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Understanding the molecular basis for response and resistance to targeted therapies in colorectal cancer

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Abstract

Active treatment options for patients with metastatic colorectal cancer (CRC) have evolved significantly over the last 20 years. During this time, the median overall survival of these patients has approximately doubled. However, the five-year survival of patients with metastatic CRC is still poor, and further research to optimise treatment is required. The use of targeted therapies has contributed to improvements in clinical outcomes in these patients. However, not all patients respond to these drugs, and they are expensive.

The central aim of this thesis was to explore ways to optimise the targeted treatment of metastatic CRC. Specifically, mechanisms of response and resistance to two targeted therapies - the BRAF inhibitor vemurafenib and the anti-angiogenic drug bevacizumab, were investigated.

Whilst treatment with single-agent BRAF inhibitors has been associated with significant improvements in response and survival outcomes in patients with advanced *BRAF* mutant malignant melanoma, results of clinical trials of BRAF inhibitors for *BRAF* mutant metastatic CRC have been unexpectedly disappointing. First, by screening a panel of *BRAF*^{V600E} CRC cell lines, cell lines which were sensitive and resistant to BRAF inhibition were identified, and these models were used to explore mechanisms of resistance to these drugs. High phosphorylated-epidermal growth factor receptor (p-EGFR) protein expression was identified to be a potential basal biomarker of sensitivity to vemurafenib. In resistant *BRAF*^{V600E} CRC cell lines, BRAF inhibitor treatment was demonstrated to induce rapid p-ERK reactivation which was not associated with increased p-EGFR expression, suggesting the presence of EGFR-independent mechanisms of inherent resistance.

Next, ways to overcome resistance of *BRAF*^{V600E} CRC to BRAF inhibitors were explored. *BRAF* mutant CRC cell lines which were relatively resistant to BRAF inhibitors were found to be broadly similarly resistant to other MAPK inhibitors, and this was also characterised by p-EGFR independent reactivation of p-ERK. This suggests that in *BRAF* mutant CRC, resistance to MAPK inhibitors is tumour- rather than drug-specific, and using combinations of multiple MAPK inhibitors is unlikely to overcome resistance. BRAF inhibitor treatment did not induce preferential RAS activation, increased p-CRAF expression, nor increased p-MEK expression in resistant *BRAF*^{V600E} CRC cell lines, suggesting that alternative mechanisms of resistance originate downstream of MEK.

These findings provide the basis for further exploration of non-EGFR mediated mechanisms of resistance, which ultimately may enable the development of new rational targeted drug combination strategies for *BRAF* mutant CRC.

Bevacizumab, an anti-human VEGF-A monoclonal antibody, is currently approved for the treatment of metastatic CRC in combination with chemotherapy. Discovery of a predictive biomarker for response to bevacizumab would enable us to identify patients who are unlikely to respond, commence them on alternative therapies sooner, and avoid unnecessary side effects. Identification of a predictive biomarker may also improve the cost effectiveness of bevacizumab use.

We explored whether single nucleotide polymorphisms (SNPs) in *VEGF-A*, *VEGFR1* and *VEGFR2* are predictive biomarkers for bevacizumab response, using archived tissue samples and clinical outcome data from participants in the MAX Phase III trial of capecitabine, bevacizumab and mitomycin in the first-line treatment of metastatic CRC. *VEGF-A* SNPs rs25648 and rs699947 were found to be prognostic, but not predictive biomarkers of survival outcomes.

Declaration

I declare that:

(i) This thesis comprises only my original work towards the Doctor of Philosophy, except where indicated in the preface;

(ii) due acknowledgement has been made in the text to all other material used; and

(iii) the thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Signed: _____ 2019

Fiona Joo Meng Chionh

Preface

(a) Acknowledgement of collaborations

I acknowledge that some of the work in this thesis was undertaken collaboratively.

Specifically:

- i. Chapters 3 and 4: Western blots were performed with the assistance of Dr. Janson Tse, Dr. Ian Luk, Analia Lesmana, or Laura Jenkins.
- ii. Chapters 3 and 4: MTS assays were performed in collaboration with Professor John Mariadason (equal contribution).
- iii. Chapter 3, Table 3.1: Exome sequencing of *BRAF*^{V600E} colorectal cancer cell lines was performed in a collaboration involving our laboratory with Associate Professor Oliver Sieber and Dr. Dmitri Mouradov.
- iv. Chapter 3, Figure 3.6: CIMP status of *BRAF*^{V600E} cell lines was determined by Associate Professor Daniel Buchanan.
- v. Chapter 3, Figure 3.13: Analysis of gene expression microarray data using Limma was performed by Dr. Nathan Hall.
- vi. Chapter 3, Figure 3.17: The cell proliferation experiment using the Roche xCELLigence System was performed with the assistance of Associate Professor Tu'Uhevaha Lino and Laura Tuohy.
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- viii. Chapter 5, Figure 5.17: Almac Xcel microarray gene expression data was obtained from Dr. Jennifer Mooi.
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- x. Chapter 5: Statistical analysis for Tables 5.1– 5.4, Tables 5.8 – 5.16, Figures 5.9 – 5.16, and Figures 5.14 – 5.16 was performed by Professor Val GebSKI. Statistical analysis for Figure 5.17 was performed by Dr. Chee Khoon Lee.

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List of abbreviations

[numeral]C	[numeral]cycles
3'UTR	3' untranslated region
5'UTR	5' untranslated region
5-AZA	5-azacytidine
ACCoRD	Analysis of Censored and Correlated Data
AE	adverse event
AJCC	American Joint Committee on Cancer
ALP	serum alkaline phosphatase
AMP	Association for Molecular Pathology
ANG	angiopoietin
ANG1	angiopoietin 1
ANG2	angiopoietin 2
APC	adenomatous polyposis coli
AS	activation segment
ASCO	American Society of Clinical Oncology
ASCP	American Society for Clinical Pathology
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
Australian PBS	Australian Pharmaceutical Benefits Scheme
BD	twice daily
BEV	bevacizumab
bFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
BORR	best overall response rate
BPC	paclitaxel + carboplatin + bevacizumab
BRAF _i	BRAF inhibitor
BSA	bovine serum albumin
BV8	prokineticin 2
C	capecitabine
CAF	circulating angiogenic factor
CAN	copy number alteration
CAP	College of American Pathologists
CAPOX	capecitabine + oxaliplatin
CB	capecitabine plus bevacizumab
CBM	capecitabine, bevacizumab and mitomycin
CCLE	Cancer Cell Line Encyclopaedia
CDK	cyclin-dependent kinase
CEC	circulating endothelial cell
CEP	circulating endothelial progenitor
CI	confidence interval
CI	combination index
CIBL	Cancer Immuno-biology Laboratory

CIMP	CpG island methylation phenotype
CIN	chromosomal instability
CL	catalytic loop
CMS	consensus molecular subtype
COSMIC	Catalogue of Somatic Mutations in Cancer
CR	complete response
CR1	conserved region 1
CR2	conserved region 2
CR2	conserved region 3
CRC	colorectal cancer
CRCSC	Colorectal Cancer Subtyping Consortium
CRD	Cys-rich domain
CRM	circumferential margin
CSF	colony-stimulating factor
CSS	cancer-specific survival
CT	computed tomography
CTLA-4	cytotoxic T-lymphocyte-associated antigen 4
D	daily
DAB	dabrafenib
DCE-MRI	dynamic contrast-enhanced magnetic resonance imaging
DFS	disease-free survival
DIF	dimerization interface
DLL-4	delta-like ligand 4
DMEM	Dulbecco's Modified Eagle Medium Nutrient Mixture
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
DTT	dithiothreitol
DUSP	dual-specificity phosphatase
EC	endothelial cell
ECM	extracellular matrix
ECOG	Eastern Cooperative Oncology Group
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
EGFRi	epidermal growth factor receptor inhibitor
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial-mesenchymal transition
EOC	epithelial ovarian carcinoma
eQTL	expression quantitative trait loci
ESMO	European Society for Medical Oncology
FA	folinic acid
FABP3	fatty acid binding protein 3
FABP7	fatty acid binding protein 7
FAP	familial adenomatous polyposis
FASN	fatty acid synthase
FBS	foetal bovine serum

FDA	Food and Drug Administration
FFPE	formalin-fixed paraffin embedded
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FLK1	foetal liver kinase-1
FLT1	Fms-like tyrosine kinase 1
FLT4	Fms-like tyrosine kinase 4
FOLFIRI	5-fluorouracil + folinic acid + irinotecan
FOLFOX	5-fluorouracil + folinic acid + oxaliplatin
FOLFOXIRI	5-fluorouracil + folinic acid + oxaliplatin + irinotecan
FU	fluorouracil
GBM	glioblastoma multiforme
GDP	guanosine-5'-diphosphate
GEF	guanine nucleotide exchange factor
GEMM	genetically engineered mouse model
GREM1	Gremlin
GSEA	gene set enrichment analysis
GTP	guanosine-5'-triphosphate
H&E	Haematoxylin and Eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	hepatocyte growth factor
HIF-1a	hypoxia-inducible factor-1a
HPSG	heparan sulphate proteoglycan
HR	hazard ratio
HRP	horseradish peroxidase
HUS	haemolytic uraemic syndrome
HWE	Hardy-Weinberg Equilibrium
ID	identification
IFL	Irinotecan + fluorouracil + leucovorin
IGF-1R	insulin-like growth factor 1 receptor
IHC	immunohistochemistry
IL-8	interleukin 8
IMPACT	immunologic multi-parameter chip technology
IPD	individual patient data
ITT	intention-to-treat
JAG1	Jagged-1
KDR	kinase insert domain receptor
K _m	Michaelis constant
KSR	kinase suppressor of RAS
K ^{trans}	volume transfer constant
LD	linkage disequilibrium
LDL	low density lipoprotein
LICR	Ludwig Institute for Cancer Research
LPS	liposaccharide
LV	leucovorin
mAb	monoclonal antibody

MAF	minor allele frequency
MBC	metastatic breast cancer
mCRC	metastatic colorectal cancer
MEKi	MEK inhibitor
mIFL	modified IFL
<i>MLH1</i>	mutL homologue 1
MMP	matrix metalloprotease
MMR-D	mismatch repair deficient
MMR-P	mismatch repair proficient
mRNA	messenger ribonucleic acid
<i>MSH2</i>	mutS homologue 2
MSI-H	microsatellite instability-high
MSS	microsatellite stable
mTOR	mammalian target of rapamycin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
muMAb	murine monoclonal antibody
mut	mutant
MVD	microvascular density
MVHP	microvesicular hyperplastic polyp
NA	not applicable
NCCN	National Comprehensive Cancer Network
NHMRC CTC	National Health and Medical Research Council Clinical Trials Centre
NRP	neuropilin
NRP1	neuropilin 1
NRP1	neuropilin 2
NS	not statistically significant
NSCLC	non-small cell lung carcinoma
NTC	non-template control
ONJCRI	Olivia Newton-John Cancer Research Institute
OR	odds ratio
ORR	objective response rate
OS	overall survival
p-[protein name]	phosphorylated [protein]
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PC	paclitaxel + carboplatin
pCR	pathological complete response
PCR	polymerase chain reaction
PD-1	programmed cell death protein-1
PDAC	pancreatic ductal adenocarcinoma
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PD-L1	programmed death-ligand 1
PDX	patient derived xenograft
PFS	progression free survival

PFS6	6-month progression-free survival
PG	pharmacogenetic
PI3K	phosphoinositide 3-kinase
PI3Ki	phosphoinositide 3-kinase inhibitor
PLC γ	phospholipase C γ
PIGF	placental growth factor
P-loop	phosphate-binding loop
PMS	phenazine methosulfate
<i>PMS2</i>	<i>PMS1</i> homologue 2
po	oral
PPC	primary peritoneal carcinoma
PR	partial response
PS	performance status
QOL	quality of life
R2PD	recommended phase 2 dose
RBD	RAS-binding domain
RCC	renal cell carcinoma
RCT	randomised controlled trial
RECIST	Response Evaluation Criteria in Solid Tumours
RFS	relapse-free survival
rhuMab	recombinant humanised monoclonal antibody
RLU	Relative Light Units
<i>RNF43</i>	Ring Finger Protein 43
RPPA	reverse phase protein array
RR	risk ratio
RSPO	R-spondin
RT	room temperature
RTK	receptor tyrosine kinase
RT-PCR	real-time polymerase chain reaction
SCC	squamous cell carcinoma
SCNA	somatic copy number alteration
SD	standard deviation
SDF1	stromal cell-derived factor 1
SDS	sodium dodecyl sulphate
shRNA	short hairpin RNA
SNP	single nucleotide polymorphism
SOS	Son of sevenless
SPRY	Sprouty
SSA/P	sessile serrated adenomas or polyp
SSA/P-D	sessile serrated adenoma or polyp with dysplasia
sVEGFR1	soluble vascular endothelial growth factor receptor 1
sVEGFR2	soluble vascular endothelial growth factor receptor 2
t-[protein name]	total [protein]
TGF-b	transforming growth factor-b
TMA	tissue microarray
TNM	Tumour, Node and Metastasis

TRAM	trametinib
Treg	T regulatory
TSA	traditional serrated adenoma
TTP	time to progression
UK	unknown
US	United States
VEGF	vascular endothelial growth factor
VEGF	vascular endothelial growth factor
VEGF-A	vascular endothelial growth factor-A
VEGF-B	vascular endothelial growth factor-B
VEGF-C	vascular endothelial growth factor-C
VEGF-D	vascular endothelial growth factor-D
VEGFR	vascular endothelial growth factor receptor
VEGFR1	vascular endothelial growth factor receptor 1
VEGFR2	vascular endothelial growth factor receptor 2
VEGFR3	vascular endothelial growth factor receptor 3
VEM	vemurafenib
VPF	vascular permeability factor
<i>v-Raf</i>	Rapidly accelerated fibrosarcoma
VRF	vascular endothelial growth factor-related factor
WHO	World Health Organisation
wt	wild-type
XELIRI	capecitabine + irinotecan
XELOX	capecitabine + oxaliplatin

1. LITERATURE REVIEW

1.1. Introduction

Worldwide, colorectal cancer (CRC) has the third-highest incidence and second-highest cancer-related mortality, accounting for over 1.8 million (10.2%) new cancer cases and over 800,000 (9.2%) cancer-related deaths in 2018 [1] (Figures 1.1A and B).

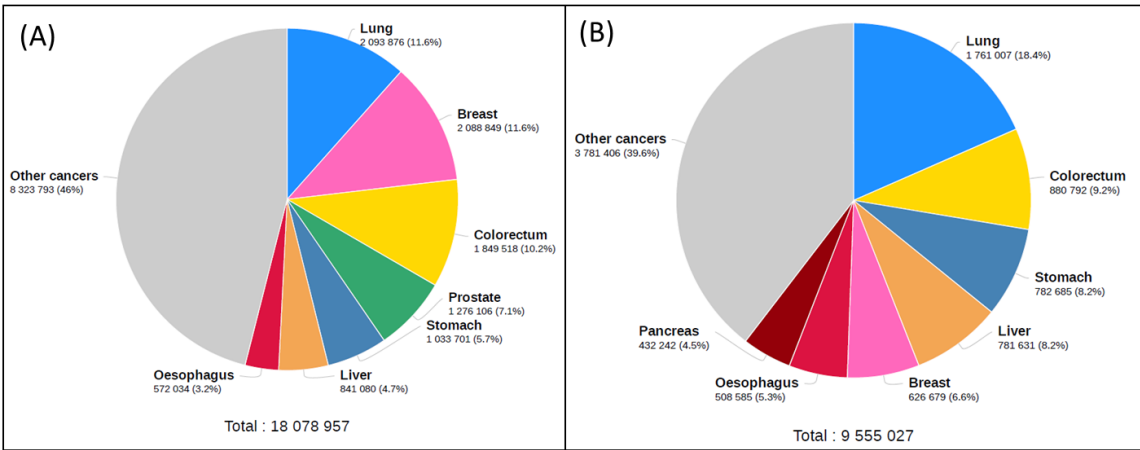


Figure 1.1. Estimated (A) incidence of and (B) mortality from cancers worldwide in 2018
Data accessed from Global Cancer Observatory (<http://gco.iarc.fr>), on 05 November 2018

Australia has one of the highest age-standardised incidence rates of CRC in the world. In 2018, more than 17,000 new CRC cancer cases were diagnosed (Figure 1.2A), and there were over 6,000 CRC cancer-related deaths, which accounted for 12.4% of all cancer-related deaths in Australia [1] (Figure 1.2B).

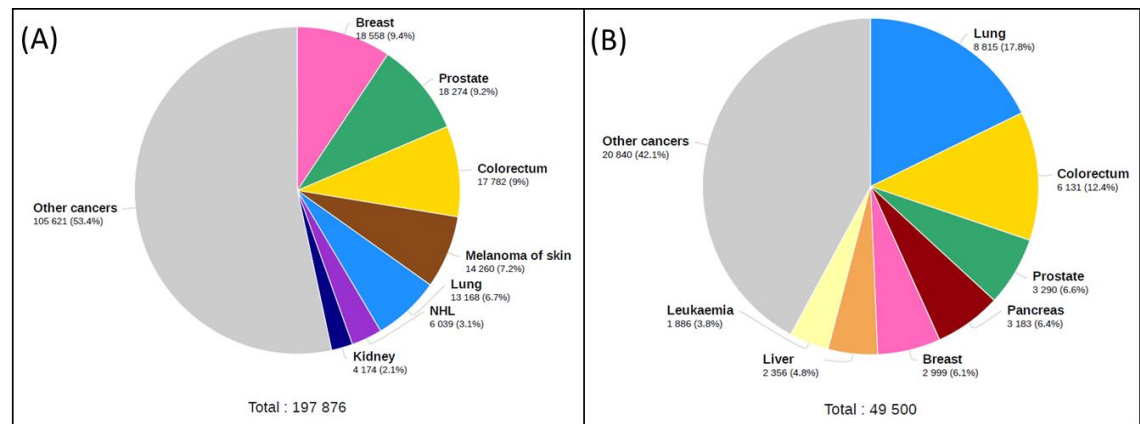


Figure 1.2. Estimated (A) incidence of and (B) mortality from cancers in Australia in 2018
Data accessed from Global Cancer Observatory (<http://gco.iarc.fr>), on 05 November 2018.

The majority of patients are diagnosed with early stage CRC without *do novo* metastatic disease (75-80%) [2], and can be potentially cured with surgical resection of the primary tumour and regional lymph nodes. Adjuvant chemotherapy with a fluoropyrimidine in combination with

oxaliplatin reduces the risk of recurrence and provides a survival advantage in patients who have stage III colon cancer (with regional lymph node involvement) [3-6] (Table 1.1). American Society of Clinical Oncology (ASCO) guidelines conclude that direct evidence from randomised controlled trials (RCTs) does not support the routine use of adjuvant chemotherapy in stage II CRC (with invasion through the muscularis propria, but without regional lymph node involvement) [7] (Table 1.1). As recommended by current National Comprehensive Cancer Network (NCCN) guidelines, decisions about the use of adjuvant chemotherapy in this setting should involve individualised physician and patient discussions of the potential benefits versus risks, including consideration of high-risk features (both clinicopathological and molecular), evidence regarding efficacy and potential toxicity, and patient preferences [8]. However, adjuvant chemotherapy is not recommended for patients with microsatellite instability-high (MSI-H) low-risk stage II colon cancer who have a better prognosis than their microsatellite stable (MSS) counterparts and who also may derive less benefit from single agent fluoropyrimidine chemotherapy [9-11], nor for patients with Stage I CRC, in whom current five-year survival rates with surgical resection alone are 95% [12].

For patients who have locally advanced rectal adenocarcinomas with the high-risk features of clinical T3-4 disease, regional nodal involvement, a threatened circumferential margin (CRM) or involved mesorectal fascia [8], or low-lying tumours [13], the current standard of care is curative intent treatment using neoadjuvant fluoropyrimidine chemotherapy concurrent with pelvic radiotherapy, surgical resection, and adjuvant chemotherapy with fluoropyrimidine and oxaliplatin [8]. For patients with rectal cancer who have been treated with initial surgical resection (including those with clinical T1-2N0M0 disease), treatment with adjuvant chemoradiotherapy or chemotherapy is recommended if there are high risk features on pathological staging [8].

Notably, fifty to sixty percent of patients diagnosed with early stage CRC subsequently develop metastatic disease [14-16], and 20 to 25% of patients with colorectal cancer present with Stage IV disease [2]. The majority (80-90%) of these patients have unresectable metastatic disease [15, 17-19]. Typically, the palliative intent treatment approach for these patients involves the sequential use of various active treatment regimens, which can be given in combination or as single agents, along with supportive care [8, 20]. The goals of treatment are to reduce tumour growth, improve symptoms, and improve survival outcomes.

Active therapeutic options for patients with metastatic CRC (mCRC) have evolved significantly over the last 20 years, and accordingly, during this time the median overall survival (OS) of patients in clinical trials has doubled to approximately 30 months [21]. These therapeutic options include three main classes of drugs: cytotoxic chemotherapy (including the fluoropyrimidines 5-fluorouracil (FU), capecitabine, and trifluridine-tipiracil, as well as oxaliplatin and irinotecan), molecularly targeted therapies, and immunotherapies. Molecularly targeted therapies include the

anti-angiogenic agents bevacizumab, ziv-aflibercept, ramucirumab, and regorafenib, and the epidermal growth factor receptor (EGFR) inhibitors cetuximab and panitumumab. Active immunotherapies for patients with MSI-H tumours include the programmed cell death protein 1 (PD-1) inhibitors pembrolizumab and nivolumab [8].

Unfortunately, despite these advances in treatment, the prognosis of patients with mCRC is still poor, with a 5-year survival of 13.8% [22], and further research to optimise treatment for these patients is required.

This thesis aims to explore ways to optimise the targeted treatment of mCRC by investigating mechanisms of response and resistance to two targeted therapies - the BRAF inhibitor vemurafenib, and the anti-angiogenic agent bevacizumab.

The biology of the pathways related to each molecularly targeted therapy will be reviewed. The role of each targeted therapy for the treatment of mCRC will be discussed, with a focus on biological mechanisms and biomarkers of response and resistance.

Whilst treatment with single-agent BRAF inhibitors have led to significant improvements in response and survival outcomes in advanced *BRAF* mutant malignant melanoma [23-32], clinical trials of BRAF inhibitors in *BRAF* mutant mCRC have had unexpectedly disappointing results [33-35]. In this thesis, we will explore mechanisms of resistance of *BRAF* mutant CRC to BRAF inhibitors, and ways of overcoming this resistance.

Whilst the addition of bevacizumab treatment to combination chemotherapy leads to longer median progression free survival (PFS) and modest improvements in OS and objective response rates (ORRs) in patients with mCRC, not all patients who are treated with the drug respond. Bevacizumab treatment is also associated with multiple side effects, which may be severe [36], and the drug is expensive [37]. Therefore, there is a clinical and economic need to identify biomarkers which are predictive of bevacizumab response [38]. In this thesis, we will explore the role of single nucleotide polymorphisms (SNPs) in vascular endothelial growth factor (VEGF) family genes as predictive biomarkers for bevacizumab in mCRC.

Table 1.1. The American Joint Committee on Cancer (AJCC) tumour, node and metastasis (TNM) staging system for colorectal cancer, 8th edition, 2017 [39]

Primary tumour (T)			
T category	T criteria		
TX	Primary tumour cannot be assessed		
T0	No evidence of primary tumour		
Tis	Carcinoma <i>in situ</i> , intramucosal carcinoma (involvement of lamina propria with no extension through muscularis mucosae)		
T1	Tumour invades the submucosa (through the muscularis mucosa but not into the muscularis propria)		
T2	Tumour invades the muscularis propria		
T3	Tumour invades through the muscularis propria into pericorectal tissues		
T4	Tumour invades the visceral peritoneum or invades or adheres to adjacent organ or structure		
T4a	Tumour invades through the visceral peritoneum (including gross perforation of the bowel through tumour and continuous invasion of tumour through areas of inflammation to the surface of the visceral peritoneum)		
T4b	Tumour directly invades or adheres to adjacent organs or structures		
Regional lymph nodes (N)			
N category	N criteria		
NX	Regional lymph nodes cannot be assessed		
N0	No regional lymph node metastasis		
N1	One to three regional lymph nodes are positive (tumour in lymph nodes measuring ≥ 0.2 mm), or any number of tumour deposits are present and all identifiable lymph nodes are negative		
N1a	One regional lymph node is positive		
N1b	Two or three regional lymph nodes are positive		
N1c	No regional lymph nodes are positive, but there are tumour deposits in the: • Subserosa • Mesentery • Non-peritonealised pericolic, or perirectal/mesorectal tissues		
N2	Four or more regional nodes are positive		
N2a	Four to six regional lymph nodes are positive		
N2b	Seven or more regional lymph nodes are positive		
Distant metastasis (M)			
M category	M criteria		
M0	No distant metastasis on investigations - no evidence of tumour in distant sites or organs.		
M1	Metastasis to one or more distant sites or organs or peritoneal metastasis is identified		
M1a	Metastasis to one site or organ is identified without peritoneal metastasis		
M1b	Metastasis to two or more sites or organs is identified without peritoneal metastasis		
M1c	Metastasis to the peritoneal surface is identified alone or with other site or organ metastases		
Stage groups			
Stage group	T category	N category	M category
0	Tis	N0	M0
I	T1-2	N0	M0
II			
IIA	T3	N0	M0
IIB	T4a	N0	M0
IIC	T4b	N0	M0
III			
IIIA	T1-2	N1/N1c	M0
	T1	N2a	M0
IIIB	T3-4a	N1/N1c	M0
	T2-3	N2a	M0
	T1-2	N2b	M0
IIIC	T4a	N2a	M0
	T3-4a	N2b	M0
	T4b	N1-2	M0
IV			
IVA	T any	N any	M1a
IVB	T any	N any	M1b
IVC	T any	N any	M1c

1.2. Targeting mutant BRAF for the treatment of metastatic colorectal cancer

1.2.1. Molecular subtypes of colorectal cancer

Colorectal cancers can be classified into two main molecular subtypes- those (the majority, ~85%) which are microsatellite stable (MSS) and characterised by chromosomal instability (CIN) with structural and numerical chromosomal abnormalities [40], and those which have microsatellite instability (MSI) (~15%) and are characterised by the accumulation of many insertion or deletion mutations at microsatellites along the genome [41] (Figure 1.3).

1.2.1.1. Chromosomal instability (CIN)

Most MSS CRCs develop through the conventional adenoma-carcinoma pathway (described in section 1.2.4.1.) and are generally non-hypermuted [42]. CIN in these CRCs arises from ongoing errors in chromosomal segregation during mitosis [43], and deregulation of multiple cancer-related pathways have been implicated in the pathogenesis [40]. For example, oncogenic signalling, replication stress, defects in centrosome replication, and telomere dysfunction have been shown to induce CIN [40].

Approximately 1% of CRCs develop from the hereditary colorectal cancer syndrome familial adenomatous polyposis (FAP) [44]. FAP is a highly penetrant autosomal-dominant disorder which is caused by a germline mutation in the adenomatous polyposis coli (*APC*) gene and is clinically characterised by the early onset (usually in adolescence) of hundreds to thousands of adenomas predominantly affecting the left colon, with almost inevitable progression to CRC by 35 to 40 years of age [45].

1.2.1.2. Microsatellite instability (MSI)

MSI CRCs develop due to defects in the deoxyribonucleic acid (DNA) mismatch repair system, caused by inactivation of mutL homologue 1 (*MLH1*), *MLH3*, mutS homologue 2 (*MSH2*), *MSH3*, *MSH6* or *PMS1* homologue 2 (*PMS2*) genes [46]. These mismatch repair deficient (MMR-D) tumours are unable to repair insertions or deletions which most commonly arise in microsatellite repeat regions (the MSI phenotype). As these repeat sequences are prone to an increased number of mutations [47, 48], MSI tumours accumulate high rates of mutations and are said to be ‘hypermuted’ [42]. This eventually leads to inactivation of tumour suppressor genes and activation of proto-oncogenes, and consequently, malignant transformation.

MSI-high CRCs are associated with the clinico-pathological features of right-sided location, mucinous differentiation and high numbers of tumour infiltrating lymphocytes [49]. In early stage CRC, MSI-H tumours are associated with a favourable prognosis [50-52], and the prognostic value of MSI is stronger in stage II than stage III CRC [53]. The prognostic value of MSI is even less clear in patients with metastatic CRC, where there is a low prevalence of MSI-H [54] and

large studies have demonstrated that an adverse prognostic impact may be driven by associated *BRAF* mutation status in the tumour [55].

Whilst 80% of MSI CRCs develop as sporadic tumours through the serrated pathway (described in section 1.2.4.2.) and are strongly associated with *BRAF* mutations [56, 57], 20% of these tumours develop from the hereditary Lynch syndrome [44]. Lynch syndrome is caused by autosomal dominant heterozygous germline mutations in one of *MLH1*, *MSH2*, *MSH6* or *PMS2*, resulting in loss of function of the protein. Lynch syndrome cancers subsequently develop following somatic loss of function of the remaining wild-type allele of the affected MMR gene [58].

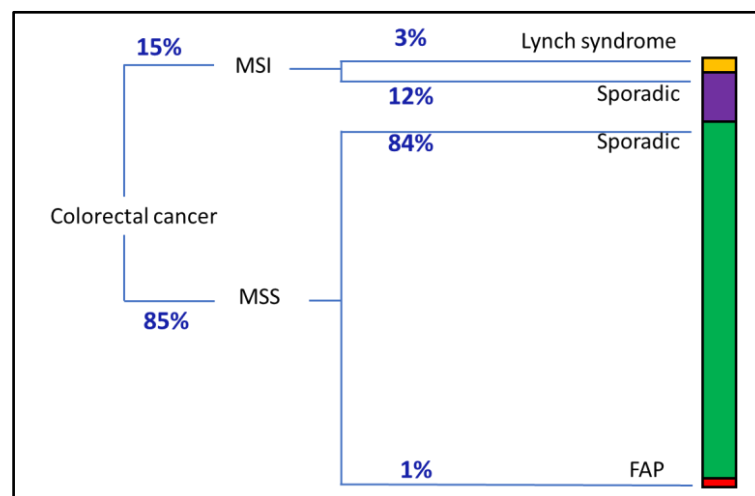


Figure 1.3. Schematic of the molecular subtypes of colorectal cancer (Modified from Brenner *et al* [44]). MSI: microsatellite instability; MSS: microsatellite stable; FAP: familial adenomatous polyposis.

1.2.2. Consensus molecular subtypes (CMS) in colorectal cancer

Recently, the international Colorectal Cancer Subtyping Consortium (CRCSC) demonstrated significant interconnectivity between six independent transcriptomic-based classification systems in CRC. This resulted in the development of a consensus molecular classification which enables classification of CRCs into one of four consensus molecular subtypes (CMSs) [59] (Figure 1.4).

Within this consensus molecular classification, MSI tumours cluster within the CMS1 group (MSI immune subtype), which is characterised by hypermutation, hypermethylation, high levels of immune infiltrate and activation, and enrichment for *BRAF* mut tumours [59]. The clinical characteristics of CMS1 tumours include female gender, right-sided tumours, presentation with higher histopathological grade, and poor survival rates after relapse [59].

The MSS/CIN tumours are sub-classified into one of three groups- CMS2 (canonical subtype), CMS3 (metabolic subtype) or CMS4 (mesenchymal subtype). Both CMS2 and CMS4 are characterised by low levels of hypermethylation and high levels of somatic copy number

alterations (SCNAs) [59]. However, CMS2 tumours are distinguished by epithelial differentiation and upregulation of *WNT* and *MYC* targets, and are predominantly left-sided [59]. In contrast, CMS4 is characterised by the activation of pathways related to epithelial-mesenchymal transition (EMT) (including transforming growth factor- β (TGF- β) activation) which is driven by the abundant stromal content [60-62], and upregulation of genes associated with extracellular matrix remodelling, complement signalling, and angiogenesis [59]. Patients with CMS4 CRCs also tend to be diagnosed at more advanced stages (III or IV), and have a worse relapse-free and overall survival [59].

Compared to CMS2 and CMS4 CRCs, CMS3 tumours are characterised by low levels of SCNAs, CpG island methylation phenotype (CIMP)-low (see section 1.2.4.2.) with intermediate levels of hypermethylation, and up to 30% have MSI and hypermutation. CMS3 CRCs are enriched for *KRAS* mutations, and are associated with metabolic deregulation, including activation of glutaminolysis and lipidogenesis [59].

CMS 1 MSI immune	CMS 2 Canonical	CMS 3 Metabolic	CMS 4 Mesenchymal	Unclassified
14%	37%	13%	23%	13%
MSI, CIMP high, hypermutation, SCNA low	SCNA high (enriched for HNF4A amplifications)	Mixed MSI status, CIMP low, SCNA low	SCNA high	Heterogeneous patterns of CMS mixtures
BRAF mutations		KRAS mutations		
Immune infiltration and activation	Epithelial differentiation, WNT and MYC activation	Metabolic deregulation	Activation of pathways related to EMT, stromal infiltration, angiogenesis	
Female, right-sided tumours, higher histopathological grade	Left-sided tumours	Diagnosed at more advanced stages (stage III-IV)		
Worse survival after relapse			Worse relapse-free and overall survival	

Figure 1.4. Consensus molecular subtypes in colorectal cancer with associated molecular, clinical, and prognostic correlates (Modified from Guinney *et al* [59]). CMS: Consensus molecular subtypes; MSI: microsatellite instability; CIMP: CpG island methylator phenotype; SCNA: somatic copy number alterations; EMT: epithelial-mesenchymal transition.

1.2.3. Transcriptomic-based characterisation of *BRAF* mutant colorectal cancer beyond CMS

Barras *et al.* recently analysed gene expression data from 218 patients with *BRAF* mut CRC, to further characterise heterogeneity within these patients [63]. Within this cohort, the authors found

that 70% of *BRAF* mut tumours were classified as CMS1, 17% as CMS4, 5% as CMS3, and 2% as CMS2. Unsupervised clustering was performed to classify patients with *BRAF* mut CRC into gene expression-based subgroups, and gene set enrichment analyses (GSEA) was used to characterise pathway activation in each subgroup. They identified two distinct subtypes, 'BM1' and 'BM2', which differed by the expression of a set of 476 genes, independently of MSI status, phosphoinositide 3-kinase (*PI3K*) mutation, gender and sidedness. All CMS4 *BRAF* mutants were classified as 'BM1', whilst CMS1 *BRAF* mutants were classified as 'BM1' or 'BM2'.

The 'BM1' subtype was molecularly characterised by KRAS/AKT pathway activation, mammalian target of rapamycin (mTOR)/4EBP deregulation, macrophage infiltration and EMT. In contrast, the 'BM2' subtype was characterised by deregulation of the cell cycle. There was no significant difference between overall and relapse-free survival between 'BM1' and 'BM2'. Notably, 'BM1' was enriched for an EGFR signature, however *EGFR* expression levels were not significantly different between 'BM1' and 'BM2'.

1.2.4. Molecular pathogenesis of colorectal cancer

There are two pathways for the development of CRC – the conventional adenoma-carcinoma pathway and the serrated neoplasia pathway.

1.2.4.1. The conventional adenoma-carcinoma pathway

The conventional adenoma-carcinoma pathway of colorectal tumorigenesis involves the stepwise accumulation of genetic and epigenetic events which lead to the formation of adenomas, and then carcinomas from normal colonic mucosa [64-66].

The first gatekeeping mutation, which most commonly occurs in the *APC* gene in the WNT pathway [67] gives the affected cells a selective growth advantage, which leads to the formation of a small adenoma [65]. Further driver events provide a growth advantage to the tumour cell, leading to further clonal expansion and increased size of the adenoma [42]. This continuing process of driver events followed by clonal expansion eventually leads to development of a malignant carcinoma [68]. The other most commonly altered pathways in this process are the MAPK, PI3K, TGF- β , and p53 pathways [46] (Figure 1.5).

Comprehensive molecular characterisation of CRCs recently reported by The Cancer Genome Atlas Network showed that genetic alterations in the WNT signalling pathway occurred in 93% of colorectal cancers, with 80% of cases involving biallelic inactivation of the *APC* gene or activating mutations of *CTNNB1*. In addition, alterations in the *SOX9*, *TCF7L2*, *AXIN2*, *FBXW7*, *ARID1A*, *FAM123B/WTX*, *FZD10*, *LRP5* and *DKK1-4* genes were also identified [46].

Genetic alterations in the RAS-RAF-MEK-ERK MAPK signalling pathway were also common, with 55% of non-hypermutated tumours having mutually exclusive alterations in *KRAS*, *NRAS* or *BRAF*, and mutations or amplifications in the *ERBB* family occurring in 13% and 53% of non-

hypermuted and hypermutated cancers, respectively. One third of tumours with genetic alterations in the MAPK pathway had co-alterations in the PI3K pathways, which included mutually exclusive mutations in *PIK3R1*, *PIK3CA* and *PTEN*, and overexpression of *IGF2* and *IRS2* [46].

In the TGF- β pathway, genetic alterations were found in *TGFBR1*, *TGFBR2*, *ACVR2A*, *ACVR1B*, *SMAD2*, *SMAD3* and *SMAD4* in 27% of non-hypermuted and 87% of hypermutated CRCs.

In the p53 pathway, genetic alterations were found in *TP53* in 59% of non-hypermuted CRCs, and *ATM* in 7% [46].

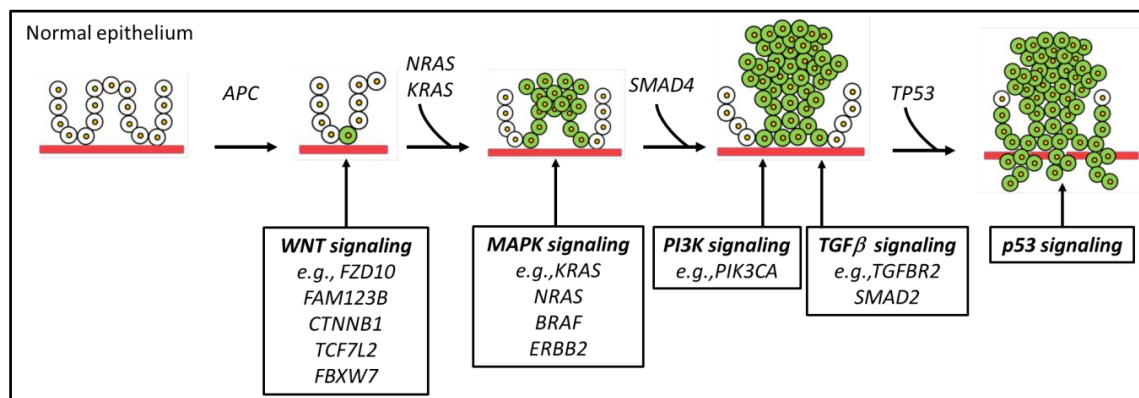


Figure 1.5. The adenoma-carcinoma sequence (Modified from Kuipers *et al* [69]). APC: adenomatous polyposis coli; TGF β : transforming growth factor β .

1.2.4.2. The serrated neoplasia pathway

The serrated neoplasia pathway for colorectal carcinogenesis is distinct from the conventional adenoma-carcinoma sequence, and has been estimated to contribute to over 15% of CRC [70-75].

The most common molecular characteristic of serrated pathway adenocarcinomas is a mutation in the *BRAF* proto-oncogene [76, 77], and these CRCs also frequently harbour hypermethylation of CpG islands on the promoter regions of tumour suppressor genes (CIMP) [70].

Sessile serrated adenomas or polyps (SSA/Ps) are precursors of serrated pathway adenocarcinomas [78], and these lesions may develop from microvesicular hyperplastic polyps (MVHPs), or directly from normal colonic mucosa without the intermediate formation of MVHPs [70, 78] (Figure 1.6). SSAs are most commonly found in the proximal colon and in older women [79, 80]. After a mean dwell time of 17 years, SSA/Ps can progress to develop dysplasia (SSA/Ps with dysplasia) and then, rapidly, carcinoma [81]. Silencing of the tumour suppressor genes *MLH-1* (in 15 to 72% of cases) [82-85], *p16INK4a* [86, 87], and *IGFBP7* [88] have been implicated in this progression. Methylation-induced silencing of *MLH1* is the best characterised, and these cases result in loss of expression of the MLH1 protein and MMR-D.

Whilst *BRAF* mutant (mut) status is an adverse prognostic factor in CRC (described in section 1.2.5.4.), *BRAF* mut MMR-D CRCs have a better prognosis than *BRAF* mut mismatch repair proficient (MMR-P) cancers, and are less likely to develop regional lymph node or distant metastases [89-91]. Higher rates of *TP53* mutations have been observed in *BRAF* mut MSS CRCs [81, 92], and this may partly explain the poorer prognosis associated with *BRAF* mut MMR-P CRCs [81]. In contrast, rates of p16 loss are similar between MMR-D and MMR-P CRCs [81] (Figure 1.6).

Less commonly, serrated adenocarcinomas arise from traditional serrated adenomas (TSAs), which account for <1% of serrated polyps [93, 94]. TSAs are associated with either *BRAF* or *KRAS* mutations [95-97], and give rise to MSS CRC [95]. Loss of *p16INK4a* due to methylation of the *CDKN2A* gene promoter has been implicated in the progression of both *BRAF* mutant and *KRAS* mutant TSAs to CRCs [86, 98], and silencing of *MGMT* by methylation has also been implicated in the *KRAS* mutant TSA pathway [99].

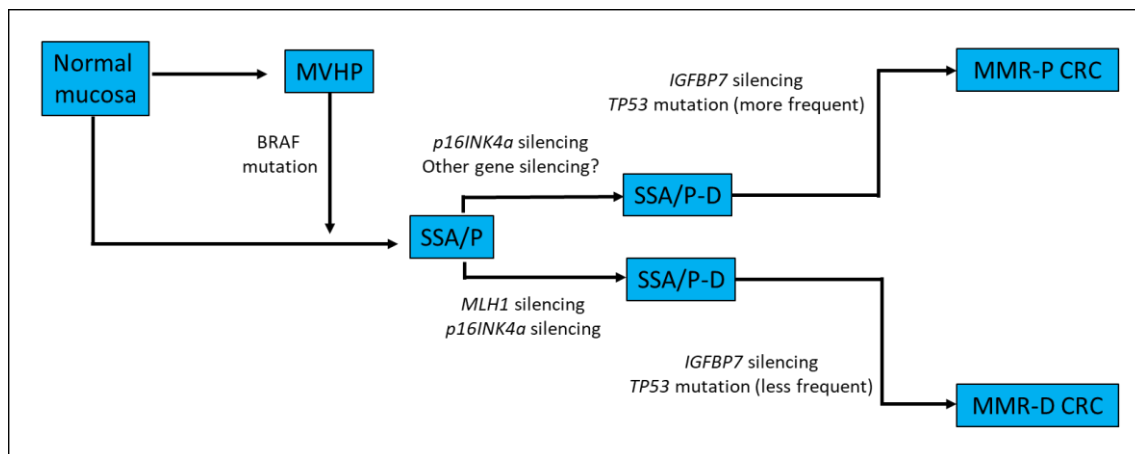


Figure 1.6. Model of the serrated neoplastic pathway for colorectal carcinogenesis (Modified from IJspeert *et al* [70]). MVHP: microvesicular hyperplastic polyp; SSA/P: sessile serrated adenoma or polyp; SSA/P-D: sessile serrated adenoma or polyp with dysplasia; MMR-P: mismatch repair proficient; MMR-D: mismatch repair deficient; CRC: colorectal cancer.

1.2.5. *BRAF* mutations and colorectal cancer

1.2.5.1. *RAF* kinases

In 1983, Rapp and colleagues first described the murine retroviral oncogene *v-Raf* (*Rapidly accelerated fibrosarcoma*) [100]. In 1984, an avian carcinoma virus was found to encode an avian homologue of *v-Raf*, the retroviral oncogene *v-Mil* [101-103]. The same year, *v-Raf* and *v-Mil* were the first oncoproteins to be identified as having serine/threonine kinase activity [104]. In 1985, human and rodent homologues of the *v-Raf* and *v-Mil* oncogenes (*c-raf*, also referred to as *CRAF* or *RAF1*) were then identified [105-108]. Two other mammalian homologues of *c-raf*, *ARAF* [109-112] and *BRAF* [113], were subsequently found in human and rodents.

1.2.5.1.1. Structure of RAF

The RAF family proteins ARAF, BRAF and CRAF share three ‘conserved regions’, conserved region (CR)1 (at the N-terminal end), CR2 and CR3 (at the C-terminal end). CR1 includes a Cys-rich domain (CRD, C1 domain) [114] and the RAS-binding domain (RBD) [115]. CR2 acts as a flexible hinge between CR1 and CR3, which is rich in serine and threonine residues. CR3 is also known as the BRAF kinase domain, and contains 6 important regions: (i) the phosphate-binding loop (P-loop) in the N-terminal which stabilises adenosine triphosphate (ATP) binding, (ii) the α C-helix, (iii) the dimerization interface (DIF), (iv) the catalytic loop (CL) which promotes phosphate transfer from ATP to BRAF substrates, (v) the Asp-Phe-Gly (DFG) motif (magnesium binding site), and (vi) the activation segment (AS, the phospho-acceptor site) [116] (Figure 1.7).

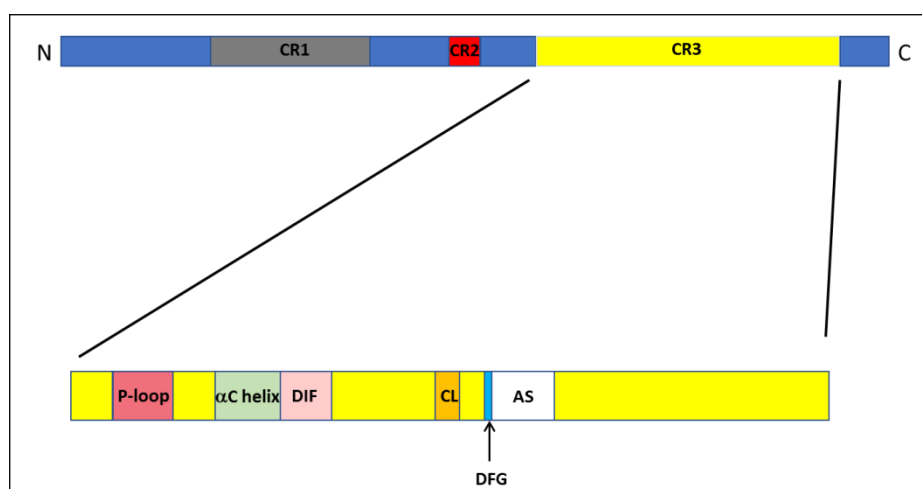


Figure 1.7. Schematic of the structure of BRAF

(Modified from Dankner *et al* [116]). CR1: conserved region 1; CR2: conserved region 2; CR3: conserved region 3; P-loop: phosphate-binding loop; DIF: dimerization interface; CL: catalytic loop; AS: activation segment.

In both normal cells and in the absence of upstream activation conditions, cytosolic BRAF exists in an inactive, monomeric state [117]. The catalytically inactive conformation of BRAF is characterised by an open configuration between the N-terminal and C-terminal, with the α C-helix of the N-terminal lobe in the OUT position, interacting with residues of the folded activation segment to stabilise it in an auto-inhibited state [118, 119].

Conversely, the catalytically active conformation of BRAF features a closed configuration between the N-terminal and C-terminal, the α C-helix in the IN position, and an unfolded activation segment [120]. This allows the α C-helix to interact with the substrate and facilitate catalysis [120]. The DFG motif remains IN in both the inactive and active conformation [118, 120].

1.2.5.1.2. RAS-RAF-MEK-ERK MAPK pathway activation

Growth factor stimulation of upstream growth factor receptors leads to activation of members of the RAS family of small GTPases (KRAS, NRAS and KRAS). Activated growth factor receptors recruit the guanine nucleotide exchange factor (GEF) Son of sevenless (SOS) to promote the release of RAS-guanosine-5'-diphosphate (GDP) from RAS, allowing guanosine-5'-triphosphate (GTP) to bind RAS, converting it to active RAS-GTP in the plasma membrane [117, 121]. RAS-GTP then binds the RBD of RAF, leading to translocation of RAF to the membrane and formation of the RAF-RAS-GTP complex [122, 123]. Auto-phosphorylation of Thr599 and Ser602 in the activation segment of RAF promotes movement into its active conformation [124], allowing homodimerisation and heterodimerisation of BRAF through the kinase domain [125, 126]. Whilst RAF is activated by dimerisation into both the homodimer and heterodimer forms, BRAF or CRAF homodimers have lower activity than BRAF-CRAF heterodimers [127]. RAF can also form side-to-side heterodimers with kinase suppressor of RAS (KSR) proteins (which share some sequence identity with RAF proteins [128], similarly resulting in RAF activation [129] (Figure 1.8).

Activated RAF leads to activation of MEK1 and MEK2 through phosphorylation of their activation segment (Ser218 and Ser222 in MEK1, and Ser222 and Ser226 in MEK 2) [130, 131]. Activated MEK in turn activates ERK1 and ERK2 through dual phosphorylation of conserved Thr and Tyr residues in their activation segment (at Thr202 and Tyr204 in ERK1, and Thr185 and Tyr187 in ERK 2) [132-134].

Activated ERK then phosphorylates hundreds of cytosolic and nuclear substrates including transcription factors, protein kinases and phosphatases, cytoskeletal elements, apoptosis regulators, and other molecules involved in signalling, which are involved in a wide range of cellular processes including proliferation, differentiation, cell cycle progression, survival and migration [135].

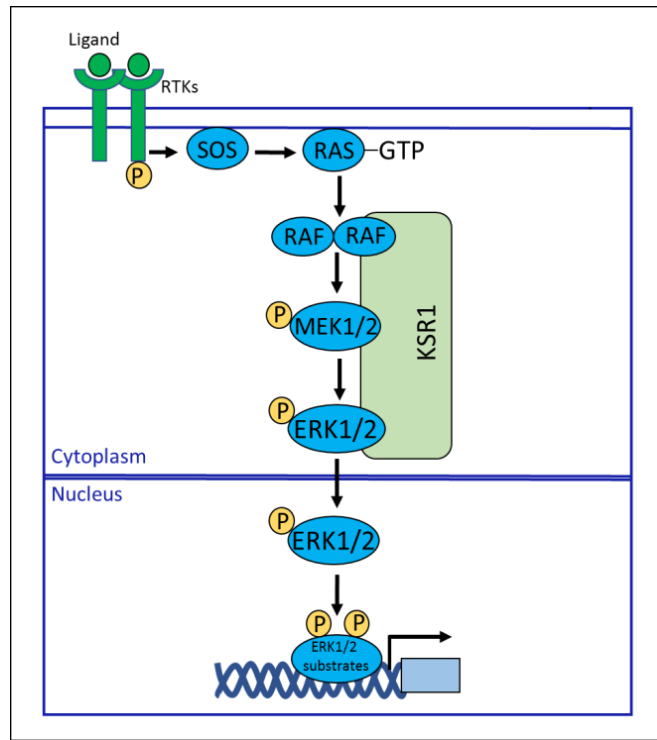


Figure 1.8. Schematic of RAS-RAF-MEK-ERK MAPK pathway activation (Modified from Caunt *et al* [121]). RTKs: receptor tyrosine kinases; SOS: Son of sevenless; GTP: guanosine-5'-triphosphate; KSR1: kinase suppressor of RAS 1.

1.2.5.1.3. Negative feedback regulation of the RAS-RAF-MEK-ERK MAPK pathway

The RAS-RAF-MEK-ERK MAPK pathway undergoes extensive regulation by negative feedback controls to maintain homeostasis [136]. These include the phosphorylation of MEK1, CRAF, BRAF, KSR1, SOS and some receptor tyrosine kinases (RTKs) by ERK and downstream kinases (such as RSK) to inhibit signalling through the pathway [137, 138] (Figure 1.9).

For example, ERK can inhibit MEK phosphorylation by phosphorylating CRAF and BRAF [127, 139, 140], and inhibit ERK phosphorylation by phosphorylating MEK1 [141, 142]. In BRAF wild-type (wt) cells, MEK inhibitor treatment leads to a decrease in ERK1 and ERK2 activity, which leads to dephosphorylation and activation of CRAF ('feedback relief'), which in turn re-activates MEK and ERK [143]. However, notably MEK inhibitor-induced MEK reactivation is not observed in *BRAF* mut cells, as *BRAF*^{V600E} proteins can signal constitutively as monomers (as described in section 1.2.5.2.1.1.) in a manner which is insensitive to both ERK-dependent inhibitory phosphorylation and Sprouty (SPRY)-related inhibition of ERK signalling (described below), and CRAF activity is low in these *BRAF* mut cells [144-146].

SPRY proteins and the dual-specificity phosphatases (DUSPs) also regulate the RAS-RAF-MEK-ERK MAPK pathway. SPRY proteins inhibit ERK signalling at multiple levels (at RTKs, SOS and the RAF catalytic domain) [147]. DUSPs act to attenuate MAPK activity in the presence of prolonged upstream activation. For example, the ERK-specific phosphatases DUSP6 and DUSP5

(localised in the cytoplasm and nuclear compartments, respectively) are induced by ERK signalling, and inactivate ERK by dephosphorylating the pT-E-pY motif [121, 148] (Figure 1.9).

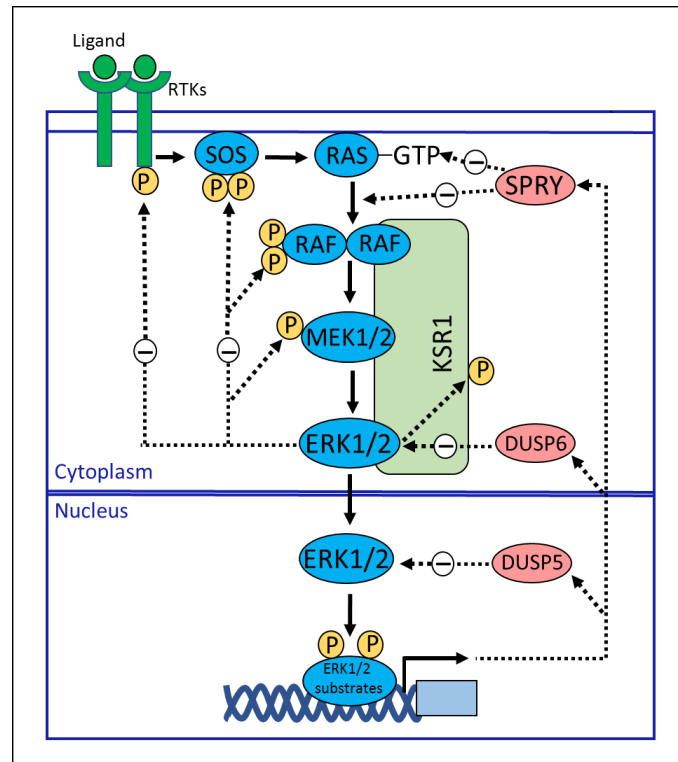


Figure 1.9. Schematic of negative feedback controls for the RAS-RAF-MEK-ERK MAPK pathway (Modified from Caunt *et al* [121]). RTKs: receptor tyrosine kinases; SOS: Son of sevenless; GTP: guanosine-5'-triphosphate; KSR1: kinase suppressor of RAS 1; SPRY: Sprouty protein; DUSP5: dual-specificity phosphatase 5; DUSP6: dual-specificity phosphatase 6.

1.2.5.2. Genetic alterations in *BRAF*

1.2.5.2.1. *BRAF* mutations

In contrast to *ARAF* and *CRAF*, which are rarely mutated in cancer, *BRAF* is mutated in multiple tumour types including hairy cell leukaemia (virtually 100%) [149], nearly all cases of papillary craniopharyngiomas [150], Langerhans cell histiocytosis (50-60%) [151], malignant melanoma (50%) [152], papillary thyroid cancers (45%) [153], colorectal cancers (10%) [56] and non-small cell lung cancers (10%) [154].

Over 20 oncogenic *BRAF* mutations have been identified in human cancers, and most of these have been identified in CRC [155-157].

BRAF mutations can be classified into three groups (Classes I to III), based on their (i) kinase activity, (ii) RAS-dependency, and (iii) dimerisation status [158, 159].

1.2.5.2.1.1. Class I *BRAF*^{V600} point mutations

BRAF^{V600D/E/K/R} are referred to as Class I *BRAF* mutations, and these are the most common *BRAF* mutations in human cancers [160]. In a recent study which performed prospective sequencing of metastatic CRC, Class I *BRAF* mutations accounted for 59% of all *BRAF* mutations found [157].

Class I *BRAF* mutations result in strong activation of *BRAF* kinase activity and constitutive activation of the MAPK pathway, independent of RAS. Constitutive activation can be dimerisation-induced, whereby the V600E substitution in the activation segment promotes formation of a salt bridge with K507 of the α C-helix and stabilises the active conformation of *BRAF*^{V600E} [119]. Alternatively, *BRAF*^{V600E} proteins can also signal constitutively in a dimerisation-independent manner as monomers [118, 158, 161-164].

The tumourigenic potential of *BRAF*^{V600E} has been validated *in vivo* in genetically engineered mouse models (GEMMs) across multiple tumour types including serrated colorectal cancer [87, 165], melanoma [166, 167], lung adenocarcinoma [168], and papillary and anaplastic thyroid cancer [169-172], as well as pancreatic ductal adenocarcinoma [173], prostate cancer [174], paediatric malignant astrocytoma [175], and soft tissue sarcoma [176].

1.2.5.2.1.2. Non-V600 *BRAF* point mutations

Non-V600 *BRAF* mutations account for 22% of *BRAF* mutations in human CRC [155]. They also account for 50-80% of *BRAF* mutations in non-small cell lung carcinoma (NSCLC) [154, 177, 178] and 5-16% of *BRAF* mutations in melanoma [179-181]. There are two main sub-groups of non-V600 *BRAF* mutations, which are classified as Class II or Class III.

Class II *BRAF* mutations form constitutively active dimers independent of RAS, and lead to intermediate to high kinase activity [158, 159]. They are predominantly located in the P-loop (e.g., R462I, I463S, G464E/V/R, and G469A/V/S) or activation segment (e.g., L597Q/R/S/V, A598C, T599I, and K601 E/N/T) [116].

Class III *BRAF* mutations are RAS dependent and are hypoactive or kinase dead, but still activate the MAPK pathway, signalling predominantly through dimerisation of CRAF [158, 159]. They are located in the P-loop (e.g., G466A/E/V/R, S467A/E/L, and G469E), catalytic loop (e.g., N581I/S) or DFG motif (D594A/E/G/H/N/V and G596A/C/D/R) [116].

1.2.5.2.1.3. Other genetic alterations in *BRAF*

1.2.5.2.1.3.1. Activating in-frame *BRAF* deletions

Activating in-frame *BRAF* deletions located in the conserved β 3/ α C-helix loop of the kinase domain of *BRAF* have also been reported, albeit rarely, in malignant melanoma, pancreatic, lung and thyroid cancers [182-184]. These deletions have been predicted to shorten the β 3/ α C-helix loop, resulting in a conformation which stabilises the α C-helix in an active IN position that

promotes dimerisation [184]. There are two different proposed mechanisms for how these deletions result in *BRAF* activation. Chen *et al.* posited that MAPK activation by *BRAF* deletions is dependent on *BRAF* homodimerisation, and in pre-clinical studies in-frame deletion mutants were consequently demonstrated to be vemurafenib-resistant but sensitive to a type II α C-helix-IN/DFG-OUT RAF inhibitor [184]. In contrast, Foster *et al.* proposed that the kinase activity of in-frame deletion mutants is independent of dimerisation, but that they are resistant to vemurafenib due to structural shifts in the α C-helix [182].

1.2.5.2.1.3.2. *RAF* fusion proteins

In addition to *BRAF* mutations, *RAF* fusion proteins have been reported in multiple different tumour types including paediatric glioma, malignant melanoma, thyroid cancer, pancreatic cancer, and NSCLC [185-191], with over twenty fusion partners for *BRAF* identified [185]. Whilst *BRAF* fusion proteins were identified in 0.3% of samples from studies using FoundationOne genomic profiling [185] and the MSK-IMPACT assays [192], the frequency of *BRAF* fusions is highly variable across tumour types and is enriched in pilocytic glioma, Spitzoid melanomas, and acinar pancreatic cancers [185]. Translation of the fusion transcript leads to truncation of the N-terminal domain [193], and the resulting *RAF* proteins either consist of the kinase domain alone or the kinase domain which has fused with a partner protein in lieu of the N-terminal domain [185]. These proteins lead to constitutive *RAF* activation by dimerisation in a similar manner to Class II *BRAF* mutants [125, 162].

1.2.5.3. Clinico-pathological and molecular features of *BRAF* mutant colorectal cancer

1.2.5.3.1. Clinico-pathological features

BRAF^{V600E} CRC is associated with specific clinico-pathological features, including female gender, age over 70 years, right-sided tumour location, and poorly differentiated and mucinous pathology [90, 194, 195]. It also has a distinct pattern of metastatic spread, characterised by higher rates of peritoneal and distant lymph node metastases and lower rates of pulmonary metastases compared to *BRAF* wt tumours [196].

Notably, in a recent retrospective cohort study, Jones *et al.* demonstrated that non-V600E *BRAF* mut metastatic CRCs have distinct clinico-pathological features [155]. Compared to those with *BRAF*^{V600E} tumours, patients with non-V600E *BRAF* mut CRC were younger (median age 58 versus 68 years), less likely to be female (46% versus 65%), and less likely to have high-grade tumours (13% versus 64%), right-sided primary tumours (37% versus 81%) and peritoneal metastases (15% versus 59%). However, there were no differences in the rates of nodal or pulmonary metastases [155].

1.2.5.3.2. Molecular features

BRAF mut CRC is strongly associated with MSI status, as 40-60% of MSI CRC but only 5-10% of MSS CRC harbour *BRAF* mutations [56, 57, 156, 194, 197-199]. As discussed in section 1.2.4.2, mutation in *BRAF* is an early event in the tumourigenesis of serrated pathway adenocarcinomas which arise from SSAs, and in cases with methylation-induced silencing of the tumour suppressor gene *MLH1* and loss of expression of the MLH1 protein, this results in tumours with a MSI-high phenotype [81].

BRAF mut CRC is also strongly associated with the CpG island methylator phenotype, with up to 92% of *BRAF* mutant CRCs being classified as CIMP-high [200]. In a recent study, Fang *et al.* reported that *BRAF*^{V600E}-stabilised MAFG initiates recruitment of a co-repressor complex to CIMP gene promoters and mediates promoter methylation and transcriptional silencing of multiple CIMP marker genes in *BRAF*^{V600E} colorectal cancer. These results demonstrated a direct role for oncogenic *BRAF* in driving CIMP, with the transcriptional repressor MAFG being the critical mediator in this process [201].

1.2.5.4. Prognostic role of *BRAF* mutations in colorectal cancer

1.2.5.4.1. Prognosis in early stage colorectal cancer

In 2008 French *et al.* reported the results of a study examining the prognostic significance of MMR-D and *BRAF*^{V600E} mutation in patients with high-risk Stage II and III colon cancer who participated in the NCIC CTG CO.9 Phase III prospective randomised trial of adjuvant high-dose versus standard-dose levamisole combined with 5-fluorouracil and leucovorin [199]. 490 of 562 enrolled patients had MMR and *BRAF* mutation status defined, and these patients were grouped into having MMR-D/*BRAF* wt, MMR-D/*BRAF* mut, MMR-P/*BRAF* wt or MMR-P/*BRAF* mut tumours. The MMR-D/*BRAF* wt group had significantly improved overall survival (OS) compared to the other groups (5 year OS 100 % versus 73%, $P = 0.002$). Within the subgroup of patients with MMR-D tumours, patients with *BRAF* wt tumours had significantly better OS than patients with *BRAF* mut tumours (5-year OS 100% versus 77%, $P = 0.001$), although there was no significant difference in disease-free survival (DFS) outcomes (5-year DFS 87% versus 77%, $P = 0.30$). There was no difference in DFS or OS by *BRAF* mutation status in patients with MMR-P tumours ($P \geq 0.57$) [199].

The results of this study have not been replicated by other subsequent studies, the results of which conversely suggest that *BRAF* mutation is an independent negative prognostic factor in stage II and III MMR-P CRC.

Roth *et al.* evaluated the prognostic value of *BRAF*^{V600E} mutation status in a translational sub-study of the PETACC-3 Phase III prospective trial, which randomised 3278 patients with Stage II or III colon cancer to adjuvant fluorouracil (FU)/folinic acid (FA) with or without irinotecan

[202]. *BRAF* mutation analysis was successful in 1307 of 1404 patients with eligible tumour specimens. The *BRAF*^{V600E} mutation was reported to be prognostic for poorer OS in patients with Stage II/III disease combined (hazard ratio (HR) 1.66; 95% CI 1.15 to 2.40) and Stage III disease alone (HR 1.76; 95% confidence interval (CI) 1.19 to 2.61), but not relapse-free survival (RFS). In an analysis restricted to the MMR-D sub-group, the stage-adjusted HR for *BRAF* mutation status was not significant (HR 1.59; 95% CI 0.65 to 3.91, *P* = 0.31), indicating that the impact of *BRAF* mutation status on OS might be restricted to MMR-P patients [202].

Most recently, Sinicrope *et al.* examined the prognostic impact of *BRAF*^{V600E} mutation in the NCCTG N0147 trial Phase III prospective trial, which randomised patients with resected Stage III colon cancer to modified FOLFOX, with or without cetuximab [203]. 2515 of 2686 randomised patients had *BRAF* mutation data. Overall, *BRAF*^{V600E} mutation was associated with worse DFS (HR 1.34; 95% CI 1.11 to 1.63, *P* = 0.0028). Among patients with MMR-D tumours, DFS was similar in those with *BRAF* mut and wt CRC (HR 1.33; 95% CI 0.87 to 2.04, *P* = .19; adjusted HR 1.58; 95% CI 0.88 to 2.82, *P* = .12), but among patients with MMR-P tumours, mutated *BRAF*^{V600E} was associated with significantly worse DFS (HR 1.51; 95% CI 1.19 to 1.92, *P* < .001; adjusted HR 1.32; 95% CI 1.01 to 1.73, *P* = .044). However, of note, the MMR by *BRAF*^{V600E} interaction test was not statistically significant (adjusted *P* = 0.93) [203].

A retrospective hospital-based study by Fariña-Sarasqueta *et al.* which included 106 patients with either stage II CRC treated with surgery alone or stage III CRC also treated with adjuvant 5FU/leucovorin (LV), found *BRAF*^{V600E} mutation was an independent prognostic factor for OS and cancer-specific survival (CSS) (HR 0.45; 95% CI 0.25 to 0.80 and HR 0.47; 95% CI 0.22 to 0.99, respectively). Stratified for MMR status, *BRAF*^{V600E} resulted in shorter survival in MMR-P patients in stage II and stage III disease (*P* = 0.011 stage II CSS and *P* = 0.016 stage III OS), but not in the MMR-D group [204].

1.2.5.4.2. Prognosis in metastatic colorectal cancer

In contrast, studies in metastatic CRC have more consistently demonstrated that *BRAF*^{V600E} is associated with poorer prognosis.

Venderbosch *et al.* examined the prognostic significance of MMR status and *BRAF*^{V600E} mutation in metastatic CRC using a pooled analysis which included 3603 of 6155 randomised patients in the CAIRO (Clinicaltrials.gov NCT00312000), CAIRO2 (Clinicaltrials.gov NCT00208546), COIN (ISRCTN 27286448) and FOCUS (ISRCTN 79877428) studies, which are four large phase III trials of first-line (+/- second-line in FOCUS) treatment [55]. Overall, median PFS and OS were significantly worse in patients *BRAF* mut compared to *BRAF* wt tumours (HR 1.34; 95% CI 1.17 to 1.54 and HR 1.91; 95% CI 1.66–2.19, respectively). When MMR-P tumours were stratified by *BRAF* mutation status, median PFS and OS were significantly decreased in patients with *BRAF* mut tumours compared with *BRAF* wt tumours (HR for PFS 1.34; 95% CI 1.10–

1.64, $P < 0.001$ and HR for OS 1.94; 95% CI 1.57–2.40, $P < 0.001$). When MMR-D tumours were stratified by *BRAF* mutation status, there was no significant survival difference in patients with *BRAF* mut tumours compared with *BRAF* wt tumours (HR for PFS 1.07; 95% CI 0.67–1.70, $P = 1.000$ and HR for OS 1.51; 95% CI 0.93–2.46, $P = 0.155$). However, the test for interaction between MMR-D and *BRAF* mut was not statistically significant for either PFS or OS [55].

The prognostic impact of *BRAF*^{V600E} mutation was also demonstrated in another pooled analysis by Bokemeyer *et al.*, which included 800 of 845 participants with *KRAS* wt metastatic CRC from the CRYSTAL (Clinicaltrials.gov NCT00154102) and OPUS trials (Clinicaltrials.gov NCT00125034), and examined the addition of cetuximab to chemotherapy in the first-line treatment of metastatic CRC [205]. *BRAF*^{V600E} mutation was associated with a worse prognosis in both the chemotherapy alone and chemotherapy with cetuximab arms, with a median overall survival of 9.9 and 14.1 months, respectively, compared to a median overall survival of 21.1 and 24.8 months in the patients with *BRAF* wt tumours. The impact of *BRAF*^{V600E} mutation in the subgroups of patients with MMR-D compared to MMR-P tumours was not reported [205].

More recently, Seligmann *et al.* examined the prognostic impact of *BRAF*^{V600E} mutation in 459 of 460 randomised patients in the PICCOLO trial (ISCRTN 93248876), which investigated irinotecan treatment with or without panitumumab in patients who had progressed on or after previous fluoropyrimidine-based chemotherapy [206]. Patients with *BRAF*^{V600E} mutation had a shorter median OS compared to patients with *BRAF* wt tumours, although this was not statistically significant (6.7 versus 10.2 months, adjusted HR 1.21; 95% CI 0.84 to 1.76; $P = 0.31$). There was no significant difference in PFS among patients with *BRAF*^{V600E} mut compared to *BRAF* wt tumours (median PFS 3.5 versus 4.0 months, adjusted HR 1.01; 95% CI 0.69 to 1.49, $P = 0.93$) [206].

In summary, there is now fairly clear evidence that the *BRAF*^{V600E} mutation confers a worse prognosis in MMR-P early-stage and metastatic CRC, but there is less data supporting the prognostic impact of *BRAF*^{V600E} mutation in MMR-D CRC.

Of note, the studies described above only examined the *BRAF*^{V600E} mutation. The recent retrospective cohort study by Jones *et al.* reported that patients with non-V600 *BRAF* mut metastatic CRC have a better prognosis compared to those with both *BRAF*^{V600E} and *BRAF* wt metastatic CRC (median OS 60.7 versus 11.4 versus 43.0 months, respectively; $P < 0.001$). Non-V600 *BRAF* mutation was also independently associated with improved overall survival in multivariate analysis (HR 0.18; 95% CI 0.10 to 0.32, $P < 0.001$) [155].

1.2.5.5. Predictive role of *BRAF* mutations in metastatic colorectal cancer

Two recent systematic reviews with meta-analyses have examined the role of *BRAF* mutation as a predictive biomarker of benefit from anti-epidermal growth factor receptor (EGFR) monoclonal antibody (mAb) treatment in patients with metastatic CRC, with conflicting conclusions.

The meta-analysis by Rowland *et al.* included pharmacogenomics sub-studies from eight randomised controlled trials of (i) standard chemotherapy alone versus the same standard chemotherapy with anti-EGFR mAb or (ii) best supportive care with or without anti-EGFR mAb [207]. The anti-EGFR mAb examined was cetuximab in four studies (CRYSTAL, OPUS, COIN and CO.17), and panitumumab in four studies (PRIME, 20050181, PICCOLO, and 20020408). Four studies were in the first-line setting (CRYSTAL, OPUS, COIN, and PRIME), one in the second-line (20050181), and three in the chemo-refractory setting (second- or further line treatment) (CO.17, PICCOLO, and 20020408) (Table 1.2).

Analysis of the effect of *BRAF* mutation on OS benefit with anti-EGFR mAbs, found the pooled hazard ratio (from 7 RCTs) for OS benefit with anti-EGFR mAb therapy was 0.97 (95% CI 0.67 to 1.41) for *RAS* wt/*BRAF* mut tumours, compared to 0.81 (95% CI 0.70 to 0.95) for *RAS* wt/*BRAF* wt tumours. However, the test for interaction examining evidence for treatment effect modification by *BRAF* mutation status was not statistically significant ($P = 0.43$).

Analysis of the effect of *BRAF* mutation on PFS benefit with anti-EGFR mAbs, found the pooled hazard ratio (from 8 RCTs) for PFS benefit with anti-EGFR mAb therapy was 0.86 (95% CI 0.61 to 1.21) for *RAS* wt/*BRAF* mut tumours, compared to 0.62 (95% CI 0.50 to 0.77) for *RAS* wt/*BRAF* wt tumours. However, the test for interaction examining evidence for treatment effect modification by *BRAF* mutation status narrowly missed statistical significance ($P = 0.07$).

Consequently, the authors concluded that there is insufficient evidence to definitively consider *BRAF* mutation status a negative predictive biomarker of survival benefit from anti-EGFR mAb therapy for metastatic CRC.

Exploratory analyses evaluating whether line of therapy was associated with a differential impact of *BRAF* mutations on anti-EGFR mAb efficacy were also performed. The analysis restricted to first-line anti-EGFR mAb therapy found minimal difference between *RAS* wt/*BRAF* wt and *RAS* wt/*BRAF* mut tumours for both OS (HR 0.87 versus 0.89, $P = 0.96$) and PFS efficacy (HR 0.75 versus 0.83, $P = 0.45$). For non-first-line anti-EGFR mAb therapy, there were non-statistically significant trends towards a difference in efficacy between *RAS* wt/*BRAF* wt and *RAS* wt/*BRAF* mut tumours for OS (HR 0.74 versus 1.06, $P = 0.38$) and PFS (HR 0.53 versus 0.84, $P = 0.05$) [207].

Table 1.2. Randomised controlled trials included in the meta-analysis examining BRAF mutation as a predictive biomarker of benefit from anti-epidermal growth factor receptor (EGFR) monoclonal antibody (mAb) treatment in patients with metastatic CRC by Rowland *et al.* [207]

Anti-EGFR mAb	Study name	Setting	Number of participants randomised	Treatment arms	Reference
Cetuximab	CRYSTAL	First-line	1198	FOLFIRI ± cetuximab	[205]
	OPUS	First-line	337	FOLFOX-4 ± cetuximab	[205]
	COIN	First-line	2445	Fluoropyrimidine chemotherapy + oxaliplatin ± cetuximab	[54]
	CO.17	Second- or further line	572	Best supportive care ± cetuximab	[208]
Panitumumab	PRIME	First-line	1183	FOLFOX-4 ± panitumumab	[209]
	20050181	Second-line	1186	FOLFIRI ± panitumumab	[210]
	PICCOLO	Second- or further line	460	Irinotecan ± panitumumab	[211]
	20020408	Third- or further line	463	Best supportive care ± panitumumab	[212]

EGFR: Epidermal growth factor receptor; mAb: monoclonal antibody; CRC: colorectal cancer; FOLFIRI: 5-fluorouracil + folinic acid + irinotecan; FOLFOX: 5-fluorouracil + folinic acid + oxaliplatin.

A second meta-analysis by Pietrantonio *et al.* included data from ten randomised controlled trials of anti-EGFR mAb therapy [213]. In addition to the 8 RCTs included in the meta-analysis by Rowland *et al.*, this meta-analysis included data from the FIRE-3 study, a phase III randomised controlled trial which compared FOLFIRI with cetuximab to FOLFIRI with bevacizumab for the first line treatment of metastatic CRC [214, 215], but did not have a FOLFIRI-alone treatment arm. The NORDIC-VII phase III randomised controlled trial of Nordic FLOX with or without cetuximab for the first line treatment of metastatic CRC was also included for the ORR outcome [216].

In analyses of efficacy which were restricted to the *BRAF* mut sub-groups in the included studies, the authors found that the addition of anti-EGFR mAb in patients with *BRAF* mut tumours did not improve OS or PFS compared with chemotherapy or best supportive care (HR 0.91; 95% CI 0.62 to 1.34, $P = 0.63$, and HR 0.88; 95% CI 0.67 to 1.14, $P = 0.33$, respectively). The ORR was also not significantly increased (ORR 1.31; 95% CI 0.83 to 2.08, $P = 0.25$). The authors concluded that “the addition of anti-EGFR MoAbs ... does not increase OS, PFS and ORR in *BRAF*-mut CRC patients” [213].

Notably, this study had important methodological limitations. In order to analyse whether *BRAF* mutation is a predictive biomarker of anti-EGFR mAb efficacy, the optimal method is to evaluate heterogeneity of effect between sub-groups using a test of interaction, however Pietrantonio *et al.*

simply restricted analyses of efficacy to the *BRAF* mut subgroup, which increases the risk of a false positive result [217-219]. Secondly, the FIRE-3 study did not have a FOLFIRI-alone treatment arm which precludes analysis of the predictive effect of *BRAF* mutation on cetuximab efficacy, yet this study was included in the meta-analysis.

Recent guidelines from both the American Society for Clinical Pathology (ASCP)/College of American Pathologists (CAP)/Association for Molecular Pathology (AMP)/American Society of Clinical Oncology (ASCO) (2017) and the European Society for Medical Oncology (ESMO) (2016) have concluded that there is insufficient evidence to recommend *BRAF*^{V600E} mutation status as a predictive molecular biomarker for response to anti-EGFR inhibitors [21, 220]. However, debate around these guidelines continues, and the role of *BRAF* mutation as a predictive biomarker of benefit from anti-EGFR therapy in patients with metastatic CRC remains a controversial issue [221, 222].

1.2.6. BRAF inhibitors

1.2.6.1. BRAF inhibitor compounds

The biochemical effect of RAF kinase inhibitors is the result of distinct allosteric mechanisms, which are determined by (i) the conformation in which they stabilize their target kinase and (ii) the cellular levels of RAS-GTP. RAF kinase inhibitors can be classified into different groups, based on both the ‘generation’ of their development and the conformation in which they stabilize their target kinase (Table 1.3).

Table 1.3. Select RAF inhibitors classified by the conformation in which they stabilize their target kinase

BRAF inhibitor	Other names	Stage of development	ClinicalTrials.gov Identifier	References
First-generation (αC-IN) RAF inhibitors				
<i>Type I (αC-helix-IN/DFG-IN)</i>				
SB590885		Pre-clinical (did not advance to clinical trials)		[223]
GDC0879		Pre-clinical (did not advance to clinical trials)		[224]
<i>Presumed Type I (αC-helix-IN/DFG-IN) [117]</i>				
L779450		Pre-clinical (did not advance to clinical trials)		[225, 226]
GW5074		Phase I	NCT03406364	[227]
<i>Type II (αC-helix-IN/DFG-OUT)</i>				
AZ628		Pre-clinical (did not advance to clinical trials)		[228, 229]
Sorafenib	Nexavar, BAY43-9006	Clinically approved - treatment of advanced renal cell carcinoma and hepatocellular carcinoma (FDA)		[230-232]
Second-generation (αC-OUT) BRAF^{V600}-selective RAF inhibitors				
<i>Type I^{1/2} (αC-helix-OUT/DFG-IN)</i>				
PLX3603	RO5212054, RG7256	Phase I completed, development discontinued	NCT01143753	[233]
Vemurafenib	Zelboraf, PLX4032, RG7204	Clinically approved - treatment of BRAF ^{V600E} metastatic melanoma and Erdheim-Chester disease (FDA)		[234, 235]
Dabrafenib	Tafinlar, GSK2118436	Clinically approved - treatment of BRAF ^{V600E/K} melanoma which is unresectable/metastatic, or for adjuvant treatment if involved lymph node(s) following complete resection; BRAF ^{V600E} metastatic NSCLC; and BRAF ^{V600E} locally advanced/metastatic anaplastic thyroid cancer with no satisfactory locoregional treatment options (in combination with trametinib) (FDA)		[236]
Encorafenib	LGX818	Clinically approved - treatment of BRAF ^{V600E/K} melanoma which is unresectable/metastatic (in combination with binimetinib) (FDA)		[158]

Table 1.3. (continued) Select RAF inhibitors classified by the conformation in which they stabilize their target kinase

BRAF inhibitor	Other names	Stage of development	ClinicalTrials.gov Identifier	References
Third-generation RAF inhibitors				
<i>Type II (αC-helix-IN/DFG-OUT)</i>				
TAK632		Pre-clinical		[237, 238]
RAF709		Pre-clinical		[239]
LY3009120		Phase I	NCT02014116	[240]
LXH254		Phase I	NCT02607813, NCT02974725 (advanced/metastatic KRAS/BRAF mut NSCLC and NRAS mut melanoma), NCT03333343 (locally advanced/metastatic EGFR mutant NSCLC)	[241]
<i>Presumed Type II (αC-helix-IN/DFG-OUT)[117]</i>				
BGB659		Pre-clinical		[158]
CCT196969 (dual pan-RAF and SRC kinase inhibitor)		Pre-clinical		[242]
CCT241161 (dual pan-RAF and SRC kinase inhibitor)		Pre-clinical		[242]
CCT3833	BAL3833	Phase I	NCT02437227	[243]
BGB283 (dual RAF and EGFR inhibitor)		Phase I	NCT02610361, NCT03641586	[244]
MLN2480	TAK580	Phase I/II	NCT02327169, NCT01425008 (metastatic melanoma), NCT03429803 (low-grade glioma-Phase I/II)	[245]
<i>Paradox breakers (αC-helix-OUT/DFG-IN/R506-OUT)</i>				
PLX7094		Pre-clinical		[246]
PLX8394		Phase I/II	NCT02428712, NCT02012231(Phase I/II)	[246]
<i>αC-helix-OUT/DFG-OUT</i>				
BI 882370		Pre-clinical		[247]
Not yet classified^a				
HM95573		Phase I	NCT02405065, NCT03118817, NCT03284502	[248]
CEP32496	RXDX-105	Phase I	NCT01877811	[249]

NSCLC: non-small cell lung cancer; EGFR: epidermal growth factor receptor; FDA: Food and Drug Administration; ^a: not yet classified due to lack of structural data.

1.2.6.1.1. First-generation RAF inhibitors

First-generation (α C-IN) RAF inhibitors were developed to target CRAF, prior to the discovery of *BRAF* mutations, and are classed into Type I and Type II RAF inhibitors [117].

1.2.6.1.1.1. First-generation type I RAF inhibitors

First-generation type I RAF inhibitors stabilise their target kinases in the α C-helix-IN/DFG-IN conformation, and include SB590885 and GDC0879, which both only reached the pre-clinical stage of development [223, 224]. L779450, which reached the pre-clinical stage of development [225, 226], and GW5074, which is being studied in a Phase I clinical trial in patients with solid tumours [227] are also presumed to have type I conformation [117].

1.2.6.1.1.2. First-generation type II RAF inhibitors

First-generation type II RAF inhibitors stabilise their target kinases in the α C-helix-IN/DFG-OUT conformation, and include AZ628, which reached the pre-clinical stage of development. In *in vitro* studies, AZ628 led to growth inhibition of *BRAF*^{V600E/G} cell lines (in particular, colon and melanoma cell lines) [228].

Sorafenib is another type II RAF inhibitor - a multi-tyrosine kinase inhibitor which targets both tumour cell proliferation and tumour angiogenesis, through inhibition of CRAF, wild-type *BRAF*, and oncogenic *BRAF*^{V600E} and the receptor tyrosine kinases vascular endothelial growth factor receptor (VEGFR)-1, VEGFR-2, VEGFR-3, platelet-derived growth factor receptor (PDGFR)- α , Flt-3, and c-Kit [231, 232]. Sorafenib is clinically approved for the treatment of advanced renal cell carcinoma and hepatocellular carcinoma.

1.2.6.1.2. Second-generation type I^{1/2} BRAF inhibitors

Second-generation type I^{1/2} (α C-OUT) BRAF inhibitors were identified using specific screens for inhibitors of *BRAF*^{V600E}, and these inhibitors stabilise their target kinases in the α C-helix-OUT/DFG-IN conformation [235].

Vemurafenib was discovered using a structure- and scaffold-based approach [234, 235], and was shown in pre-clinical studies to potently inhibit proliferation and MEK/ERK phosphorylation in multiple melanoma cell lines and one breast cancer cell line with *BRAF*^{V600} mutations (but not wt *BRAF* or non-V600 mut *BRAF*), and tumour xenograft models of *BRAF*^{V600E} melanoma [250]. Vemurafenib was the first second-generation BRAF inhibitor to be studied in clinical trials, and is now approved for the treatment of *BRAF*^{V600E} metastatic melanoma and Erdheim-Chester disease.

Dabrafenib, another second-generation BRAF inhibitor, was discovered using a structure-activity-relationship approach [236]. In *in vitro* studies, dabrafenib inhibited MEK/ERK phosphorylation, and selectively inhibits growth in *BRAF* mut cell lines. In a panel of 195 cell lines including multiple tumour types, 80% of *BRAF*^{V600E} cell lines, 60% of non-V600E *BRAF* mut cell lines, 12% of *RAS/RAF* wt cell lines, and 0% of *RAS* mut cell lines were sensitive to dabrafenib treatment. Dabrafenib also demonstrated *in vivo* activity in *BRAF*^{V600E} melanoma and colorectal cancer xenograft models [251]. Dabrafenib was subsequently approved for the treatment of *BRAF*^{V600E/K} melanoma which is unresectable or metastatic, or for adjuvant treatment in cases with involved lymph node(s) following complete resection. Dabrafenib has also been approved for the treatment of *BRAF*^{V600E} metastatic NSCLC and *BRAF*^{V600E} locally advanced/metastatic anaplastic thyroid cancer when used in combination with the MEK inhibitor trametinib.

Encorafenib is a second-generation RAF inhibitor which was shown in pre-clinical studies to have high potency and slow off-rate from *BRAF*^{V600E}, which may translate to prolonged target inhibition [158]. Encorafenib was reported to have anti-tumour activity in multiple *BRAF* mut human tumour xenograft models [252]. Encorafenib has been clinically approved for the treatment of unresectable/metastatic *BRAF*^{V600E/K} melanoma when used in combination with the MEK inhibitor binimetinib.

Clinical trials using the BRAF inhibitors vemurafenib, dabrafenib and encorafenib in melanoma and metastatic CRC are described in detail in section 1.2.9.

PLX3603 is another second-generation BRAF inhibitor, which was shown in *in vitro* studies to inhibit cellular proliferation and MAPK signalling in *BRAF*^{V600E} cell lines more potently than vemurafenib, with similar anti-tumour activity in *BRAF*^{V600E} xenograft models with lower doses and exposures [233]. PLX3603 progressed to a completed Phase I clinical trial in patients with *BRAF*^{V600} advanced solid tumours (NCT01143753), with a favourable efficacy and safety profile. However, the large pill burden required to achieve desired exposure, and associated concerns about patient compliance, led to discontinuation of its development [253].

1.2.6.1.2.1. Allosteric mechanisms of intrinsic RAF dimer resistance to second-generation α C-OUT type I^{1/2} BRAF inhibitors

As discussed in section 1.2.5.1.1., the catalytically inactive conformation of BRAF is characterised by the α C-helix being stabilised in the OUT position. Karoulia *et al.* demonstrated that for second-generation α C-OUT type I^{1/2} BRAF inhibitors such as vemurafenib, stabilisation of the α C-helix in this inactive OUT position is not sterically possible for both protomers in a mutant BRAF dimer. This is because binding of the BRAF inhibitor to the first protomer does not allow movement of the α C-helix of the second protomer to the OUT position without breaking

the mutant BRAF dimer, and instead it is stabilised to the IN position. This leads to ‘negative allostery’, whereby the affinity of BRAF inhibitor for the second protomer is reduced [118]. This represents an allosteric mechanism of resistance of dimeric mutant BRAF to the second-generation α C-OUT type I^{1/2} BRAF inhibitors.

1.2.6.1.3. Third-generation BRAF inhibitors

Third-generation BRAF inhibitors can be divided into two main classes – (i) Type II (α C-IN/DFG-OUT) inhibitors and (ii) ‘paradox breakers’.

1.2.6.1.3.1. Third-generation type II BRAF inhibitors

Third-generation type II BRAF inhibitors stabilise their target kinases in the α C-IN/DFG-OUT conformation. In contrast to the second generation type I^{1/2} BRAF inhibitors, third-generation type II BRAF inhibitors bind both protomers of dimeric RAF with similar affinity. Consequently, they equipotently inhibit both monomeric and dimeric RAF [118]. As these RAF inhibitors suppress both BRAF and CRAF dimers, they are also considered to be ‘pan-RAF’ inhibitors [238, 240, 242].

This class of inhibitors includes TAK632 and RAF709, which are both in the pre-clinical phase of development [237-239]. Pre-clinical studies have demonstrated that TAK632 has potent anti-proliferative effects in multiple cancer cell lines with *BRAF*, *KRAS* and *NRAS* mutations, as well as *BRAF* mut melanoma cells with acquired resistance to BRAF inhibitors through *NRAS* mutation or BRAF truncation [118, 238]. RAF709 has been shown to inhibit MAPK signalling and have anti-tumour activity both *in vitro* and *in vivo* in multiple tumour types with *BRAF* or *RAS* mutations [239].

LY3009120 has been shown to have activity against *BRAF*, *NRAS* and *KRAS* mut melanoma, lung and colorectal cancer *in vitro* and *in vivo*, as well as in vemurafenib-resistant melanoma cell lines [240, 254, 255]. LXH254 is a highly-selective BRAF and CRAF inhibitor which has been reported to inhibit MAPK kinase signalling in *BRAF*, *NRAS* and *KRAS* mut tumour models, leading to tumour regression in pre-clinical studies [241]. LY3009120 and LXH254 are being studied in Phase I clinical trials in patients with advanced solid tumours (LY3009120 and LXH254), and in patients with advanced/metastatic *KRAS/BRAF* mut NSCLC or *NRAS* mut melanoma, and locally advanced/metastatic *EGFR* mutant NSCLC (LXH254).

Other third-generation BRAF inhibitors which are presumed to have type II conformation include BGB659, CCT196969 and CCT241161, which are in the pre-clinical phase of development [117]. BGB659 has been reported to inhibit ERK signalling driven by mutant RAF monomers and dimers at doses at which it does not inhibit signalling in normal cells, which could translate to a wide index in clinical practice [158]. CCT196969 and CCT24116 are two dual pan-RAF and SRC kinase inhibitors which have been shown to inhibit ERK signalling in *BRAF* and *NRAS* mutant

melanoma as well as BRAF- and BRAF/MEK inhibitor resistant melanoma *in vitro* and in PDX models [242].

CCT3833 and BGB283 are being studied in Phase I clinical trials in patients with advanced solid tumours. CCT3833 is a dual pan-RAF and SRC kinase inhibitor which was shown in pre-clinical studies to be effective in both *BRAF* mutant and *KRAS* mutant cancers (including pancreatic ductal adenocarcinoma, CRC and NSCLC) [243]. BGB283 is a dual RAF and EGFR inhibitor which preferentially inhibits tumour cells with *BRAF*^{V600E} and *EGFR* mutation/amplification. It has been reported to inhibit EGFR reactivation and EGFR-mediated cell proliferation *in vitro* and inhibit tumour growth *in vivo* in *BRAF*^{V600E} CRC xenograft and patient derived xenograft (PDX) models [244].

MLN2480 (TAK580) is a third-generation type II BRAF inhibitor which has been demonstrated in pre-clinical studies to not lead to paradoxical activation (see section 1.2.6.1.3.2.1.) and to also have good blood-brain-barrier penetrance, and therefore has potential as a treatment of *BRAF* mutant brain tumours [245]. It is currently being studied in Phase I clinical trials in patients with advanced solid tumours and metastatic melanoma, and a Phase I/II trial in low-grade glioma.

1.2.6.1.3.2. Third-generation ‘paradox breakers’

1.2.6.1.3.2.1. Paradoxical activation

In 1999, Hall-Jackson *et al.* published the first evidence that ATP-competitive RAF inhibitors can paradoxically increase activity of wild type RAF kinases, in experiments using the first-generation RAF inhibitor ZM336372 [256].

A decade later, three groups demonstrated that inhibition of RAF kinases using ATP-competitive RAF inhibitors leads to ‘paradoxical activation’ of CRAF, subsequent downstream activation of the MAPK pathway, proliferation of *RAS* mutant cells *in vitro*, and in some cases, increased tumour growth *in vivo* [164, 257, 258]. Each of these groups described distinct mechanisms driving the paradoxical activation, albeit with some overlapping themes.

Heidorn *et al.* reported that in the presence of oncogenic or growth-factor activated *RAS*, selective chemical or genetic inhibition of BRAF induces binding of wild type BRAF to CRAF, which leads to CRAF hyperactivation and increased MEK-ERK signalling. In contrast to BRAF-selective inhibitors, pan-RAF inhibitors induced CRAF activation, but not MEK-ERK signalling, possibility due to the dual-targeting of both BRAF and CRAF. This group proposed that in *RAS* mutant cells, BRAF is auto-inhibited due to auto-phosphorylation or phosphorylation of a partner protein which maintains it in an inactive conformation. Following BRAF inhibition, BRAF is relieved from its auto-inhibited state, is recruited to the plasma membrane by *RAS*, and forms a stable complex with CRAF, acting as a scaffold which enhances CRAF activation [257].

Hatzivassiliou *et al.* demonstrated that in *KRAS* mut and *RAS/RAF* wt tumours, RAF inhibitors activate wt RAF isoforms in a RAS-dependent manner. This group proposed that binding of RAF inhibitors to the ATP-binding pocket leads to direct conformational effects on the RAF kinase domain, which induces RAF dimerisation and translocation of CRAF to the membrane, where it interacts with RAS-GTP, leading to downstream activation of the RAF-MEK-ERK pathway. This process is independent of kinase inhibition. In *BRAF*^{V600E} tumours, paradoxical activation is not observed, because the pathway is sensitised to RAF inhibition due to a higher ATP apparent Michaelis constant (*K*_m) of the mutant *BRAF*^{V600E} protein, and lack of CRAF activation [258].

Poulikakos *et al.* showed that ATP-competitive RAF inhibitors enhance ERK signalling in *BRAF* wt cells, through an allosteric mechanism which requires direct binding of the BRAF inhibitor to the ATP-binding site of one protomer and is dependent on RAS activity. This group posited that binding of a RAF inhibitor to one protomer of a RAF homodimer (CRAF-CRAF) or heterodimer (CRAF-BRAF) results in inhibition of the catalytic activity of the inhibitor-bound protomer, and transactivation of the drug-free protomer. In *BRAF*^{V600E} tumours, paradoxical activation is not observed, because levels of RAS activation are insufficient to support transactivation of wt RAF, and the dominant effect of the drug is inhibition of BRAF [164].

More recently, Holderfield *et al.* proposed that paradoxical activation is ATP-dependent and directly associated with kinase activity. This group identified an auto-phosphorylation site in the phosphate-binding loop (P-loop) of RAF which is phosphorylated in auto-inhibited wt RAF under physiological conditions. Catalytic inhibition of RAF, including with pan-RAF inhibitors, relieves RAF autoinhibition and increases RAF kinase activity. BRAF oncogenes with point mutations in the P-loop bypass the auto-inhibitory effect, hence RAF inhibitors do not lead to paradoxical activation in *BRAF* mut cells. They proposed that this mechanism is dominant to the conformational effects of RAF inhibitors binding RAF dimers, although RAS activity and dimerisation are likely to still have a role [259].

1.2.6.1.3.2.2. Clinical consequences of paradoxical activation

The clinical consequences of paradoxical activation became evident when patients treated with the second-generation type I^{1/2} BRAF inhibitors vemurafenib and dabrafenib developed cutaneous lesions including cutaneous squamous cell carcinomas and keratoacanthomas, which frequently contained *RAS* mutations [260, 261]. These have been reported at rates of 20-26%, and 6-11% for vemurafenib and dabrafenib, respectively [262].

New primary melanomas may also develop in patients treated with vemurafenib or dabrafenib. In a study of 22 new or recently altered cutaneous melanocytic lesions in 19 patients being treated with selective BRAF inhibitors for *BRAF* mut metastatic melanoma, all were *BRAF* wt with one demonstrating an *NRAS* mutation and 12 were new primary melanomas [263].

Two case reports have also described the progression or development of *BRAF* wt *RAS* mut non-melanoma malignancies during treatment with selective BRAF inhibitors. Callahan *et al.* reported a case of a patient who experienced accelerated progression of a pre-existing clinically undiagnosed *RAS* mut chronic myelomonocytic leukaemia during treatment with vemurafenib for metastatic *BRAF* mut melanoma, consistent with RAS-dependent paradoxical activation by BRAF inhibitor treatment [264]. We reported a case of a patient with a past history of a stage II adenocarcinoma of the ascending colon resected 3 years previously without subsequent adjuvant chemotherapy, who developed metastatic *RAS* mut CRC during treatment for metastatic melanoma with dabrafenib, consistent with BRAF inhibitor mediated paradoxical activation and growth promotion of dormant *RAS* mut CRC [265].

1.2.6.1.3.2.3. Therapeutic consequences of paradoxical activation

Paradoxical activation also has potential consequences for therapeutic efficacy. For example, in *BRAF* mut malignant melanoma, one described mechanism of acquired resistance to BRAF inhibitors is the acquisition of activating *NRAS* mutations [162, 266]. As described above, activating *RAS* mutations would lead to paradoxical activation of the MAPK pathway which would, in turn, likely be exacerbated by the BRAF inhibitor treatment.

1.2.6.1.3.2.4. Paradox breakers (α C-helix-OUT/DFG-IN/R506-OUT BRAF inhibitors)

Third-generation BRAF inhibitors termed ‘paradox breakers’ were developed in order to overcome paradoxical activation. ‘Paradox breakers’ are BRAF inhibitor analogues with variable terminal sulphonamide and sulfamide substitutions, which were selected based on their potent inhibition of p-ERK in *BRAF*^{V600E} cells but low potency in inducing p-ERK activation in *RAS* mut cell lines [246], and include PLX7094 and PLX8394.

Recent work by Karoulia *et al.* proposed that greater degrees of movement of R506 in the α C-helix away from the OUT position are associated with greater promotion of RAF inhibitor-induced RAF-RAS-GTP interaction and dimerization, and therefore paradoxical activation [118]. α C-IN BRAF inhibitors displace R506 from the OUT position, and promote RAF-RAS-GTP interaction. Moreover, the position of both the α C-helix and DFG motif impact on the interaction of RAF with MEK. RAF inhibitors which stabilise a conformation closer to the native active kinase state of RAF (i.e., α C-IN/DFG-IN, as described in section 1.2.5.1.1.) promote formation of RAF-MEK complexes allosterically. Hence, type I α C-IN/DFG-IN inhibitors lead to greater paradoxical activation than type II α C-IN/DFG-OUT inhibitors [118]. Whilst the second-generation α C-OUT type I^{1/2} BRAF inhibitors dabrafenib and vemurafenib stabilise the α C-helix in an overall OUT position, these inhibitors displace the R506 residue towards the IN position and consequently also promote RAF/RAS-GTP interaction and dimerization [118]. However, as α C-OUT RAF inhibitors disrupt the RAF-MEK complex [120], they are expected to lead to less paradoxical activation than α C-IN inhibitors. In contrast to these classes of RAF inhibitors,

PLX7904 interacts strongly with the R506 residue to stabilise the α C-helix into the OUT position [246], and therefore does not promote BRAF-CRAF dimerisation or paradoxical activation [118, 246].

PLX7094 has reached the pre-clinical phase of development, and has been demonstrated *in vitro* to inhibit growth in $BRAF^{V600E}$ melanoma and CRC cell lines, and also overcome dimerisation-mediated and secondary RAS mutation-mediated RAF inhibitor resistance. It also does not increase p-ERK levels in breast and squamous cell carcinoma cell lines with upstream activation of RAS secondary to over-expression of EGFR and HER2, respectively. Similar results were shown *in vivo*, with PLX7094 having anti-tumour effects in a $BRAF^{V600E}$ CRC xenograft model, but not accelerating tumour growth of a xenograft model of a squamous cell carcinoma with a HRAS mutation [246]. PLX8394, its optimised analogue, is being studied in Phase I/II clinical trials in patients with advanced solid tumours.

1.2.6.1.3.3. Other third generation BRAF inhibitors

BI 882370 is an α C-helix-OUT/DFG-OUT RAF inhibitor which is in the pre-clinical phase of development, and has been shown to have efficacy *in vivo* in mouse models of $BRAF$ mutant melanoma and CRC [247]. BI 882370 stabilizes the DGF motif in an inactive conformation by placing Phe595 of the DFG motif just below the pyrrolopyridine moiety.

HM95573 and CEP32496 are other RAF inhibitors which have not yet been structurally classified [117], but are being studied in Phase I studies in advanced solid tumours. HM95573 has been reported to have anti-tumour activity both *in vitro* and *in vivo* in $BRAF$ mutant, $KRAS$ mutant, and $NRAS$ mutant cell lines and xenograft models respectively, in multiple tumour types [248]. CEP32496 has been shown to have efficacy *in vivo* in $BRAF^{V600E}$ melanoma and CRC xenograft models [249].

1.2.6.2. Predicted efficacy of RAF inhibitors in tumours with non-V600E BRAF mutations

1.2.6.2.1. Non-V600E class I $BRAF^{V600}$ point mutations

Given that non-V600E Class I $BRAF^{V600}$ point mutant forms are catalytically active as monomers independent of RAS (as described in section 1.2.5.2.1.1.), they have been shown to be sensitive to inhibition by currently clinically approved α C-OUT RAF inhibitors in pre-clinical studies [118, 158].

1.2.6.2.2. Non-V600 BRAF point mutations

In contrast, as the catalytic activity of non-V600 $BRAF$ point mutant forms is fully or partially dependent on dimerisation (as described in section 1.2.5.2.1.2.), they have been demonstrated to be resistant to current clinically approved α C-OUT RAF inhibitors including vemurafenib in pre-clinical studies, and α C-IN RAF inhibitors may be a potentially effective alternative for these tumours [118, 158, 161, 267]. Interestingly, some *in vitro* and clinical studies have reported

responsiveness of non-V600 *BRAF* class II mutants to α C-OUT RAF inhibitors [268-270], whilst other have not [271-274], and further studies are required to examine potential molecular mechanisms to explain these different observations [116].

As discussed in section 1.2.5.2.1.2., non-V600 *BRAF* class III mutants signal in a RAS-dependent manner, signal predominantly through CRAF dimers and have impaired kinase activity. The RAS dependency has led to the hypotheses that in non-V600E *BRAF* class III mutants, the requirement for upstream RTK signalling could be exploited as a therapeutic strategy [275]. Some have proposed that EGFR inhibition [159] or pan-ErbB inhibition [275] may be optimal treatment approaches. The use of EGFR inhibition in class III *BRAF* mutant CRCs has however, thus far, had mixed results in clinical practice [159, 276, 277].

1.2.6.2.3. Other genetic alterations in *BRAF*

1.2.6.2.3.1. Activating in-frame *BRAF* deletion mutations

As discussed in section 1.2.5.2.1.3.1., there are conflicting proposed mechanisms for how activating *BRAF* deletion mutations lead to catalytic activation [182, 184]. However, based on the mechanisms proposed, tumours with in-frame *BRAF* deletions are expected to be resistant to current clinically approved α C-OUT RAF inhibitors, but predicted to respond to third-generation type II *BRAF* inhibitors which equipotently inhibit both monomeric and dimeric RAF [182, 184].

1.2.6.2.3.2. *RAF* fusion proteins

As *RAF* fusion proteins promote constitutive activation by dimerisation (as described in section 1.2.5.2.1.3.2.), they are also predicted to be resistant to α C-OUT *BRAF* inhibitors, and potentially more sensitive to α C-IN RAF inhibitors [117].

1.2.7. Results of clinical trials of single-agent second-generation *BRAF* inhibitors

1.2.7.1. Results of clinical trials of single-agent second generation *BRAF* inhibitors in malignant melanoma

1.2.7.1.1. Results of clinical trials of single-agent vemurafenib in malignant melanoma

The first “proof-of-concept” clinical trial validating the efficacy of second generation α C-OUT *BRAF* inhibitors was the Phase I study of vemurafenib in patients with metastatic cancer [23]. The dose-escalation phase included 55 patients with any tumour type. *BRAF*^{V600E} mutation status was not mandated, although the study population was enriched for *BRAF*^{V600E} melanoma, with 16 of 21 melanoma patients treated at the ≥ 240 mg twice daily dose level having a *BRAF*^{V600E} mutation. In the dose-expansion phase, 32 patients with *BRAF*^{V600E} melanoma were treated with the recommended phase 2 dose of 960 mg oral (po) twice daily (BD). The efficacy data was promising, with 10 partial responses (PRs) and 1 complete response (CR) observed in patients with *BRAF*^{V600E} melanoma in the dose-escalation phase. In the dose-expansion phase, there were

24 PRs and 2 CRs observed, with an estimated PFS of over 7 months. The adverse event (AE) data was also encouraging, with 89% of all side effects being only grade 1 or 2, and the most common $\geq$ grade 2 AEs being arthralgia, rash and nausea. Grade 3 cutaneous squamous cell carcinoma (SCC) (keratoacanthoma type) was observed in 21% of patients [23].

These results were further confirmed in the BRIM-2 study, an open-label multi-centre single-arm phase II trial of vemurafenib in patients with $BRAF^{V600E}$ metastatic melanoma who had received at least one prior line of therapy [24]. The study enrolled 132 patients and was positive for its primary endpoint of best overall response rate (BORR), which was 52.3% (95% CI 43-61%; 2.3% CRs and 50% PRs). The median PFS was 6.2 months (95% CI 5.6 to 6.8 months). The most common AEs, which were mostly Grade 1-2 and predominantly reversible, were arthralgia, rash, photosensitivity, fatigue, alopecia, pruritis and skin papilloma, and the most common Grade 3 AE was cutaneous SCC (24.2%) [24].

Finally, the BRIM-3 study was a pivotal multi-centre international phase III randomised clinical trial which compared vemurafenib to dacarbazine, the only chemotherapeutic agent approved by the FDA for the treatment of metastatic melanoma which was previously reported to lead to ORRs of 7-12% and median OS of 5.6 to 7.8 months in phase 3 trials [278-281]. Participants had unresectable, treatment-naïve stage IIIC or IV $BRAF^{V600E}$ melanoma, and the co-primary endpoints of the study were PFS and OS. The study randomised 675 patients from 104 centres in 12 countries, including 20 patients with non-V600E $BRAF$ mut melanoma (19 with V600K and 1 with V600D). Patients were randomised in a 1:1 ratio to receive vemurafenib 960 mg po BD or dacarbazine. After an interim analysis which favoured vemurafenib for both PFS and OS, the protocol was amended to permit cross-over from the dacarbazine arm to the vemurafenib arm [25].

After a median follow-up of 12.5 months on vemurafenib and 9.5 months on dacarbazine, 83 (25%) of 338 patients in the dacarbazine group had crossed over to vemurafenib. Overall, the median OS and PFS (censored at cross-over) were both significantly longer in the vemurafenib compared to the dacarbazine arm (OS: 13.6 months, 95% CI 12.0 to 15.2 months versus 9.7 months, 95% CI 7.9 to 12.8 months; HR 0.70; 95% CI 0.57 to 0.87, $P = 0.0008$, and PFS: 6.9 months, 95% CI 6.1 to 7.0 months versus 1.6 months, 95% CI 1.6 to 2.1 months; HR 0.38; 95% CI 0.32 to 0.46, $P < 0.0001$). Among the patients with $BRAF^{V600E}$ melanoma, the median OS and PFS were significantly longer in the vemurafenib compared to the dacarbazine group (OS: 13.3 months, 95% CI 11.9 to 14.9 months versus 10.0 months, 95% CI 8.0 to 14.0 months; HR 0.75; 95% CI 0.60 to 0.93, $P = 0.0085$, and PFS: 6.9 months, 95% CI 6.2 to 7.0 months versus 1.6 months, 95% CI 1.6 to 2.1 months; HR 0.39; 95% CI 0.33 to 0.47, $P < 0.0001$). For the patients with $BRAF^{V600E}$ melanoma, the ORR was 59% (173/598 patients) for vemurafenib and 11%

(34/298) for dacarbazine. Similar to BRIM-2, the most common AEs in the vemurafenib arm, which were mostly Grade 1-2, were arthralgia, rash, fatigue, and photosensitivity, and the most common Grade 3 AE was cutaneous SCC (19%) [26].

The results of the final OS data for BRIM-3 were recently reported after a median follow-up of 13.4 months on vemurafenib and 9.2 months on dacarbazine, at which point 84 of 338 patients (25%) in the dacarbazine group had crossed over to vemurafenib [27]. The median OS (censored at cross-over) remained significantly longer in the vemurafenib compared to the dacarbazine arm (OS: 13.6 months, 95% CI 12.0 to 15.4 months versus 9.7 months, 95% CI 7.9 to 12.8 months; HR 0.81; 95% CI 0.67 to 0.98, $P = 0.03$) [27].

1.2.7.1.2. Studies of single-agent dabrafenib for malignant melanoma

Similar impressive results were observed in studies of single-agent dabrafenib for malignant melanoma.

The phase I study of dabrafenib in patients with melanoma included a dose-escalation phase which enrolled patients with advanced solid tumours, and a dose-expansion phase with three cohorts – metastatic melanoma, asymptomatic untreated melanoma brain metastases and non-melanoma solid tumours [28]. The study enrolled 184 patients, of whom 156 had melanoma, and all but 3 melanoma patients had *BRAF* mut disease. *BRAF* mutation was initially not an inclusion criterion, but later became mandatory due to lack of activity observed in the 3 patients with *BRAF* wt melanoma. At the recommended phase 2 dose (R2PD) of 150 mg po BD, the overall confirmed objective response rate (ORR) was 50% (18/36 patients) in those with *BRAF*^{V600E/K} disease, and 56% (25/27 patients) in the subset with *BRAF*^{V600E} disease. Median PFS was comparable in patients with *BRAF*^{V600E} and *BRAF*^{V600K} tumours (5.5 months versus 5.6 months). In ten patients with melanoma and untreated brain metastases who received the RP2D, nine had a decrease in size of brain lesions - four achieved complete resolution of the brain metastases. In patients with assessable non-melanoma solid tumours, PRs were observed in 2 out of 9 patients (confirmed) with papillary thyroid cancer, 1 out of 1 patient (unconfirmed) with NSCLC, and 1 out of 9 patients (confirmed) with CRC. 76% of patients had no drug-related AEs greater than Grade 1-2, and the most common grade ≥ 2 AEs were cutaneous SCC or keratoacanthoma (11%), fatigue (8%) and pyrexia (6%) [28].

The clinical activity and favourable safety profile of dabrafenib were confirmed in BREAK-2, a multi-centre, international, single-arm phase II study of dabrafenib in patients with *BRAF*^{V600E/K} metastatic melanoma, of whom 90% had prior treatment [29]. This study enrolled 76 patients with *BRAF*^{V600E} and 16 patients with *BRAF*^{V600K} malignant melanoma. The ORR was 59% (45/76 patients) in those with *BRAF*^{V600E} melanoma, and 13% (2/16 patients) in those with *BRAF*^{V600K} melanoma. However, median PFS and OS were similar in patients with *BRAF*^{V600E} and *BRAF*^{V600K}

melanoma (6.3 months and 13.1 months for $BRAF^{V600E}$ melanoma, and 4.5 months and 12.9 months for $BRAF^{V600K}$ melanoma, respectively). Updated survival data for patients with $BRAF^{V600E}$ melanoma from the cut-off date of 17th June 2016 were a 5-year PFS of 11%, and 5-year OS of 20%. The most common AEs were arthralgia (33%), hyperkeratosis (27%) and pyrexia (24%) [30].

Finally, the BREAK-3 study was an open-label phase 3 randomised controlled trial which randomised patients with stage IV or unresectable stage III $BRAF^{V600E}$ melanoma in a 3:1 ratio to receive dabrafenib 150 mg po BD or dacarbazine [31]. The primary endpoint of the study was PFS. After a median follow-up of 18.6 months on vemurafenib and 12.8 months on dacarbazine, 37 of 63 patients (59%) in the dacarbazine arm had crossed over to dabrafenib treatment [30]. In the intention-to-treat (ITT) analysis of the dabrafenib versus dacarbazine arms, 5-year PFS and OS were 12% versus 3%, and 24% versus 22% respectively. Of note, OS results are likely to have been confounded by crossover of patients from the dacarbazine arm to the dabrafenib arm. The safety profile observed was similar to BREAK-2 [31].

1.2.7.1.3. Studies of single-agent encorafenib for malignant melanoma

Results of a phase I trial of single-agent encorafenib in patients with BRAF inhibitor –naïve or –pretreated $BRAF^{V600}$ stage IIIB or IV melanoma have also been recently reported [32]. 54 patients were enrolled in the dose-escalation phase, from whom the R2PD was determined at 450 mg po D. A further 35 patients (17 BRAF inhibitor-naïve and 18 BRAF inhibitor-pretreated) were treated in the dose-expansion phase (melanoma cohort). Among BRAF inhibitor-naïve and BRAF inhibitor-pretreated patients, the ORR and median PFS were 60% and 12.4 months (95% CI 7.4–not reached) and 22% and 1.9 months (95% CI, 0.9–3.7), respectively, confirming the clinical activity of single-agent second-generation BRAF inhibitors in malignant melanoma. In the dose-expansion phase, the most common drug-related AEs were myalgia (63%), nausea (57%), and palmar–plantar erythrodysesthesia (54%) [32].

1.2.7.2. Results of clinical trials of single-agent BRAF inhibitors in metastatic colorectal cancer

Given the successful results observed with the use of single-agent second-generation BRAF inhibitors in malignant melanoma, it was hoped that these drugs would demonstrate similar success for the treatment of $BRAF^{V600E}$ metastatic CRC, however clinical trials have shown unexpectedly disappointing results (Table 1.4).

Kopetz *et al.* reported the results of a phase II multi-centre international pilot study of vemurafenib in patients with $BRAF^{V600E}$ metastatic CRC, which enrolled 21 patients from the US and Australia, 20 of whom had prior chemotherapy for metastatic disease, and 15 (71%) of whom

had received 3 or more lines of previous therapy [33] (Table 1.4). Of 19 patients who had assessable disease, none had CRs, and only one patient (5%) achieved a confirmed PR. Ten patients, however, had some degree of tumour shrinkage on their first planned computed tomography (CT) scan. The median PFS and OS were 2.1 months (range, 0.4 to 11.6 months) and 7.7 months (range, 1.4 to 13.1 months), respectively [33].

Similarly, a phase II “basket study” of vemurafenib in patients with $BRAF^{V600}$ non-melanoma cancers included 10 patients with $BRAF^{V600}$ metastatic CRC, including 8 patients with $BRAF^{V600E}$ and 2 patients with $BRAF^{V600K}$ disease who were treated with single-agent vemurafenib prior to a protocol amendment which changed the treatment to combination vemurafenib and cetuximab [34] (Table 1.4). 70% of these patients had received 3 or more lines of previous therapy. No objective complete or partial responses were observed, and the median PFS and OS were 4.5 months (95% CI 1.8 to 5.5 months) and 9.3 months (95% CI 5.6 months – not reached), respectively [34].

Efficacy results from the colorectal cancer expansion cohort of the phase I trial of encorafenib described in section 1.2.7.1.3. were similarly disappointing [35] (Table 1.4). This cohort included 18 patients with $BRAF^{V600E}$ non-resectable, advanced CRC for whom there was no existing effective therapy. Gomez-Roca *et al.* reported no confirmed objective complete or partial responses (unconfirmed CRs/PRs were reported in 5/16 (31%) patients), and the median PFS was 4.0 months (95% CI 1.8 to 5.6 months) [35].

Table 1.4. Efficacy data from clinical trials of BRAF inhibitors in metastatic colorectal cancer which have results

Drugs	ClinicalTrials.gov Identifier	Phase	Arms	BRAF mutation	Pre-treatment	Sample size	ORR (n responder/n total, %)	Median PFS (months)	Median OS (months)	Completion status	Reference
Single-agent BRAFi											
Vemurafenib	NCT00405587	II	NA	BRAF ^{V600E}	71% of patients had ≥ 3 lines of previous therapy	21	1/19 (5%) confirmed ^a	2.1 (range, 0.4 to 11.6)	7.7 (range, 1.4 to 13.1)	Completed, with results	[33]
Encorafenib	NCT01436656	I, dose-expansion	NA	BRAF ^{V600E}	Median 3 lines of previous therapy (range, 1 to 8), 1 patient had prior treatment with vemurafenib.	18	0/16 (0%) confirmed, 5/16 (31%) including unconfirmed ^a			Completed, with results	[35]
						n = 6 received encorafenib 300 mg po D	0/5 (0%) confirmed, 1/5 (20%) including unconfirmed ^a	2.3 (95% CI 1.7 to 7.2)	Not reported		
						n = 12 received encorafenib 450 mg po D	0/11 (0%) ^a confirmed, 4/11 (35%) including unconfirmed	4.0 (95% CI 1.8 to 5.5)	Not reported		
BRAFi + EGFRi											
Vemurafenib ± cetuximab	NCT01524978 (MO28072)	II - "basket" study	Vemurafenib alone	BRAF ^{V600} (8/10 BRAF ^{V600E} ; 2/10 BRAF ^{V600} UK)	7/10 (70%) of patients had ≥ 3 lines of previous therapy.	10	0/10 (0%) ^a	4.5 (95% CI 1.0 to 5.5)	9.3 (95% CI 5.6 to not reached)	Completed, with results	[34]
			Vemurafenib + cetuximab	BRAF ^{V600} (24/27 BRAF ^{V600E} ; 3/27 BRAF ^{V600} UK)	11/27 (41%) of patients had ≥ 3 lines of previous therapy. 12/27 (44%) received prior anti-EGFR antibody	27	1/27 (4%, 95% CI <1 to 20) ^a	3.7 (95% CI 1.8 to 5.1)	7.1 (95% CI 4.4 to not reached)		
Vemurafenib + panitumumab	NCT01791309	Pilot study	Single arm	BRAF ^{V600E}	8/15 (53%) and 7/15 (57%) had 1 and 2 lines of previous therapy, respectively	15	2/12 (17%) ^a	3.2 (95% CI 1.6 to 5.3)	7.6 (95% CI 2.1 to not reached)	Completed, with results	[282]

Table 1.4. (continued) Efficacy data from clinical trials of BRAF inhibitors in metastatic colorectal cancer which have results

Drugs	ClinicalTrials.gov Identifier	Phase	Arms	BRAF mutation	Pre-treatment	Sample size	ORR (<i>n</i> responder/ <i>n</i> total, %)	Median PFS (months)	Median OS (months)	Completion status	Reference
Vemurafenib + erlotinib	NA (ACTRN126140004 86628, EViCT)	I/II	Single arm	<i>BRAF</i> ^{V600E}	50% and 41% had 1 and 2 lines of previous therapy, respectively	30	5/23 (22%) confirmed, 9/23 (39%) including unconfirmed ^a	Not reported	Not reported	Active, not recruiting. Interim results published as abstract	[283]
BRAFi + MEKi											
Dabrafenib + trametinib	NCT01072175	I/II	Single arm	<i>BRAF</i> ^{V600E}	36/43 (84%) of patients had ≥ 2 lines of previous therapy	43	3/43 (7%) confirmed; 5/43 (12%) including unconfirmed	3.5 (95% CI 3.4 to 4.0)	Not reported	Completed, with results for <i>BRAF</i> ^{V600E} -mutant mCRC expansion cohort in Part B	[284]
BRAFi + EGFRi ± MEKi											
Dabrafenib, panitumumab, trametinib combination	NCT01750918	I/II	Arm 1: Dabrafenib + panitumumab	<i>BRAF</i> ^{V600E}	8/20 (40%) had ≥ 2 lines of previous therapy	20	2/20 (10%) confirmed ^a	3.5 (95% CI 2.8 to 5.8)	13.2 (95% CI 6.7 to 22.0)	Active, not recruiting. Interim results published as journal article.	[285]
			Arm 2: Dabrafenib + panitumumab + trametinib		43/91 (47%) had ≥ 2 lines of previous therapy	91	19/86 (22%) confirmed ^a	4.2 (95% CI 4.0 to 5.6)	9.1 (95% CI 7.6 to 20.0)		
			Arm 3: Panitumumab + trametinib		16/31 (52%) had ≥ 2 lines of previous therapy	31	0/29 (0%) ^a	2.6 (95% CI 1.4 to 2.8 m)	8.2 (95% CI 6.5 to 9.4)		

Table 1.4. (continued) Efficacy data from clinical trials of BRAF inhibitors in metastatic colorectal cancer which have results

Drugs	ClinicalTrials.gov Identifier	Phase	Arms	BRAF mutation	Pre-treatment	Sample size	ORR (<i>n</i> responder/ <i>n</i> total, %)	Median PFS (months)	Median OS (months)	Completion status	Reference
BRAFi + EGFRi ± PI3Ki											
Encorafenib + cetuximab ± alpelisib (BYL719)	NCT01719380	Ib, dose-escalation	Encorafenib + Cetuximab	BRAF mutant (52/54 (96%) BRAF ^{V600E})	19/26 (73%) had ≥ 2 lines of previous therapy	26	5/24 (21%) confirmed ^a			Completed, with results	[286]
			Encorafenib + Cetuximab + Alpelisib		18/28 (64%) had ≥ 2 lines of previous therapy	28	5/27 (19%) confirmed ^a				
Encorafenib + cetuximab ± alpelisib (BYL719)	NCT01719380	II	Encorafenib + Cetuximab	BRAF mutant	Median 2 lines of previous therapy	50	11/46 (24%) confirmed ^a	5.4 (95% CI 4.1 to 7.2)	15.2	Active, not recruiting. Interim results published as abstract.	[287]
			Encorafenib + Cetuximab + Alpelisib		Median 2 lines of previous therapy	52	14/49 (29%) confirmed ^a	4.2 (95% CI 3.4 to 5.4)	Not reached		
Irinotecan + EGFRi ± BRAFi											
Irinotecan + cetuximab + vemurafenib	NCT01787500	Ib	Single arm	BRAF ^{V600E}	Median 2 lines of previous therapy (range, 1 to 4), 1 patient had prior treatment with a BRAF inhibitor	19	6/17 (35%, 95% CI 14 to 62%) ^a , all confirmed	7.7 (95% CI 3.1 to not reached)	Not reported	Completed, with results	[288]
Irinotecan + cetuximab ± vemurafenib	NCT02164916	II	Irinotecan + cetuximab	BRAF ^{V600}	Not reported	50	4%	2.0 (95% CI 1.8 to 2.1)	Not reported	Active, not recruiting. Interim results published as abstract.	[289]
			Irinotecan + cetuximab + vemurafenib			49	16%	4.4 (95% CI 3.6 to 5.7)	Not reported		

ORR: objective response rate; PFS: progression-free survival; OS: overall survival; CI: confidence interval; BRAFi: BRAF inhibitor; MEKi: MEK inhibitor; EGFRi: epidermal growth factor receptor inhibitor; PI3Ki: Phosphoinositide 3-kinase inhibitor; UK: unknown; mCRC: metastatic colorectal cancer; ^a: denominator for ORR refers to the number of patients in whom treatment response was measured.

1.2.8. Mechanisms of resistance of colorectal cancer to BRAF inhibitors

Multiple pre-clinical studies and translational sub-studies within clinical trials have been performed in an effort to explore the molecular mechanisms underlying the poor responses to second generation BRAF inhibitors in *BRAF* mut colorectal cancer, compared to melanoma.

1.2.8.1. Previous studies examining basal biomarkers of resistance of *BRAF*^{V600E} colorectal cancer to BRAF inhibitors

1.2.8.1.1. The PI3K pathway

Mao *et al.* performed a proteomic analysis which compared *BRAF* mut melanoma and CRC using reverse phase protein arrays (RPPAs) in a panel of cell lines [290]. *BRAF* mut CRC cell lines demonstrated higher activation of phosphorylated (p)-Akt and p-p70 S6K and higher expression of eIF4E, indicating greater PI3K pathway activation in *BRAF* mut CRC compared to melanoma. *BRAF*^{V600E} CRC cell lines which were PTEN null or *PIK3CA* mut were more resistant to treatment with PLX4720 (a precursor of vemurafenib) *in vitro*, and *PTEN* knockdown reduced sensitivity of *BRAF* mut CRC cells to colony formation and cell proliferation. Furthermore, combining PLX4720 with the PI3K inhibitors LY294002 or GDC0941 led to synergistic growth inhibition *in vitro*, and combining PLX4720 with the Akt inhibitor MK2206 led to greater tumour growth inhibition compared to PLX4720 alone *in vivo* [290]. Collectively, these results suggested that parallel activation of the PI3K/AKT pathway is a mechanism of inherent resistance to BRAF inhibitors in *BRAF*^{V600E} CRC, and that the combination of BRAF inhibitors with PI3K/AKT inhibitors could be used to improve outcomes.

Translational sub-studies within two reported clinical trials, including one study of BRAF inhibitor alone [33], have explored the relationship between basal PI3K pathway activation and vemurafenib resistance. In the phase II study of single-agent vemurafenib in patients with *BRAF*^{V600E} metastatic CRC (described in section 1.2.7.2.), Kopetz *et al.* reported that 4 out of 14 evaluable tumours had PI3K pathway activation characterised by complete loss of PTEN expression using IHC or the presence of *PIK3CA*/*AKT* mutations, however there was no association between PI3K pathway activation and PFS [33]. In a Phase I/II single-arm clinical trial which examined the combination of the BRAF inhibitor dabrafenib with the MEK inhibitor trametinib in *BRAF*^{V600E} CRC [284] (Table 1.4) (described in detail in section 1.2.9.1.2.), Corcoran *et al.* performed mutational analysis on formalin-fixed paraffin embedded (FFPE) primary tumour samples available from 15 of 43 enrolled patients, and found PI3K activation characterised by mutations in *PIK3CA* at hotspots in exon 9 and 20 (5/15 patients), *PIK3R1* (0/15 patients), or *PTEN* (2/15 patients) in a total of 7 of the 15 patients. Interestingly, 3 of the 5 patients who achieved a PR or CR had a tumour which harboured a *PIK3CA* mutation, and all except one patient with a *PIK3CA* mutation had a decrease in target lesion size by Response Evaluation Criteria in Solid Tumours (RECIST) criteria, suggesting improved responses with *PIK3CA*

mutation. Two of 4 PDX models successfully generated from participants' pre-treatment biopsies had activating *PIK3CA* mutations, and these regressed following treatment with the dabrafenib /trametinib combination [284]. Whilst these results appear to contradict the pre-clinical study described above, this translational study was limited by a very small sample size. PTEN loss was also analysed using immunohistochemistry (IHC), and identified in 4 out of 19 evaluable patient samples, however there was no association between PTEN status and PFS [284].

1.2.8.1.2. Microsatellite instability (MSI)

Translational sub-studies within four reported clinical trials, including one study of BRAF inhibitor alone [33], have also explored the relationship between MSI/MSS status and inherent vemurafenib resistance.

In the phase II study of single-agent vemurafenib in patients with *BRAF*^{V600E} metastatic CRC (described in section 1.2.7.2.), Kopetz *et al.* reported that 4 out of 15 evaluable tumours were MSI-H as defined by complete loss of hMLH1 protein expression and methylation of hMLH1 in the promoter region, however MSI-H was not associated with radiographic response [33]. In the Phase I/II single-arm clinical trial of dabrafenib with trametinib in *BRAF*^{V600E} CRC [284] (Table 1.4) (described in detail in section 1.2.9.1.2.), Corcoran *et al.* reported that of 24 evaluable specimen results for MSI analysis, 8 were MSI, however there was no significant difference in PFS between patient with MSI and MSS tumours. Similarly, in a Phase Ib study of vemurafenib combined with irinotecan and the EGFR inhibitor cetuximab [288] (Table 1.4) (described in detail in section 1.2.9.1.4), Hong *et al.* performed MSI analysis in tumours from 14 of 19 enrolled patients, and found that there was no significant association between MSI and response [288].

However, recently published interim results of a Phase I/II clinical trial which examined combinations of the BRAF inhibitor dabrafenib (D), EGFR inhibitor panitumumab (P), and MEK inhibitor trametinib (T) [285] (Table 1.4) (described in detail in section 1.2.9.1.2.) suggest improved outcomes in patients with MSI-high/MMR-D tumours. Among 78 patients in the D+T+P arm (86%) for whom information on MSI/MMR status and clinical outcomes were available, a trend to a higher response rate in patients with MSI-high/MMR-D tumours was observed compared to those with MSS/MMR-P tumours (46%, 5/11 patients; 95% CI 17 to 77%, versus 27%, 18/67 patients; 95% CI 17 to 39%), although this was not statistically significant. Similarly, there was a trend towards a better PFS in patients with MSI tumours among the patients who received D+T+P (HR 2.624; 95% CI 0.997 to 6.907, log rank *P* = 0.0449). Of note, this translational data was related to the triple combination of D+T+P and not a BRAF inhibitor alone, and the study design precludes determination of whether the effects are predictive or prognostic.

1.2.8.1.3. CpG Island Methylation Phenotype (CIMP)

Exploiting the observation that *BRAF* mut CRC is strongly associated with the CpG Island Methylation Phenotype (CIMP) which leads to epigenetic silencing of multiple genes, the study

by Mao *et al.* described in section 1.2.8.1.1. also examined combining PLX4720 with the DNA methyltransferase inhibitor 5-azacytidine (5-AZA) in *BRAF*^{V600E} cell lines [290]. They found that this combination led to synergistic growth inhibition *in vitro*, and *in vivo* experiments using a HT29 cell line xenograft confirmed enhanced tumour regression with the combination of PLX4720 and 5-AZA (used to pre-prime *in vitro*, with subsequent continued treatment *in vivo*), compared to BRAF inhibitor alone. 5-AZA inhibited phosphorylation of Akt, and the authors demonstrated that this was potentially mediated by Akt modulators including *IGFBP3* and *PTEN* which are commonly cited as being under epigenetic control [291-293]. The authors concluded that whilst these pre-clinical results suggest that combining BRAF inhibitors with epigenetic therapy may represent a targeted treatment approach, demethylator therapy requires several months to fully inhibit aberrant methylation. This may be unachievable in practice, given the rapidly progressive nature of *BRAF* mut CRC [290].

However, in the phase II study of single-agent vemurafenib in patients with *BRAF*^{V600E} metastatic CRC (described in section 1.2.7.2.), Kopetz *et al.* reported that an exploratory analysis of CIMP assessed using methylation analysis of six-gene panel (*MINT1*, *MIN2*, *MIN31*, *p16*, *p14* and *MLH1*) [294] showed no correlation between CIMP status and clinical activity [33].

1.2.8.1.4. Basal levels of epidermal growth factor receptor (EGFR)

Previous pre-clinical studies have hypothesised that high basal expression of EGFR or p-EGFR may be predictive of resistance to BRAF inhibitor monotherapy in multiple *BRAF* mutant cancers [295], including *BRAF* mut CRCs [296].

Prahallad *et al.* examined a panel of *BRAF*^{V600E} malignant melanoma, colon cancer and thyroid cancer cell lines, and found that while melanoma cell lines expressed low levels of EGFR (and p-EGFR) on western blot, the majority of CRC cell lines (8 out of 10) expressed high levels of EGFR. The two EGFR^{low} CRC cell lines were almost as sensitive to the melanoma cell line A375 in short-term cell viability assays and long-term colony formation assays, leading to the hypothesis that basal EGFR levels determine response to BRAF inhibitors [295].

Corcoran *et al.* found higher levels of EGFR and p-EGFR expression in a panel of four *BRAF*^{V600E} CRC cell lines, compared to four *BRAF*^{V600E} melanoma cell lines. Analysis of p-EGFR levels assessed using IHC on patient FFPE tissue samples found that a greater proportion of *BRAF*^{V600E} CRC compared to melanoma cell lines had high p-EGFR expression levels (60 versus 18%), leading to the hypothesis that basal p-EGFR levels may determine which *BRAF* mut CRCs may be resistant to BRAF inhibition. Using phospho-RTK arrays, Corcoran *et al.* also found that *BRAF* mut CRC cells expressed higher basal levels of other phosphorylated RTKs (including p-HER2, p-MET and p-IGF1R) compared to *BRAF* mut melanoma, and hypothesised that these could also potentially mediate the relative resistance to BRAF inhibitors observed in *BRAF* mut CRC compared to melanoma [296].

Of note, neither of these pre-clinical studies specifically examined the association between total (t-)EGFR or p-EGFR expression and vemurafenib sensitivity within a panel of *BRAF*^{V600E} CRC cell lines.

Translational sub-studies within two reported clinical trials, including one study of BRAF inhibitor alone [33], also explored the relationship between t-EGFR expression and inherent vemurafenib resistance, however found no association.

In the phase II study of single-agent vemurafenib in patients with *BRAF*^{V600E} metastatic CRC (described in section 1.2.7.2.), Kopetz *et al.* reported that expression of t-EGFR determined by IHC and dichotomised as present/absent, was not associated with tumour response [33]. In the Phase I/II single-arm clinical trial of dabrafenib with trametinib in *BRAF*^{V600E} CRC (Table 1.4) (described in detail in section 1.2.9.1.2.), Corcoran *et al.* reported that among 22 patients with evaluable specimens, analysis of t-EGFR by IHC did not correlate with PFS [284].

Of note, neither of these translational sub-studies specifically examined the association between p-EGFR expression and inherent vemurafenib resistance in participants' tumour specimens.

1.2.8.2. Studies examining mechanisms of resistance of *BRAF*^{V600E} colorectal cancer to BRAF inhibitors

1.2.8.2.1. EGFR-mediated mechanisms of resistance

Whilst two previous studies have described EGFR-mediated mechanisms of resistance of *BRAF*^{V600E} CRC to BRAF inhibitors, some of their findings are conflicting and the postulated underlying mechanisms are distinct.

Prahallad *et al.* performed an RNA-interference based genetic screen using a short hairpin RNA (shRNA) library of human kinases and kinase-related genes to identify genes which, when inhibited, conferred sensitivity to vemurafenib in *BRAF*^{V600E} CRC cells [295]. Three independent shRNA vectors targeting the EGFR were found to confer sensitivity to vemurafenib treatment. Subsequent experiments in three *BRAF*^{V600E} CRC cell lines (WiDr, VACO432 and KM20) showed that combining EGFR inhibitors (the anti-EGFR monoclonal antibody cetuximab, and EGFR tyrosine kinase inhibitors gefitinib and erlotinib) with vemurafenib resulted in synergistic growth inhibition and induction of apoptosis *in vitro* and additively inhibited tumour growth in xenograft models.

Mechanistically, the authors also observed that while treatment of these cell lines with vemurafenib alone led to a rapid, ligand-dependent, increase in p-EGFR (Tyr1068) and p-Akt, combining cetuximab/gefitinib with vemurafenib prevented this effect.

The authors went on to hypothesise that the underlying mechanism of resistance is dependent on phosphatase-mediated feedback regulation of EGFR. In *Xenopus*, ERK kinases can

phosphorylate and activate the phosphatase Cdc25c [297]. In the authors' proposed model, BRAF inhibition leads to inhibition of MEK and ERK kinases, which results in reduced activation of CDC25C. This in turn leads to increased p-EGFR as a result of decreased de-phosphorylation [295].

In comparison, Corcoran *et al.* observed that whilst vemurafenib treatment led to sustained suppression of p-ERK in *BRAF*^{V600} melanoma, in *BRAF* mut CRC cell lines p-ERK re-accumulated after 24 hours, consistent with MAPK pathway reactivation. This was associated with upregulation of p-CRAF (Ser338) and increased activated GTP-RAS [296].

However, in contrast to the findings of Prahallad *et al.*, vemurafenib treatment failed to induce p-EGFR in *BRAF*^{V600E} CRC cells, with in fact a slight decrease in p-EGFR and t-EGFR levels observed after 24 to 48 hours. Instead, using phospho-RTK arrays, the authors observed that vemurafenib induced phosphorylation of IGF1R. Surprisingly, however, combination of vemurafenib with an EGFR inhibitor (gefitinib) or a dual EGFR/HER2 inhibitor (lapatinib) led to more complete suppression of p-ERK than the combination of vemurafenib with an IGF1R inhibitor (NVP-AEW541). Combination of vemurafenib with an EGFR inhibitor also suppressed vemurafenib-mediated induction of RAS-GTP and p-CRAF, led to decreased cell viability *in vitro*, and additively inhibited tumour growth in xenograft models.

The authors postulated that the lack of vemurafenib-induced p-EGFR upregulation may be explained by EGFR already being active prior to vemurafenib treatment in *BRAF*^{V600E} CRC cells. They hypothesised that vemurafenib treatment does not lead to EGFR activation, but instead increases the ability of EGFR to effectively signal downstream in the MAPK pathway, possibly through suppression of the negative MAPK pathway feedback regulator Sprouty4 (Spry4) [296].

1.2.8.2.2. Parallel adaptive activation of the Wnt/ β -catenin pathway

In a recent study, Chen *et al.* reported that parallel adaptive signalling through upregulation of the Wnt/ β -catenin pathway may be an additional mechanism of BRAF inhibitor resistance in *BRAF*^{V600E} CRC [298]. First- (GDC0879), second- (vemurafenib/dabrafenib), and third-generation (PLX7904 and LY3009120) BRAF inhibitors led to FAK-mediated upregulation of Wnt/ β -catenin signalling *in vitro* and/or *in vivo*. FAK activation was demonstrated to be independent of MAPK activation, and was not inhibited by treatment with an ERK inhibitor, EGFR inhibitor, or MEK inhibitor. Co-targeting of BRAF with the Wnt inhibitor IGC-001 or the FAK inhibitor PF562271 had synergistic anti-tumour effects *in vitro* and *in vivo*. The authors observed elevated levels of the Wnt pathway targets SOX9 (a stem-cell transcriptional regulator) and LGR5 (an intestinal stem cell marker) following BRAF inhibitor treatment, and hypothesised

that hyperactivation of Wnt signalling may contribute to resistance to BRAF inhibitor treatment by enhancing colon cancer stemness through an increase in the stem cell pool [298].

1.2.8.2.3. The PI3K pathway

Coffee *et al.* examined the effects of BRAF inhibitor treatment (using the first-generation α C-INRA inhibitor GDC0879) on PI3K/mTOR signalling in *BRAF*^{V600E} CRC, both in cell line models *in vitro* and using a novel GEMM for MSS *BRAF*^{V600E} CRC which morphologically recapitulated the *BRAF*^{V600E} sessile serrated CRC pathway [299]. GDC0879 treatment led to sustained (but, notably, not upregulated) p-Akt and p-S6 protein expression both *in vitro* and *in vivo*, which was suppressed when the BRAF inhibitor was combined with the PI3K/mTOR inhibitor NVP-BEZ235. Compared to single-agent BRAF inhibitor, combination treatment with GDC0879 and NVP-BEZ235 resulted in decreased cell viability *in vitro* and tumour regression *in vivo*. Concurrent BRAF and PI3K/mTOR blockade was found to induce apoptosis both *in vitro* and *in vivo*, relative to BRAF inhibitor alone [299], supporting the approach of combining PI3K/AKT inhibitors to BRAF inhibitors for the treatment of *BRAF*^{V600E} CRC.

1.2.9. Results of clinical trials of BRAF inhibitors in combination with other therapies in metastatic colorectal cancer

Based on the results of the pre-clinical studies examining basal biomarkers and mechanisms of resistance to single-agent BRAF inhibitors, Phase I and II clinical trials examining relevant drug combinations have been performed (Table 1.4).

Overall, the clinical trials examining the doublet combination of BRAF inhibitor and EGFR inhibitor have shown only a modest improvement in efficacy compared to single agent BRAF inhibitor, with ORRs ranging from 4 to 24% (with unconfirmed ORRs up to 39%) (Table 1.4). Similarly, the median PFS in patients treated with these combinations have ranged from 3.2 to 5.4 months (Table 1.4).

Clinical trials examining triplet combinations of a BRAF inhibitor and an EGFR inhibitor, with either a MEK inhibitor, a PI3K inhibitor or chemotherapy have reported slightly greater efficacy than the doublet BRAF/EGFR inhibitor combinations, with ORRs ranging from 16 to 35%, and median PFS ranging from 4.2 to 7.7 months (Table 1.4). However, response rates even with combination therapy remain lower than those observed with single agent BRAF inhibitor treatment in melanoma.

1.2.9.1. Clinical trials including the combination of BRAF inhibitor and EGFR inhibitor for BRAF mutant colorectal cancer

Based on the results of pre-clinical studies suggesting EGFR may drive resistance of *BRAF*^{V600E} CRC to BRAF inhibitors (described in section 1.2.8.2.1.) and *in vivo* models showing increased anti-tumour activity with combination BRAF and EGFR inhibition [250], multiple clinical trials

testing the combination of BRAF and EGFR inhibitors for metastatic *BRAF* mut CRC were initiated (Table 1.4).

1.2.9.1.1. Clinical trials combining BRAF inhibitor and EGFR inhibitor for *BRAF* mutant colorectal cancer

The phase II “basket study” of vemurafenib in patients with *BRAF*^{V600} non-melanoma cancers, described in section 1.2.7.2., underwent a protocol amendment to include assessment of the combination of **vemurafenib and cetuximab** [34]. Thereafter, 27 patients with *BRAF*^{V600} CRC were treated with this combination (including 24 patients with *BRAF*^{V600E} and 3 patients with *BRAF*^{V600} unknown), of whom 11 (41%) of patients were heavily pre-treated with 3 or more lines of previous therapy (Table 1.4). A PR was observed in only one out of 27 evaluable patients (4%, 95 % CI <1 to 20), and the median PFS and OS were 3.7 months (95% CI 1.8 to 5.1 months) and 7.1 months (95% CI 4.4 months – not reached), respectively [34].

Yaeger *et al.* reported the results of a single-arm pilot study which tested the combination of **vemurafenib and the anti-EGFR monoclonal antibody panitumumab** in 15 patients with *BRAF*^{V600E} CRC [282] (Table 1.4). This cohort of patients were less heavily pre-treated, with 8 out of 15 (53%) and 7 out of 15 (57%) having had 1 and 2 lines of previous therapy, respectively. Partial responses were observed in 2 out of 12 (17%) evaluable patients, and the median PFS and OS were 3.2 months (95% CI 1.6 to 5.3 months) and 7.6 months (95% CI 2.1 to not reached), respectively [282].

The EViCT study is a single-arm phase I/II trial testing the combination of **vemurafenib and erlotinib** [283] (Table 1.4). Of 30 patients with *BRAF*^{V600E} CRC who were enrolled, 50% and 41% had received 1 and 2 previous lines of therapy, respectively. Published interim results for this study reported confirmed PRs in 5 out of 23 (22%) evaluable patients (the response rate when including unconfirmed PRs was 9 out of 23 patients, 39%) [283].

1.2.9.1.2. Clinical trials combining (i) BRAF inhibitor with MEK inhibitor, and (ii) BRAF inhibitor and EGFR inhibitor with or without MEK inhibitor for *BRAF* mutant colorectal cancer

(i) BRAF inhibitor with MEK inhibitor for *BRAF* mutant colorectal cancer

Corcoran *et al.* reported results of the *BRAF*^{V600}-mutant metastatic CRC expansion cohort of a single-arm phase I/II study which examined the combination of **dabrafenib and trametinib** [284] (Table 1.4). The rationale for the testing of this combination was based on prior pre-clinical data from Corcoran *et al.* showing that vemurafenib-induced reactivation of p-ERK could be suppressed by the addition of the MEK inhibitor, AZD6244 (selumetinib) [296], and clinical evidence from the BRF113220 and COLUMBUS trials which demonstrated improved efficacy

with combined BRAF/MEK inhibition compared to BRAF inhibition alone in *BRAF* mutant melanoma [300-303].

Among 43 enrolled patients in this study, of whom 36/43 (84%) of patients had 2 or more lines of previous therapy, confirmed PRs were observed in 3 out of 43 patients (7%) (the response rate when including unconfirmed PRs was 5 out of 43 patients, 12%). The median PFS was 3.5 months (95% CI 3.4 to 4.0 months). The authors hypothesised that the modest response rate observed may have been due to suboptimal suppression of the MAPK pathway [284].

(ii) BRAF, EGFR, and MEK inhibitor combinations for *BRAF* mutant colorectal cancer

Subsequently, Corcoran *et al.* reported the results of a three-arm phase I/II study which examined combined BRAF/EGFR inhibition, combined MEK/ EGFR inhibition, and combined BRAF/EGFR/MEK inhibition in patients with metastatic *BRAF*^{V600E} CRC [285] (Table 1.4).

In Arm 1 of this study, which tested **dabrafenib and panitumumab**, 8 out of 20 (40%) patients had received 2 or more lines of previous therapy. The confirmed ORR was 2 out of 20 evaluable patients (10%), including one PR and one CR. The median PFS and OS in this arm were 3.5 months (95% CI 2.8 to 5.8 months) and 13.2 months (95% CI 6.7 to 22.0 months), respectively.

In Arm 2 of this study, which tested **dabrafenib with panitumumab and trametinib**, 43 out of 91 (47%) patients had 2 or more lines of previous therapy. The confirmed ORR increased to 19 out of 86 evaluable patients (22%), including 18 PRs and one CR. The median PFS and OS in this arm were 4.2 months (95% CI 4.0 to 5.6 months) and 9.1 months (95% CI 7.6 to 20.0 months), respectively [285].

Studies without results

The BEACON CRC study (NCT02928224) is a Phase III trial aiming to enrol 645 patients with *BRAF*^{V600E} metastatic CRC who have received 1 or 2 lines of prior therapy. This study is examining the combination of encorafenib, cetuximab, and the MEK inhibitor binimetinib (triplet arm), encorafenib and cetuximab (doublet arm), and irinotecan or FOLFIRI (investigator's choice) and cetuximab (control arm) (Table 1.5).

The ANCHOR-CRC study (NCT03693170) is a single-arm phase II trial testing the triplet combination of encorafenib with cetuximab and binimetinib in patients with previously untreated *BRAF*^{V600E} metastatic CRC, and aims to recruit 90 patients (Table 1.5).

1.2.9.1.3. Clinical trials combining BRAF inhibitor and EGFR inhibitor, with or without PI3K inhibitor for *BRAF* mutant colorectal cancer

Two clinical trials have examined the combination of BRAF inhibitor and EGFR inhibitor, with or without PI3K inhibitor for *BRAF*^{V600E} CRC. The rationale for the testing of these combinations

include previous pre-clinical data firstly suggesting that activation of the PI3K/AKT pathway is a mechanism of resistance to BRAF inhibitors in *BRAF*^{V600E} CRC [290] (described in section 1.2.8.1.1.) and secondly, supporting the efficacy of the triplet combination both *in vitro* and *in vivo* [304].

A phase Ib dose-escalation study which examined encorafenib and cetuximab, with or without the PI3K inhibitor alpelisib (BYL719) included 54 patients with *BRAF* mut CRC (96% *BRAF*^{V600E}) [286] (Table 1.4). In the arm testing ***encorafenib and cetuximab***, 19 out of 26 (73%) enrolled patients had 2 or more lines of previous therapy. The confirmed ORR was 5 out of 24 evaluable patients (21%), including four PRs and one CR. In the arm testing ***encorafenib and cetuximab with alpelisib***, 18 out of 28 (64%) enrolled patients had 2 or more lines of previous therapy. There were confirmed PRs in 5 out of 27 evaluable patients (19%), and no CRs observed [286].

A subsequent phase II study which examined the same drug combinations included 102 patients with *BRAF* mut CRC who had a median of 2 lines of previous therapy, and the interim results suggest slightly higher ORRs than in the phase Ib study [287] (Table 1.4). In the arm testing ***encorafenib and cetuximab***, the confirmed ORR was 11 out of 46 evaluable patients (24%), and the median PFS and OS in this arm were 5.4 months (95% CI 4.1 to 7.2 months) and 15.2 months, respectively. In the arm testing ***encorafenib and cetuximab with alpelisib***, the confirmed ORR was 14 out of 49 evaluable patients (29%), and the median PFS in this arm was 4.2 months (95% CI 3.4 to 5.4 months). Median OS was not reached [287].

1.2.9.1.4. Clinical trials combining irinotecan cytotoxic chemotherapy and EGFR inhibitor, with or without BRAF inhibitor for *BRAF* mutant colorectal cancer

Two clinical trials have also examined the combination of irinotecan cytotoxic chemotherapy and EGFR inhibitor, with or without BRAF inhibitor for *BRAF* mut CRC. The rationale for the testing of these combinations include previous pre-clinical data showing increased tumour efficacy with the triplet combination of cetuximab, vemurafenib and irinotecan compared to cetuximab and vemurafenib, cetuximab and irinotecan, or vemurafenib and irinotecan *in vivo* [250].

A single-arm phase Ib study which examined ***irinotecan with cetuximab and vemurafenib*** enrolled 19 patients with *BRAF*^{V600E} CRC who had a median of 2 lines of previous therapy [288] (Table 1.4). Confirmed PRs were observed in 6 out of 17 evaluable patients (35%, 95% CI 14 to 62%), and the median PFS was 7.7 months (95% CI 3.1 to not reached).

A subsequent phase II study examined irinotecan and cetuximab, with or without vemurafenib, and has enrolled a total of 99 patients with *BRAF*^{V600} mut CRC [289] (Table 1.4). Interim results suggest a lower ORR and shorter PFS than in the phase Ib study for the arm testing ***irinotecan***

with cetuximab and vemurafenib, which recruited 49 patients. The reported ORR was 16%, and the median PFS was 4.4 months (95% CI 3.6 to 5.7 months).

Studies without results

Another small single-arm phase II trial (NCT03727763) is recruiting patients (estimated $n = 30$) with $BRAF^{V600E}$ extended RAS wild-type advanced/recurrent CRC (with no restriction on the number of previous lines of therapy), and testing the combination of vemurafenib, cetuximab, and FOLFIRI (Table 1.5).

1.2.9.1.5. Clinical trial combining BRAF inhibitor, EGFR inhibitor, and Wnt signalling inhibitor for BRAF mutant colorectal cancer

Study without results

As described in section 1.2.8.2.2., there is pre-clinical data suggesting that parallel adaptive signalling through upregulation of the Wnt/ β -catenin pathway may be a mechanism of BRAF inhibitor resistance in $BRAF^{V600E}$ CRC [298]. Ring Finger Protein 43(*RNF43*) mutations and R-spondin (RSPO) fusions activate Wnt signalling through ligand-dependent activation of this pathway [305]. A single arm phase I/II trial (NCT02278133) is examining the combination of encorafenib with cetuximab and the Wnt signalling inhibitor WNT974 in patients with $BRAF^{V600E}$ KRAS wt metastatic CRC with *RNF43* mutations and/or RSPO fusions, aiming to recruit 20 patients.

1.2.9.2. Clinical trial combining BRAF inhibitor, MEK inhibitor, and PD-1 inhibitor for BRAF mutant colorectal cancer

Study without results

There is a strong association between *BRAF* mutations in CRC and sporadic microsatellite instability (discussed in section 1.2.5.3.2.). Moreover, there is evidence supporting increased immunogenicity and increased responsiveness of MSI CRC to immune checkpoint inhibitors [306, 307], as well as data suggesting synergy between MAPK inhibitors and immune checkpoint inhibitors in *BRAF* mut melanoma [308]. Accordingly, the combination of MAPK inhibitors with immunotherapy is also being tested in a current clinical trial in *BRAF* mut CRC.

A single arm Phase II study (NCT03668431) is testing the combination of dabrafenib with trametinib and the PD-1 inhibitor PDR001 in 25 patients with $BRAF^{V600E}$ metastatic CRC (with no restriction on the number of previous lines of therapy) (Table 1.5).

1.2.9.3. Clinical trial combining BRAF inhibitor, MEK inhibitor, and CDK4/CDK6 inhibitor for BRAF mutant colorectal cancer

Study without results

Recent pre-clinical data has suggested that combined inhibition of RAF and cyclin-dependent kinase (CDK) 4/6 leads to additive or synergistic effects in tumours with *KRAS*, *NRAS* or *BRAF* mutations, or cyclin D activation [309]. A phase II study (NCT01543698) is examining the combination of encorafenib with binimetinib and the CDK4/CDK6 inhibitor LEE011 (triplet arm) or encorafenib and binimetinib (doublet arm) in patients with *BRAF*^{V600} non-resectable advanced metastatic CRC or locally advanced/metastatic melanoma, with a planned 179 patients to be enrolled (Table 1.5).

Table 1.5. Clinical trials of BRAF inhibitors in metastatic colorectal cancer which do not have results

Drugs	ClinicalTrials.gov Identifier	Phase	Setting	Arms	Number of participants	Completion status
BRAFi + EGFRi ± MEKi						
Encorafenib + cetuximab ± binimetinib	NCT02928224 (BEACON CRC)	III	<i>BRAF</i> ^{V600E} mCRC, 1 or 2 prior lines of therapy	Triplet arm: Encorafenib + cetuximab + binimetinib	645 (estimated)	Active, recruiting
				Doublet arm: Encorafenib + cetuximab		
				Control arm: Irinotecan or FOLFIRI, + cetuximab (investigator's choice)		
Encorafenib + cetuximab + binimetinib	NCT03693170 (ANCHOR-CRC)	II	Previously untreated <i>BRAF</i> ^{V600E} mCRC	Single arm	90 (estimated)	Not yet recruiting
BRAFi + EGFRi + irinotecan-based chemotherapy						
Vemurafenib + cetuximab + FOLFIRI	NCT03727763	II	<i>BRAF</i> ^{V600E} extended <i>RAS</i> wild-type advanced/recurrent CRC, no restriction on previous lines of therapy	Single arm	30 (estimated)	Active, recruiting
BRAFi + EGFRi + Wnt signalling inhibitor						
Encorafenib + cetuximab + WNT974	NCT02278133	I/II	<i>BRAF</i> ^{V600E} <i>KRAS</i> wild-type mCRC with <i>RNF43</i> mutation and/or <i>RSPO</i> fusion, ≥ 1 prior line of therapy	Single arm	20 (actual)	Completed
BRAFi + MEKi + PD-1 inhibitor						
Dabrafenib + trametinib + PDR001	NCT03668431	II	<i>BRAF</i> ^{V600E} mCRC, no restriction on previous lines of therapy	Single arm	25 (estimated)	Active, recruiting
BRAFi + MEKi + CDK4/CDK6 inhibitor						
Encorafenib + binimetinib + LEE011	NCT01543698	II	<i>BRAF</i> ^{V600E} non-resectable advanced mCRC or locally advanced/metastatic melanoma (or any other indication upon agreement with the Sponsor, whose disease has progressed despite previous antineoplastic therapy or for whom no further effective standard therapy is available)	Triplet arm: Encorafenib + binimetinib + LEE011	179 (estimated)	Active, not recruiting
				Doublet arm: Encorafenib + binimetinib		

BRAFi: BRAF inhibitor; EGFRi: epidermal growth factor receptor inhibitor; MEKi: MEK inhibitor; CRC: colorectal cancer; FOLFIRI: 5-fluorouracil + folinic acid + irinotecan; mCRC: metastatic colorectal cancer; *RNF43*: Ring Finger Protein 43; *RSPO*: R-spondin; PD-1: programmed cell death-protein 1; CDK4: cyclin-dependent kinase 4; CDK6: cyclin-dependent kinase 6.

1.2.10. Summary

A review of the literature regarding responses and mechanisms of resistance of *BRAF* mutant CRC to BRAF inhibitors has revealed several issues.

Whilst the use of BRAF inhibitors has led to significant improvements in response and survival outcomes in advanced *BRAF* mutant malignant melanoma, clinical trials of these drugs in *BRAF* mutant mCRC have had less successful results.

Multiple pre-clinical studies have investigated potential mechanisms of resistance of *BRAF*^{V600E} CRC to BRAF inhibitors. Basal levels of parallel PI3K/AKT pathway activation have been found to be associated with resistance. Furthermore, rapid upregulation of p-EGFR and p-Akt, MAPK pathway activation associated with an increased ability of EGFR to effectively signal downstream, and parallel adaptive signalling through upregulation of the Wnt/ β -catenin pathway have been identified as potential mechanisms of resistance following BRAF inhibitor treatment.

Based on these findings, Phase I and II clinical trials examining relevant drug combinations have been performed. Treatment with the doublet combination of BRAF inhibitor and EGFR inhibitor led to only modest improvements in efficacy compared to single agent BRAF inhibitor. Triplet combinations of a BRAF inhibitor and an EGFR inhibitor, with either a MEK inhibitor, a PI3K inhibitor or chemotherapy, had slightly greater efficacy than the doublet BRAF/EGFR inhibitor combinations. However, the response rates with these combinations were still lower than those observed with single agent BRAF inhibitor treatment in melanoma. Results of a phase I/II trial which examined the triplet combination of BRAF inhibitor and EGFR inhibitor with a Wnt signalling inhibitor are not yet available.

In Chapter 3 of this thesis we report the results of our pre-clinical studies which comprehensively explored mechanisms of resistance of *BRAF* mutant CRC to BRAF inhibitors. In Chapter 4 we describe the results of further pre-clinical studies which investigated ways to overcome resistance of *BRAF*^{V600E} CRC to BRAF inhibitors.

1.3. Bevacizumab for the treatment of metastatic colorectal cancer

1.3.1. Angiogenesis and anti-angiogenics for cancer treatment

1.3.1.1. Role of angiogenesis in the pathogenesis of cancer

Tumours need to establish a vascular supply in order to receive adequate oxygen, metabolites and nutrients [310]. This is achieved through ‘angiogenesis’, the process of vessel sprouting or growth from pre-existing vessels [310].

The first observations that the growth of human tumours was associated with increased vascularity were reported over a century ago [311]. Then, in 1939, Ide *et al.* were the first to hypothesise that tumours may release factors which stimulate blood vessel growth [312]. In 1945, Algire *et al.* further built on this concept and proposed that the acquisition by tumour cells of the ability to induce growth of a rich vascular supply is a significant step in tumourigenesis [313]. Eighteen years later, in 1963, Folkman *et al.* demonstrated evidence that lack of neovascularization is associated with severe restriction of tumour growth [314]. In 1968, Greenblatt and Shubik [315] and Ehrmann and Knoth [316] provided early evidence that diffusible factors released by tumour cells mediate neovascularisation.

Subsequently, in a seminal ‘New England Journal of Medicine’ publication in 1971, Folkman hypothesised that inhibition of angiogenesis, or ‘anti-angiogenesis’, could represent a new therapeutic approach for cancer [317]. This spurred widespread efforts focused on the isolation of angiogenesis factors [311] and the discovery of angiogenesis inhibitors [318].

It was not until the discovery and characterization of vascular endothelial growth factor A (VEGF-A, or VEGF) in the 1980’s, and the subsequent development in 1997 of Bevacizumab (Avastin, Roche), a humanized anti-VEGF-A monoclonal antibody which blocks binding of VEGF-A to its key receptors, that proof-of-principle of a clinically relevant anti-cancer therapy targeting a known tumour angiogenic factor was demonstrated.

1.3.1.2. The vascular endothelial growth factor (VEGF) family

1.3.1.2.1. Vascular endothelial growth factors (VEGFs)

Members of the vascular endothelial growth factor (VEGF) family, which include VEGFs A–D and placental growth factor (PlGF), are key mediators of angiogenesis and lymphangiogenesis [319]. Lymphangiogenesis, the generation of new lymphatic vessels [320–322], is now recognised as a process which actively promotes metastatic tumour spread [323], and is mediated by lymphangiogenic growth factors that are produced and secreted by tumours, stromal cells, tumour-infiltrating macrophages, or activated platelets [324–327].

VEGFs A-D are structurally related dimeric polypeptides which have distinct patterns of expression, receptor specificity and functions [328]. The VEGF family signals predominantly through the receptor tyrosine kinases (RTKs) VEGF receptor (VEGFR)-1 (also known as Fms-like tyrosine kinase 1 or FLT1 [329, 330]), VEGFR-2 (also known as foetal liver kinase-1 or FLK1, and kinase insert domain receptor or KDR [331-333]), and VEGFR-3 (also known as Fms-like tyrosine kinase 4 or FLT4), which are expressed on endothelial and tumour cells [334]. They also interact with the co-receptors neuropilin (NRP)1 and NRP2, as well as other receptors including integrins [334].

Canonical activation of VEGFRs involves binding to VEGF ligands, which induces homodimerisation or heterodimerisation of VEGFRs, and activation and *trans*-phosphorylation of tyrosine residues on the intracellular domain of the receptor [335]. This results in the activation of various intracellular signalling pathways which mediate immediate responses including vascular permeability, and delayed responses that require gene regulation including endothelial cell survival, migration and proliferation [335]. Non-canonical VEGFR activation is initiated by non-VEGF-dependent activation of VEGFRs, such as through shear stress, and binding by the bone morphogenetic protein (BMP) antagonist Gremlin (GREM1), β -galactoside-binding proteins (galactins), lactate and low density lipoproteins (LDLs) [336-340].

1.3.1.2.2. VEGF-A

1.3.1.2.2.1. Identification of VEGF

The earliest identification of a protein in the VEGF family was reported in 1983, when Senger *et al.* discovered a factor in the ascitic fluid of guinea pigs, hamsters and mice which increased vascular permeability, which they named “vascular permeability factor” (VPF) [341]. Six years later, Ferrara *et al.* isolated and sequenced an endothelial-cell mitogen that could induce angiogenesis, which they named “vascular endothelial growth factor” (VEGF, now termed ‘VEGF-A’) [342], and this was found to be identical to VPF [342-344].

1.3.1.2.2.2. Properties of VEGF-A

VEGF-A, the target of Bevacizumab, is the most well-characterised pro-angiogenic ligand in the VEGF family [319]. VEGF-A signalling is an essential requirement for normal vasculogenesis and angiogenesis, as demonstrated by the embryonic lethality of mice lacking a single *VEGF-A* allele [345, 346]. In tumours, VEGF-A is predominantly secreted by tumour cells, but can also be secreted by tumour-associated stroma [347, 348]. The primary targets of the potent mitogenic activity of VEGF-A are the micro- and macrovascular endothelial cells in arteries, veins, and lymphatics [342, 344, 349-353].

VEGF-A is expressed by multiple tumours, including lung [354], breast [355], colorectal [356-358], pancreatic [359], renal [360], and ovarian cancers [361], and has been reported to be more highly expressed in hypoxic tumour cells adjacent to areas of necrosis [362, 363]. Hypoxia, which is a major driver of tumour angiogenesis [364], induces VEGF-A expression through mediators including hypoxia-inducible factor-1 α (HIF-1 α) [365, 366].

The gene encoding human VEGF-A has eight exons, separated by seven introns [367, 368], and multiple isoforms of VEGF-A are generated from alternative exon splicing or proteolytic processing [369]. The principal VEGF-A isoforms produced from exon splicing are VEGF₁₂₁, VEGF₁₆₅ (the predominant, most physiologically relevant isoform [370]), VEGF₁₈₉ and VEGF₂₀₆ [371]; the proteolytic cleavage of VEGF₁₆₅ results in VEGF₁₁₀ or VEGF₁₁₃, which are biologically active [369]. Less frequent splice variants include VEGF₁₄₅, VEGF₁₈₃, VEGF₁₆₂ and VEGF_{165b} [372].

These different isoforms have varying bioavailability and affinity for heparan sulphate proteoglycans (HSPGs) in the extracellular matrix (ECM) [373, 374]. Whilst VEGF₁₂₁ lacks heparin-binding properties and is freely diffusible [373], VEGF₁₆₅ has intermediate heparin affinity and bioavailability [374]. VEGF₁₈₉ and VEGF₂₀₆ have greater heparin affinity than VEGF₁₆₅, and are almost completely sequestered by HSPGs in the ECM [373, 374].

Importantly, the heparin-binding VEGF-A isoforms have a crucial role in the initiation of vascular branching during the development of the vascular system [375, 376]. Indeed, mice expressing only VEGF₁₂₀ (the mouse homologue of human VEGF₁₂₁) die perinatally with evidence of ischaemic cardiomyopathy and multiorgan failure [377]. Furthermore, VEGF-A gradients which are mediated by the balance between diffusible and HSPG-bound VEGF-A have a major role in vasculogenesis [319]. A VEGF-A gradient, driven by the heparin-binding isoforms, induces the formation of motile endothelial cells (ECs) termed ‘tip cells’, which lead new vessel sprout formation [372]. A VEGF-A gradient also induces neighbouring stalk EC proliferation at an opening in the basement membrane [375, 376]. These stalk ECs establish the vessel lumen, and support sprout elongation [310].

This process is additionally regulated by other signalling molecules, including matrix metalloproteases (MMPs), angiopoietin 2 (ANG2), and the Notch pathway. The onset of sprouting requires proteolytic degradation of the basement membrane which is mediated by MMPs [310], and mural cell detachment which is stimulated by ANG2 [378, 379]. VEGF-A and Notch also co-operate in a feedback loop to establish a “branching pattern” of vascularisation [310]. VEGF-A/VEGFR2 upregulates the Notch ligand delta-like ligand 4 (DLL-4) in tip cells,

and activates Notch signalling. This suppresses the tip phenotype in adjacent stalk ECs by downregulating VEGFR2, VEGFR3 and NRP1, and upregulating VEGFR1, thereby suppressing excess angiogenesis and promoting orderly vessel formation [380-383]. Jagged-1 (JAG1), an opposing Notch ligand which is expressed at high levels in stalk cells, further regulates angiogenesis by inhibiting DLL-4-Notch interactions and promoting the emergence of new tip cells and proliferation [384].

The effects of VEGF-A on EC differentiation, proliferation, migration and vasculogenesis are primarily mediated by the RTK VEGFR2 [385], which induces intracellular signalling pathways including the phospholipase C γ (PLC γ)–ERK1/2 pathway [386, 387], the PI3K–AKT–mTOR pathway [388], and the SRC pathway [335] (Figure 1.10). The essential role of VEGFR2 in vasculogenesis is also demonstrated by the embryonic lethality of *VEGFR2* null mice between E8.5 and E9.5 days due to failure to develop yolk-sac blood-islands [389]. VEGFR2 also mediates VEGF-A induced vascular permeability [390]. Whilst VEGFR1 binds to VEGF-A with higher affinity than VEGFR2 [391], VEGF-A induces weaker tyrosine phosphorylation of VEGFR1 than VEGFR2 [328]. Moreover, expression of a soluble, alternatively spliced variant of VEGFR1 (s-VEGFR1) has been shown to act as a VEGF trap [386], leading to the hypothesis that VEGFR1 acts primarily as a decoy receptor for VEGF-A, modulating vascular development by regulating the levels of VEGF-A available to bind and activate VEGFR2 [392]. Consistent with this model, it has been observed that *VEGFR1* null mouse embryos die at E8.9 to E9.0 from vascular overgrowth and disorganisation [393] (Figure 1.10).

The VEGF co-receptors NRP1 and NRP2 are transmembrane proteins with a small cytoplasmic domain which lacks catalytic function [394]. NRP1 and NRP2 bind to VEGFs, as well as the class 3 semaphorins (axonal guidance molecules which also bind to plexin receptors) [395, 396]. VEGF-A binds to NRP1 with a 50-fold stronger affinity than NRP2 [397]. This binding occurs simultaneously with VEGFRs, leading to the formation of VEGF-A/VEGFR/NRP1 complexes on ECs [397-399]. Whilst NRP1 is predominantly expressed in arteries and acts primarily as a co-receptor for VEGFR1/2, NRP2 is preferentially expressed in veins and lymphatics and acts a co-receptor for VEGFR3 [400-403] (Figure 1.10).

NRP1 has a crucial role in VEGFR2 intracellular trafficking, which regulates arteriogenic ERK activation [404, 405]. It also regulates VEGF-A induced activation of p38 MAPK and organization of ECs into branched, pericyte-associated vessels [406]. Conversely, NRP1 can also modulate angiogenesis by binding to VEGF-A/VEGFR2 complexes *in trans* (i.e., on adjacent ECs), arresting VEGFR2 endocytosis, and suppressing angiogenic signalling [407]. Whilst NRP1 null mice die *in utero* due to cardiovascular and central nervous system defects [408, 409], it has been observed that mutations in NRP1 in the sequence that encode the VEGF-A binding site or

which lead to loss of the cytoplasmic domain, are associated with normal vasculogenesis and angiogenesis [410, 411]. This may be explained by observations that NRP1 can function with the TGF- β receptor to regulate vascular sprouting independent of VEGFR2 [412, 413]. Whilst NRP2 has been shown to enhance VEGF-C signalling through both VEGFR2 and VEGFR3 *in vitro* [414], *nrp2*^{+/-}*-vegfr2*^{+/-} trans-heterozygous mutant mice have normal lymphatic development. In contrast, *nrp2*^{+/-}*-vegfr3*^{+/-} trans-heterozygous mutant mice have significant defects in lymphatic sprouting, indicating that NRP2 mediates VEGF-C induced lymphangiogenesis *in vivo* in a VEGFR3-dependent manner [415] (Figure 1.10).

Integrins, in particular integrin- β 1 and integrin- β 3, also interact with VEGFR2 and play an important role in modulating VEGFR2 activation and signalling. VEGF-A regulates the association between VEGFR2 and integrin- β 3, which is required for full activation of VEGFR2 [416], and VEGFR2 activation in turn induces the tyrosine phosphorylation of integrin- β 3, which further enhances the VEGFR2-integrin- β 3 interaction [417]. Cross-talk between VEGFR2 and integrin- β 3 is facilitated by SRC kinase [418] and the small GTPase RAP1B [419]. Furthermore, ECM-bound VEGF-A isoforms which are exposed to ECs form VEGFR2-integrin- β 1 complexes, which leads to the re-distribution of integrin- β 1 to focal adhesions and prolonged activation of VEGFR2 [420].

1.3.1.2.3. Other VEGF ligands and receptors

VEGF-B (also known as VEGF-related factor or VRF), one of the least well characterised VEGF family members [421], is another ligand for VEGFR1 and NRP1 [422] which is expressed in a wide variety of tissues including ECs, mural cells, brown fat, skin, lung, placenta, brain and retina [423, 424]. Binding of VEGF-B does not lead to optimal activation of the VEGFR1 receptor (compared to PlGF, see below), and therefore it is a weak activator of VEGFR1 signalling [335] (Figure 1.10).

In contrast to VEGF-A, VEGF-B deficient mice are healthy and do not have vascular defects [425-427], indicating that in contrast to VEGF-A, VEGF-B is redundant for vasculogenesis [335]. Instead, VEGF-B has predominantly been implicated in cardiovascular disease, where it has a role in fatty uptake in cardiac ECs and revascularisation following myocardial infarction [425-428].

VEGF-B is expressed in a wide range of tumours including CRC [429, 430], hepatocellular carcinoma [431], breast cancer [432, 433], lung cancer [434, 435], and renal cell carcinoma [436]. Whilst there is some evidence that VEGF-B can induce cell motility and invasion in CRC [437], it has also been reported that overexpression of VEGF-B in a mouse model of pancreatic

neuroendocrine carcinoma suppressed tumour growth [438], highlighting the need for further research into the definitive role of VEGF-B in tumour biology.

Placental growth factor (PlGF) is a ligand for VEGFR1, NRP1 and NRP2 [439, 440] (Figure 1.10). Similar to VEGF-B, PlGF-deficient mice are healthy, suggesting that PlGF is dispensable for normal vascular development [441]. PlGF may enhance VEGF-A signalling to stimulate angiogenesis through multiple mechanisms, including the displacement of VEGF-A from VEGFR1 to allow increased activation of VEGFR2 [442], by inducing upregulation of VEGF-A [443], and by inducing crosstalk between VEGFR1 and VEGFR2 which in turn increases VEGFR2 signalling [444]. There is evidence that PlGF can also signal directly through VEGFR1 in a VEGF-A independent manner, regulating expression of a distinct subset of pro-angiogenic genes [444, 445]. However, studies investigating the role of PlGF in driving tumour angiogenesis and growth using multiple tumour models have shown conflicting findings [446, 447]. It has therefore been proposed that the role of PlGF in cancer may be dependent on the expression of other VEGF family members or receptors on endothelial, tumour or immune cells [448]. Furthermore, the efficacy of anti-PlGF treatment may be limited to VEGFR1 expressing tumours, as it has been shown to require inhibition of PlGF/VEGFR1 signalling in tumour cells [449].

VEGF-C and -D are highly related polypeptides which undergo proteolytic processing to generate mature forms of the growth factors which have a high affinity for both VEGFR2 and VEGFR3 [450-453] (Figure 1.10).

VEGF-C null mouse embryos develop an oedematous phenotype and die between E15.5 to 17.5 [454], indicating that VEGF-C is critical for early lymphatic development. Furthermore, the binding of VEGF-C to VEGFR2 and VEGFR3 promotes VEGFR2/VEGFR3 heterodimerisation [455, 456], activating Akt signalling which is required for both embryonic and adult lymphangiogenesis [457]. This process requires involves formation of complexes between the VEGFR2/VEGFR3 heterodimer and the co-receptor NRP1 [458]. VEGF-C is expressed in a wide range of tumours, including thyroid [459-461], gastric [462-467], lung [435, 468-470], colorectal [471-474], oesophageal [475, 476] and breast cancers [477, 478]. Multiple studies in mouse models have demonstrated that VEGF-C promotes tumour angiogenesis and lymphangiogenesis *in vivo*, and also promotes tumour growth and metastasis [320, 479, 480].

VEGF-D is believed to have a more subtle role in regulating lymphangiogenesis than VEGF-C [481]. Experiments inactivating the *VEGF-D* gene have shown that whilst it is not critical for the development of the lymphatic system [482-484], it has roles in the modulation of dermal and pulmonary lymphatics [482, 485]. VEGF-D has also been shown to regulate blood vascular development through modulation of the activity of the SOX18 transcription factor [486]. It is

expressed in a range of tumour types including ovarian [487], colorectal [488], and gastric cancers [489]. VEGF-D has been shown to promote angiogenesis and lymphangiogenesis in solid tumours, and to promote tumour growth and metastases in a range of mouse models [483, 490-494].

VEGFR3 is primarily expressed on lymphatic ECs [495] where its activation is sufficient to induce lymphangiogenesis [496], however, it is also expressed on capillary and venous ECs, in neuronal progenitors, macrophages and osteoblasts [497, 498]. VEGFR3 is essential for the development of both the blood and lymphatic vasculature [455]. Targeted inactivation of both alleles of the *VEGFR3* gene in mouse embryos leads to death at E10-11 due to defective cardiovascular remodelling [499], and point mutations in VEGFR3 result in an inactive tyrosine kinase leading to hereditary lymphedema [500-502]. VEGFR3 contributes to angiogenic signalling by promoting conversion of the phenotype of ECs at fusion points of blood vessel sprouts from a tip cell to a stalk cell [503]. The proteolytically processed mature forms of VEGF-C and -D can also activate VEGFR2 to promote angiogenesis [450, 451, 490, 504-508] (Figure 1.10).

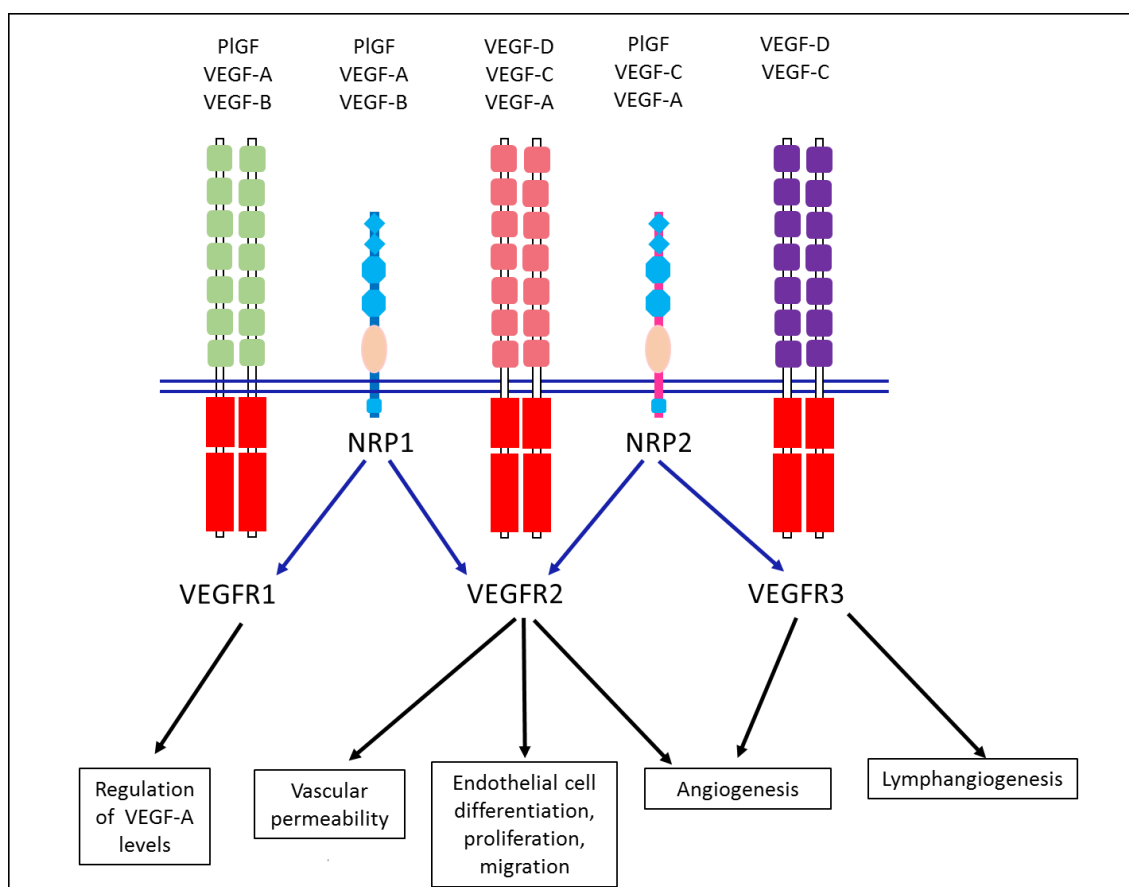


Figure 1.10. Schematic of the interaction between VEGF family members with VEGF receptors and NRP co-receptors

(Modified from Stacker *et al* [481]). PIGF: placental growth factor; VEGF-A: vascular endothelial growth factor-A; VEGF-B: vascular endothelial growth factor-B; VEGF-D: vascular endothelial growth factor-D; VEGF-C: vascular endothelial growth factor-C; VEGFR1: vascular endothelial growth factor receptor 1; NRP1: neuropilin 1; VEGFR2: vascular endothelial growth factor receptor 2; NRP2: neuropilin 2; VEGFR3: vascular endothelial growth factor receptor 3.

1.3.1.3. Development of bevacizumab

In 1993, A.4.6.1., a murine anti-human VEGF-A monoclonal antibody (muMAb) which neutralises the bioactive forms of VEGF-A, was shown to inhibit the growth of human xenograft tumours *in vivo* [509], and its efficacy in other tumour models was confirmed in further studies over the subsequent few years [510, 511]. In 1997, the A.4.6.1. muMAb was subsequently humanised by transferring six of its complementarity-determining regions to a consensus human framework [512], which resulted in the recombinant humanised anti-VEGF monoclonal antibody (rhuMAb) Bevacizumab (Avastin). Bevacizumab was shown to inhibit VEGF-A induced proliferation of ECs *in vitro* and tumour growth *in vivo* with similar potency and efficacy as the original muMAb A.4.6.1 [512], and subsequently entered clinical trials for the treatment of cancer [371].

Since then, multiple other anti-angiogenic agents have been approved for cancer treatment [513]. These include small-molecule tyrosine kinase inhibitors targeting VEGFRs (e.g., axitinib, cabozantinib, lenvatinib, pazopanib, regorafenib, sorafenib, sunitinib, vandetanib); a monoclonal antibody targeting the extracellular domain of VEGF-R2 (ramucirumab) [514, 515]; and a 'VEGF-trap' fusion protein with VEGF-binding portions from the extracellular domains of human VEGFR-1 and -2 fused to the Fc portion of human immunoglobulin G1, which neutralise VEGF-A, VEGF-B and PlGF (ziv-aflibercept) [516]. However, the focus of this thesis is on the anti-angiogenic agent bevacizumab, and specifically, biomarkers of response and resistance to this drug in patients with mCRC.

Bevacizumab requires the addition of cytotoxic chemotherapy to exert a beneficial survival effect in metastatic colorectal carcinoma [517-519]. Whilst the mechanism by which bevacizumab potentiates the efficacy of cytotoxic chemotherapy is not completely understood [518, 520], there are multiple hypotheses. One is that 'vascular normalisation' increases blood perfusion, thereby alleviating tumour hypoxia, increasing the delivery or improving the distribution of the concurrent treatments, and increasing the accessibility of anti-tumour immune cells to the tumour [520, 521]. Anti-angiogenics may also act as chemo-sensitisers by inhibiting chemotherapy-induced mobilisation and 'tumour-homing' of pro-angiogenic bone-marrow derived circulating endothelial progenitor cells [522], or by enhancing chemotherapy-induced damage to endothelial cells [523, 524].

1.3.1.4. Clinical trials of bevacizumab in metastatic colorectal cancer

Multiple randomised clinical trials comparing the efficacy of bevacizumab combined with chemotherapy to chemotherapy alone for the treatment of metastatic CRC have been performed. The majority of these studies have been in the first-line setting ($n = 9$), and have examined the combination of bevacizumab with irinotecan-based chemotherapy (AVF2107g [519, 525], Stathopoulos *et al.* [526], ARTIST [527]), with oxaliplatin-based chemotherapy (NO16966 [528]), with either irinotecan- or oxaliplatin- based chemotherapy (ITACA [529]), with 5-fluorouracil (AVF0780 [530], AVF2192 [531]), or with capecitabine (MAX [532], AVEX [533]) (Table 1.6). Two studies were in the second-line setting, and examined the combination of bevacizumab with FOLFOX4 (E3200 [534]), and irinotecan- or oxaliplatin-based chemotherapy (ML18147 [535]). Most studies included participants aged 18 years or over, however two studies (AVF2192 and AVEX) in the first-line setting specifically examined a more elderly population, restricting eligibility to participants aged 65 years and over (AVF2192), or 70 years and over (AVEX), respectively (Table 1.6). The efficacy results for these trials are shown in Table 1.6.

A recent systematic review and meta-analysis pooled the results of all nine first-line trials, and found that compared to chemotherapy alone, bevacizumab with chemotherapy led to a modest improvement in objective response rates (ORR) (risk ratio (RR) 0.89; 95% CI 0.82 to 0.96, $P =$

0.003), a longer PFS (HR 0.69; 95% CI 0.63 to 0.75, $P < 0.00001$), and a modest improvement in OS (HR 0.87; 95% CI 0.80 to 0.95, $P = 0.002$) [536]. There was substantial heterogeneity between the included studies for the ORR and PFS outcomes ($\text{Chi}^2 = 23.57$, $P = 0.003$; $I^2 = 66\%$, and $\text{Chi}^2 = 27.57$, $P = 0.0003$; $I^2 = 75\%$, respectively) and moderate heterogeneity between the included studies for the OS outcome ($\text{Chi}^2 = 15.08$, $P = 0.03$; $I^2 = 54\%$). There were higher rates of Grade ≥ 3 hypertension (RR 3.56, 95% CI 2.58 to 4.92, $P < 0.00001$), proteinuria (RR 1.89; 95% CI 1.26 to 2.84, $P = 0.002$), gastrointestinal perforation (RR 3.63; 95% CI 1.31 to 10.09, $P = 0.01$), thromboembolic events (RR 1.44; 95% CI 1.20 to 1.73, $P = 0.0001$), and bleeding (RR 1.81; 95% CI 1.22 to 2.67, $P = 0.003$) in participants treated with bevacizumab and chemotherapy, compared to chemotherapy alone [536].

In the second-line setting, the Phase 3 Eastern Cooperative Oncology Group (ECOG) E3200 study, randomised patients to oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) without bevacizumab, FOLFOX4 with bevacizumab, or bevacizumab alone [534]. The addition of bevacizumab to FOLFOX4 chemotherapy was associated with improvements in ORR (22.7% versus 8.6%, $P < 0.0001$), PFS (median PFS 7.3 *versus* 4.7 months; HR 0.61, $P < 0.0001$), and the primary end-point, OS (12.9 *versus* 10.8 months; HR 0.75, $P = 0.0011$) compared to chemotherapy alone [534]. The ML18147 study assessed continuation of bevacizumab with standard second-line chemotherapy versus chemotherapy alone, after first progression in patients with mCRC [535]. The combination chemotherapy regimen given was determined by the regimen which had been given in the first-line setting - i.e., participants who were given first-line oxaliplatin-based treatment were switched to second-line irinotecan-based treatment, and vice versa. Continuation of bevacizumab in addition to combination chemotherapy was not associated with an improvement in ORR (5 versus 4%, $P = 0.31$), but was associated with a longer PFS (median PFS 5.7 versus 4.1 months; HR 0.68, 95% CI 0.59 to 0.78, $P < 0.0001$) and a modest improvement in the primary end-point, OS (median OS 11.2 versus 9.8 months; HR 0.81, 95% CI 0.69 to 0.94, $P = 0.0062$) [535].

Bevacizumab has been approved for the first- or second-line treatment of mCRC in combination with chemotherapy, by the Australian Pharmaceutical Benefits Scheme (PBS) and the United States (US) Food and Drug Administration (FDA). It has also been approved by the FDA as a continuing treatment in combination with second-line chemotherapy in patients who have progressed on a first-line bevacizumab-containing regimen.

Table 1.6. Efficacy data from randomised clinical trials of bevacizumab and chemotherapy versus chemotherapy alone in patients with metastatic colorectal cancer

Study	ClinicalTrials.gov Identifier; alternative names	Phase	Primary endpoint/s	Patient characteristics	Treatment arms	Number of randomised patients	ORR (% , P-value)	PFS		OS		References
								Median (months)	HR (P-value or CI)	Median (months, P-value)	HR (95% CI), P-value	
FIRST-LINE												
Irinotecan-based chemotherapy												
Hurwitz <i>et al.</i>	AVF2107g; NCT00109070	III	OS	ECOG PS 0-1, ≥ 18 years old	IFL + placebo	411	34.8	6.2		15.6		[519, 525]
					IFL + BEV	402	44.8	10.6	0.54, <i>P</i> < 0.001	20.3	0.66, <i>P</i> < 0.001	
Stathopoulos <i>et al.</i>		III	ORR, OS	WHO PS 0-1, ≥ 18 years old	Arm A: IFL	114	36.8			22 (95% CI 18.1 to 25.9 months)		[526]
					Arm B: IFL + BEV	108	35.2	Not reported	Not reported	25 (95% CI 18.1 to 31.9 months), <i>P</i> = 0.14	Not reported	
Guan <i>et al.</i>	ARTIST; BO20696; NCT00642577	III	PFS rate at 6 months, PFS	ECOG PS 0-1, ≥ 18 years old	mIFL	72	17	4.2 (95% CI 3.7 to 4.9 months)		13.4 (95% CI 9.7 to 17.2 months)		[527]
					mIFL + BEV	142	35, <i>P</i> = 0.013	8.3 (95% CI 7.4 to 8.9 months)	0.44 (95% CI 0.31 to 0.63), <i>P</i> < 0.001	18.7 (95% CI 15.8 to 19.6 months), <i>P</i> = 0.014	0.62 (95% CI 0.41 to 0.95), <i>P</i> = 0.014	
Oxaliplatin-based chemotherapy												
Saltz <i>et al.</i>	NO16966; NCT00069095	III	PFS	ECOG PS 0-1, ≥ 18 years old	FOLFOX-4/ XELOX + placebo	701	49	8		19.9		[528]
					FOLFOX-4 /XELOX + BEV	699	47, <i>P</i> = 0.31	9.4	0.83 (97.5% CI 0.71 to 0.95), <i>P</i> = 0.0023	21.3	0.89 (97.5% CI 0.76 to 1.03), <i>P</i> = 0.077	
Irinotecan- or oxaliplatin-based chemotherapy												
Passardi <i>et al.</i>	ITACA; NCT01878422	III	PFS	ECOG PS 0-2 (0-1 if ≥ 70 years old), ≥ 18 years old	Arm B: FOLFIRI/FOLFOX 4 (investigator's discretion)	194	50.5	8.4 (95% CI 7.2 to 9.0 months)		21.3 (95% CI 19.9 to 24.1 months)		[529]
					Arm A: FOLFIRI/FOLFOX 4 (investigator's discretion) + BEV	176	50.6, <i>P</i> = 0.87	9.6 (95% CI 8.2 to 10.3 months)	0.86 (95% CI 0.70 to 1.07), <i>P</i> = 0.18 ^a	20.8 (95% CI 15.9 to 23.2 months)	1.13 (95% CI 0.89 to 1.43), <i>P</i> = 0.317 ^a	

Table 1.6. (continued) Efficacy data from randomised clinical trials of bevacizumab and chemotherapy versus chemotherapy alone in patients with metastatic colorectal cancer

Study	ClinicalTrials.gov Identifier; alternative names	Phase	Primary endpoint/s	Patient characteristics	Treatment arms	Number of randomised patients	ORR (% , <i>P</i> -value)	PFS		OS		References
								Median (months)	HR (P-value or CI)	Median (months, <i>P</i> -value)	HR (95% CI), <i>P</i> -value	
Chemotherapy with 5-fluorouracil alone												
Kabbinavar <i>et al.</i>	AVF0780	II	TTP, ORR	ECOG PS 0-1, ≥ 18 years old	FU/LV	36	17 (reference)	5.2 ^b	reference	13.8		[530]
					FU/LV + BEV 5 mg/kg q2w	35	40, <i>P</i> = 0.029	9 ^b	0.46, <i>P</i> = 0.005 ^b	21.5		
					FU/LV + BEV 10 mg/kg q2w	33	24, <i>P</i> = 0.434	7.2 ^b	0.66, <i>P</i> = 0.22 ^b	16.1		
Kabbinavar <i>et al.</i>	AVF2192; NCT00109226	II	OS	ECOG PS 1-2, ≥ 65 years old	FU/LV + placebo	105	15.2	5.5		12.9		[531]
					FU/LV + BEV	104	26, <i>P</i> = 0.055	9.2	0.50 (95% CI 0.34 to 0.73), <i>P</i> = 0.0002	16.6	0.79 (95% CI 0.56 to 1.10), <i>P</i> = 0.16	
Chemotherapy with capecitabine alone												
Tebbutt <i>et al.</i>	MAX; NCT00294359	III	PFS	ECOG PS 0-2, ≥ 18 years old	Capecitabine	156	30.3 (reference)	5.7	reference	18.9	reference	[532]
					Capecitabine + BEV	157	38.1, <i>P</i> = 0.16	8.5	0.63 (95% CI 0.50 to 0.79), <i>P</i> < 0.001	18.9	0.88 (95%CI 0.68 to 1.13), <i>P</i> = 0.31 ^c	
					Capecitabine + BEV + Mitomycin-C	158	45.9, <i>P</i> = 0.006	8.4	0.59 (95% CI 0.47 to 0.75), <i>P</i> < 0.001	16.4	0.94 (95% CI 0.73 to 1.21), <i>P</i> = 0.64 ^c	
Cunningham <i>et al.</i>	AVEX; MO19286; NCT00484939	III	PFS	ECOG PS 0-2, ≥ 70 years old	Capecitabine	140	10	5.1 months (95% CI 4.2 to 6.3 months)		16.8 (95% CI 12.6 to 20.1 months)		[533]
					Capecitabine + BEV	140	19, <i>P</i> = 0.04	9.1 (95% CI 7.3 to 11.4 months)	0.53 (95% CI 0.41 to 0.69), <i>P</i> < 0.0001	20.7 (95% CI 17.0 to 26.0 months)	0.79 (95% CI 0.57 to 1.09), <i>P</i> = 0.18	
SECOND-LINE												
Oxaliplatin-based chemotherapy												
Giantonio <i>et al.</i>	E3200; NCT00025337	III	OS	ECOG PS 0-2, ≥ 18 years old	FOLFOX4	291	8.6 (reference)	4.7	reference	10.8	reference	[534]
					FOLFOX4 + BEV	286	22.7, <i>P</i> < 0.0001	7.3	0.61 , <i>P</i> < 0.0001	12.9	0.75, <i>P</i> = 0.0011	
					BEV alone	243 ^d	3.3	2.7		10.2		

Table 1.6. (continued) Efficacy data from randomised clinical trials of bevacizumab and chemotherapy versus chemotherapy alone in patients with metastatic colorectal cancer

Study	ClinicalTrials.gov Identifier; alternative names	Phase	Primary endpoint/s	Patient characteristics	Treatment arms	Number of randomised patients	ORR (% , P-value)	PFS		OS		References
								Median (months)	HR (P-value or CI)	Median (months, P-value)	HR (95% CI), P-value	
Irinotecan- or oxaliplatin-based chemotherapy (switch of chemotherapy from first-line, bevacizumab continued beyond first progression)												
Bennouna <i>et al.</i>	ML18147 (previously AIO KRK 0504); NCT00700102	III	OS	ECOG PS 0-2, ≥ 18 years old	Irinotecan-/Oxaliplatin- based chemotherapy ^e	411	4	4.1 (95% CI 3.7 to 4.4 months)		9.8 (95% CI 8.9 to 10.7 months)		[535]
					Irinotecan-/Oxaliplatin- based chemotherapy ^e + BEV	409	5, P = 0.31	5.7 (9.5% CI 5.2 to 6.2 months)	0.68 (95% CI 0.59 to 0.78), P < 0.0001	11.2 (95% CI 10.4 to 12.2 months)	0.81 (95% CI 0.69 to 0.94 months), P = 0.0062	

ORR: Objective response rate; PFS: progression-free survival; OS: overall survival; HR: Hazard ratio; CI: confidence interval; ECOG PS: Eastern Cooperative Oncology Group performance status; IFL: Irinotecan + fluorouracil + leucovorin; BEV: bevacizumab; WHO: World Health Organisation; mIFL: modified IFL; FOLFOX: 5-fluorouracil + folinic acid + oxaliplatin; XELOX: capecitabine + oxaliplatin; FOLFIRI: 5-fluorouracil + folinic acid + irinotecan; TTP: time to progression; FU: fluorouracil; LV: leucovorin; ^a: adjusted for centre, chemotherapy regimen and KRAS status; ^b: for TTP; ^c: multivariate analysis, adjusted for ECOG PS, neutrophils, alkaline phosphatase (ALP), prior radiotherapy, and prior tumour resected; ^d: bevacizumab alone arm closed to accrual in 2003, after interim analysis showed inferior survival; ^e: with infusional or bolus fluorouracil, or oral capecitabine at investigator's discretion

1.3.2. Mechanisms of resistance to Bevacizumab in colorectal cancer

Emerging evidence suggests that there are multiple mechanisms of resistance to anti-angiogenic agents, and that these may be drug- and tumour-dependent [537].

1.3.2.1. Induction of compensatory signalling pathways and pro-angiogenic factors

In addition to VEGF and its receptors (discussed in section 1.3.1.2.1.), multiple other pro-angiogenic signalling pathways have been identified as having an important role in the regulation of tumour angiogenesis, and induction of tumour growth [538]. These include the platelet-derived growth factor (PDGF) family [539-544], fibroblast growth factor (FGF) family [545-548], angiopoietin (ANG)/TIE2 receptor family [379, 549], the hepatocyte growth factor (HGF)/MET signalling pathway [550], EGFR [551] and insulin-like growth factor 1 receptor (IGF-1R) [551].

Resistance mechanisms based on the ‘classic’ induction of compensatory signalling pathways involving some of these families have been identified. Chronic inhibition of VEGF-A and VEGFR2 can lead to induction of proteins including FGF and FGF receptors (FGFRs) [552, 553], angiopoietin 1 (ANG1) [554] and ANG2 [555], and the MET oncogene [556], which sustain angiogenesis and induce tumour growth.

1.3.2.2. Changes in dependence of VEGF family members and receptors

VEGF-C and VEGF-D, ligands for VEGFR2 and VEGFR3, can promote tumour angiogenesis and lymphangiogenesis and have been found to be associated with resistance to anti-angiogenic agents including bevacizumab [557-562]. VEGF-C and VEGF-D may act as alternate ligands to VEGF-A for VEGFR2 binding, and hence promote tumour angiogenesis [448, 560], or alternatively may contribute to a larger pool of circulating angiogenic factors which may collectively re-establish angiogenesis [561].

Anti-VEGF therapy also has been shown to lead to the upregulation of PlGF [563]. Accordingly, treatment with an anti-PlGF antibody has been shown to suppress tumour growth and angiogenesis, and enhance VEGFR blockade or VEGF-trap treatment [447, 563].

Other proposed mechanisms of resistance related to VEGF family members include switches in the dominant VEGF-A isoform (VEGF₁₂₁, VEGF₁₆₅, or VEGF₁₈₉) or decreased VEGF-A dependence in ECs due to downregulation of VEGFR2 [564, 565].

1.3.2.3. Induction of compensatory pro-angiogenic factors

Anti-angiogenic agents can stimulate the production of chemo-attractants for neutrophils and macrophages, which in turn recruit myeloid cells and other cells which promote immune tolerance and angiogenesis [537, 566]. These chemo-attractants include stromal cell-derived factor 1 (SDF1, or CXCL12), and colony-stimulating factors (CSFs) G-CSF (or CSF3) and CSF2 [537,

567]. CSF3, the master regulator of neutrophil production, induces STAT3-dependent upregulation of BV8 (prokineticin 2) expression [568], which stimulates proliferation of ECs and angiogenesis in tumours [569].

1.3.2.4. Metabolic, stress, or immune responses

The disruption of tumour angiogenesis and perfusion by anti-VEGF-A therapy can lead to metabolic, stress, or immune responses in cancer cells which enable them to survive.

There are dual adaptive responses that tumours can demonstrate upon treatment with anti-angiogenic agents [537]. One adaptive response is ‘vascular normalisation’, which is associated with correction of tumour hypoxia (normoxia) due to increased blood perfusion [521]. The second adaptive response is vascular pruning, which leads to increased hypoxia [565]. The type of response elicited has important implications for specific mechanisms of resistance, including those related to the metabolic state of the tumour and immune cell recruitment [537].

Tumour cells have higher glucose uptake than their normal counterparts, and this is metabolised through aerobic glycolysis, resulting in high lactate levels even in the presence of oxygen-rich conditions (Warburg effect) [570]. There is evidence that anti-angiogenic agents which induce vascular normalisation and hypoxia correction are associated with a switch in metabolism. Whilst downregulation of glycolysis mediated through decreased HIF-1 α and AKT signalling is observed, tumour growth is sustained through increased uptake of ketones and fatty acids and upregulation of mitochondrial metabolism [571].

In contrast, tumour hypoxia is associated with an immunosuppressive phenotype. This can be associated with HIF-1 α -mediated recruitment of T regulatory (Treg) cells, which suppress self-reactive T cells and protect the host from auto-immunity. As discussed in section 1.3.1.2.2.2., hypoxia induces upregulation of HIF-1 α , which in turn increases VEGF-A secretion. VEGF-A binds to NRP1 expressed on Treg cells, and consequently, NRP1 guides Treg cells into the tumour [572]. HIF-1 α can also induce the secretion of TGF- β secretion by tumour cells, which acts as a chemo-attractant for Treg cell recruitment into tumours [573]. HIF-1 α also promotes an immunosuppressive environment by decreasing the susceptibility of tumour cells to T-cell mediated killing [574], and by inducing autophagy of tumour cells, which is associated with resistance to immune-mediated lysis by cytotoxic T-cells [575]. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death-1 (PD-1) are negative regulators of T-cell immune function [576]. HIF-1 α has been demonstrated to promote immune escape by inducing the expression of CTLA-4 and programmed death-ligand 1 (PD-L1), the ligand for PD-1, on CD8⁺ T-cells and hypoxic tumour cells, respectively [577, 578].

Furthermore, hypoxia may pre-condition the re-growth of tumour cells following withdrawal of anti-angiogenic treatment [579]. Hypoxia induces upregulation of fatty acid binding protein

(FABP)3 and FABP7 in a HIF-1 α dependent manner, which leads to fatty acid uptake and lipid droplet accumulation, which are essential for cell survival in hypoxia-reoxygenation [580]. Withdrawal of anti-angiogenic treatment is associated with a metabolic shift from glycolytic metabolism to *de novo* lipogenesis and accordingly, inhibition of fatty acid synthase (FASN) can reverse tumour growth associated with anti-angiogenic withdrawal [581].

Another resistance mechanism involves a multi-compartmental model [537], with metabolic compartmentalisation of tumour epithelial cells into hypoxic and normoxic regions [582]. These regions are in ‘metabolic symbiosis’, whereby tumour cells in hypoxic regions import and metabolise glucose through glycolysis, which is associated with upregulation of the glucose transporter GLUT1 and the lactate exporter MCT4. In contrast, tumour cells in normoxic regions import and catabolise lactate through oxidative metabolism, which is associated with upregulation of the lactate importer MCT1 [582-585]. Notably, lactate catabolism increases glutamine metabolism, and is associated with upregulation of mTOR signalling which can be suppressed by treatment with mTOR inhibitors [583].

1.3.2.5. Circumventing dependence on angiogenesis

Tumours can circumvent their dependence on angiogenesis through ‘vascular co-option’, whereby tumour cells grow along pre-existing vasculature [565, 579, 586, 587]. This has been implicated as a resistance mechanism in patients with colorectal cancer liver metastases [587], melanoma [588], metastatic lung cancer [589] and glioblastoma [590, 591].

Similarly, tumour cells may utilise “vasculogenic mimicry”, which involves the dysregulation of their lineage-specific phenotype, and transdifferentiation into other cell types, including endothelial-like cells. These endothelial-like cells form vessel-like networks which mimic the pattern of endothelial-lined vascular networks [592], between which a viable blood flow can be established [593]. Vasculogenic mimicry has been reported in multiple tumour types [594] including melanoma [595-598], breast [593, 599, 600], prostate [601, 602], ovarian [603, 604], and lung carcinomas [605].

Some tumours may be resistant to anti-angiogenic agents due to intrinsic hypovascularity [606]. An example is pancreatic ductal adenocarcinomas (PDACs), which are characterised by both hypovascularity and an abundant desmoplastic stroma, which primes these tumours to survive in a hypoxic microenvironment [607-609].

1.3.3. Biomarkers of response and resistance to bevacizumab in colorectal cancer

1.3.3.1. The need for biomarkers of response and resistance to bevacizumab in colorectal cancer

A biomarker is defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a

therapeutic intervention” [610]. The two main types of biomarkers which are relevant to cancer treatment are prognostic and predictive biomarkers.

Prognostic biomarkers provide information about a patient’s overall outcome, regardless of the treatment received. In contrast, predictive biomarkers provide information about response to a therapeutic intervention. Predictive biomarkers can therefore be used to select for patients who are likely to respond to a specific treatment [611, 612].

Distinguishing between prognostic and predictive markers has important implications for the design of clinical trials which incorporate retrospective biomarker studies. Identification of prognostic biomarkers is more straightforward and can be analysed using series of patients given either placebo or standard treatment, including cohort or single-arm studies [613]. However, identification of predictive biomarkers requires clinical trials which include a control arm receiving placebo or standard treatment [614]. This ensures that any baseline prognostic impact of the biomarker can also be taken into account. Randomisation is also essential, so that patients who receive the treatment for which the biomarker is hypothesised to be predictive are comparable to those who receive placebo or standard treatment [613, 614]. A biomarker is predictive if the treatment effect (experimental compared with standard) is different for patients who are biomarker-positive, compared with patients who are biomarker-negative, and there is a statistical interaction between the treatment effect and the biomarker status [614]. Valid retrospective analysis of a predictive biomarker from an appropriately designed RCT also requires tissue samples to be available for analysis for a large majority of the study population, in order to avoid selection bias [613].

Whilst the addition of bevacizumab treatment to combination chemotherapy leads to longer median PFS and modest improvements in OS and ORR in patients with mCRC, not all patients who are treated with the drug respond (Table 1.6). In addition, the drug is costly – in the MAX clinical trial, the average cost per patient from the beginning of treatment until the time of progression was AUD 44,169 for patients treated with capecitabine and bevacizumab, compared to AUD 14,577 for patients treated with capecitabine alone [37]. Moreover, bevacizumab treatment is associated with multiple side effects, which may be severe (grade 3 or more). The most common grade 3 – 4 AEs associated with bevacizumab treatment include venous thromboembolism (8-12%), hypertension (2-11%), arterial thromboembolism (3-4%), proteinuria (1-4%), bleeding (0-3%), gastrointestinal perforation (0-2%), and neutropenia (0-1%) [36]. Other potentially serious AEs include reversible posterior leuco-encephalopathy syndrome (which although rare, manifests clinically with neurological symptoms including confusion, lethargy, seizures, and visual disturbances [615]) and impaired wound healing due to inhibition of neovascularisation [36].

Therefore, there is both a clinical and economic need to identify biomarkers which are predictive of bevacizumab response [38]. Predictive biomarkers would enable us to identify patients who are unlikely to respond, firstly so that they can be commenced on alternative therapies sooner and, secondly, to avoid unnecessary side effects. Identification of a predictive biomarker which selects a subset of patients who derive greater benefit from bevacizumab could also improve the cost effectiveness of bevacizumab use in mCRC [37].

1.3.3.2. Biomarkers of response and resistance to bevacizumab

Multiple potential predictive biomarkers of bevacizumab response, including basal biomarkers which are measured pre-treatment (section 1.3.3.2.1.) and treatment-related biomarkers which are measured during treatment (section 1.3.3.2.2.) have been investigated. However, despite extensive efforts, a validated predictive biomarker for bevacizumab treatment outcome has not yet been identified [38].

1.3.3.2.1. Basal biomarkers

1.3.3.2.1.1. Circulating angiogenic factors

Many previous studies have examined circulating angiogenic factors (CAFs) (including VEGF-A, VEGF-C, VEGF-D, short VEGF-A isoforms, soluble VEGFR1 (sVEGFR1), sVEGFR2, basic fibroblast growth factor (bFGF), PlGF, PDGF, stromal cell-derived factor 1 (SDF1), interleukin 8 (IL-8) and ANG2) as potential predictive biomarkers of bevacizumab outcome [38].

High baseline circulating VEGF-A levels have been proposed to be a predictive biomarker of bevacizumab treatment as they may reflect dependency of the tumour vasculature on VEGF-A [38]. Of note, the majority of previous studies examining pre-treatment VEGF-A levels have not included a non-bevacizumab control arm, precluding the distinction between its prognostic and predictive value as a biomarker [38]. A study by Hegde *et al.* analysed baseline plasma VEGF-A levels from six Phase 3 RCTs with non-bevacizumab control arms- the AVF2107 (NCT00109070) study in mCRC, E4599 (NCT00021060) and AVAiL (BO17704; NCT00806923) studies in recurrent/advanced NSCLC, AVOREN (NCT00738530) and AVF2938 (NCT00081614, erlotinib with bevacizumab versus bevacizumab alone) studies in metastatic renal cell carcinoma. Whilst pre-treatment VEGF-A was prognostic for outcome in metastatic colorectal, lung, and renal cell cancers, it was not predictive for bevacizumab-based treatment benefit [616].

Levels of circulating short VEGF-A isoforms have also been studied as a potential predictive biomarker for bevacizumab. It has been proposed that short VEGF-A isoforms which, as

discussed in section 1.3.1.2.2.2., are highly diffusible over long distances, may be more reflective of tumour-secreted VEGF-A [38]. Retrospective analyses of several Phase 3 RCTs in HER-2 negative metastatic breast cancer (AVADO), advanced gastric (AVAGAST), and metastatic pancreatic cancer (AViTA) using a novel immunologic multi-parameter chip technology (IMPACT) enzyme-linked immunosorbent assay (ELISA) which preferentially detects short VEGF-A isoforms had promising results which suggested prognostic and predictive effects of pre-treatment circulating short VEGF-A isoforms in bevacizumab-treated patients [617-619]. However subsequently, the phase 3 MERiDiAN RCT (NCT01663727), a prospective biomarker evaluation study, failed to validate pre-treatment circulating short VEGF-A isoforms as a predictive biomarker in HER-2 negative locally recurrent or metastatic breast cancer (PFS treatment-by-VEGF-A interaction $P = 0.46$) [620].

It has been proposed that high levels of pre-treatment s-VEGFR1 sequester tumour-derived VEGF-A, acting as an endogenous trap, and may potentially reduce the benefits of bevacizumab-induced VEGF-A neutralisation [38, 521]. Whilst high s-VEGFR1 levels have been found to be inversely correlated with outcome in patients treated with bevacizumab for rectal and HER-2 negative breast cancer, these studies have not included a non-bevacizumab control arm [621, 622].

1.3.3.2.1.2. Circulating endothelial cells (CECs) and progenitor cells

Circulating endothelial cells (CECs) include mature apoptotic cells arising from the endothelial wall, or immature circulating endothelial progenitors (CEPs) which are derived from the bone marrow [623]. It has been hypothesised that increased levels of CEPs reflect active tumour angiogenesis, and therefore could be predictive of response to anti-angiogenic treatments [624, 625]. Studies examining basal levels of CECs and CEPs as biomarkers for bevacizumab treatment have shown conflicting results, and the majority have lacked a non-bevacizumab control arm [626-631].

1.3.3.2.1.3. In situ biomarkers

Multiple studies have examined the expression of VEGF family and pathway genes using immunohistochemistry (IHC), real-time polymerase chain reaction (RT-PCR) and *in situ* hybridisation as potential biomarkers for bevacizumab. Some significant associations have been reported, however, most of these have not been validated. For example, in the NO16966 Phase 3 RCT in mCRC, high density of CD31+ vessels which are indicative of microvascular density (MVD), a surrogate marker of tumour angiogenesis [38], were associated with a trend to bevacizumab-treatment benefit [632]. However, in the AVF2107g study, microvascular density measured using CD 34+ stained sections was not found to be prognostic or predictive of bevacizumab-treatment benefit [633]. Our laboratory demonstrated that in the MAX trial, high VEGF-D expression using IHC was predictive of less benefit from bevacizumab treatment for

both PFS and OS [560]. However, in the CAIRO-2 study, we found no difference in PFS or OS according to VEGF-D expression (although all patients in the control arm were treated with bevacizumab and chemotherapy) [560]. Importantly, it has been shown that bevacizumab does not inhibit the interaction of VEGF with NRPs [634], and therefore higher NRP expression/levels may be associated with less benefit from bevacizumab treatment, and vice versa [334]. Indeed, in the AVAGAST study, low baseline expression of NRP-1 measured using IHC was associated with a trend towards greater benefit from bevacizumab for OS (interaction $P = 0.06$) [618]. This was supported by biomarker analyses in the NO16966 study in mCRC and the AVF2119g study in metastatic breast cancer [632, 635], which showed that low NRP-1 expression was associated with trends to increased benefit from bevacizumab treatment for PFS.

1.3.3.2.1.4. Consensus molecular subtype (CMS) classification

More recently, the consensus molecular subtype (CMS) classification of CRC (described in section 1.2.2.) has been investigated as a potential prognostic and predictive biomarker in mCRC. CMS analyses were performed in both the FIRE-3 and CALGB 80405 studies, which were Phase 3 RCTs comparing the addition of cetuximab versus bevacizumab to first-line combination chemotherapy in patients with *KRAS* wt metastatic CRC [636, 637]. CMS classification was found to be a strong prognostic biomarker in both studies, and both studies found that CMS2 was associated with the best prognosis, and CMS1 with the worst [636, 637]. Our laboratory recently published results of CMS analysis in the MAX trial [638]. Consistent with the results of the FIRE-3 and CALGB 80405 analyses, CMS was a significant prognostic biomarker for OS, and CMS2 and CMS1 were associated with the best and worst prognosis, respectively. Furthermore, there was a significant interaction between CMS and bevacizumab-treatment outcome in PFS. Patients with tumours classified as CMS2, and possibly CMS3 (sample size, $n = 28$), benefited more from the addition of bevacizumab to capecitabine treatment compared to the other CMS groups. These findings are yet to be validated in another phase 3 RCT. Moreover, the biological basis for the association between the CMS subgroups and bevacizumab-treatment benefit has not yet been determined. It is, however, interesting to note that CMS2 and CMS3 subgroups share the feature of highly proliferative epithelial tumours, whilst CMS1 and CMS4 are associated with high immune and stromal infiltration, respectively [639].

1.3.3.2.1.5. Somatic copy number alteration (SCNAs)

Interestingly, Smeets *et al.* recently published results of an ANGIOPREDICT Consortium study which examined genome-wide copy number alterations (CNAs) as potential predictive biomarkers of bevacizumab response in mCRC [640]. Using unsupervised hierarchical clustering of CNA profiles from 908 patients with mCRC, the authors classified tumours into 3 CNA clusters. Cluster 1 had almost no CNAs, but was enriched for high somatic mutational burden, including MSI tumours, and *POLE*, *POLD1*, *BRAF*, and *PIK3CA* mutations. Cluster 3 was characterised by the highest numbers of CNAs, and *TP53* mutations were observed more

frequently in Clusters 2 and 3. As expected (based on the known frequencies of SCNAs in CMS1-4 [59], there was enrichment of CMS1/3 in CNA cluster 1 and CMS2/4 in Clusters 2 and 3. In a retrospective cohort of 215 patients with mCRC who had whole-genome sequencing data, of whom 195 received treated with bevacizumab, the authors found that patients with tumours in Clusters 2 and 3 had better survival outcomes with bevacizumab treatment [640]. Whilst these findings are interesting, this was a retrospective study and, and as such, the findings need to be validated in appropriately designed RCTs. Such studies may provide valuable insight into the relative impact of CNA clusters alone, compared to CMS classification, as predictive biomarkers for bevacizumab treatment.

1.3.3.2.2. Treatment-related biomarkers

1.3.3.2.2.1. Treatment-related changes in circulating angiogenic factor levels

Treatment-related changes in CAF levels have also been examined in several studies [38]. Findings from studies examining changes in circulating VEGF-A levels (all with small sample sizes and without a non-bevacizumab control arm) have been inconsistent [38]. Whilst treatment-related changes in PlGF [621, 641, 642], bFGF [643], PDGF-BB [641], VEGF-C [561], and VEGF-D [561] have been associated with outcome in patients treated with bevacizumab, these studies have been small and lacking a non-bevacizumab control arm [38].

1.3.3.2.2.2. Treatment-related changes in CECs and progenitor cells

Studies of treatment-related changes in CECs and CEPs as biomarkers for bevacizumab treatment have shown conflicting results, and the majority have lacked a non-bevacizumab control arm [627, 629, 630, 644, 645].

1.3.3.2.2.3. Hypertension

Hypertension, a common side effect of bevacizumab, has also been investigated as a potential predictive biomarker for bevacizumab-treatment benefit. Whilst several phase 3 RCTs suggested an association between hypertension and benefit from bevacizumab treatment in metastatic breast cancer (E2100) [646], advanced non-squamous NSCLC (E4599) [647], and metastatic renal cell carcinoma (CALGB90206) [648], a large retrospective biomarker analysis across 6 RCTs including 4 different tumour types (AVF2107g and NO16966 in mCRC, AVADO and RIBBON-1 in metastatic breast cancer, AVAiL in NSCLC, and AVOREN in metastatic renal cell carcinoma) showed that in 5 of the 6 studies, hypertension during treatment was neither prognostic nor predictive [649]. However, in the AVF2107g study, HT was predictive for significantly improved PFS and OS in bevacizumab-treated patients [649].

1.3.3.2.4. Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI)

As anti-VEGF-A agents inhibit tumour angiogenesis, investigators have explored parameters from dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) as imaging biomarkers for bevacizumab response. Whilst multiple studies have demonstrated significant changes in DCE-MRI parameters, including a reduction in volume transfer constant (K^{trans} , a composite measure of permeability, capillary surface area and flow) following bevacizumab treatment [650-657], these have been exploratory, early-phase studies.

1.3.4. Single nucleotide polymorphisms (SNPs) in VEGF family genes as biomarkers of response and resistance to bevacizumab in metastatic colorectal cancer

1.3.4.1. Rationale for investigating SNPs in VEGF family genes as biomarkers

Common causes for therapeutic heterogeneity include pharmacokinetic, somatic, or germline variability [658]. In the case of bevacizumab treatment, clinically used doses of bevacizumab exceed those required to eliminate circulating VEGF [659]. Furthermore, bevacizumab is not required to be metabolised for activation or clearance [660]. As such, pharmacokinetic variability is unlikely to account for the heterogeneity in response observed with bevacizumab treatment.

Furthermore, whilst somatic variation has been commonly investigated as a biomarker of response to other targeted therapies in oncology, this is less relevant to biomarker discovery for anti-angiogenic therapies [658]. This is because although VEGF-mediated autocrine and paracrine signalling is initiated by tumour cells [334], bevacizumab is believed to exert its effects through targeting of VEGF-A mediated angiogenesis involving host endothelial cells which are influenced by germline genetic variability [658, 661, 662]. Accordingly, multiple studies have focused on whether single nucleotide polymorphisms (SNPs) in VEGF family genes, including *VEGF-A*, *VEGFR1*, and *VEGFR2*, are predictive of bevacizumab-treatment benefit [38].

1.3.4.2. SNPs in VEGF family genes as potential biomarkers of response and resistance to bevacizumab

Single nucleotide polymorphisms (SNPs) are single nucleotide variations in a genome, which typically have a minor allele frequency of > 1% in a population. SNPs account for >90% of human genetic variation, and it is estimated that there are > 10 million SNPs in the human genome [663].

VEGF-A is a highly polymorphic gene which has multiple well-established SNPs, predominantly located within the promoter, 5' untranslated region (5'UTR), and 3' untranslated region (3'UTR) [664]. However, there are no common non-synonymous SNPs within the VEGF genes [658]. There are also multiple SNPs associated with the genes for its receptors, *VEGFR-1* and *-2* [665].

Table 1.7 and Table 1.8 show the results of previous studies in the literature which have examined the association between SNPs in *VEGF-A*, *VEGFR1* and *VEGFR2* and efficacy outcomes in

patients treated with bevacizumab and chemotherapy for mCRC, and other tumour types, respectively.

Notably, only one of the studies performed in patients with mCRC (ITACa [666], Ulivi *et al.*, Table 1.6 and Table 1.7) was a Phase III randomised controlled trial which had a non-bevacizumab control arm, and therefore had a suitable study design for retrospective predictive biomarker analysis. In this first-line study, participants were randomised to FOLFIRI or FOLFOX4 combination chemotherapy (chosen at the investigator's discretion), with or without bevacizumab. 237 out of 376 (63%) randomised patients were included in the biomarker analysis, which used peripheral blood samples in 153 patients and FFPE tumour samples in 84 patients. Of five candidate SNPs in *VEGF-A* examined (rs699947, rs833061, rs1570360, rs2010963, and rs3025039), two were found to be associated with efficacy outcomes. Using Cox proportional-hazards models adjusted for gender, age, performance status, *KRAS* mutation status, tumour location and chemotherapy regimen, the *VEGF-A* rs3025039 TT genotype was found to be associated with a shorter median PFS and OS than C-variants (7.8 versus 10.2 months, $P = 0.036$; and 8.6 versus 22.7 months, $P = 0.007$, respectively) in the bevacizumab-containing arm, but not the chemotherapy-only arm. However, tests for interaction between treatment effect and SNP genotype were not significant for PFS (results of tests for interaction were not described for the OS outcome). Of note, only 3 patients had the *VEGF-A* rs3025039 TT genotype, limiting power of the analyses. In addition, the *VEGF-A* rs2010963 GC genotype was associated with an inferior ORR compared to other genotypes ($P = 0.017$) [666].

A meta-analysis of individual patient data from six Phase III randomised controlled trials with non-bevacizumab control arms included one trial in mCRC (NO16966 [528], which had the co-primary objective of comparing the effect on PFS of bevacizumab versus placebo when combined with XELOX or FOLFOX-4 chemotherapy). This meta-analysis also included trials in metastatic pancreatic cancer (AViTA), recurrent/advanced NSCLC (AVAiL), metastatic renal cell carcinoma (AVOREN), metastatic breast cancer (AVADO), and advanced gastric cancer (AVAGAST) cancer [667] (Table 1.8). Overall, only 1402 of 5209 (27%) patients in the 6 trials were included in the biomarker analysis. The proportion of patients who consented to the genetic sub-studies from the individual clinical trials ranged from 11.4% (in AVAiL) to 47.5% (in AVADO), and included 43.5% of patients in the NO16966 mCRC study (Table 1.8). Among the 195 SNPs in VEGF pathway genes that were analysed, *VEGF-A* rs699946 G-allele was reported to be associated with an inferior PFS in the bevacizumab arm, but not the placebo arm (allelic HR 1.27; 95% CI 1.08 to 1.49, $P = 0.0034$), however the test for interaction between treatment effect and SNP genotype was not statistically significant [667].

Study designs for the other previous biomarker studies which have examined SNPs in VEGF family members in mCRC include: Phase 3 RCTs with bevacizumab treatment in both arms ($n =$

3) [668-670], a phase 3 RCT in which the control arm included bevacizumab and the experimental arm included cetuximab treatment ($n = 1$) [671], single-arm Phase 2 studies ($n = 3$) [642, 672, 673], single-arm pilot or pharmacogenetic studies ($n = 2$) [674, 675], a study combining two single-arm Phase 2 studies and a patient registry ($n = 1$) [676], and retrospective studies ($n = 2$) [677, 678].

As shown in Tables 1.7 and 1.8, previous biomarker studies which have examined SNPs in VEGF family members have each identified a range of different SNPs in *VEGF-A*, *VEGFR1* and *VEGFR2* which are associated with efficacy outcomes. Whilst some of these associations have been replicated in one or more other studies, other studies have shown no association or conflicting results. As such, no individual SNP has been consistently validated across all studies either within a tumour type, or across tumour types. For example, for *VEGF-A* rs3025039, C-variants have been associated with superior efficacy outcomes in some studies of patients with metastatic CRC [666] and breast cancer in the neo-adjuvant setting [679], but poorer efficacy outcomes in advanced NSCLC [680] and metastatic breast cancer [681], whilst no association has been found in other studies of patients with mCRC [642, 669-671, 674, 676-678], advanced NSCLC [682], metastatic breast cancer [617, 646], ovarian cancer [683, 684], head and neck cancer [685], or glioblastoma multiforme [686].

Table 1.7. Studies examining the association between SNPs in *VEGF-A*, *VEGFR1* and *VEGFR2* and efficacy outcomes with bevacizumab in metastatic colorectal carcinoma

Study	Clinical trial name; Clinicaltrials.gov or other identifier	Phase	Treatment line	Sample size (n), n with tissue analysed	Treatment arms	Randomisation to non-BEV control arm	Tissue used	Genetic variants examined	Clinical associations with SNPs examined	Association/s with clinical outcome	REF
Ulivi <i>et al.</i>	ITACa; NCT01878422	III	First-line	376 (237)	Arm A: FOLFIRI/FOLFOX4 (investigator's discretion) + BEV Arm B: FOLFIRI/FOLFOX4 (investigator's discretion)	Yes	Peripheral blood (n = 153) and FFPE tumour samples (n = 84)	<i>VEGF-A</i> : -2578C>A (rs699947), -1498C>T (rs833061), -1154G>A (rs1570360), -634C>G (rs2010963), +936C>T (rs3025039)	PFS, ORR, OS	<i>VEGF-A</i> rs3025039 : TT had a shorter median PFS and OS than C-variants (7.8 vs 10.2 months, $P = 0.036$; and 8.6 vs 22.7 months, $P = 0.007^a$, respectively) in Arm A. Tests for interaction were not significant for PFS ^b . <i>VEGF-A</i> rs2010963 : GC had an inferior ORR compared to other genotypes ($P = 0.017$).	[666]
Lenz <i>et al.</i>	TRIBE; NCT00719797	III	First-line	508 (385; n = 163/256 from Arm A, and n = 222/252 from Arm B)	Arm A: FOLFIRI + BEV Arm B: FOLFOXIRI + BEV <i>Analyses performed in both arms combined, and individually</i>	No	Not specified in abstract	37,043 coding SNPs with MAF > 1%, using Illumina HumanExome array	OS	<i>VEGFR2</i> rs10008360 : Associated with inferior OS. For combined Arms A & B, HR 5.76, 95% CI 2.66 to 12.46, $P = 8.8 \times 10^{-6}$; for Arm B, HR 9.81, 95% CI 3.59 to 26.81, $P = 8.52 \times 10^{-6}$.	[668]
Hansen <i>et al.</i>	Nordic ACT; NCT00598156	III	First-line	249 (218)	Arm 1: FOLFOX/XELOX or FOLFIRI/XELIRI (investigator's discretion) + BEV, then randomised to maintenance BEV alone FOLFOX/XELOX or FOLFIRI/XELIRI (investigator's discretion) + BEV, then randomised to maintenance BEV + erlotinib <i>Both arms combined for analysis</i>	No	Peripheral blood	<i>VEGF-A</i> : -2578 C/A (rs699947), -1498 C/T (-460 C/T) (rs833061), -1154 G/A (rs1570360), -634 G/C (405 G/C) (rs2010963), 936 C/T (rs3025039); <i>VEGFR1</i> : 319 C/A (rs9582036); <i>VEGFR2</i> : -604 T/C (rs2071559; 1192 C/T (rs2305948), 1719 T/A (rs1870377)	ORR	<i>VEGFR1</i> rs9582036 : C-variants had an inferior ORR to AA (39% vs 56%, $P = 0.015$)	[669]

Table 1.7. (continued) Studies examining the association between SNPs in *VEGF-A*, *VEGFR1* and *VEGFR2* and efficacy outcomes with bevacizumab in metastatic colorectal carcinoma

Study	Clinical trial name; Clinicaltrials.gov or other identifier	Phase	Treatment line	Sample size (n), n with tissue analysed	Treatment arms	Randomisation to non-BEV control arm	Tissue used	Genetic variants examined	Clinical associations with SNPs examined	Association/s with clinical outcome	REF
Koutras <i>et al.</i>	HeCOG 6A/05; ACTRN1261000 0270011	III	First-line	302 (173)	Arm A: XELIRI-BEV Arm B: FOLFIRI-BEV <i>Both arms combined for analysis</i>	No	Peripheral blood	<i>VEGF-A</i> : 1154 (rs1570360), +936 (rs3025039), 634 (rs2010963), 2578 (rs699947) and 1498 (rs833061)	PFS, OS, ORR	<i>VEGF-A</i> rs1570360 : GG was associated with inferior OS compared to A-variants (HR 1.62, 95% CI 1.09 to 2.40, $P = 0.017$)	[670]
Pander <i>et al.</i>	CAIRO2; NCT00208546	III	First-line	368 (279)	Control arm : CAPOX-BEV <i>Only the control arm was included in the analysis</i>	No	Peripheral blood	<i>VEGF-A</i> : -1154G>A (rs1570360), 405G>C (rs2010963), 936C>T (rs3025039), -2578C>A (rs699947), -460C>T (rs833061)	PFS	No association between any examined SNPs and PFS	[671]
Loupakis <i>et al.</i>	PROVETTA; NCT01363739	II (non-randomised)	First-line	424 (424)	Single arm: FOLFIRI + BEV	No	Peripheral blood	<i>VEGF-A</i> : rs833061, rs699946, rs9582036, rs7993418; <i>VEGFR1</i> : rs11133360, rs12505758, rs2305948	PFS, OS, ORR (Prospectively powered for primary outcome of association of <i>VEGF-A</i> rs833061 TT with PFS)	<i>VEGFR2</i> rs12505758 : C-variants had shorter PFS than TT (HR 1.36, 95% CI 1.05 to 1.75), $P = 0.015$ (not after correction for multiple testing)	[672]
Loupakis <i>et al.</i>	FOIB; NCT01163396; EudraCT number 2006-001007-11	II (non-randomised)	First-line	57 (57)	Single arm: FOLFOXIRI + BEV	No	Peripheral blood	<i>VEGF-A</i> : 2578A/C (rs699947), 1498C/T (rs833061), 1154A/G (rs1570360), 634C/G (rs2010963), 936C/T (rs3025039); <i>VEGFR2</i> : 604A/G (rs2071559), 1192C/T (rs2305948), 1719A/T (rs1870377)	PFS	No association between any examined SNPs and PFS	[642]

Table 1.7. (continued) Studies examining the association between SNPs in *VEGF-A*, *VEGFR1* and *VEGFR2* and efficacy outcomes with bevacizumab in metastatic colorectal carcinoma

Study	Clinical trial name; Clinicaltrials.gov or other identifier	Phase	Treatment line	Sample size (n), n with tissue analysed	Treatment arms	Randomisation to non-BEV control arm	Tissue used	Genetic variants examined	Clinical associations with SNPs examined	Association/s with clinical outcome	REF
Becouarn <i>et al.</i>	NCT00467142; EUDRACT-2006-003901-22	II (non-randomised)	First-line	62 (45)	Single arm: FOLFIRI + BEV	No	Peripheral blood	<i>VEGF-A</i> : rs699947 (−2578C >A), rs2010963 (−634G > C), and rs25648 (S178S, formerly known as -7C > T)	PFS, OS	<i>VEGF-A</i> rs25648 : C-variants had a longer OS ($P = 0.01$)	[673]
Abajo <i>et al.</i>		Pilot	Second-line ^c	59 (59)	Single arm: Gemcitabine + irinotecan + BEV	No	Peripheral blood	<i>VEGF-A</i> : -2578C>A (rs699947), -460T>C (rs833061), +405G>C (rs2010963), +936 C>T (rs3025039), 3' near G>A (rs10434)	TTP	No significant association between any <i>individual</i> examined SNPs and TTP. Patients with at least one of <i>VEGF-A</i> rs699947 AA, rs833061 CC, or rs2010963 GG genotypes had a longer median TTP than those without (8.8 vs 4.5 months, $P = 0.043$).	[674]
Formica <i>et al.</i>		PG	First-line	40 (40)	Single arm: FOLFIRI + BEV	No	Peripheral blood	<i>VEGF-A</i> : rs699947, rs35569394, rs1005230, rs35864111, rs833061, rs13207351, rs1570360, rs2010963	PFS, ORR, OS	<i>VEGF-A</i> rs13207351 : GG had a shorter PFS than A-variants (8.9 vs 15.4 months, $P = 0.007$). <i>VEGF-A</i> rs1570360 : GG had a shorter PFS than A-variants (9.8 vs 16 months, $P = 0.03$). <i>VEGF-A</i> rs2010963 : GG had a higher ORR than C-variants (64% vs 14%, $P = 0.03$).	[675]

Table 1.7. (continued) Studies examining the association between SNPs in *VEGF-A*, *VEGFR1* and *VEGFR2* and efficacy outcomes with bevacizumab in metastatic colorectal carcinoma

Study	Clinical trial name; Clinicaltrials.gov or other identifier	Phase	Treatment line	Sample size (n), n with tissue analysed	Treatment arms	Randomisation to non-BEV control arm	Tissue used	Genetic variants examined	Clinical associations with SNPs examined	Association/s with clinical outcome	REF
Sohn <i>et al.</i>	2 Phase II trials and 1 patient registry			Total 129 (125)							[676]
		Trial 1: Phase II	First-line		Single arm: CAPOX + BEV	No	Peripheral blood	VEGF-A: -2578 A/C (rs699947), -1498 (-460) C/T (rs833061), -1154 A/G (rs1570360), -634 (+405) C/G (rs2010963), +936 C/T (rs3025039); VEGFR1: rs9513070, rs9554316, rs9554320, 319 A/C (rs9582036); VEGFR2: IVS25-92 A/G (rs1531289), +889 C/T (rs2305948), +1416 A/T (rs1870377)	ORR , PFS, OS	VEGF-A rs833061: TT had a superior ORR to C-variants (75.9 vs 50.8%, P = 0.008). VEGFR1 rs9513070 AA was associated with longer median PFS and OS compared to G-variants (8.7 vs 6.6 months, P = 0.001, and 26.4 vs 16.1 months, P = 0.038, respectively).	
	Trial 2: NCT 0086234	Trial 2: Phase II	Second-line		Single arm: oxaliplatin- or irinotecan- based doublet chemotherapy with 5FU or capecitabine + BEV, chemotherapy switch after first-line.	No					
Gerger <i>et al.</i>		Retrospective hospital-based	First-line	132 (132)	FOLFOX/XELOX + BEV	NA	Peripheral blood	VEGF-A: rs2010963, rs3025039, rs1570360, rs833061, rs699947; VEGFR2: rs2305948, rs2071559	PFS, OS, ORR	VEGFR2 rs2305948: CC had an inferior ORR to T-variants (P = 0.024)	[677]
Loupakis <i>et al.</i>		Retrospective hospital-based	First-line	218 (218)	111 patients treated with FOLFIRI + BEV	No (historical control)	Peripheral blood	VEGF-A: -2578 C/A (rs699947), -1498 C/T (rs833061), +405 C/G (rs2010963) and + 936 C/T (rs3025039)	PFS, OS, ORR	VEGF-A rs833061 TT was associated with shorter median PFS than C-variants (7.5 vs 11.1 months, HR 2.13, 95% CI 1.41 to 5.10, P = 0.0027).	[678]
					107 historical controls treated with first-line FOLFIRI						

SNPs: single nucleotide polymorphisms; REF: reference/s; *VEGF-A*: vascular endothelial growth factor-A; *VEGFR1*: vascular endothelial growth factor receptor 1; *VEGFR2*: vascular endothelial growth factor receptor 2; FOLFIRI: 5-fluorouracil + folinic acid + irinotecan; FOLFOX: 5-fluorouracil + folinic acid + oxaliplatin; BEV: bevacizumab; FFPE: formalin-fixed paraffin embedded; PFS: progression-free survival, OS: overall survival, ORR: objective response rate; FOLFIRI: 5-fluorouracil + folinic acid + oxaliplatin + irinotecan; MAF: minor allele frequency; HR: hazard ratio; CI: confidence interval; XELOX: capecitabine + oxaliplatin; XELIRI: capecitabine + irinotecan; CAPOX: capecitabine + oxaliplatin; TTP: time to progression; PG: pharmacogenetic; NA: not applicable; ^a: adjusted for chemotherapy, gender, age, KRAS status, tumour location; ^b: results of tests for interaction were not specified for OS; ^cprogressed after one oxaliplatin/ fluoropyrimidine based chemotherapy regimen for mCRC.

Table 1.8. Studies examining the association between SNPs in *VEGF-A*, *VEGFR1* and *VEGFR2* and efficacy outcomes with bevacizumab in other tumour types

Tumour type	Study	Clinical trial name; Clinicaltrials.gov or other identifier	Phase	Treatment line	Sample size (n), n with tissue analysed	Treatment arms	Randomisation to non-BEV control arm	Tissue used	Genetic variants examined	Clinical associations with SNPs examined	Association/s with clinical outcome	REF
Lung	Zhang <i>et al.</i>	ECOG 4599; NCT00021060	III	First-line (recurrent or advanced (Stage IIIB/IV) NSCLC)	878 (133; n = 66/434 from BPC and n = 67/444 from PC)	Paclitaxel + carboplatin + BEV (BPC) Paclitaxel + carboplatin (PC)	Yes	Peripheral blood	Not specified in abstract	PFS, OS, ORR	VEGF-A rs2010963 (634C>G): Tests for interaction between genotype and treatment effect were statistically significant ($P < 0.05$) for OS	[687]
	Dy <i>et al.</i>	NCCTG N0821; NCT00798603	II	First-line (Stage IIIB/IV non-squamous NSCLC)	65 (57)	Single arm: Carboplatin + pemetrexed + BEV, followed by pemetrexed + BEV maintenance ^c	No	Peripheral blood	20 SNPs in <i>VEGF-A</i> , 7 SNPs in <i>VEGFR1</i> , 16 SNPs in <i>VEGFR2</i> , 8 SNPs in <i>VEGF-R3</i>	PFS6, ORR, PFS, OS	VEGF-A rs3025018: G-variants were associated with shorter PFS and OS than C/C (HR 6.9, 95% CI 2.21 to 21.3, $P = 0.0001$), and HR 6.8, 95% CI 2.14 to 21.53, $P = 0.0002$, respectively). VEGFR2 rs7671745: A-variants were associated with superior ORR and PFS6 than G/G (OR 4.0, 95% CI 1.11 to 14.47, $P = 0.029$, and OR 4.062, 95% CI 1.29 to 12.78, $P = 0.014$, respectively).	[688]

Table 1.8. (continued) Studies examining the association between SNPs in *VEGF-A*, *VEGFR1* and *VEGFR2* and efficacy outcomes with bevacizumab in other tumour types

Tumour type	Study	Clinical trial name; Clinicaltrials.gov or other identifier	Phase	Treatment line	Sample size (n), n with tissue analysed	Treatment arms	Randomisation to non-BEV control arm	Tissue used	Genetic variants examined	Clinical associations with SNPs examined	Association/s with clinical outcome	REF
Lung continued	Pallaud <i>et al.</i>	ABIGAIL; BO21015; NCT00700180	II	First-line (recurrent or advanced (Stage IIIB/IV) non-squamous NSCLC)	303 (245; n = 125/154 from BEV 7.5 mg/kg arm and n = 120/149 from BEV 15 mg/kg arm) ^a	Carboplatin/ gemcitabine or carboplatin/ paclitaxel (investigator's discretion) + BEV 7.5 mg/kg Carboplatin/ gemcitabine or carboplatin/ paclitaxel (investigator's discretion) + BEV 15 mg/kg	No	Peripheral blood	<i>VEGF-A</i> : c.-2578 C>A (rs699947), c.+936 C>T (rs3025039), c.-1154 A>G (rs1570360), c.-460 >C; c.-1498 >C (CT) (rs833061), c.+405/c.-634 (CG) (rs2010963); <i>VEGFR1</i> : 519 C>T, rs9582036, rs9554316; <i>VEGFR2</i> : -604 T>C (rs2071559), c.1192 G>A (rs2305948), c.1718 A>T (rs1870377), +4422(AC)11-14 (rs3034659)	ORR, PFS, OS	<i>VEGF-A</i> rs2010963 : CC had superior ORR to G-variants (OR 0.63; 95% CI 0.41–0.9; <i>P</i> = 0.038). <i>VEGF-A</i> rs833061 : TT had superior ORR to C-variants (OR 1.53, 95% CI 1.03 to 2.28, <i>P</i> = 0.036). <i>VEGF-A</i> rs699947 : AA had superior ORR compared to C-variants (OR 1.53, 95% CI 1.03 to 2.27, <i>P</i> = 0.037). <i>VEGFR1</i> rs9554316 : TT was associated with shorter PFS and OS than G-variants (HR 1.66, 95% CI 1.04 to 1.78, <i>P</i> = 0.024, and HR 1.40, 95% CI 1.02 to 1.90, <i>P</i> = 0.035, respectively).	[682]
	Massuti <i>et al.</i>	ANGIOMET; NCT01814163	PG	First-line (advanced unresectable, metastatic or recurrent non-squamous NSCLC)	200 (168)	Single arm: Carboplatin + paclitaxel + BEV, followed by maintenance BEV	No	Peripheral blood	Not specified in abstract	ORR, PFS, OS	<i>VEGF-R1</i> rs9582036 : CC was associated with shorter PFS (<i>P</i> = 0.01) and OS (<i>p</i> = 0.01) than A-variants. <i>VEGF-A</i> rs3025039 : CC was associated with shorter OS compared with T-variants (<i>P</i> = 0.009).	[680]

Table 1.8. (continued) Studies examining the association between SNPs in *VEGF-A*, *VEGFR1* and *VEGFR2* and efficacy outcomes with bevacizumab in other tumour types

Tumour type	Study	Clinical trial name; Clinicaltrials.gov or other identifier	Phase	Treatment line	Sample size (n), n with tissue analysed	Treatment arms	Randomisation to non-BEV control arm	Tissue used	Genetic variants examined	Clinical associations with SNPs examined	Association/s with clinical outcome	REF
Breast	Schneider <i>et al.</i>	ECOG 2100; NCT00028990	III	First-line (HER-2 negative MBC)	722 (363; n = 180/368 from experimental arm and n = 183/354 from control arm)	Experimental arm: Paclitaxel + BEV	Yes	FFPE tumour tissue	VEGF-A: -2578 C/A (rs699947), -1498 C/T (rs833061), -1154 G/A (rs1570360), -634 G/C (rs2010963), 936 C/T (rs3025039); VEGFR2: 889 G/A (V297I) rs2305948; 1416 A/T (Q472H) rs1870377	ORR, PFS, OS	VEGF-A rs699947: AA was associated with longer median OS than C-variants (HR 0.58, 95% CI 0.36 to 0.93, P = 0.023), and VEGF-A rs1570360: AA was associated with longer median OS than G-variants (HR 0.62, 95% CI 0.46 to 0.83, P = 0.001)	[646]
	Hein <i>et al.</i>	GeparQuinto; GBG 44; NCT00567554	III	Neoadjuvant HER-2 negative breast cancer	1948 (1453; n = 724/974 from control arm and n = 7 729/974 (BEV arm)	Control arm: Epirubicin + cyclophosphamide (4C)- docetaxel (4C) (EC-T)						
						BEV arm: Epirubicin + cyclophosphamide (4C)- docetaxel (4C) + BEV (EC-T + BEV)						
							Yes	Peripheral blood	71 SNPs in genes in the VEGF pathway: VEGF-A, PIGF, VEGF-B, VEGF-C, VEGF-D, VEGFR1, VEGFR2, VEGFR3, NRP1	pCR	Homozygous common genotypes for VEGF-A rs833058 (G/G), rs699947 (A/A), rs3025039 (C/C), rs3025030 (G/G), and VEGFR1 rs7995976 (C/C) were significantly associated with higher ORs for pCR (not after correction for multiple testing).	[679]

Table 1.8. (continued) Studies examining the association between SNPs in *VEGF-A*, *VEGFR1* and *VEGFR2* and efficacy outcomes with bevacizumab in other tumour types

Tumour type	Study	Clinical trial name; Clinicaltrials.gov or other identifier	Phase	Treatment line	Sample size (n), n with tissue analysed	Treatment arms	Randomisation to non-BEV control arm	Tissue used	Genetic variants examined	Clinical associations with SNPs examined	Association/s with clinical outcome	REF
Breast continued	Miles <i>et al.</i>	AVADO; BO17708; NCT00333775	III	First-line (HER-2 negative MBC)	736 (396; n = 129/241 from chemotherapy + placebo arm, n = 129/248 from BEV 7.5 mg/kg arm, n = 138/247 from BEV 15 mg/kg arm) ^a	Docetaxel + placebo	Yes	Peripheral blood	<i>VEGF-A</i> : rs699947, rs2010963, rs3025039, rs833061, rs1570360; <i>VEGFR1</i> : rs9554316, rs9582036; <i>VEGFR2</i> : rs1870377, rs2305948, rs2071559, rs3034659	PFS, OS	<i>VEGF-A</i> rs699947 : C allele associated with longer PFS in the placebo arm only (allelic HR 0.73, <i>P</i> = 0.043), and treatment by genotype interaction in the BEV 7.5 mg/kg arm (<i>P</i> = 0.018). <i>VEGF-A</i> rs2010963 : Associated with PFS in placebo arm only (allelic HR 1.50, <i>P</i> = 0.027), and treatment by genotype interaction in the BEV 7.5 mg/kg arm (<i>P</i> = 0.046) ^e No association between these SNPs and PFS in the BEV 15 mg/kg arm.	[617]
						Docetaxel + BEV 7.5 mg/kg						
						Docetaxel + BEV 15 mg/kg						
	Etienne-Grimaldi <i>et al.</i>	MO19391; ATHENA Study; EUDRACT number 2006-002529-21	II	First-line (HER-2 negative locally recurrent breast cancer or MBC)	2251 (137)	Single arm: BEV with chemotherapy (78% taxane-based, investigator's discretion)	No	Peripheral blood	<i>VEGF-A</i> : rs699947, rs833061, rs1570360, rs2010963, rs3025039	ORR, TTP, OS	<i>VEGF-A</i> rs3025039 : T-variants associated with longer TTP (median TTP 11.5 months (95% CI 10.2 to 25.8) in CT or TT patients vs 9.7 months (95% CI 7.8 to 12) in CC patients, log-rank <i>P</i> = 0.022)	[681]

Table 1.8. (continued) Studies examining the association between SNPs in *VEGF-A*, *VEGFR1* and *VEGFR2* and efficacy outcomes with bevacizumab in other tumour types

Tumour type	Study	Clinical trial name; Clinicaltrials.gov or other identifier	Phase	Treatment line	Sample size (n), n with tissue analysed	Treatment arms	Randomisation to non-BEV control arm	Tissue used	Genetic variants examined	Clinical associations with SNPs examined	Association/s with clinical outcome	REF
Breast continued	Makhoul <i>et al.</i>	2 Phase II trials										
		Trial 1: NCT00203502	Trial 1: II	Neoadjuvant Stage IIA-B/IIA-C breast cancer	39 (37) ^b	Single arm: Docetaxel + cyclophosphamide + BEV (4C) - docetaxel (4C), post-operative BEV	No (Two separate single-arm studies analysed)	Peripheral blood	833 SNPs on the HumanOmni2.5-8v1-1 BeadChip, and 1460 SNPs on the HumanOmni5-4 BeadChip system with MAF > 5%, including <i>VEGF-A</i>	pCR	<i>VEGF-A</i> rs833068 and rs833069 : Minor allele counts were associated with increased pCR (OR 3.67, 95% CI 1.16 to 11.60, <i>P</i> = 0.027 and OR 4.95, 95% CI 1.37 to 17.9, <i>P</i> = 0.015, respectively) ^c	[689]
			Trial 2: II	Breast cancer, not specified	55 (38)	Single arm: Docetaxel and cyclophosphamide						
Ovarian	Schultheis <i>et al.</i>	PH-II-45	II	Second- or third-line recurrent EOC or PPC	70 (53)	Single arm: cyclophosphamide + BEV	No	Peripheral blood	<i>VEGF-A</i> : C+405G/C-634G (rs2010963), C+936T (rs3025039); <i>VEGFR2</i> : 11-14 AC repeat +4422 ARNT binding site	ORR, PFS, OS	No significant association between any examined SNPs and PFS	[683]
	Smerdel <i>et al.</i>		PG	Fourth-line or further EOC	38 (38)	Single arm: BEV alone	No	Peripheral blood	<i>VEGF-A</i> : -2578 C/A (rs699947), -1154 G/A (rs1570360), -460C/T (rs833061), +405G/C (rs2010963), +936 C/T (rs3025039)	ORR, PFS, OS	No significant association between any examined SNPs and efficacy outcomes	[690]
Head and neck	Argiris <i>et al.</i>		II	First-line	40 (28)	Single arm: Pemetrexed + BEV	No	Peripheral blood	<i>VEGF-A</i> : rs699947, rs2010963, rs3025039	TTP, OS	<i>VEGF-A</i> G634C (rs2010963) : Significant association with TTP and OS (data not shown). Only one patient had GG genotype, and outcomes were similar for GC and CC genotypes.	[685]

Table 1.8. (continued) Studies examining the association between SNPs in *VEGF-A*, *VEGFR1* and *VEGFR2* and efficacy outcomes with bevacizumab in other tumour types

Tumour type	Study	Clinical trial name; Clinicaltrials.gov or other identifier	Phase	Treatment line	Sample size (n), n with tissue analysed	Treatment arms	Randomisation to non-BEV control arm	Tissue used	Genetic variants examined	Clinical associations with SNPs examined	Association/s with clinical outcome	REF
GBM	Galanis <i>et al.</i>	NCCTG N0776	II	First-line	54 (54)	Group A: Sorafenib 200 mg po BD + BEV	No	Peripheral blood	<i>VEGF-A</i> : rs699947, rs1005230, rs833061, rs1570360, rs2010963, rs25648, rs3025039, and rs10434; <i>VEGFR2</i> : rs2071559, rs2305948, rs1870377, and rs2219471	PFS6	Homozygous minor allele for <i>VEGF-A</i> rs699947 and rs833061 and heterozygous genotype for <i>VEGF-R2</i> rs2071559 associated with superior PFS6. Homozygous minor allele for <i>VEGF-A</i> rs1005230 and rs1570360 associated with inferior PFS6 (HRs not specified).	[686]
						Group B: Sorafenib 200 mg po D + BEV <i>Both arms combined for analysis</i>						
Pancreatic	Lambrechts <i>et al.</i>	AVITA; BO17706; NCT01214720	III	First-line	607 (154; n = 77/306 from control arm and n = 77/301 from BEV arm ^a)	Gemcitabine + erlotinib	Yes	Peripheral blood	157 SNPs, including in the following VEGF family genes: <i>VEGF-A</i> , <i>PIGF</i> , <i>VEGF-B</i> , <i>VEGF-C</i> , <i>VEGF-D</i> , <i>VEGFR1</i> , <i>VEGFR2</i>	PFS, OS	<i>VEGFR1</i> rs9582036 : A allele associated with improved OS and PFS in the BEV arm. Tests for interaction between genotype and treatment effect were statistically significant ($P = 0.041$) for OS. <i>VEGFR1</i> rs9554316 (G allele associated with improved OS and PFS), rs9513070 and rs9554320 (data not shown) associated with OS in the BEV arm (not after correction for multiple testing)	[691]
						Gemcitabine + erlotinib + BEV						
Renal cell carcinoma	Lambrechts <i>et al.</i>	AVOREN; BO17705; NCT00738530	III	First-line	649 (110; n = 51/322 from control arm and n = 59/327 from BEV arm ^a)	Interferon α -2a + placebo	Yes	Peripheral blood	157 SNPs, including in the following VEGF family genes: <i>VEGF-A</i> , <i>PIGF</i> , <i>VEGF-B</i> , <i>VEGF-C</i> , <i>VEGF-D</i> , <i>VEGFR1</i> , <i>VEGFR2</i>	PFS	<i>VEGFR1</i> rs7993418 : T allele was associated with improved PFS in the BEV arm. <i>VEGFR1</i> rs9554316 T allele and rs9513070 A allele were also associated with improved PFS in the BEV arm.	[691]
						Interferon α -2a + BEV						

Table 1.8. (continued) Studies examining the association between SNPs in *VEGF-A*, *VEGFR1* and *VEGFR2* and efficacy outcomes with bevacizumab in other tumour types

Tumour type	Study	Clinical trial name; Clinicaltrials.gov or other identifier	Phase	Treatment line	Sample size (n), n with tissue analysed	Treatment arms	Randomisation to non-BEV control arm	Tissue used	Genetic variants examined	Clinical associations with SNPs examined	Association/s with clinical outcome	REF
Multiple	de Haas <i>et al.</i>	NO16966 in mCRC, AVAGAST (BO20904) in advanced gastric cancer, AVAiL (BO17704) in recurrent/advanced NSCLC, AViTA in metastatic pancreatic cancer, AVOREN in metastatic RCC, AVADO in HER-2 negative MBC	IPD meta-analysis of 6 phase III RCTs	Various	5209 (1402, n = 716 in BEV arm, n = 686 in placebo arm) ^{a, d} • NO16966: 610/1400 ^d (43.5%) • AVAGAST: 289/774 ^d (37%) • AVAiL: 119/1043 ^d (11.4%) • AViTA: 161/607 ^d (26.5%) • AVOREN: 108/649 ^d (16.6%) • AVADO: 350/736 ^d (47.6%)	Various, placebo arm	Yes	Peripheral blood	195 SNPs, including in the following VEGF family genes: <i>VEGF-A</i> , <i>PLGF</i> , <i>VEGF-B</i> , <i>VEGF-C</i> , <i>VEGF-D</i> , <i>VEGFR1</i> , <i>VEGFR2</i> , <i>VEGFR3</i>	PFS, OS	VEGF-A rs699946: G-allele associated with inferior PFS in the BEV arm, however the test for interaction between genotype and treatment effect was not statistically significant.	[667]
						Various, BEV-containing arm						
Multiple	Lambrechts <i>et al.</i>	NO16966 in mCRC, AVAiL (BO17704) in recurrent /advanced NSCLC, AViTA in metastatic pancreatic cancer, AVOREN in metastatic RCC, AVADO in HER-2 negative MBC	IPD meta-analysis of 5 phase III RCTs note overlap from de Haas <i>et al.</i> study	Various	Germline DNA available from n = 1346	Various, placebo arm	Yes	Not specified	158 SNPs	PFS, OS	VEGF-A rs699946: associated with improved PFS in the BEV arm (allelic HR 1.27, 95% CI 1.08 to 1.49, P = 0.003) VEGFR2 rs11133360: T- allele associated with improved PFS in BEV arm	[692]
						Various, BEV-containing arm						
Advanced solid tumours	Falchook <i>et al.</i>		I	Metastatic or advanced solid tumours not amenable to standard therapy	115 (31 to 36 patients, depending on SNP)	Single arm: sorafenib + BEV	No	Peripheral blood or FFPE tumour samples	<i>VEGF-A</i> -2578 (rs699947), -1498 (rs833061), -1154 (rs1570360), -634 (rs2010963)	ORR, TTP	No significant association between any examined SNPs and ORR or TTP	[693]

SNPs: single nucleotide polymorphisms; REF: reference/s; *VEGF-A*: vascular endothelial growth factor-A; *VEGFR1*: vascular endothelial growth factor receptor 1; *VEGFR2*: vascular endothelial growth factor receptor 2; NSCLC: non-small cell lung carcinoma; BPC: paclitaxel + carboplatin + bevacizumab; PC: paclitaxel + carboplatin; BEV: bevacizumab; PFS: progression-free survival, OS: overall survival, ORR: objective response rate; *VEGF-R3*: vascular endothelial growth factor receptor 1; PFS6: 6-month progression-free survival; OR: odds ratio; PG: pharmacogenetic; MBC: metastatic breast cancer; FFPE: formalin-fixed paraffin embedded; *PIGF*: platelet growth factor; *VEGF-B*: vascular endothelial growth factor-B, *VEGF-C*: vascular endothelial growth factor-C; *VEGF-D*: vascular endothelial growth factor-D; *NRP1*: neuropilin 1; pCR: pathological complete response; TTP: time to progression; C: cycles; MAF: minor allele frequency; EOC: epithelial ovarian carcinoma; PPC: primary peritoneal carcinoma; GBM: glioblastoma multiforme; BD: twice daily; D: daily; mCRC: metastatic colorectal carcinoma; RCC: renal cell carcinoma; IPD: individual patient data; RCTs: randomised controlled trials; ^a: SNPs only analysed in Caucasian patients, ^b: only those of African American and European American ethnicity included; ^c: adjusted for race and bevacizumab; ^d: numerator is number of patients who participated in the clinical study; denominator is number of patients who consented to the genetic substudy.

SNPs in *VEGF* family genes may impact on clinical efficacy outcomes if they lead to functional effects on the production or expression of the corresponding ligands and receptors [658]. The mechanism for these functional effects will differ depending on the location of the SNP [694]. For example, SNPs in the promoter region may modulate gene expression by affecting binding of transcription factors [695], hence modifying promoter activity, or by altering DNA methylation [696]. SNPs in the 5'UTR can alter gene expression through alterations in translation efficiency [697], or by modifying transcriptional activity [698]. In contrast, SNPs in the 3'UTR can alter gene expression through changes in messenger ribonucleic acid (mRNA) degradation and translation [694]. Whilst non-synonymous exonic SNPs do not alter the amino acid sequence of the encoded protein, they can alter protein expression indirectly [694], for example, due to codon usage bias and hence translational efficiency [691, 699]. Introns contain multiple functional elements, including intron splice enhancers and silencers which regulate alternate splicing [700, 701] and trans-splicing elements [702], and therefore intronic SNPs can alter gene expression through aberrant splicing [703], and have also been reported to be associated with long-range gene regulation [704, 705].

Table 1.9 shows the results of previous studies which have shown an association between SNPs in *VEGF-A* and *VEGFR2* and gene or protein expression of the corresponding ligand or receptor. Some of these SNPs (e.g., *VEGF-A* rs699946 and rs699947) have also been demonstrated to be located in expression quantitative trait loci (eQTL), supporting the findings that these variants are associated with *VEGF-A* expression levels [667, 679].

Table 1.9. Studies showing an association between SNPs in *VEGF-A* and *VEGFR2* and gene or protein expression

Gene	SNP	Reported association/s	
<i>VEGF-A</i>	rs699946	AA genotype associated with lower <i>VEGF-A</i> mRNA expression <i>in vitro</i> and lower plasma VEGF-A levels in healthy individuals [667]	
	rs699947	- AA genotype associated with lower baseline platelet-normalised serum circulating VEGF-A levels in patients with mCRC [674] - C-variants (in combination with the rs1570360 GG genotype) associated with higher VEGF-A levels in supernatant of PBMCs from healthy volunteers [706] - CC genotype associated with higher VEGF-A levels in PBMCs of renal transplant recipients [707]	
	rs833061	- C-variants associated with higher <i>VEGF-A</i> mRNA expression in primary CRC tissue [708] - C-allele associated with increased VEGF-A expression in CRC tumour tissue assessed using IHC, and higher serum VEGF-A levels in patients with CRC [709] - T-allele associated with lower VEGF-A protein levels in normal colorectal tissue, using ELISA [710] - T-allele associated with lower serum VEGF-A levels in healthy controls [711]	CC genotype associated with lower baseline platelet-normalised serum circulating VEGF-A in patients with mCRC [674]
	rs1570360	- GG genotype (in combination with rs699947 C-variants) associated with higher VEGF-A levels in supernatant of PBMCs from healthy volunteers [706] - GG genotype associated with higher VEGF-A levels in PBMC of renal transplant recipients [707] - A-allele associated with lower VEGF-A protein levels <i>in vitro</i> [712]	
	rs2010963	- C-variants had higher <i>VEGF-A</i> mRNA expression than GG in normal tissue of patients with clear cell RCC [713] - CC genotype associated with higher plasma VEGF-A levels at baseline in patients with GBM [714] - CC genotype associated with higher serum VEGF-A levels in healthy subjects [715] - CC genotype associated with higher <i>VEGF-A</i> mRNA expression in the placenta of women with pre-eclampsia [716] - G-allele associated with lower VEGF-A protein levels <i>in vitro</i> [712]	- C-allele associated with lower VEGF-A protein levels in normal colorectal tissue, using ELISA [710] - G-allele associated with higher VEGF-A levels in LPS-stimulated PBMCs [717]
	rs25648	- T-variants associated with higher <i>VEGF-A</i> mRNA expression in primary CRC tissue [708] - TT genotype associated with higher serum VEGF-A levels from a population-based sample [718]	

Table 1.9. (continued) Studies showing an association between SNPs in *VEGF-A* and *VEGFR2* and gene or protein expression

Gene	SNP	Reported association/s
<i>VEGFR2</i>	rs7655964	G-allele associated with higher <i>VEGFR2</i> mRNA levels in NSCLC tumour samples [719]
	rs1870377	- A-allele associated with lower <i>VEGFR2</i> mRNA levels in resectable NSCLC tumour samples [719] - A-allele significantly associated with lower levels of serum <i>VEGFR2</i> at baseline in patients who had acute coronary syndrome [720]
	rs7667298	A-allele associated with lower <i>VEGFR2</i> mRNA levels in resectable NSCLC tumour samples [719]
	rs2071559	T-allele associated with higher <i>VEGFR2</i> expression assessed using IHC in prostate epithelial cells, in patients with prostate carcinoma or nodular hyperplasia [721]
	rs1551641	A-allele associated with higher <i>VEGFR2</i> mRNA levels in resectable NSCLC tumour samples [719]

SNPs: single nucleotide polymorphisms; *VEGF-A*: vascular endothelial growth factor-A; *VEGFR2*: vascular endothelial growth factor receptor 2; mCRC: metastatic colorectal carcinoma; PBMCs: peripheral blood mononuclear cells; IHC: immunohistochemistry; ELISA: enzyme-linked immunosorbent assay; RCC: renal cell carcinoma; GBM: glioblastoma multiforme; LPS: liposaccharide; NSCLC: non-small cell lung carcinoma.

Moreover, SNPs in *VEGF* family genes may impact on clinical efficacy outcomes if they are associated with variability in microvascular density (MVD), a surrogate marker of tumour angiogenesis. For example, the *VEGF-A* rs833061T-allele has been associated with greater MVD in lung cancer FFPE specimens [722], and the *VEGF-R2* rs2305948 CC genotype and rs2071559 CC genotype have been associated with lower and higher MVD scores than their alternative genotypes in CRC specimens, respectively [665]. Finally, SNPs in *VEGF* family genes have also been linked to other clinical outcomes such as bevacizumab-related hypertension [723], and susceptibility to this toxicity may also be a direct result of common genetic variability in corresponding *VEGF* genes. For example, *VEGFR2* rs1870377 minor allele has been associated with increased hypertension in patients with solid tumours treated with anti-angiogenic agents including sorafenib and/or bevacizumab [724], and the *VEGFR2* rs2305949 C-allele has been associated with increased incidence of bevacizumab-induced hypertension in patients with advanced solid tumours [723].

1.3.5. Summary

Bevacizumab is an active drug in metastatic CRC, and is approved for treatment when given in combination with chemotherapy. This literature review revealed that, whilst the addition of bevacizumab to chemotherapy was demonstrated to improve median PFS in multiple studies, the benefit for OS has been only modest. In addition, the drug is costly, and is associated with multiple side effects. Discovery of a predictive biomarker would enable us to commence alternative therapies and avoid unnecessary side effects in patients who are unlikely to respond, and to improve the cost effectiveness of bevacizumab use by identifying a subset of patients who are more likely to respond.

A review of the literature has revealed multiple previous studies which have explored potential predictive biomarkers of bevacizumab response. These studies have examined potential basal biomarkers including circulating angiogenic factors, circulating endothelial cells and progenitor cells, *in situ* biomarkers (immunohistochemistry, real-time PCR, and *in situ* hybridisation), consensus molecular subtypes (CMS), and somatic copy number alterations (SCNAs), as well as treatment-related biomarkers including hypertension and dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI). However, a validated predictive biomarker for bevacizumab treatment outcome has not yet been identified.

Bevacizumab, a recombinant humanised anti-VEGF monoclonal antibody, is believed to exert its effects by targeting VEGF-A mediated angiogenesis which involves host endothelial cells influenced by germline genetic variability. Therefore, germline variability in *VEGF-A* and its receptors, *VEGFR1* and *VEGFR2*, may be important determinants of heterogeneity in response to bevacizumab. Single nucleotide polymorphisms (SNPs) in these *VEGF* family genes may impact on clinical efficacy outcomes if they lead to functional effects on the production or expression of the corresponding ligands and receptors. Indeed, this literature review identified multiple studies which reported an association between SNPs in *VEGF* family genes, and gene or protein expression of the corresponding ligand or receptor.

The ideal study design for retrospective predictive biomarker analysis is a randomised controlled trial with a non-bevacizumab control arm. A review of the literature revealed that only one of the studies which has examined the association between SNPs in *VEGF-A*, *VEGFR1*, and *VEGFR2*, and efficacy outcomes in patients treated with bevacizumab and chemotherapy for mCRC had this study design.

The MAX clinical trial was a Phase III randomised controlled trial which randomised patients with metastatic CRC to receive first-line treatment with capecitabine (the non-bevacizumab control arm), capecitabine plus bevacizumab, or capecitabine, bevacizumab and mitomycin. In

Chapter 5 of this thesis we report the results of our translational study which examined whether SNPs in *VEGF-A*, *VEGFR1* and *VEGFR2* were predictive biomarkers for bevacizumab response in the MAX clinical trial.

2. MATERIALS AND METHODS

2.1. Cell culture

2.1.1 Cell lines

All cell lines used in this thesis, and information about their origin, properties and source are listed in Table 2.1.

Table 2.1. Origin, properties, and source of human cancer cell lines used in this thesis

Cell line	Origin	Properties	Source	Reference
Human colorectal cancer cell lines				
Co-115	Adenocarcinoma of the ascending colon	Adherent	Diego Arango, Vall d'Hebron Research Institute, Spain	[725]
COLO201	Metastatic cells from ascitic fluid in a patient with primary adenocarcinoma of the colon ^a	Suspension, with some loosely adherent cells	ATCC	[726]
COLO205	Metastatic cells from ascitic fluid in a patient with primary adenocarcinoma of the colon ^a	Mixed adherent and suspension	ATCC	[726]
HCT116 ^{RasWT} (HAF1)	HCT116 isogenic cell line (adenocarcinoma of the colon), genotype KRAS2 +/-	Adherent	Bert Vogelstein, Baltimore, USA	[727]
HCT116 ^{RasMut} (HAE6)	HCT116 isogenic cell line (adenocarcinoma of the colon), genotype KRAS2 D13/-	Adherent	Bert Vogelstein, Baltimore, USA	[727]
HT29	Adenocarcinoma of the colon	Adherent	ATCC	[728]
LIM2405	Adenocarcinoma of the caecum	Adherent	LICR, Parkville	[729]
LIM2551	Adenocarcinoma of the transverse colon	Loose aggregates	LICR, Parkville	[730]
LS411N	Adenocarcinoma of the caecum	Adherent	Oliver Sieber, LICR, Parkville	[731]
RKO	Adenocarcinoma of the colon	Adherent	ATCC	[732]
SW1417	Adenocarcinoma of the colon	Adherent	Robyn Ward, University of New South Wales	[733]
VACO5	Adenocarcinoma of the caecum	Adherent	Oliver Sieber, LICR, Parkville	[734]
VACO432	Adenocarcinoma of the colon	Adherent	Bert Vogelstein, Baltimore, USA	[735]
Human malignant melanoma cell lines				
LMME28	Malignant melanoma	Adherent	CIBL, ONJCRI	[736]
LMME64	Malignant melanoma ^b	Adherent	CIBL, ONJCRI	[736]
LMME70	Malignant melanoma ^b	Adherent	CIBL, ONJCRI	[736]
SK-MEL-3	Malignant melanoma, from lymph node	Adherent	CIBL, ONJCRI	[733, 737]
SK-MEL-28	Malignant melanoma	Adherent	CIBL, ONJCRI	[738]

ATCC: American Type Culture Collection; LICR: Ludwig Institute for Cancer Research; CIBL: Cancer Immuno-biology Laboratory; ONJCRI: Olivia Newton-John Cancer Research Institute; ^aDerived from ascites of the same patient; ^bDerived from different tumours of the same patient.

2.1.2. Maintaining and passaging cell lines

Cell culture work was performed in a Class II biohazard hood. All cell lines were maintained in Dulbecco's Modified Eagle Medium Nutrient Mixture (DMEM/F-12) (Gibco®, Life Technologies, Australia), which was supplemented with 10% foetal bovine serum GOLD (FBS) (PAA Laboratories, Australia), 1% 1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer solution (HEPES) (Gibco®, Life Technologies, Australia) and 1% 10,000 U/mL Penicillin Streptomycin (Gibco®, Life Technologies, Australia), in a 37°C incubator with 5% CO₂.

Cells were passaged when approximately 80% confluence in a T75 or T175 cell culture flask (BD Biosciences, USA) was reached.

Adherent cell lines were passaged by aspirating the old growth medium, washing with Dulbecco's phosphate-buffered saline (DPBS, no calcium, magnesium or phenol red; 2.67 mM KCl, 1.47 mM KH₂PO₄, 137.93 mM NaCl, 8.06 mM Na₂HPO₄·7H₂O) (Gibco®, Life Technologies, Australia) and incubating with 3 mL or 7 mL (for T75 and T175 flasks, respectively) of TrypLE™ Express Enzyme Trypsin Replacement (Life Technologies, Australia) at 37°C until cells detached from the flask surface. Fresh growth medium was added to the cells (7 mL or 13 mL for T75 and T175 flasks, respectively), and the cells were passaged at a ratio of 1:10 to 1:5, depending on the cell line.

Cell lines with mixed adherent and suspension properties were passaged by collecting the old growth medium with suspension cells in a 15 mL or 50 mL Falcon® conical centrifuge tube (Corning Life Sciences, USA), centrifuging the cell suspension at 100 x *g* for 5-10 minutes, and aspirating the growth medium supernatant to the level of the cells. The resulting cell pellet was gently resuspended in fresh growth medium. The adherent cells in the flask were washed with DPBS (no calcium, magnesium or phenol red) (Gibco®, Life Technologies, Australia), incubated with TrypLE™ Express Enzyme Trypsin Replacement (Life Technologies, Australia) at 37°C until cells detached from the flask surface, and fresh growth medium was added to the cells as described above. The growth media with cells from the suspension component and from the adherent component were combined in a single cell culture flask, and cells were passaged at a ratio of 1:10 to 1:5.

2.1.3. Long-term storage of cell lines

Cell lines grown in cell culture flasks were cryopreserved in 1 mL of freezing solution comprising 50 v/v% growth medium (as described in 2.1.2), 40 v/v% FBS and 10 v/v% dimethyl sulfoxide (DMSO), aliquoted into 2 mL internal threaded Corning® cryogenic vials (Corning Life Sciences, USA), and stored in liquid nitrogen.

To thaw cell lines, cryogenic vials were removed from the liquid nitrogen tanks and thawed rapidly in a 37°C water bath for 1-2 minutes. Thawed cells were resuspended in 9 mL of pre-warmed fresh growth medium (as described in section 2.1.2.) in a 15 mL conical centrifuge tube (Corning Life Sciences, USA), and centrifuged at 1000 rpm for 5 minutes. The supernatant was aspirated, cells were resuspended in 10 mL of fresh growth medium, and maintained in a 37°C incubator with 5% CO₂.

2.1.4. Mycoplasma testing

Cell lines were tested for Mycoplasma upon receipt from external sources, if mycoplasma status was unclear or unknown, and prior to long term storage.

Mycoplasma screening was performed by the Cell Bank Services at the Ludwig Institute for Cancer Research (Austin Hospital, Melbourne), and subsequently the Olivia Newton-John Cancer Research Institute, using the MycoAlert® Mycoplasma Detection Kit (Lonza, Australia).

This assay was performed on a 5 mL sample of supernatant taken from a cell culture flask containing cells which had been cultured for a minimum of 14 days, to confluence, in antibiotic-free media.

2.1.5. Cell counting and seeding

To perform cell counting, cells were detached from their cell culture flasks or plates using TrypLE™ Express Enzyme Trypsin Replacement (Life Technologies, Australia), and resuspended in growth medium.

Ten µL of the cell suspension was loaded into the counting chamber of a TC10™ counting slide (Bio-Rad Laboratories, Australia), which was inserted into a TC10™ Automated Cell Counter (Bio-Rad Laboratories, Australia). The cell counter automatically calculated the total number of cells per mL.

The optimum seeding density for cell lines was assessed by determining the number of cells required to achieve approximately 80% confluence in the appropriate cell culture plate after the same number of days as the duration of the experiment. The optimum seeding density was used in all subsequent experiments.

2.2. Drug treatments

After cell lines were seeded into cell culture plates, the cells were incubated for 24 hours at 37°C in 5% CO₂, prior to treatment with drugs or vehicle control.

All drugs used in this thesis, and information about their form, diluent, storage conditions and supplier are listed in Table 2.2.

Table 2.2. Form, diluent, storage condition, and supplier of drugs used in this thesis

Compound name	Form	Diluent	Storage condition	Supplier
Vemurafenib (PLX4032, RG7204)	Powder	DMSO	-20°C	Roche, USA and Selleck Chemicals, USA
Dabrafenib mesylate (GSK2118436B)	Powder	DMSO	-20°C	ChemieTek, USA
PLX8394 (PB03)	Powder	DMSO	-20°C	Plexxikon, USA
Trametinib	Powder	DMSO	-20°C	Selleck Chemicals, USA
Cetuximab	5 mg/mL solution	Normal saline (sodium chloride, 0.9% solution)	4°C	Merck Serono, Australia
SCH772984	Powder	DMSO	-20°C	ApexBio, USA

DMSO: Dimethyl sulfoxide (Sigma-Aldrich, USA)

For experiments where human cancer cell lines were treated with drug/s and a control condition, the control condition utilised the same concentration of vehicle (DMSO or saline) as the drug condition/s.

2.3. DNA and RNA techniques

2.3.1. RNA extraction

Following treatment with drug or vehicle control, growth medium was aspirated from the cell culture plate and cells were washed with DPBS (no calcium, magnesium or phenol red) (Gibco®, Life Technologies, Australia). Cells were harvested by gentle scraping with a Falcon cell scraper (Corning Life Sciences, USA), collected in an Axygen 1.7 mL microcentrifuge tube (Corning Life Sciences, USA), and centrifuged at 300 x *g* for 3 minutes at 4°C.

Total RNA was isolated from approximately 1 x 10⁶ cultured cells using the High Pure RNA Isolation Kit (Roche Life Sciences, USA). Cell pellets were resuspended in 200 µL PBS, and the suspension was mixed with 400 µL Lysis/Binding buffer and vortexed for 15 s. The sample was transferred to a High Pure Filter Tube, which was centrifuged at 8,000 x *g* for 15 s, and the flowthrough in the Collection Tube was discarded.

For each sample, a mixture of 10 µL DNase I and 90 µL DNase Incubation Buffer was added to the filter fleece and incubated for 15 min at room temperature.

The High Pure Filter Tube was washed in a three-step process: (i) 500 µL of Wash Buffer I was added to the upper reservoir of the Filter Tube, which was centrifuged for 15 s at 8,000 x *g*, (ii) 500 µL of Wash Buffer II was added to the upper reservoir of the Filter Tube, which was

centrifuged for 15 s at 8,000 x *g*, and (iii) 200 µL of Wash Buffer II was added to the upper reservoir of the Filter Tube, which was centrifuged for 2 min at 13,000 x *g*. The flowthrough in the Collection Tube was discarded after each centrifugation step.

RNA was eluted by inserting the Filter Tube into a sterile Axygen 1.7 mL microcentrifuge tube (Corning Life Sciences, USA), adding 50 – 100 µL of Elution Buffer to the upper reservoir of the Filter Tube, and centrifuging it for 1 min at 8,000 x *g*. The eluted RNA was collected in the microcentrifuge tube, and either used immediately in experiments or stored at -80°C for later use.

2.3.2. Extraction of genomic DNA from cell pellets

Total DNA was purified from cultured cells using the DNeasy® Blood and Tissue kit (Qiagen, Germany) according to the manufacturer's instructions. In brief, a cell pellet with up to 5×10^6 cells was resuspended in phosphate-buffered saline (PBS), mixed with proteinase K and buffer, and incubated at 56°C for 10 minutes. Ethanol was added to the mixture, which was further vortexed and transferred into a supplied spin column, and then centrifuged at room temperature. After two further steps of adding buffer to the spin column followed by centrifuging at room temperature, the spin column was placed in a 2 mL microcentrifuge tube. Elution buffer was added to the DNeasy spin column membrane, incubated for 1 minute at room temperature, and centrifuged. The eluted genomic DNA was stored at -80°C for future use.

2.3.3. Determining RNA or DNA concentration

The NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA) was used to determine the nucleic acid (RNA or DNA) concentration of samples, and their purity.

In brief, 2 µL of an appropriate buffer (the solution which the molecule of interest was suspended in) was loaded and measured first, to establish a blank. Subsequently, 2 µL of each sample was loaded and measured, with the pedestals thoroughly cleaned using a dry laboratory wipe between each sample. The spectrophotometer automatically measured the nucleic acid concentration in ng/µL. The ratio of absorbance at 260 nm and 280 nm (260/280 ratio) was used to assess nucleic acid purity. For RNA, a 260/280 ratio of ~ 2.0 was considered to reflect high purity and suitability for use in subsequent experiments. For DNA, a 260/280 ratio of ~ 1.8 was considered to reflect high purity and suitability for use in subsequent experiments.

2.4. Gene expression profiling

2.4.1. Gene expression microarray

Gene expression profiling was performed on 11 *BRAF*^{V600E} CRC cell lines treated with vemurafenib (5 µM) or DMSO control for 6 hours, using the Affymetrix HG-U133- Plus-2 Human Genome Array platform at the Peter MacCallum Cancer Centre genomics core facility.

2.4.2. Analysis of gene expression microarray

Affymetrix microarray CEL files were imported into Partek Genomics Suite version 6.6 (Partek, Missouri USA) and normalised using the Robust Multiarray Averaging (RMA) method. Normalised data was analysed using the Limma package, which uses linear models to determine differential expression in microarray data [739, 740]. The moderated paired t-test was utilised to allow the cell line pair effects to be considered in the linear model. Affymetrix probes were determined to be differentially expressed between DMSO control and vemurafenib treatment if the adjusted *P*-value was < 0.05 (this takes into account a false discovery correction).

2.5. Western blot

2.5.1. Cell lysate preparation

2.5.1.1. Cell pelleting

Following vehicle or drug treatment, the media was aspirated and cells were washed twice in cold DPBS. Cells were then harvested by scraping on ice with a Falcon cell scraper (Corning Life Sciences, USA) and transferred to a microcentrifuge tube. The cells were pelleted by centrifugation at 300 x *g* at 4°C for 15 minutes in a table-top centrifuge (Heraeus Fresco 17 Centrifuge, Thermo Scientific, USA). Following removal of supernatant, cell pellets were lysed immediately for use in Western Blot, or stored at -80°C for future use.

2.5.1.2. Cell lysis

Cells were lysed in NP-40 lysis buffer (1% IGEPAL CA-630 pH 7.4, 50 mM Tris-HCl, 150 mM NaCl, 0.5% sodium deoxycholate, 3M urea (all from Sigma-Aldrich, USA) and 1mM ethylenediaminetetraacetic acid (EDTA, Thermo Fisher Scientific, USA), and supplemented with a cOmplete™ Protease Cocktail Inhibitor tablet (one tablet per 50 mL lysis buffer; Roche Diagnostics, Germany) and a PhosSTOP™ phosphatase inhibitor tablet (one tablet per 10 mL lysis buffer; Roche Diagnostics, Germany). Cell pellets were resuspended in the lysis buffer, vortexed and incubated for a minimum of 15 minutes on ice. The cell lysate was centrifuged at 17,000 x *g* at 4°C for 15 minutes. The supernatant containing the extracted proteins was transferred to a fresh microcentrifuge tube for immediate protein quantification.

2.5.2. Protein quantification

The concentration of the extracted proteins was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA). Briefly, diluted bovine serum albumin (BSA) standards were prepared using the Albumin Standard ampoules provided. The BCA working reagent was prepared by combining BCA Reagents A and B at room temperature. BSA standards, lysis buffer blanks, and samples were pipetted into individual wells of a Falcon® 96-well flat bottom microplate (BD Falcon, Australia), BCA working reagent was added to each well, and the microplate was mixed thoroughly on a Stuart microtitre plate shaker (Bibby Scientific, UK). The

microplate was incubated at 37°C for 15 minutes, and cooled to room temperature. Absorbance was measured at 562 nm using a VERSA_{max} micro-plate reader (Molecular Devices, USA), and protein concentrations were determined using standard curves generated from blank-corrected BSA standards.

2.5.3. Protein gel electrophoresis

2.5.3.1. Sample preparation

Samples were prepared for gel electrophoresis by mixing the samples with NuPAGE[®] LDS sample Buffer (4X) (Thermo Fisher Scientific, USA) and NuPAGE[®] reducing agent (10X), and heating at 95°C for 8 minutes in a QBD1 dry block heating system (Grant Instruments, UK).

2.5.3.2. Gel Electrophoresis

For the majority of Western Blot experiments, gel electrophoresis was performed using NuPAGE[®] Novex[®] 4-12% Bis-Tris Mini or Midi Protein Gels in an XCell SureLock[™] Mini-Cell or an XCell4 SureLock[™] Midi-Cell Electrophoresis System (Life Technologies, USA), respectively. The Upper and Lower Buffer Chambers of the electrophoresis system were filled with 1X Running Buffer, prepared by mixing 20X NuPAGE[®] MES or MOPS SDS running buffer (Life Technologies, USA) with deionized water. Protein molecular weight markers (Novex[®] Sharp Pre-stained Protein Standards, Life Technologies, USA) and samples were loaded onto the gel, and NuPAGE[®] Antioxidant (Life Technologies, USA) was added to the 1X Running Buffer in the Upper Chamber of the electrophoresis system just prior to commencing electrophoresis.

For Western Blot experiments examining expression of phospho-mTOR and total mTOR, NuPAGE[®] Novex[®] 3-8% Tris-Acetate Mini Protein Gels, NuPAGE[®] Tris-Acetate SDS running buffer, and HiMark[™] Pre-stained protein standards (all from Life Technologies, USA) were used.

2.5.4. Protein transfer, blocking, and probing with primary and secondary antibodies

The iBlot[®] Gel Transfer Device or iBlot[™] 2 Gel Transfer Device (Invitrogen, USA) were used to perform dry blotting of proteins from mini- or- midi- gels using iBlot[®] Gel Transfer Stacks with nitrocellulose transfer membranes (mini or regular size, respectively) (Life Technologies, USA).

1X PBS was prepared by mixing 10X PBS (Ambion, USA) with deionized water. PBS-T was prepared with 1X PBS and 0.1% Tween[®] 20 (Sigma-Ultra, USA).

Membranes were blocked using Odyssey[®] Blocking Buffer (Li-Cor, USA) for one hour at room temperature, and then incubated with primary antibodies diluted in a solution of 50% Odyssey[®] Blocking Buffer and 50% PBS-T with 0.1% Tween 20, using the conditions described in Table 2.3.

Membranes were washed four times for 5 minutes each in PBS-T, and incubated with the species-appropriate fluorescent IRDye secondary antibody (Table 2.4) which was diluted in 50% Odyssey[®] Blocking Buffer/50% PBS-T solution with 0.1% Tween 20 and 0.01% sodium dodecyl sulphate (SDS)(Sigma- Aldrich, USA) for one hour at room temperature, protected from light.

Membranes were washed three times for 5 minutes in PBS-T and once for 5 minutes in PBS, then dried and stored, protected from light.

Table 2.3. Primary antibodies used in Western Blots

Primary Antibody	Isotype/ Source	Supplier and product number	Antibody concentration	Condition	
				Temperature	Incubation time
Anti-phospho-EGFR (Tyr1068)	Rabbit IgG	Cell Signalling, USA (#3777)	1:500	4°C	Overnight
Anti-EGFR	Rabbit IgG	Cell Signalling, USA (#4267)	1:750	4°C	Overnight
Anti-phospho-CRAF (Ser338)	Rabbit IgG	Cell Signalling, USA (#9427)	1:500	4°C	Overnight
Anti-phospho-MEK 1/2 (Ser217/221)	Rabbit IgG	Cell Signalling, USA (#9154)	1:2000	4°C	Overnight
Anti-MEK1/2	Mouse IgG1	Cell Signalling, USA (#4694)	1:1000	4°C	Overnight
Anti-phospho-ERK1/2 (Thr202/Tyr204)	Rabbit IgG	Cell Signalling, USA (#4370)	1:1000	4°C	Overnight
Anti-ERK1/2	Mouse IgG1	Cell Signalling, USA (#9107)	1:1000	4°C	Overnight
Anti-phospho-p90RSK (Thr359/Ser363)	Rabbit	Cell Signalling, USA (#9344)	1:500	4°C	Overnight
Anti-phospho-FRA1 (Ser265)	Rabbit IgG	Cell Signalling, USA (#5841)	1:2000	4°C	Overnight
Anti-phospho-mTOR (Ser2448)	Rabbit IgG	Cell Signalling, USA (#2976)	1:500	4°C	Overnight
Anti-mTOR	Rabbit IgG	Cell Signalling, USA (#2983)	1:1000	4°C	Overnight
Anti-phospho-Akt (Ser473)	Rabbit IgG	Cell Signalling, USA (#4060)	1:500	4°C	Overnight
Anti-Akt1	Rabbit IgG	Cell Signalling, USA (#2938)	1:750	4°C	Overnight
Anti-phospho-S6 (Ser240/244)	Rabbit IgG	Cell Signalling, USA (#5364)	1:2500	4°C	Overnight
Anti-S6	Mouse IgG1	Cell Signalling, USA (#2317)	1:1250	4°C	Overnight
Anti-PTEN	Rabbit IgG	Cell Signalling, USA (#9559)	1:1000	4°C	Overnight
Anti-beta-Actin	Mouse IgG2a	Sigma-Aldrich, USA (A5316)	1:15,000	4°C	Overnight
Anti-beta-Tubulin	Rabbit IgG	Abcam, USA (ab6046)	1:30,000	4°C	Overnight

IgG: Immunoglobulin G; EGFR: epidermal growth factor receptor; mTOR: mammalian target of rapamycin.

Table 2.4. Secondary antibodies used in Western Blots

Secondary Antibody	Supplier	Antibody concentration	Condition	
			Temperature	Incubation time
IRDye® 680RD Goat anti-Mouse	Li-Cor®, USA	1:25,000	RT	1 hour
IRDye® 800CW Goat anti-Rabbit	Li-Cor®, USA	1:15,000	RT	1 hour

RT: room temperature.

2.5.5. Protein detection and quantitative analysis of Western Blots

Western blot membranes were imaged on the Odyssey Infrared Imager (Li-Cor®, USA). Quantitative analysis of western blots was performed using Image Studio Lite software (Version 5.x) (Li-Cor®, USA). Bands for proteins of interest were individually analysed, and background corrected values for each band were obtained by subtracting the median value of the pixels in the background segment. Background corrected values for each protein of interest were divided by the background corrected values for the corresponding loading control (e.g., beta-actin or beta-tubulin), as indicated in the legends. For quantitative analyses where expression of phospho-proteins were normalised to expression of the corresponding total protein, firstly background corrected values for the phospho-protein were divided by the background corrected values for the corresponding loading control (resulting in the value 'x'). Secondly, background corrected values for the total protein were divided by the background corrected values for the corresponding loading control (resulting in the value 'y'). Normalisation was then performed by dividing 'x' by 'y'.

2.6. Cell growth and viability assays

2.6.1. MTS assay

Cell viability was assessed using the colourimetric Cell Titer 96® AQueous Non-radioactive Cell Proliferation Assay. This assay utilised solutions of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega, Australia) and the electron coupling reagent phenazine methosulfate (PMS) (Sigma-Aldrich, USA).

The MTS solution was prepared by dissolving 2 mg/mL of MTS reagent powder in DPBS. The pH of the MTS solution was adjusted to between 6.0 and 6.5 using 1M HCl, and filtered into a sterile, light protected container using a 0.2 µm Acrodisc filter (Pall Corporation, USA). The PMS solution was prepared by dissolving 0.92 mg/ml of PMS in DPBS. Both solutions were stored at -20°C, and thawed prior to use. A combined MTS/PMS solution comprising 100 µL of PMS solution for every 2 mL of MTS solution was used for the MTS assay.

Cells were seeded into Falcon® 96-well flat bottom microplates at the optimum seeding density for $t = 72$ hr absorbance measurements (in quadruplicate for each condition), and incubated at 37°C in 5% CO_2 for 24 hrs. Additionally, cells were seeded into a 96-well microplate at the optimum seeding density for $t = 0$ hr (t_0) absorbance measurements (in quadruplicate for each cell line), and incubated at 37°C in 5% CO_2 for 24 hrs.

After 24 hours of incubation, the $t = 0$ hr microplate was used for determination of t_0 absorbance values for each cell line. The combined MTS/PMS solution (20 μL) was added to each well of the $t = 0$ hr plate and incubated at 37°C in 5% CO_2 for 90 minutes. Absorbance was assessed at 490 nm using a VERSA_{max} Microplate Reader and Softmax Pro 4.8 software (both from Molecular Devices, USA). Absorbance at the reference wavelength of 630 nm was also determined as a background, and the value subtracted from the 490 nm reading. Media was gently aspirated from the $t = 72$ hr microplates using a glass pipette under gentle suction or by directly pipetting out the media, and cells treated in quadruplicate with vehicle control and drug conditions.

After a further 72 hours of incubation at 37°C in 5% CO_2 , the 490 nm and 630 nm absorbances were assessed in the $t = 72$ hr 96-well microplates, as above. The differing relative rates of cell growth for each cell line was adjusted for in the analysis by subtracting the t_0 absorbance for a cell line from the $t = 72$ hr absorbances in the vehicle control and treatment conditions for that cell line. Cell viability results for each condition were presented as percentage cell proliferation relative to vehicle control.

2.6.2. Analysis of drug interactions

Analysis of synergy between two drugs was performed using Compusyn software (ComboSyn, Inc., USA). For each cell line, the MTS assay was used to obtain cell proliferation data for each drug at four different drug treatment concentrations, and for the combination of the two drugs at one drug treatment concentration.

The Chou & Talalay method was used to calculate a combination index (CI), which quantifies synergism or antagonism for two drugs:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2}$$

$$= \frac{(D)_1}{(D_m)_1 \left[\frac{f_a}{1-f_a} \right]^{\frac{1}{m_1}}} + \frac{(D)_2}{(D_m)_2 \left[\frac{f_a}{1-f_a} \right]^{\frac{1}{m_2}}}$$

, where D denotes dose,

$(D_x)_1$ is the dose for drug 1 alone that inhibits a system $x\%$,

$(D_x)_2$ is the dose for drug 2 alone that inhibits a system $x\%$, and

$(D)_1 + (D)_2$ in combination inhibit the system $x\%$.

f_a is the fraction affected by D (i.e., percentage inhibition /100),

D_m is the 'median-effect dose' that inhibits the system under study by 50%, and

m is the coefficient signifying the shape of the dose effect relationship ($m = 1$, > 1 , and < 1 indicate hyperbolic, sigmoidal and flat sigmoidal dose-effect curves, respectively) [741].

CI values of < 1 , $= 1$ and > 1 were considered synergistic, additive and antagonistic, respectively [742].

F_a - CI drug combination plots (CI on the y-axis as a function of effect levels (f_a) on the x-axis) were generated automatically using the Compusyn software [741].

2.6.3. GI₅₀ analysis

The GI₅₀ for vemurafenib was calculated for the *BRAF*^{V600E} colorectal cancer and melanoma cell lines. The GI₅₀ was defined as the drug concentration that results in a 50% inhibition of the net cell proliferation relative to control cell growth [743, 744], and was calculated using Prism 5 (GraphPad Software, USA).

2.6.4. xCELLigence real-time cell analyser

The Roche xCELLigence System uses E-plates which contain sensor electrode arrays in each of the 96 wells. When cells are present on top of these electrodes, they affect the ionic environment at the electrode-solution interface, resulting in an increase in electrical impedance. The more cells attached to the electrodes, the larger the increase in electric impedance. The Cell Index is a parameter which is derived as a relative change in the measured electrical impedance, and is a quantitative measure of cell numbers present in a well. Therefore, the Cell Index can be used to measure cell proliferation. The Real-Time Cell Analyzer (RTCA) continuously measures electrical impedance data, and thus can perform real-time monitoring of cell proliferation.

The Roche xCELLigence System was used for real-time monitoring of cell proliferation of *BRAF*^{V600E} colorectal cancer and melanoma cell lines, after treatment with DMSO control or vemurafenib (1 μ M). Cell lines were seeded in quadruplicate at the optimal seeding density in an E-Plate 96 (Roche Diagnostics, GmbH). The Real-Time Cell Analyzer MP instrument (Roche Diagnostics, GmbH), together with the E-Plate 96, was placed in a cell culture incubator maintained at 37°C with 5% CO₂, and continuous electrical impedance measurements were taken hourly for 8 days. Cell Index values were plotted against time for each cell line, using RTCA software version 1.2.1 (Roche Diagnostics, GmbH).

2.7. RAS activation assay

2.7.1. RAS activation assay

RAS activation was measured using the commercially available RAS GTPase ELISA kit (chemiluminescent) (ab13460; Abcam, Cambridge, USA). The following samples were used: the positive control HCT116^{RasMut} (HAE6, genotype *KRAS2* D13/-) with its isogenic cell line HCT116^{RasWT} (HAF1, genotype *KRAS2* +/-), the positive control EGF treated HeLa whole cell extract (provided in the kit), and the samples SW1417, COLO201, LIM2551, LS411N, RKO, VACO432, and SKMEL28 which had been treated with DMSO control or vemurafenib 5 μ M for 6 hours.

Reagents and the supplied positive control were prepared as per manufacturer's instructions. For sample collection, following treatment with DMSO control or vemurafenib, growth medium was aspirated from the cell culture plate and cells were washed with DPBS (no calcium, magnesium or phenol red) (Gibco®, Life Technologies, Australia). Cells were harvested as described above (in section 2.5.1.1) and cell pellets incubated in Complete Lysis/Binding Buffer for 15 minutes at 4°C. After vortexing the tube for 10 seconds, and further centrifuging for 10 minutes at 14,000 rpm at 4°C, the supernatant was collected. The Bradford Assay (Quickstart 1x dye reagent; Bio-Rad, California, USA) was used to performed protein quantification of these test extracts, as per manufacturer's instructions.

The RAS activation assay was performed per manufacturer's instructions, as summarised below:

1. Ras binding

GST-Raf-RBD (2 μ g diluted in 50 μ L of Complete Lysis/Binding Buffer) was added to each well, and the plate was incubated for 1 hour at 4°C with mild agitation. Each well was washed 3 times with 200 μ L 1X Wash Buffer, and the controls, samples and blanks were added to the wells in duplicate. For the HeLa whole cell extract positive control, 25 μ g was diluted in 50 μ L of Complete Lysis/Binding Buffer per well. For the HCT116 control and other samples 100 μ g was diluted in 50 μ L of Complete Lysis/Binding Buffer per well. For the blanks, 50 μ L of Complete Lysis/Binding Buffer only was added to each well. The plate was incubated for 1 hour at room temperature with mild agitation, followed by washing of each well 3 times with 200 μ L 1X Wash Buffer.

2. Binding of primary antibody

H-Ras antibody (50 μ L diluted 1:500 in 1X Antibody Binding Buffer) was added to each well. The plate was incubated for 1 hour at room temperature without agitation, followed by washing of each well 3 times with 200 μ L 1X Wash Buffer.

3. Binding of secondary antibody

Horseradish peroxidase (HRP)-conjugated secondary antibody (50 µL diluted 1:5000 in 1X Antibody Binding Buffer) was added to each well. The plate was incubated for 1 hour at room temperature without agitation, followed by washing of each well 4 times with 200 µL 1X Wash Buffer.

4. Chemiluminescence detection

Chemiluminescent Working Solution (50 µL) was added to each well, and chemiluminescence was read using the SpectraMax L Microplate Reader using SoftMax Pro Software (both from Molecular Devices, USA).

2.7.2. Computation of RAS activity

To calculate RAS activity in Relative Light Units (RLU) for each control and sample, the mean of the reading from the two blanks was initially subtracted from the reading for each well. The mean and standard deviation were then calculated using the duplicates for each control and sample.

2.8. Determination of PI3K pathway activation status in *BRAF*^{V600E} colorectal cancer cell lines

We systematically searched for established mutations in *mTOR*, *PIK3R1*, *PIK3CA* and *PTEN* using (i) mutational data from the whole exome screening of cell lines in the Catalogue of Somatic Mutations in Cancer (COSMIC, version 86, Genome version GRCh38) (accessed at <https://cancer.sanger.ac.uk/cosmic>), (ii) mutational data from the targeted massively parallel sequencing and mass spectrometric genotyping of cell lines from the Cancer Cell Line Encyclopaedia (CCLE, accessed at <https://portals.broadinstitute.org/ccle>) [745, 746], and (iii) data from the whole exome-sequencing of colorectal cancer cell lines previously performed by our laboratory in collaboration with the Sieber Laboratory (Systems Biology and Personalised Medicine Division, Walter and Eliza Hall Institute of Medical Research, Parkville, Australia) [747], as well as (iv) cell line mutation data from the American Type Culture Collection (ATCC) (accessed at <https://www.atcc.org/>) [733].

Additionally, loss of PTEN protein expression was measured by performing western blot for the PTEN protein in the cell lines.

2.9. CpG Island Methylation Phenotype testing of *BRAF*^{V600E} cell lines

CpG Island Methylation Phenotype (CIMP) testing was performed on genomic DNA extracted from CRC cell lines, using a quantitative DNA methylation assay with a five-gene panel (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, *SOCS1*) [200]. CIMP testing was performed by A/Prof Dan Buchanan at the QIMR Berghofer Medical Research Institute, Queensland.

2.10. Microsatellite instability characterisation of *BRAF*^{V600E} cell lines

Information on MSI characterisation of *BRAF*^{V600E} CRC cell lines was obtained from the published paper by Medico *et al.* who performed MSI testing using the MSI Analysis System kit (Promega) [748].

2.11. Statistical analysis of pre-clinical data

Associations between independent variables with 2 levels and continuous variables were calculated using the Mann Whitney test. Associations between interval variables and continuous variables were calculated using Spearman's correlation.

2.12. MAX trial biological substudy

2.12.1. MAX clinical trial design

The MAX (Mitomycin, Avastin, and Xeloda) clinical trial was an investigator-initiated, open-label, multicentre (in Australia, New Zealand, and the United Kingdom), randomised Phase III study of capecitabine, bevacizumab and mitomycin in the first-line treatment of metastatic colorectal cancer, the results of which have been previously published [532].

2.12.1.1. Ethics approval

Institutional ethics approval was obtained for the study, and the patients provided written informed consent to participate. The study was performed in accordance with the Declaration of Helsinki.

2.12.1.2. Patient eligibility

Eligibility criteria included: age 18 years or older; histologically confirmed diagnosis of colorectal adenocarcinoma; measurable or non-measurable unresectable metastatic disease; judged by the investigator to be suitable for capecitabine monotherapy; adequate bone marrow, renal function, and hepatic function; Eastern Cooperative Oncology Group (ECOG) performance status (PS) of ≤ 2 ; and a life expectancy of at least 12 weeks. Adjuvant chemotherapy (≥ 6 months prior to relapse) was permitted, however no other prior chemotherapy was allowed.

Exclusion criteria included: uncontrolled clinically significant cardiac disease, hypertension, arrhythmias, or angina pectoris; acute myocardial infarction or cerebrovascular accident within 12 months; regular aspirin use of greater than 325 mg per day or regular use of non-steroidal anti-inflammatory drugs; central nervous system metastases; active bleeding disorders within 3 months; major surgical procedure within 28 days; a serious non-healing wound, ulcer, or bone fracture; 24-hour urinary protein of > 2 g in 24 hours; pregnancy; and prior history of other malignancy (except those treated with curative intent > 2 years previously without evidence of relapse) [532].

2.12.1.3. Patient evaluation for efficacy outcomes

A contrast enhanced computed tomography (CT) scan of the thorax, abdomen, and pelvis was performed within 21 days before the first study treatment, and then every 6 weeks on study. Response rates (RRs) were assessed according to RECIST, version 1.0 [749].

Patients underwent clinical assessment with history and physical examination whilst receiving study treatment. After study treatment ceased, patients had a clinical assessment every 12 weeks until death.

2.12.1.4. Treatment regimens

Patients were stratified by age (< 65 years vs $\geq$ 65 years), ECOG PS (0 or 1 vs 2), capecitabine dose (1 vs 1.25 g/m² twice daily), and institution, and randomly assigned in a 1:1:1 ratio to receive treatment with capecitabine (C), capecitabine plus bevacizumab (CB), or capecitabine, bevacizumab and mitomycin (CBM). Treatment was planned to continue until confirmed disease progression. The doses of treatments used are included in Table 2.5:

Table 2.5. Treatment doses of drugs in the MAX clinical trial

Drug treatment	Dosage	Comments
Capecitabine	1.25 g/m ² oral twice daily, day 1 to 14, every 3 weeks	• A lower starting dose (i.e., 1 g/m² oral twice daily) could be used by investigators for patients considered at risk of toxicity • Patients with creatinine clearance rates of 30 to 50 mL/min were commenced at a dose of 1g/m² oral twice daily
Bevacizumab	7.5 mg/kg intravenous on day 1, every 3 weeks	
Mitomycin	• 7 mg/m² intravenous on day 1 (maximum dose 14 mg), every 6 weeks for four treatments (i.e., 24 weeks) • After 24 weeks, patients continued with capecitabine and bevacizumab treatment	• Mitomycin treatment was ceased after 24 weeks to minimise the risk of HUS.

HUS: haemolytic uraemic syndrome

2.12.1.5. Statistical design and analysis

The primary endpoint of the study was progression free survival (PFS), which was defined as disease progression according to RECIST, or death from any cause. The two planned primary comparisons were (i) C versus CB and (ii) C versus CBM. Sample size calculations determined that 450 patients ($n = 150$ per arm) would provide 80% power to detect at least a 2.5 month

improvement in PFS, from 5.5 months to 8 months (a 33% relative reduction in risk), with 36 months for accrual and 12 months of additional follow-up. To preserve an overall significance level of 5%, a two-tailed 2.5% significance level for each was used.

Secondary endpoints included overall survival (OS), RR and quality of life (QOL).

For the PFS and OS endpoints, Kaplan-Meier curves were generated and compared using log-rank tests. Cox proportional hazards models were used to explore and adjust for significant prognostic co-variables for these endpoints. Pre-specified subgroup analyses were performed to ascertain whether any clinico-pathological characteristics were predictive of bevacizumab treatment efficacy, using Cox regression models with the treatment co-variate (pooled CB and CBM vs C), clinico-pathological characteristic of interest, and their interaction. The proportions of patients with complete or partial tumour responses in each treatment group were compared using χ^2 tests.

All comparisons were performed using intent to treat analyses, without adjustment for multiple comparisons. Analyses were performed using SAS (version 9.2; SAS Institute, Cary, NC) or ACCoRD (Analysis of Censored and Correlated Data).

2.12.2 MAX biological substudy – single nucleotide polymorphisms (SNPs) in vascular endothelial growth factor (VEGF) family genes as prognostic and predictive biomarkers

2.12.2.1 Ethics approval

The MAX biological substudy examining single nucleotide polymorphisms (SNPs) in vascular endothelial growth factor (VEGF) family genes as prognostic and predictive biomarkers was an exploratory *post-hoc* translational study.

At enrolment all participants in the MAX clinical trial were provided with the opportunity to consent to participate in translational research, which required tissue banking of their archived formalin-fixed paraffin embedded (FFPE) tissue specimens. Separate ethics approval was obtained for the translational sub-study.

2.12.2.2. Acquisition and storage of patient tissue specimens

Archived FFPE tissue specimens collected at the time of diagnosis and associated histopathology reports were retrieved from the pathology departments at the hospital where participants were diagnosed. FFPE tissue blocks were then stored centrally at Austin Health, Melbourne, Australia.

2.12.2.3. Blinding

Staff at the central storage site for tumour specimens at Austin Health, Melbourne, Australia, and all investigators performing scientific assays using these specimens for the MAX biological substudies, were blinded to patient identification and outcomes. Archived FFPE tissue specimens

were only identified by trial identification (ID) number. All data on the MAX clinical trial outcomes was stored at a separate site - the National Health and Medical Research Council (NHMRC) Clinical Trials Centre (CTC) (Camperdown, New South Wales, Australia).

2.12.2.4. Extraction of genomic DNA from FFPE patient specimens

2.12.2.4.1. Tissue cores

2 x 4 µm sections were cut from the FFPE tissue blocks using a rotary microtome, and these sections were placed on glass slides for Haematoxylin and Eosin (H&E) staining.

Where available (in order of preference), a core of normal tissue, colorectal adenoma tissue, or colorectal tumour tissue were extracted using a tissue microarray (TMA) Mark II Arrayer, (Beecher Instruments, USA), using the H&E slide to guide the coring procedure.

For the first fifty cases (ordered consecutively by patient ID) where both colorectal tumour tissue and adjacent normal colorectal epithelial tissue were available, cores of matching tumour and normal tissue were extracted.

2.12.2.4.2. Genomic DNA extraction

Genomic DNA was extracted from the tissue cores using the QIAamp ® DNA FFPE Tissue Kit (Qiagen, Germany), as per manufacturer's instructions. In brief, six steps were performed as follows:

1. Removal of paraffin from the tissue cores

Excess paraffin was trimmed from tissue cores using a new scalpel blade. The remaining tissue was placed in a microcentrifuge tube and dissolved in xylene, then centrifuged at full speed for 2 minutes before removing the supernatant, leaving a tissue pellet.

The tissue pellet was mixed with 100% ethanol to extract any residual xylene from the sample, and then centrifuged at full speed for 2 minutes, before removing supernatant, leaving a tissue pellet. The tissue pellet was incubated for 10 minutes at room temperature in the open microcentrifuge tube to enable evaporation of residual ethanol.

2. Lysis of sample under denaturing conditions

The tissue pellet was resuspended and mixed in 180 µL Buffer ATL and 20 µL proteinase K, then incubated at 56°C in a QBD1 dry block heating system for at least 1 hour, until completely lysed.

3. Reversal of formalin crosslinking by heating

The sample was incubated at 90°C in the heating block for 1 further hour, to partially reverse formaldehyde modification of nucleic acids. Two µL of RNase A (100 mg/mL stock) was incubated with each sample for 2 minutes at room temperature.

4. Binding of DNA to the membrane

Each sample was mixed with 200 µL of Buffer AL and 200 µL of 100% ethanol, to yield a homogenous solution.

5. Washing to remove residual contaminants

Buffers AW1 and AW2 were reconstituted at room temperature by adding 25 mL and 30 mL of 100% ethanol to the bottles of concentrate, respectively.

Each lysate sample was transferred to a QIAamp MinElute column and washed in three repeated stages which involved centrifuging at 6000 x *g* for 1 minute, discarding the flow-through, and adding fresh wash buffer. Finally, the column was centrifuged at full speed for 3 minutes to completely dry the membrane and remove any ethanol carryover.

6. Elution of DNA from the membrane

The QIAamp MinElute column was then placed in a clean microcentrifuge tube, and 20-100 µL of Buffer ATE was applied to the centre of the membrane and incubated at room temperature for 5 minutes. The microcentrifuge tube together with the QIAamp MinElute column was then centrifuged at full speed for 1 minute to elute genomic DNA, and the column discarded. The eluted genomic DNA was used immediately, or stored at -80°C for future use.

2.12.2.5. SNP genotyping assays

SNP genotyping was performed using Taqman® SNP genotyping assays (Applied Biosystems, USA) on the ViiA™ 7 Real Time PCR system (Applied Biosystems, USA).

Candidate SNPs in *VEGF-A*, *VEGFR1*, and *VEGFR2* were selected on the basis of previous demonstration of an effect on corresponding VEGF ligand or receptor function and/or expression, and/or an association with clinical outcomes. Selected SNPs also had a global minor allele frequency (MAF) of 10% or more.

The context sequences and primer sequences for the Taqman® SNP genotyping assays used are listed in Table 2.6. For the context sequences, the alleles denoted **[1/2]** correspond to VIC reporter-labelled Allele 1 and FAM reporter-labelled Allele 2, respectively.

Table 2.6. Context sequences for Taqman® SNP genotyping assays

Gene	SNP	Context sequence/primer sequences	Taqman ® Assay ID
VEGF-A	rs699946	TTGTTTAAATTCTCTGACAGAGACA[A/G]ACTGT CCCGGGGGCGGGGAAGGCA	C___1647395_10
	rs699947	GCCAGCTGTAGGCCAGACCCTGGCA[A/C]GATCT GGGTGGATAATCAGACTGAC	C___8311602_10
	rs833061	GAGTGTGTGCGTGTGGGGTTGAGGG[C/T]GTTGG AGCGGGGAGAAGGCCAGGGG	C___1647381_10
	rs1570360	AGCCCGGGCCCCGAGCCGCGTGTGGA[A/G]GGGC TGAGGCTCGCCTGTCCCCGCC	C___1647379_10
		Forward primer (5' to 3'): TCACTTCGGACCGGGC Reverse primer (5' to 3'): CCGCTACCAGCCGACTTTTAA Reporter 1 (first allele/G, VIC): CTCAGCCCCTCCACAC Reporter 2 (second allele/A, 6-FAM): CCTCAGCCCTTCCACAC	Not applicable- Custom made
	rs2010963	CGCGCGGGCGTGCAGCAGCGAAAG[C/G]GACA GGGGCAAAGTGAGTGACCTGC	C___8311614_10
	rs25648	GCCGCGCCGGCCCCGGTCGGGCCTC[C/T]GAAAC CATGAACCTTCTGCTGTCTT	C___791476_10
VEGFR1	rs3025039	Forward primer (5' to 3'): ACTCCGGCGGAAGCATTC Reverse primer (5' to 3'): AGCAAGAAAAATAAAATGGCGAATCCA Reporter 1 (first allele/C, VIC): CAAGAGGGACCGTGCTG Reporter 2 (second allele/T, 6-FAM): AAGAGGGACCATGCTG	Not applicable- Custom made
	rs9513071	TTGCCCAACTTGTGGCCACGGGCT[A/G]CATGA ATCCCAGGATGGCTTTGAGT	C___30362252_10
	rs9554316	AAAGACATCATTCGATTTTTTTTCT[G/T]GAACTC AATGGAATTCTCAGCCCCC	C___32231224_20
	rs7993418	GGCTCATGAACTTGAAAGCATTTAC[A/G]TATCT AATGAAGAAACAGAAAGAAT	C___1910654_10
	rs9582036	TGTGCCCAGCAACAATAGCCTTCTT[A/C]CAAAA TGTATACTAAAGTATTTAGG	C___1910658_10
VEGFR1	rs9554320	TGTGTGTAGCTGATCATTCTCCCC[A/C]GCACTG CAAAGCCGCTGTTACAAAC	C___32231227_10

Table 2.6. (continued) Context sequences for Taqman® SNP genotyping assays

Gene	SNP	Context sequence/primer sequences	Taqman ® Assay ID
VEGFR2	rs12505758	ATGTCAACATATCTCGATGCAATAC[C/T]TGGCA TCATAGATTGGCAGAGTAA	C__1673865_10
	rs7655964	GTGTATATGTACCACATTTTTTTT[A/C]TCCCAC TGACCTTCTATTATGAAAA	C__26111282_10
	rs1870377	GGTATGGGTTTGTCACTGAGACAGC[A/T]TGGCT ATAAGAAAGAGATAACAGCG	C__11895315_20
	rs2305948	TACAATCCTTGGTCACTCCGGGTTA[C/T]ACCATC TATAGTTAAGGTGCTCAAA	C__22271999_20
	rs2305949	CCCTAGAGCAAGTAAATTGAAAAAA[C/T]AGAA CATGAGAGAGCAAATAAGCCT	C__16192173_20
	rs11133360	TTCACATTGCTATGCCCAACACATC[C/T]CATCA AGCATTCTCAGAGTGCTTCA	C__26111278_10
	rs7667298	GCAGAGGAAACGCAGCGACCACACA[C/T]TGAC CGCTCTCCCGGGTCCCGGGA	C__28992770_10
	rs2071559	GAAAACGCACTTGCCAGTTCGCCA[A/G]CATTC CCGCTATTTCCCAAAATATT	C__15869271_10
	rs1551641	TTGGCAGAGTCTCAATGAAATCAGT[C/T]TACCA CATTCCCTCCCACACAGCAC	Not applicable- Custom made

SNP: single nucleotide polymorphism; *VEGF-A*: vascular endothelial growth factor-A; *VEGFR1*: vascular endothelial growth factor receptor 1; *VEGFR2*: vascular endothelial growth factor receptor 2

Quantification of extracted genomic DNA was performed as described in section 2.3.3. For each SNP assay, 5 ng of genomic DNA from each trial participant was diluted in 2.25 µL nuclease-free water, and mixed thoroughly with 2.75 µL of reaction mix consisting of 2X Taqman ® GTXpress Master Mix (Applied Biosystems, USA) and 20X TaqMan genotyping assay mix (Applied Biosystems, USA). Samples were then loaded into MicroAmp® Optical 384-Well Reaction Plates with Barcode (Applied Biosystems, USA), with each plate including at least one non-template control (NTC) where genomic DNA was substituted with nuclease-free water. The plates were vortexed, centrifuged and PCR reactions performed on the ViiA™ 7 Real-Time PCR System with the following conditions: (i) a pre-read stage at 60°C for 30 seconds, (ii) a hold stage with an initial DNA polymerase activation step (95°C for 20 seconds), (iii) a PCR stage with 40 to 60 amplification cycles using a two-step cycling protocol of denaturing at 95°C for 1 second and annealing and extension at 60°C for 20 seconds, and (iv) a post-read stage at 60°C for 30 seconds.

2.12.2.6. Analysis of SNP genotyping assays

After completing the run, experiments were analysed using the Applied Biosystems® ViiA™ 7 Real-Time PCR System software (Applied Biosystems, USA). In brief, Allelic Discrimination Plots produced by the software contrast the normalised reporter dye fluorescence (Rn) for the allele-specific probes of each SNP assay. These plots were used to identify which of the three possible genotypes, (i) homozygous Allele 1, (ii) homozygous Allele 2, and (iii) heterozygous Allele 1/2, applied to each participant sample. These genotypes cluster (i) along the x-axis of the plot, (ii) along the y-axis of the plot, and (iii) mid-way between the homozygote clusters, respectively. The NTC lies at the bottom-left corner of the plot, and undetermined samples may lie anywhere on the plot. The genotyping optimization tool was used to reveal traces to determine the ideal cycle for clustering. Genotyping experiments were performed in duplicate as a minimum.

2.12.2.7. Hardy-Weinberg Equilibrium and Minor Allele Frequency

The Hardy-Weinberg principle is a fundamental population genetics principle which states that genotype frequencies at a locus are determined by allele frequencies, and that the genotype and allele frequencies remain unaltered over generations, assuming that there is no mutation, migration, selection and deviation from random mating [750, 751]. In this state, the population is in Hardy-Weinberg Equilibrium (HWE) [752]. Genotypes *AA*, *AB* and *BB* of a bi-allelic genetic marker are expected to occur with frequencies of p^2 , $2pq$, and q^2 , where p and $q = 1 - p$ are the allele frequencies for alleles *A* and *B*, respectively [753]. As most large, randomly mating populations are expected to be in HWE [754], significant deviations from HWE are often due to genotyping errors [752]. Hence, testing for departure from HWE is widely used to detect genotyping errors [755-759].

We tested each SNP for departure from HWE, and those with significant deviations were excluded from further analyses. The null hypothesis for these tests is that the genotype is in HWE in the population. The HWE *P*-value (the probability that deviation from HWE could be explained by chance) was calculated for each SNP using the Haploview 4.2 software, which utilises the exact test for computation [760]. A Bonferroni correction was applied to a significance level of $P > 0.05$, to correct for multiple testing [750].

The Minor Allele Frequency (MAF) of a SNP is defined as the allele frequency of the second most common allele [761]. The MAF for each SNP was calculated using the Haploview 4.2 software [760].

2.12.2.8. *VEGF family gene and protein expression analysis using Almac Xcel microarray and Bio-plex® arrays*

2.12.2.8.1. Almac Xcel microarray

Gene expression profiling of MAX study samples was performed using FFPE samples of primary tumours from participants. H&E stained sections were reviewed by a pathologist and macro-dissected to obtain tumour-rich tissue, from which RNA was extracted and converted to cDNA for microarray hybridisation. Gene expression profiling was performed using the Almac Xcel microarray (Almac, Craigavon, UK), using Affymetrix GeneChip technology optimised for use with FFPE tissue, as previously described by our laboratory [638]. Whilst over 97,000 transcripts were interrogated using this microarray, only the data for *VEGF-A* gene expression was used in the analysis of associations with the *VEGF-A* SNP genotypes associated with PFS and/or OS.

2.12.2.8.2. Bio-plex® arrays

Pro-angiogenic protein expression levels were analysed using FFPE samples of primary tumours from MAX study participants, as previously described [762]. In brief, protein lysates were prepared from FFPE sections of primary tumour, quantified using the EZQ protein assay kit (Life Technologies, Carlsbad, CA), and assayed for IL-6, IL-8, bFGF, PDGF-BB, and VEGF-A protein expression using a custom multiplex Bio-Plex® array on the Bio-Plex® 200 System instrument (Bio-Rad, USA). Standard curves were generated using a five-parameter logistic regression model, and results were expressed in pg/mL. Only the data for VEGF-A protein expression was used in the analysis of associations with *VEGF-A* SNP genotypes which were found to be associated with PFS and/or OS.

2.12.3. Statistical analysis of the MAX biological substudy

Statistical analysis was performed using the statistical package ACCoRD (Analysis of Censored and Correlated Data) version 8.58, Boffin Software, Eastwood NSW. All reported *P*-values are two-sided.

2.12.3.1. *Statistical analysis of concordance in genotype frequencies in matched normal and tumour tissue*

In the first fifty cases (ordered consecutively by patient ID) where both tumour tissue and adjacent normal tissue were available, SNP genotyping was performed using the matched normal and tumour tissue. For each SNP, statistical analysis of concordance in the frequencies of each genotype (homozygous major allele, homozygous minor allele, and heterozygous major and minor alleles) in normal compared to tumour tissue was calculated using Kendall's τ (a measure of concordance for ordered categories), in cases where full genotypes in both normal and tumour tissue were determined. This measure is a correlation, with high values signifying strong association (in terms of frequencies) of normal and tumour tissue groups.

2.12.3.2. Statistical analysis of survival outcomes

Statistical analysis for the survival time-to-event outcomes PFS and OS was performed using the proportional hazards model.

For each SNP, two comparisons were performed to determine the association between the SNP genotype and survival outcome (i.e., to determine whether SNP genotype was a prognostic biomarker). In the first comparison, outcomes in the participants with a homozygous major allele genotype were compared to outcomes in the participants without a homozygous major allele genotype (i.e., composite of participants with homozygous minor allele and heterozygous major and minor allele genotypes). In the second comparison, outcomes in the participants with a homozygous minor allele genotype were compared to outcomes in the participants without a homozygous minor allele genotype (i.e., composite of participants with homozygous major allele and heterozygous major and minor allele genotypes).

Those SNPs showing a univariate association with survival outcomes were included in a model containing a treatment covariate ('CB and CBM' vs C), and a 'SNP by treatment' interaction, to determine whether SNP genotype was a predictive biomarker for resistance to Bevacizumab. These SNPs were also tested in a multivariate model containing baseline factors which were shown to be associated with survival outcomes in the primary MAX study [532]. These covariates were treatment received, ECOG performance status, neutrophils, bilirubin and alkaline phosphatase levels, prior radiotherapy, whether the primary tumour was resected and the number of metastatic sites. Variables which were significantly associated with the survival outcomes in the multivariate model were included in the final model.

In further analyses, additionally, a multivariate model containing *KRAS* and *BRAF* mutation status along with the other baseline factors was tested. Variables which were significantly associated with the survival outcomes were included in the final model.

2.12.3.3. Statistical analysis of response rate

Statistical analyses were performed to determine the association between SNP genotype and treatment group in patients who achieved an objective response (best response CR/PR) and those who did not (best response SD/PD). A logistic regression model with treatment covariate, SNP genotype status and their interaction was used to assess whether SNP genotype was predictive of an effect of bevacizumab on ORR.

2.12.3.4. Statistical analysis of association between SNP genotype and corresponding VEGF-A gene and protein expression

For SNPs which were found to be associated with survival outcomes, the association between SNP genotype and corresponding log2 transformed gene expression data from the Almac Xcel microarray and Bioplex® suspension array analyses were analysed using t-tests.

3. RESULTS – Mechanisms of resistance of $BRAF^{V600E}$ colorectal cancer to BRAF inhibitors

3.1. Introduction

Clinical trials of single-agent BRAF inhibitors for the treatment of advanced *BRAF* mutant malignant melanoma had impressive results, with significant improvements in response and survival outcomes [23-32]. In contrast, clinical trials of BRAF inhibitors in *BRAF* mutant mCRC had unexpectedly disappointing results [33-35]. In the phase II multi-centre international pilot study of vemurafenib in patients with $BRAF^{V600E}$ metastatic CRC, only one of 19 patients (5%) with assessable disease achieved a confirmed PR, although ten further patients had some degree of tumour shrinkage on their first planned CT scan [33] (Table 1.4).

In this chapter, I present the results of comprehensive pre-clinical studies which explore mechanisms of resistance of $BRAF^{V600E}$ CRC to BRAF inhibitors.

3.2. $BRAF^{V600E}$ colorectal cancer cell lines can be relatively sensitive or resistant to BRAF inhibitors

Firstly, we determined whether the clinical variability in tumour shrinkage in patients with metastatic CRC treated with vemurafenib on the Phase II clinical trial [33] was also reflected *in vitro* in $BRAF^{V600E}$ CRC cell lines.

To assess whether there were $BRAF^{V600E}$ CRC cell lines which were relatively sensitive and relatively resistant to vemurafenib, we examined growth inhibition of 11 $BRAF^{V600E}$ CRC cell lines and 5 $BRAF^{V600E}$ malignant melanoma cell lines after 72 hours treatment with DMSO control or vemurafenib at a range of doses (0.001 μ M to 5 μ M), using MTS assays (Figure 3.1).

An *in vitro* vemurafenib dose of 5 μ M is comparable to efficacious achievable human plasma drug concentrations [763]. At this dose, we found marked differences in growth inhibition among the $BRAF^{V600E}$ CRC cell lines. Growth inhibition relative to control ranged from -0.21 ± 0.14 (mean $\pm$ standard deviation (SD)) in the most sensitive cell line, SW1417, to 0.44 ± 0.14 in the most resistant cell line, VACO432 (Figure 3.1E). Whilst vemurafenib had cytotoxic effects (interpreted based on bars falling below the $t = 0$ hr baseline) on three $BRAF^{V600E}$ CRC cell lines (SW1417, COLO201, and COLO205), its effect on the other eight cell lines was only cytostatic (Figure 3.1E).

Interestingly, we also observed that the most sensitive $BRAF^{V600E}$ CRC cell lines had comparable growth inhibition to the $BRAF^{V600E}$ melanoma cell lines, where the mean growth inhibition ranged from -0.40 ± 0.14 (mean $\pm$ SD) to 0.05 ± 0.16 . Vemurafenib had cytotoxic effects on four out of the five $BRAF^{V600E}$ melanoma cell lines (LMMEL28, SKMEL28, LMMEL70, and SKMEL3, but not LMMEL64).

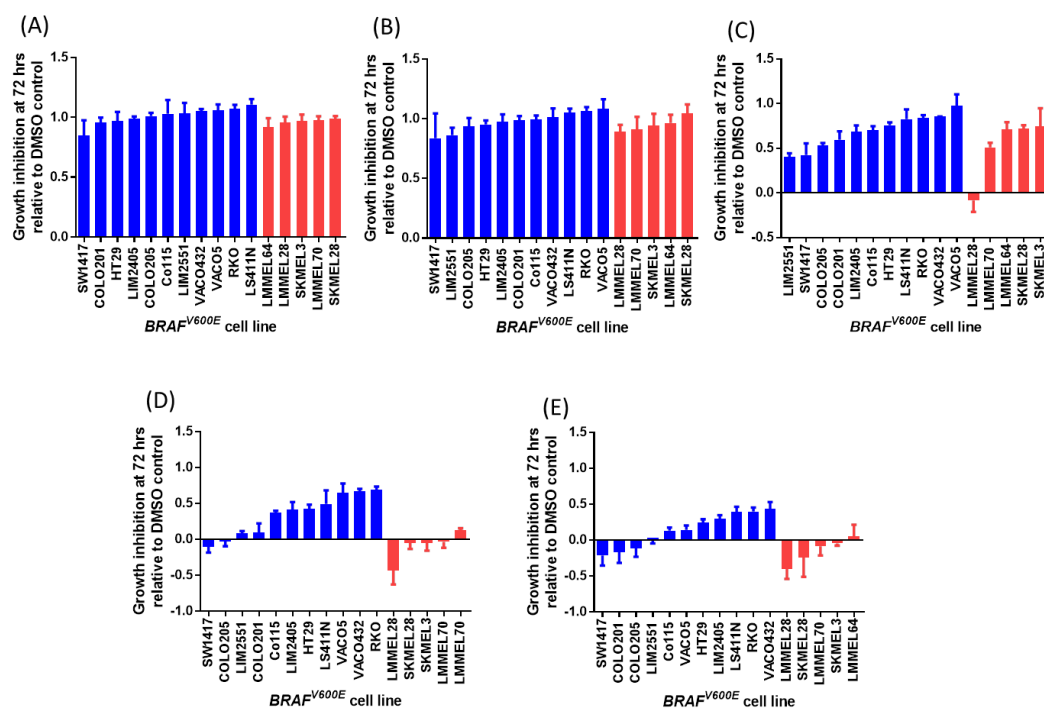


Figure 3.1. MTS assays showing growth inhibition of 11 *BRAF*^{V600E} colorectal carcinoma cell lines and 5 *BRAF*^{V600E} malignant melanoma cell lines after 72 hours treatment with DMSO control or vemurafenib (A) 0.001 uM, (B) 0.01 uM, (C) 0.1 uM, (D) 1 uM, and (E) 5 uM. Data shown are the mean and SD for growth inhibition after vemurafenib treatment, relative to DMSO control. Data is from three biological replicate experiments, each performed in technical quadruplicate.

3.3. Investigating potential biomarkers of *BRAF* inhibitor resistance in *BRAF*^{V600E} colorectal cancer cell lines

3.3.1. Basal characteristics of *BRAF*^{V600E} colorectal cancer cell lines

To investigate potential basal characteristics of *BRAF*^{V600E} mCRC which may be associated with response to vemurafenib, we first considered potential resistance mechanisms that may be common to CRC and malignant melanoma, as well as other distinct features of CRC not shared by malignant melanoma.

3.3.1.1. *PI3K* pathway activation status

A subset of *BRAF* mutant CRCs and melanomas harbor parallel activation of the *PI3K* pathway, which some studies have reported to be a determinant of response to *BRAF* inhibitors. Specifically, *PTEN* loss, which occurs in approximately 20% of *BRAF* mutant melanoma [152], has been reported to be a mechanism of inherent resistance in those tumours [764]. Notably, *PTEN* mutations also co-occur in 22% of *BRAF* mutant CRC [290].

Constitutive activation of the PI3K signaling pathway in human cancers can occur through activating mutations in exons 9 and 20 of the *PIK3CA* gene [765]. Whilst *PIK3CA* mutations are rare in *BRAF* mutant melanoma [152], co-mutation occurs in 13% of *BRAF* mutant CRC [290]. Mao *et al.* previously found that *BRAF* mutant CRC cell lines with *PIK3CA* mutations or PTEN loss were more resistant to the BRAF inhibitor PLX4720 [290].

Mutations in *PIK3R1* are also associated with PI3K pathway activation in CRC [46], due to a decreased ability to inhibit the PI3K p110a subunit [766, 767]. Similarly, there are *MTOR* mutations which activate the PI3K pathway [768, 769].

To assess whether there was heterogeneity in PI3K activation status among the 11 *BRAF*^{V600E} CRC cell lines which may account for their differential sensitivity to vemurafenib, we assessed PI3K pathway activation status in the cell lines by systematically searching for established mutations in *mTOR*, *PIK3R1*, *PIK3CA*, and *PTEN*. We searched mutational data from the Catalogue of Somatic Mutations in Cancer (COSMIC) [770], the Cancer Cell Line Encyclopaedia (CCLE) [745, 746] and the American Type Culture Collection (ATCC) [733], and our own data from whole exome-sequencing of a large panel of colorectal cancer cell lines [747].

We also measured loss of PTEN protein expression by performing western blot for the PTEN protein in the cell lines (Figure 3.2, Table 3.1).

Of note, of the 8 *BRAF*^{V600E} CRC cell lines which had been characterised by sequencing from COSMIC, CCLE and/or Mouradov *et al.*, all 8 harboured an established functional mutation in *mTOR*, indel in *PIK3R1*, mutation in *PIK3CA*, or an indel in *PTEN* (Table 3.1).

Table 3.1: Data on mutations and deletions in key genes in the PI3K pathway and loss of PTEN protein expression, for the 11 *BRAF*^{V600E} CRC cell lines

<i>BRAF</i> ^{V600E} CRC cell line	Characterised by sequencing	Mutation/deletion in key gene in PI3K pathway							PTEN protein expression from Western blot
		Gene	Mutation/ Indel	Type of Mutation/ Indel	Heterozygous/ homozygous	CDS mutation	AA mutation	Source/s	
LIM2551	Yes	<i>PIK3CA</i>	Mutation	Substitution-Missense	Heterozygous	c.3140A>G	p.H1047R	COSMIC [770], Mouradov <i>et al.</i> [747]	Wild type
SW1417	Yes	<i>PIK3R1</i>	Indel	Deletion	Homozygous	c.1_2175del2175	No information	ATCC [733]	Wild type
COLO205	Yes	<i>MTOR</i>	Mutation	Substitution-Missense	Heterozygous	c.3578C>T	p.P1193L	COSMIC [770], CCLE [745, 746], Mouradov <i>et al.</i> [747]	Wild type
COLO201	Yes	<i>MTOR</i>	Mutation	Substitution-Missense	Heterozygous	c.3578C>T	p.P1193L	COSMIC [770], CCLE [745, 746], Mouradov <i>et al.</i> [747]	Wild type
Co115	No	NA							Null
LIM2405	Yes	<i>PTEN</i>	Indel	Frameshift-insertion	Homozygous	c.862_863insA	p.V290fs*8	COSMIC [770], Mouradov <i>et al.</i> [747]	Null
HT29	Yes	<i>PIK3CA</i>	Mutation	Substitution-Missense	Heterozygous	c.1345C>A	p.P449T	COSMIC [770], CCLE [745, 746], Mouradov <i>et al.</i> [747], ATCC [733]	Wild type
LS411N	Yes	<i>PTEN</i>	Indel	Frameshift-deletion	Heterozygous	c.310delT	p.C105fs*8	COSMIC [770]	Null
		<i>PTEN</i>	Indel	Frameshift-deletion	Heterozygous	c.487delA	p.K164fs*3	COSMIC [770], CCLE [745, 746]	
		<i>PTEN</i>	Indel	Frameshift-deletion	No information	No information	p.F104fs	CCLE [745, 746]	
VACO5	No	NA							Wild type
RKO	Yes	<i>PIK3CA</i>	Mutation	Substitution-Missense	Heterozygous	c.3140A>G	p.H1047R	COSMIC [770], CCLE [745, 746], Mouradov <i>et al.</i> [747], ATCC [733]	Wild type
VACO432	No	NA							Wild type

CRC: colorectal cancer; NA: not applicable.

Of the remaining 3 cell lines which had not been characterised by sequencing, western blot analysis of the PTEN protein found Co115 to be PTEN null (Figure 3.2). VACO432 has been reported to harbour a heterozygous P124T mutation in *PIK3CA* [727], however, this has not been characterised as being an activating mutation [299], and there was insufficient data to definitively determine the PI3K pathway activation status of the VACO5 cell line.

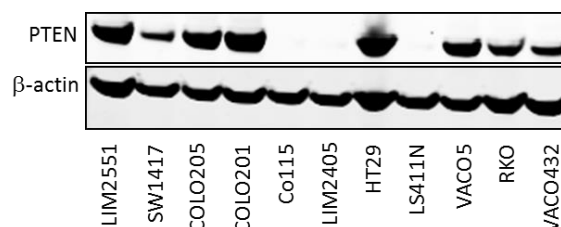


Figure 3.2. Western blot of basal PTEN protein expression in the 11 *BRAF*^{V600E} colorectal carcinoma cell lines

Therefore, as the vast majority of *BRAF* mutant CRC cell lines also harboured simultaneous activation of the PI3K pathway, a definitive analysis could not be performed. Nevertheless, our data indicate that even among cell lines with concurrent mutation of *BRAF* and activation of PI3K signalling, variable responses to vemurafenib are evident, suggesting that alternate mechanisms must account for the differential sensitivity to vemurafenib observed in the 11 *BRAF*^{V600E} CRC cell lines.

To confirm these findings, we examined whether basal expression of key proteins in the PI3K/AKT/mTOR signalling pathway were associated with sensitivity of *BRAF*^{V600E} CRC cell lines to vemurafenib.

We calculated the GI₅₀ for vemurafenib in the 11 *BRAF*^{V600E} CRC cell lines using growth inhibition data from MTS assays after 72 hours treatment with DMSO control or vemurafenib at doses of 0.001 uM, 0.01 uM, 0.1 uM, 1 uM, and 5 uM (Figure 3.3). We then performed western blot of p-Akt, p-mTOR, and the downstream effector p-S6, ordered by increasing GI₅₀ for vemurafenib (Figure 3.4).

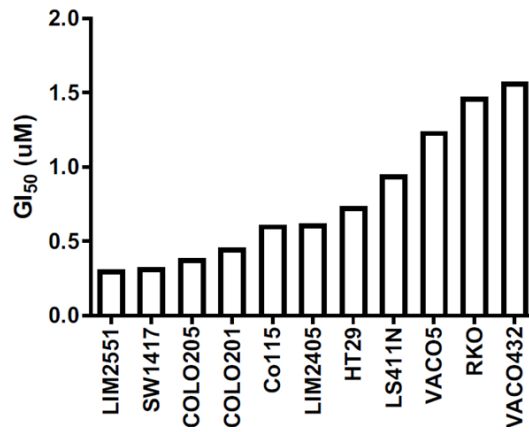


Figure 3.3. *BRAF*^{V600E} colorectal carcinoma cell lines ordered by GI₅₀ for vemurafenib (from lowest on the left, to highest on the right)

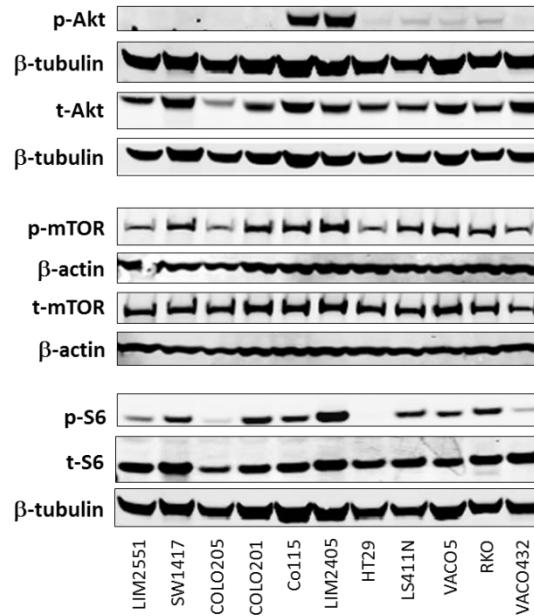


Figure 3.4. Western blots of basal expression levels of proteins in the PI3K/AKT/mTOR signaling pathway in *BRAF*^{V600E} colorectal carcinoma cell lines, ordered by GI₅₀ for vemurafenib (from lowest on the left, to highest on the right).

There was no significant correlation between the GI₅₀ for vemurafenib and basal protein expression of PTEN ($r = -0.15$, $P = 0.67$), p-Akt/t-Akt ($r = 0.33$, $P = 0.33$), p-mTOR/t-mTOR ($r = 0.32$, $P = 0.34$), or p-S6/t-S6 ($r = 0.055$, $P = 0.88$) in the 11 *BRAF*^{V600E} CRC cell lines (Figure 3.5 A-D), confirming that mechanisms other than basal activation of the PI3K pathway account for the differential sensitivity to vemurafenib observed in the 11 *BRAF*^{V600E} CRC cell lines.

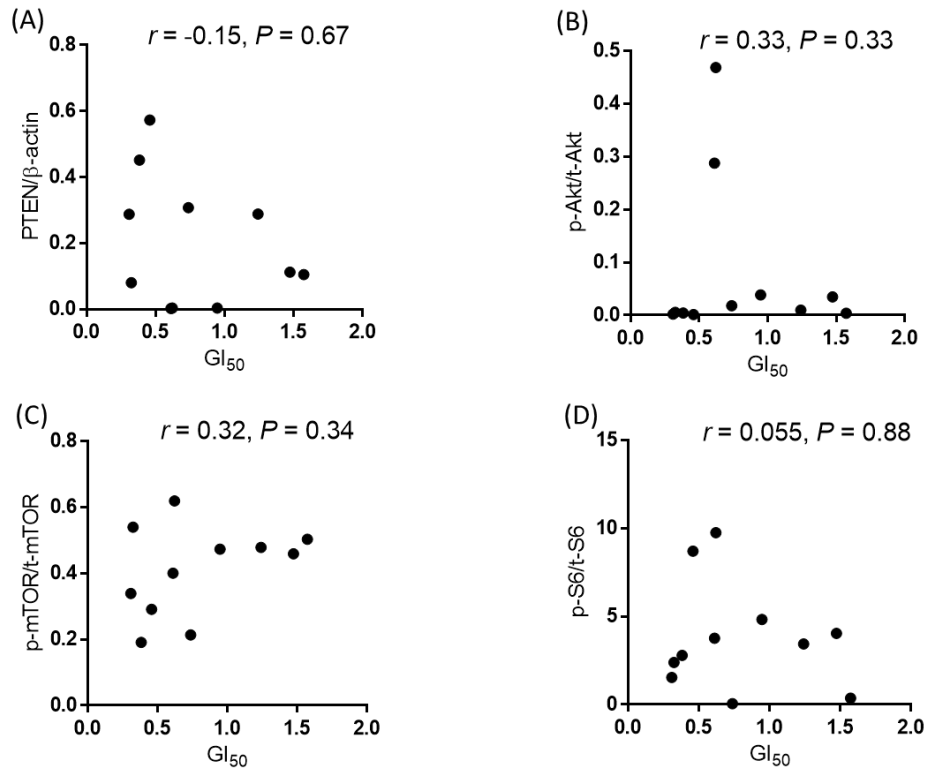


Figure 3.5. Analysis of correlation between GI_{50} for vemurafenib, and levels of (A) PTEN normalised to β -actin, (B) p-Akt/t-Akt, (C) p-mTOR/t-mTOR, and (D) p-S6/t-S6 from western blot quantitative analysis in 11 $BRAF^{V600E}$ colorectal carcinoma cell lines (Spearman's correlation).

3.3.1.2. Microsatellite Instability (MSI)

As discussed in section 1.2.5.3.2., there is a strong association between *BRAF* mutations in CRC and sporadic microsatellite instability (MSI) [57, 771]. Furthermore, MSI-high (MSI-H) is found much more commonly in CRC (~20% in colon adenocarcinoma) than malignant melanoma (0.64% in cutaneous melanoma) [772]. A recent clinical trial reported that in patients with *BRAF*^{V600E} CRC who were treated with a combination of the BRAF inhibitor dabrafenib, MEK inhibitor trametinib, and EGFR inhibitor panitumumab, there was a trend to increased response rates and progression-free survival in patients who had MSI-high/MMR-D compared to MSS/MMR-P tumours [285].

We examined whether there was an association between MSI status (Figure 3.6) and sensitivity of *BRAF*^{V600E} CRC cell lines to vemurafenib. MSS *BRAF*^{V600E} CRC cell lines were more sensitive to vemurafenib overall (median GI₅₀ 0.42, range 0.33 to 0.74 vs 0.95, range 0.31 to 1.57 for MSI cell lines), however this difference was not statistically significant (*P* = 0.16) (Figure 3.7A).

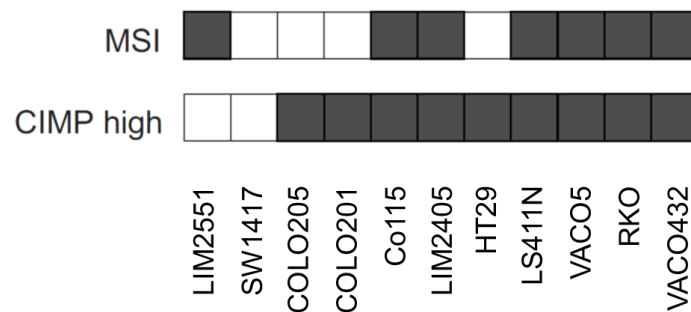


Figure 3.6. *BRAF*^{V600E} colorectal carcinoma cell lines and associated MSI status and CIMP status. Cell lines ordered by GI₅₀ for vemurafenib (from lowest on the left, to highest on the right). Boxes shaded grey denote MSI and CIMP high cell lines.

3.3.1.3. CpG island methylated phenotype (CIMP)

The CpG island methylated phenotype (CIMP) denotes a subset of CRCs which have a high frequency of aberrant DNA methylation of select CpG islands [200]. Whilst aberrant hypermethylation is not associated with *BRAF* mutant melanoma [773], CIMP-positive CRCs have been shown to encompass almost all CRCs with *BRAF* mutations, and are also associated with MSI-H [200].

We examined whether there was an association between CIMP status and sensitivity of *BRAF*^{V600E} CRC cell lines to vemurafenib. Of note, the majority of the *BRAF*^{V600E} CRC cell lines were CIMP high (9 out of 11, 82%) (Figure 3.6). Notably, CIMP-low cell lines were more sensitive to

vemurafenib (median GI₅₀ 0.32, range 0.31 to 0.33 vs 0.74, range 0.38 to 1.57 for CIMP-high cell lines; $P = 0.036$) (Figure 3.7B).

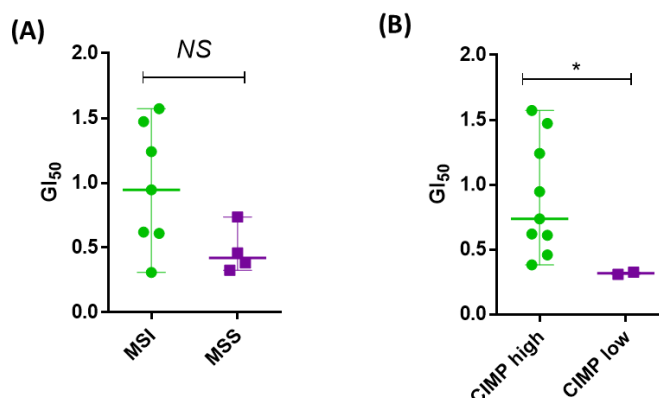


Figure 3.7. Association between GI₅₀ values for vemurafenib and (A) MSI/MSS status, and (B) CIMP status in the $BRAF^{V600E}$ colorectal carcinoma cell lines (Mann-Whitney test). Data shown are the median and range. NS: not statistically significant, $P \geq 0.05$.

3.3.1.4. Basal expression levels of proteins in the RAS/RAF/MEK/ERK signalling pathway

We also hypothesised that higher basal levels of activation of the RAS/RAF/MEK/ERK pathway may be associated with resistance to growth inhibition by vemurafenib in $BRAF^{V600E}$ CRC, as higher concentrations of drug may be required to suppress signalling.

To explore this, we performed Western blot of p-MEK, t-MEK, p-ERK, t-ERK, and the downstream ERK target protein p-FRA1 in each of the untreated $BRAF^{V600E}$ CRC cell lines (Figure 3.8). FRA-1 (FOSL1) is a member of the Fos family of transcription factors which heterodimerises with other AP-1 components [774] to form the transcriptional regulator, AP-1 [775]. Activation of the RAS/RAF/MEK/ERK pathway leads to transcriptional activation of the *FRA-1* gene [776]. Kinases in this pathway also phosphorylate the FRA-1 protein product, which results in its stabilisation and accumulation [775]. Therefore, phosphorylated-FRA1 protein expression was examined as a readout of downstream ERK activity.

We examined the correlation between basal expression of the phospho-proteins and the GI₅₀ for vemurafenib (Figure 3.9). Basal p-MEK and p-ERK expression was high in the majority of the $BRAF^{V600E}$ CRC cell lines, consistent with constitutive activation of the RAS/RAF/MEK/ERK pathway in these cell lines (Figure 3.8). There was no significant correlation between basal protein expression of p-MEK/t-MEK and p-ERK/t-ERK in the cell lines ($r = -0.073$, $P = 0.84$). There was also marked variability in basal p-FRA1 expression amongst the cell lines.

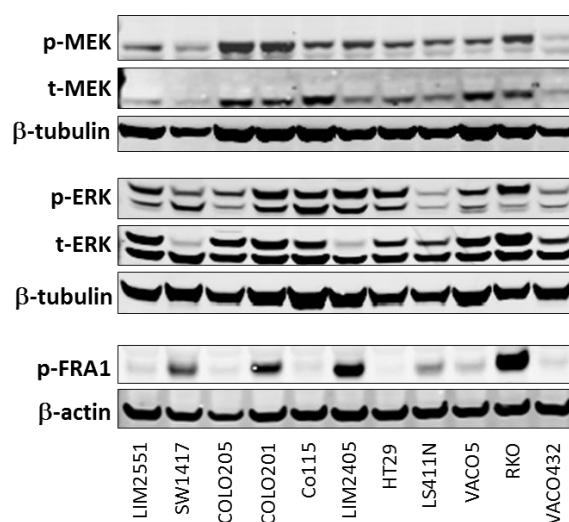


Figure 3.8. Western blots of basal expression levels of proteins in the RAS/RAF/MEK/ERK MAPK signaling pathway in $BRAF^{V600E}$ colorectal carcinoma cell lines. Ordered by GI_{50} for vemurafenib (from lowest on the left, to highest on the right).

There was no significant correlation between the GI_{50} for vemurafenib and basal protein expression of p-MEK/t-MEK ($r = -0.26$, $P = 0.43$), p-ERK/t-ERK ($r = -0.22$, $P = 0.52$), or p-FRA1 normalised to β -actin ($r = 0.14$, $P = 0.69$) in the 11 $BRAF^{V600E}$ CRC cell lines (Figure 3.9).

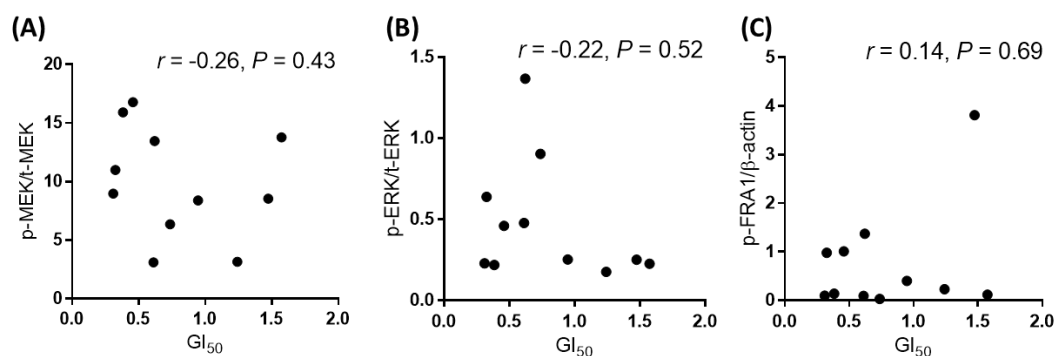


Figure 3.9. Analysis of correlation between GI_{50} for vemurafenib, and levels of (A) p-MEK/t-MEK, (B) p-ERK/t-ERK, and (C) p-FRA1 normalised to β -actin, from western blot quantitative analysis in 11 $BRAF^{V600E}$ colorectal carcinoma cell lines (Spearman's correlation).

3.3.1.5. Phospho-EGFR

The epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase which can signal to and activate the RAS-RAF-MEK-MAPK pathway [777]. Previously, it has been hypothesised that high basal expression of EGFR or p-EGFR may be predictive of resistance to BRAF inhibitor monotherapy in $BRAF$ mutant cancers, including CRCs [295, 296].

We therefore performed western blot of p-EGFR and t-EGFR in the 11 $BRAF^{V600E}$ CRC cell lines and 5 $BRAF^{V600E}$ melanoma cell lines (Figure 3.10). Consistent with previous reports, we confirmed that basal expression of both p-EGFR and t-EGFR were significantly higher in the

BRAF^{V600E} CRC cell lines compared to melanoma cell lines (Figure 3.11). The median level of p-EGFR in CRC cell lines was 0.13, range 0.012 to 0.38, and in melanoma was 0.0054, range 0.0029 to 0.01; $P = 0.005$. For t-EGFR, the median level of expression normalized to β -actin in CRC was 1.05, range 0.61 to 2.91, compared to 0.0058, range 0.0019 to 0.15 in melanoma, $P = 0.005$.

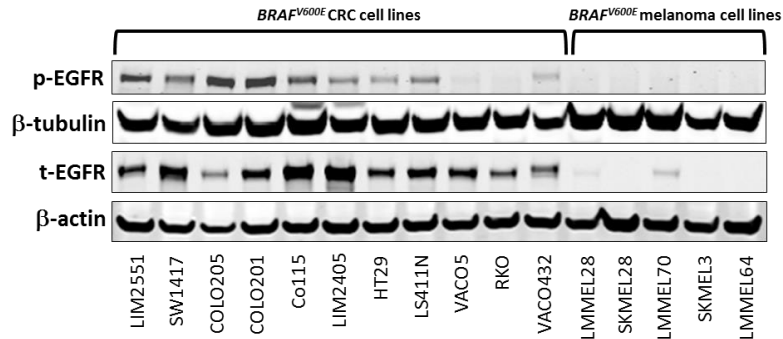


Figure 3.10. Western blots of basal expression levels of p-EGFR and t-EGFR in *BRAF*^{V600E} colorectal carcinoma and melanoma cell lines. The cell lines for each tumour type are ordered by GI₅₀ for vemurafenib (from lowest on the left, to highest on the right).

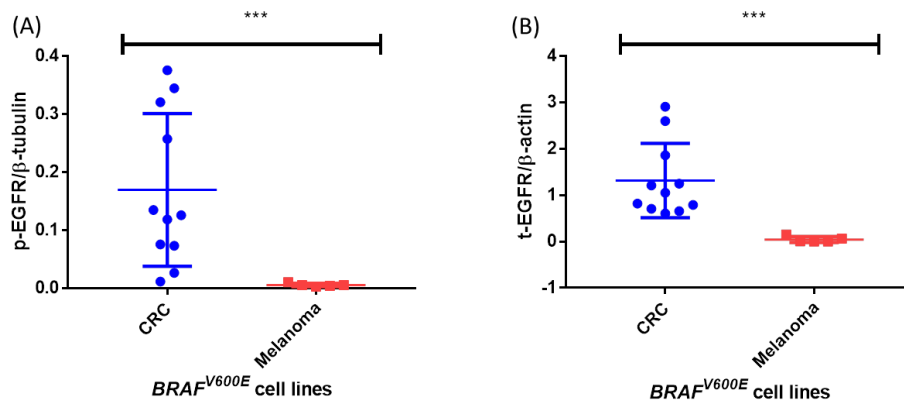


Figure 3.11. Comparison of levels of (A) p-EGFR normalised to β -tubulin and (B) t-EGFR normalised to β -actin, from western blot quantitative analysis in colorectal carcinoma versus melanoma *BRAF*^{V600E} cell lines (Mann-Whitney test). Data shown are the median and range, calculated using mean of $n = 2$ biological replicates.

Notably, we found that within the *BRAF*^{V600E} CRC cell lines, there was a significant negative correlation between basal levels of p-EGFR expression and GI₅₀ for vemurafenib ($r = -0.85$, $P = 0.0018$), suggesting that in *BRAF*^{V600E} CRC cell lines higher basal p-EGFR protein expression was associated with sensitivity to vemurafenib (Figure 3.12A).

When basal protein expression of p-EGFR (normalised to β -tubulin) was adjusted for corresponding t-EGFR levels (normalised to β -actin) in the cell lines, there was still a significant negative correlation with GI₅₀ for vemurafenib ($r = -0.71$, $P = 0.018$) (Figure 3.12B). In

comparison, there was no significant correlation between basal protein expression of t-EGFR and GI_{50} for vemurafenib ($r = -0.38$, $P = 0.25$) (Figure 3.12C).

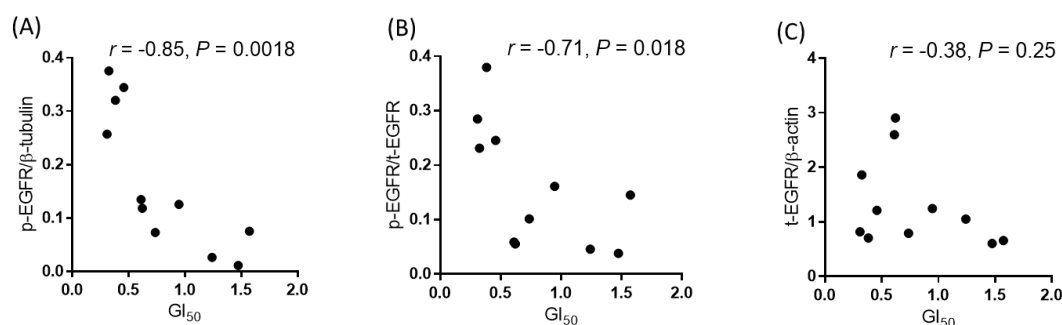


Figure 3.12. Analysis of correlation between GI_{50} for vemurafenib, and levels of (A) p-EGFR normalised to tubulin, (B) ‘p-EGFR normalised to β-tubulin/t-EGFR normalised to β-actin’ and (C) t-EGFR normalised to β-actin, from western blot quantitative analysis in 11 $BRAF^{V600E}$ colorectal carcinoma cell lines (Spearman’s correlation). Correlation calculated using mean of $n = 2$ biological replicates.

3.4. Mechanisms of resistance to BRAF inhibitors in $BRAF^{V600E}$ colorectal cancer

3.4.1. Differential sensitivity of $BRAF^{V600E}$ colorectal cancer cell lines to vemurafenib may involve the MAPK pathway

To explore potential mechanisms of the differential vemurafenib sensitivity observed in the $BRAF^{V600E}$ CRC cell lines, we treated the 11 $BRAF^{V600E}$ CRC cell lines with vemurafenib (5 uM) or DMSO control for 6 hours, and used mRNA extracted from these samples in gene expression microarrays using the Affymetrix U133 Human Genome Array platform.

Gene expression analysis revealed a total of 99 genes which had significant differential expression after vemurafenib treatment compared to treatment with DMSO control, in the five most vemurafenib-sensitive $BRAF^{V600E}$ CRC cell lines (with the lowest GI_{50}) *but not* the five most vemurafenib-resistant $BRAF^{V600E}$ CRC cell lines (with the highest GI_{50}). This included 41 significantly upregulated genes, and 58 significantly downregulated genes (Figure 3.13).

Notably, 12 of the 58 (20.6%) significantly downregulated genes were related to the MAPK pathway (Figure 3.13). We therefore hypothesised that vemurafenib treatment may lead to greater suppression and/or more sustained suppression of the MAPK pathway in vemurafenib-sensitive compared to vemurafenib-resistant $BRAF^{V600E}$ CRC cell lines.

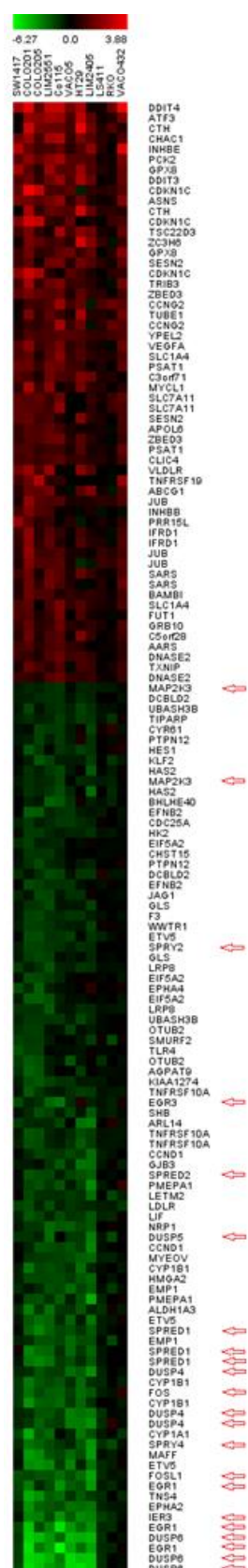


Figure 3.13. Heat map of the genes with significant differential expression in the five most vemurafenib-sensitive colorectal carcinoma cell lines but not the five most vemurafenib-resistant colorectal carcinoma cell lines, after vemurafenib 5 uM treatment versus DMSO control. Data is from gene expression analysis of Affymetrix U133 Human Genome Arrays. Red arrows indicate genes related to the MAPK pathway.

3.4.2. Association between p-ERK feedback and BRAF inhibitor resistance in *BRAF*^{V600E} colorectal cancer cell lines

3.4.2.1. Vemurafenib treatment induces rapid and sustained p-ERK reactivation in resistant *BRAF*^{V600E} colorectal cancer cell lines

To examine whether the differential inhibition of the RAS/RAF/MEK/ERK signalling pathway between sensitive and resistant *BRAF*^{V600E} CRC cell lines was due to differential inhibition of the pathway or to differences in the kinetics of pathway reactivation following drug treatment, we performed time course experiments. The three *BRAF*^{V600E} CRC cell lines which were most sensitive (SW1417, COLO201 and LIM2551) and most resistant (VACO432, RKO and LS411N) to vemurafenib were treated with vemurafenib (5 μ M) or DMSO control for 15 minutes to 24 hours and p-ERK levels assessed by western blot. We also included the two most sensitive *BRAF*^{V600E} melanoma cell lines for comparison.

We found that in the three most vemurafenib-resistant *BRAF*^{V600E} CRC cell lines, treatment with vemurafenib led to initial p-ERK suppression. However, rapid and sustained p-ERK reactivation occurred after 3 hours of treatment (Figure 3.14A).

In contrast, in the three most vemurafenib-sensitive *BRAF*^{V600E} CRC cell lines, vemurafenib 5 μ M treatment led to relatively greater suppression of p-ERK, which was also more sustained (Figure 3.14B). Similarly, p-ERK remained suppressed for 24 hours in both of the *BRAF*^{V600E} melanoma cell lines treated with vemurafenib (Figure 3.14C).

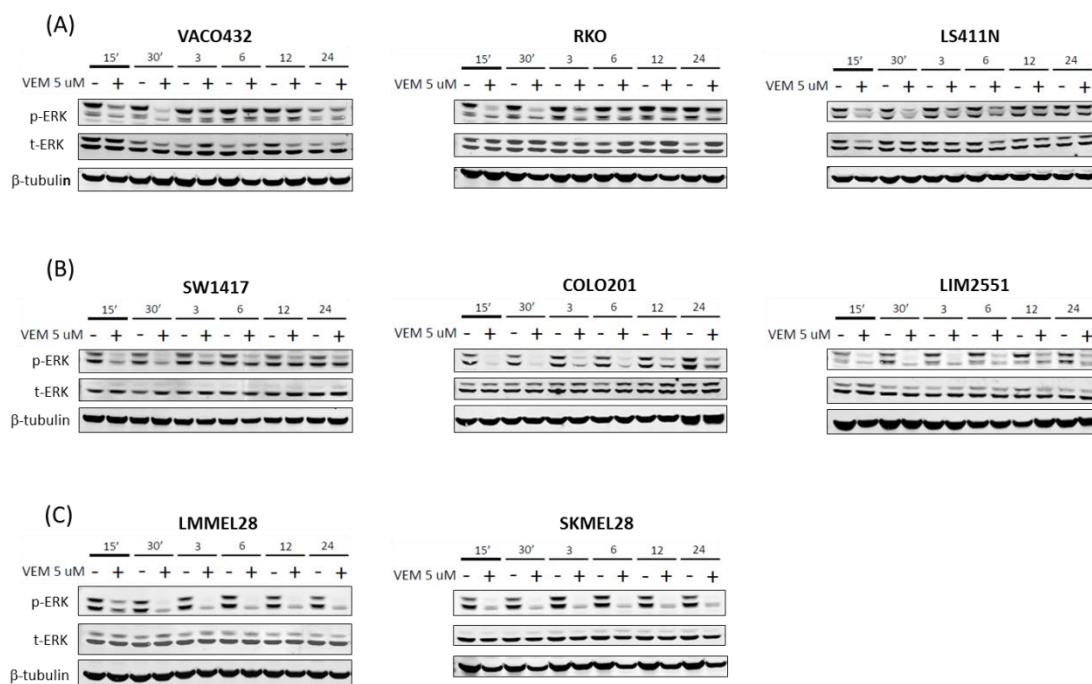


Figure 3.14. Western blots of p-ERK in timecourse experiments (15 min to 24 hrs) of treatment with DMSO control or vemurafenib 5 uM in (A) 3 vemurafenib-resistant colorectal carcinoma cell lines, (B) 3 vemurafenib-sensitive colorectal carcinoma cell lines, and (C) 2 melanoma cell lines. VEM: vemurafenib.

To confirm these findings and directly compare p-ERK reactivation in sensitive and resistant *BRAF*^{V600E} CRC cell lines, sensitive and resistant cell lines were treated with vemurafenib for 6, 24 or 72 hours and p-ERK levels examined in a single western blot.

Consistent with the time course experiments, p-ERK levels were strongly suppressed in the three most vemurafenib-sensitive CRC cell lines and in the melanoma cell lines, while minimal suppression was observed in the three most vemurafenib-resistant CRC cell lines, at all three time points (Figure 3.15).

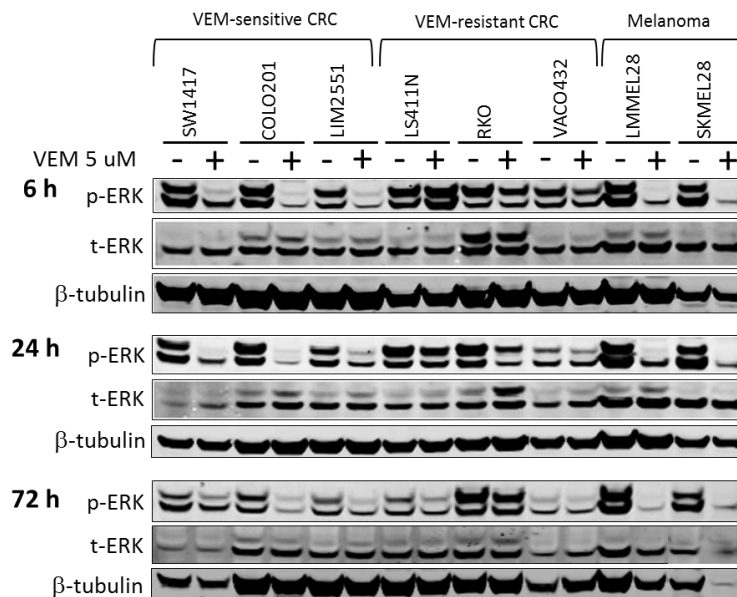


Figure 3.15. Western blots of p-ERK after treatment with DMSO control or vemurafenib 5 uM for 6, 24 and 72 hours in the 3 most vemurafenib-resistant colorectal carcinoma cell lines, 3 most vemurafenib-sensitive colorectal carcinoma cell lines, and 2 melanoma cell lines. CRC: colorectal carcinoma; VEM: vemurafenib.

3.4.2.2. Vemurafenib treatment induces sustained suppression of p-FRA1, a downstream target of p-ERK, in sensitive compared to resistant $BRAF^{V600E}$ colorectal cancer cell lines

To examine whether the more rapid reactivation of p-ERK observed in vemurafenib-resistant $BRAF^{V600E}$ CRC cell lines treated with vemurafenib also led to reactivation of downstream targets of p-ERK, we performed western blots of the nuclear target of p-ERK, p-FRA1, after 6 hours of treatment with vemurafenib (5 uM) or DMSO control.

Consistent with the sustained suppression of p-ERK in sensitive cell lines, p-FRA levels were suppressed after 6 to 24 hours, and remained suppressed at 72 hours in the vemurafenib-sensitive CRC and melanoma cell lines. In comparison, while p-FRA1 was suppressed at 6 to 24 hours in the vemurafenib-resistant CRC cell lines, the magnitude of suppression was markedly reduced at 72 hours, consistent with the reactivation of p-ERK in these lines (Figure 3.16).

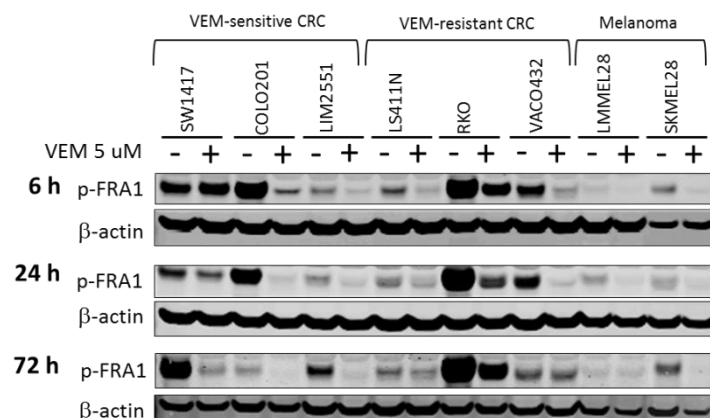


Figure 3.16. Western blots of p-FRA1 after treatment with DMSO control or vemurafenib 5 uM for 6, 24 and 72 hours in 3 most vemurafenib-resistant colorectal carcinoma cell lines, 3 most vemurafenib-sensitive colorectal carcinoma cell lines, and 2 melanoma cell lines. CRC: colorectal carcinoma; VEM: vemurafenib.

3.4.2.3. Kinetics of vemurafenib-induced p-ERK suppression in resistant $BRAF^{V600E}$ colorectal cancer cell lines are correlated with effects on cell proliferation

To assess whether the initial suppression of p-ERK followed by its rapid reactivation after vemurafenib treatment was associated with similar patterns of cell growth inhibition in vemurafenib-resistant $BRAF^{V600E}$ CRC cell lines, we used the Roche xCELLigence System to perform real-time monitoring of cell proliferation over 144 hours in two sensitive and two resistant cell lines.

Consistent with the patterns of p-ERK suppression, vemurafenib induced a modest growth inhibitory response in the two resistant cell lines, and after over 72 hours of treatment, the curves for the vemurafenib-treated lines crossed and exceeded the curves for the DMSO control (Figure 3.17A).

In contrast, in the two vemurafenib-sensitive CRC and melanoma cell lines, there was a sustained growth inhibitory response, which was maintained until the end of the monitoring period (Figure 3.17 B, C).

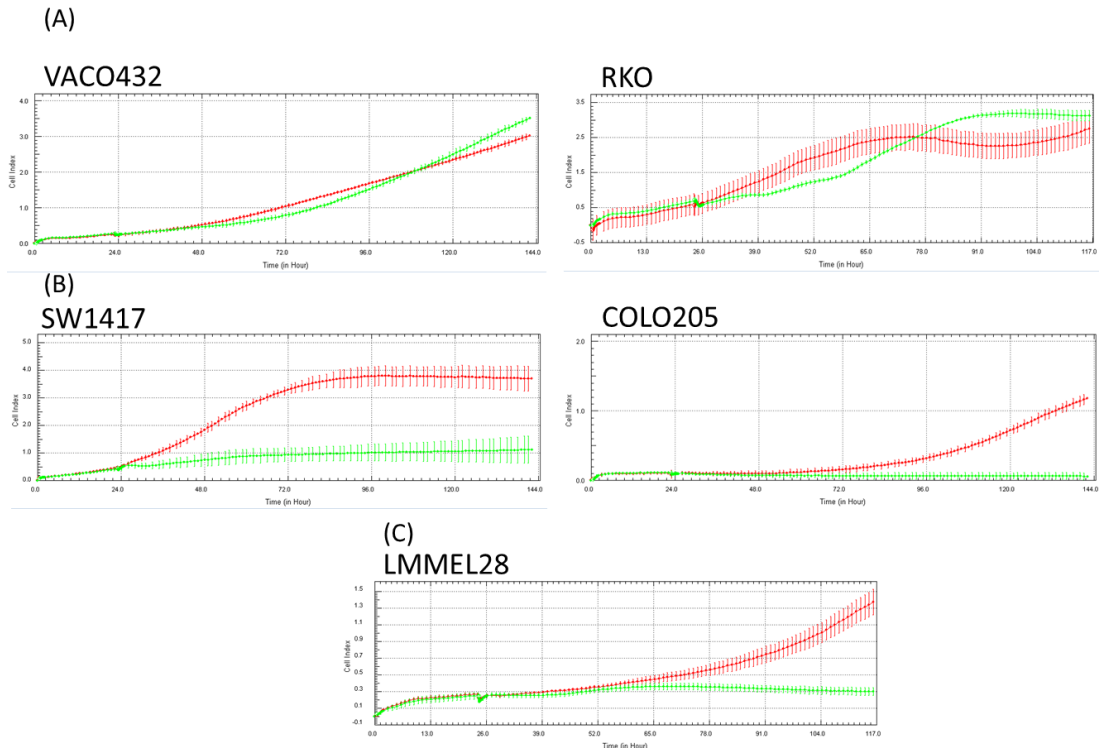


Figure 3.17. Cell Index data over time for (A) 2 vemurafenib-resistant colorectal carcinoma cell lines, (B) 2 vemurafenib-sensitive colorectal carcinoma cell lines, and a melanoma cell line. Experiment duration was 144 hours in the VACO432, SW1417, and COLO205 cell lines, and 120 hours in the RKO and LMMEL28 cell lines. Red – cells treated with DMSO control; green – cells treated with vemurafenib 1 uM.

Collectively, these findings demonstrate that in resistant *BRAF*^{V600E} CRC cell lines, vemurafenib treatment induces rapid and sustained p-ERK reactivation associated with resistance to vemurafenib treatment demonstrated by a lack of growth inhibitory response after 72 to 96 hours. In contrast, in vemurafenib-sensitive CRC and melanoma cell lines, vemurafenib treatment induces p-ERK suppression which is associated with a sustained growth inhibitory response.

3.4.2.4. *p-ERK reactivation is not linked to increased p-EGFR expression in vemurafenib-resistant *BRAF*^{V600E} CRC cell lines*

Prahallad *et al.* previously observed an upregulation of p-EGFR in 3 *BRAF*^{V600E} CRC cell lines (WiDr, VACO432 and KM20), after treatment with vemurafenib, and proposed that resistance of *BRAF*^{V600E} CRC cell lines to vemurafenib is triggered by feedback-relief mediated activation of p-EGFR [295]. Notably however, other studies have failed to confirm this finding (Corcoran *et al.*), instead observing a slight decrease in p-EGFR and t-EGFR expression following vemurafenib treatment [296].

Therefore, to determine whether p-ERK reactivation in vemurafenib-resistant *BRAF*^{V600E} CRC cell lines is associated with increased p-EGFR expression, we examined p-EGFR levels in the three most vemurafenib-sensitive and resistant cell lines, as well as melanoma cell lines treated with vemurafenib (5 uM) for 6 and 24 hours.

These experiments revealed upregulation of p-EGFR in two vemurafenib-sensitive *BRAF*^{V600E} CRC cell lines (COLO201 and LIM2551) after vemurafenib treatment. In comparison, consistent with the findings shown in Figure 3.10, basal levels of p-EGFR were markedly lower overall in the resistant CRC cell lines and no induction of p-EGFR was observed, collectively suggesting that resistance to vemurafenib in *BRAF*^{V600E} CRC cell lines is not driven by upregulation of p-EGFR (Figure 3.18). As expected, p-EGFR levels were basally low and no induction following vemurafenib treatment was observed in the two melanoma lines.

Corresponding western blots of p-Akt showed that at 6 hours and 24 hours, there was up-regulation of p-Akt in some vemurafenib-sensitive and vemurafenib-resistant *BRAF*^{V600E} CRC cell lines (SW1417, LIM2551, LS411N and VACO432; and SW1417, LIM2551, respectively), however this did not mirror the exact same cell lines in which p-EGFR upregulation was observed (Figure 3.18).

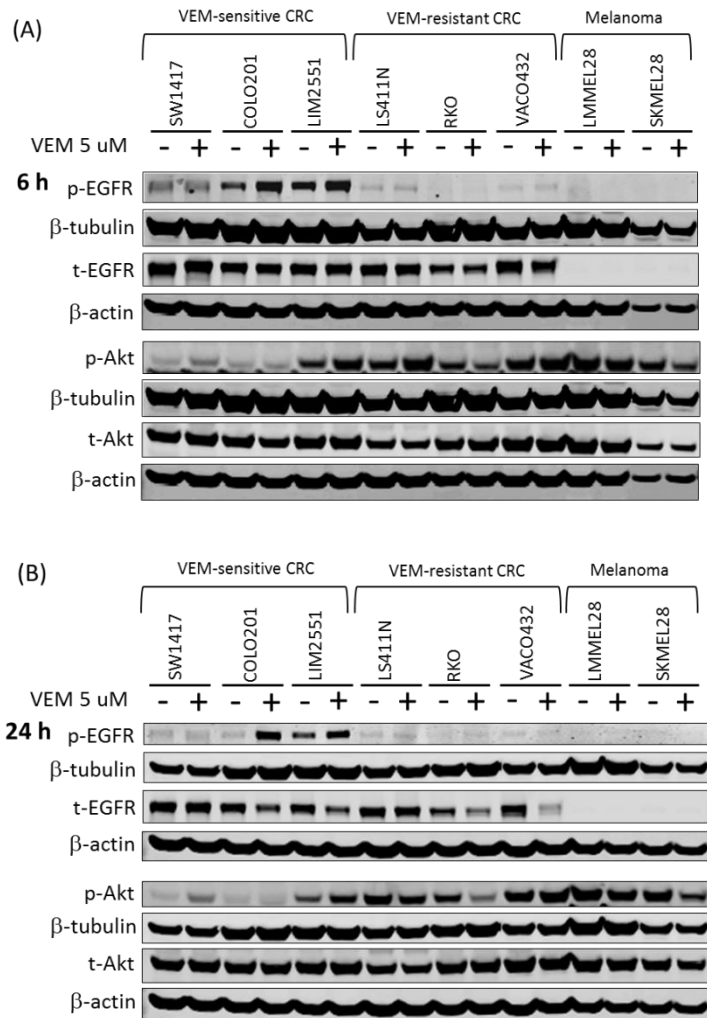


Figure 3.18. Western blots of p-EGFR and p-Akt after treatment with DMSO control or vemurafenib 5 uM for (A) 6 hours and (B) 24 hours in the 3 most vemurafenib-resistant colorectal carcinoma cell lines, 3 most vemurafenib-sensitive colorectal carcinoma cell lines, and 2 melanoma cell lines. CRC: colorectal carcinoma; VEM: vemurafenib.

To confirm these findings in resistant cell lines over more extended time courses, we examined p-EGFR induction in the three resistant cell lines treated with vemurafenib for 15 minutes to 24 hours. No significant increase in p-EGFR levels was observed after treatment with vemurafenib at any point in the time course, confirming that the reinduction of p-ERK is not linked to reinduction of p-EGFR in these cell lines (Figure 3.19).

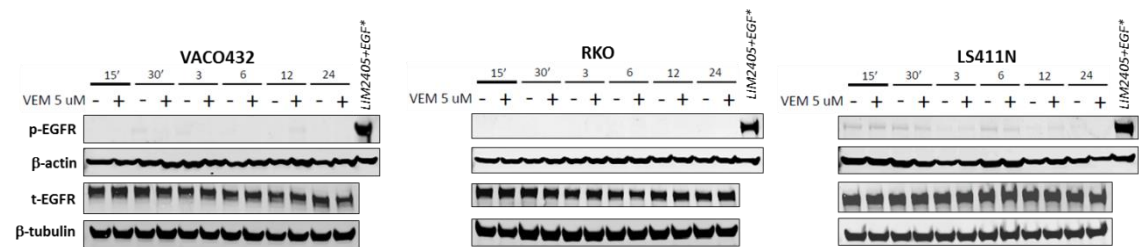


Figure 3.19. Western blots of p-EGFR in timecourse experiments (15 min to 24 hrs) of treatment with DMSO control or vemurafenib 5 uM in the 3 most vemurafenib-resistant colorectal carcinoma cell lines. VEM: vemurafenib. *Positive control: LIM2405 cell lines treated with EGF 100 ng/mL for 10 min.

3.4.2.5. Dabrafenib treatment is associated with p-ERK reactivation in vemurafenib-resistant *BRAF*^{V600E} colorectal cancer cell lines, and there is no association between this p-ERK reactivation and increased p-EGFR expression

To confirm that the differential response among *BRAF* mutant CRCs to vemurafenib is not drug-specific, we performed similar experiments using another selective BRAF inhibitor, dabrafenib.

Firstly, we examined growth inhibition of the three most vemurafenib-sensitive and resistant *BRAF*^{V600E} CRC cell lines and the two *BRAF*^{V600E} melanoma cell lines after 72 hours treatment with dabrafenib (1 uM) using MTS assays..

These results confirmed that the spectrum of sensitivity to dabrafenib closely reflected the sensitivity spectrum to vemurafenib, indicating that these cell lines harbor inherent generic differences in response to BRAF inhibitors (Figure 3.20).

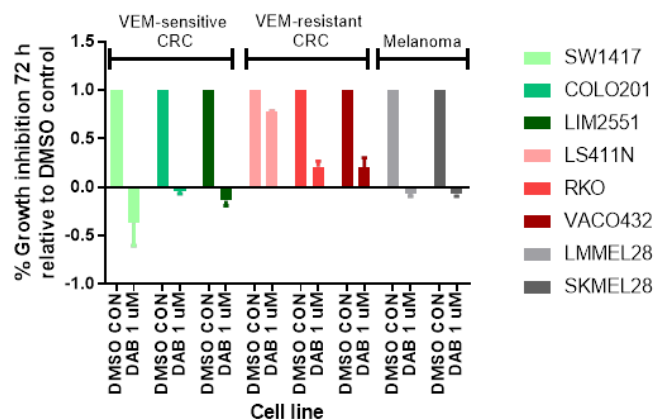


Figure 3.20. MTS assays showing growth inhibition of the 3 most vemurafenib-resistant colorectal carcinoma cell lines, 3 most vemurafenib-sensitive colorectal carcinoma cell lines, and 2 melanoma cell lines, after 72 hours treatment with DMSO control or dabrafenib 1 uM. Data shown are the mean and SD for growth inhibition after dabrafenib treatment relative to DMSO control. Data is from two biological replicate experiments, each performed in technical quadruplicate. CRC: colorectal cancer; CON: control; DAB: dabrafenib; VEM: vemurafenib.

We next performed Western blots of p-EGFR, p-Akt and p-ERK after 24 hours of treatment with dabrafenib (1 uM) or DMSO in the three most vemurafenib-sensitive and resistant cell lines, and the two *BRAF*^{V600E} melanoma cell lines.

Our findings confirmed that, similar to vemurafenib treatment, dabrafenib preferentially suppressed p-ERK in the vemurafenib-sensitive *BRAF*^{V600E} CRC cell lines (Figure 3.21). Lack of p-ERK suppression in vemurafenib-resistant cell lines was not associated with p-EGFR upregulation; in fact the vemurafenib-sensitive cell line COLO201 was the only cell line where p-EGFR upregulation was observed. Similarly, p-Akt expression did not consistently mirror p-EGFR expression in the cell lines following dabrafenib treatment (Figure 3.21).

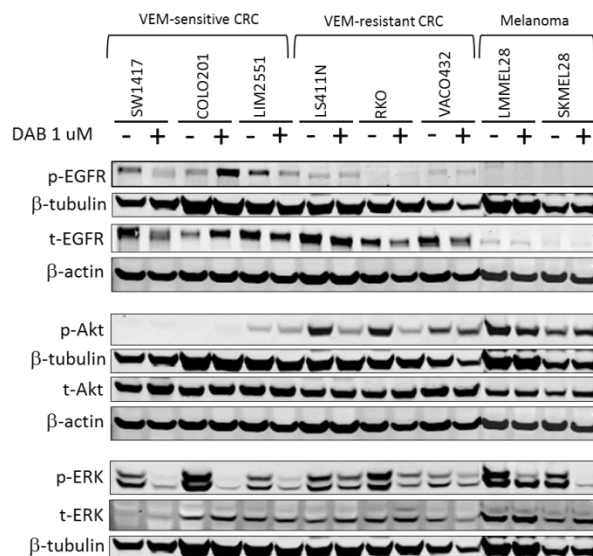


Figure 3.21. Western blots of p-EGFR, p-Akt and p-ERK after treatment with DMSO control or dabrafenib 1 uM for 24 hours in the 3 most vemurafenib-resistant colorectal carcinoma cell lines, 3 most vemurafenib-sensitive colorectal carcinoma cell lines, and 2 melanoma cell lines. CRC: colorectal carcinoma; DAB: dabrafenib; VEM: vemurafenib.

3.4.2.6. Vemurafenib-resistant $BRAF^{V600E}$ colorectal cell lines are similarly resistant to the next generation paradox breaker BRAF inhibitor PLX8394/PB03

An adverse effect of BRAF inhibitors such as vemurafenib and dabrafenib is that in *BRAF* wt cells that harbor either mutations in *RAS* or express high levels of receptor tyrosine kinases (RTKs), they can cause the paradoxical activation of MAPK signalling through enhancing the formation of *BRAF/CRAF* heterodimers or *CRAF/CRAF* homodimers [164, 257, 258]. To overcome this, next-generation RAF inhibitors referred to as “paradox breakers” have been developed which inhibit wild-type and mutant *BRAF* without causing paradoxical activation of the MAPK pathway in the presence of oncogenic *RAS* or RTK overexpression [246] (reviewed in section 1.2.6.1.3.2.). The lead compound in this class is PLX8394.

We performed MTS assays in these cell lines to examine growth inhibition after 72 hours treatment with PLX8394 (5 uM). These results demonstrated that vemurafenib-resistant *BRAF*^{V600E} CRC cell lines remained relatively resistant to PLX8394 compared to the vemurafenib-sensitive *BRAF*^{V600E} CRC cell lines (Figure 3.22).

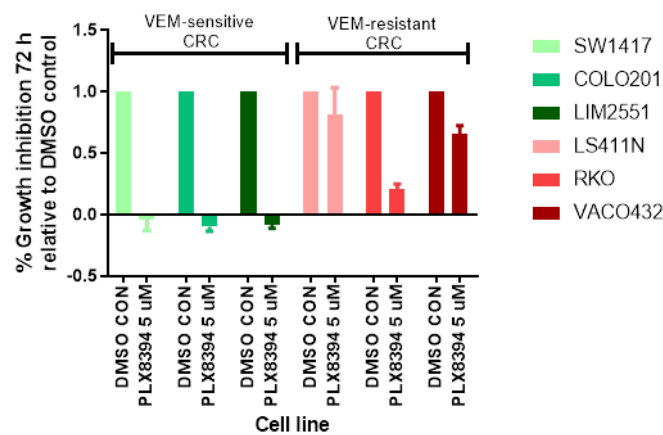


Figure 3.22. MTS assays showing growth inhibition of the 3 most vemurafenib-resistant CRC cell lines and 3 most vemurafenib-sensitive CRC cell lines, after 72 hours treatment with DMSO control or PLX8394 5 uM. Data shown are the mean and SD for growth inhibition after PLX8394 treatment, relative to DMSO control. Data is from two biological replicate experiments, each performed in technical quadruplicate. VEM: vemurafenib.

Furthermore, western blots of these cell lines treated with PLX8394 for 24 hours revealed reduced suppression of p-ERK in the vemurafenib-resistant *BRAF*^{V600E} CRC cell lines (Figure 3.23).

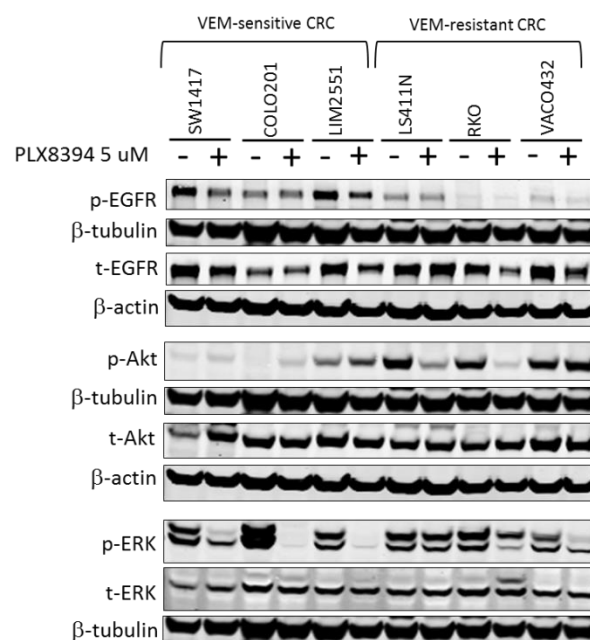


Figure 3.23. Western blots of p-EGFR, p-Akt and p-ERK after treatment with DMSO control or PLX8394 5 uM for 24 hours in the 3 most vemurafenib-resistant colorectal carcinoma cell lines and 3 most vemurafenib-sensitive colorectal carcinoma cell lines. CRC: colorectal carcinoma; VEM: vemurafenib.

To confirm that the reduced suppression of p-ERK in the resistant cell lines was due to p-ERK reactivation, we also performed time course experiments in the three most vemurafenib-resistant *BRAF*^{V600E} CRC cell lines. These showed that, similar to vemurafenib treatment, PLX8394

induced initial suppression of p-ERK which was followed by rapid reactivation in all three resistant cell lines after 3 to 6 hours (Figure 3.24).

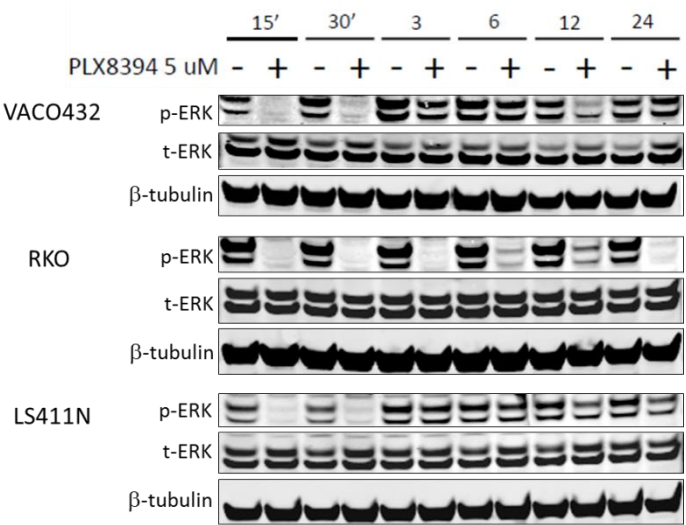


Figure 3.24. Western blots of p-ERK in timecourse experiments (15 min to 24 hrs) of treatment with DMSO control or PLX8394 5 uM in the 3 vemurafenib-resistant colorectal carcinoma cell lines (VACO432, RKO, and LS411N).

Notably, there was no upregulation of p-EGFR after PLX8394 treatment in any of the BRAF^{V600E} CRC cell lines. Additionally, p-Akt expression did not mirror p-EGFR expression in the cell lines following PLX8394 treatment (Figure 3.23).

3.5. Discussion

This chapter described the results of pre-clinical studies in which I explored mechanisms of resistance of BRAF^{V600E} CRC to BRAF inhibitors.

3.5.1. Basal biomarkers of resistance of BRAF^{V600E} colorectal cancer to BRAF inhibitors

Among a broad panel of BRAF^{V600E} CRC cell lines, I found that some are relatively sensitive whilst others are relatively resistant to vemurafenib treatment *in vitro*. This is consistent with clinical data from the phase II trial of vemurafenib in patients with BRAF^{V600E} metastatic CRC reported by Kopetz *et al.*, where the waterfall plot demonstrated significant variability in individual participants’ tumour responses [33].

Next, exploiting the variation in vemurafenib sensitivity observed among the panel of BRAF^{V600E} CRC cell lines, I investigated potential basal biomarkers of BRAF inhibitor resistance. I found that all BRAF^{V600E} CRC cell lines which had been characterised by sequencing also harboured simultaneous activation of the PI3K pathway, defined by the presence of established mutations in *mTOR*, *PIK3R1*, *PIK3CA*, or *PTEN*. This is in contrast with the findings by Mao *et al.*, who found PI3K pathway activation in 70% of the cell lines studied, however their study only considered *PIK3CA* mut and *PTEN* null status [290]. I also observed variable sensitivity to vemurafenib

among the cell lines which had concurrent mutation of *BRAF* and activation of the PI3K pathway, suggesting that alternate mechanisms account for the differential response. Supporting these findings, I also found that there was no significant association between basal expression of key proteins in the PI3K/AKT/mTOR signalling pathway and vemurafenib sensitivity.

Although MSS *BRAF*^{V600E} CRC cell lines were more sensitive to vemurafenib overall, this difference was not statistically significant. This is consistent with the results of most of the previous translational sub-studies investigating the association between MSI status and *BRAF* inhibitor response [33, 284, 288].

I found a significant association between CIMP-low status and sensitivity to vemurafenib. This is in contrast to the results of the translational sub-study from the phase II trial of vemurafenib in patients with *BRAF*^{V600E} metastatic CRC reported by Kopetz *et al.*, who used a different gene-panel for CIMP analysis, where no correlation between CIMP and clinical activity was found [33]. Regardless, the low prevalence of CIMP-low cases within *BRAF*^{V600E} CRC [200] may limit its clinical utility as a biomarker of vemurafenib response.

I found no significant correlation between basal expression of key proteins in the RAS/RAF/MEK/ERK signalling pathway and vemurafenib sensitivity. Moreover, consistent with previous studies by Prahallad *et al.* and Corcoran *et al.*, I found that basal expression of both p-EGFR and t-EGFR were significantly higher in the *BRAF*^{V600E} CRC cell lines compared to melanoma cell lines [295, 296].

Based on the differences in EGFR expression that they observed in *BRAF*^{V600E} CRC compared to melanoma, Prahallad *et al.* and Corcoran *et al.* previously hypothesised that expression of EGFR or p-EGFR may be predictive of resistance to *BRAF* inhibitor monotherapy in multiple *BRAF* mutant cancers [295] and, specifically, might predict which *BRAF* mut CRCs may be resistant to *BRAF* inhibition [285]. However, notably, neither of these pre-clinical studies specifically examined the association between t-EGFR and p-EGFR expression and vemurafenib sensitivity within a panel of *BRAF*^{V600E} CRC cell lines, as I have. Similarly, exploratory translational studies within two reported clinical trials of single agent vemurafenib [33] and combination dabrafenib/trametinib in *BRAF* mut CRC [284], examined associations between vemurafenib response and t-EGFR, but not p-EGFR expression, by IHC. Most interestingly, I found that within our panel of *BRAF*^{V600E} CRC cell lines, higher basal p-EGFR protein expression was in fact associated with inherent sensitivity to vemurafenib. However, it is unclear whether high p-EGFR directly contributes to vemurafenib sensitivity, and this requires further study.

One limitation of these data is that assessments of sensitivity and resistance to vemurafenib were performed using a single 2-dimensional cell proliferation assay (MTS assay). Further research could also include experiments to confirm that the spectrum of sensitivity in the cell lines is

similar when using 3-dimensional cell proliferation assays or *in vivo*. Furthermore, protein quantification was performed using analysis of western blots. Quantitative protein analysis of western blots using secondary antibodies directly labelled with near-infrared (IR) dyes has been demonstrated to be reproducible, with high sensitivity, and a wide quantitative linear range compared to conventional chemiluminescent techniques [778-780]. However, limitations of this method still include the potential for results to be affected by deviation of data from proportional linear models (in particular, for high abundance proteins), and problems with normalisation if levels of loading controls change under different biological and experimental conditions [781]. Further planned research includes analysis using an alternative method of protein quantification (e.g., reverse phase protein arrays) to confirm these findings.

3.5.2. Mechanisms of inherent resistance of $BRAF^{V600E}$ colorectal cancer to BRAF inhibitors

To further explore the differential sensitivity of $BRAF^{V600E}$ CRC cell lines to vemurafenib, I adopted a different approach to previous pre-clinical studies, and compared MAPK pathway suppression between the most vemurafenib-sensitive and -resistant cell lines.

This analysis revealed that, in contrast to vemurafenib-sensitive CRC cell lines, vemurafenib induced rapid and sustained p-ERK reactivation in resistant $BRAF^{V600E}$ CRC cell lines. Consistent with MAPK pathway reactivation, this was associated with re-expression of the downstream MAPK target, p-FRA1. These findings are consistent with results from Corcoran *et al.*, who also observed vemurafenib-induced p-ERK reaccumulation in some $BRAF^{V600E}$ CRC cell lines, although in contrast to our study, this was not directly linked to vemurafenib-resistant cell lines [296].

Notably, in my study, p-ERK reactivation was not linked to increased p-EGFR expression in any vemurafenib-resistant $BRAF^{V600E}$ CRC cell lines. In fact, I observed vemurafenib-induced upregulation of p-EGFR in two vemurafenib-sensitive cell lines. Furthermore, I did not observe an association between vemurafenib-induced levels of p-EGFR and p-Akt expression. This is in contrast to the findings reported by Prahallad *et al.*, who proposed that vemurafenib-resistance in $BRAF$ mut CRC is mediated by phosphatase-mediated feedback reactivation of p-EGFR, which is associated with increased expression of p-Akt and suppression of p-ERK [295]. It is also in contrast to Corcoran *et al.* who did not observe vemurafenib-induced upregulation of p-EGFR in any examined cell lines, but observed increased p-Akt after vemurafenib treatment [296]. Importantly, my findings suggest that there are likely to be p-EGFR independent mechanisms of resistance of $BRAF^{V600E}$ CRC to BRAF inhibitors.

I confirmed these findings using another clinically relevant second-generation type I^{1/2} BRAF inhibitor, dabrafenib, and the third generation ‘paradox breaker’ PLX8394. Vemurafenib-resistant $BRAF^{V600E}$ CRC cell lines were similarly resistant to these other BRAF inhibitors, and resistance was also associated with p-ERK reactivation, but not increased p-EGFR or p-Akt expression.

4. RESULTS – Overcoming resistance of $BRAF^{V600E}$ colorectal cancer to BRAF inhibitors

4.1. Introduction

In this chapter, I investigate ways to overcome resistance of $BRAF^{V600E}$ CRC to BRAF inhibitors.

It has been hypothesised that incomplete suppression of the MAPK pathway may underpin resistance of $BRAF^{V600E}$ CRC to BRAF inhibitor monotherapy, leading to studies combining BRAF inhibitors with other MAPK pathway inhibitors [782]. To investigate whether this is an optimal approach, I examine whether the vemurafenib-induced p-ERK reactivation and resistance which is observed in vemurafenib-resistant $BRAF^{V600E}$ CRC cell lines is also observed in these same cell lines after treatment with other MAPK pathway inhibitors, and whether these cell lines are also resistant to the combination of vemurafenib and other MAPK inhibitors.

Combination treatment of BRAF inhibitors with EGFR inhibitors is being extensively explored clinically (discussed in section 1.2.9.1.). However, in Chapter 3, I demonstrated that p-ERK reactivation in vemurafenib-resistant $BRAF^{V600E}$ CRC cell lines is not associated with increased p-EGFR expression. Therefore, in this chapter, I explore the hypothesis that $BRAF^{V600E}$ CRC cell lines which are resistant to vemurafenib will be similarly resistant to the combination of vemurafenib and an EGFR inhibitor.

Finally, I explore where BRAF inhibitor-induced p-ERK reactivation may originate.

4.2. BRAF-inhibitor resistant $BRAF^{V600E}$ colorectal cancer are also resistant to other MAPK pathway inhibitors

To investigate whether combining BRAF inhibitors with other MAPK kinase inhibitors is the optimal approach to overcome resistance of $BRAF^{V600E}$ CRC to BRAF inhibitors, we theorised that it would be prudent to firstly investigate whether the p-ERK reactivation and resistance which is observed in vemurafenib-resistant $BRAF^{V600E}$ CRC cell lines is also observed in these same cell lines after treatment with other MAPK pathway inhibitors.

4.2.1. Vemurafenib-resistant $BRAF^{V600E}$ colorectal cancer cell lines are similarly resistant to a MEK inhibitor (Trametinib)

Trametinib (GSK1120212 or JTP-74057) is an allosteric MEK1/2 inhibitor which inhibits the phosphorylation of MEK1/2 by RAF [121, 783].

We first determined whether the response of $BRAF^{V600E}$ CRC cell lines to trametinib was similar to their differential response to vemurafenib.

Cells were treated with DMSO or trametinib 10 nM for 72 hours and growth inhibition was assessed by MTS assays. This revealed that vemurafenib-resistant $BRAF^{V600E}$ CRC cell lines were

also relatively resistant to trametinib compared to vemurafenib-sensitive *BRAF*^{V600E} CRC cell lines, in particular, SW1417 and LIM2551 (Figure 4.1).

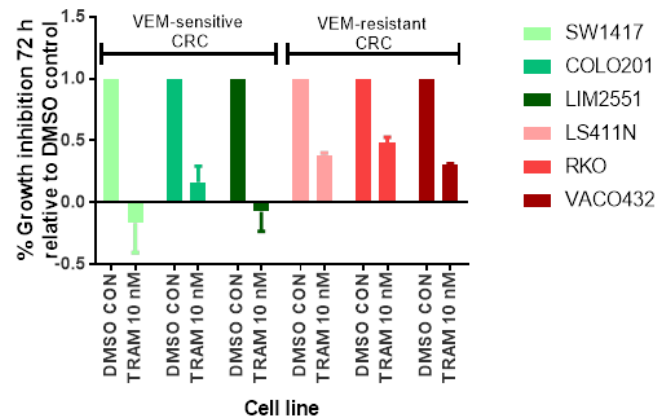


Figure 4.1. MTS assays showing growth inhibition of the 3 most vemurafenib-resistant colorectal carcinoma cell lines and 3 most vemurafenib-sensitive colorectal carcinoma cell lines, after 72 hours treatment with DMSO control or trametinib 10 nM. Data shown are the mean and SD for growth inhibition after trametinib treatment, relative to DMSO control. Data is from two biological replicate experiments, each performed in technical quadruplicate. CRC: colorectal carcinoma; TRAM: trametinib; VEM: vemurafenib.

To address whether this was reflected in the extent of MAPK pathway inhibition, we examined the effect of trametinib (10 nM) treatment on p-ERK suppression after 24 hours in the same cell lines. Mirroring the effects on cell proliferation, p-ERK was markedly more suppressed in the three vemurafenib-sensitive *BRAF*^{V600E} CRC cell lines compared to the resistant lines (Figure 4.2).

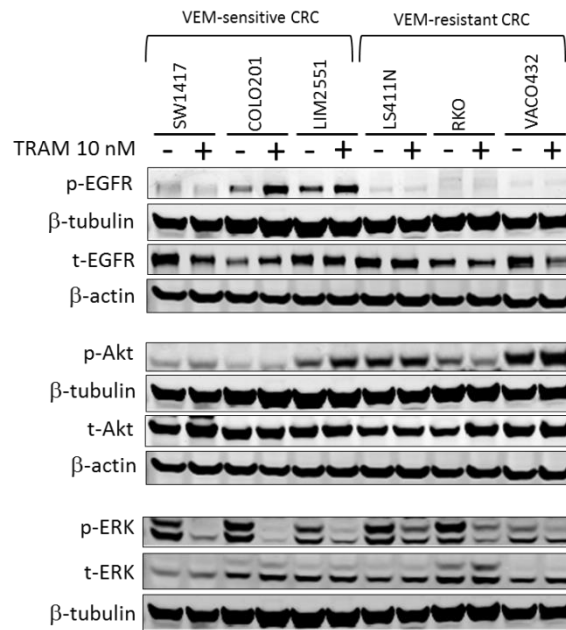


Figure 4.2. Western blots of p-ERK, p-EGFR and p-Akt after treatment with DMSO control or trametinib 10 nM for 24 hours in the 3 most vemurafenib-resistant colorectal carcinoma cell lines and 3 most vemurafenib-sensitive colorectal carcinoma cell lines. CRC: colorectal carcinoma; TRAM: trametinib; VEM: vemurafenib.

Finally, to determine whether the reduced suppression of p-ERK in the three most vemurafenib-resistant *BRAF*^{V600E} CRC cell lines was due to reactivation of p-ERK, cells were treated with DMSO or trametinib (10 nM) for 15 minutes to 24 hours and p-ERK levels determined by western blot. As observed for vemurafenib treatment, p-ERK was initially suppressed by trametinib, with expression levels reaching a nadir at 30 minutes (RKO) or 3 hours (VACO432 and LS411N), followed by some reactivation (Figure 4.3).

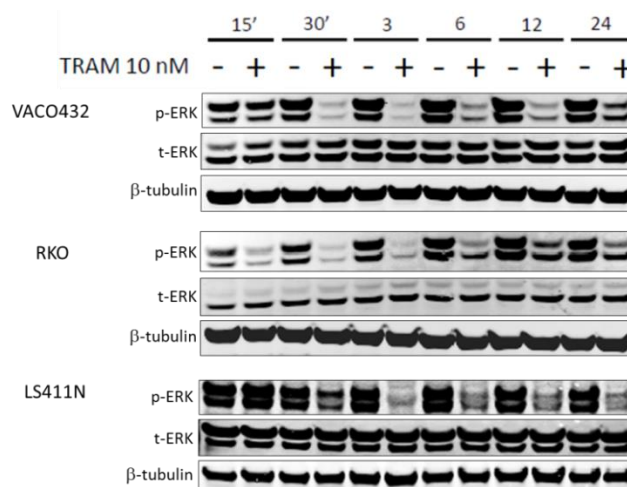


Figure 4.3. Western blots of p-ERK in timecourse experiments (15 min to 24 hrs) of treatment with DMSO control or trametinib 10 nM in the 3 vemurafenib-resistant CRC cell lines (VACO432, RKO, and LS411N). TRAM: trametinib.

As observed for vemurafenib treatment, the reactivation of p-ERK in resistant cell lines was not associated with p-EGFR upregulation; in fact, p-EGFR upregulation was observed in two of the vemurafenib-sensitive cell lines (COLO201 and LIM2551), but not in any of the vemurafenib-resistant cell lines. Similarly, p-Akt expression only mirrored p-EGFR upregulation in the LIM2551 cell line (Figure 4.2).

4.2.2. Vemurafenib-resistant $BRAF^{V600E}$ colorectal cancer cell lines demonstrate delayed p-ERK reactivation with ERK inhibitor (SCH772984) treatment

Given that p-ERK reactivation is a common response of vemurafenib-resistant $BRAF^{V600E}$ CRC cell lines to treatment with multiple inhibitors of the MAPK pathway, we hypothesised that treatment with an ERK inhibitor may be an effective treatment in these cell lines.

SCH772984 is a specific inhibitor of ERK 1/2 which leads to ATP-competitive inhibition of ERK1/2 catalytic activity, and also stabilises or induces a conformational state in ERK which prevents its activation by MEK [784].

4.2.2.1. The ERK inhibitor SCH772984 suppresses p-ERK expression similarly in vemurafenib-sensitive and resistant $BRAF^{V600E}$ colorectal cancer cell lines after 24 hours of treatment

Treatment of the three most vemurafenib-sensitive and resistant $BRAF^{V600E}$ CRC cell lines with SCH772984 (200 nM) for 24 hours revealed that p-ERK was similarly suppressed in all of the cell lines (Figure 4.4A).

4.2.3. p-ERK reactivation is evident in vemurafenib-resistant $BRAF^{V600E}$ CRC cell lines after 72 hours of treatment with the ERK inhibitor SCH772984, and is associated with p-RSK1 reactivation and resistance

To determine if p-ERK became reactivated at a later time point, we examined the extent of p-ERK suppression after 72 hours. These analyses revealed p-ERK reactivation in vemurafenib-resistant cell lines after 72 hours of treatment with SCH772984 (200 nM). Some p-ERK reactivation was also observed in the vemurafenib-sensitive $BRAF^{V600E}$ CRC cell lines, albeit to a lesser extent than observed in resistant cells, in particular, LS411N and VACO432 (Figure 4.4B).

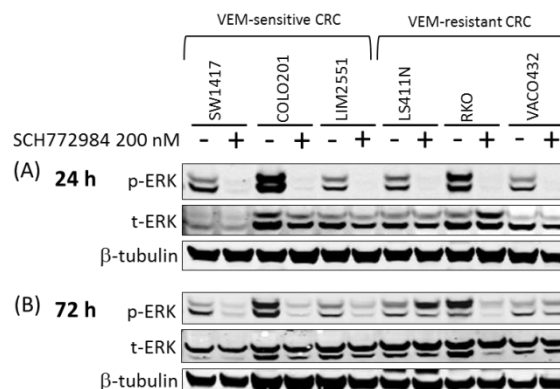


Figure 4.4. Western blots of p-ERK after treatment with DMSO control or SCH772984 200 nM for (A) 24 and (B) 72 hours in the 3 most vemurafenib-resistant colorectal carcinoma cell lines and 3 most vemurafenib-sensitive colorectal carcinoma cell lines. CRC: colorectal carcinoma; VEM: vemurafenib.

As observed for vemurafenib and trametinib treatment, p-ERK reactivation was not consistently associated with p-EGFR upregulation, which was only observed in one of the vemurafenib-resistant cell lines, LS411N, after 72 hours of treatment. On the other hand, p-Akt was selectively upregulated in the vemurafenib-resistant cell lines after SCH772984 treatment, an effect which was not observed in response to vemurafenib or trametinib treatment (Figure 4.5). These findings suggest that activation of p-Akt may also play a role in resistance to ERK inhibitors in these cell lines.

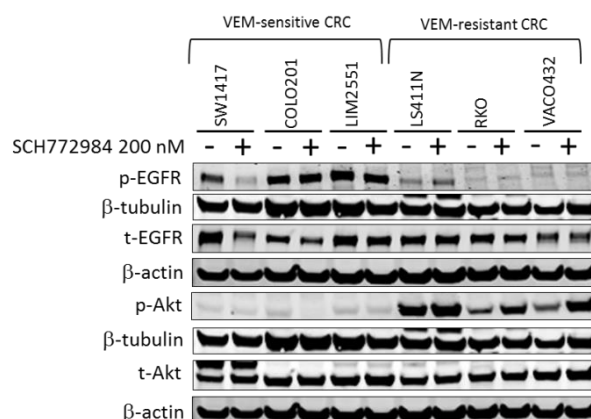


Figure 4.5. Western blots of p-EGFR and p-Akt after treatment with DMSO control or SCH772984 200 nM for 72 hours in the 3 most vemurafenib-resistant colorectal carcinoma cell lines and 3 most vemurafenib-sensitive colorectal carcinoma cell lines. CRC: colorectal carcinoma; VEM: vemurafenib.

Consistent with the reactivation of p-ERK, MTS assays after 72 hours confirmed that the vemurafenib-resistant *BRAF*^{V600E} CRC cell lines were, overall, relatively more resistant to ERK inhibition compared to the vemurafenib-sensitive *BRAF*^{V600E} CRC cell lines, with the exception of VACO432 (Figure 4.6).

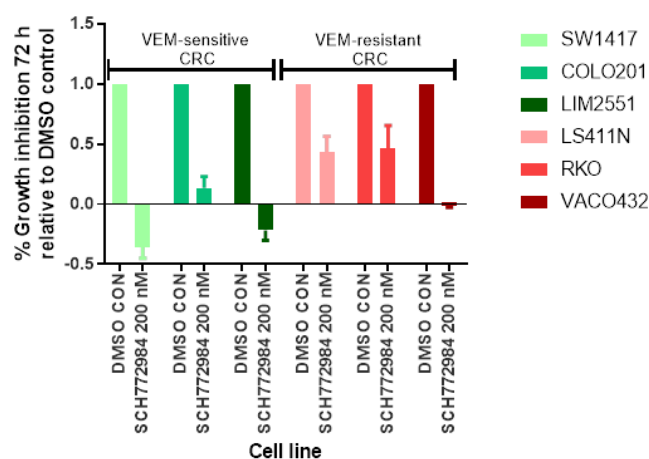


Figure 4.6. MTS assays showing growth inhibition of the 3 most vemurafenib-resistant colorectal carcinoma cell lines and 3 most vemurafenib-sensitive colorectal carcinoma cell lines, after 72 hours treatment with DMSO control or SCH772984 200 nM. Data shown are the mean and SD for growth inhibition after SCH772984 treatment, relative to DMSO control. Data is from two biological replicate experiments, each performed in technical quadruplicate. CRC: colorectal carcinoma; VEM: vemurafenib.

A previous study had demonstrated that SCH772984 treatment is also associated with delayed p-ERK reactivation in *BRAF*^{V600E} melanoma cell lines after 36 hours, despite sustained suppression of the cytosolic p-ERK target, p-RSK1 [784]. However, in *BRAF*^{V600E} CRC cell lines, p-ERK reactivation was accompanied by downstream reactivation of p-RSK1 after 72 hours of treatment in most of the cell lines, with the exception of SW1417 and RKO (Figure 4.7).

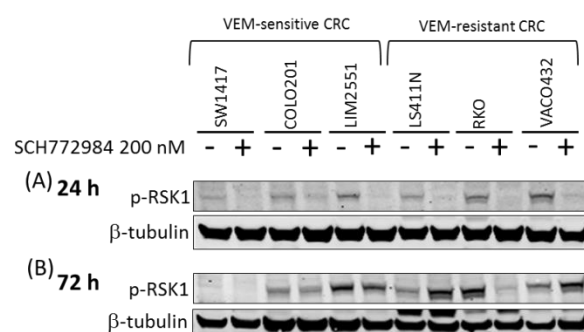


Figure 4.7. Western blots of p-RSK1 after treatment with DMSO control or SCH772984 200 nM for (A) 24 and (B) 72 hours in the 3 most vemurafenib-resistant colorectal carcinoma cell lines and 3 most vemurafenib-sensitive colorectal carcinoma cell lines. CRC: colorectal carcinoma; VEM: vemurafenib.

These experiments using a MEK inhibitor (Trametinib) and ERK inhibitor (SCH772984) therefore demonstrate that *BRAF*^{V600E} CRC cell lines which were relatively resistant to BRAF-inhibitors were similarly resistant to these other MAPK pathway inhibitors. This suggests that the mechanisms of resistance to BRAF inhibitors are not drug-specific, but rather are cell-line specific.

4.3. Exploring combination therapies to overcome resistance of *BRAF*^{V600E} colorectal cancer cell lines to BRAF inhibitors

4.3.1. Combining vemurafenib with a MEK inhibitor is not effective in overcoming resistance of *BRAF*^{V600E} colorectal cancer to BRAF inhibitors

Given our observation that BRAF inhibitor-resistant CRC cell lines were also relatively resistant to single-agent inhibitors of other components of the MAPK pathway, we further hypothesised that these cell lines would also be more resistant to the combination of vemurafenib and other MAPK inhibitors (e.g., trametinib and SCH772984). To address this, we first treated vemurafenib-resistant *BRAF* mutant CRC cells with a combination of vemurafenib 5 μ M and trametinib 10 nM.

Western blot analysis of the three most vemurafenib-sensitive and resistant *BRAF*^{V600E} CRC cell lines treated with single agent vemurafenib (5 μ M), trametinib (10 nM), or the combination of vemurafenib and trametinib demonstrated preferential suppression of p-ERK in the 3 sensitive cell lines for each of these conditions. In the vemurafenib-resistant cell lines, p-ERK levels were suppressed more after single agent trametinib compared to vemurafenib treatment, but were not further suppressed by combination treatment with vemurafenib and trametinib compared to trametinib alone (Figure 4.8).

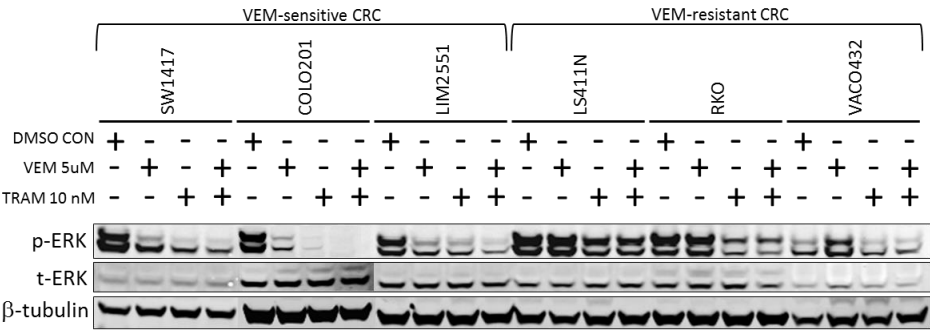


Figure 4.8. Western blots of p-ERK after treatment with DMSO control, vemurafenib 5 μ M, trametinib 10 nM, or vemurafenib 5 μ M + trametinib 10 nM for 24 hours in the 3 most vemurafenib-resistant colorectal carcinoma cell lines and 3 most vemurafenib-sensitive colorectal carcinoma cell lines. CRC: colorectal carcinoma; TRAM: trametinib; VEM: vemurafenib.

MTS assays of cells treated with DMSO, vemurafenib alone, trametinib alone, or vemurafenib and trametinib for 72 hours confirmed that vemurafenib-resistant *BRAF*^{V600E} CRC cell lines were relatively more resistant to each of these conditions compared to the vemurafenib-sensitive *BRAF*^{V600E} CRC cell lines. Furthermore, in the vemurafenib-resistant cell lines, there was only a small additional increase in growth inhibition after combination treatment compared to cells treated with trametinib alone (Figure 4.9).

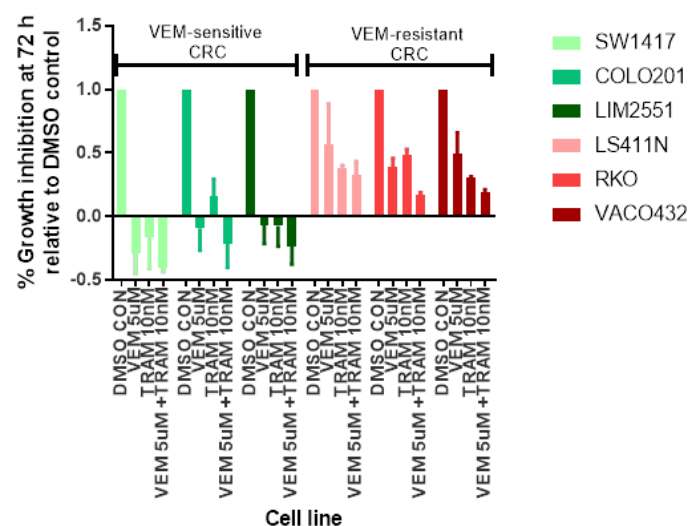


Figure 4.9. MTS assays showing growth inhibition of the 3 most vemurafenib-resistant colorectal carcinoma cell lines and 3 most vemurafenib-sensitive colorectal carcinoma cell lines, after 72 hours treatment with DMSO control, vemurafenib 5 uM, trametinib 10 nM, and vemurafenib 5 uM + trametinib 10 nM. Data shown are the mean and SD for growth inhibition after treatment conditions, relative to DMSO control. Data is from two biological replicate experiments, each performed in technical quadruplicate. CRC: colorectal carcinoma; TRAM: trametinib; VEM: vemurafenib.

To confirm these findings statistically, we calculated the combination index (CI) values using the Chou Talalay method [742]. The CI value was > 1 (indicating lack of synergy) in all cell lines except for RKO, where the CI value was 0.96, indicating a synergistic but close to additive effect (Figure 4.10). The CI value was not consistently lower in vemurafenib-resistant cell lines (range, 0.96 to 2.76) than in vemurafenib-sensitive cell lines (range, 1.03 to 2.60) (Figure 4.10).

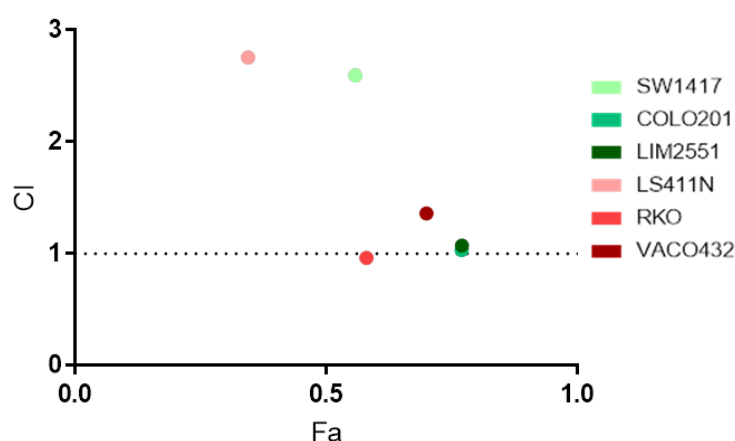


Figure 4.10. F_a -CI plots of Chou and Talalay for the combination of vemurafenib 5 uM and trametinib 10 nM, using growth inhibition data from MTS assays after 72 hours treatment with DMSO control, vemurafenib (0.1, 1, 2.5 and 5 uM), trametinib (0.1, 1, 5, 10 nM), and vemurafenib 5 uM + trametinib 10 nM. CI: combination index; F_a : fraction affected. $CI < 1$ is synergistic, $CI = 1$ is additive, and $CI > 1$ is antagonistic. Data are from three biological replicate experiments, each performed in technical quadruplicate.

4.3.2. Combining vemurafenib with an EGFR inhibitor is not effective in all vemurafenib-resistant *BRAF*^{V600E} colorectal cancer cell lines

Combination treatment of BRAF inhibitors with EGFR inhibitors is being extensively explored clinically (discussed in section 1.2.9.1.). However, given our findings that p-ERK reactivation in vemurafenib-resistant *BRAF*^{V600E} CRC cell lines is not associated with increased p-EGFR expression, we hypothesised that *BRAF*^{V600E} CRC cell lines which are resistant to vemurafenib would be similarly resistant to the combination of vemurafenib and an EGFR inhibitor.

To address this, the three most vemurafenib-sensitive and three most vemurafenib-resistant *BRAF*^{V600E} CRC cell lines were treated with DMSO control, vemurafenib alone (5 uM), cetuximab alone (20 µg/mL), or vemurafenib in combination with cetuximab for 24 hours and p-ERK levels determined by western blot. Combination treatment with vemurafenib and cetuximab resulted in modestly greater suppression of p-ERK in the 3 vemurafenib-sensitive cell lines, and in the vemurafenib-resistant VACO432 cell line. As expected, cetuximab treatment alone had no effect on p-ERK levels in vemurafenib-sensitive or resistant cell lines (Figure 4.11).

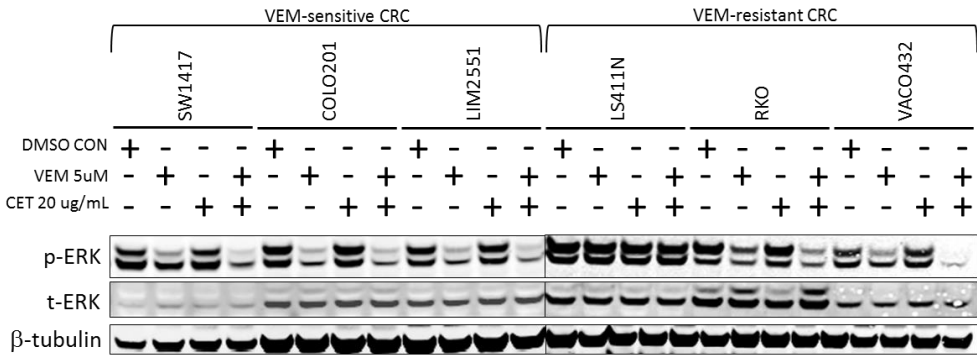


Figure 4.11. Western blots of p-ERK after treatment with DMSO control, vemurafenib 5 uM, cetuximab 20 µg/mL, and vemurafenib 5 uM + cetuximab 20 µg/mL for 24 hours in the 3 most vemurafenib-resistant colorectal carcinoma cell lines and 3 most vemurafenib-sensitive colorectal carcinoma cell lines. CRC: colorectal carcinoma; CET: cetuximab; VEM: vemurafenib

Similar to our previous experiments using single-agent vemurafenib, p-EGFR was increased following 24 hours of vemurafenib treatment in two vemurafenib-sensitive *BRAF*^{V600E} CRC cell lines (COLO201 and LIM2551), but not in any of the three vemurafenib-resistant cell lines. In these two vemurafenib-sensitive cell lines, the upregulation of p-EGFR was partially suppressed by combination treatment with vemurafenib and cetuximab. Interestingly, of the vemurafenib-resistant cell lines, the combination of vemurafenib and cetuximab only led to suppression of p-EGFR expression in the VACO432 cell line. Corresponding Western blots of p-Akt showed that p-Akt expression did not mirror p-EGFR expression in most of the *BRAF*^{V600E} CRC cell lines, with the exception of VACO432 (Figure 4.12).

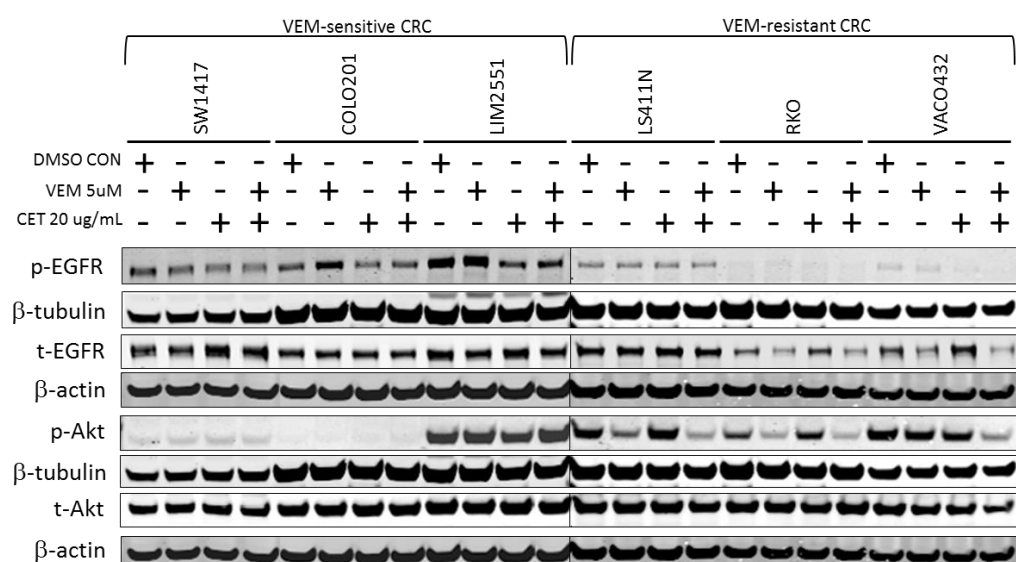


Figure 4.12. Western blots of p-EGFR and p-Akt after treatment with DMSO control, vemurafenib 5 uM, cetuximab 20 µg/mL, and vemurafenib 5 uM + cetuximab 20 µg/mL for 24 hours in the 3 most vemurafenib-resistant colorectal carcinoma cell lines and 3 most vemurafenib-sensitive colorectal carcinoma cell lines. CRC: colorectal carcinoma; CET: cetuximab; VEM: vemurafenib.

To determine the effect of combination treatment on cell proliferation, we performed MTS assays over 72 hours in cells treated with DMSO, vemurafenib alone (5 uM), cetuximab alone (20 µg/mL), or vemurafenib and cetuximab. As expected, cetuximab alone had minimal effect on growth inhibition in all *BRAF* mutant cell lines, but suppressed proliferation of the *KRAS/BRAF* wt CRC cell line LIM1215, confirming efficacy of the drug (Figure 4.13). The addition of cetuximab to vemurafenib was able to convert only one vemurafenib-resistant *BRAF*^{V600E} CRC cell line (VACO432) to a level of sensitivity similar to that observed in the vemurafenib-sensitive cell lines. In the two other vemurafenib-resistant cell lines (RKO and LS411N), there was only a small additional increase in growth inhibition after combination treatment compared to cells treated with vemurafenib alone (Figure 4.13).

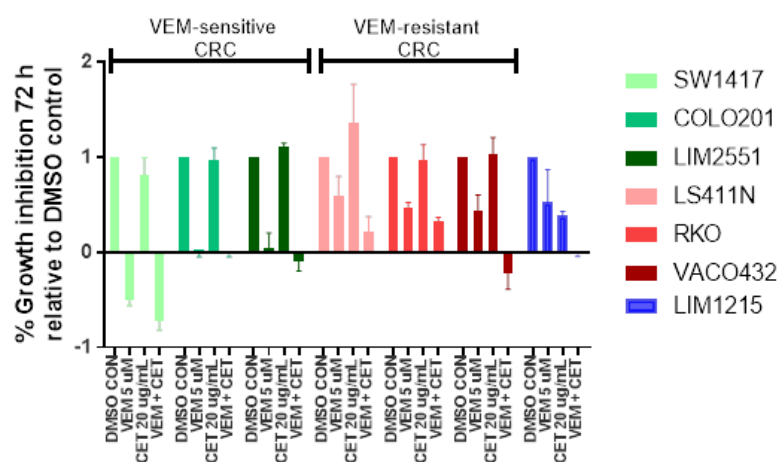


Figure 4.13. MTS assays showing growth inhibition of the 3 most vemurafenib-resistant colorectal carcinoma cell lines and 3 most vemurafenib-sensitive colorectal carcinoma cell lines, and the *KRAS/BRAF* wt CRC cell line LIM1215, after 72 hours treatment with DMSO control, vemurafenib 5 uM, cetuximab 20 μ g/mL, and vemurafenib 5 uM + cetuximab 20 μ g/mL. Data shown are the mean and SD for growth inhibition after treatment conditions, relative to DMSO control. Data is from two biological replicate experiments, each performed in technical quadruplicate. CRC: colorectal carcinoma; CET: cetuximab; VEM: vemurafenib.

To examine whether the combination of vemurafenib and cetuximab was synergistic, we calculated the CI values using the Chou Talalay method in all 6 cell lines. The CI value was > 1 (indicating lack of synergy) in all cell lines except for LS411N, where the CI value was 0.96, indicating a synergistic but close to additive effect, and VACO432, where the CI value was 0.031, indicating synergy (Figure 4.14). The CI value was consistently lower in vemurafenib-resistant cell lines (range, 0.032 to 470.88) than in vemurafenib-sensitive cell lines (range, 896.17 to ∞) (Figure 4.14).

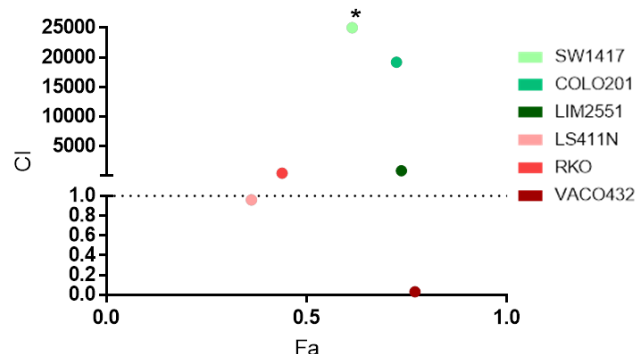


Figure 4.14. F_a -CI plots of Chou and Talalay for the combination of vemurafenib 5 uM and cetuximab 20 μ g/mL, using growth inhibition data from MTS assays after 72 hours treatment with DMSO control, vemurafenib (0.1, 1, 2.5 and 5 uM), cetuximab (0.1, 1, 10 and 20 μ g/mL), and vemurafenib 5 uM + cetuximab 20 μ g/mL. Data are from three biological replicate experiments, each performed in technical quadruplicate. CI: combination index; F_a : fraction affected. $CI < 1$ is synergistic, $CI = 1$ is additive, and $CI > 1$ is antagonistic. * SW1417 $CI = \infty$.

These findings demonstrate that combination treatment with a BRAF inhibitor and an EGFR inhibitor is more effective than single agent BRAF inhibitor treatment in some, but not all *BRAF*^{V600E} CRC cell lines.

4.4. Determining the point in the MAPK pathway at which p-ERK reactivation following BRAF inhibitor treatment originates

4.4.1. RAS is not preferentially activated following vemurafenib treatment in vemurafenib-resistant *BRAF*^{V600E} colorectal cancer cell lines

To further explore the mechanisms of resistance to BRAF inhibitors, we performed experiments to determine the point in the MAPK signalling pathway from which p-ERK reactivation originates in vemurafenib-resistant *BRAF*^{V600E} CRC cell lines. We postulated that if p-ERK reactivation is driven by reactivation of RTK signalling, RAS would be activated following vemurafenib treatment, and this would occur preferentially in vemurafenib-resistant cell lines.

We therefore performed RAS activation assays in the 3 most vemurafenib-sensitive and resistant *BRAF*^{V600E} CRC cell lines, and one *BRAF*^{V600E} melanoma cell line, treated with DMSO control or vemurafenib 5 μ M for 6 hours. In addition, the HCT116^{RasMut} (HAE6, genotype *KRAS2* D13/-) cell line and its isogenic cell line HCT116^{RasWT} (HAF1, genotype *KRAS2* +/-) were used as positive and negative controls respectively, and HeLa cells treated with EGF were used as a further positive control.

As expected, there were very high levels of RAS activity in the HCT116^{RasMut} positive control compared to HCT116^{RasWT}, and high levels of RAS activity in the HeLa cells treated with EGF (Figure 4.15). Vemurafenib treatment induced a small increase in RAS activation in all of the *BRAF*^{V600E} CRC cell lines except for COLO201 (Figure 4.15).

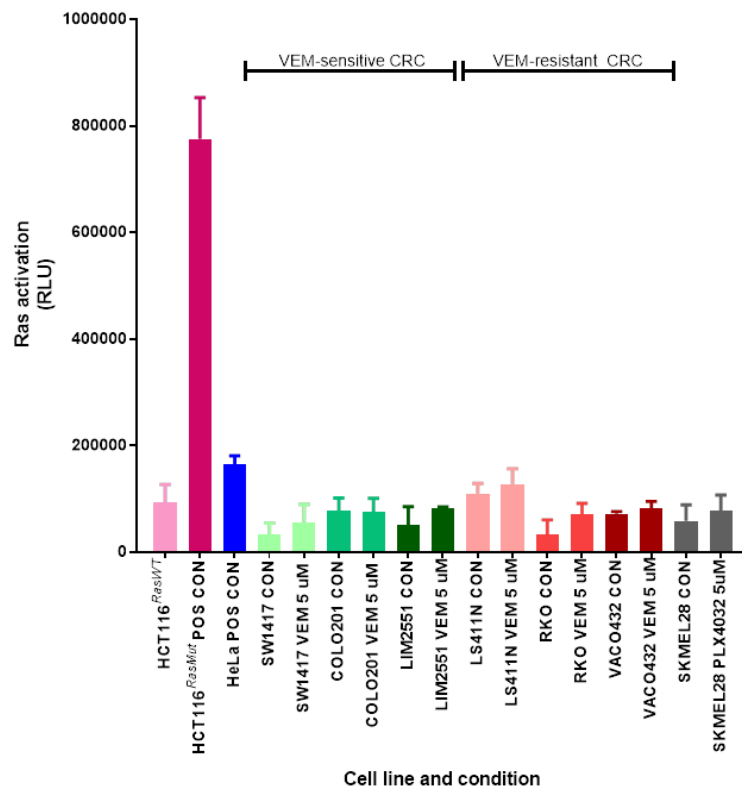


Figure 4.15. RAS activation of the 3 most vemurafenib-resistant colorectal carcinoma cell lines and 3 most vemurafenib-sensitive colorectal carcinoma cell lines, after 6 hours treatment with DMSO control or vemurafenib 5 uM. Positive controls are lysates from untreated HCT116^{RasMut} and EGF-treated HeLa cell lines. CON: control; POS CON: positive control; CRC: colorectal carcinoma; VEM: vemurafenib; RLU: relative light units.

The increase in RAS activation in the *BRAF*^{V600E} CRC cell lines was similar to that observed in the *BRAF*^{V600E} melanoma cell line SKMEL28. There was no significant difference between the fold-change in RAS activation between vemurafenib-sensitive and vemurafenib-resistant *BRAF*^{V600E} CRC cell lines ($P > 0.99$) (Figure 4.16).

This suggests that p-ERK reactivation associated with BRAF inhibitor treatment does not originate through activation of RAS.

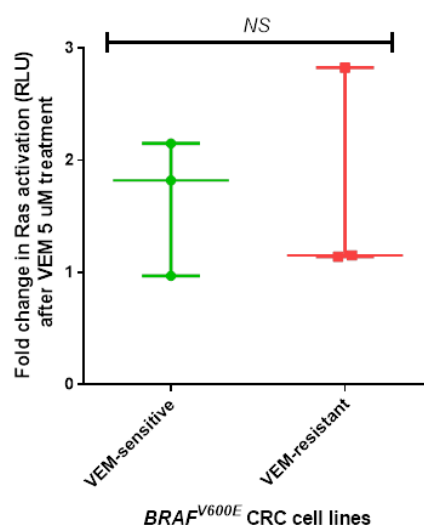


Figure 4.16. Comparison of fold-change in Ras activation (in RLU) before and after vemurafenib 5 uM treatment, in the 3 most vemurafenib-resistant colorectal carcinoma cell lines and 3 most vemurafenib-sensitive colorectal carcinoma cell lines (Mann-Whitney test). Data shown are the median and range for fold change in Ras activation after vemurafenib 5 uM treatment in each group of cell lines. RLU: Relative Light Units; VEM: vemurafenib; CRC: colorectal carcinoma; NS: not statistically significant, $P \geq 0.05$.

4.4.2. p-CRAF expression is not preferentially upregulated after vemurafenib treatment in vemurafenib-resistant *BRAF*^{V600E} colorectal cancer cell lines

Next, we focused downstream of RAS in the RAS/RAF/MEK/ERK MAPK pathway, and examined levels of activated p-CRAF in the vemurafenib-sensitive and vemurafenib-resistant *BRAF*^{V600E} CRC cell lines.

BRAF^{V600E} CRC cell lines were treated with vemurafenib (5 uM) or DMSO for 24 hours and phosphorylated CRAF (Ser338) determined by western blot. These analyses revealed that p-CRAF was not preferentially suppressed in vemurafenib-sensitive cell lines. There was a small increase (SW1417, RKO and VACO432) or no change (LIM2551 and LS411N) in p-CRAF expression in both vemurafenib-sensitive and vemurafenib-resistant cell lines, and suppression of p-CRAF in one vemurafenib-sensitive cell line (COLO201) (Figure 4.17).

Collectively, these findings do not establish a clear link between p-ERK reactivation and phosphorylation of CRAF in *BRAF*^{V600E} CRC cell lines. However, basal levels of p-CRAF appeared higher in vemurafenib-resistant cell lines overall, suggesting that further investigation of this mechanism is warranted.

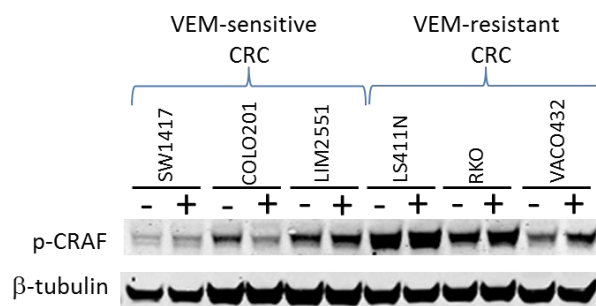


Figure 4.17. Western blots of p-CRAF after treatment with DMSO control and vemurafenib 5 uM for 24 hours in the 3 most vemurafenib-resistant colorectal carcinoma cell lines and 3 most vemurafenib-sensitive colorectal carcinoma cell lines. CRC: colorectal carcinoma; VEM: vemurafenib.

4.4.3. p-MEK is suppressed to a similar extent in vemurafenib-sensitive and resistant *BRAF*^{V600E} CRC cell lines

To further explore the level at which p-ERK reactivation originates in vemurafenib-resistant *BRAF*^{V600E} CRC cell lines, we examined levels of p-MEK in the vemurafenib-sensitive and vemurafenib-resistant *BRAF*^{V600E} CRC cell lines. We expected that, if p-ERK reactivation originates above the level of ERK, p-MEK would be preferentially reactivated following vemurafenib treatment in vemurafenib-resistant CRC cell lines.

To address this, cells were treated with vemurafenib (5 uM) or DMSO for 24 hours and p-MEK levels determined by western blot. These analyses revealed that p-MEK was robustly suppressed following vemurafenib treatment in both vemurafenib-sensitive and resistant cell lines (Figure 4.18A). Similar findings were obtained following treatment of these cell lines with a second BRAF inhibitor, dabrafenib (1 uM) (Figure 4.18B).

Collectively, these findings suggest that p-ERK reactivation in vemurafenib-resistant *BRAF*^{V600E} CRC cell lines does not originate at the level of MEK.

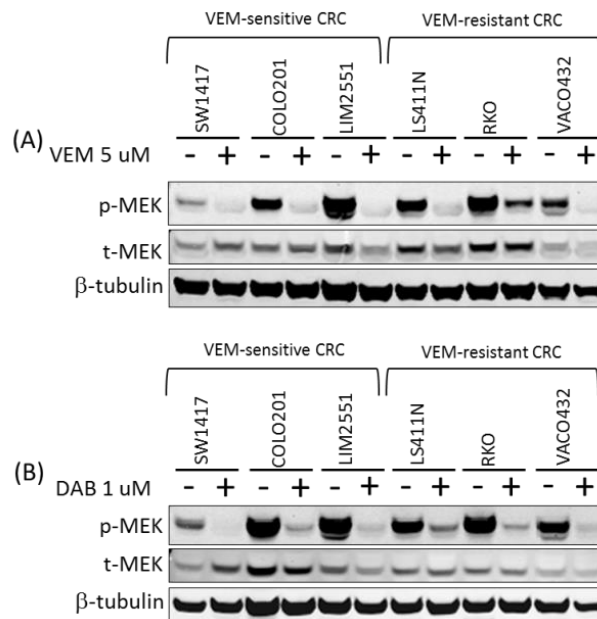


Figure 4.18. Western blots of p-MEK after treatment with DMSO control and (A) vemurafenib 5 uM or (B) dabrafenib 1 uM for 24 hours in the 3 most vemurafenib-resistant colorectal carcinoma cell lines and 3 most vemurafenib-sensitive colorectal carcinoma cell lines. CRC: colorectal carcinoma; VEM: vemurafenib; DAB: dabrafenib.

4.5. Discussion

In this chapter, I investigated ways to overcome resistance of $BRAF^{V600E}$ CRC to BRAF inhibitors.

4.5.1. BRAF-inhibitor resistant $BRAF^{V600E}$ colorectal cancer are also resistant to other MAPK pathway inhibitors

Previously, Corcoran *et al.* hypothesised that incomplete MAPK pathway inhibition may underlie BRAF inhibition resistance in $BRAF$ mut CRC [296]. This hypothesis was based on findings from Bollag *et al.*, who had examined paired tumour biopsies from a cohort of patients with metastatic melanoma in a Phase I study of PLX4032 [23] and found that the degree of MAPK pathway inhibition (measured by cytoplasmic p-ERK levels) in day 14 biopsies compared to pre-treatment biopsies was directly associated with tumour regression [234].

This hypothesis led to studies combining BRAF inhibitor with other MAPK inhibitors for $BRAF^{V600E}$ CRC in the pre-clinical and clinical setting [284, 296]. To investigate whether this is an optimal approach, I theorised that it would be prudent to investigate whether the p-ERK reactivation observed in vemurafenib-resistant $BRAF^{V600E}$ CRC cell lines is also observed after treatment with other MAPK pathway inhibitors.

Interestingly, I found that vemurafenib-resistant $BRAF^{V600E}$ CRC cell lines were similarly resistant to the MEK inhibitor trametinib. Furthermore, p-ERK was rapidly reactivated following

trametinib treatment in these cell lines, and this was not associated with increased expression of p-EGFR or p-Akt.

As I found that p-ERK reactivation was a common response in vemurafenib-resistant *BRAF*^{V600E} CRC cell lines after treatment with multiple different inhibitors of the MAPK pathway, I also treated these cell lines with the ERK inhibitor SCH772984. Notably, vemurafenib-resistant *BRAF*^{V600E} CRC cell lines, with the exception of VACO432, were also similarly resistant to SCH772984 treatment. p-ERK was also reactivated in these cell lines, albeit with a delayed onset (72 hours) compared to treatment with other MAPK pathway inhibitors. As with the other MAPK pathway inhibitors, p-ERK reactivation was not consistently associated with p-EGFR upregulation, and p-Akt expression did not mirror p-EGFR expression. However, as p-Akt was also observed to be upregulated at 72 hours, it is possible that upregulation of p-Akt may also contribute to resistance of vemurafenib-resistant *BRAF*^{V600E} CRC cell lines to SCH772984. This could be explored in further research, by comparing growth inhibition of these cell lines after ERK inhibitor treatment, with and without inhibition of p-Akt. My finding that the use of an ERK inhibitor cannot completely overcome inherent resistance to BRAF inhibitors is supported by the results of the Phase I trial of the ERK1/2 inhibitor ulixertinib (BVD-523), which found no responses among 11 evaluable patients with *BRAF* mut CRC [785]. The VACO432 cell line was sensitive to SCH772984, despite a clear increase in p-ERK levels 72 hours after treatment. One possible explanation for this observation is that p-ERK levels, although induced at 72 hours, remain suppressed at time points such as 48 hours - this requires detailed time course analyses to resolve. As discussed in section 4.2.2., SCH772984 has dual mechanisms of action, both inhibiting ERK 1/2 kinase activity and preventing ERK1/2 phosphorylation by MEK. Therefore, another possibility is that despite the increase in p-ERK levels, SCH772984 is inhibiting ERK1/2 kinase activity sufficiently to result in growth inhibition in this cell line.

Given my observation that BRAF inhibitor-resistant CRC cell lines were also relatively resistant to single-agent inhibitors of other components of the MAPK pathway, I further hypothesised that these cell lines would also be more resistant to the combination of vemurafenib and other MAPK inhibitors.

Indeed, vemurafenib-resistant *BRAF*^{V600E} CRC cell lines were relatively more resistant to the combination of vemurafenib and trametinib compared to the vemurafenib-sensitive *BRAF*^{V600E} CRC cell lines. I observed a lack of synergy with this combination in all cell lines except for RKO, where a synergistic but close to additive effect was observed. p-ERK levels were more suppressed after single agent trametinib compared to vemurafenib treatment, but were not further suppressed by combination treatment with vemurafenib and trametinib compared to trametinib alone. These findings are consistent with the modest response rate (7%) observed in the Phase I/II clinical trial of dabrafenib and trametinib in *BRAF*^{V600} mut CRC [284]. Similarly, the

accompanying pharmacodynamic analysis of paired pre-treatment and day 15 treatment biopsies found significantly less suppression of p-ERK expression in patients with *BRAF* mut CRC treated with vemurafenib and trametinib compared to melanoma patients treated with dabrafenib alone [284]. Whilst Ahronian *et al.* proposed that incomplete MAPK inhibition limits the efficacy of this combination in *BRAF* mut CRC [782], I posit that *BRAF* mut CRCs which are resistant to vemurafenib demonstrate broadly similar resistance to other MAPK inhibitors which, at least in some cases, is also characterised by p-EGFR independent reactivation of p-ERK. This indicates that the resistance is tumour- rather than drug-specific, which has important implications for future pre-clinical and clinical research, as combining multiple MAPK inhibitors is unlikely to be the optimal approach.

4.5.2. Combining vemurafenib with an EGFR inhibitor is not effective in all vemurafenib-resistant *BRAF*^{V600E} colorectal cancer cell lines

In section 3.4.2.4., I found that p-ERK reactivation in vemurafenib-resistant *BRAF*^{V600E} CRC cell lines was not associated with increased p-EGFR expression. To further explore whether the difference in inherent vemurafenib resistance among *BRAF*^{V600E} CRC cell lines was due to an increased capacity to reactivate EGFR, I examined growth inhibition when vemurafenib was combined with cetuximab. This combination was not effective in all vemurafenib-resistant *BRAF*^{V600E} CRC cell lines, and was only able to convert one of the three vemurafenib-resistant lines (VACO432) to a level of sensitivity similar to that observed in the vemurafenib-sensitive cell lines. Therefore, the ability to reactivate EGFR may only explain the basis of inherent vemurafenib resistance in a subset of *BRAF*^{V600E} CRC cell lines. At this stage, it is not clear what is unique about the VACO432 cell line that makes it sensitive to the combination of vemurafenib with cetuximab, which was synergistic in this specific cell line. My future research will include proteomic profiling of all of the *BRAF*^{V600E} CRC cell lines, pre- and post-treatment with vemurafenib and cetuximab. This analysis may shed light on the molecular basis for sensitivity to the combination in this cell line.

4.5.3. Determining the point at which p-ERK reactivation following BRAF inhibitor treatment originates

To further explore alternative non-EGFR mediated mechanisms of resistance of *BRAF* mut CRC to *BRAF* inhibitors, I investigated where in the MAPK pathway *BRAF* inhibitor-induced p-ERK reactivation may originate.

Interestingly, I found that while vemurafenib treatment modestly activated RAS in almost all *BRAF*^{V600E} cell lines, RAS was not preferentially activated in vemurafenib-resistant cell lines. This suggests that reactivation of p-ERK following vemurafenib treatment does not originate through activation of RAS. Similarly, whilst p-CRAF expression was increased following vemurafenib treatment in some *BRAF*^{V600E} CRC cell lines, it was not preferentially upregulated in

vemurafenib-resistant $BRAF^{V600E}$ CRC cell lines. Finally, I observed that p-MEK is suppressed to a similar extent by vemurafenib and dabrafenib treatment in vemurafenib-sensitive and resistant $BRAF^{V600E}$ CRC cell lines, suggesting that p-ERK reactivation in vemurafenib-resistant $BRAF^{V600E}$ CRC cell lines does not originate at the level of MEK.

Corcoran *et al.* proposed that in $BRAF$ mut CRC, the mechanism of resistance to BRAF inhibitors appears to involve RAS activation and p-CRAF induction, based on their observation of higher levels of RAS-GTP and p-CRAF in vemurafenib-treated $BRAF$ mut CRC compared to melanoma. Of note, this group examined p-CRAF levels in four $BRAF$ mut CRC cell lines (SW1417, HT29, WiDr – a derivative of HT29, and LS411N) [296]. Based on our growth inhibition data, the SW1417, HT29, and LS411N cell lines demonstrate relatively high, intermediate, and low sensitivity to vemurafenib, respectively (Figure 3.1). However, the degree of p-CRAF induction demonstrated by Corcoran *et al.* was not clearly different between these cell lines. Moreover, in the same study, an increase in RAS-GTP following vemurafenib treatment was only observed in the HT29 cell line [296].

Hence, my findings are novel and raise the possibility that alternative non-EGFR mediated mechanisms of resistance to BRAF inhibitors may originate downstream of MEK. Potential mechanisms of non-canonical ERK activation include involvement of parallel pathways which crosstalk with the RAS-RAF-MEK-ERK MAPK (RAS-MAPK) pathway, or negative regulators of the RAS-MAPK pathway.

There is evidence that the RAS-MAPK pathway is involved in crosstalk with multiple pathways including other MAPK (JNK and p38) pathways [786], and the PI3K-mTOR [787], TGF- β [788, 789], Wnt- β -catenin [790, 791] and Hedgehog pathways [792]. Modes of crosstalk include cross-inhibition, cross-activation, and pathway convergence on substrates [787], and these may involve processes downstream of MEK. For example, in RAS-MAPK and PI3K-mTOR pathway convergence, activated ERK, RSK, AKT and S6K often act on the same substrate [787]. An example of JNK-MAPK and RAS-MAPK pathway cross-inhibition is where JNK can directly dephosphorylate ERK, at least partially independent of RAF and MEK [793, 794]. Negative feedback regulators of the RAS-MAPK pathway such as DUSPs (described in section 1.2.5.1.3.) may also be involved. For example, the ERK-specific phosphatases DUSP6 and DUSP5 are induced by ERK signalling, and inactivate ERK by dephosphorylating the pT-E-pY motif [121, 148].

In my future research, I will aim to further localise where ERK reactivation in vemurafenib-resistant $BRAF^{V600E}$ CRC cell lines originates in a systematic, unbiased manner by analysing the proteins and phospho-proteins which are up- or down- regulated on reverse phase protein array following vemurafenib treatment.

5. RESULTS – Biomarkers of response and resistance to bevacizumab in patients with metastatic colorectal cancer

5.1. Introduction

The addition of bevacizumab, a murine anti-human VEGF-A monoclonal antibody, to combination chemotherapy results in longer median PFS and modest improvements in OS and ORR in patients with mCRC [536]. However, not all patients who are treated with the drug respond [536]. In addition, the drug is costly and associated with multiple side effects [36].

Discovery of a predictive biomarker of bevacizumab response would enable us to identify patients who are unlikely to respond. These patients could be commenced on alternative therapies sooner, and avoid unnecessary side effects associated with bevacizumab treatment. Identification of a predictive biomarker could also improve the cost effectiveness of bevacizumab use in mCRC [37].

Multiple potential predictive biomarkers of bevacizumab response have been investigated. These include circulating angiogenic factors, circulating endothelial cells and progenitor cells, *in situ* biomarkers, consensus molecular subtype (CMS) subgroups, somatic copy number alteration (SCNAs), hypertension, and dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) (discussed in section 1.3.3.2.). However, despite extensive efforts, a validated predictive biomarker has not yet been identified [38].

Single nucleotide polymorphisms (SNPs) (single nucleotide variations in a genome with a minor allele frequency of > 1% in a population) account for over 90% of human genetic variation [663]. *VEGF-A* is a highly polymorphic gene which has multiple well-established SNPs [658], and there are also multiple SNPs associated with the genes for its receptors, *VEGFR-1* and *-2* [665]. SNPs in *VEGF* family genes may impact on clinical efficacy outcomes if they lead to functional effects on the production or expression of the corresponding ligands and receptors [658]. I hypothesise that germline variability in *VEGF-A*, *VEGFR1* and *VEGFR2* may be important determinants of heterogeneity in response to bevacizumab.

In this chapter, I explore whether SNPs in *VEGF-A*, *VEGFR1* and *VEGFR2* are predictive biomarkers for bevacizumab response, using archived tissue samples and clinical outcome data from participants in the MAX Phase III trial of capecitabine, bevacizumab and mitomycin in the first-line treatment of mCRC [532].

5.2. MAX clinical trial efficacy results

The MAX clinical trial randomised 471 patients in a 1:1:1 ratio to receive treatment with capecitabine (C), capecitabine plus bevacizumab (CB), or capecitabine, bevacizumab and

mitomycin (CBM). 156 patients were randomised to receive C, 157 patients were randomised to receive CB, and 158 patients were randomised to receive CBM [532].(Figure 5.1).

The trial was positive for its primary endpoint of PFS, and the results have been published [532]. In summary, adding bevacizumab, with or without mitomycin, to capecitabine chemotherapy led to an improved PFS in patients with metastatic colorectal cancer. The median PFS for patients treated with C, CB, and CBM was 5.7 months, 8.5 months and 8.4 months, respectively (CB vs C: HR 0.63; 95% CI, 0.50 to 0.79, $P < 0.001$; CBM vs C: HR 0.59; 95% CI, 0.47 to 0.75, $P < 0.001$).

After a median follow-up of 31 months, there was no significant difference in the secondary endpoint, OS (the median OS was 18.9 months for C and CB, and 16.4 months for CBM).

In assessable patients, the confirmed ORR was better with CBM than C ($P = 0.006$), but not better with CB than C ($P = 0.16$) [532].

5.3. Characteristics of the patients in the VEGF family SNP biological sub-study

Of the 471 randomised participants in the MAX clinical trial, 389 participants consented to participation in biological sub-studies. Of these, archived FFPE specimens from 325 participants were available for examination of VEGF family SNPs (69% of the total study population) (Figure 5.1).

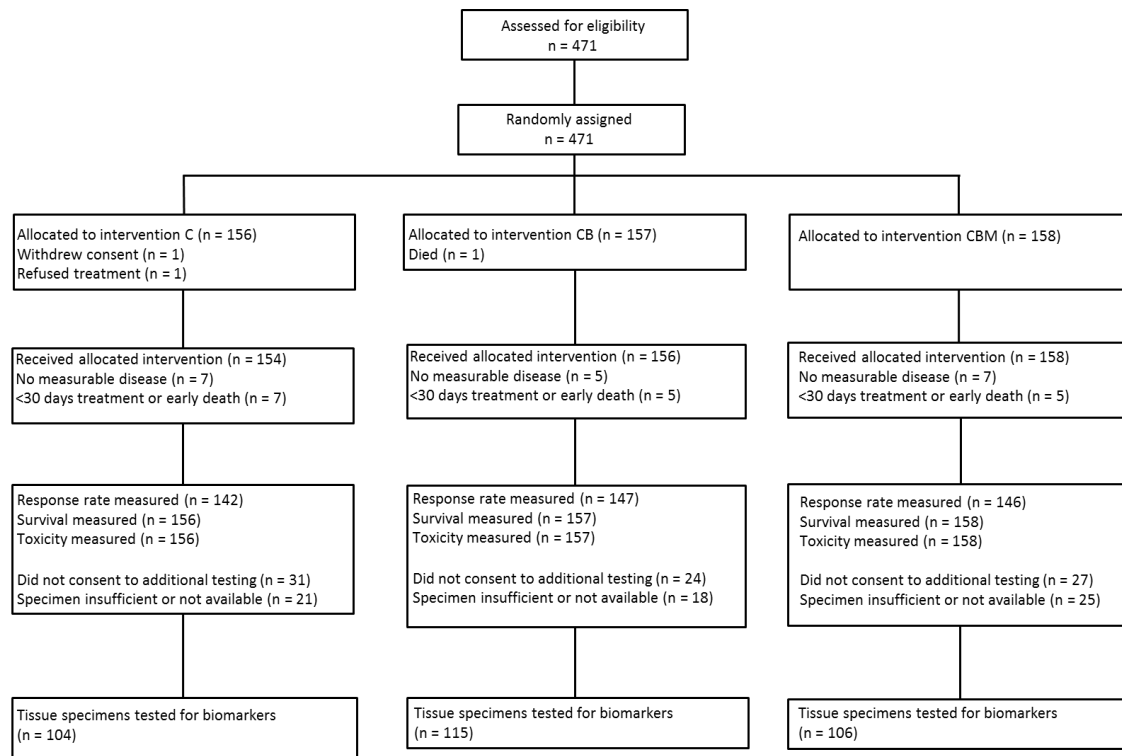


Figure 5.1. CONSORT diagram for the MAX clinical trial *VEGF* family SNP biomarker substudy

The baseline characteristics in the total study population versus the SNP biomarker study population were comparable (Table 5.1).

In addition, the proportion of patients randomised to the C, CB and CBM arms, and the clinical outcomes of patients randomised to these treatment arms were also comparable between the total study population and the SNP biomarker study population (Tables 5.2 - 5.4).

Table 5.1. Baseline characteristics in the total and SNP biomarker study population

Characteristic	Total study population (n = 471)	SNP biomarker study population (n = 325)
Age (years)		
Median	67	68
Range	32 - 86	31 - 85
Gender (n, (%))		
Male	295 (63)	200 (62)
ECOG performance status (n, (%))		
0	263 (56)	187 (58)
1	178 (38)	118 (36)
2	30 (6)	20 (6)
Capecitabine dosage (n, (%))		
1 g/m ² BD	315 (67)	208 (64)
1.25 g/m ² BD	156 (33)	117 (36)
Prior adjuvant treatment (n, (%))		
Chemotherapy	104 (22)	70 (22)
Radiotherapy	59 (13)	32 (10)
Primary site (n, (%))		
Right sided (caecum, ascending colon, transverse colon)	124 (26)	94 (29)
Left sided (descending colon, sigmoid, recto-sigmoid)	209 (44)	148 (46)
Rectum	107 (23)	68 (21)
Other	27 (6)	14 (4)
Uncertain	4 (1)	1 (0.3)
Primary tumour resected (n, (%))		
Yes	371 (79)	280 (86)
Any metastases resected (n, (%))		
Yes	45 (10)	29 (9)
Extent of disease at baseline (n, (%))		
Local disease (colon or rectum)	169 (36)	103 (32)
Liver metastases	353 (75)	240 (74)
Lymph node metastases	219 (46)	148 (46)
Lung metastases	185 (39)	126 (39)
Bone metastases	18 (4)	11 (3)
Peritoneal metastases	84 (18)	54 (17)
Other metastases	50 (11)	37 (11)

SNP: single nucleotide polymorphism; ECOG: Eastern Cooperative Oncology Group; BD: twice daily.

Table 5.2. Number and proportion of patients randomised to the C, CB and CBM arms in the total and SNP biomarker study population

Treatment arm	Total study population (n, (%)) (n = 471)	SNP biomarker study population (n, (%)) (n = 325)
C	156 (33)	104 (32)
CB	157 (33)	115 (35)
CBM	158 (34)	106 (33)

SNP: single nucleotide polymorphism; C: capecitabine; CB: capecitabine plus bevacizumab; CBM: capecitabine, bevacizumab and mitomycin.

Table 5.3. Progression-free survival outcomes in the total and SNP biomarker study population

Treatment arm	Median PFS (months)	Total study population			SNP biomarker population		
		HR	95% CI	P value	HR	95% CI	P value
C	5.7	1.00	-	-	1.00	-	-
CB	8.5	0.62	0.49 to 0.79	< 0.001	0.67	0.57 to 0.89	0.005
CBM	8.4	0.59	0.47 to 0.75	< 0.001	0.66	0.49 to 0.87	0.004
CB + CBM	8.4	0.61	0.48 to 0.74	< 0.001	0.66	0.52 to 0.85	0.001

SNP: single nucleotide polymorphism; PFS: progression-free survival; C: capecitabine; CB: capecitabine plus bevacizumab; CBM: capecitabine, bevacizumab and mitomycin; HR: hazard ratio; CI: confidence interval.

Table 5.4. Overall survival outcomes in the total and SNP biomarker study population

Treatment arm	Median OS (months)	Total study population			SNP biomarker population		
		HR	95% CI	P value	HR	95% CI	P value
C	18.9	1.00	-	-	1.00	-	-
CB	18.9	0.86	0.67 to 1.11	0.25	0.96	0.70 to 1.31	0.78
CBM	16.4	1.00	0.78 to 1.29	0.97	1.00	0.73 to 1.38	0.98
CB + CBM	17.3	0.93	0.75 to 1.16	0.54	0.98	0.74 to 1.28	0.86

SNP: single nucleotide polymorphism; OS: overall survival; C: capecitabine; CB: capecitabine plus bevacizumab; CBM: capecitabine, bevacizumab and mitomycin; HR: hazard ratio; CI: confidence interval.

5.4. SNP genotyping results

5.4.1. Archived FFPE tissue available for SNP genotyping

Of the archived FFPE specimens from 325 participants which were available for SNP genotyping, normal tissue was available for 164 participants (51%). This was normal colorectal epithelial tissue for all but one participant, who had normal liver tissue available.

Colorectal adenoma tissue was available for seven participants (2%), and colorectal carcinoma tissue was available for the remaining 154 participants (47%). Of these 154 participants with tumour tissue available for SNP genotyping, the majority were from the primary tumour (137, 90%) (Figure 5.2).

Of the 17 participants with only secondary tumour tissue available for SNP genotyping, metastatic sites included liver ($n = 6$), omentum ($n = 4$), and pelvic, abdominal wall, bone, lung, skin, orbital and gastric tissue ($n = 1$ for each site).

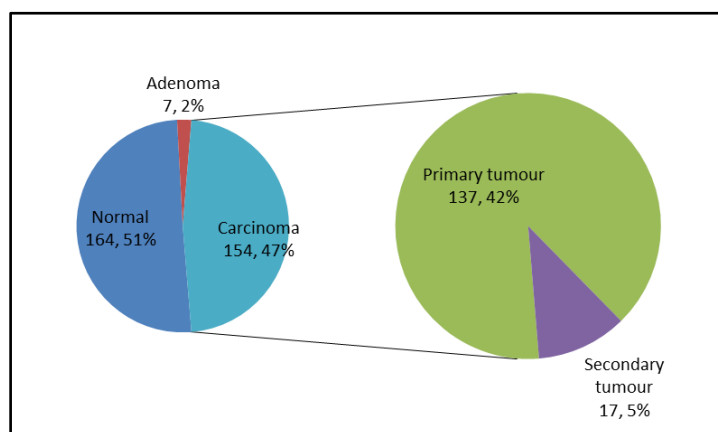


Figure 5.2. Pie chart showing the type of archived formalin-fixed paraffin embedded tissue available for SNP genotyping

5.4.2. SNP genotyping

5.4.2.1. Analysis of SNPs in *VEGF-A*, *VEGFR1 (FLT-1)* and *VEGFR2 (KDR)*

A total of 21 SNPs were genotyped – 7 in *VEGF-A* (rs699946, rs699947, rs833061, rs1570360, rs2010963, rs25648, rs3025039), 5 in *VEGFR1* (rs9513070, rs9554316, rs7993418, rs9582036, rs9554320) and 9 in *VEGFR2* (rs12505758, rs7655964, rs1870377, rs2305948, rs2305949, rs11133360, rs7667298, rs2071559, rs1551641). These SNPs were selected based on previous demonstration of an effect on corresponding VEGF ligand or receptor mRNA or protein expression, and/or an association with clinical outcomes.

These SNPs were assessed for deviation from Hardy-Weinberg Equilibrium (HWE) using a corrected Hardy-Weinberg P -value of $P > (0.05/21)$ or 0.0024. 16 SNPs which were in HWE

were used in subsequent analyses. These included 6 SNPs in *VEGF-A*, 4 SNPs in *VEGF-R1* and 6 SNPs in *VEGF-R2* (Table 5.5). The location of these SNPs is shown in Figures 5.3 - 5.5, and their location relative to the gene is shown in Table 5.5.

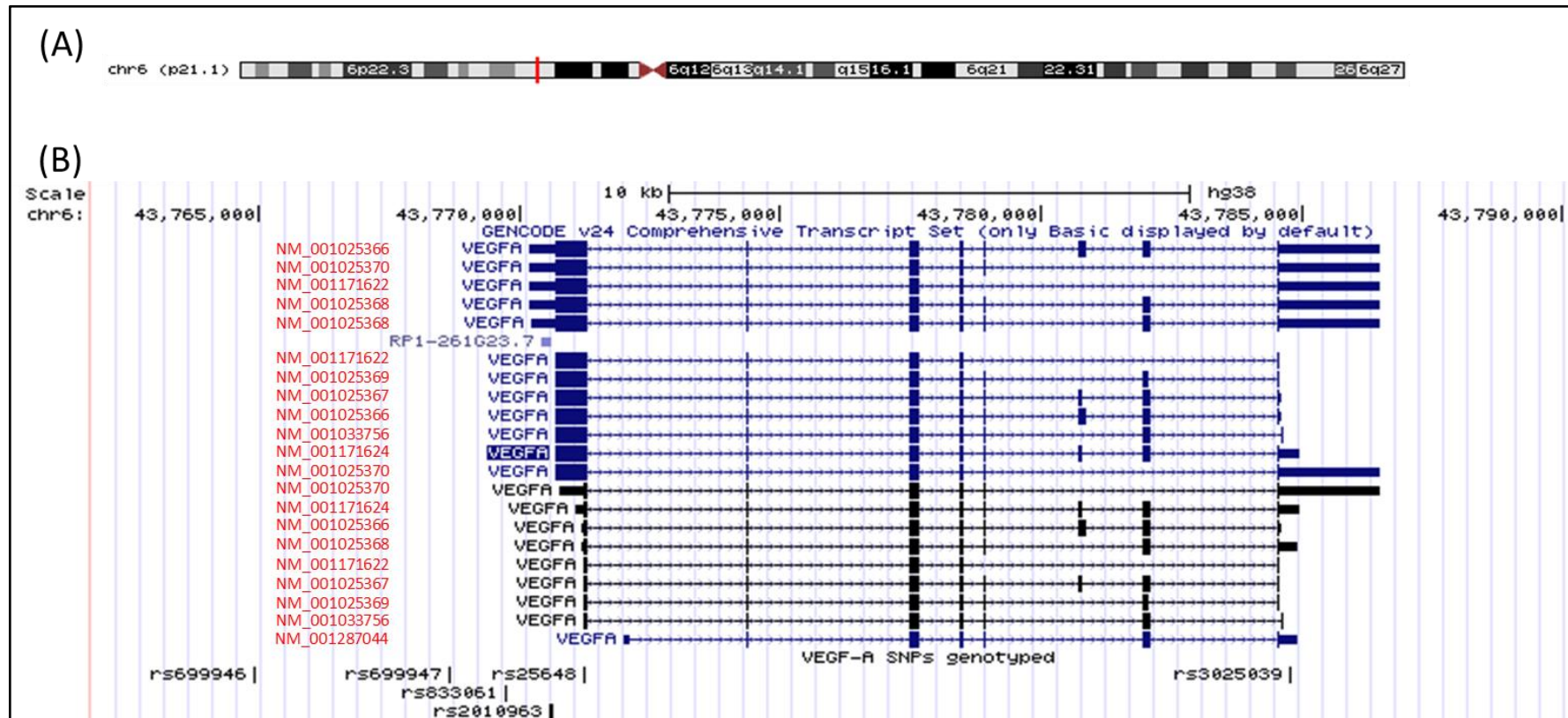


Figure 5.3. (A) Location of *VEGF-A* gene on chromosome 6, denoted by vertical red line; (B) Location of analysed SNPs in the *VEGF-A* gene. Location of SNPs were mapped using sequences from dbSNP 150, in the UCSC Genome Browser on the Human Dec. 2013 (GRCh38/hg38) Assembly. RefSeq accession numbers for the mRNA transcript are in red.

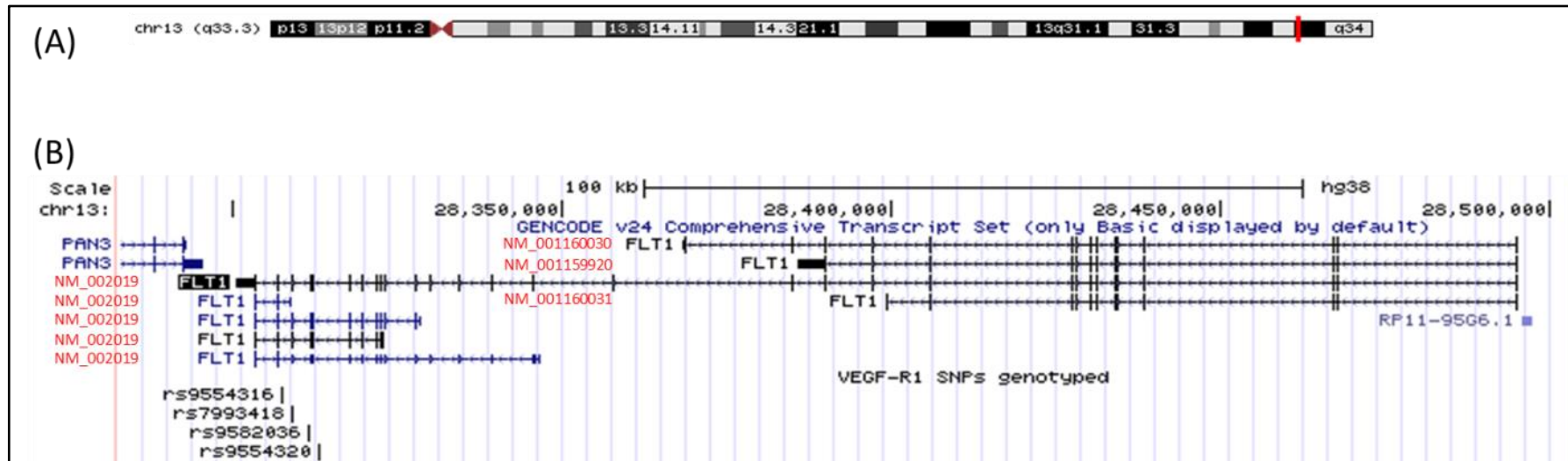


Figure 5.4. (A) Location of *VEGFR1* gene on chromosome 13, denoted by vertical red line; (B) Location of analysed SNPs in the *VEGFR1* gene. Location of SNPs were mapped using sequences from dbSNP 150, in the UCSC Genome Browser on the Human Dec. 2013 (GRCh38/hg38) Assembly. RefSeq accession numbers for the mRNA transcript are in red.

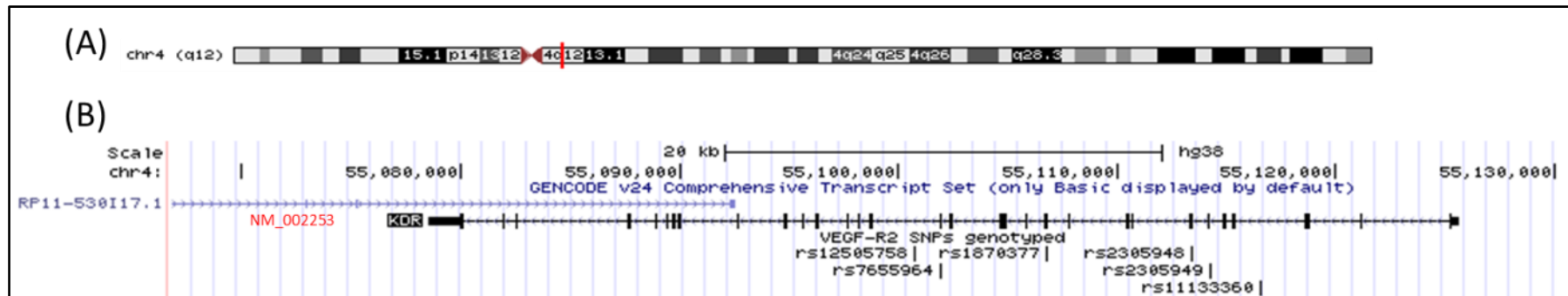


Figure 5.5. (A) Location of *VEGFR2* gene on chromosome 4, denoted by vertical red line; (B) Location of analysed SNPs in the *VEGFR2* gene

Location of SNPs were mapped using sequences from dbSNP 150, in the UCSC Genome Browser on the Human Dec. 2013 (GRCh38/hg38) Assembly. RefSeq accession numbers for the mRNA transcript are in red.

Table 5.5. Location of the analysed SNPs in relation to the gene

Gene	SNP	Location ^a	RefSeq accession number for corresponding mRNA transcript ^a
VEGF-A	rs699946	Not described	
	rs699947	Near gene (5' end)/promoter [664]	NM_001025366.2, NM_001025367.2, NM_001025368.2, NM_001025369.2, NM_001025370.2, NM_001033756.2, NM_001171622.1, NM_001171623.1, NM_001171624.1, NM_001171625.1, NM_001171626.1, NM_001171627.1, NM_001171628.1, NM_001171629.1, NM_001171630.1, NM_001204384.1, NM_001204385.1, NM_001317010.1, NM_003376.5
	rs833061	Near gene (5' end)/promoter [664]	NM_001025366.2, NM_001025367.2, NM_001025368.2, NM_001025369.2, NM_001025370.2, NM_001033756.2, NM_001171622.1, NM_001171623.1, NM_001171624.1, NM_001171625.1, NM_001171626.1, NM_001171627.1, NM_001171628.1, NM_001171629.1, NM_001171630.1, NM_001204384.1, NM_001204385.1, NM_001317010.1, NM_003376.5
	rs2010963	5'UTR [664]	NM_001025366.2, NM_001025367.2, NM_001025368.2, NM_001025369.2, NM_001025370.2, NM_001033756.2, NM_001171622.1, NM_001171623.1, NM_001171624.1, NM_001171625.1, NM_001171626.1, NM_001171627.1, NM_001171628.1, NM_001171629.1, NM_001171630.1, NM_001204384.1, NM_001204385.1, NM_001317010.1, NM_003376.5
		Near gene (5' end)	NM_001287044.1
	rs25648	Exon- coding sequence, synonymous substitution	NM_001025366.2, NM_001025367.2, NM_001025368.2, NM_001025369.2, NM_001025370.2, NM_001033756.2, NM_001171622.1, NM_001204385.1, NM_003376.5
		5'UTR [664]	NM_001171623.1, NM_001171624.1, NM_001171625.1, NM_001171626.1, NM_001171627.1, NM_001171628.1, NM_001171629.1, NM_001171630.1, NM_001204384.1, NM_001317010.1
		Near gene (5' end)	NM_001287044.1
	rs3025039	3'UTR	NM_001025366.2, NM_001025367.2, NM_001025368.2, NM_001025369.2, NM_001025370.2, NM_001033756.2, NM_001171622.1, NM_001171623.1, NM_001171624.1, NM_001171625.1, NM_001171626.1, NM_001171627.1, NM_001171628.1, NM_001171629.1, NM_001171630.1, NM_001204384.1, NM_001204385.1, NM_001287044.1, NM_001317010.1, NM_003376.5

Table 5.5. (continued) Location of the analysed SNPs in relation to the gene

Gene	SNP	Location ^a	RefSeq accession number for corresponding mRNA transcript ^a
<i>VEGFR1</i> (<i>FLT-1</i>)	rs9554316	Intron	NM_002019.4, XM_017020485.1
	rs7993418	Exon- coding sequence, synonymous substitution	NM_002019.4, XM_017020485.1
	rs9582036	Intron	NM_002019.4, XM_017020485.1
	rs9554320	Intron	NM_002019.4, XM_017020485.1
<i>VEGFR2</i> (<i>KDR</i>)	rs12505758	Intron	NM_002253.2
	rs7655964	Intron	NM_002253.2
	rs1870377	Exon- missense mutation	NM_002253.2
	rs2305948	Exon- missense mutation	NM_002253.2
	rs2305949	Intron	NM_002253.2
	rs11133360	Intron	NM_002253.2

^a Location and RefSeq accession numbers for the corresponding mRNA transcript taken from the Gene Model table in the NCBI dbSNP Short Genetic Variations database, using the assembly GRCh38.p7. Retrieved from (<https://www.ncbi.nlm.nih.gov/snp>), accessed October 31 2018.

As shown in Table 5.6, the calculated minor allele frequency (MAF) for all of the SNPs analysed was 10% or greater, providing reassurance that they would have sufficient impact as a potential biomarker at the population level.

Table 5.6. Calculated minor allele frequency for the analysed SNPs

Gene	SNP	Minor Allele Frequency
VEGF-A	rs699946	0.138
	rs699947	0.47
	rs833061	0.482
	rs2010963	0.308
	rs25648	0.125
	rs3025039	0.14
VEGFR1 (FLT-1)	rs9554316	0.19
	rs7993418	0.197
	rs9582036	0.273
	rs9554320	0.414
VEGFR2 (KDR)	rs12505758	0.112
	rs7655964	0.315
	rs1870377	0.252
	rs2305948	0.1
	rs2305949	0.192
	rs11133360	0.431

SNP: single nucleotide polymorphism

5.4.2.2. Success of genotyping

Of the 16 SNPs analysed, genotyping experiments successfully identified the full genotype (i.e., Homozygous Major Allele, Homozygous Minor Allele, or Heterozygous Major Allele/Minor Allele), in $\geq 84\%$ of cases for all of the SNPs genotyped (Figure 5.6, Figure 5.7, Table 5.7).

In some cases, whilst it was possible to partially identify the genotype (i.e., having the Major Allele or the Minor Allele), it was not possible to clearly determine both alleles from the Allelic Discrimination Plot (Figure 5.8), even after performing multiple replicates and using a range of

amplification cycles (between 40 and 60). The proportion of cases with 'Partial genotype known' was not more than 7% for any SNP (Figure 5.6, Table 5.7).

In other cases, it was not possible to determine the genotype, due to discordant genotyping calls between replicates, the reactions failing to amplify, or due to having insufficient genomic DNA template available to perform the experiment. The proportion of cases with 'Undetermined genotype' was not more than 9% for any SNP (Figure 5.6, Figure 5.7, Table 5.7).

Examples of full, partial and undetermined genotype calls on an Allelic Discrimination Plot are shown in Figure 5.8.

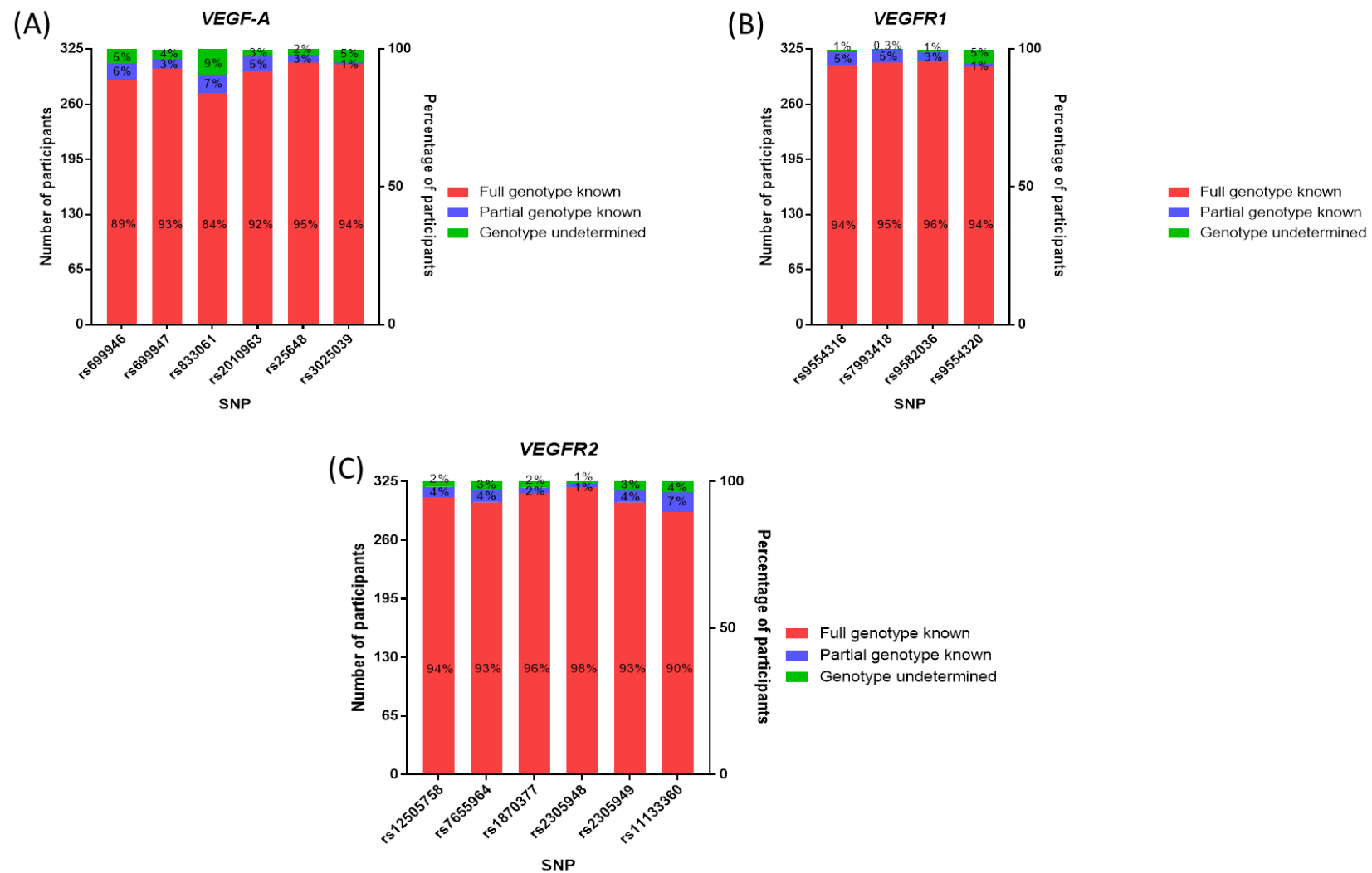


Figure 5.6. Success of genotyping for the analysed SNPs in *VEGF-A*, *VEGFR1*, and *VEGFR2*

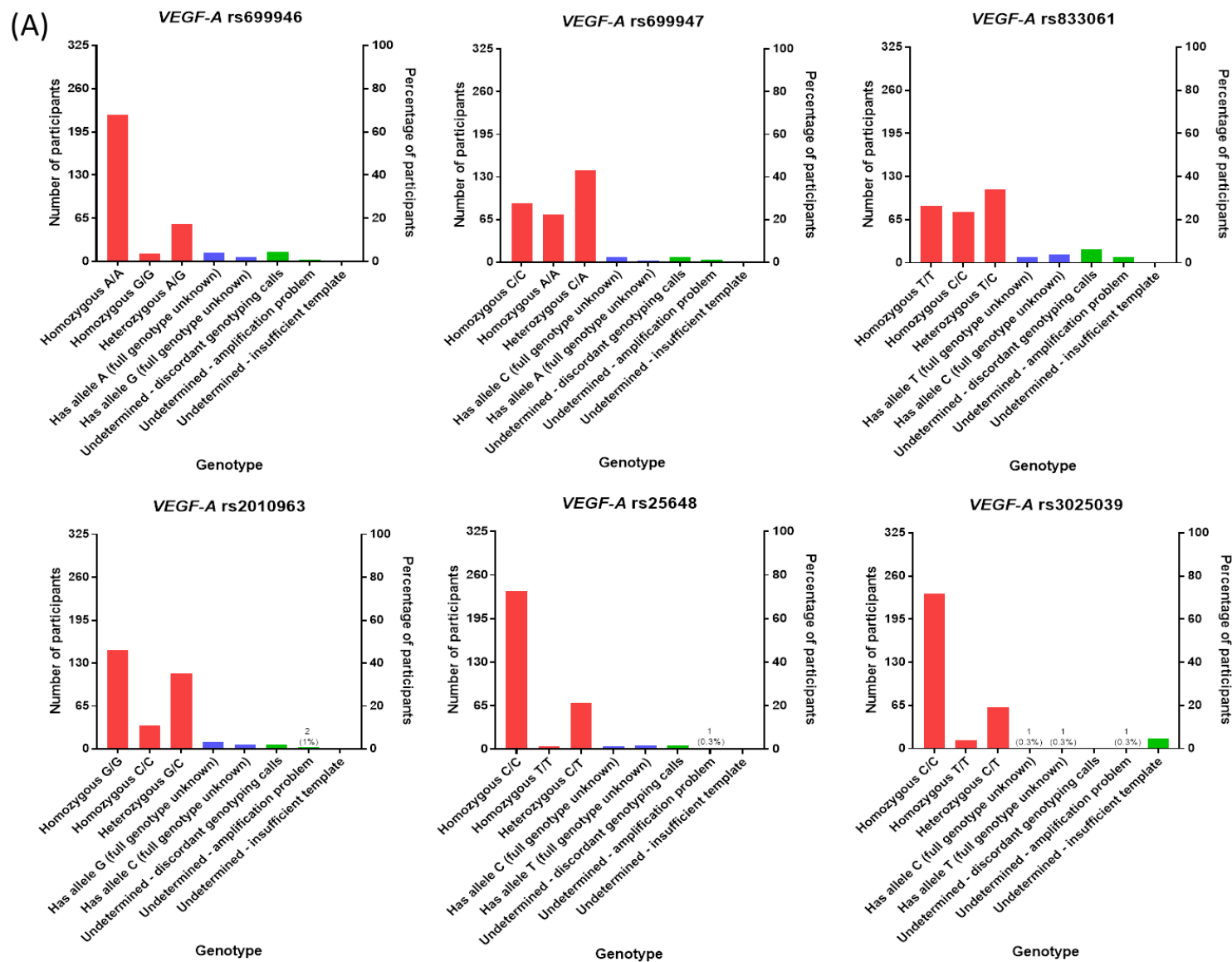


Figure 5.7. (A) Results of genotyping for the analysed SNPs in *VEGF-A*

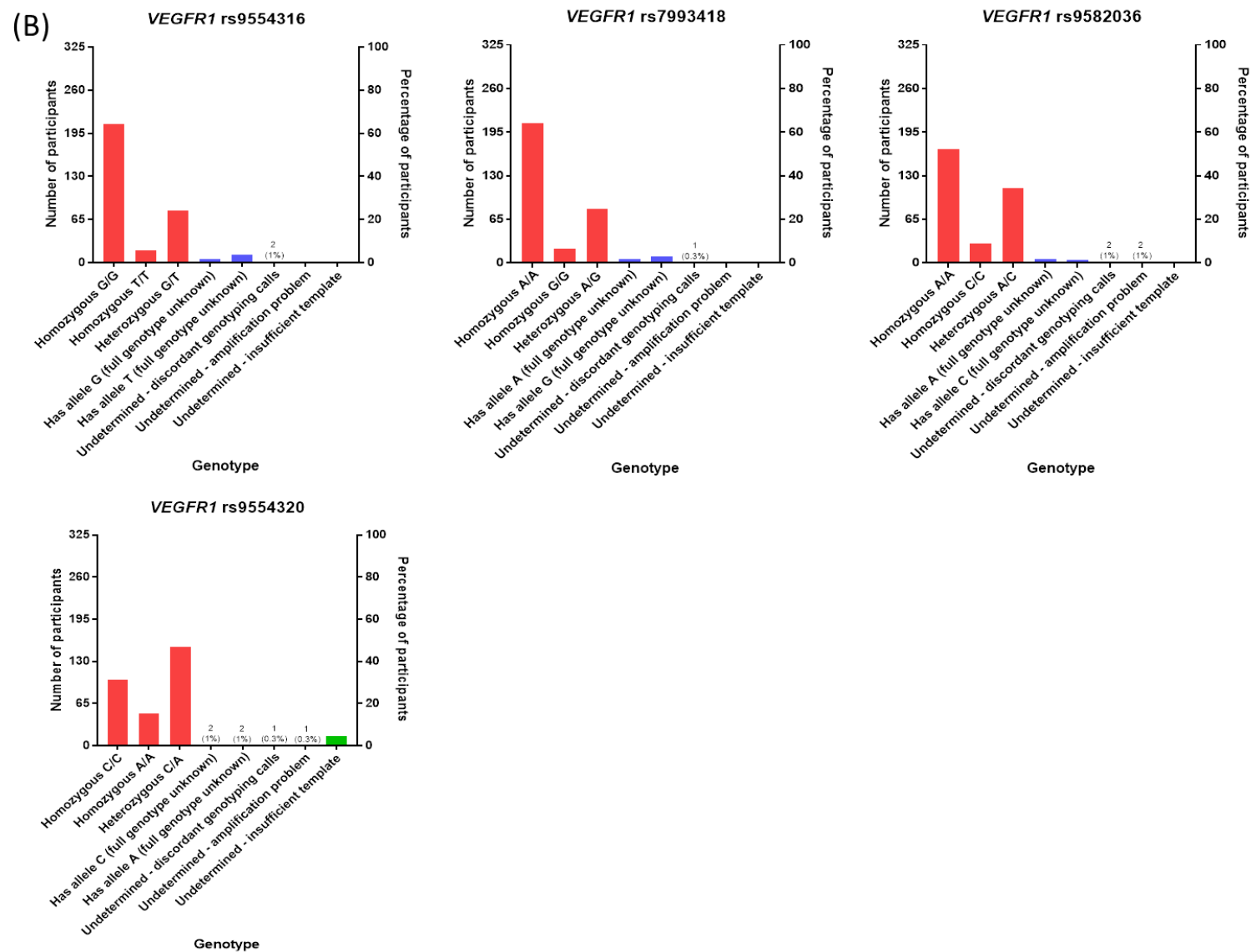


Figure 5.7. (B) Results of genotyping for the analysed SNPs in *VEGFR1*

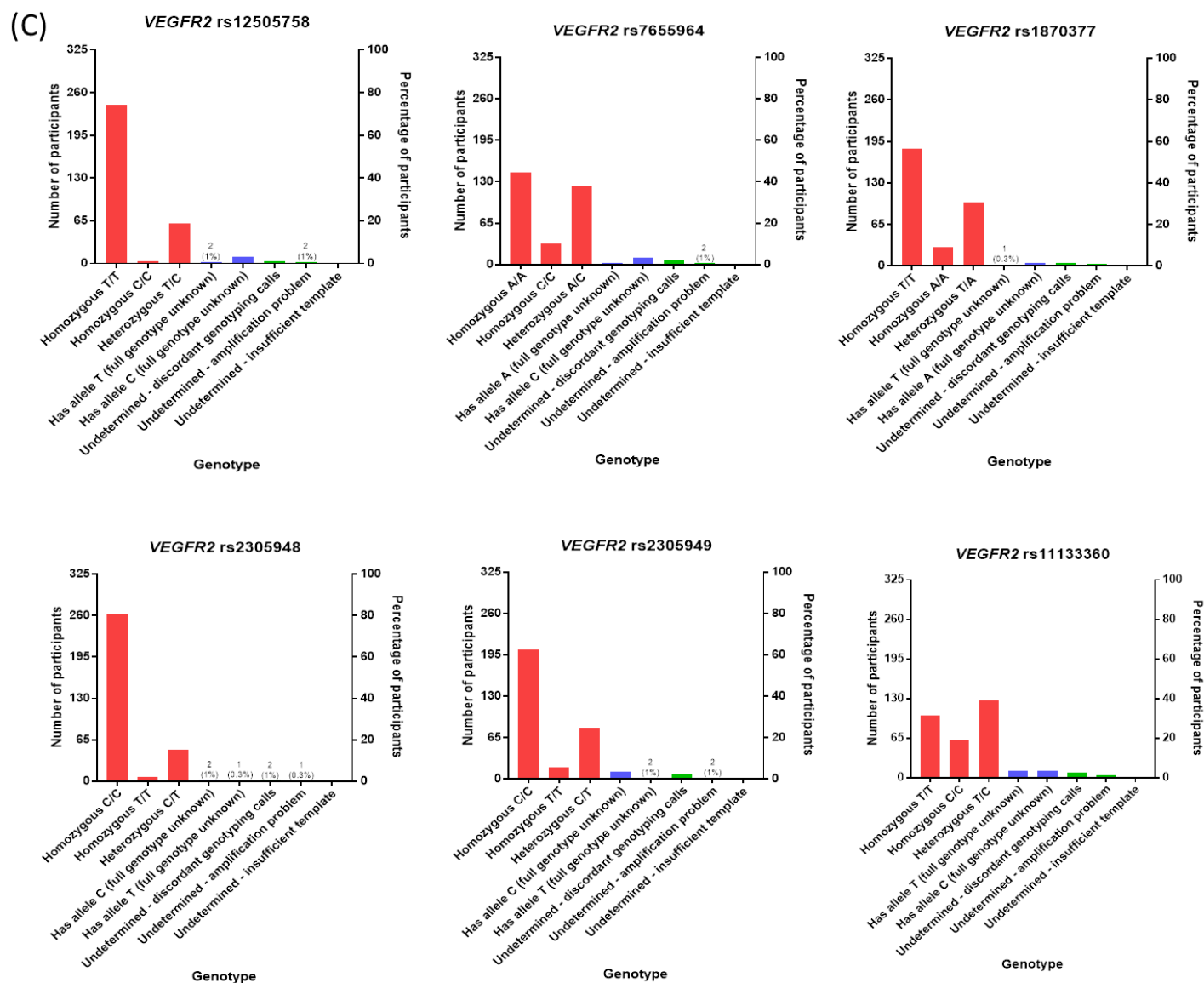


Figure 5.7. (C) Results of genotyping for the analysed SNPs in *VEGFR2*

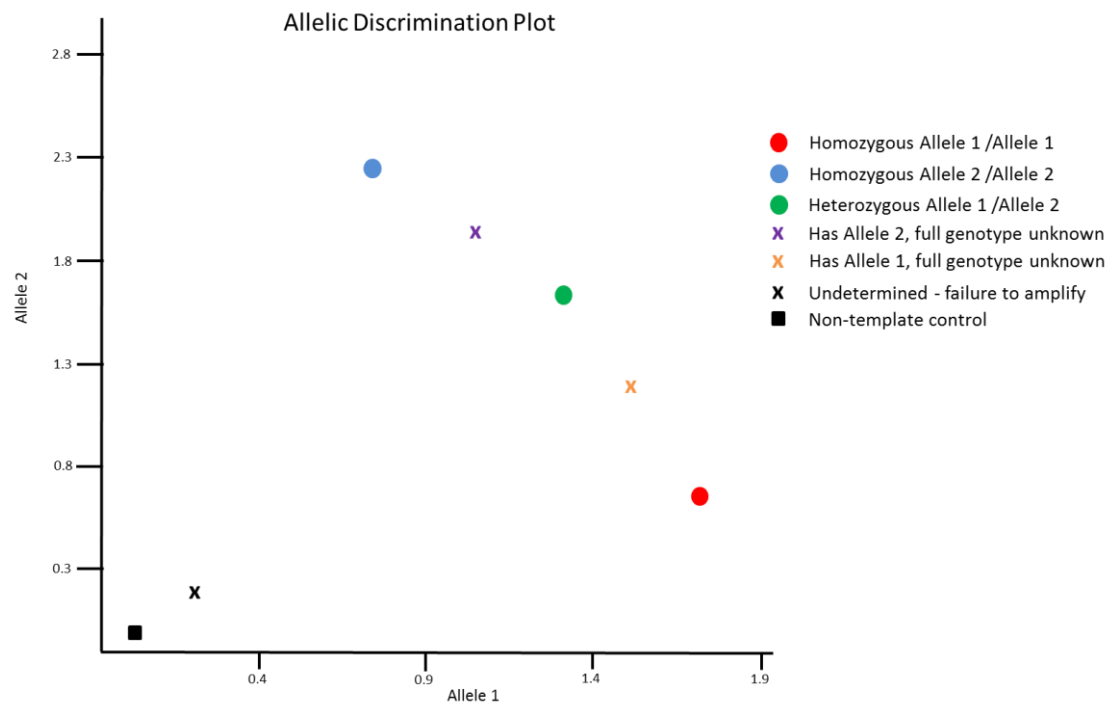


Figure 5.8. Examples of genotype calls on an Allelic Discrimination plot

Table 5.7. Results of SNP genotyping for 16 SNPs in *VEGF-A*, *VEGFR1* and *VEGFR2*

SNP information				Genotype (n, (%))										
				Full genotype known				Partial genotype known			Undetermined genotype			
Gene	SNP	Major allele	Minor allele	Homozygous major allele	Homozygous minor allele	Heterozygous major/ minor alleles	Total number and proportion	Has major allele	Has minor allele	Total number and proportion	Discordant genotyping calls	Amplification problem	Insufficient template to perform genotyping	Total number and proportion
<i>VEGF-A</i>	rs699946	A	G	221 (68)	12 (4)	56 (17)	289 (89)	13 (4)	6 (2)	19 (6)	14 (4)	3 (1)	0 (0)	17 (5)
	rs699947	C	A	90 (28)	72 (22)	140 (43)	302 (93)	8 (2)	3 (1)	11 (3)	8 (2)	4 (1)	0 (0)	12 (4)
	rs833061	T	C	86 (26)	76 (23)	111 (34)	273 (84)	9 (3)	13 (4)	22 (7)	21 (6)	9 (3)	0 (0)	30 (9)
	rs2010963	G	C	150 (46)	35 (11)	114 (35)	299 (92)	11 (3)	6 (2)	17 (5)	7 (2)	2 (1)	0 (0)	9 (3)
	rs25648	C	T	236 (73)	4 (1)	69 (21)	309 (95)	4 (1)	5 (2)	9 (3)	6 (2)	1 (0.3)	0 (0)	7 (2)
	rs3025039	C	T	233 (72)	12 (4)	62 (19)	307 (94)	1 (0.3)	1 (0.3)	2 (1)	0 (0)	1 (0.3)	15 (5)	16 (5)
<i>VEGFR1</i>	rs9554316	G	T	209 (64)	19 (6)	78 (24)	306 (94)	5 (2)	12 (4)	17 (5)	2 (1)	0 (0)	0 (0)	2 (1)
	rs7993418	A	G	208 (64)	21 (6)	80 (25)	309 (95)	5 (2)	10 (3)	15 (5)	1 (0.3)	0 (0)	0 (0)	1 (0.4)
	rs9582036	A	C	170 (52)	29 (9)	112 (34)	311 (96)	6 (2)	4 (1)	10 (3)	2 (1)	2 (1)	0 (0)	4 (1)
	rs9554320	C	A	102 (31)	50 (15)	152 (47)	304 (94)	2 (1)	2 (1)	4 (1)	1 (0.3)	1 (0.3)	15 (5)	17 (5)
<i>VEGFR2</i>	rs12505758	T	C	242 (74)	4 (1)	61 (19)	307 (94)	2 (1)	10 (3)	12 (4)	4 (1)	2 (1)	0 (0)	6 (2)
	rs7655964	A	C	145 (45)	33 (10)	124 (38)	302 (93)	3 (1)	11 (3)	14 (4)	7 (2)	2 (1)	0 (0)	9 (3)
	rs1870377	T	A	184 (57)	29 (9)	99 (30)	312 (96)	1 (0.3)	5 (2)	6 (2)	4 (1)	3 (1)	0 (0)	7 (2)
	rs2305948	C	T	262 (81)	7 (2)	50 (15)	319 (98)	2 (1)	1 (0.3)	3 (1)	2 (1)	1 (0.3)	0 (0)	3 (1)
	rs2305949	C	T	204 (63)	18 (6)	80 (25)	302 (93)	11 (3)	2 (1)	13 (4)	8 (2)	2 (1)	0 (0)	10 (3)
	rs11133360	T	C	102 (31)	62 (19)	127 (39)	291 (90)	11 (3)	11 (3)	22 (7)	8 (2)	4 (1)	0 (0)	12 (4)

SNP: single nucleotide polymorphism.

5.4.2.3. Concordance between SNP genotypes in normal and tumour patient tissue

As described in section 5.4.1., 164 of 325 participants (51%) had normal tissue which was available and preferentially used for SNP genotyping; of the remaining participants without available normal tissue, colorectal carcinoma tissue was used for 154 participants (47%) and colorectal adenoma tissue was used in seven participants (2%).

We proposed that it would be reasonable to use SNP genotypes determined from either normal or non-normal tissue if a preliminary analysis revealed moderate or strong concordance in SNP genotypes derived from matched normal and tumour samples. For the first fifty cases (ordered consecutively by patient ID) where both tumour tissue and adjacent normal tissue were available, we assessed the concordance of SNP genotypes generated from these tissues (where the full genotype was determined for both samples).

We found that concordance was strong (Kendall's $\tau > 0.70$) for all SNPs analysed, with the exception of rs12505758 in *VEGF-R2*, where concordance was moderate (Kendall's $\tau > 0.40$) (Table 5.8). Therefore, all 16 SNPs were used in analyses.

Table 5.8. Concordance in SNP genotype between matched colorectal normal and tumour tissue

Gene	SNP	Kendall's τ for concordance	<i>n</i> (out of 50) with full genotype determined for both normal and tumour tissue
<i>VEGF-A</i>	rs699946	1	40
	rs699947	0.94	43
	rs833061	0.84	38
	rs2010963	0.89	44
	rs25648	0.95	49
	rs3025039	0.95	47
<i>VEGFR1</i> (<i>FLT-1</i>)	rs9554316	0.97	44
	rs7993418	0.91	46
	rs9582036	0.87	43
	rs9554320	0.86	48
<i>VEGFR2</i> (<i>KDR</i>)	rs12505758	0.46	48
	rs7655964	0.85	46
	rs1870377	0.81	46
	rs2305948	0.91	47
	rs2305949	0.86	43
	rs11133360	0.79	42

SNP: single nucleotide polymorphism.

5.5. Association between SNPs and progression-free survival (PFS)

5.5.1. SNPs in VEGF family members as prognostic biomarkers for the PFS outcome

5.5.1.1. Univariate associations between SNPs in VEGF family members and the PFS outcome

In the first analysis of univariate associations between SNPs in VEGF family members and PFS, we compared outcomes in the participants with a homozygous major allele genotype to outcomes in the participants without homozygous major allele (i.e., composite of participants with the genotypes homozygous minor allele and heterozygous major and minor alleles) (Figure 5.9, Table 5.9). For the *VEGF-A* rs699946 SNP, there were significant differences between PFS in participants with the AA genotype, compared to the GG and AG genotype. The median PFS was 7.5 months among participants with the *VEGF-A* rs699946 AA genotype, compared to 8.8 months among those with the GG and AG genotype (log rank $P = 0.024$, Figure 5.10). The hazard ratio (HR) for AA vs GG/AG was 1.40; 95% CI 1.04 to 1.87, $P = 0.025$.

There were also significant differences between PFS in participants with the *VEGF-A* rs25648 CC genotype, compared to the TT and CT genotypes. The median PFS was 8.6 months among participants with the *VEGF-A* rs25648 CC genotype, compared to 6.0 months among those with the TT and CT genotypes (log rank $P = 0.002$, Figure 5.11). The HR for CC vs TT/CT was 0.65; 95% CI 0.49 to 0.85, $P = 0.002$.

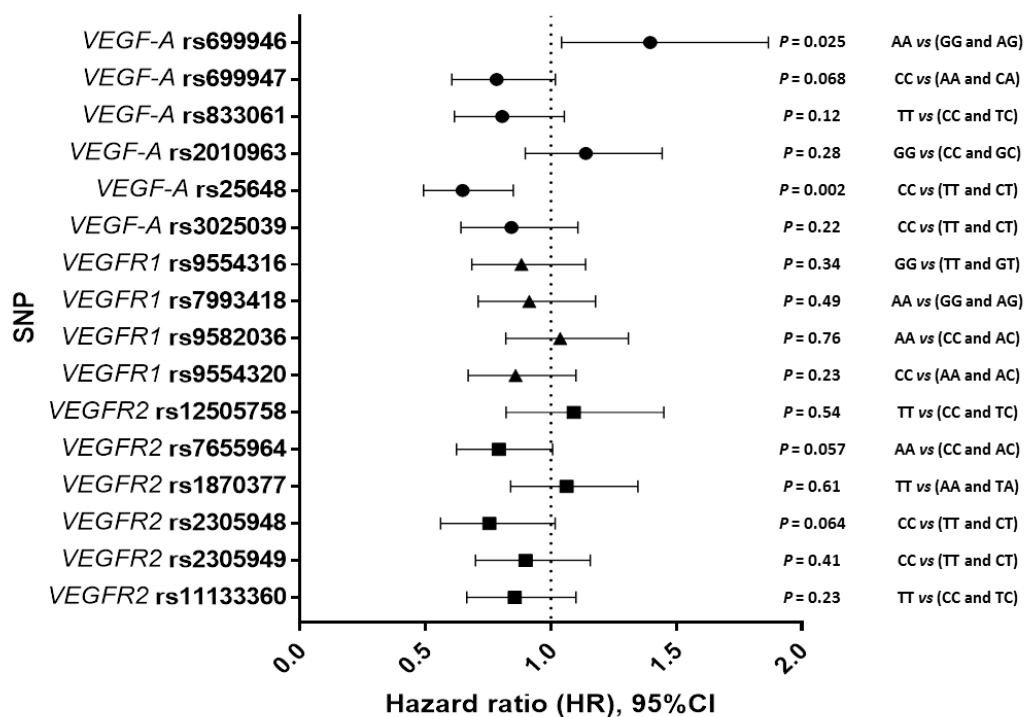


Figure 5.9. Forest plot showing Hazard Ratio (HR) with 95% CI for the comparison of PFS in participants with a homozygous major allele genotype versus without a homozygous major allele genotype

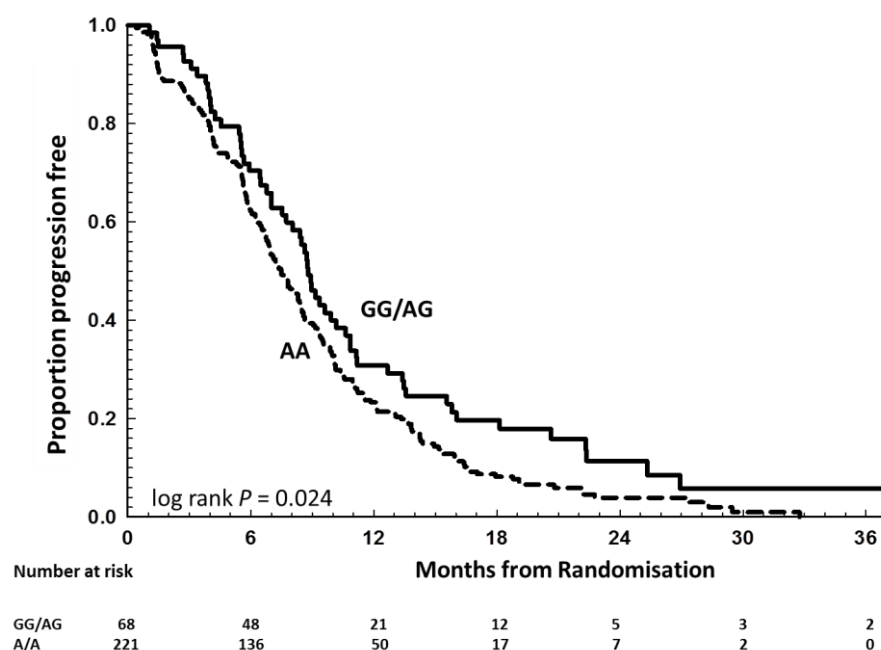


Figure 5.10. Kaplan-Meier curves of PFS for all patients according to the comparison of the VEGF-A rs699946 AA genotype to the GG and AG genotypes

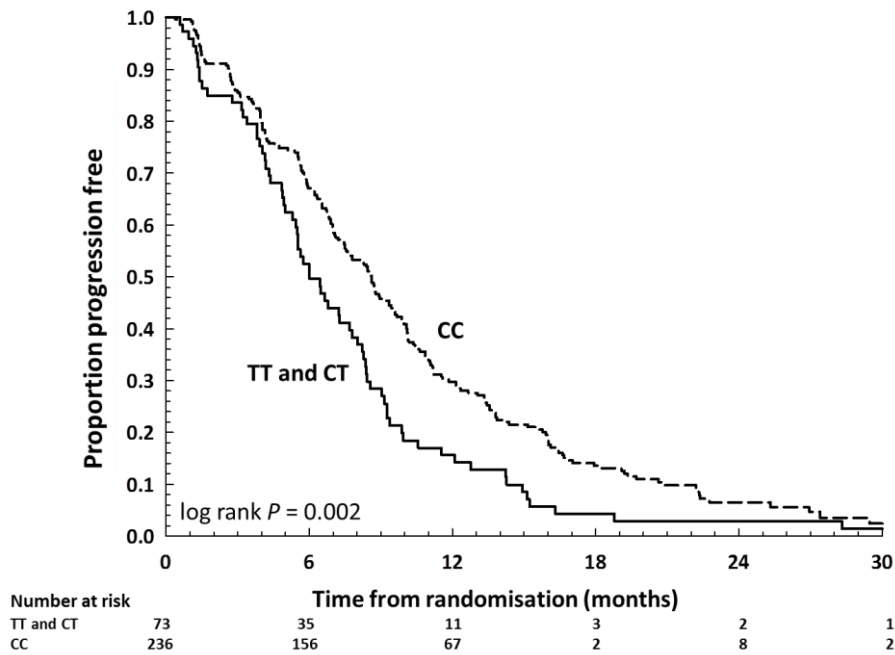


Figure 5.11. Kaplan-Meier curves of PFS for all patients according to the comparison of the *VEGF-A* rs25648 CC genotype to the TT and CT genotypes

In the second analysis of univariate associations between SNPs in *VEGF* family members and PFS, we compared outcomes in the participants with a homozygous minor allele genotype to outcomes in the participants without a homozygous minor allele genotype (i.e., composite of participants with homozygous major allele and heterozygous major and minor allele genotypes) (Figure 5.12, Table 5.9). There were significant differences between PFS in participants with the *VEGF-A* rs699947 AA genotype, compared to the CC and CA genotypes. The median PFS was 6.8 months among participants with the *VEGF-A* rs699947 AA genotype, compared to 8.42 months among those with the CC and CA genotypes (log rank $P = 0.047$, Figure 5.13). The HR for AA vs CC/CA was 1.32, 95% CI 1.002 to 1.738, $P = 0.048$.

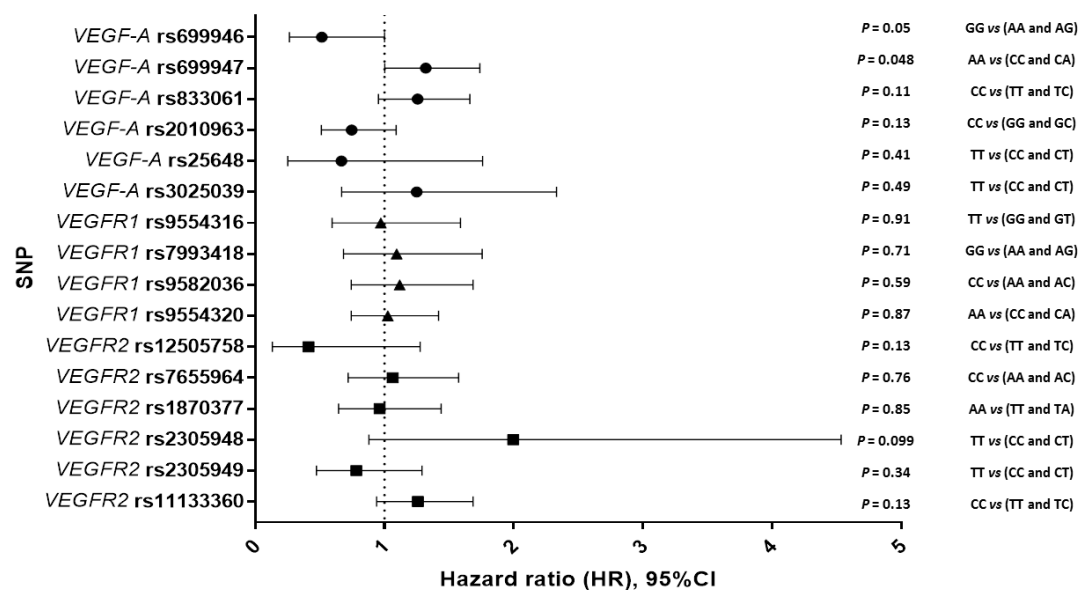


Figure 5.12. Forest plot showing Hazard Ratio (HR) with 95% CI for the comparison of PFS in participants with a homozygous minor allele genotype versus without a homozygous minor allele genotype

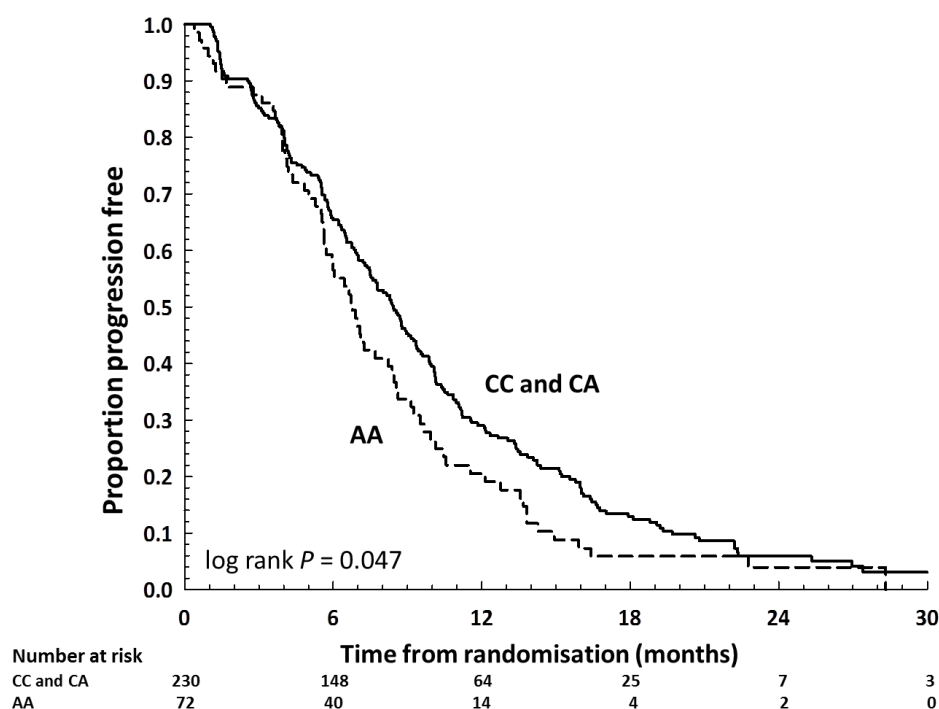


Figure 5.13. Kaplan-Meier curves of PFS for all patients according to the comparison of the VEGF-A rs699947 AA genotype to the CC and CA genotype

Table 5.9. Univariate associations between SNPs in VEGF family members and progression-free survival outcomes

Gene	SNP	Major allele	Minor allele	Homozygous major allele genotype versus without a homozygous major allele genotype					Homozygous minor allele genotype versus without a homozygous minor allele genotype				
				Hazard ratio (95% confidence interval)	P-value	Number of participants with homozygous major allele genotype	Number of participants without a homozygous major allele genotype	Total number of participants in analysis	Hazard ratio (95% confidence interval)	P-value	Number of participants with homozygous minor allele genotype	Number of participants without a homozygous minor allele genotype	Total number of participants in analysis
VEGF-A	rs699946	A	G	1.40 (1.04 to 1.87)	P = 0.025	221	68	289	0.52 (0.27 to 1.00)	P = 0.05	12	277	289
	rs699947	C	A	0.78 (0.61 to 1.02)	P = 0.07	90	212	302	1.32 (1.00 to 1.74)	P = 0.048	72	230	302
	rs833061	T	C	0.81 (0.62 to 1.05)	P = 0.12	86	187	273	1.26 (0.95 to 1.66)	P = 0.11	76	197	273
	rs2010963	G	C	1.14 (0.90 to 1.44)	P = 0.28	150	149	299	0.75 (0.51 to 1.09)	P = 0.13	35	264	299
	rs25648	C	T	0.65 (0.49 to 0.85)	P = 0.002	236	73	309	0.67 (0.25 to 1.76)	P = 0.41	4	305	309
	rs3025039	C	T	0.84 (0.64 to 1.11)	P = 0.22	233	74	307	1.25 (0.67 to 2.33)	P = 0.49	12	295	307
VEGFR1	rs9554316	G	T	0.88 (0.69 to 1.14)	P = 0.34	209	97	306	0.97 (0.60 to 1.59)	P = 0.91	19	287	306
	rs7993418	A	G	0.91 (0.71 to 1.18)	P = 0.49	208	101	309	1.09 (0.68 to 1.76)	P = 0.71	21	288	309
	rs9582036	A	C	1.04 (0.82 to 1.31)	P = 0.76	170	141	311	1.12 (0.74 to 1.69)	P = 0.59	29	282	311
	rs9554320	C	A	0.86 (0.67 to 1.10)	P = 0.23	102	202	304	1.03 (0.82 to 1.31)	P = 0.76	50	254	304
VEGFR2	rs12505758	T	C	1.09 (0.82 to 1.45)	P = 0.54	242	65	307	0.41 (0.13 to 1.28)	P = 0.13	4	303	307
	rs7655964	A	C	0.79 (0.62 to 1.01)	P = 0.057	145	157	302	1.06 (0.72 to 1.57)	P = 0.76	33	269	302
	rs1870377	T	A	1.06 (0.84 to 1.35)	P = 0.61	184	128	312	0.96 (0.64 to 1.44)	P = 0.85	29	283	312
	rs2305948	C	T	0.76 (0.56 to 1.02)	P = 0.06	262	57	319	2.00 (0.88 to 4.53)	P = 0.099	7	312	319
	rs2305949	C	T	0.90 (0.70 to 1.16)	P = 0.41	204	98	302	0.78 (0.47 to 1.29)	P = 0.34	18	284	302
	rs11133360	T	C	0.86 (0.67 to 1.10)	P = 0.23	102	189	291	1.26 (0.94 to 1.69)	P = 0.13	62	229	291

SNP: single nucleotide polymorphism

5.5.1.2. Associations between SNPs in VEGF family members and the PFS outcome in multivariable models

SNPs which were shown to have a univariate association with PFS were also tested in a multivariable model containing baseline factors which had been associated with survival outcomes in the primary MAX study [532]. Of these, the only SNPs which remained significant independent prognostic factors for PFS were *VEGF-A* rs25648 CC (for the comparison of homozygous major allele genotype versus without a homozygous major allele genotype) and *VEGF-A* rs699947 AA (for the comparison of homozygous minor allele genotype versus without a homozygous minor allele genotype).

In the multivariate model, the adjusted hazard ratio for *VEGF-A* rs25648 CC compared to (TT and CT) was 0.61 (95% CI 0.46 to 0.81), $P = 0.001$ when the covariates neutrophils and serum bilirubin were treated as continuous variables. The adjusted hazard ratio was 0.62 (95% CI 0.47 to 0.82), $P = 0.001$ when the covariates neutrophils and serum bilirubin were treated as categorical variables and dichotomised into the category levels ' $\geq 8 \times 10^9/L$ vs $< 8 \times 10^9/L$ ' and ' $\geq 14 \mu\text{mol/L}$ vs $< 14 \mu\text{mol/L}$ ', respectively (Table 5.10).

In the multivariate model, the adjusted hazard ratio for *VEGF-A* rs699947 AA compared to (CC and CA) was 1.39 (95% CI 1.05 to 1.83), $P = 0.02$ when the covariates neutrophils and serum bilirubin were treated as continuous variables. The adjusted hazard ratio was 1.50 (95% CI 1.13 to 1.99), $P = 0.005$ when the covariates neutrophils and serum bilirubin were treated as categorical variables as described above (Table 5.11).

Table 5.10. Results of the final multivariate model for *VEGF-A* rs25648 CC compared to TT and CT genotypes and the PFS outcome

Variable	First analysis				Second analysis			
	Variable details	Hazard ratio (95% confidence interval), P -value for PFS outcome			Variable details	Hazard ratio (95% confidence interval), P -value for PFS outcome		
<i>VEGF-A</i> rs25648	Homozygous CC vs (TT and CT)	0.61 (0.46 to 0.81)	$P = 0.001$		Homozygous CC vs (TT and CT)	0.62 (0.47 to 0.82)	$P = 0.001$	
Bevacizumab treatment	(CB + CBM) vs C	0.59 (0.45 to 0.76)	$P < 0.001$		(CB + CBM) vs C	0.59 (0.46 to 0.77)	$P < 0.001$	
ECOG performance status	≥ 1 vs 0	1.65 (1.35 to 2.01)	$P < 0.001$		≥ 1 vs 0	1.63 (1.34 to 1.99)	$P = 0.001$	
Neutrophils	Continuous variable, $\times 10^9/L$	1.09 (1.04 to 1.15)	$P = 0.001$		$\geq 8 \times 10^9/L$ vs $< 8 \times 10^9/L$	1.60 (1.14 to 2.26)	$P = 0.007$	
Serum bilirubin	Continuous variable, $\mu\text{mol/L}$	1.04 (1.01 to 1.07)	$P = 0.005$		$\geq 14 \mu\text{mol/L}$ vs $< 14 \mu\text{mol/L}$	1.53 (1.13 to 2.06)	$P = 0.006$	

PFS: progression-free survival; CB: capecitabine plus bevacizumab; CBM: capecitabine, bevacizumab and mitomycin; C: capecitabine; ECOG: Eastern Cooperative Oncology Group.

Table 5.11. Results of the final multivariate model for *VEGF-A* rs699947 AA compared to CC and CA genotypes and the PFS outcome

Variable	First analysis				Second analysis	
	Variable details	Hazard ratio (95% confidence interval), <i>P</i> -value for PFS outcome		Variable details	Hazard ratio (95% confidence interval), <i>P</i> -value for PFS outcome	
<i>VEGF-A</i> rs699947	Homozygous AA vs (CC and CA)	1.39 (1.05 to 1.83)	<i>P</i> = 0.02	Homozygous AA vs (CC and CA)	1.50 (1.13 to 1.99)	<i>P</i> = 0.005
Bevacizumab treatment	(CB + CBM) vs C	0.61 (0.47 to 0.79)	<i>P</i> < 0.001	(CB + CBM) vs C	0.62 (0.48 to 0.80)	<i>P</i> < 0.001
ECOG performance status	≥ 1 vs 0	1.69 (1.38 to 2.08)	<i>P</i> < 0.001	≥ 1 vs 0	1.69 (1.39 to 2.07)	<i>P</i> < 0.001
Neutrophils	Continuous variable, x 10 ⁹ /L	1.09 (1.04 to 1.15)	<i>P</i> = 0.001	≥ 8 x 10 ⁹ /L vs < 8 x 10 ⁹ /L	1.60 (1.14 to 2.25)	<i>P</i> = 0.007
Serum bilirubin	Continuous variable, μmol/L	1.03 (1.01 to 1.06)	<i>P</i> = 0.015	≥ 14 μmol/L vs < 14 μmol/L	1.53 (1.12 – 2.08)	<i>P</i> = 0.007

PFS: progression-free survival; CB: capecitabine plus bevacizumab; CBM: capecitabine, bevacizumab and mitomycin; C: capecitabine; ECOG: Eastern Cooperative Oncology Group.

5.5.1.3. Associations between SNPs in *VEGF* family members and the PFS outcome in multivariate models with *KRAS* and *BRAF* mutation status

Mutations in *KRAS* and *BRAF* are believed to be poorly prognostic of poorer survival in colorectal cancer. Whilst the prognostic value of *KRAS* mutations is controversial [202, 795-801], *BRAF* mutations are more consistently associated with poor prognosis, particularly in metastatic disease [90, 194, 205, 799, 802-805].

The association between *VEGF-A* rs25648 CC compared to TT and CT genotypes, and *VEGF-A* rs699947 AA compared to CC and CA genotypes, and PFS were further analysed in multivariate models which included the baseline factors which were associated with survival outcomes in the primary MAX study [532], as well as *KRAS* and *BRAF* mutation status.

Neither *KRAS* nor *BRAF* mutation status were significantly associated with the PFS outcome in the multivariate model, and therefore were not included the final model.

5.5.2. SNPs in *VEGF* family members as predictive biomarkers for PFS

SNPs which were shown to have a univariate association with PFS were also included in a model containing a treatment covariate (CB and CBM vs C), and a ‘SNP by treatment’ interaction. The ‘SNP by treatment interaction’ was not statistically significant for *VEGF-A* rs699946, rs25648, or rs699947. Therefore, none of these SNPs were predictive biomarkers for PFS with bevacizumab treatment in the MAX clinical trial.

5.6. Association between SNPs and overall survival (OS)

5.6.1. SNPs in VEGF family members as prognostic biomarkers for the OS outcome

5.6.1.1. Univariate associations between SNPs in VEGF family members and the OS outcome

In the first analysis of univariate associations between SNPs in VEGF family members and OS, we compared outcomes in the participants with a homozygous major allele genotype to outcomes in the participants without homozygous major allele (i.e., composite of participants with genotypes homozygous minor allele and heterozygous major and minor alleles) (Figure 5.14, Table 5.12).

There were significant differences between OS in participants with the *VEGF-A* rs25648 CC genotype, compared to the TT and CT genotypes. The median OS was 21.4 months among participants with the *VEGF-A* rs25648 CC genotype, compared to 17.3 months among those with the TT and CT genotypes (log rank $P = 0.019$, Figure 5.15). The HR for CC vs TT/CT was 0.70, 95% CI 0.52 to 0.94, $P = 0.019$.

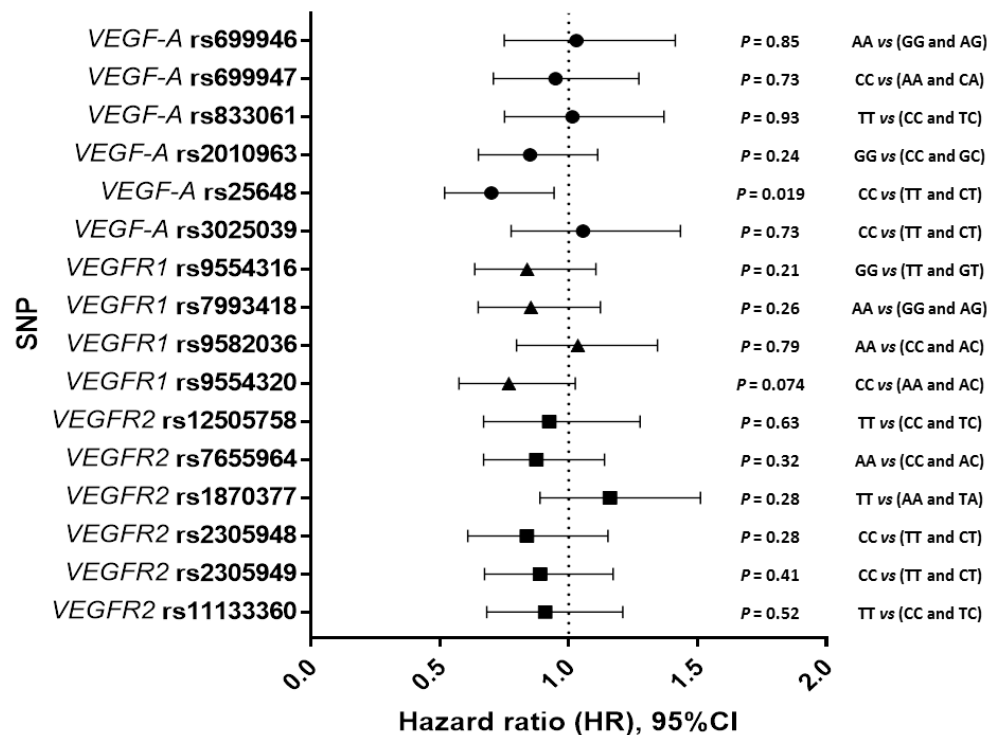


Figure 5.14. Forest plot showing Hazard Ratio (HR) with 95% CI for the comparison of OS in participants with a homozygous major allele genotype versus without a homozygous major allele genotype

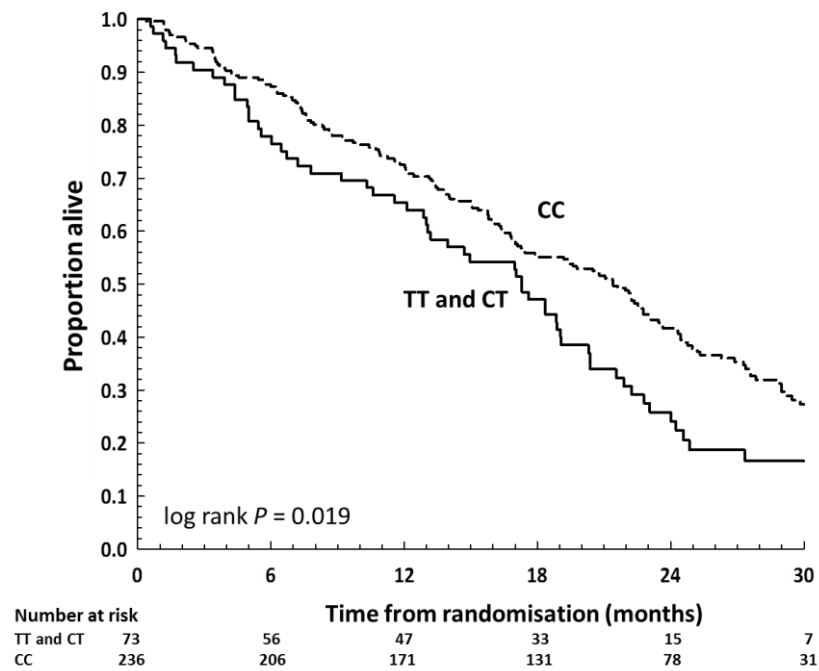


Figure 5.15. Kaplan-Meier curves of OS for all patients according to the comparison of the *VEGF-A* rs25648 CC genotype to the TT and CT genotypes

In the second analysis of univariate associations between SNPs in VEGF family members and OS, we compared outcomes in the participants with a homozygous minor allele genotype to outcomes in the participants without a homozygous minor allele genotype (i.e., composite of participants with homozygous major allele and heterozygous major and minor allele genotypes). There were no significant differences between OS in participants with these different genotypes for any of the analysed SNPs (Figure 5.16, Table 5.12).

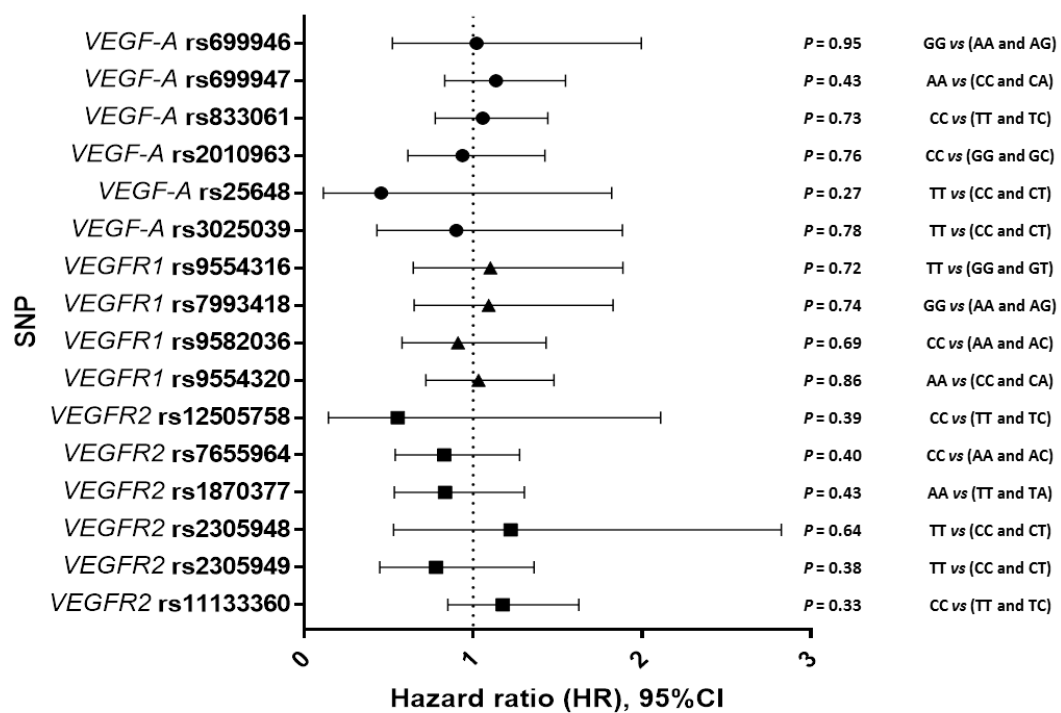


Figure 5.16. Forest plot showing Hazard Ratio (HR) with 95% CI for the comparison of OS in participants with a homozygous minor allele genotype versus without a homozygous minor allele genotype

Table 5.12. Univariate associations between SNPs in VEGF family members and overall survival outcomes

Gene	SNP	Major allele	Minor allele	Homozygous major allele genotype versus without a homozygous major allele genotype					Homozygous minor allele genotype versus without a homozygous minor allele genotype				
				Hazard ratio (95% confidence interval)	P-value	Number of participants with homozygous major allele genotype	Number of participants without a homozygous major allele genotype	Total number of participants in analysis	Hazard ratio (95% confidence interval)	P-value	Number of participants with homozygous major allele genotype	Number of participants without a homozygous major allele genotype	Total number of participants in analysis
VEGF-A	rs699946	A	G	1.03 (0.75 to 1.41)	$P = 0.85$	221	68	289	1.02 (0.52 to 2.00)	$P = 0.95$	12	277	289
	rs699947	C	A	0.95 (0.71 to 1.27)	$P = 0.73$	90	212	302	1.14 (0.83 to 1.55)	$P = 0.43$	72	230	302
	rs833061	T	C	1.02 (0.75 to 1.27)	$P = 0.93$	86	187	273	1.06 (0.78 to 1.44)	$P = 0.73$	76	197	273
	rs2010963	G	C	0.85 (0.65 to 1.11)	$P = 0.24$	150	149	299	0.94 (0.62 to 1.43)	$P = 0.76$	35	264	299
	rs25648	C	T	0.70 (0.52 to 0.94)	$P = 0.019$	236	73	309	0.46 (0.11 to 1.82)	$P = 0.27$	4	305	309
	rs3025039	C	T	1.06 (0.78 to 1.43)	$P = 0.73$	233	74	307	0.90 (0.43 to 1.88)	$P = 0.78$	12	295	307
VEGFR1	rs9554316	G	T	0.84 (0.64 to 1.11)	$P = 0.21$	209	97	306	1.10 (0.65 to 1.89)	$P = 0.72$	19	287	306
	rs7993418	A	G	0.85 (0.65 to 1.12)	$P = 0.26$	208	101	309	1.09 (0.65 to 1.83)	$P = 0.74$	21	288	309
	rs9582036	A	C	1.04 (0.80 to 1.35)	$P = 0.79$	170	141	311	0.91 (0.58 to 1.43)	$P = 0.69$	29	282	311
	rs9554320	C	A	0.77 (0.58 to 1.03)	$P = 0.074$	102	202	304	1.03 (0.72 to 1.48)	$P = 0.86$	50	254	304
VEGFR2	rs12505758	T	C	0.93 (0.67 to 1.28)	$P = 0.63$	242	65	307	0.55 (0.15 to 2.11)	$P = 0.39$	4	303	307
	rs7655964	A	C	0.88 (0.67 to 1.14)	$P = 0.32$	145	157	302	0.83 (0.54 to 1.28)	$P = 0.40$	33	269	302
	rs1870377	T	A	1.16 (0.89 to 1.51)	$P = 0.28$	184	128	312	0.83 (0.53 to 1.30)	$P = 0.43$	29	283	312
	rs2305948	C	T	0.84 (0.61 to 1.15)	$P = 0.28$	262	57	319	1.22 (0.53 to 2.83)	$P = 0.64$	7	312	319
	rs2305949	C	T	0.89 (0.67 to 1.17)	$P = 0.41$	204	98	302	0.78 (0.45 to 1.36)	$P = 0.38$	18	284	302
	rs11133360	T	C	0.91 (0.68 to 1.21)	$P = 0.52$	102	189	291	1.18 (0.85 to 1.63)	$P = 0.33$	62	229	291

SNP: single nucleotide polymorphism.

5.6.1.2. Associations between SNPs in VEGF family members and the OS outcome in multivariable models

The only SNP which demonstrated a univariate association with the PFS outcome, *VEGF-A* rs25648 CC (for the comparison of homozygous major allele genotype versus without a homozygous major allele genotype) was tested in a multivariable model containing baseline factors which were associated with survival outcomes in the primary MAX study [532], and the association remained significant.

In the first multivariate analysis where the covariates neutrophils and serum alkaline phosphatase were treated as continuous variables, the adjusted hazard ratio for *VEGF-A* rs25648 CC compared to (TT and CT) was 0.66 (95% CI 0.49 to 0.89), $P = 0.007$. Of note, in the multivariable model, the HR for serum alkaline phosphatase (ALP) was 1.001 (95% CI 1.000 to 1.002) (Table 5.13). As this hazard ratio is not considered clinically significant, a further analysis was performed excluding serum ALP from the multivariate model (Table 5.13), and in this model the adjusted HR for *VEGF-A* rs25648 CC compared to (TT and CT) was 0.65 (95% CI 0.48 to 0.87), $P = 0.004$.

Table 5.13. Results of the final multivariable model for *VEGF-A* rs25648 CC compared to TT and CT genotypes and the OS outcome –first analysis with neutrophils and serum alkaline phosphatase as continuous variables

Variable	Variable details	With serum alkaline phosphatase (ALP)		Without serum alkaline phosphatase (ALP)	
		Hazard ratio (95% confidence interval), P -value for OS outcome		Hazard ratio (95% confidence interval), P -value for OS outcome	
<i>VEGF-A</i> rs25648	Homozygous CC vs (TT and CT)	0.66 (0.49 to 0.89)	$P = 0.007$	0.65 (0.48 to 0.87)	$P = 0.004$
ECOG performance status	≥ 1 vs 0	1.95 (1.56 to 2.44)	$P < 0.001$	2.04 (1.64 to 2.55)	$P < 0.001$
Neutrophils	Continuous variable, $\times 10^9/L$	1.13 (1.07 to 1.20)	$P < 0.001$	1.16 (1.10 to 1.22)	$P < 0.001$
Serum ALP	Continuous variable, U/L	1.001 (1.000 to 1.002)	$P = 0.002$		

OS: overall survival; ALP: alkaline phosphatase; ECOG: Eastern Cooperative Oncology Group.

In a second multivariate analysis where the covariates neutrophils were treated as categorical variables and dichotomised into the category levels ' $\geq 8 \times 10^9/L$ vs $< 8 \times 10^9/L$ ' and ' ≥ 140 U/L vs < 140 U/L, respectively, the adjusted hazard ratio for *VEGF-A* rs25648 CC compared to (TT and CT) was 0.68 (95% CI 0.50 to 0.92), $P = 0.011$. In the multivariable model, the HR for serum alkaline phosphatase (ALP) was 1.002 (95% CI 1.000 to 1.003) (Table 5.14), and was again not considered clinically significant. In a further analysis performed which excluded serum ALP from the multivariable model, the adjusted HR for *VEGF-A* rs25648 CC compared to (TT and CT) was 0.67 (95% CI 0.49 to 0.90), $P = 0.008$ (Table 5.14).

Table 5.14. Results of the final multivariable model for VEGF-A rs25648 CC compared to TT and CT genotypes and the OS outcome – second analysis with neutrophils and serum alkaline phosphatase as categorical variables

Variable	Variable details	With serum ALP		Without serum ALP	
		Hazard ratio (95% confidence interval), <i>P</i> -value for OS outcome		Hazard ratio (95% confidence interval), <i>P</i> -value for OS outcome	
VEGF-A rs25648	Homozygous CC vs (TT and CT)	0.68 (0.50 to 0.92)	<i>P</i> = 0.011	0.67 (0.49 to 0.90)	<i>P</i> = 0.008
ECOG performance status	≥ 1 vs 0	2.08 (1.68 to 2.38)	<i>P</i> < 0.001	2.07 (1.66 to 2.56)	<i>P</i> < 0.001
Neutrophils	≥ 8 x 10 ⁹ /L vs < 8 x 10 ⁹ /L	1.85 (1.27 to 2.70)	<i>P</i> = 0.001	1.84 (1.27 to 2.68)	<i>P</i> = 0.001
Serum ALP	≥ 140 U/L vs < 140 U/L	1.002 (1.000 to 1.003)	<i>P</i> = 0.028		

OS: overall survival; ALP: alkaline phosphatase; ECOG: Eastern Cooperative Oncology Group.

5.6.1.3. Associations between SNPs in VEGF family members and the OS outcome in multivariable models with KRAS and BRAF mutation status

The association between VEGF-A rs25648 CC compared to TT and CT genotypes and OS was further analysed in multivariable models which included the baseline factors which were associated with survival outcomes in the primary MAX study [532], as well as *KRAS* and *BRAF* mutation status.

BRAF, but not *KRAS*, mutation status was significantly associated with OS in the multivariate model. When *BRAF* mutation status was included in the multivariate model, the covariate ‘serum ALP’ was no longer significantly associated with OS, and therefore was not included in this model. The inclusion of *BRAF* mutation status in the multivariable model did not significantly change the adjusted HR for VEGF-A rs25648 CC compared to TT and CT genotypes and OS (Table 5.15), which remained an independent prognostic factor. The HR for VEGF-A rs25648 CC compared to TT and CT genotypes was 0.68 (95% CI 0.50 to 0.93), *P* = 0.014 when neutrophils were treated as a continuous variable, and 0.70 (95% CI 0.52 to 0.96), *P* = 0.024 when neutrophils were treated as a categorical variable.

Table 5.15. Results of the multivariable model including *BRAF* mutation status model for *VEGF-A* rs25648 CC compared to TT and CT genotypes and the OS outcome

Variable	First analysis				Second analysis	
	Variable details	Hazard ratio (95% confidence interval), <i>P</i> -value for OS outcome		Variable details	Hazard ratio (95% confidence interval), <i>P</i> -value for OS outcome	
<i>VEGF-A</i> rs25648	Homozygous CC vs (TT and CT)	0.68 (0.50 to 0.93)	<i>P</i> = 0.014	Homozygous CC vs (TT and CT)	0.70 (0.52 to 0.96)	<i>P</i> = 0.024
ECOG performance status	≥ 1 vs 0	2.07 (1.65 to 2.60)	<i>P</i> < 0.001	≥ 1 vs 0	2.10 (1.68 to 2.61)	<i>P</i> < 0.001
<i>BRAF</i> mutation status	Wild type vs mutant	0.45 (0.30 to 0.69)	<i>P</i> < 0.001	Wild type vs mutant	0.49 (0.32 to 0.75)	<i>P</i> = 0.001
Neutrophils	Continuous variable, $\times 10^9/L$	1.17 (1.10 to 1.23)	<i>P</i> < 0.001	$\geq 8 \times 10^9/L$ vs $< 8 \times 10^9/L$	1.85 (1.26 to 2.72)	<i>P</i> = 0.002

OS: overall survival; ECOG: Eastern Cooperative Oncology Group.

5.6.2. SNPs in VEGF family members as predictive biomarkers for OS

VEGF-A rs25648, which was shown to have a univariate association with OS, was also included in a model containing a treatment covariate (CB and CBM vs C), and a ‘SNP by treatment’ interaction. The ‘SNP by treatment interaction’ was not statistically significant (*P* = 0.54), and hence *VEGF-A* rs25648 was not a predictive biomarker for OS with Bevacizumab treatment in the MAX clinical trial.

5.7. Association between SNPs and objective response rate (ORR)

The effect of the addition of bevacizumab on ORR was not significantly greater among the patients with *VEGF-A* rs25648 CC compared to (TT and CT) (*P* = 0.22 for the interaction between SNP genotype and the assigned treatment), *VEGF-A* rs699947 (CC and CA) compared to AA (*P* = 0.67 for the interaction between SNP genotype and the assigned treatment), or *VEGF-A* rs699946 CC compared to (TT and CT) (*P* = 0.85 for the interaction between SNP genotype and the assigned treatment) (Table 5.16).

Table 5.16. Objective response rate by *VEGF-A* rs25648, *VEGF-A* rs699947 and genotype

Treatment	VEGF-A 25648			VEGF-A 699947		
	CC genotype (%)	TT and CT genotypes (%)	P-value ^a	CC and CA genotypes (%)	AA genotype (%)	P-value ^a
C	29 (83)	6 (17)	0.22	26 (74)	9 (26)	0.67
CB	29 (73)	11 (27)		29 (76)	9 (24)	
CBM	40 (87)	6 (13)		37 (82)	8 (18)	
Treatment	VEGF-A 699946					
	CC genotype (%)	TT and CT genotypes (%)	P-value ^a			
C	26 (79)	7 (21)	0.85			
CB	29 (78)	8 (22)				
CBM	31 (74)	11 (26)				

C: capecitabine; CB: capecitabine plus bevacizumab; CBM: capecitabine, bevacizumab and mitomycin.

^aP-value for interaction between SNP biomarker status and the assigned treatment (C vs CB and CBM)

5.8. Association between *VEGF-A* expression and SNP genotypes

We hypothesised that the *VEGF-A* SNP genotypes which were found to be associated with PFS and/or OS, may have effects on these survival outcomes through modulating expression of *VEGF-A*. Specifically, we hypothesised that *VEGF-A* SNP genotypes which were associated with higher levels of *VEGF-A* gene or protein expression may be associated with a worse prognosis.

5.8.1. Association between *VEGF-A* gene expression and SNP genotypes

To explore this hypothesis, we firstly utilised data from gene expression profiling of FFPE samples of primary tumours from MAX study participants, which had been previously obtained using the Almac Xcel microarray [638]. This data was available for 239 of 309 (77%) participants who had a full genotype known for *VEGF-A* rs25648, and 234 of 302 (77%) participants who had a full genotype known for *VEGF-A* rs699947.

The mean *VEGF-A* gene expression level was higher in those with the better prognosis *VEGF-A* rs25648 CC genotype compared to the TT and CT genotypes (mean 0.00091 versus -0.020), though this difference was not statistically significant ($P = 0.77$).

Similarly, the mean *VEGF-A* gene expression level was higher in those with the better prognosis *VEGF-A* rs699947 CC and CA genotypes compared to the AA genotype (mean 0.0012 versus -0.022), however again this difference was not statistically significant ($P = 0.74$) (Figure 5.17).

Therefore, *VEGF-A* SNP genotypes which were associated with higher levels of *VEGF-A* mRNA expression were not associated with a worse prognosis.

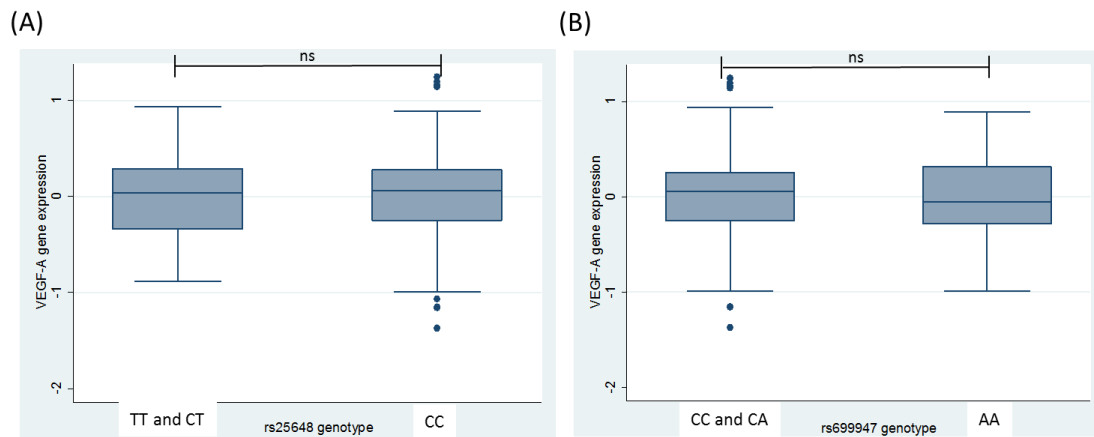


Figure 5.17. Box and whiskers plot of *VEGF-A* gene expression and a) *VEGF-A* rs25648 and b) *VEGF-A* rs699947 genotypes
ns: not significant.

5.8.2. Association between VEGF-A protein expression and SNP genotypes

Secondly, we utilised VEGF-A protein expression data from FFPE samples of primary tumours from MAX study participants, which had been previously obtained using Bio-plex® arrays [762].

The mean VEGF-A protein expression level was higher in those with the better prognosis *VEGF-A* rs25648 CC genotype compared to the TT and CT genotypes (mean 6.26 versus 5.80), however this difference was not statistically significant ($P = 0.38$).

The mean VEGF-A protein expression level was lower in those with the better prognosis *VEGF-A* rs699947 CC and CA genotypes compared to the AA genotype (mean 6.04 versus 6.26), although this difference was not statistically significant ($P = 0.66$) (Figure 5.18).

Therefore, from these two correlative gene expression and protein expression analyses, only the *VEGF-A* rs699947 AA genotype which was associated with higher levels of VEGF-A protein expression was associated with a worse prognosis, although the difference in levels compared to the CC and CA genotypes was not statistically significant.

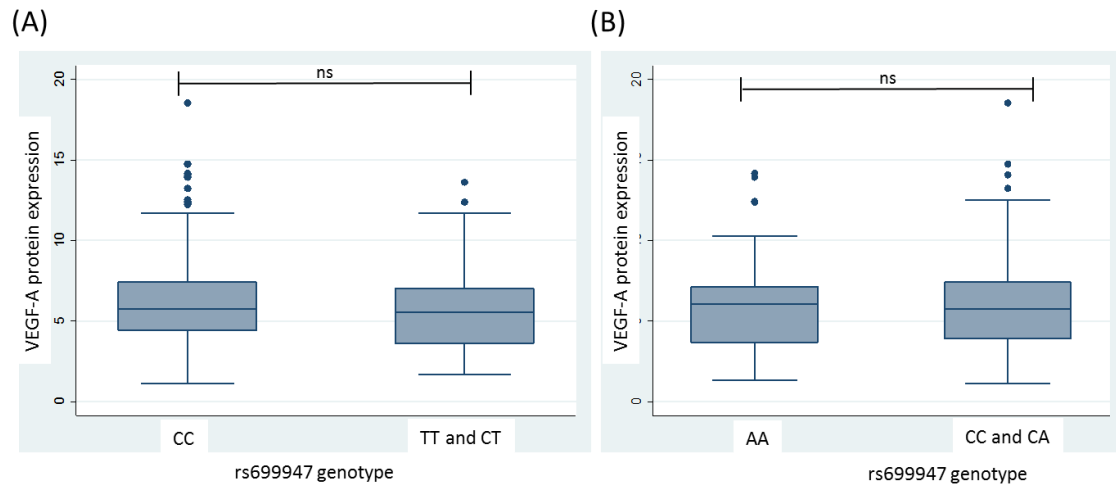


Figure 5.18. Box and whiskers plot of VEGF-A protein expression and a) *VEGF-A* rs25648 and b) *VEGF-A* rs699947 genotypes
ns: not significant.

5.9. Discussion

5.9.1. Summary of results

In the MAX study, I found that the *VEGF-A* rs25648 CC genotype was associated with improved PFS and OS outcomes, and that the *VEGF-A* rs699947 AA genotype was associated with shorter PFS in patients treated with first-line chemotherapy with or without bevacizumab for mCRC. However, whilst these SNPs were prognostic, neither were predictive of bevacizumab treatment effect on PFS and OS, nor ORR, in patients with mCRC.

5.9.2. Strengths of the SNP biomarker study

The MAX study was a Phase 3 randomised controlled trial which included a non-bevacizumab chemotherapy-only control arm, and therefore had the ideal study design for incorporation of a retrospective predictive biomarker study. I identified only one other biomarker study which examined SNPs in *VEGF-A*, *VEGFR1* and *VEGFR2* as predictive biomarkers of bevacizumab outcome in patients with mCRC (Ulivi *et al.* [666]) which also had this optimal study design. The study by Ulivi *et al.* also did not identify any SNPs in VEGF family members or receptors as predictive biomarkers of bevacizumab response, however found that the *VEGF-A* rs3025039 TT genotype was associated with poorer PFS and OS, and that the *VEGF-A* rs2010963 GC genotype was associated with an inferior ORR.

In my study, the baseline characteristics of the patients in the SNP biomarker study population were comparable to those in the total MAX study population. Moreover, in my SNP biomarker study, patient FFPE tissue samples were available for biomarker analysis in a high proportion of patients (69%), therefore reducing selection bias [613]. This is in contrast to previous randomised

phase III bevacizumab predictive biomarker studies in mCRC, where tumour samples have been available for analysis in lower proportions (18, 34, 63 and 49%) of patients [632, 633, 666, 806].

5.9.3. Limitations of the SNP biomarker study

However, this study did have some limitations. Firstly, this was a *post-hoc* retrospective analysis of multiple genetic biomarkers, and as such, was exploratory in nature. Therefore, there is a possibility that my findings may have been related to a random effect. Secondly, I genotyped SNPs using a candidate approach, based on demonstration of a previous functional effect on corresponding gene and/or protein expression or previous association with clinical outcomes. An alternative approach for selecting SNPs to genotype is SNP tagging. This approach is based on the premise that in a population of individuals, there is a high degree of correlation among nearby allelic variations (linkage disequilibrium, LD), and therefore most SNPs may be omitted for more cost-effective and efficient genotyping [807, 808]. Instead, a minimum set of representative ‘tag SNPs’ which can capture the genomic diversity in a population is selected by using methods which either exploit prior information about haplotype block structure (block-based methods) [809], or haplotype-reconstruction-based or LD-based (block-free) methods [807]. This approach has the advantage of being more systematic and may lead to the testing of SNPs which were not identified in previous studies [691]. Thirdly, genetic variants may be associated with different disease risk in different ethnic populations [810], and race or ethnicity may be a confounder in SNP biomarker studies [682]. The heterogeneity of effect by race/ethnicity may be due to variation in LD among populations [810], allelic heterogeneity (different causal variants at the same locus influencing a trait across populations) [811], or gene-gene and gene-environment interactions [812]. Information on the race or ethnicity of participants was not collected in the MAX clinical trial. Therefore, I did not restrict analyses to an ethnically homogenous population, nor adjust for ethnicity in the analysis, which are approaches which have been used in some previous genetic biomarker studies of bevacizumab [617, 667, 682, 689, 691].

5.9.4. Discussion of the results

My finding that the *VEGF-A* rs25648 CC genotype was associated with both improved PFS and OS in the MAX study is supported by the results of one phase II single-arm study of patients treated with first-line FOLFIRI and bevacizumab for mCRC (Becouarn *et al.*), where participants with *VEGF-A* rs25648 C-variants had a longer OS than those with alternative genotypes [673].

In contrast, my finding that the *VEGF-A* rs699947 AA genotype was associated with shorter PFS than C-variants in the MAX study conflicts with the results of most of the pre-existing studies in the literature which had identified significant associations between this SNP and prognosis. In one single-arm pilot study of 59 patients with mCRC who were treated with second-line gemcitabine, irinotecan and bevacizumab, Abajo *et al.* reported that participants with at least one of the *VEGF-A* rs699947 AA, rs833061 CC, or rs2010963 GG genotypes had a longer median

TTP than those without these genotypes [674]. In studies of patients with recurrent or advanced NSCLC (Pallaud *et al.*) [682], glioblastoma (Galanis *et al.*) [686], and metastatic breast cancer (Schneider *et al.*) [646], the *VEGF-A* rs699947 AA genotype was associated with a superior ORR, 6-month PFS, and median OS, respectively. However, in one phase III study of patients treated with first-line docetaxel with or without bevacizumab for metastatic breast cancer, similar to my study, Miles *et al.* reported that the *VEGF-A* rs699947 C-allele was a good prognostic factor and associated with longer PFS in the placebo arm [617]. Reasons for these conflicting results may include heterogeneous patient populations, and differences in tumour vasculature between different cancer types or following treatment with different regimens [38].

Having identified associations between specific SNPs in *VEGF* family genes and patient outcomes, I considered how these SNPs may alter VEGF-A function to bring about this effect. One possibility was that, being located in the 5'UTR (rs25648) and promoter (rs699947), these SNPs may impact levels of VEGF-A mRNA or protein expression. However, I found no significant associations between *VEGF-A* gene expression (measured using the Almac Xcel gene expression microarray) or *VEGF-A* protein expression (measured using Bio-plex® arrays) and *VEGF-A* SNP genotypes.

Notably, the FFPE tissue samples which I used to measure VEGF-A mRNA and protein expression were from participants' primary tumours. This is because the majority of patients did not have tissue samples from their metastatic tumour available for mRNA extraction - a common situation in biomarker studies in patients with metastatic disease [625]. As angiogenesis is a complex, dynamic and adaptive process which varies over time [38, 625], VEGF-A expression in the primary tumour may not be reflective of expression levels in the metastatic tumour at the time of study participation, which is the time at which prognosis was examined. This may explain the observed lack of association between VEGF-A gene and protein expression and *VEGF-A* SNP genotypes. However, in 47 (10%) MAX study participants for whom matched primary and metastatic tumour samples were available, I found a statistically significant moderate positive correlation between VEGF-A mRNA expression in the primary and metastatic tumour (data not shown). Studies examining the association between *VEGF-A* mRNA expression in matched primary and metastatic tumour from a larger number of patients are required to further address this.

A further possibility is that the SNPs which I have identified as prognostic biomarkers exert their prognostic impact by affecting the expression of other genes in the vicinity of *VEGF-A* (for example, by acting as a distal enhancer).

6. GENERAL DISCUSSION

This thesis aimed to explore ways to optimise the targeted treatment of metastatic CRC, by investigating mechanisms of response and resistance to two targeted therapies - the BRAF inhibitor vemurafenib and the anti-angiogenic drug bevacizumab.

Patients with metastatic *BRAF*^{V600E} CRC have a very poor prognosis, and these patients are in urgent need of new treatment options. Given the successful results observed in clinical trials of BRAF inhibitors in *BRAF* mut malignant melanoma [23-32], it was hoped that these drugs would have similar efficacy in patients with *BRAF*^{V600E} metastatic CRC. However, results were unexpectedly disappointing [33-35]. Whilst two previous pre-clinical studies described EGFR-mediated mechanisms of rapid resistance of *BRAF*^{V600E} CRC to BRAF inhibitors [295, 296], subsequent clinical trials testing the combination of BRAF inhibitors with EGFR inhibitors reported only modest improvements in efficacy compared to BRAF inhibitors alone. While trials of this combination are ongoing (e.g., the phase III BEACON CRC trial), our findings suggest that there are also EGFR-independent mechanisms of resistance.

In chapters 3 and 4 of this thesis, I explored basal biomarkers and mechanisms of resistance of *BRAF*^{V600E} CRC to BRAF inhibitors, and investigated ways to overcome this resistance. My research included multiple novel findings which have provided important new biological insight into the molecular basis of resistance of *BRAF* mut CRC to BRAF inhibitors, and more broadly, MAPK inhibitors.

First, I identified high p-EGFR protein expression to be a potential basal biomarker of sensitivity to vemurafenib in *BRAF* mut CRC. Whether high p-EGFR directly contributes to vemurafenib sensitivity is unclear, and requires further study. In my future research, I will seek to validate this finding by examining the association between tumour p-EGFR expression and response in clinical trial cohorts treated with BRAF inhibitor alone or in combination with other therapies. The identification of a validated basal biomarker of sensitivity or resistance to vemurafenib has significant clinical implications. Basal biomarkers which can be measured in patients' tumour specimens prior to commencing treatment have the potential to avoid the need for repeated invasive tests including biopsies, which are associated with significant risks. Importantly, they may identify which patients are unlikely to respond, and enable these patients to be commenced on alternative treatment (including clinical trials or immunotherapy) earlier.

Second, I demonstrated that in resistant *BRAF*^{V600E} CRC cell lines, BRAF inhibitor treatment induces rapid p-ERK reactivation which is not associated with increased p-EGFR or p-Akt expression, suggesting the presence of EGFR-independent mechanisms of inherent resistance. Indeed, I found that the combination of vemurafenib with cetuximab was not effective in all

vemurafenib-resistant *BRAF*^{V600E} CRC cell lines, and was only able to convert one vemurafenib-resistant line (VACO432) to a level of sensitivity similar to that observed in the vemurafenib-sensitive cell lines. The variability in inherent vemurafenib resistance among *BRAF*^{V600E} CRC cell lines is therefore likely to involve multiple mechanisms, as it cannot be explained by reactivation of EGFR alone. My future research will include proteomic profiling of the *BRAF*^{V600E} CRC cell lines, both pre- and post-treatment with vemurafenib and cetuximab, which may provide us with insight into the molecular basis for sensitivity to the combination in the VACO432 cell line.

Third, I found that *BRAF* mut CRCs which are relatively resistant to BRAF inhibitors demonstrate broadly similar resistance to other MAPK inhibitors, which at least in some cases, is also characterised by p-EGFR independent reactivation of p-ERK. This suggests that in *BRAF* mut CRC, resistance to MAPK inhibitors is tumour- rather than drug-specific, and presents a novel paradigm for *BRAF* mut CRC with important implications for future pre-clinical and clinical research, in that combining multiple MAPK inhibitors is unlikely to be the optimal approach. Indeed, I found that vemurafenib-resistant *BRAF*^{V600E} CRC cell lines were relatively more resistant to the combination of vemurafenib and trametinib compared to vemurafenib-sensitive *BRAF*^{V600E} CRC cell lines.

Finally, I found that BRAF inhibitor treatment does not induce preferential RAS activation, increased p-CRAF expression, nor increased p-MEK expression in resistant *BRAF*^{V600E} CRC cell lines. These findings are also novel and raise the possibility that alternative non-EGFR mediated mechanisms of resistance to BRAF inhibitors originate below the level of MEK. My future research plan includes further localisation of where MAPK reactivation in resistant cell lines originates by analysing reverse phase protein array data in *BRAF* mut CRC cell lines which have been treated with vemurafenib.

Collectively, these data provide the basis for further exploration of non-EGFR mediated mechanisms of resistance to BRAF inhibitors in future research.

Bevacizumab is currently approved for the treatment of mCRC in combination with chemotherapy. However, not all patients who are treated with bevacizumab respond, and the drug is costly and associated with multiple side effects. Discovery of a predictive biomarker for response to bevacizumab would enable us to identify patients who are unlikely to respond, commence them on alternative therapies sooner, and avoid unnecessary side effects. Identification of a predictive biomarker may also improve the cost effectiveness of bevacizumab use.

In Chapter 5 of this thesis, I explored whether SNPs in *VEGF-A*, *VEGFR1* and *VEGFR2* are predictive biomarkers for bevacizumab response, using archived tissue samples and clinical outcome data from participants in the MAX Phase III trial of first-line chemotherapy with or without bevacizumab for mCRC.

I found that *VEGF-A* SNPs rs25648 and rs699947 were prognostic biomarkers for survival outcomes in these patients. The *VEGF-A* rs25648 CC genotype was associated with improved PFS and OS outcomes, and the *VEGF-A* rs699947 AA genotype was associated with shorter PFS. However, neither of these SNPs were predictive of bevacizumab treatment effect on PFS and OS, nor ORR. *VEGF-A* SNP genotypes which were linked to a worse prognosis were not associated with higher levels of VEGF-A gene or protein expression measured in patients' primary tumour samples. As this was an exploratory study, our findings – in particular, for rs25648, which has been less well examined than rs699947 in bevacizumab biomarker studies - should be validated in additional cohorts.

In summary, the findings from my thesis have significantly advanced our understanding of mechanisms of resistance to BRAF inhibitors in *BRAF* mut CRC, and genetic biomarkers for response to bevacizumab in mCRC. Additional research is required to validate and further explore these findings. In particular, my thesis identified that further exploration of non-EGFR mediated mechanisms of resistance to BRAF inhibitors is an important avenue to pursue in future research, and ultimately may enable the development of new rational targeted drug combination strategies which are urgently needed for patients with *BRAF* mut mCRC.

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8. APPENDIX

Western blots used in key figures, with details of primary and secondary antibodies used:

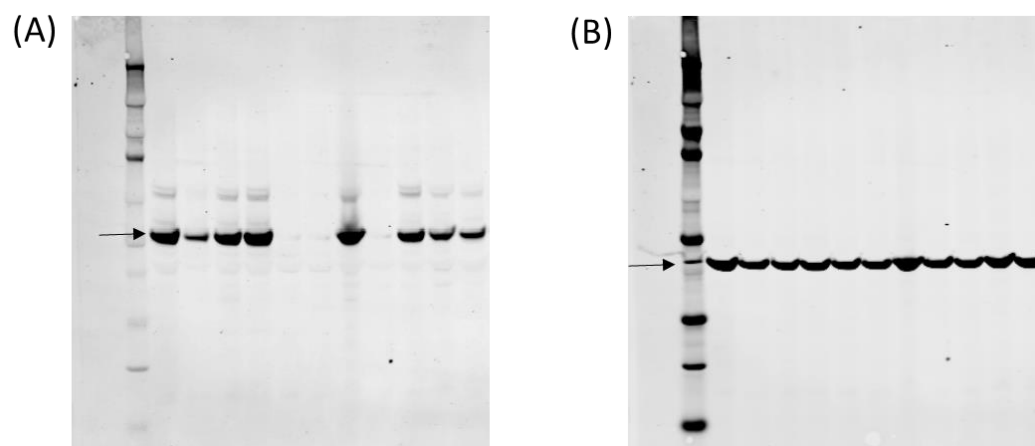


Figure 8.1. Western blots used in Figure 3.2. (A) Primary antibody anti-PTEN, secondary antibody IRDye® 800CW Goat anti-Rabbit; (B) primary antibody anti- β -Actin, secondary antibody: IRDye® 680RD Goat anti-Mouse.

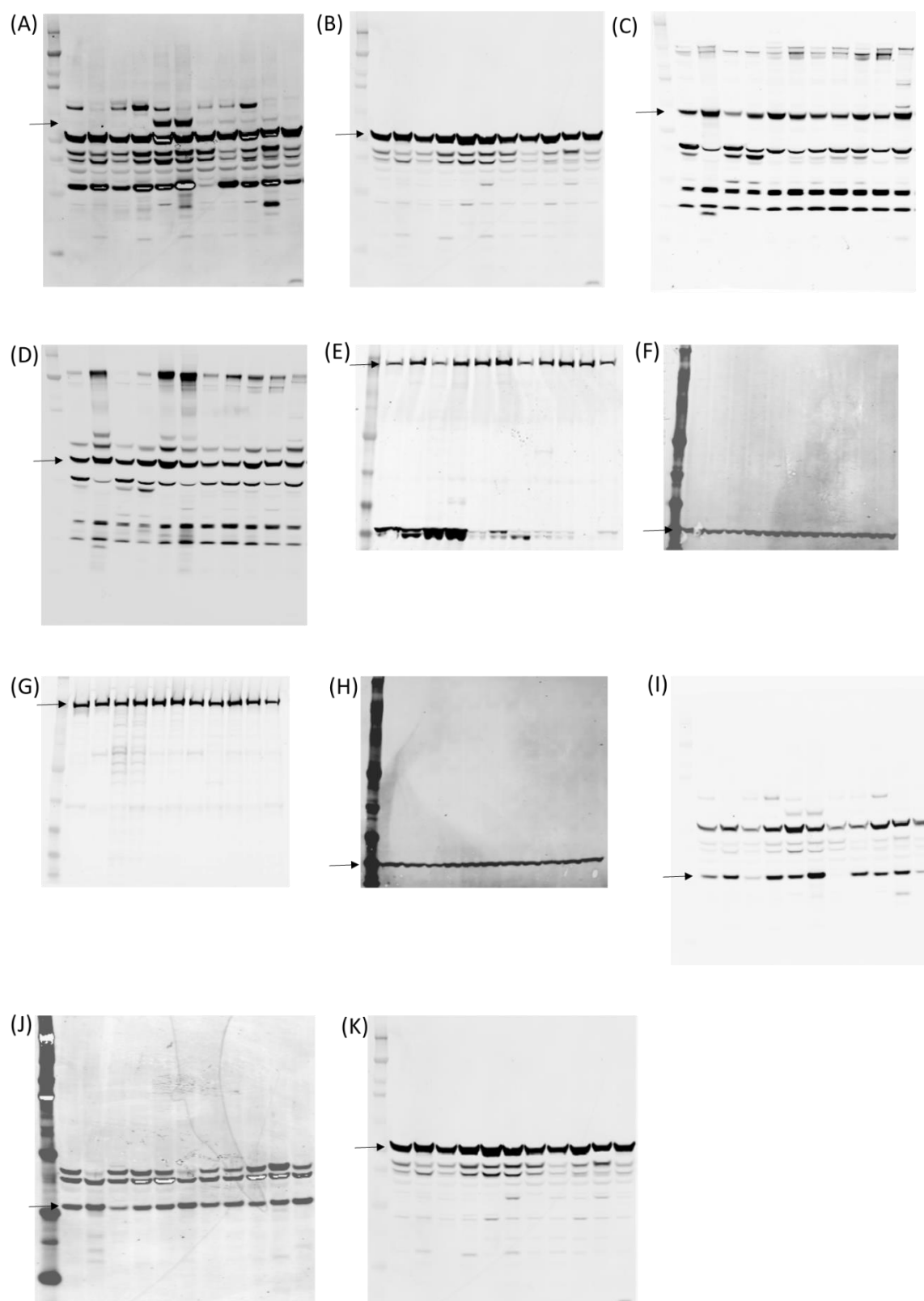


Figure 8.2. Western blots used in Figure 3.4. (A) Primary antibody anti-phospho-Akt, secondary antibody IRDye® 800CW Goat anti-Rabbit; (B) primary antibody anti- β -Tubulin, secondary antibody IRDye® 800CW Goat anti-Rabbit; (C) primary antibody anti-Akt1; secondary antibody IRDye® 800CW Goat anti-Rabbit; (D) primary antibody anti- β -Tubulin, secondary antibody IRDye® 800CW Goat anti-Rabbit; (E) primary antibody anti-phospho-mTOR, secondary antibody IRDye® 800CW Goat anti-Rabbit; (F) primary antibody anti-phospho-mTOR, secondary antibody IRDye® 800CW Goat anti-Rabbit; (G) primary antibody anti-phospho-Akt, secondary antibody IRDye® 800CW Goat anti-Rabbit; (H) primary antibody anti-phospho-mTOR, secondary antibody IRDye® 800CW Goat anti-Rabbit; (I) primary antibody anti-phospho-Akt, secondary antibody IRDye® 800CW Goat anti-Rabbit; (J) primary antibody anti-phospho-mTOR, secondary antibody IRDye® 800CW Goat anti-Rabbit; (K) primary antibody anti-phospho-Akt, secondary antibody IRDye® 800CW Goat anti-Rabbit.

antibody IRDye[®] 800CW Goat anti-Rabbit; (F) primary antibody anti- β -Actin, secondary antibody IRDye[®] 680RD Goat anti-Mouse; (G) primary antibody anti-mTOR, secondary antibody IRDye[®] 800CW Goat anti-Rabbit; (H) primary antibody anti- β -Actin, secondary antibody IRDye[®] 680RD Goat anti-Mouse; (I) primary antibody anti-phospho-S6, secondary antibody IRDye[®] 800CW Goat anti-Rabbit; (J) primary antibody anti-S6, secondary antibody IRDye[®] 680RD Goat anti-Mouse; (K) primary antibody anti- β -Tubulin, secondary antibody IRDye[®] 800CW Goat anti-Rabbit.

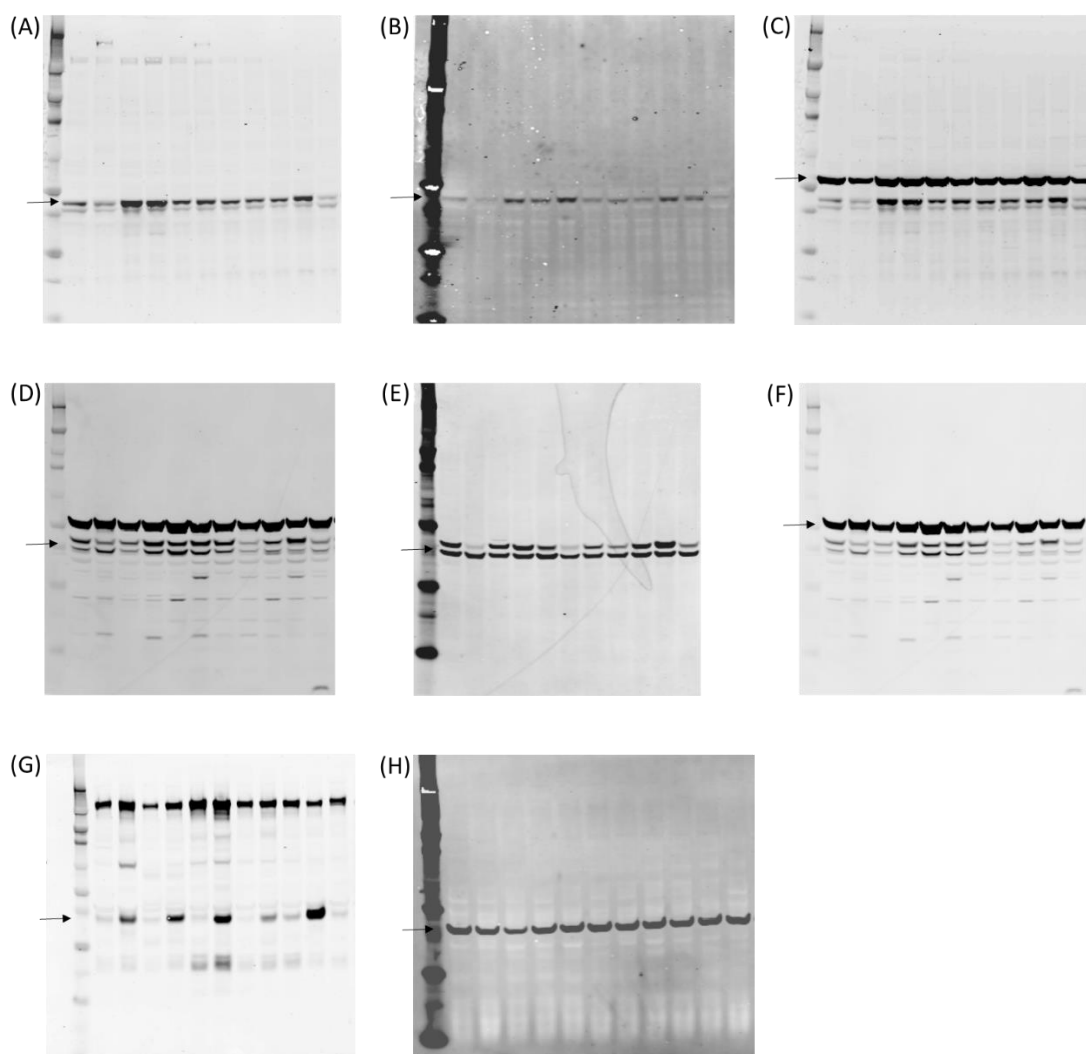


Figure 8.3. Western blots used in Figure 3.8. (A) Primary antibody anti-phospho-MEK1/2, secondary antibody IRDye® 800CW Goat anti-Rabbit; (B) primary antibody anti-MEK1/2, secondary antibody IRDye® 680RD Goat anti-Mouse; (C) primary antibody anti- β -Tubulin, secondary antibody IRDye® 800CW Goat anti-Rabbit; (D) primary antibody anti-phospho-ERK1/2, secondary antibody IRDye® 800CW Goat anti-Rabbit; (E) primary antibody anti-ERK1/2, secondary antibody IRDye® 680RD Goat anti-Mouse; (F) primary antibody anti- β -Tubulin, secondary antibody IRDye® 800CW Goat anti-Rabbit; (G) primary antibody anti-phospho-FRA1, secondary antibody IRDye® 800CW Goat anti-Rabbit; (H) primary antibody anti- β -Actin, secondary antibody IRDye® 680RD Goat anti-Mouse.

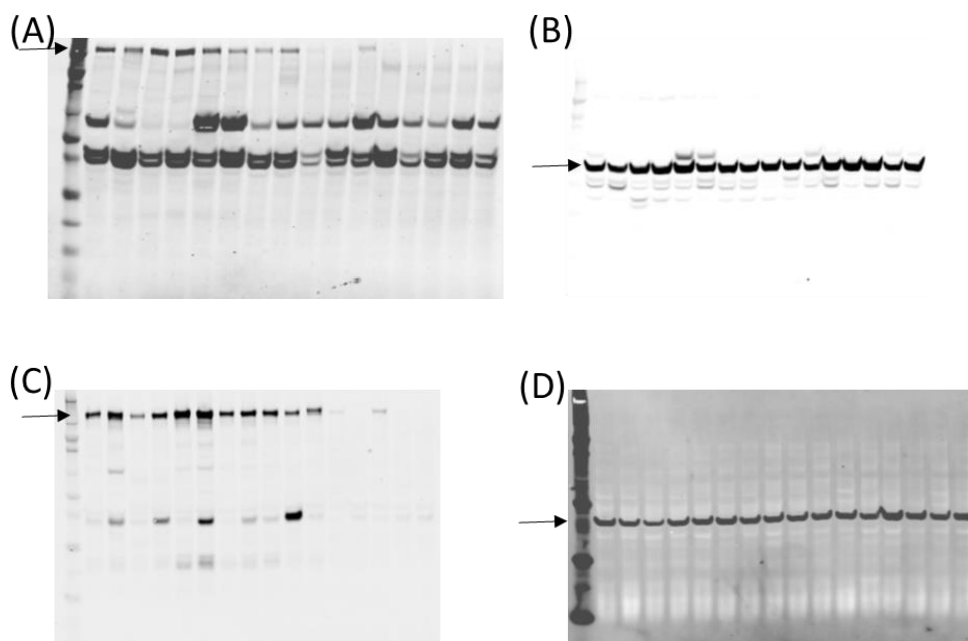


Figure 8.4. Western blots used in Figure 3.10. (A) Primary antibody anti-phospho-EGFR, secondary antibody IRDye[®] 800CW Goat anti-Rabbit; (B) primary antibody anti-β-Tubulin, secondary antibody IRDye[®] 800CW Goat anti-Rabbit; (C) primary antibody anti-EGFR, secondary antibody IRDye[®] 800CW Goat anti-Rabbit; (D) primary antibody anti-β-Actin, secondary antibody IRDye[®] 680RD Goat anti-Mouse.

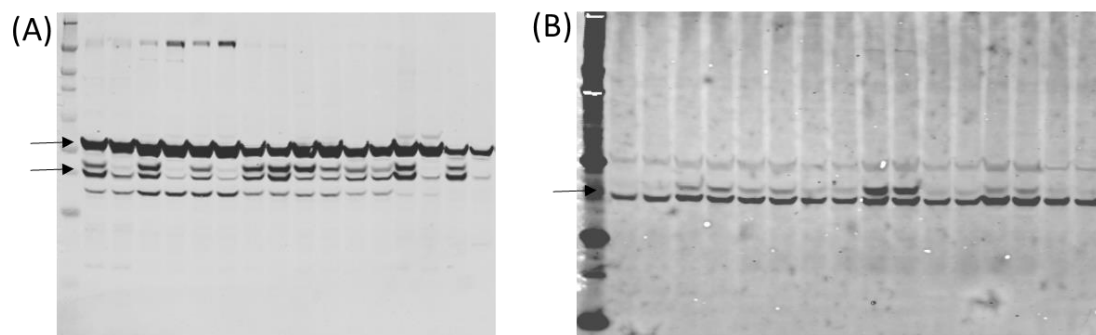


Figure 8.5. Western blots used in Figure 3.15 (A). (A) Primary antibody anti-phospho-ERK1/2 (bottom arrow) and anti-β-Tubulin (top arrow), secondary antibody IRDye® 800CW Goat anti-Rabbit; (B) primary antibody anti-ERK1/2, secondary antibody IRDye® 680RD Goat anti-Mouse.

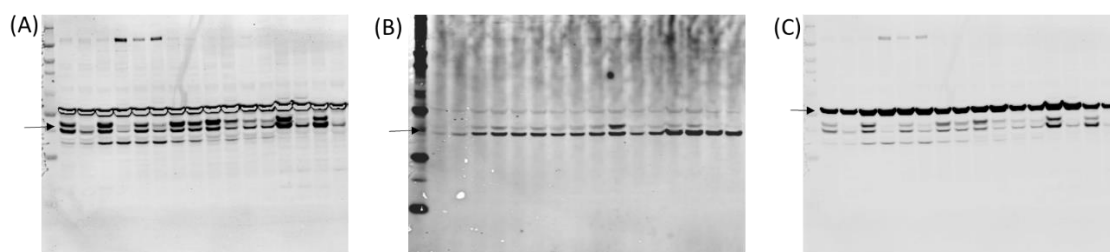


Figure 8.6. Western blots used for Figure 3.15 (B). (A) Primary antibody anti-phospho-ERK1/2, secondary antibody IRDye[®] 800CW Goat anti-Rabbit; (B) primary antibody anti-ERK1/2, secondary antibody IRDye[®] 680RD Goat anti-Mouse; (C) primary antibody anti- β -Tubulin, secondary antibody IRDye[®] 800CW Goat anti-Rabbit.

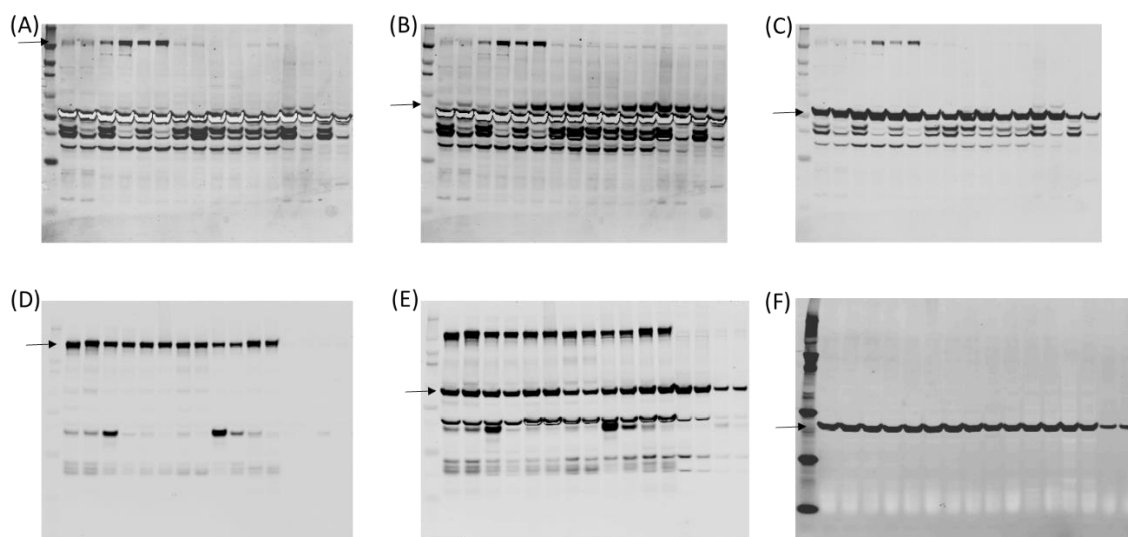


Figure 8.7. Western blots used in Figure 3.18 (A). (A) Primary antibody anti-phospho-EGFR, secondary antibody IRDye® 800CW Goat anti-Rabbit; (B) primary antibody anti-phospho-Akt, secondary antibody IRDye® 800CW Goat anti-Rabbit; (C) primary antibody anti- β -Tubulin, secondary antibody IRDye® 800CW Goat anti-Rabbit; (D) primary antibody anti-EGFR, secondary antibody IRDye® 800CW Goat anti-Rabbit; (E) primary antibody anti-Akt1; secondary antibody IRDye® 800CW Goat anti-Rabbit; (F) primary antibody anti- β -Actin, secondary antibody IRDye® 680RD Goat anti-Mouse.

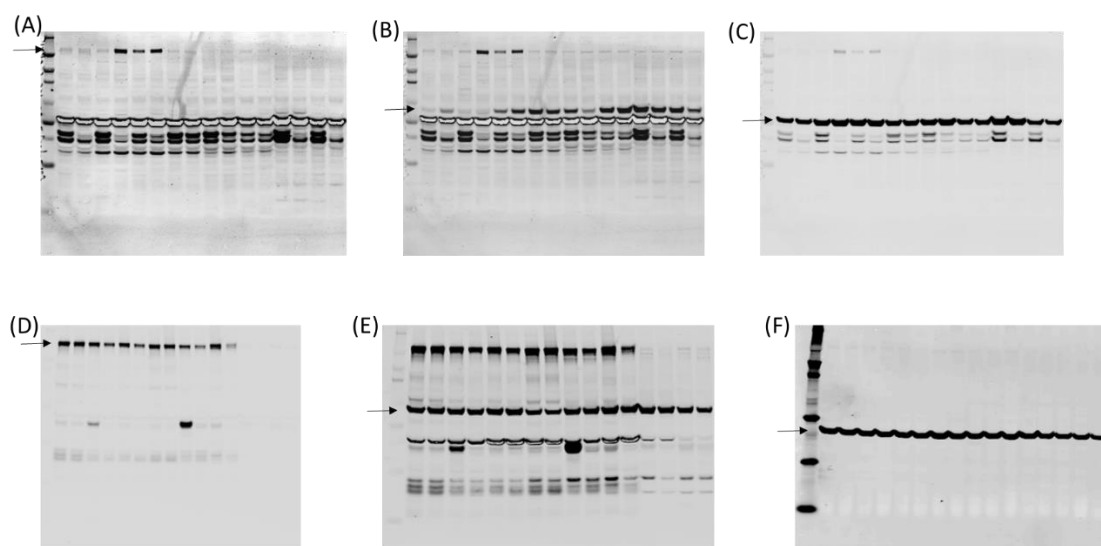


Figure 8.8. Western blots used in Figure 3.18 (B). (A) Primary antibody anti-phospho-EGFR, secondary antibody IRDye® 800CW Goat anti-Rabbit; (B) primary antibody anti-phospho-Akt, secondary antibody IRDye® 800CW Goat anti-Rabbit; (C) primary antibody anti- β -Tubulin, secondary antibody IRDye® 800CW Goat anti-Rabbit; (D) primary antibody anti-EGFR, secondary antibody IRDye® 800CW Goat anti-Rabbit; (E) primary antibody anti-Akt1; secondary antibody IRDye® 800CW Goat anti-Rabbit; (F) primary antibody anti- β -Actin, secondary antibody IRDye® 680RD Goat anti-Mouse.

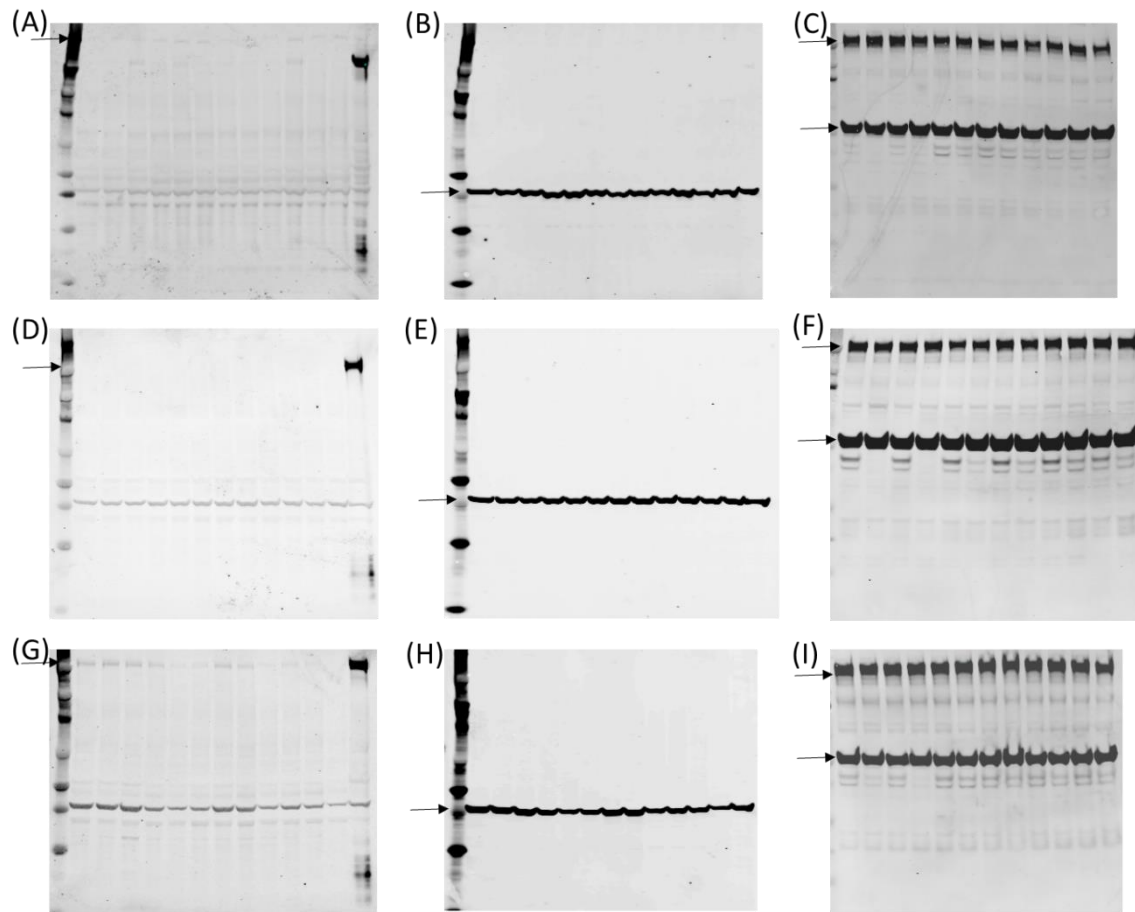


Figure 8.9. Western blots used in Figure 3.19. (A, D, and G) Primary antibody anti-phospho-EGFR, secondary antibody IRDye® 800CW Goat anti-Rabbit, for VACO432, RKO, and LS411N, respectively; (B, E, and H) primary antibody anti-β-Actin, secondary antibody IRDye® 680RD Goat anti-Mouse, for VACO432, RKO, and LS411N, respectively; (C, F, and I) primary antibody anti-EGFR (top arrow) and anti-β-Tubulin (bottom arrow), secondary antibody IRDye® 800CW Goat anti-Rabbit, for VACO432, RKO, and LS411N, respectively.

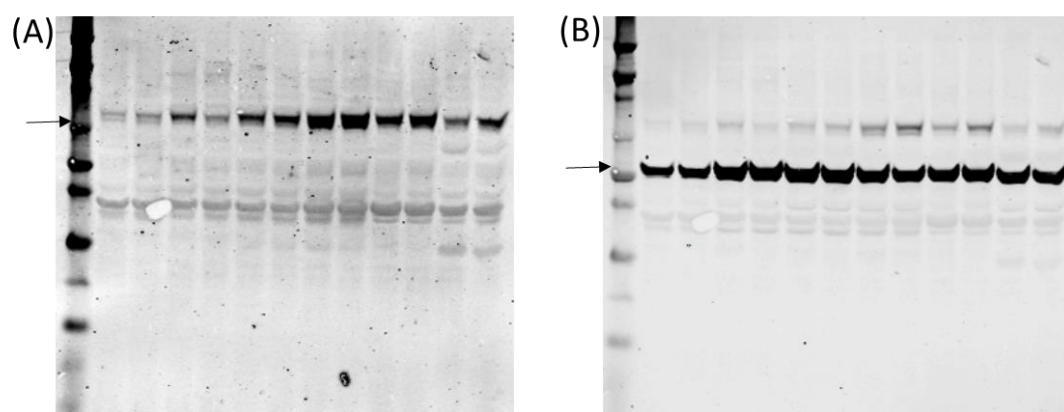


Figure 8.10. Western blots used in Figure 4.17. (A) Primary antibody anti-phospho-CRAF, secondary antibody IRDye[®] 800CW Goat anti-Rabbit; (B) primary antibody anti- β -Tubulin, secondary antibody IRDye[®] 800CW Goat anti-Rabbit.

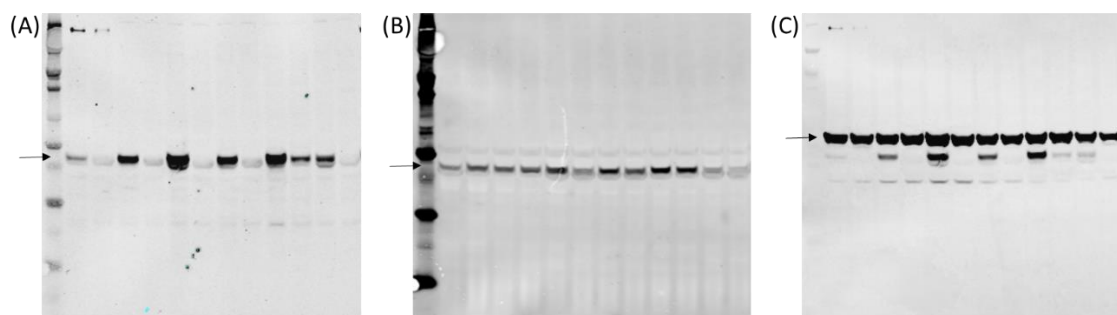


Figure 8.11. Western blots used in Figure 4.18. (A) Primary antibody anti-phospho-MEK 1/2, secondary antibody IRDye[®] 800CW Goat anti-Rabbit; (B) primary antibody anti-MEK1/2, secondary antibody IRDye[®] 680RD Goat anti-Mouse; (C) primary antibody anti- β -Tubulin, secondary antibody IRDye[®] 800CW Goat anti-Rabbit.