF3	GABRA3	NFE2L1	SGTA	PIP5K1A
PDCD6IP	ARHGDIB	GATA3	NFKBIE	SH3GL1	CDC42BPA
SLC17A4	ALDH7A1	GCH1	NFRKB	SHMT1	PPFIA1
SLC25A13	AXL	B4GALT1	NNAT	SIPA1	CNTNAP1
DCAF7	BCL6	GMFB	NOTCH4	SIX3	DGKZ

CHST1	VAT1	KLHL3	LGR4	CSRNP3	MRPL10
PDXK	DPYSL4	RGS17	SMPD3	NAA50	TSHZ2
TNKS	YKT6	VPS4A	SOX6	LIMD2	PTRH1
PER2	KIF1C	SLCO3A1	PACS1	SGPP1	ZBTB46
SLC7A6	TOB2	DBNL	LMBR1L	SEH1L	SOGA1
CLDN1	ZMYND11	TFCP2L1	FOXJ2	AIF1L	VTI1A
UNC119	HPSE	SNX15	Sep-03	RPS6KL1	FAM101A
CCNE2	CPSF6	ALG6	KIAA1217	BCO2	RAD9B
PCSK7	NXPH3	SNX12	NRIP3	ZNF644	C2CD4A
DGKI	HRH3	CDIP1	SAR1A	CCDC135	ZSCAN29
LPAR2	FKBP9	EHD4	TMEM63C	TMEM79	CDAN1
XPR1	PLEKHA6	AK3	ASPHD2	ZDHHHC16	CMTM4
MTA2	RALY	SIDT2	ZNF304	LZTS2	MGAT5B
NUMBL	PCNX	LEF1	RHOJ	SYVN1	RTN4RL1
KLF4	SPEN	CLDN18	NCEH1	TNRC18	LSM14B
ATG5	FAIM2	KCNK9	TP53INP2	LOXL3	FAM117B
SLC25A27	PALLD	MEX3C	RPRD1B	PTPN5	SPICE1
CHST10	MAST3	HEMK1	NTN4	ADO	MBLAC2
EI24	ERC1	HPCAL4	C2orf43	CNTNAP4	RBM33
FAM131B	ATG2A	ZCCHC17	PAPPA2	FGD3	FBXO16
MLEC	ZZEF1	GALNT7	MOAP1	C9orf69	C9orf62
ATG13	NCDN	CLIC5	ABCG4	ZNF551	TSTD2
RNF144A	ATG4B	TOLLIP	NSD1	LRRC46	ARHGAP36
TATDN2	OTUD3	NEURL1B	COPS7B	DUOXA1	TMTC3
ZNF623	ANKS1A	ADAMTSL4	C16orf58	SLC39A13	GPR156
TRANK1	ICOSLG	MIER2	C6orf106	ELMSAN1	OLIG3
SGSM2	PHF15	TBC1D13	METTL4	CHMP7	CADM4
ARHGAP44	TENC1	GATAD2A	MARCKSL1	ORAI3	FAM76A
PTBP3	CRTC1	SWT1	DBNDD1	ORMDL3	TMEM201
GNPDA1	AHCYL2	SIDT1	PLEKHF1	MED8	MED19
TOM1	ZNF281	TMEM104	FAT4	LMTK3	DOK6
DNM1L	PSD4	TTC19	ARSJ	CCDC85A	FGD2
DPP3	PPIL2	SEMA4C	VTCN1	TSEN15	PXDC1
PREB	TMEM184B	STX17	ZYG11B	FAM129A	GPR111
LHFPL2	DAK	RALGPS2	AAGAB	BTBD11	TMEM130
CALCOCO2	PPP1R16B	MSL2	ZFHX4	C14orf28	CNOT6L
FSTL3	FAM127B	C22orf26	ZNF672	ANKRD13B	FNDCC5

ZDHHC23	BAD	CXCR1	PFKFB3	SPN	TMEFF1
CALHM1	BAX	IL9R	PGAM1	SPRR2G	JRK
TXNDC8	BBS1	INPP5B	PGD	TRIM21	STX11
GK5	BIK	KCNC3	SERPINA1	SSTR3	DGAT1
TMED8	BMP1	KCNJ8	PIK3R2	SSX5	B3GALNT1
CES4A	MPPED1	LETM1	PKP2	STAT3	TNFSF14
FAM126B	CALU	LHCGR	POLG	STX1A	CDKL1
RGMB	CD74	LMX1B	PPARG	SURF6	TSC22D1
TPRN	CDH13	LRP4	PPL	SYN2	PROZ
MMAB	CHM	LRPAP1	PPP2R4	TAGLN	CACNA1I
C6orf222	LYST	SMAD7	PPP2R5C	TAL2	FAM127A
GLYATL3	CHRNA7	MBP	PRKCQ	TCF19	P4HA2
UBE2NL	KLF6	METTTL1	PTAFR	VPS72	RPS6KA4
TMEM200B	CX3CR1	MFAP4	PTK6	THBD	CLDN9
C15orf54	DGKB	MID1	PTPRB	THRA	SYNGR2
C5orf63	ACE	MMP17	PVRL2	TK1	SYNGR1
C9orf170	DHCR7	MOCS1	RAD9A	TRIO	LRRFIP1
CUEDC1	DLX1	MTAP	RPL15	TUBA3C	PDLIM7
TRIM67	DLX3	MUC1	RPL28	TUFT1	GPR56
GPX8	DPYD	MYH6	RPS24	TULP3	NRXN2
SPRN	ECE1	MYL4	VPS52	UBA1	PICK1
BTBD18	MARK2	MYO6	SCNN1G	UBE2I	ROCK2
GMNC	EPB41	NAIP	SRL	UBTF	H6PD
ANHX	ETV6	NEDD9	SDC1	UROS	TRAF4
AREGB	FKBP1A	NEUROD2	SEC13	VEGFA	AATK
PLIN4	FUCA2	NF2	ITSN1	WNT7B	RGS6
SHISA7	FUT5	NFKBIA	ST3GAL1	XRCC2	RALGPS1
MEF2BNB	GABRG3	NGFR	SLC4A2	ZFPL1	FAM53B
DDTL	GP5	NOP2	SLC6A3	ZNF2	LRRC14
MSANTD3-	GPS2	NOTCH3	SLC6A6	ZSCAN9	KIAA0355
TMEFF1	HLX	PNP	SLC7A1	MAFK	VPRBP
APLNR	HSPA5	OAZ2	SLC12A3	KDM5D	SNPH
ALOX5	HTR2A	OSM	SLC16A1	PLA2G6	STARD8
ALX3	HUS1	FURIN	SMARCC1	PKP4	PIEZO1
ATM	IFIT1	PAX7	SMPD1	KCNAB2	BMS1
ATP6V1E1	IGSF1	PCDHGC3	SNAPC2	LGR5	SPOCK2
ATP6V0B	IL1RN	PCYT1A	SNCG	AKR7A2	PLEKHM1

FARP2	IQCE	MRPL30	KIAA1143	FLJ22184	FCHO2
RBM19	WSCD1	RASL12	ZNF512B	C2orf44	SLC22A12
SEC16A	DNAJC16	GMIP	SHROOM4	PNPLA3	C5orf55
ZBTB40	ANGEL1	PHF21A	RPTOR	DDHD1	TWIST2
P2RY14	SPECC1L	WAC	HACE1	TIGD6	COMTD1
TSSC4	RHOQ	THEM6	Mar-04	NETO2	SDR9C7
ATP9A	SF3B3	SNX9	ZSWIM5	SYT15	FSD2
UST	DAAM2	RTFDC1	ZBTB4	ACAD11	EARS2
ENAM	DAPK2	MBD3	CPNE5	CAMKK1	GJD3
ZNF263	PRND	CSNK1G1	DENND1A	PPAPDC1B	SLC5A10
PSME3	BCL2L13	CYCS	PRX	GLYR1	MFSD12
AP3S2	TP53TG3	ING3	GPR107	MPV17L2	HSPB6
TIMM17B	TBC1D22A	DYM	GBA3	FAM83A	SGPP2
SPEG	TMEM59L	BSPRY	NGB	TMEM141	PPM1M
NMUR1	ZNF324	PPP1R14D	OVOL2	SMIM3	ADAT2
TMEM5	MYRIP	MTMR10	TMEM35	SHANK3	COL26A1
SPON1	SNED1	ZSCAN2	CDH23	KIAA1671	NDUFAF6
CIB2	TENM4	LAMTOR1	CLSTN2	FAM207A	MUC17
ARFGEF1	RAI14	RBM28	C10orf54	NKD1	ASB11
SORBS1	KLK13	TRIM62	MMP25	NKD2	ASB6
MXD4	WIPI2	NAT10	KLHL25	CHRFAM7A	WDR66
CERS1	MTG2	WDR33	INF2	ZNF628	LYSMD4
PRSS21	FBXO25	SLC22A15	PLA2G2F	SYS1	EME1
NISCH	TSPAN17	CSGALNACT2	MTHFSD	UCN2	DHRS13
PROSC	FBXO10	TNFRSF19	PLEKHG2	UNC5A	B3GALNT2
RNF24	CNNM3	SAGE1	KRI1	ZNF766	LELP1
PMF1	ZNF285	STAB2	TTPAL	C12orf29	LOC149373
CBX3	UQCRQ	SMPD4	MAP7D3	SHKBP1	WFDC5
KLRK1	CACNG4	TSR1	ZNF329	PEX11G	YDJC
SAMD4A	COMMD9	PARVA	ARMC5	ZBTB47	CHADL
EMC1	ZDHHC8	GSDMB	ALG13	ABCC12	CDPF1
SWAP70	UBIAD1	TMEM234	UBA5	OPN4	GAREML
KDM4C	SLC39A3	SLC2A9	HDAC11	TGS1	SLC16A14
TRIM35	IRAK4	AKIP1	LPCAT1	SLC46A1	PRELID2
TBC1D2B	MRT04	ARNTL2	MAP6D1	C12orf57	AMOTL1
NFASC	NAGPA	RHBG	MZT2B	LENG8	TTLL11
SLC35D1	FAM203A	ZMIZ1	ATHL1	LRRC56	LOC158434

ZNF780B	ZNF707	LOC100506115	EMX2	PNN	CHAF1B
PAQR7	ZFP41	C15orf38-AP3S2	ETV1	PPM1B	EEA1
CHST13	TSPAN33	KLRC4-KLRK1	EVI2A	PRKAR2B	SUPT3H
DACT2	SMTNL2	RPL36A-	F13A1	EIF2AK2	SORBS2
C10orf91	TGM6	HNRNPH2	FCGR2B	RELN	BHLHE40
ACTL10	OTOP3	FXVD6-FXVD2	FOXG1	PTPRE	PPAP2B
POLR3H	C15orf38	ZNF664-	GHR	RAP1B	PER3
EME2	ZNF530	FAM101A	GLI3	RBBP4	CPNE3
C1orf100	C11orf34	LOC101060457	GNL1	ATXN2	PRPF4B
ANKRD23	GPR142	LOC101060464	GSK3B	SRSF4	NEURL
KLHDC8B	SPATA12	LOC101928192	NRG1	SHMT2	EBAG9
TMEM184A	POLN	LOC101928195	HOXA2	SLC5A5	REPS2
CERS3	KRTAP10-7	LOC101928268	HPRT1	SNAP25	DCLK1
TMEM136	RTL1	LOC101928589	HRH1	SOAT1	KIF3B
TPCN2	LOC388282	LOC101928722	HSPA9	SOX5	PSMF1
CYB561A3	BLOC1S3	LOC101928841	IL12B	SPAG1	ADAMTS2
FAM162B	ZNF324B	LOC101928843	ITGA1	TBXAS1	PPIP5K1
ZBTB9	ZNF662	LOC101929306	ITGAV	KLF10	ULK2
RNF182	MAP3K15	LOC101929396	KAL1	TK2	CEP104
BRAT1	ZNF805	LOC101929970	KCNC1	TRAF5	DNAJC6
ZSCAN23	GRID2IP	LOC101930060	KIF2A	TRPC6	GOLGA5
SLC29A4	C14orf180	ACO1	SH2D1A	TSNAX	ACOT8
POLR2J2	GRAPL	ADCYAP1R1	MAP2	TWIST1	WASF2
MBLAC1	C1orf226	BIRC3	CTAGE5	UBE2V2	RCAN2
RASGEF1C	CENPP	ATP6V1A	OAZ1	YY1	RTN3
C3orf43	FAM127C	ATP6V0A1	OCRL	SF1	NDRG1
ALS2CL	GIMAP6	ATP7B	OMG	CNBP	SLC9A6
P4HA3	POLR2J3	CA1	PAX3	ZSCAN20	B3GNT2
C14orf182	BOLA2	CBLB	PCBP1	ZNF84	NUP50
PROX2	CYB5RL	CD44	PCBP2	TRIM25	NCKAP1
HEXDC	TP53TG3C	CTSC	SERPINA5	ZNF189	MMP24
PTRF	PPAN-P2RY11	FOXN3	CDK17	ZNF195	YWHAQ
ZNF844	FAM203B	CREBL2	PDGFA	PXDN	VAX1
C19orf77	SFTPA2	CRHR2	PDPK1	RAB7A	RER1
RSPO1	TP53TG3D	DHX9	PIP4K2A	SLC39A7	CD160
DOK7	TP53TG3B	DLG1	PLP1	NCOA4	POLR3A
SH3PXD2B	LOC100131094	EIF4E	PMM2	NRIP1	ERLIN2

SEC23IP	GOLIM4	MYO5C	JPH4	NAALADL2	GPR21
SLC6A14	RRP7A	KLHL9	C1orf198	FRMD3	HNRNPC
GABARAP	CCDC113	KCNQ5	ATAD1	ZNF660	IL1A
MTF2	SCHIP1	ASAH2	KBTBD6	CNPY1	LY75
FNDC3A	NOX4	SMARCAD1	EFCAB11	MSL1	MBL2
SLITRK3	F11R	NIPAL3	FRMD7	LANCL3	MMP8
LIMCH1	DESI2	CLK4	KIAA2013	FAM101B	MX2
MYT1L	HSPA14	PCDH19	ZNF697	THEMIS	NBN
PDS5B	AMZ2	CGN	KIAA1919	RASL11A	NDUFAB1
SMG1	GSKIP	MIB1	SPECC1	NOTCH2NL	NDUFC1
EFR3A	RAB6B	SH3RF1	UBE2Q2	RBM12B	NDUFV3
PRRC2C	ARMCX3	ZBTB26	ARHGAP12	SHC4	NINJ2
PLCB1	FAM49B	BRINP2	GALNT13	ANKRD20A3	NSF
EHBP1	TRIM33	HEATR6	FBXO32	PALM2-AKAP2	NUP98
SASH1	RAPGEF6	DMRTB1	MRFAP1L1	ZNF674	P2RY1
KIAA0895	IL20RA	MCCC2	NAA30	TMEM232	PCM1
LARP1	SSH1	SLC39A8	CD109	ASAH2B	PCNT
RYBP	TRMT13	C5orf28	ZNF280B	NAT2	PLCL1
ABCA5	HEATR5B	PARP12	ZNF558	ACTN2	POU2AF1
ICMT	PUS7	FNDC3B	C1orf52	ARF6	PRB1
SKIV2L2	FAM63B	TMEM135	RNF38	ARSA	PRB3
LEMD3	RSBN1	ATP13A3	NFXL1	ATP6V1G2	KLK7
PATZ1	ZNHIT6	RPAP3	FAM84B	AVPR1A	PTPRK
AMACR	LY6K	VASH2	AWAT2	NKX3-2	PTPRN2
PITPNB	DPP8	NIPAL2	FAM122B	BMP2	RABIF
LAMP5	RBM23	SETD6	ADAL	BTD	RBL2
POU2F3	TMEM33	FOXRED2	FNDC7	CAPN1	RLBP1
C3orf17	INTS10	CTC1	TOR1AIP2	CD2	RPGR
METTL21B	RCBTB1	TM2D3	RNF168	CDC5L	RPS20
SENPA6	ASXL2	TET1	ZNF800	CLN5	RPS29
FGFR10P2	LIN7C	PRRT1	CXorf22	CR1	RRM1
FBXL5	ABHD10	CAB39L	UBR1	CTNNA1	SLC1A6
FBXO3	IMPACT	ADAMTS12	CREG2	CYP19A1	SLC2A5
LATS2	PRPF40A	HSDL1	DENND6A	DCC	SLC12A1
ANKRD1	NDC1	ITCH	C9orf91	DFFA	SLC18A1
GHITM	HHAT	TMTC1	HNRNPUL2	DMRT1	SNAPC3
PCDH17	SCN3B	ZIC4	C8orf46	EIF2S1	SNRPE

SOX12	ARHGEF17	BBS9	PDSS2	FHDC1	IBA57
AKR1D1	CD302	NKIRAS1	EPPIN	C6orf7	FLCN
SRP68	RBM8A	MRPL13	CHMP1B	SLC25A21	RANBP3L
SSBP1	SLC23A2	CLEC2D	TMEM159	ANKRD40	MICU2
STXBP3	NAALAD2	C11orf21	SNX14	RCCD1	ZUFSP
TACC1	GNE	WRAP73	CSRP2BP	ALKBH8	FAM217A
THRSP	TSPAN1	FAM135B	ZFP14	TTC30A	STXBP4
TLL2	NAMPT	CUTC	ZNF71	ADAMTSL1	GPATCH11
TMOD1	ZNF443	CRYL1	MCUR1	FBXO44	TMEM151A
TPMT	RRAGB	ABHD5	CLSPN	TP53RK	C16orf54
TPO	MICU1	SH3GLB1	TP53AIP1	ZNF257	DTWD2
TSHR	BTN3A3	GTSE1	DCLRE1C	BTBD9	CTAGE6
TUFM	N4BP2L2	MPP6	CERK	WDR31	ANKRD62
WT1	SLC25A17	TMEM8B	DDX31	GBP4	ZNF677
XG	TRAF3IP2	CHIC1	P2RY12	CHCHD1	FAM73A
ZNF20	ZBTB6	CNTN5	Mar-07	TARSL2	ZKSCAN4
ZNF45	WASF3	MIS18A	AGXT2	C2CD4C	C15orf59
ZNF140	EDAR	C21orf91	CASD1	FBLN7	C4orf40
ZNF143	TOMM34	GDAP1	FAM134A	ZFP42	ZNF705A
MTERF	AKAP2	MTRF1L	GNPTAB	C9orf57	ZNF716
GCM1	FASTKD2	TRPM7	MCPH1	ALG10B	CTAGE15
SLC25A12	DAAM1	GDAP2	HSPBAP1	ZNF597	CTAGE9
FADD	PDXDC1	FKBP14	C3orf52	ZNF785	C7orf73
RIOK3	AVL9	GPATCH1	CLMN	ZNF417	ZBTB8A
SYNJ2	MDN1	SLC6A15	CCDC102B	SGOL1	TLCD2
USP13	SEL1L3	OGFOD1	SIKE1	TMEM65	ZNF705D
ZMYM6	USP22	TMEM140	PVRL4	CLECL1	CTAGE4
STK17B	SYNM	AP1AR	TMEM133	CLEC14A	CTAGE8
MED27	ACAP2	DNAJA4	ING5	OR7D2	PET117
MED20	ATL3	ETNK1	ZNF527	OLFML2A	IQCJ-SCHIP1
RNF14	CLIC4	GPATCH2L	PARD6G	PGAM5	LY75-CD302
ARHGEF10	POLDIP2	VIMP	RTN4IP1	DZIP1L	LOC101927622
BCLAF1	WSB1	CCDC47	ZNF518B	TCTEX1D1	LOC101928493

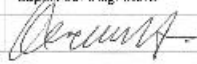
Appendix 2

Complimentary material: 24 h record of GSCs differentiation

<https://figshare.com/s/8ba3b335fc39c8833dae>

Appendix 3

declaration forms

 Declaration for a thesis with publication		
PhD and MPhil students may include a primary research publication in their thesis in lieu of a chapter if: - The student contributed greater than 50% of the content in the publication and is the "primary author", in the student was responsible primarily for the planning, execution and preparation of the work for publication - The student has approval to include the publication in their thesis from their Advisory Committee - It is a primary publication that reports on original research conducted by the student during their enrolment - The initial draft of the work was written by the student and any subsequent editing in response to co-authors and editors reviews was performed by the student - The publication is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in the thesis Students must submit this form, along with Co-author authorisation forms completed by each co-author, when the thesis is submitted to the Thesis Examination System: https://res.app.unimelb.edu.au/. If you are including multiple publications in your thesis you will need to complete a separate form for each publication. Further information on this policy is available at: gradresearch.unimelb.edu.au/preparing-my-thesis/thesis-with-publication		
A. PUBLICATION DETAILS (to be completed by the student)		
Full title	A comprehensive meta-analysis of correlation models in glioma as potential diagnostic biomarker	
Authors	Mac, Nguyen HT, Luu RB, Sykes, Goggs A, Partridge L, Kaye AH, Norrish AP	
Student's contribution (%)	70%	
Journal or book name	PLOS One	
Volume/page numbers	Volume 3 Issue 2 e0189452	
Status	☐ Accepted and In press ☒ Published ☐ In progress	Date accepted/ published 14th Feb 2018
B. STUDENT'S DECLARATION		
I declare that the publication above meets the requirements to be included in the thesis		
Student's name	Student's signature	Date (dd/mm/yy)
Chenka Ma		05/09/2018
C. PRINCIPAL SUPERVISOR'S DECLARATION		
I declare that:		
- The information above is accurate - The advisory committee has met and agreed to the inclusion of this publication in the student's thesis - All of the co-authors of the publication have reviewed the above information and have agreed to its veracity 'Co-Author Authorisation' forms for each co-author are attached.		
Supervisor's name	Supervisor's signature	Date (dd/mm/yy)
A. MOROKOFF		25/09/18



Co-author authorisation form

All co-authors must complete this form. By signing below co-authors agree to the listed publication being included in the student's thesis and that the student contributed greater than 50% of the content of the publication and is the "primary author" ie. the student was responsible primarily for the planning, execution and preparation of the work for publication.

In cases where all members of a large consortium are listed as authors of a publication, only those that actively collaborated with the student on material contained within the thesis should complete this form. This form is to be used in conjunction with the *Declaration for a thesis with publication form*.

Students must submit this form, along with the *Declaration for thesis with publication form*, when the thesis is submitted to the Thesis Examination System: <https://tes.app.unimelb.edu.au/>

Further information on this policy and the requirements is available at:
gradresearch.unimelb.edu.au/preparing-my-thesis/thesis-with-publication

A. PUBLICATION DETAILS (to be completed by the student)

Full title *A comprehensive meta-analysis of circadian miRNAs in glioma as potential biomarkers*
Authors *Ma C, Nguyen HPT, Luuor RB, Stylianou S, Grojos A, Paradiso L, Kaye AH, Morokoff AP*
Student's contribution (%) *90%*
Journal or book name *PLOS One*
Volume/page numbers *Volume 13 Issue 2 00189452*
Status ☐ Accepted and In-press ☒ Published ☐ In progress
Date accepted/published *14th Feb 2018*

B. CO-AUTHOR'S DECLARATION (to be completed by the collaborator)

I authorise the inclusion of this publication in the student's thesis and certify that:

- the declaration made by the student on the *Declaration for a thesis with publication form* correctly reflects the extent of the student's contribution to this work;
- the student contributed greater than 50% of the content of the publication and is the "primary author" ie. the student was responsible primarily for the planning, execution and preparation of the work for publication.

Co-author's name

Co-author's signature

Date (dd/mm/yy)

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27 Aug 2018

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Last Updated 19 October 2017

20/7/18