Biomarkers of resistance to anti-*EGFR* in wild type *KRAS/BRAF* colorectal cancer cell lines

Thesis submitted for the degree of Doctor of Philosophy

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"Be not afraid of greatness. Some are born great, some achieve greatness, and others have greatness thrust upon them."

~ William Shakespeare

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ABSTRACT

Colorectal cancer (CRC) is a leading cause of cancer death worldwide and despite significant improvement the median survival remains relatively poor. The use of targeted therapies like cetuximab and panitumumab inhibiting the epidermal growth factor receptor (*EGFR*) offer promise in improving patient outcomes. However, a high proportion of CRC patients show resistance to anti-*EGFR* therapy. Biomarkers such as mutant *KRAS* or *BRAF* predict resistance to anti-*EGFR* therapy in only a subset of patients and we hypothesise that other biomarkers for resistance to *EGFR* targeted therapies exist. The studies presented in this thesis aimed to determine other biomarkers of resistance to anti-*EGFR* therapy in wild type *KRAS* and *BRAF* CRC cell lines.

Following RT-Profiler Array analysis, the 3 most significantly upregulated genes amongst the 3 anti-*EGFR* resistant CRC cell lines (SNU-C1, SW48 and COLO-320DM) were chosen as candidate biomarkers of resistance: *HBEGF* (heparin-binding epidermal growth factor-like growth factor), *EGR1* (early growth response protein 1) and *AKT3* (protein kinase B gamma) were validated using qRT-PCR. *HBEGF* is a member of EGF-like growth factor family is a potent inducer of tumour growth, angiogenesis, and implicated in metastasis. *EGR1* is a transcription factor implicated in cell growth, survival, transformation, tumour progression. *AKT3* is a serine/threonine kinase and a downstream mediator of *PI3K-AKT-mTOR* pathway resulting in cell proliferation, cell survival and angiogenesis. *HBEGF* was knocked down by 79.4% in SNUC1, *EGR1* was knocked down by 85.6% in SW48 and *AKT3* was knocked down by 95.3% in COLO-320DM, as validated by qRT-PCR and western blot. Following knockdown, these cell lines were treated with anti-*EGFR*, and SNU-C1 had proliferation rate of 49.1% (83.8% before knockdown), SW48 yielded proliferation rate

of 46.9% (70% before knockdown) and COLO-320DM had proliferation rate of 64.1% (68.3% before knockdown). This suggests that the resistant phenotype of these cell lines was reversed. The expression of these markers was also elucidated using immunohistochemistry on mCRC primary tumour tissues from 10 patients that had undergone cetuximab monotherapy. Some 50% of these patients had overexpression of two or more of these markers, and these patients did not respond to cetuximab, suggesting that these overexpressed biomarkers might be involved in circumventing cetuximab to confer resistance.

One of the studies presented in this thesis also explored the *KRAS G13D* phenomenon and the effect of cetuximab and panitumumab on cell lines harbouring different mutational status. Previous clinical studies have demonstrated that a proportion of *KRAS G13D* harbouring tumour patients respond to the anti-*EGFR* therapies, and a large proportion of *KRAS* WT patients do not respond. After treatment with cetuximab or panitumumab, the *KRAS G13D* mutant cell lines showed intermediate sensitivity to both treatments, between the resistant *KRAS G12V* mutant cell line and the sensitive WT *KRAS* cell line. One of the *G13D* cell lines was significantly more sensitive to panitumumab than to cetuximab. This study demonstrated that specific *KRAS* mutation determines the responsiveness to anti-*EGFR* monoclonal antibody treatment, corresponding to previously reported clinical observations.

In conclusion, the studies presented in this thesis have demonstrated that components of *EGFR* signalling cascade have emerged as important biomarkers of resistance for anti-*EGFR* targeted therapies. Further assessment of the molecular mechanisms that dictate this resistance and identification of other specific biomarkers for these agents will provide valuable information to identify the most effective therapy for primary and mCRC patients.

Conference presentations

Shalini Sree Kumar, Jennifer Hardingham. SHC1 and SRC up-regulation contributes to resistance in SW48 treated with anti-*EGFR*. *Poster presentation: Research Day 2011, Basil Hetzel Institute, Adelaide, South Australia, Australia.*

Shalini Sree Kumar, Timothy Price, Jennifer Hardingham. Biomarkers of resistance to anti-*EGFR* therapy in colorectal cancer. *Poster presentation: 24th Lorne Cancer Conference, Lorne, Victoria, Australia.*

Shalini Sree Kumar, Timothy Price, Jennifer Hardingham. *KRAS G13D* mutant colon cancer cell lines - resistant or sensitive to anti-*EGFR* antibody? *Poster presentation: Australasian Gastro-Intestinal Trials Group 14th Annual Scientific Meeting, Sydney, New South Wales, Australia.*

Shalini Sree Kumar, Timothy Price, Jennifer Hardingham. Validation of predictive biomarkers of resistance to anti-*EGFR* in wild type *KRAS*/*BRAF* colorectal cancer cell lines. *Oral presentation: Research Day 2012, Basil Hetzel Institute, Adelaide, South Australia, Australia.*

Shalini Sree Kumar, Timothy Price, Jennifer Hardingham. Validation of predictive biomarkers of resistance to anti-*EGFR* in wild type *KRAS*/*BRAF* colorectal cancer cell lines. *Poster presentation: 6th Australian Health and Medical Research Congress 2012, Adelaide, South Australia, Australia.*

Shalini Sree Kumar, Timothy Price, Jennifer Hardingham. Biomarkers of resistance to anti-*EGFR* in wild type *KRAS*/*BRAF* colorectal cancer cell lines. *Poster presentation: Centre of Personalised Cancer Medicine Symposium 2013, Adelaide, South Australia, Australia.*

Shalini Sree Kumar, Timothy Price, Jennifer Hardingham. Biomarkers of resistance to anti-*EGFR* in wild type *KRAS*/*BRAF* colorectal cancer cell lines. *Poster presentation: Faculty of Health Sciences Postgraduate Research Conference 2013, Adelaide, South Australia, Australia.*

Shalini Sree Kumar, Timothy Price, Omar Mohyieldin, Matthew Borg, Amanda Townsend, Jennifer Hardingham. *KRAS G13D* mutations associated with sensitivity to cetuximab or panitumumab treatment in colorectal cancer cell lines. *Poster presentation: European Cancer Congress 2013, Amsterdam, Netherlands.*

Shalini Sree Kumar, Timothy Price, Jennifer Hardingham. Biomarkers of resistance to anti-*EGFR* in wild type *KRAS*/*BRAF* colorectal cancer cell lines. *Poster presentation: European Cancer Congress 2013, Amsterdam, Netherlands.*

Shalini Sree Kumar, Timothy Price, Jennifer Hardingham. Biomarkers of resistance to anti-*EGFR* in wild type *KRAS*/*BRAF* colorectal cancer cell lines. *Poster presentation: Research Day 2013, Basil Hetzel Institute, Adelaide, South Australia, Australia.*

Prizes awarded

- SCHOOL OF MEDICAL SCIENCES TRAVEL AWARD: Recipient of School of Medical Sciences travel award, AUD$ 2750. Awarded by University of Adelaide, Australia to attend and present at the 2013 European Cancer Congress in Amsterdam and laboratory meetings in University of Amsterdam and Institute of Cancer Research, University of London.

- WINNER FOR BEST POSTER PRESENTATION: Best poster winner in the 2013 Research Day conference, Basil Hetzel Institute for Translational Health Research, Woodville, Australia.

CHAPTER 1:

LITERATURE REVIEW

1.1 INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer worldwide and the second leading cause of cancer-related death in the Western world (Roda and Cervantes 2010). In 2012, CRC was reported to be the second most common cancer in Australia after prostate cancer for men and breast cancer for women with 15,840 new cases being reported in Australia. More than half of the cases were in men and about 44.7% were in women (AIHW 2012). CRC is one of the most curable cancers if it is detected at an early stage.

In 1991, the incidence rate of bowel cancer for males was 76 per 100,000. This number increased to 80 per 100,000 in 2000 and declined in the following years. In 2012, it was expected to fall to 73 per 100,000, which is a 4.7% decrease from the rate in 1991 (Figure 1). For females, the incidence rate of colorectal cancer varied between 51 and 55 per 100,000 from 1991 to 2009. It was significantly lower than that for males during this entire period. This may be related to differences in the behaviour that increases the risk of colorectal cancer as well as the differing effect obesity has in males and females (Center, Jemal et al. 2009). The rate is expected to be stable between 2010 and 2012.

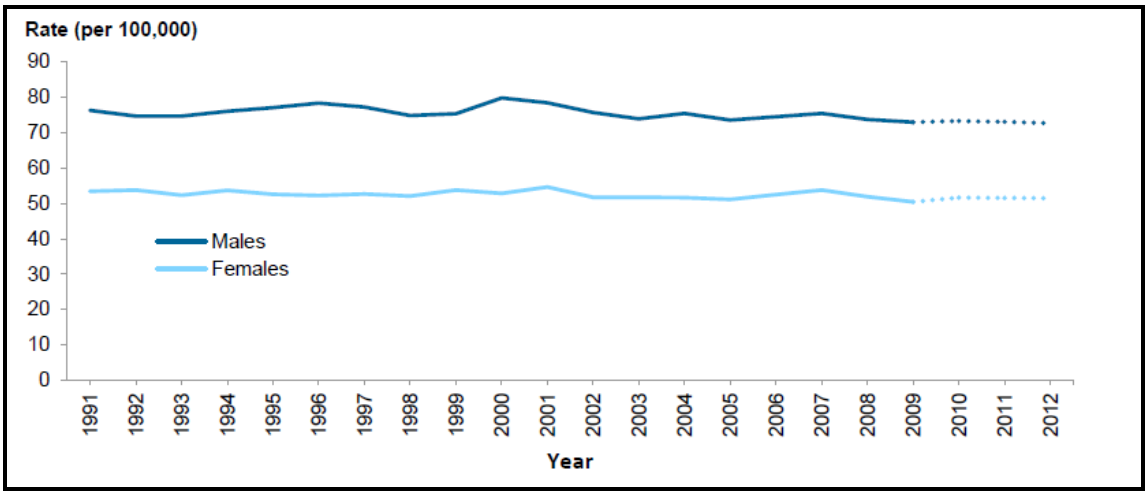

Figure 1: Trends in incidence of bowel cancer, Australia, 1991 to 2009 with estimates to 2012. (Source: Cancer in Australia: an overview, 2012)

The age-standardised mortality rate of bowel cancer decreased for males and females. Between 1991 and 2010, it fell by 41% for males (from 34 to 20 per 100,000) and 45% for females (from 24 to 13 per 100,000). The reasons for the continued decline may be due to earlier detection of pre-cancerous polyps and improved treatment (AIHW 2012).

There are three chemotherapy drugs available presently for the treatment of metastatic CRC (mCRC) - 5-fluorouracil, oxaliplatin, and irinotecan, and five targeted drugs that includes cetuximab and panitumumab (epidermal growth factor receptor [*EGFR*] antibodies), bevacizumab and aflibercept (vascular endothelial growth factor [VEGF] inhibitors), and regorafenib (a multikinase inhibitor) (Schmoll and Stein 2014). There have been improvements over the years in the treatment of mCRC in the form of targeted therapies that provide additional benefit to standard chemotherapeutic treatments. One of the important targets that have emerged for mCRC treatment is the *EGFR* (Peeters and Price 2012). *EGFR* (*HER1*/*erbB1*), a 170-kDa surface receptor, is a member of the class I superfamily of receptor tyrosine kinases, also comprising *HER2* (*erbB2*), *HER3* (*erbB3*) and *HER4* (*erbB4*). All of these comprise of a ligand-binding region, a cytoplasmic intracellular tyrosine-kinase domain and a single membrane-

spanning region (Herbst 2004). *EGFR*-mediated signaling is dysregulated in many human carcinomas, including colon, lung, prostate, breast and ovary. *EGFR* signaling involves two main intracellular pathways, one of which is the *KRAS* signaling cascade, activating *BRAF*, which triggers the *MAPK*s. The membrane localisation of the lipid kinase *PIK3CA* is opposed by promoting *AKT1* phosphorylation, which in turn activates a parallel intracellular pathway (Sartore-Bianchi, Martini et al. 2009). These pathways are responsible for many cellular responses, such as differentiation, apoptosis, proliferation and migration. Mutations and gene amplification or loss, can lead to the downstream constitutive activation of various downstream pathways, such as the *MAPK*, *PI3K–AKT* and *STAT* pathways, contributing to carcinogenesis in these tumours. Such aberrant factors may emerge as therapeutic targets (Citri and Yarden 2006).

1.2 *EGFR*-TARGETED THERAPIES FOR CRC

One of the most important targeted therapies in CRC treatment involves the *EGFR*, which controls signalling pathways that are involved in cell differentiation, proliferation and angiogenesis. Two anti-*EGFR* monoclonal antibodies that are currently approved by US Food and Drug Administration and European Medicines Agency for the treatment of CRC are cetuximab and panitumumab. Both monoclonal antibodies are directed against the ligand-binding site of *EGFR* and competitively inhibiting ligand-induced activation, hence leading to the inhibition of *EGFR*-induced cell growth, survival, and proliferation. Unfortunately, only a subset of CRC patients benefit from *EGFR* inhibition and therefore more in-depth research is needed to identify predictive markers that enable specific therapy tailored for the individual patient.

1.2.1 Monoclonal antibodies

Cetuximab (Erbitux, Merck-Serono KgaA), a chimeric mouse-human mAb, is the first anti-*EGFR* mAb approved to be used clinically for mCRC treatment. It was evaluated in combination with chemotherapeutic drugs (Cunningham, Humblet et al. 2004; Sobrero, Maurel et al. 2008; Van Cutsem, Kohne et al. 2009) and also as monotherapy (Jonker, O'Callaghan et al. 2007; Van Cutsem, Peeters et al. 2007). It inhibits several signalling pathways that includes the *RAS-RAF-MEK-ERK*, *STAT* and *PI3K/AKT* pathways. Panitumumab (Vectibix, Amgen), a fully humanised mAb, has demonstrated monotherapeutic efficacy in patients with chemotherapy-refractory mCRC (Van Cutsem, Peeters et al. 2007) and as combination with chemotherapeutic drugs in first and second lines of mCRC treatment (Douillard, Siena et al. 2010; Peeters, Price et al. 2010). The binding of this agent to *EGFR* internalises the receptor–antibody complex and prevents downstream signalling, which in turn induces apoptosis, and inhibits proliferation and tumour growth. Cetuximab and panitumumab have similar objective response rates of approximately 10% when used as monotherapy for non-molecularly tested chemotherapy-refractory mCRCs expressing *EGFR* (Cunningham, Humblet et al. 2004; Saltz, Meropol et al. 2004; Jonker, O'Callaghan et al. 2007; Van Cutsem, Peeters et al. 2007; Hecht, Mitchell et al. 2009). However, because panitumumab is a fully humanised antibody, it is likely to be less immunogenic and seldom perpetrates any severe infusion reactions (Van Cutsem, Peeters et al. 2007). About 22% of patients treated with cetuximab experience such severe infusion reactions, however this is dependent on the geographical region the patients hail from, and they appear to be associated with pre-existing IgE antibodies against the oligosaccharide component of the cetuximab molecule, galactose-alpha-1,3-galactose (Chung, Mirakhur et al. 2008). Efficacy of cetuximab, an IgG1 antibody, is also

mediated by antibody-dependant cell-mediated cytotoxicity (ADCC) via IgG Fcγ receptor engagement (Kimura, Sakai et al. 2007).

Chemotherapy-refractory patients particularly to fluoropyrimidine, irinotecan and oxaliplatin produce response rates of about 12% when treated with single agent cetuximab (Saltz, Meropol et al. 2004). Patients that are refractory to irinotecan can be resensitised with the addition of cetuximab treatment to irinotecan (Cunningham, Humblet et al. 2004). The CRYSTAL study showed that both response rates and progression-free survival were increased when cetuximab was added to FOLFIRI; similarly, the response rates were seen to improve when cetuximab was added to FOLFOX according to the OPUS study (Bokemeyer, Bondarenko et al. 2009). However, the MRC COIN study concluded that for patients with widespread metastatic disease, combining cetuximab treatment with oxaliplatin and capecitabine in the first-line chemotherapy was not recommended (Maughan, Adams et al. 2011). Furthermore, the analysis of the NORDIC-VII trial showed in first-line treatment of mCRC, there was no significant benefit when cetuximab was added to the Nordic FLOX (fluorouracil/folinic acid and oxaliplatin) regimen (Tveit, Guren et al. 2012).

1.2.2 Small molecule tyrosine kinase inhibitors

Gefitinib and erlonitib, the other *EGFR* small molecule tyrosine kinase inhibitors have shown disappointing response rates (0–1%) in mCRC and, when administered in combination with chemotherapeutic drugs, the toxicity is increased without any improvement in the efficacy of the combination therapy (Kuo, Cho et al. 2005; Rothenberg, LaFleur et al. 2005; Townsley, Major et al. 2006).

1.3 PREDICTIVE BIOMARKERS WITHIN THE *EGFR* SIGNALLING PATHWAY

Activating mutations in *KRAS* exon 2 particularly at codons 12 and 13 was the first established molecular marker that affects clinical response to cetuximab and panitumumab, both *EGFR*-targeted mAbs (Lievre, Bachet et al. 2006; Karapetis, Khambata-Ford et al. 2008). This mutation was predictive of non-response to cetuximab treatment, either administered as monotherapy or as combination therapy with irinotecan or oxaliplatin (Bokemeyer, Bondarenko et al. 2009). Other effectors of *EGFR* signalling are suggested to be predictive of anti-*EGFR* treatment outcome although there remains uncertainty and they are not routinely used in clinical practice. Mutations in other *KRAS* codons or in *NRAS* have recently been reported to be predictive of resistance (Douillard, Oliner et al. 2013; Peeters, Douillard et al. 2013). Activating mutation in *PI3K* exons 9 or 20, and *PTEN* loss have also been linked with poor outcome for WT *KRAS* tumours-harbouring patients (Di Nicolantonio, Martini et al. 2008; Sartore-Bianchi, Martini et al. 2009). *BRAF* has been shown to be a prognostic marker for worse overall survival (OS) in CRC (Roth, Tejpar et al. 2010; Price, Hardingham et al. 2011).

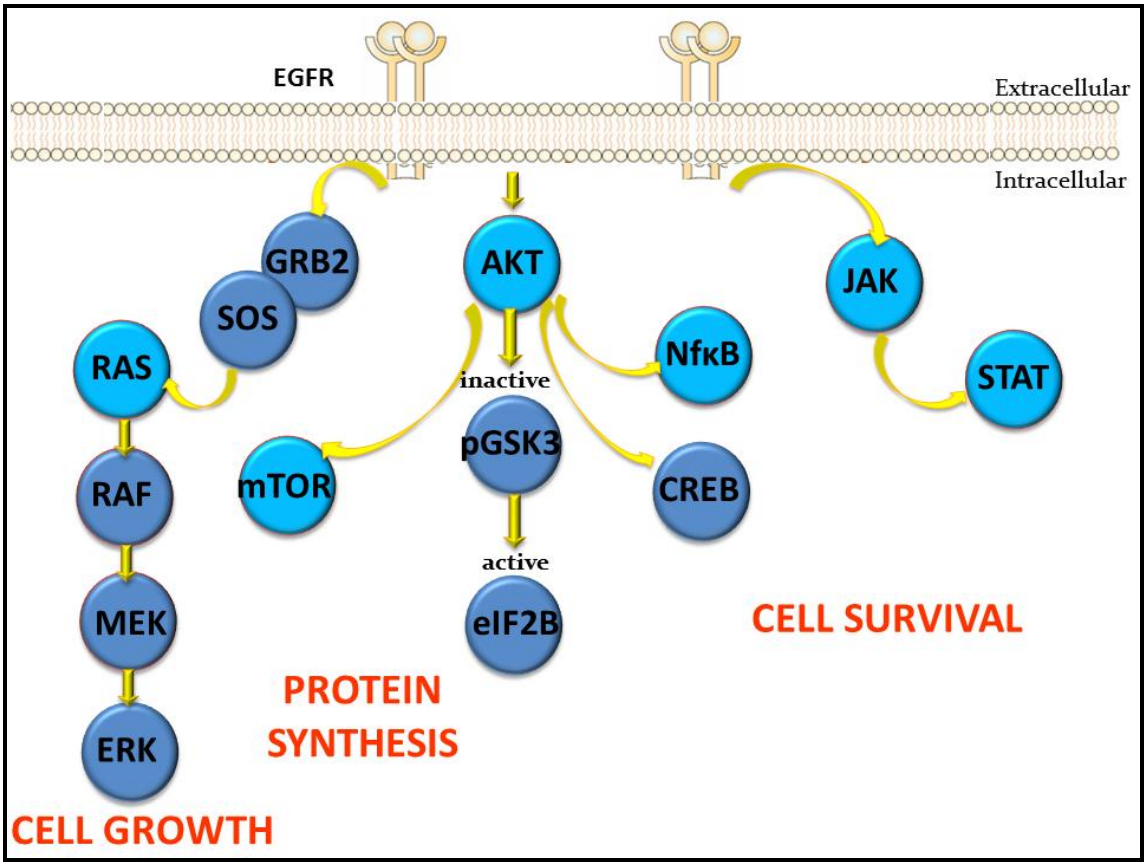

Figure 7: Schematic of the *EGFR* signalling pathway and the downstream intracellular cascades and the genes involved.

1.4 *EGFR* EXPRESSION

The overexpression of *EGFR* expression is usually determined by immunohistochemistry method, has been found to be associated with tumour progression and poor survival in various malignancies, such as head and neck carcinoma (Chang and Califano 2008). *EGFR* has been reported to be overexpressed in CRCs in the range of 25% to 82% and some studies report protein overexpression (defined as 2+ and/or 3+ staining or in >50% of cells) in 35 to 49% of cases (McKay, Murray et al. 2002; Resnick, Routhier et al. 2004). The clinical significance of *EGFR* overexpression in CRCs however is uncertain. One study of 249 CRCs reported an association of *EGFR* overexpression with tumour grade (poor differentiation) (P =

0.014) (McKay, Murray et al. 2002), another group found no association with grade in 134 tumours (Resnick, Routhier et al. 2004).

Due to the known expression of *EGFR* in CRC, a phase II trial of cetuximab in patients with refractory *EGFR* positive (assessed by immunohistochemistry) CRC was undertaken (Saltz, Meropol et al. 2004). The results were promising, however, it was soon discovered that there was no correlation between *EGFR* expression in the tumour and the response to therapy (Cunningham, Humblet et al. 2004; Chung, Shia et al. 2005). In the study by Chung et al., 25% patients with *EGFR*-negative tumours who received cetuximab-irinotecan combination therapy achieved a partial response with a greater than 50% reduction in the size of measurable lesions (Chung, Shia et al. 2005). A similar response rate was also reported in a separate cetuximab-plus-irinotecan clinical trial in *EGFR*-positive patients (Cunningham, Humblet et al. 2004). As a result, cetuximab is now administered without the need for *EGFR* expression testing.

The undefined significance of *EGFR* expression as a prognostic indicator may be related to the variability in the methodology used to detect *EGFR*. Most studies use immunohistochemistry to detect *EGFR* expression in CRCs. Immunohistochemistry is highly dependent on the antibody that is used, the staining protocols, selection of scoring methods, and selection of cut-off values. Until a standard method of *EGFR* staining and reporting is adopted, the significance of *EGFR* protein expression in colorectal cancer will remain ambiguous.

1.5 *EGFR* LIGANDS

Epiregulin and amphiregulin are the principal ligands for *EGFR*. Overexpression of these ligands may lead to promotion of tumour growth and survival by an autocrine loop (Jacobs, De Roock et al. 2009). The expression of these ligands have been reported by several studies to be correlated with sensitivity to cetuximab monotherapy. Results from the study showed a PFS that was statistically longer among patients with high epiregulin expression. However, the use of amphiregulin or epiregulin expression profile does not result in selecting the sub-group of patients that benefit from cetuximab treatment (Khambata-Ford, Garrett et al. 2007).

1.6 *EGFR* GENE MUTATIONS AND GENE COPY NUMBER

Recent studies have showed that an increased *EGFR* gene copy number (GCN) analysed using FISH technique, is potentially a predictor of response for mCRC patients when treated with *EGFR*-targeted monoclonal antibodies. The study by Moroni et al. was the first to demonstrate an association between the increased *EGFR* GCN with favourable response to anti-*EGFR* therapy amongst mCRC patients harbouring WT *KRAS* (Moroni, Veronese et al. 2005). A systematic review and meta-analysis by Jiang et al. was carried out to elucidate the association between the variations of *EGFR* GCN with the survival outcomes of mCRC patients receiving *EGFR*-targeted therapy, either cetuximab or panitumumab (Jiang, Li et al. 2013). Ten studies involving 776 patients on OS, eight studies involving 893 patients on PFS, and three studies on time-to-progression (TTP) were critically analysed. Increased GCN was reported to be significantly associated with improved OS amongst patients treated with anti-*EGFR* mAbs (HR = 0.62; 95% CI 0.50-0.77; $P < 0.001$). Increased GCN was also seen to be

significantly associated with improved PFS (HR = 0.65; 95% CI 0.47-0.89; P = 0.008); however no significant TTP benefit for high *EGFR* GCN patients was found (HR = 0.71; 95% CI 0.44-1.14; P = 0.157) (Jiang, Li et al. 2013). The meta analyses of these studies confirms that increased *EGFR* GCN is associated with OS and PFS benefit, albeit moderately. Similarly, *EGFR* GCN has also been assessed as a potential predictive marker of response in non-small-cell lung cancer patients when treated with tyrosine kinase inhibitors (TKIs), which showed an association between increased *EGFR* GCN with improved survival outcomes of the patients (Dahabreh, Linardou et al. 2011). A recent meta-analysis by Yang *et al.* to differentiate the objective response rate (ORR) between mCRC patients with high *EGFR* GCN with those with no increased *EGFR* GCN demonstrated a trend towards higher ORR in patients with high *EGFR* GCN (Yang, Shen et al. 2012). Results from this study demonstrated the association between increased *EGFR* GCN with improved survival outcomes amongst mCRC patients treated with anti-*EGFR* therapies (Yang, Shen et al. 2012). This implies that not only *EGFR* GCN is a potentially effective predictive marker, but also a valuable prognostic marker.

1.7 PREDICTIVE BIOMARKERS FOR ANTI-*EGFR* THERAPIES AND CLINICAL DATA

1.7.1 *KRAS* Mutations

KRAS, a proto-oncogene that encodes a 21 kDa guanosine triphosphate (*GTP*)/guanosine diphosphate (*GDP*) binding protein, regulates cellular response to many extracellular stimuli. *RAS* recruits the oncogenic protein *RAF* after binding and activation by *GTP*, and this phosphorylates mitogen-activated protein kinase (*MAP2K*),

initiating *MAPK* signalling that leads to cell proliferation, differentiation and survival (Schubbert, Shannon et al. 2007). *KRAS* is the most commonly mutated gene in the *EGFR* pathway, and it is mutated in 35–45% of colorectal adenocarcinomas. This mutation is known to be an early event in colon tumorigenesis (Andreyev, Norman et al. 2001). Approximately 90% of *KRAS* gene mutations are detected in exon 2 in codons 12 (70%) and 13 (20%), and less in exon 3 in codon 61 (5%) and in exon 4 in codon 146 (5%). Interestingly, mutations resulting in the glycine-to-valine substitution at the catalytic site are associated with a more aggressive tumour which impacts on survival outcome (Andreyev, Norman et al. 2001).

In the most recent analysis stemming from the phase III PRIME study, the effect of panitumumab-FOLFOX versus FOLFOX on PFS and OS in mCRC patients based on *RAS* (*KRAS* or *NRAS* exon 2, 3 and 4) and *BRAF* mutation status was elucidated. In the WT *RAS* group, both PFS and OS was significantly increased in the panitumumab arm versus FOLFOX arm (median PFS 10.1 months vs 7.9 months $p < 0.01$); median OS was 26 months vs 20.2 months, ($p = 0.04$). A similar increase in PFS and OS was found in the panitumumab arm for patients WT for both *RAS* and *BRAF*: median PFS 10.8 months vs 9.2 months $p < 0.01$); median OS 28.3 months vs 20.9 months $p = 0.02$) (Douillard, Oliner et al. 2013).

An important finding of the PRIME trial was the inferior outcome in *KRAS* mutant (MT) patients treated with panitumumab. These concerning findings are supported by inferior outcomes in anti-*EGFR* mAbs treated *RAS* MT patients in the OPUS (Douillard, Oliner et al. 2013) and PICCOLO trials (Seymour, Brown et al. 2013). Given the updated PRIME results demonstrate inferior outcomes extend to patients with exon 3 and 4 *KRAS* MT and *NRAS* MT, there is a strong case these biomarkers should be included in routine clinical practice, leading to a new definition of

"*RAS* MT". A recent hypothesis suggests non-mutant isoforms of *RAS* may play a role in suppressing the mutant *RAS* isoform. In the setting of mutant *KRAS*, WT *NRAS* and *HRAS* continue to play an important role in modulating downstream signalling. These WT *RAS* isoforms have an inhibitory effect on oncogenic mutant *KRAS*. Inhibition of *EGFR* inhibits the function of WT *RAS* isoforms and may remove its inhibitory effect on mutant *RAS*, paradoxically leading to an increase in *MAPK* signalling and cell proliferation (Young, Lou et al. 2013).

Findings of a meta-analysis of *KRAS* status that included 10 randomised controlled trials in advanced CRC treated with anti-*EGFR* mAbs and chemotherapy showed a trend towards worse PFS in patients with *KRAS* mutations (Adelstein, Dobbins et al. 2011), and 3/10 trials showed a statistically significant detrimental effect of combination therapy (Tol, Koopman et al. 2009; Douillard, Siena et al. 2010; Bokemeyer, Bondarenko et al. 2011). Such drug-specific adverse interactions has led to a notion that cetuximab would be better when paired with irinotecan than with other drugs (De Roock, Claes et al. 2010).

The PICCOLO randomised clinical trial of second line therapy for mCRC randomised molecularly unselected patients to irinotecan with or without panitumumab (Seymour, Brown et al. 2013). Enrolment was later restricted to *KRAS* exon 2 and 3 WT patients only. *KRAS* exon 4, *BRAF*, *NRAS*, *PIK3CA* mutations were assessed and outcomes analysed retrospectively by mutation status. No difference in OS (primary end-point) was observed in *KRAS* WT patients treated with panitumumab compared to irinotecan alone (HR 1.01, 95% CI 0·83–1·23; p=0·91) but those in the irinotecan + panitumumab group had longer PFS (p=0·015) and a greater ORR (34% vs 12% p<0·0001) than did individuals in the irinotecan group. The secondary analysis of patients with any mutation in *KRAS*, *NRAS*, *BRAF*, or *PIK3CA*, showed that

panitumumab treatment had an adverse effect on OS (HR 1·64; 95% CI 1·14–2·34, p = 0.028) (Seymour, Brown et al. 2013).

Recently, however, a systematic review that included a meta-analysis showed that in patients with the *KRAS* codon 13 (*G13D*) mutation showed a better response to cetuximab treatment compared to patients with *KRAS* codon 12 mutations (Mao, Huang et al. 2013). In a retrospective analysis across 3 treatment arms with and without panitumumab, mutant *KRAS* codon 12 or 13 patients did not elicit any benefit from panitumumab therapy, suggesting that this therapy should be restricted to WT *KRAS* mCRC patients (Peeters, Douillard et al. 2013).

1.7.1.1 *KRAS G13D* Phenomenon

KRAS mutations are found in approximately 60% of all CRC tumours. Of those, about 79% involve codon 12, whereas a codon 13 mutation is found in 17.6% of patients (http://www.sanger.ac.uk/genetics/CGP/cosmic). *In vitro* data suggest that *KRAS* codon 13 mutations have a weaker transforming activity than codon 12 mutations (Guerrero, Casanova et al. 2000) and some clinical reports also suggest that some of these patients do respond to cetuximab (De Roock, Piessevaux et al. 2008).

In the retrospective study by De Roock *et al.* (De Roock, Jonker et al. 2010), it was evident that a proportion of patients with *KRAS G13D* mutation responded to cetuximab. The largest retrospective analysis conducted by Peeters *et al*. (Peeters, Douillard et al. 2013) evaluating three phase III trial studies involving the alternate anti-*EGFR* drug panitumumab (first line, second line and monotherapy) revealed that *KRAS G13D* was unfavourably associated with panitumumab treatment effects on OS but not on PFS or response rate. These differing results may reflect subtle differences between

the two antibodies to *EGFR*, for example chimeric versus fully humanised. There is also a report of activity of panitumumab following cetuximab failure adding further evidence to potential differences in activity (Saif, Kaley et al. 2010).

Based on these retrospective studies and the conflicting results, we aimed to explore in a pre-clinical colorectal cancer cell line model the sensitivity and/or resistance to both cetuximab and panitumumab treatment, and to investigate the correlation of the *KRAS* mutational status of the colorectal cancer cell lines to the responsiveness to these agents. The published version of our study is presented in Chapter 3.

1.7.2 *NRAS* Mutations

Neuroblastoma *RAS* viral oncogene homolog (*NRAS*), a member of the *RAS* oncogene family, is mapped to chromosome 1 (Malumbres and Barbacid 2003). The mutation rate of *NRAS* in CRC is 3–5% in the exon 2 in codons 12 and 13 and in the exon 3 in codon 61. The presence of *NRAS* mutations is associated with a lack of response to cetuximab therapy (De Roock, Claes et al. 2010; Peeters, Oliner et al. 2013).

In the randomised phase III study by Peeters et al., multigene sequencing of genes including *KRAS*, *NRAS*, *EGFR*, *BRAF*, *PTEN*, *PIK3CA*, and *AKT* was done to investigate whether *EGFR* pathway mutations predicted response to monotherapy with panitumumab (n=320). Among patients with WT *KRAS* (codons 12/13/61) and WT *NRAS* (n = 138), treatment with panitumumab was associated with improved PFS (HR, 0.39; 95% CI, 0.27–0.56; $p < 0.001$). In patients with wild-type *KRAS* and mutant *NRAS* tumours, assigned to panitumumab therapy in either the randomised (n = 11) or extension study (n = 9), there was a lack of response and a lack of improved PFS

(Peeters, Oliner et al. 2013). Among WT *KRAS* patients, a Cox proportional hazards model with *NRAS* status and treatment, gave an interaction p value of 0.076 which was suggestive only (Peeters, Oliner et al. 2013). Nonetheless, these results were consistent with the hypothesis that *NRAS* mutations may limit the efficacy of panitumumab.

Analysis of *NRAS* mutations maybe important though as shown by Douillard et al., where patients with *NRAS* mutations had poorer median OS and PFS compared to the WT RAS group (Douillard, Oliner et al. 2013). The PICCOLO study also assessed tests of interaction with mutation status (*KRAS*, *BRAF*, *NRAS* and *PIK3CA* mutations versus all WT) as a predictive biomarker of the effect of panitumumab-irinotecan treatment on OS, PFS and ORR. The interaction test was positive for all of the three outcome measures: OS (P = 0.03), PFS (P = 0.02) and ORR (P = 0.001), but there was no between-group OS difference. For *NRAS*-mutated tumours, there was a suggestion that administration of panitumumab was harmful. However, the small sample number (n = 29) was insufficient to detect or negate the interactions between mutation status and treatment effect (Douillard, Oliner et al. 2013). Mutations in *NRAS* are low-prevalence mutations, their true predictive or prognostic value must be confirmed in larger studies.

1.7.3 *BRAF* Mutations

BRAF is a kinase that is located downstream of *KRAS* in the *EGFR* signal transduction pathway. The most commonly observed mutation for *BRAF* is a DNA missense mutation that leads to a valine to glutamic acid amino acid substitution (V600E)., *BRAF* mutation is involved in the receptor-independent aberrant activation of the *MEK-ERK* pathway and also CRC carcinogenesis. *BRAF* mutations in CRC have a

relatively low frequency, about 10% (Price, Hardingham et al. 2011; Douillard, Oliner et al. 2013).

Di Nicolantonio *et al.* have retrospectively analysed time to progression (TTP), OS and also *KRAS* and *BRAF* mutational status in 113 mCRC patients treated with cetuximab or panitumumab. Compared with WT, *BRAF*-mutated patients showed a significantly shorter PFS ($p = 0.011$) and OS ($p < 0.0001$) suggesting it was necessary to have WT *BRAF* to respond to anti-*EGFR* antibodies. In *BRAF*-mutated CRC cell lines, the sensitivity to *EGFR* inhibitors was restored by sorafenib, the multi-kinase inhibitor (Di Nicolantonio, Martini et al. 2008).

In WT *KRAS* patients, *BRAF*-mutated individuals have shown worse PFS and OS outcome. *BRAF* was prognostic for OS in patients with microsatellite instability (MSI) low (MSI-L) and stable (MSI-S) tumours. Amongst the MSI-H (high) subpopulation, no prognostic value of *KRAS* or *BRAF* mutation was found for PFS or OS (Roth, Tejpar et al. 2010). In the OPUS study, WT *KRAS*/WT *BRAF* patients showed a significant difference in PFS and OS between FOLFOX-4 with cetuximab versus without. In the MT *BRAF*/WT *KRAS* patients the difference was not significant. Nevertheless these patients seemed to benefit from the cetuximab addition, with an increase in the OS, despite the small sample size (Douillard, Oliner et al. 2013). Other studies have shown *BRAF* mutation to be prognostic for poorer outcome in mCRC independently of treatment (Price, Hardingham et al. 2011; Van Cutsem, Kohne et al. 2011; Safaee Ardekani, Jafarnejad et al. 2012; Douillard, Oliner et al. 2013).

Recently, the PRIME study (panitumumab-FOLFOX versus FOLFOX), showed that for mCRC patients with MT *BRAF* the treatment hazard ratios for PFS and OS were inconsistent with *BRAF* as a predictive biomarker (median PFS was 6.1 months in the panitumumab arm vs 5.4 months in FOLFOX arm; median OS 10.5

months vs 9.2 months respectively) (Douillard, Oliner et al. 2013). However Peeters *et al.* showed in 408 patients treated with panitumumab monotherapy there was a trend to PFS benefit in the 18 *BRAF* MT patients (Peeters, Oliner et al. 2013). There is still uncertainty of the value of *BRAF* mutation as a predictive biomarker.

1.7.4 *PIK3CA* Mutations and *PTEN* Loss

ErbB activates the *PI3K-AKT* signalling pathway and may be oncogenically deregulated by activating mutations of *PIK3CA* p110 subunit or by inactivation of *PTEN* phosphatase. The p110 subunit of *PI3K* is encoded by the oncogene *PIK3CA*, which is activated via the interaction with the RAS protein (Yarden 2001). The role of the dysregulation of *PIK3CA*/*PTEN* signalling with regards to the response to targeted therapy has been investigated in breast cancer (Nagata, Lan et al. 2004), glioblastoma (Mellinghoff, Wang et al. 2005) and mCRC (Ogino, Nosho et al. 2009; Bardelli and Siena 2010; Wilson, Labonte et al. 2010). *PIK3CA* mutations occur in 14% to 18% of CRCs and mostly involve the hotspots on exons 9 and 20 (Ogino, Nosho et al. 2009; De Roock, Claes et al. 2010). The *PTEN* gene encodes protein tyrosine phosphatase enzyme (*PTEN*), dephosphorylating phosphatidylinositol-3,4,5 triphosphate (PIP3) and subsequently inhibiting function of *PI3K* (Hynes and Lane 2005). *PTEN* loss results in the constitutive activation of *PI3K-AKT* pathway. *PTEN* mutations have been reported in 13%-18% of colon cancers and the loss of heterozygosity (LOH) of the *PTEN* locus has been shown in 17%-19% of colon cancers (Zhou, Loukola et al. 2002; Nassif, Lobo et al. 2004). Multivariate analyses from a study of 302 mCRC patients from the MAX trial showed that loss of *PTEN* by copy number PCR was not prognostic for PFS or OS (Price, Hardingham et al. 2013).

PIK3CA exon 20 mutations are reported to be associated with poor outcome after cetuximab treatment (De Roock, Claes et al. 2010). In a pre-clinical study, *PIK3CA* mutation and the expression status of *PTEN* was associated with response of CRC cells to cetuximab treatment, distinguishing resistant and sensitive cell lines (Jhawer, Goel et al. 2008). CRC cell lines harbouring activating *PIK3CA* mutations or *PTEN* loss (*PTEN* null) were reported to be more resistant to cetuximab than cell lines expressing WT *PIK3CA* /WT *PTEN*. *PIK3CA* mutant/*PTEN* null and *RAS*/*BRAF* mutant cell lines were highly resistant to cetuximab compared with cell lines without the dual mutations/*PTEN* loss, indicating that the constitutive and simultaneous activation of the *RAS* and *PIK3CA* pathways result in resistance to this treatment (Jhawer, Goel et al. 2008). This pre-clinical study provided evidence that the *PI3K* mutation status should be considered before anti-*EGFR* targeted treatments. Sartore-Bianchi et al. found that the activating *PIK3CA* mutations in 15 of the 110 patients (13.6%) that were treated with cetuximab or panitumumab-based regimens in first- to fourth-line treatment were associated with lack of response to anti-*EGFR* mAbs compared to a response rate of 23% in the 95 patients with WT *PIK3CA* ($p = 0.03$). When only WT *KRAS* patients were analysed, the statistical correlation was stronger ($p = 0.016$). In terms of PFS, patients harbouring *PIK3CA* mutations had a worse clinical outcome ($p = 0.004$) (Sartore-Bianchi, Martini et al. 2009). Souglakos et al. also reported that PFS was significantly lower among 92 patients with *PIK3CA* mutations treated with salvage chemotherapy and cetuximab (2.5 months versus 3.9 months; HR 2.1; 95% CI 1.2-3.9; $p = 0.01$) (Souglakos, Philips et al. 2009). A study by Prenen et al. highlighted some conflicting findings: 200 irinotecan-refractory patients treated with cetuximab as monotherapy or in combination with irinotecan were analysed for *PIK3CA* mutations. Of 23/200 (12%) tumours with such a mutation, 5 patients had an objective response to

cetuximab, and 18 did not (p = 0.78). This does not support an association between *PIK3CA* mutations and resistance to cetuximab in chemotherapy-refractory mCRC. The median PFS (24 vs. 18 weeks; p = 0.760) and OS (45 vs. 39 weeks; p = 0.698) did not differ significantly between *PIK3CA* mutant and WT patients (Prenen, Tejpar et al. 2010). These data suggest that the activating mutations of *PI3K* are not involved in causing resistance to cetuximab.

A larger dataset showed that among 356 *KRAS* WT chemorefractory patients treated with cetuximab, those with mutant *PIK3CA* had significantly lower ORR compared with WT *PIK3CA* (17.7% vs. 37.7%; HR, 0.35; 95% CI, 0.13–0.83; p = 0.015), although there was no significant difference in PFS (median PFS, 18 vs. 24 weeks; HR, 1.30; 95% CI, 0.91–1.86; p = 0.17) and OS (median OS, 39 vs. 51 weeks; HR, 1.41; 95% CI, 0.96–2.06; p = 0.09). When compared with WT *PIK3CA*, *PIK3CA* exon 20 mutations had a negative effect on ORR (36.8 vs 0%; p = 0.029), HR (0·00, 95% CI 0.00–0·.9; p = 0·029), PFS (median, 11.5 vs. 24 weeks; HR, 2.52; 95% CI, 1.33–4.78; p = 0.013) and OS (median, 34 vs. 51 weeks; HR, 3.29; p = 0.0057). However, exon 9 *PIK3CA* mutations were associated with *KRAS* mutations and had no significant effect on ORR (28.6% vs. 36.3% in WT; p = 0.47) (De Roock, Claes et al. 2010). These data highlight *PIK3CA* exon 20 mutation in being predictor of resistance to cetuximab and panitumumab treatment, but due to its low frequency, will require further confirmation.

Pre-clinical data have also shown that in CRC cell lines, the loss of *PTEN* confers resistance to cetuximab-induced apoptosis (Jhawer, Goel et al. 2008). In one study, none of 11 patients with low *PTEN* expression responded to a combination of cetuximab and irinotecan, whereas 10 of 16 patients (63%) with *PTEN* protein expression had a partial response (Frattini, Saletti et al. 2007). In another study, *PTEN*

loss was associated with lack of response to cetuximab and panitumumab (p = 0.001) among 81 evaluated tumours (Sartore-Bianchi, Martini et al. 2009). *PTEN* loss was also significantly associated with shorter PFS (p = 0.007), and *PTEN* loss combined with *PIK3CA* mutation showed worse OS (p = 0.005). These studies used IHC for *PTEN* expression which is difficult to standardise due to both staining and inter-observer variability thus limiting the ability to make any firm conclusions in these small studies.

1.8 HYPOTHESIS AND AIMS OF PROJECT

The research hypothesis of this project is differentially expressed genes between anti-*EGFR*-resistant and -sensitive cell lines will provide additional biomarkers of resistance to *EGFR*-targeted therapy. The null hypothesis of this project is differentially expressed genes between anti-*EGFR*-resistant and –sensitive cell lines will not provide any additional biomarkers of resistance to *EGFR*-targeted therapy.

The specific aims of this project are:

1. To identify WT *KRAS*/WT *BRAF* colorectal cancer cell lines resistant or sensitive to anti-*EGFR* treatment.
2. To identify candidate biomarkers using RT Profiler Array (QIAGEN Human *EGFR* Pathway-focused Array plates).
3. To validate the differential expression of candidate biomarkers using qRT-PCR.
4. To use siRNA to knockdown one or more overexpressed genes in the resistant CRC cell lines, and validate knockdown using qRT-PCR and western blot.

5. To determine effect of knockdown of individual biomarkers in resistant cells on response to anti-*EGFR* therapy using proliferation assays.

6. To elucidate expression of these biomarkers of resistance on CRC patient samples using immunohistochemistry and correlate the expression with the patients' response to cetuximab therapy.

Additional information

A condensed version of this literature review was published as “Predictive biomarkers of response to anti-*EGF* receptor monoclonal antibody therapies” in Colorectal Cancer 2014, 3(2):223-232.

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CHAPTER 2:

MATERIAL AND METHODS

2.1 MATERIALS

Cell culture

DMEM medium (Gibco, USA); Penicillin-Streptomycin (Gibco, USA); Foetal Bovine Serum (Gibco, USA); GlutaMax (Invitrogen, USA); DMSO (Sigma, USA); EDTA; PBS

Disposable pipettes (10 mL, 5 mL) (Becton Dickinson, USA)

15 mL polypropylene Falcon 2097 conical tubes (Becton Dickinson, USA)

Culture flask T25 (25 cm3), T75 (75 cm3) (Greiner Bio-One, Germany)

96-well and 24-well plates (Corning, USA)

Cell lines

SW48, SNU-C1, COLO-320DM, CACO2, SW948, LoVo, HCT-116, T84 (ATCC, USA), LIM1215 (a kind gift from the Ludwig Institute, Melbourne)

Proliferation assay

CellTiter 96® AQueous Assay kit (Promega, USA)

MTS/PMS solution (Promega, USA)

RNA extraction

PureLink® RNA Mini kit (Life Technologies, USA)

Taqman Gene Expression Assays

Validated primers for the genes *HBEGF*, *EGR1* and *AKT3* were purchased from Life Technologies (USA)

siRNA

Validated *HBEGF*, *EGR1* and *AKT3* siRNA complexes were purchased from Dharmacon (USA); ONTarget Plus Non-Targetting siRNA complex (Dharmacon, USA); siGlo Red Transfection Indicator (Dharmacon, USA); DharmaFECT transfection reagent (Dharmacon, USA); siRNA resuspension buffer (Dharmacon, USA)

qRT-PCR

Superscript III First Strand cDNA Synthesis kit (Life Technologies, USA)

RT Profiler Array Human *EGF/PDGF* pathway kit (SA Biosciences, Australia)

Protein retrieval

RIPA buffer (Thermo Scientific, USA); Halt Protease Inhibitor Cocktail (Pierce, USA)

Precision Plus Dual Colour Protein Standards (Biorad, USA), sizes in kDa: 10, 15, 20, 25, 37, 50, 75, 100, 150, 250

Protein quantification (EZQ)

EZQ Assay (Life Technologies, USA)

Western blot

1° antibodies: *HBEGF*: mouse monoclonal, used at 1:1000 (ab66792, Abcam, USA)

EGR1: mouse monoclonal, used at 1:1000 (ab55160, Abcam, USA)

AKT3: mouse monoclonal, used at 1:1000 (ab13919, Abcam, USA)

β-actin: mouse monoclonal, used at 1:2000 (ab8229, Abcam, USA)

2° antibody: Goat anti-mouse IgG HRP-conjugated, used at 1:2000 (Bio-Rad, USA)

Precision Plus Protein WesternC Chemiluminescent Standards (Bio-Rad, USA), sizes in kDa: 10, 15, 20, 25, 37, 50, 75, 100, 150, 250

Streptactin-HRP Conjugate for chemiluminescent detection (Bio-Rad, USA)

Sample buffer

RIPA buffer (Thermo Scientific, USA); 1X Laemmli buffer (Bio-Rad, USA)

Running buffer

Pre-mixed 10X Tris/glycine/SDS buffer (Bio-Rad, USA)

1X PBS buffer

10 PBS tablets per 1 L of milliQ water

10X TBS

24.2g Tris-HCl (Sigma, USA) and 80g NaCl in 1L of milliQ water, mixed until dissolved with a flea. pH adjusted to 7.6 with HCl/NaOH

TBST

0.05% Tween-20 (Sigma, USA) in 1X PBS

TBSTM

5% skimmed milk in TBST

Gel

Mini-PROTEAN TGX stain-free gel (Bio-Rad, USA)

Immunohistochemistry

Xylene (Sigma, USA); Absolute Ethanol (Sigma, USA)

Target Retrieval Solution Tris-EDTA pH 9 (DAKO, Denmark)

1X TBS

1X TBST

Pap pen (DAKO, Denmark)

1° antibodies: *HBEGF*: mouse monoclonal, used at 1:100 (ab66792, Abcam, USA)

EGR1: mouse monoclonal, used at 1:100 (ab55160, Abcam, USA)

AKT3: mouse monoclonal, used at 1:100 (ab13919, Abcam, USA)

Isotype controls: IgG_1: mouse monoclonal, used at 1:100 (ab81032, Abcam, USA)

IgG_{2A}: mouse monoclonal, used at 1:100 (MAB003, R&D Systems, USA)

Peroxidazed Solution 1 (Biocare Medical, USA)

Background Sniper solution (Biocare Medical, USA)

MACH3 mouse probe and MACH3 mouse HRP polymer (Biocare Medical, USA)

Betazoid DAB Chromogen (Biocare Medical, USA)

Haematoxylin (Sigma, USA)

Depex mounting agent (Sigma, USA)

2.2 METHODS

2.2.1 Cell culture

Five CRC cell lines, SW48, CaCo2, SNU-C1, SW948, COLO-320DM, were purchased from ATCC American Type Culture Collection (ATCC, USA). Another CRC cell line, LIM1215 was a kind gift from the Ludwig Institute, Melbourne, Australia. These cell lines were chosen due to their *KRAS* exon 2 and *BRAF* wild type status. The cell lines was cultured in 75 mL tissue culture flasks (Greiner Bio-One, Germany) in DMEM media supplemented with 10% fetal bovine serum, 100 U/mL Penicillin, 100 µg/mL Streptomycin, and 1% GlutaMax (all from Life Technologies, USA). The cells were cultured according to the protocol provided by ATCC and were found to be free of contamination throughout the experimental procedure.

2.2.2 Proliferation assay to determine resistance or sensitivity to anti-*EGFR*

The CellTiter 96® AQueous Assay kit (Promega, USA) kit was used to assess the resistance/sensitivity of these colorectal cancer cell lines to anti-*EGFR* sc-120 antibody (Santa Cruz Biotechnology, USA). The cells were seeded into 96-well plates (Greiner Bio-one, Germany) at a concentration of 5 x 10^3 cells/mL in total volume of 100 µL media and incubated for 24 hours in a 5% CO_2 humidified atmosphere at 37°C. Following this, 2 µg of anti-*EGFR* antibody was administered to the cells. 2 µg was determined as the saturation point for the antibody using flow cytometry previously in the laboratory. Another set of cells on the plate was treated with 2 µg of monoclonal mouse IgG_{2A} isotype control antibody (R&D Systems, USA) as a negative control designed to measure the level of non-specific background signal. The cells were plated in triplicate. The plate was incubated for 72 hours in the 5% CO_2 37°C incubator as per

manufacturer's protocol (Promega Cell Titer Proliferation Assay, USA). Following the 72 hours incubation, the cells were treated with 20 µL of the MTS/PMS solution and incubated for a further 1.5 hours. The absorbance was then read and analysed using the Fluostar OPTIMA instrument (BMG Labtech, USA) at a wavelength of 490 nm.

2.2.3 RNA extraction, purification, integrity testing, estimation of concentration

RNA was extracted and purified from all 6 colorectal cancer cell lines using the PureLink RNA Mini Kit (Life Technologies, USA). Cells were disrupted by adding 350 µL Lysis buffer with 2-mercaptoethanol (Sigma, USA) and the mixture was pipetted up and down. The lysate was homogenised by passing it through a blunt 21-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe at least 5 times.

One volume of 70 % ethanol was added to the homogenised lysate and it was mixed well by pipetting. Up to 700 µL of the sample was transferred, including any precipitate that may have formed, into the spin cartridge. The lid was closed gently and centrifuged for 15 seconds at 12000 g. The flow-through was discarded and the collection tube was reused for the next step. 700 µL of Wash Buffer 1 was added to the spin cartridge. The lid was closed gently and centrifuged for 15 seconds at 12000 g to wash the spin column membrane. The flow-through and collection tube were discarded. The spin cartridge was placed into a fresh collection tube. 500 µL of Wash Buffer II with ethanol was added to the spin cartridge. The lid was closed gently and centrifuged for 15 seconds at 12000 g. The flow-through was discarded. The collection tube was reused for the next step. 500 µL of Wash Buffer II with ethanol was added again to the spin cartridge. The lid was closed gently and centrifuged for 15 seconds at 12000 g.

The spin cartridge was placed in a new 2 mL collection tube and the old collection tube was discarded with the flow-through. The lid was gently closed and centrifuged at full speed for 1 minute. This step was performed to eliminate any possible carryover of Wash Buffer II with ethanol, or if residual flow-through remains on the outside of the spin cartridge after the last step. The spin cartridge was placed in a new 1.5 mL collection tube. 50 µL of RNase-free water was added directly to the spin column membrane and incubated at room temperature for 1 minute. The lid was closed gently and centrifuged for 2 minutes at 12000 g to elute the RNA.

RNA concentration was quantified by using the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). RNA integrity was determined using the Agilent 2100 Bioanalyser (Agilent Technologies, USA).

2.2.4 Reverse transcription

500 ng RNA was reverse-transcribed using Superscript III First Strand cDNA Synthesis kit (Invitrogen, USA) as per manufacturer's protocol. Briefly, the reagents in the kit were thawed and centrifuged for 15 seconds to bring the contents to the bottom of the tubes. 500 ng RNA was added to 1 µL of 50 µM oligo (dT) primer, 1 µL 20mM dNTP mix and RNase-free water to top up to total volume of 10 µL. The mix was then pipetted up and down gently and centrifuged briefly. This mix was incubated for 5 minutes at 65°C and immediately placed on ice for at least 1 minute. Meanwhile the cDNA synthesis mix for one reaction was prepared with 2 µL 10X RT buffer, 4 µL 25 mM $MgCl_2$, 2 µl 0.1 M DTT, 1 µL RNase-OUT (40 U/µL), and 1 µL Superscript III RT (200 U/µL) for a total volume of 10 µL. 10 µL cDNA synthesis mix was added to each tube containing 10 µL RNA mixture and mixed gently. The tubes were then briefly

centrifuged to collect the mixture. The tubes were incubated at 50°C for exactly 50 minutes. The reactions were stopped immediately by incubating at 85°C for 5 minutes and the tubes were chilled on ice. The tubes were briefly centrifuged to collect the mixture. 1 µL RNase H was added to each tube and incubated further at 37°C for 20 minutes. Reactions were placed on ice and proceeded with the real-time PCR protocol.

2.2.5 Real-time PCR: Gene expression analysis

The resulting cDNA was used in the RT Profiler Array Human *EGF/PDGF* pathway kit. Each array plate contains a panel of 84 pathway-focused genes. The RT^2 SYBR Green Mastermix was briefly centrifuged for 10-15 seconds to bring the contents to the bottom of the tube. As the RT^2 SYBR Green Master mix contains HotStart DNA *Taq* Polymerase that is active only after heat activation, reactions were prepared at room temperature (15-25°C). The PCR components mix were prepared in a 5 mL tube: 1350 µL of 2x RT^2 SYBR Green Master mix, 102 µL of cDNA synthesis reaction, 1248 µL of RNase-free water to make total volume of 2700 µL for one reaction. The PCR components mix were dispensed into the RT^2 Profiler PCR Array according to the format formulated for 96 well plates. Pipet tips were changed following each pipetting step to avoid cross-contamination between the wells. The RT^2 Profiler PCR Array plate was carefully removed from its sealed bag. 25 µL PCR components mix was added to each well of the RT^2 Profiler PCR Array using an electronic pipettor.

The RT^2 Profiler PCR Array was tightly sealed with optical thin-wall 8-cap strips. The plate was centrifuged for 1 minute at 1000 g at room temperature (15-25°C) to remove bubbles. The plate was visually inspected from underneath to ensure no bubbles were present in the wells. The RT^2 Profiler PCR Array was placed on ice while the PCR

cycling program was set up. The real-time cycler (Biorad CFX96) was programmed according to these conditions:

95°C for 10 minutes
95°C for 15 seconds
60°C for 1 minute
The cycle was repeated for 39 more times

Data was collected at the end of the run and results were analysed.

2.2.6 Validation of biomarkers

Biomarkers were validated using Taqman gene expression assays. Each cDNA sample was run in triplicate. Briefly, for one reaction, 7.5 µL 2X Gene Expression Mastermix, SSOFast probes supermix (Biorad, USA), 0.75 µL Taqman primer conjugated with FAM dye for *HBEGF*, *AKT3* and *EGR1* and 4.75 µL ultra-pure water were mixed in an eppendorf tube in a clean pressure-controlled RNA-DNA PCR room. 13 µL of this mixture was pipetted into each PCR tube using an electronic multiple dispensing pipette, 2 µL of cDNA sample was added to each PCR tube and briefly spun down. PCR was run on the Biorad CFX 96 machine, programmed as follows:

95°C for 30 seconds
95°C for 5 seconds
60°C for 10 seconds
The cycle was repeated for 44 more times

Reference gene used was *HPRT1*, as it has been validated as the best reference gene for colorectal cancer gene expression studies. The results for all of the colorectal cancer cell lines were normalised against *HPRT1* and plotted in scatter plot form using GraphPad Prism 6 program.

2.2.7 Silencing of candidate biomarkers in *EGFR*-resistant cell lines using siRNA

Each experiment included the following samples in triplicate:

a) Untreated cells

b) Positive control siRNA (targetting the reporter gene)

c) Negative control siRNA (non-targetting)

The following steps were performed in a laminar flow cell culture hood using sterile techniques. The colorectal cancer cells were diluted in antibiotic-free complete medium to achieve 1 x 10^5 cells in 100 µL of solution. 100 µL of cells were plated into each well of a 24-well plate. The cells were incubated at 37°C with 5% CO_2 overnight.

For siRNA resuspension, 5X siRNA buffer was diluted to 1X siRNA buffer by mixing four volumes of sterile RNase-free water with one volume of 5X siRNA buffer. The solution was pipetted 3-5 times, avoiding the introduction of bubbles. The solution was then placed on an orbital mixer/shaker for 30 minutes at room temperature. siRNA is then resuspended to a convenient stock concentration. The lyophilised siRNA concentration provided by the manufacturer was 5 nmol. This was resuspended with 500 µL of 1X siRNA buffer for a final concentration of 10 µM stock solution.

siRNA targeting *HBEGF*, *EGR1* and *AKT3* were purchased from Dharmacon (Thermo Fisher Scientific, USA). Cells were transfected with siRNA and DharmaFECT transfection reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, cells were seeded in a 24-well-plate at a density of 1×10^5 cells/well and incubated for 24 hours before the transfection. The siRNA complexes were mixed with the DharmaFECT transfection reagent in serum-free DMEM medium and were incubated at room temperature for 20 minutes to form a complex. The transfection mixtures were added to each well and topped up with DMEM medium containing 10% FBS at a final concentration of 10 nM *HBEGF*, 10 nM *EGR1* siRNA and 50 nM *AKT3* siRNA. A set of cells were treated with just DharmaFECT transfection reagent and medium as the "mock transfection" group, another set of cells were treated with non-targeting siRNA complex as "negative control" and the last set of cells were seeded in just complete DMEM medium as "untreated" group. Cells were all plated in triplicate and repeated at least three times. Seventy-two hours after the transfection, cells were collected for RNA isolation, and ninety-six hours after the transfection, cells were collected for protein isolation.

2.2.8 Proliferation assay after siRNA administration

The CellTiter 96® AQueous Assay kit (Promega, USA) kit was used to assess the resistance/sensitivity of these siRNA-treated cells to anti-*EGFR* antibody (Santa Cruz Biotechnology, USA). The cells (siRNA-treated, non-targeting-siRNA-treated cells, mock transfected cells and untreated cells and blank cells with just medium) were seeded in 6 wells each into 96-well plates (Greiner Bio-one, Germany) at 5×10^3 cells/mL in total volume of 100 µL media and incubated for 24 hours in a 5% CO_2 humidified atmosphere at 37°C. Following this, 2 µg of anti-*EGFR* antibody was

administered to the cells. Another set of siRNA-treated cells on the plate was treated with 2 µg of monoclonal mouse IgG_{2A} isotype control antibody (R&D Systems, USA) to measure the level of non-specific background signal. The plate was incubated for 24 hours in the CO_2 37°C incubator as per protocol (Promega Cell Titer Proliferation Assay, USA). Following incubation, the cells and blank wells were treated with 20 µL of the MTS/PMS solution and incubated for a further 1.5 hours. The absorbance was read at a wavelength of 490nm and analysed using the Fluostar OPTIMA instrument (BMG Labtech, USA).

2.2.9 Protein extracts

Cells were harvested in RIPA lysis buffer [25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS]. Cells were scraped and collected into microfuge tubes, cleared by centrifugation (10 minutes at 12000 g), and the supernatant was retained in a fresh eppendorf tube. The protein concentration of the cell extracts was determined using EZQ Assay (Life Technologies, USA).

2.2.10 Protein collection and estimation of concentration using EZQ assay

Protein from the cells was collected and its concentration was determined using the EZQ Assay. The protocol is as follows:

Protein standards preparation

a) The ovalbumin was used to make protein standards for the assay. 200 µL of buffer was added to one vial containing 2 mg of ovalbumin and mixed well to make a 10 mg/mL stock solution. The buffer used was the same as that was used for the experimental samples. The aliquots of the stock solution were dispensed into

microcentrifuge tubes and stored at ≤-20°C for future use. The buffer used was 1D buffer. Briefly, to make 1X buffer mix, 1.25 mL 1M Tris-HCl pH 6.8, 4 mL 10% sodium dodecyl sulphate (SDS), 2 mL glycerol and 7.2 g urea. It was made up to 20 mL in ultra-pure water. For every 10 mL of 1X buffer, 1 protease inhibitor tablet (Roche, USA) was dissolved in it. The buffer was aliquoted in 200 µL and stored in -20°C. Prior to use, 0.45 µL Benzonase (at 330 units/µL) was added per mL of buffer and incubated for 10 minutes at room temperature. DTT was added to 1% just before electrophoresis.

b) The standards were prepared by making serial dilutions of the 10 mg/mL ovalbumin stock solution. The dilution buffer used was the same as that was used for the experimental samples. At least 5 concentrations were used to cover the range expected for the experimental samples. The full effective protein concentration range for this assay is ~0.02 – 5 mg/mL. The highest concentration used was 5 mg/mL and it was serially diluted to get a range with the lowest concentration of 0.07 mg/mL. Volumes of 1 µL were used in this assay.

EZQ 96-well Microplate Cassette was prepared:

a) The microplate was placed face down on a clean surface. Wearing gloves, a sheet of assay paper was placed over the microplate and the paper was aligned with the inner tabs of the top, bottom, and left sides of the plate. One corner of the paper was snipped to identify the orientation.

b) The stainless steel backing plate was inserted into the microplate whilst holding the backing plate so that the flexible bar is at the top. The bottom tabs of the backing plate were placed along the bottom, inner edge of the microplate. Gentle pressure was applied to the flexible bar and the top edge of the plate was guided into position.

The top tabs were fitted into the top, inner edge of the microplate. The pressure on the flexible bar was released and the assay paper was checked to ensure it is securely in place.

Protein standards and samples were loaded:

a) 1 µL volume of each protein standard and each experimental sample was applied to separate wells of the microplate assembly. Protein standards and experimental samples were applied to the assay paper in triplicate. A 1 µL sample of the buffer alone was included to serve as a no-protein control. The samples were gently dispensed from the pipette tip onto the paper without touching the pipette tip to the paper.
b) The protein standards and experimental samples on the paper were allowed to completely dry.

The protein standards and samples were stained:

a) Wearing gloves, the assay paper was removed from the cassette by depressing the spring arm of the backing plate and the backing plate was tilted up and away from the assay paper. The protein-spotted assay paper was removed.
b) The protein spots were fixed and washed. About 40 mL of methanol was poured into a plastic staining tray. The plastic tray that was used was slightly larger than the assay paper. The protein-spotted assay paper was placed into the methanol and washed with gentle agitation for 5 minutes. This step removed contaminating substances including urea, SDS, reducing agents, salts and dyes that may be present.
c) After washing, the protein-spotted assay paper was dried.

d) 40 mL of the EZQ protein quantitation reagent was poured into a staining tray. The protein-spotted assay paper was placed into the stain solution and agitated gently on an orbital shaker for 30 minutes.

e) After staining, the assay paper was rinsed for 1 – 2 minutes in rinse buffer (10 % methanol and 7 % acetic acid). The wash was repeated twice, for a total of three rinses.

f) The assay paper was dried on a clean, flat surface and the protein spots were analysed using the Fluostar OPTIMA instrument (BMG Labtech, USA).

The results were read and analysed:

a) The dried paper was placed back into the microplate and the backing plate was secured. The stained protein spots were analysed in the Flour OPTIMA reader using excitation/emission settings of ~485/590 nm. The microplate reader was programmed to take multiple samplings or readings of each well for optimal results.

b) The fluorescence values of the experimental samples and standards were calculated by subtracting the fluorescence value of the no-protein control. A standard curve was created by plotting the corrected fluorescence values of the standards versus the corresponding protein concentration. The concentration of the experimental samples was determined from the standard curve.

2.2.11 Western blot and immunostaining

Cells extracts containing 30 µg of total protein prepared in Laemmli loading buffer (Bio-Rad, USA) were separated by 12% sodium dodecyl sulfate polycrylamide gel electrophoresis (SDS/PAGE) using the Mini-PROTEAN TGX stain-free gel (Bio-Rad, USA) and the resolved proteins were transferred electrophoretically to polyvinylidene

difluoride (PVDF) membranes (Bio-Rad, USA) and visualised and analysed by chemiluminescence using mouse monoclonal anti-*HBEGF* antibody (Bio-Rad, USA), mouse monoclonal anti-*EGR1* antibody (Abcam, USA), mouse monoclonal anti-*AKT3* antibody (Abcam, USA) all at 1:1000 dilution, mouse monoclonal anti-actin antibody (Sigma, USA) and Goat anti-mouse horseradish peroxidase secondary antibody (Bio-Rad, USA) at 1:2000 dilution. Equal protein loading was confirmed by the Gel Doc system (Bio-Rad, USA). Chemiluminescent protein standards (Bio-Rad, USA) were used to identify molecular weights of proteins. After blocking with tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST) and 5% skimmed milk (TBSTM) for 1 hour on the shaker at room temperature, the membranes were incubated with the antibodies in TBSTM overnight on the shaker at 4°C.

The next day, after the membranes were washed on the shaker 3 times for 10 minutes in a small tray containing TBSTM, they were incubated for an hour on the shaker at room temperature with secondary goat anti-mouse horse radish peroxidase (HRP) antibody at 1:2000 for the primary antibodies and with streptactin HRP-conjugate antibody at 1:10000 for the chemiluminescent protein standards. After the incubations, the membranes were removed and washed on the shaker 5 times for 10 minutes in a small tray of TBST at room temperature.

Actin antibody was added at 1:2000 to no less than 3 mL of TBSTM in a 50 mL falcon tube. Using forceps, the membrane was carefully added to the falcon tube, with no air bubbles between the back of the membrane and the tube. The membrane was incubated on the roller for 1 hour at room temperature. After incubation, membrane was removed from falcon tube and washed 3 times for 10 minutes in a small tray of TBSTM on the

shaker at room temperature. Secondary goat anti-mouse horse radish peroxidase (HRP) antibody at 1:2000 was added to no less than 3ml of TBSTM in a 50ml falcon tube. Using forceps, the membrane was carefully added to the falcon tube, as before. The membrane was incubated on the roller for 1 hour at room temperature. After incubation, membrane was removed from falcon tube and washed 5 times for 10 minutes in a small tray of TBST on the shaker at room temperature.

Chemiluminescent detection was performed in accordance with the manufacturer's instructions (Bio-Rad, USA). The ECL solution was prepared using the Clarity Western ECL Substrate (Bio-Rad, USA) by mixing the substrate components in a 1:1 ratio in a clean tray. The *HBEGF*/*EGR1*/*AKT3* signals were analysed using the ImageQuant LAS 4000 equipment (GE Healthcare Life Sciences, USA) and quantified using ImagePro Plus software version 4.0 and normalised to that of actin. Blots were performed in triplicate.

2.2.12 Immunohistochemistry

The slides with tumour sections (5 µm) were placed on the hot plate at 60°C for 2 hours. The slides were then removed and deparaffinised in xylene (3 changes) and rehydrated through graded alcohol for 5 minutes in each solution:

100% ethanol 1

100% ethanol 2

90% ethanol

70% ethanol

MilliQ water

MilliQ water

Antigen/epitope retrieval was performed by microwave-heating sections in Target Retrieval Solution Tris-EDTA buffer pH 9 (DAKO, Denmark) for 20 minutes. The slides were left to cool in room temperature for 20 minutes and transferred to TBS solution to wash off the retrieval solution. Slides were placed in a humidified chamber for the staining steps. The tissues were circled with a pap pen (DAKO, Denmark) to minimise reagent volumes required for staining. Following blocking of nonspecific binding using the Peroxidazed 1 solution (Biocare Medical, USA) and Background Sniper solution (Biocare Medical, USA), sections were washed with TBST to remove blocker and incubated with either mouse anti-*HBEGF* (Abcam, USA) at a dilution of 1:100, mouse anti-*EGR1* (Abcam, USA) at a dilution of 1:100 or mouse anti-*AKT3* (Abcam, USA) at a dilution of 1:100 at room temperature for 30 minutes. MACH3 mouse probe and MACH3 mouse HRP polymer (Biocare Medical, USA) were added to the slides and incubated for 10 minutes each. After further washing with TBS, sections were incubated with Betazoid DAB chromogen (Biocare Medical, USA) for 5 minutes at room temperature. Appropriate isotype controls IgG_1 and IgG_{2A} were used as negative controls. The sections were then counterstained with freshly prepared and sterile-filtered haematoxylin (at 1:4 dilution) for 8 seconds before dehydration and mounted with Depex. Dehydration steps were as follows:

100% ethanol for 3 dips

Xylene 1 for 5 minutes

Xylene 2 for 5 minutes

Xylene 3 for 5 minutes

As previously described (Loupakis, Pollina et al. 2009), intensity was scored according to a four-tier system: 0, no staining; 1, weak; 2, moderate; and 3, strong. One, two or three additional points were attributed if the percentage of positive was <25%, 25–50% or >50%, respectively. The specimens with a cumulative score of ≥4 were characterised as positive (Loupakis, Pollina et al. 2009). The stained slides were scored by a pathologist and compared with its respective matched normal sections.

2.2.13 Statistical methods

All statistical analyses was done using GraphPad Prism 6 program. Statistical method used for the proliferation assay, RT Profiler Arrays and Taqman gene expression assays was Student's *t*-test, where $p < 0.05$ was deemed significant. Statistical method used for comparison of qRT-PCR expression levels in the siRNA studies was ANOVA.

CHAPTER 3: *KRAS G13D* MUTATION AND SENSITIVITY TO CETUXIMAB OR PANITUMUMAB IN A COLORECTAL CANCER CELL LINE MODEL

Additional information

Currently, *KRAS* is the only established predictive marker for mCRC patients receiving *EGFR*-targeted therapies, such as cetuximab and panitumumab. However, studies have demonstrated that a large proportion of *KRAS* WT patients do not respond to anti-*EGFR* therapy. In order to improve the clinical outcome, it is imperative to understand the response of different types of *KRAS* mutated tumours to the anti-*EGFR* therapies, which will allow a more personalised approach to patient treatment. The response to these anti-*EGFR*-targeted therapies has not previously been explored pre-clinically. The studies described herein aimed to investigate the effect of anti-*EGFR* monoclonal antibody therapies on cell lines that harbor *KRAS G12V* mutation, *KRAS G13D* mutation and cell line with WT *KRAS*. The specific aims of the study were to determine the sensitivity and/or resistance of CRC cell lines with *KRAS G13D* mutation compared to CRC cell lines with *KRAS G12V* mutation and *KRAS* WT to cetuximab and panitumumab treatment. Additionally, these studies aimed to investigate the correlation of the *KRAS* mutational status of the CRC cell lines to the responsiveness to cetuximab and panitumumab. This published paper appears as "*KRAS G13D* Mutation and Sensitivity to Cetuximab or Panitumumab in a Colorectal Cancer Cell Line Model" in Gastrointestinal Cancer Research 2014, 7:23-26.

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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

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Contribution to the Paper	Performed all experiments, analysed data, interpreted data and wrote the manuscript.		
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Contribution to the Paper	Manuscript evaluation.		
Signature		Date	24/11/2014

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Contribution to the Paper	Principal supervisor, data interpretation, manuscript editing and acted as corresponding author.		
Signature		Date	24/11/2014

KRAS G13D Mutation and Sensitivity to Cetuximab or Panitumumab in a Colorectal Cancer Cell Line Model

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ABSTRACT

BACKGROUND: The treatment of metastatic colorectal cancer (mCRC) includes drugs targeting the epidermal growth factor receptor (EGFR). Mutation in codon 12 or 13 in the Kirsten rat sarcoma viral oncogene homolog (KRAS) gene, downstream of the EGFR, evokes constitutive activation of the RAS/RAF/MAPK signaling pathway and correlates with resistance to anti-EGFR monoclonal antibody (mAb) therapies. However, a retrospective study reported that a proportion of patients with the KRAS G13D mutation may respond to cetuximab. A similar analysis for panitumumab was not as conclusive. We sought to determine the sensitivity of CRC cell lines to cetuximab or panitumumab treatment and to investigate the correlation of the KRAS mutational status of the CRC cell lines to the responsiveness to cetuximab or panitumumab.

METHODS: To determine the responsiveness of CRC cell lines to cetuximab or panitumumab, cell lines were treated with an optimized concentration of each mAb, and proliferation assays were conducted.

RESULTS: After treatment with cetuximab or panitumumab, at the optimum concentration of 8 μg/well, the KRAS G13D mutant cell lines HCT-116, LoVo, and T84 showed intermediate sensitivity to both treatments, between the resistant KRAS G12V mutant cell line SW480 and the sensitive KRAS wild-type cell line LIM1215. One of the G13D cell lines was significantly more sensitive to panitumumab than to cetuximab ($P = .02$).

CONCLUSION: The specific KRAS mutation determines the responsiveness to anti-EGFR monoclonal antibody treatment, corresponding to reported clinical observations.

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The treatment of metastatic colorectal cancer (mCRC) has improved over recent years, with targeted therapies providing additional benefit to standard chemotherapy. The two most important targets for mCRC treatment are epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF). Mutation of the KRAS gene is now known to be predictive of nonresponse to EGFR-targeted mAb therapy, either as monotherapy or in combination with irinotecan- or oxaliplatin-based chemotherapy.[1,2]

Cetuximab (a chimeric human-murine IgG1 mAb) and panitumumab (a fully humanized IgG2 mAb) target the EGFR and act by binding to the EGFR on tumor cells, blocking the downstream intracellular signaling pathways. A member of this downstream cascade is KRAS, and evidence has suggested that patients with KRAS mutations do not benefit from the addition of cetuximab or panitumumab, either alone or in addition to standard chemotherapy.[2,3] Mutation of KRAS results in constitutive downstream activation of the EGFR pathway, propagating further signaling events and making the EGFR inhibitors ineffective. A retrospective analysis of early trials of cetuximab therapy suggested that KRAS exon 2 mutation occurs in 27–43% of patients with mCRC tumors, and the reported objective response rate (ORR) was 0 in this group.[4] These reports also established superior ORRs with EGFR inhibitors in wild-type (WT) KRAS tumors.[5,6] Subsequent analysis of large randomized trials involving both cetuximab and panitumumab have confirmed the predictive nature of the KRAS mutation.[4] As a consequence, KRAS testing has been made mandatory for patients with mCRC before treatment with

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cetuximab or panitumumab.[7] However, there is growing evidence of the existence of an array of mutations that in turn influence the responsiveness to an anti-EGFR treatment, and their roles are not fully understood.[4] Overall KRAS mutations, if they include exons 3 and 4 in addition to 2, are likely to be found in approximately 45–55% of all colorectal cancer specimens.[8]

In a retrospective study by De Roock et al,[9] it was evident that a proportion of patients with KRAS G13D mutation do respond to cetuximab. The largest retrospective analysis, conducted by Peeters et al,[10] to evaluate three phase III trial studies involving the alternate anti-EGFR drug panitumumab (first line, second line, and monotherapy) revealed that KRAS G13D was unfavorably associated with panitumumab treatment effects on overall survival (OS) but not on progression-free survival (PFS) or response rate. These discrepant results may reflect subtle differences between the two antibodies to EGFR—for example, chimeric vs. fully humanized. There is also a report of activity of panitumumab after cetuximab failure, adding further evidence to potential differences in activity.[11]

Based on these retrospective studies and the conflicting results, we sought to explore, in a preclinical CRC cell line model, the sensitivity and/or resistance to both cetuximab and panitumumab treatment and to investigate the correlation of the KRAS mutational status of the CRC lines to the responsiveness to these agents.

MATERIALS AND METHODS

Cell Lines and Reagents

CRC lines, HCT-116, T84, LoVo (all KRAS G13D mutant), and SW480 (KRAS G12V mutant) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). LIM1215 CRC line (KRAS WT) was a kind gift from the Ludwig Institute (Melbourne, Australia). The cell lines were cultured in 75-mL tissue culture flasks (Greiner Bio-One, Frickenhausen, Germany) in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 200 μg/mL glutamine (all from Gibco-Life Technologies, Grand Island, NY, USA) at 37°C and 5% CO_2, according to the protocol provided by ATCC. The cell lines were tested with the MycoAlert mycoplasma detection kit (Lonza, Sydney, NSW, Australia) and were found to be free of mycoplasma contamination throughout the experimental procedure.

Monoclonal Antibodies

Cetuximab (Erbitux; Merck Serono, Frenchs Forest, NSW, Australia) at 5 mg/mL and panitumumab (Vectibix; Amgen, Thousand Oaks, CA, USA) at 20 mg/mL were generously provided by the Medical Oncology Unit Pharmacy of The Queen Elizabeth Hospital, Adelaide, Australia.

Proliferation Assay and Optimization of Antibody Concentrations

The CellTiter 96® AQueous nonradioactive cell proliferation assay kit (Promega, Madison, WI, USA) was used to assess the resistance and sensitivity of these CRC lines to the monoclonal antibodies. The cells were seeded into 96-well plates (Greiner Bio-one) at 5×10^3 cells/mL in a total volume of 100 μL medium and incubated for 24 hours in a 5% CO_2 humidified atmosphere at 37°C. The cells were treated in triplicate with cetuximab or panitumumab at concentrations of 0.5–10 μg per well, to determine the optimal concentrations for the proliferation assay. Another set of cells on the plate was treated in triplicate with 10 μg of monoclonal mouse IgG1 or IgG_{2A} isotype control antibody (R&D Systems, Minneapolis, MN, USA) as a negative control. The plates were incubated for 72 hours at 37°C in a 5% CO_2 incubator, and all wells were then treated with 20 μL of MTS/PMS solution and incubated for a further 1.5 hours. The absorbance was read with the Fluostar Optima instrument (BMG Labtech, Durham, NC, USA) at a wavelength of 490 nm. Proliferation was recorded as a percentage of that obtained for the isotype control antibody-treated cells (100%).

Statistical Analysis

Statistical analyses were performed with Students *t*-test in the Prism 6 program (GraphPad Software, La Jolla, CA, USA) with $P < .05$ deemed significant.

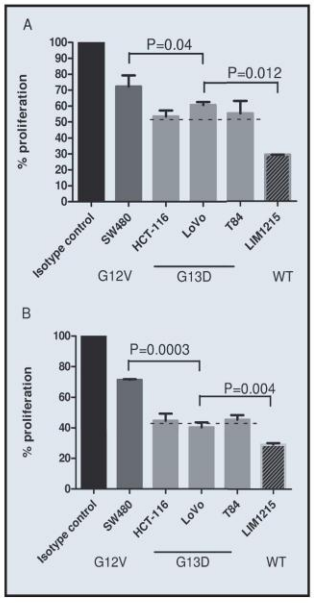

Figure 1. Response of KRAS G12V, G13D and WT cell lines to (A) 8 μg cetuximab treatment and (B) 8 μg panitumumab treatment. Dashed line: mean proliferation, as a percentage of isotype control antibody treatment for the G13D cell lines.

RESULTS

Optimization of Antibody Concentrations

The optimal treatment concentration to provide the maximum inhibition of proliferation for all the cell lines for cetuximab and panitumumab was determined to be 8 μg/well (Supplementary Figures 1–10).

Correlation Between KRAS Status and Responsiveness to Cetuximab or Panitumumab Treatment

After cetuximab treatment (Figure 1A), the G13D cell lines, with a mean proliferation rate of 53.5% relative to the isotype control, were significantly more sensitive than SW480 (KRAS G12V mutation) with a proliferation rate of 72.3% ($P = .04$), but were less sensitive than LIM1215 (KRAS WT) (proliferation 29.5%; $P = .012$). After panitumumab treatment (Figure 1B), the G13D cell lines (mean proliferation, 44.7%) again showed intermediate sensitivity between SW480 (proliferation, 71.4%; $P = .0003$) and LIM1215 (proliferation, 29%; $P = .004$). Comparing the efficacy of cetuximab

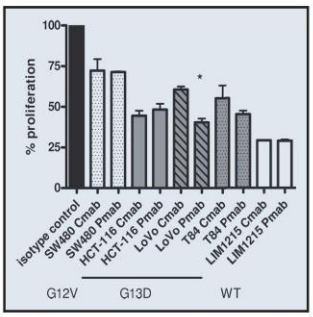

Figure 2. Percentage proliferation relative to isotype control antibody, comparing cetuximab to panitumumab for each of the cell lines. *Significance at $P = .02$.

vs. panitumumab within each of the 5 cell-lines showed that only LoVo (G13D) had a significant difference in response, being more sensitive to panitumumab ($P = .02$) (Figure 2).

DISCUSSION

Anti-EGFR mAbs are effective at prolonging disease-free survival and OS and in improving response rates in patients with KRAS WT mCRC.[1,12] Mutation in KRAS exon 2, which leads to EGFR-independent, constitutive activation of RAS, was shown to predict for resistance to both anti-EGFR agents (cetuximab and panitumumab), and subsequently this was confirmed in the retrospective KRAS analysis of the two pivotal studies of anti-EGFR therapy.[2,3]

In our *in vitro* study, the SW480 CRC line with the KRAS G12V mutation had the highest proliferation rate, showing resistance to both the anti-EGFR mAbs cetuximab and panitumumab, when compared with other cell lines with the KRAS G13D mutation and KRAS WT cells. The 3 cell lines with KRAS G13D (HCT-116, LoVo, and T84), showed an intermediate level of responsiveness to cetuximab and panitumumab, with the proliferation rate decreasing as the treatment concentration increased, indicating a dose–response activity. However, the responsiveness was not as great as LIM1215, a KRAS WT cell line, which had the lowest proliferation rate when treated with cetuximab and panitumumab. The KRAS WT cell line showed the highest response rate to both cetuximab and panitumumab, as expected.

Both antibodies are likely to have a similar activity *in vitro*, although there are differences in their action in vivo, probably because they are different immunoglobulin isotypes. Panitumumab, like other IgG2 antibodies, was thought not to mediate antibody-dependent cellular cytotoxicity (ADCC), a recognized action of IgG1 antibodies, including cetuximab,[13] although a recent report has demonstrated that panitumumab may in fact induce ADCC via myeloid effector cells *in vitro*, a mechanism that may contribute to its efficacy in vivo.[14] Further, a report of panitumumab activity after cetuximab failure again suggested some differences in the mechanism of action.[15]

The possibility of a difference between these drugs has been further highlighted by the differential response in the G13D mutant subgroup in retrospective analyses: De Roock et al[9] reported that patients ($n = 571$) receiving any cetuximab-based treatment regardless of concomitant chemotherapy, showed a statistically significant longer OS and PFS in those with KRAS G13D mutations than did patients with any other KRAS mutation (OS, 7.6 months, 95% CI, 5.7–20.5, and PFS, 4.0 months, 95% CI, 1.9–6.2 vs. OS, 5.7 months, 95% CI, 4.9–6.8, and PFS, 1.9 months, 95% CI, 1.8–2.8).[9] These findings were supported in a recent systematic review and meta-analysis of 10 studies of cetuximab treatment in patients with mCRC. Those with KRAS G13D tumors had a significantly higher ORR and longer PFS and OS than did patients with KRAS codon 12-mutated tumors, and lower ORR with shorter PFS and OS than patients with KRAS WT tumors.[16] These data thus support a better prognosis for patients with G13D mutations, in keeping with the results in our cell line analysis. However, clinical outcomes for panitumumab therapy appear to be less well defined: Peeters et al[10] concluded from their pooled analysis of randomized trials for 1053 KRAS mutant patients treated with panitumumab that although no mutant KRAS allele was consistently shown to be a significant predictive factor for PFS or OS, patients with mutant KRAS codon 12 or 13 mCRC tumors were unlikely to benefit from panitumumab therapy.[10] In contrast, our *in vitro* study suggests that G13D mutant cells show sensitivity to panitumumab that falls between the sensitive WT cells ($P = .004$) and the resistant G12V cells ($P = .0003$).

A look at the potential differences in codon 12 vs. 13 mutations shows that our overall results concur with those in an *in vitro* study done by Guerrero and colleagues in 2000.[17] They found that the malignant potential of tumor cells may be influenced, not only by the presence or absence of RAS mutations, but also by its molecular nature. KRAS codon 13 mutation was reported to have a reduced transforming capacity, as compared with KRAS codon 12 mutation in experimental systems. Their study suggested that tumors carrying KRAS codon 13 mutations are less aggressive than those with codon 12 mutations. Also, colorectal tumors carrying KRAS codon 12 mutations showed a lower apoptotic rate than did tumors lacking this mutation and conferred a more aggressive tumor phenotype by altering the threshold of apoptotic induction; in contrast KRAS codon 13 mutations reduced this threshold. Their results indicate that cells carrying KRAS codon 13 mutations or overexpressing the WT allele may have reduced survival and could be selected against in the adenoma-to-carcinoma transition.[17] This possibility would explain the higher proliferative capacity of the codon 12 G12V cell line in our study, which exhibited definite resistance to cetuximab and panitumumab when compared with the G13D cell lines, which showed a degree of sensitivity to both anti-EGFR drugs. It has recently been established that negative feedback involving EGFR mediates cross-talk between the RAS-MEK-ERK pathway and the parallel AKT pathway through phosphorylation of key signaling molecules and that this activation of AKT varies depending on specific mutations including in KRAS and BRAF. Furthermore, this study highlighted the need for combined inhibition of MEK and EGFR to inactivate both pathway end points.[18] This variability in the activation of AKT, depending on the particular KRAS mutation, explains the discrepant growth inhibition responses to EGFR inhibition in tumor cells with differing KRAS mutations in our study.

CONCLUSIONS

In our preclinical CRC cell line model, there were differences in response to anti-EGFR treatments, depending on the particular KRAS mutation. SW480 with KRAS G12V

mutation was resistant to both cetuximab and panitumumab, whereas all of the KRAS G13D-mutated cell lines showed intermediate sensitivity to both treatments. Of note, one of the G13D cell lines showed greater sensitivity to panitumumab than to cetuximab. Further prospective data are needed to clarify the role of the KRAS G13D mutation for anti-EGFR-based treatments in mCRC; an Australian-led international trial, the AGITG ICECREAM study (ACTRN12612000901808) is currently under way. This is a randomized phase II study of cetuximab alone or in combination with irinotecan in mCRC patients with KRAS WT and in patients harboring a G13D mutation. Results from such trials in the future should inform the selection of patients for anti-EGFR therapy and the need for additional targeted therapies, based on genotype, so that a more personalized approach to treatment can be realized.

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Disclosures of Potential Conflicts of Interest

Timothy Price has been an uncompensated member of advisory boards for Merck and Amgen. All other authors declare no potential conflicts of interest.

CHAPTER 4:

BIOMARKERS OF RESISTANCE TO ANTI-*EGFR* MONOCLONAL ANTIBODY TREATMENT IN WT *KRAS*/*BRAF* COLORECTAL CANCER CELL LINES

4.1 INTRODUCTION

CRC is the third most common cancer worldwide and the second leading cause of cancer-related death in the Western world (Roda and Cervantes 2010). CRC is the second most common cancer in both men and women in Australia with over 15,000 cases diagnosed in 2012, and with some 4,000 deaths, it is the second most common cause of cancer related deaths. More than half of the cases occur in men with 45.2% of cases reported in women (AIHW 2012). CRC is one of the most curable cancers if it is detected at an early stage highlighting the importance of screening, however a significant number still present with more advanced disease.

The treatment of mCRC has improved over the recent years, with targeted therapies providing additional benefit to standard chemotherapy. One important target for mCRC treatment is *EGFR*. *EGFR* and the signalling pathway are considered targets for mCRC. Tumour *EGFR* expression predicts aggressive disease and poor outcome in mCRC patients (Karameris, Kanavaros et al. 1993). *EGFR* expression by immunohistochemistry has not ultimately correlated well with the clinical benefit of *EGFR*-targeted treatment in some retrospective analyses (Chung, Shia et al. 2005). Nonetheless, many of the studies upon which the utility of *EGFR* inhibitors has been justified were restricted to *EGFR*-positive patients (Saltz, Meropol et al. 2004; Lenz, Van Cutsem et al. 2006). Classes of *EGFR* inhibitors include monoclonal antibodies and small-molecule tyrosine kinase inhibitors, of which the former group has so far proven more useful.

Mutation of the downstream *KRAS* gene is predictive of non-response to *EGFR*-targeted monoclonal antibody therapy, either as monotherapy or in combination with irinotecan- or oxiplatin-based chemotherapy (Bokemeyer *et al.* 2007) and this has been the most important predictor thus far. Very recent evidence has highlighted that

additional *RAS* mutations beyond *KRAS* exon 2 also predict non-response (Douillard, Oliner et al. 2013). *BRAF* mutations also appear to affect sensitivity to *EGFR* inhibitors at least when used as single agent therapy (Roth, Tejpar et al. 2010). Both these mutations have emerged to be major prognostic markers for poor survival in CRC, in addition to predicting response to *EGFR*-targeted therapy (Roth, Tejpar et al. 2010).

The rationale for use of the *EGFR* as a target antigen for specific anti-cancer therapies is based on its role in cancer cell growth through cellular proliferation, , angiogenesis, and inhibition of apoptosis (Mendelsohn 2002). *EGFR* is dysregulated in several malignant disorders including lung, breast, colorectal, head and neck, prostate, pancreatic and other cancers (Salomon, Brandt et al. 1995). Mechanisms involved in the activation of *EGFR* include receptor overexpression (Hirsch, Varella-Garcia et al. 2003), autocrine activation by overproduction of ligands (Hackel, Zwick et al. 1999), ligand independent activation through other receptor systems (Liu, Aguirre Ghiso et al. 2002) and mutant receptors resulting in ligand-independent activation.

Despite improving patient selection via *KRAS*/extended *RAS* testing, clinical trials continue to show that a proportion of mCRC patients with WT *KRAS*/WT *BRAF* status treated with anti-*EGFR* antibody (cetuximab) do not respond as the tumours may be inherently resistant or developed resistance during therapy. Thus, it is important to determine candidate biomarkers to predict response to these targeted antibody therapies. In this study, we have determined the sensitivity or resistance of colorectal cancer cell lines to anti-*EGFR* treatment, identified a panel of candidate biomarkers using pathway focused array plates, elucidated the effect of siRNA targeted against these biomarkers in proliferation of CRC cells and determined the expression of these markers in mCRC patients that have been administered with cetuximab monotherapy.

4.2 MATERIAL AND METHODS

4.2.1 Cell lines and reagents

SW48, CaCo2, SNU-C1, SW948, COLO-320DM and LIM1215 were cultured in DMEM media supplemented with 10% fetal bovine serum, 100 U/mL Penicillin, 100 µg/mL Streptomycin, and 1% GlutaMax (all from Life Technologies, USA), according to the protocol provided by ATCC.

4.2.2 Proliferation assay

The CellTiter 96® AQueous Assay kit (Promega, USA) kit was used to assess the resistance/sensitivity of these colorectal cancer cell lines to anti-*EGFR* antibody (Santa Cruz Biotechnology, USA). Briefly, the cells were seeded into 96-well plates (Greiner Bio-one, Germany) and 2µg of anti-*EGFR* antibody was administered to the cells. Following the 72 hours incubation of the plate in the CO_2 37°C incubator as per protocol (Promega Cell Titer Proliferation Assay, USA), the cells were treated with 20µL of the MTS/PMS solution and the absorbance was read at a wavelength of 490nm and analysed using the Fluostar OPTIMA instrument (BMG Labtech, USA).

4.2.3 Quantitative PCR

500 ng of extracted RNA from the cell lines was reverse-transcribed first using RT First Strand Kit (SA Biosciences, USA) as per manufacturer's protocol. Briefly, the reagents in the kit were mixed with the RNA (genomic DNA elimination mix) mixed well by pipetting. Reactions were placed in the PCR Thermal Cycler (Bio Rad, USA) and proceeded with the real-time PCR protocol.

4.2.4 Real-time PCR: Gene expression analysis

The resulting cDNA obtained from the cell lines was used in the RT Profiler Array Human *EGF/PDGF* pathway kit (QIAGEN, USA). Each array plate contains a panel of 84 pathway-focused genes. Briefly, the PCR components mix were dispensed into the RT^2 Profiler PCR Array according to the format formulated for 96 well plates. The RT^2 Profiler PCR Array was tightly sealed with optical thin-wall 8-cap strips and placed in the CFX96™ Real-Time PCR Detection System (Biorad, USA). Data was collected at the end of the run and results were analysed.

4.2.5 Validation of biomarkers

Biomarkers were validated using Taqman gene expression assays. Each cDNA sample was run in triplicate. The results for all of the CRC cell lines were normalised against *HPRT1* and plotted in scatter plot form using GraphPad Prism 6 program.

4.2.6 Protein extracts

Briefly cells were harvested in RIPA lysis buffer [25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS] and the protein concentration of the cell extracts was determined using EZQ Assay (Invitrogen, USA).

4.2.7 Protein concentration estimated using EZQ assay

Briefly, 1 µL volume of each protein standard and each experimental sample was applied in triplicate to separate wells of the microplate assembly. The protein-spotted assay paper was placed into the methanol and washed with gentle agitation for 5 minutes. Following that, 40 mL of the EZQ protein quantitation reagent was poured into a staining tray and agitated gently on an orbital shaker for 30 minutes. The assay paper

was rinsed for 1 – 2 minutes in rinse buffer (10 % methanol and 7 % acetic acid) and analysed using excitation/emission settings of ~485/590 nm using the Fluostar OPTIMA instrument (BMG Labtech, USA).

4.2.8 Validation of siRNA using qRT—PCR and Western blot

Briefly, siRNA targetting *HBEGF*, *EGR1* and *AKT3* were purchased from Dharmacon (Thermo Fisher Scientific, USA). Cells were transfected with siRNA and DharmaFECT transfection reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Cells were all plated in triplicate and repeated at least three times. Seventy-two hours after the transfection, cells were collected for RNA isolation, and ninety-six hours after the transfection, cells were collected for protein isolation.

4.2.9 Proliferation assay after siRNA administration

The CellTiter 96® AQueous Assay kit (Promega, USA) kit was used to assess the resistance/sensitivity of these siRNA-treated cells to anti-*EGFR* antibody (Santa Cruz Biotechnology, USA). The cells were seeded in 6 wells each into 96-well plates (Greiner Bio-one, Germany) and treated with 2 µg of anti-*EGFR* antibody. The following day, . the cells were treated with 20 µL of the MTS/PMS solution and the absorbance was read at a wavelength of 490nm and analysed using the Fluostar OPTIMA instrument (BMG Labtech, USA).

4.2.10 Western blot

Cells extracts containing 30 µg of total protein prepared in Laemmli loading buffer were separated by 12% sodium dodecyl sulfate polycrylamide gel electrophoresis (SDS/PAGE) using the Mini-PROTEAN TGX stain-free gel (Bio-Rad, USA) and the

resolved proteins were transferred electrophoretically to PVDF membranes (Bio-Rad, USA) and visualised and analysed by chemiluminescence. using mouse monoclonal anti-*HBEGF* antibody (Bio-Rad, USA), mouse monoclonal anti-*EGR1* antibody (Abcam, USA), mouse monoclonal anti-*AKT3* antibody (Abcam, USA) all at 1:1000 dilution, and mouse monoclonal anti-actin antibody (Sigma, USA) and Goat anti-mouse horseradish peroxidase secondary antibody (Bio-Rad, USA) were used at 1:2000 dilution. Blots were analysed using the ImageQuant LAS 4000 equipment (GE Healthcare Life Sciences, USA) and quantified using ImagePro Plus software version 4.0 and normalised to that of actin. Blots were performed at least three times.

4.2.11 Patient samples

This was a pilot study of a retrospective analysis aiming to explore the predictive value of biomarkers analysis in the outcome of patients with mCRC treated with cetuximab monotherapy. The expression for *HBEGF*, *EGR1* and *AKT3* were determined in 10 mCRC patients from 3 different trials: The NCIC Clinical Trials Group (NCIC CTG) and the Australasian Gastrointestinal Trials Group (AGITG) CO.17, NCIC CTG/AGITG CO.20 and Victoria Cancer Council's Dual Inhibition of *EGFR* Signalling Using the Combination of Cetuximab (Erbitux®) and Erlotinib (Tarceva®) in Patients With Chemotherapy-Refractory Colorectal Cancer (DUX). Patients from the CO.17 cohort were recruited before *KRAS* testing was routine, patients from CO.20 cohort were chosen for the *KRAS* wild type status and *KRAS* testing for patients from the DUX trial was performed outside the public hospital system and the results were unobtainable. The primary objective of all of these trials were to elucidate the effect of cetuximab monotherapy on survival among mCRC patients in whom all chemotherapy had failed and for whom no other standard anti-cancer therapy was available. None of

the patients had received any previous therapy with monoclonal antibodies directed against *EGFR*. Treatment was continued until death, occurrence of any adverse events or side effects, tumour progression, worsening symptoms of cancer or request for discontinuation by the patient. The markers' expression was matched with their respective matched normal sections. Patients' response to cetuximab was blinded until every slide were analysed and scored for biomarker expression values.

4.2.12 Immunohistochemistry

Briefly, antigen/epitope retrieval was performed by microwave-heating the sections (5µm) in Target Retrieval Solution Tris-EDTA buffer pH 9 (DAKO, Denmark) for 20 minutes and thereafter the slides were placed in a humidified chamber for the staining steps. Respective sections were incubated with mouse anti-*HBEGF* (Abcam, USA) at a dilution of 1:50, mouse anti-*EGR1* (Abcam, USA) at a dilution of 1:100 or mouse anti-*AKT3* (Abcam, USA) at a dilution of 1:100 at room temperature for 30 minutes. The sections were then counterstained with haematoxylin (at 1:10 dilution) for 8 seconds before dehydration and mounting. The intensity of the stains was scored as previously described by Loupakis *et al.* (Loupakis, Pollina et al. 2009).

4.2.13 Statistical methods

All statistical analyses was done using GraphPad Prism 6 program (GraphPad, USA). Statistical method used for the proliferation assay was student *t*-test, where $p<0.05$ was deemed significant. Statistical method used for RT Profiler Arrays was *t*-test. Statistical method used for the Taqman gene expression assays was student *t*-test.

4.3 RESULTS

4.3.1 Proliferation assay

SW48 has been reported to not harbour any known mutations that may cause resistance to anti-*EGFR* treatment such as *NRAS*, Harvey rat sarcoma viral oncogene homologue (*HRAS)* and *KRAS* exon 3 (https://cansar.icr.ac.uk/cansar/cell-lines/SW48/mutations/). SNU-C1 has been reported to harbour *HRAS*-like suppressor (*HRASLS)* and *HRASLS5* mutations (https://cansar.icr.ac.uk/cansar/cell-lines/SNU-C1/mutations/). COLO-320DM harbours no reported mutations (https://cansar.icr.ac.uk/cansar/cell-lines/COLO-320-DM/mutations/). After 72 hours incubation, SW48 and SNU-C1 and COLO-320DM cell lines were resistant to anti-*EGFR* treatment, with a mean of 70%, 83.8% and 68.3% cell proliferation respectively while LIM1215, CaCo2 and SW948 were sensitive to the treatment with 18.6%, 42% and 29.1% proliferation respectively. The cut-off point 50% used to determine resistance or sensitivity of the CRC cell lines to anti-*EGFR* treatment was derived from the study described by Jhawer *et al.* (Jhawer, Goel et al. 2008).

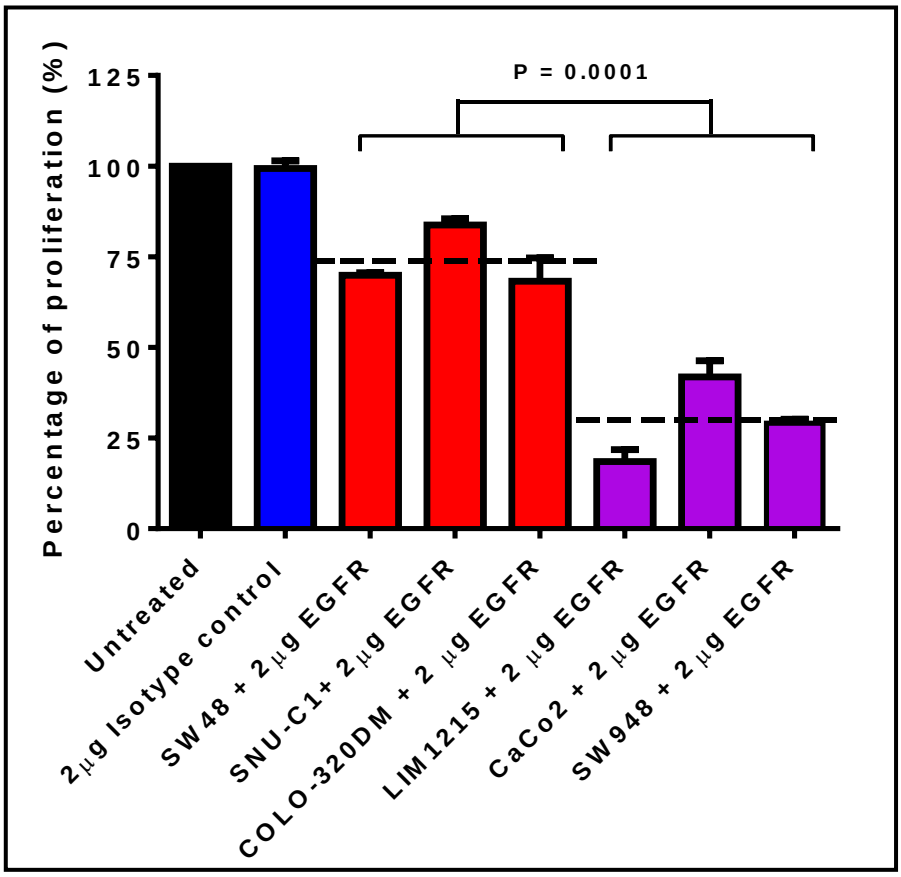

Figure 1: Proliferation assay of 6 colorectal cell lines after 72 hours of anti-*EGFR* treatment. The first three cell lines SW48, SNU-C1 and COLO-320DM showed higher proliferation rate compared to LIM1215, CaCo2 and SW948 (P = 0.0001). Dashed lines show the mean of each group.

4.3.2 Upregulation of biomarkers in anti-*EGFR* resistant cell lines

Each of the anti-*EGFR* resistant cell lines were compared with all of the sensitive CRC cell lines, and each resistant cell line had 3 combination array results (for example for SW48: SW48 vs LIM1215, SW48 vs CACO2, SW48 vs SW948). In total, 9 combinations of the RT Profiler array results were analysed using the RT^2 Profiler Array PCR Data Analysis version 3.5 software available online to determine candidate biomarkers that were upregulated in anti-*EGFR* resistant CRC cell lines. Candidate biomarkers were chosen according to these criteria: those upregulated in resistant cell lines in common in most of the comparisons, fold regulation must be more than 3, and

the differences were statistically significant ($p<0.05$). The top 3 biomarkers that fulfilled these criteria were chosen: *HBEGF*, *EGR1* and *AKT3*. The fold regulation and p-values for these biomarkers are given in Table 1.

Table 1: Top three upregulated genes in the resistant cell lines, alongside the number of combinations with upregulation, fold changes and p-values.

Gene	Number of combinations with upregulation	Fold regulation	p-value
EGR1	6	5.8	0.0005
		4.9	0.0009
		33	0.0003
		85	0.000001
		18.8	0
		8.9	0.0003
HBEGF	5	13.5	0.0004
		3.4	0
		4.9	0.001
		3.8	0.0001
		21.4	0.001
AKT3	5	41.5	0.00003
		15.6	0.000001
		5.4	0.02
		10.5	0.003
		120	0.000001

4.3.3 Validation of upregulated biomarkers

The 3 candidate biomarkers were validated using qRT-PCR. This experiment was repeated three times for each cell line. When compared to all of the sensitive cell lines *HBEGF* was found to be upregulated in SNUC1, *EGR1* was found to be upregulated in SW48 while. *AKT3* was found to be upregulated in COLO320-DM. The levels of *HBEGF*, *EGR1* and *AKT3* in all of the sensitive lines were lower compared to the resistant cell lines. The fold changes and p-values for these overexpressed biomarkers in the respective resistant CRC cell line are listed in Table 2.

Table 2: qRT-PCR validation of array results of overexpressed biomarkers in resistant cell lines and fold changes.

Resistant cell line	Overexpressed biomarker	Fold changes	P-value
SW48	*EGR1*	19.8	P < 0.0001
SNUC1	*HBEGF*	22.9	P = 0.0283
COLO-320DM	*AKT3*	122.5	P < 0.0001

4.3.4 siRNA knockdown of overexpressed biomarkers in resistant cell lines, validated by qRT-PCR

Overexpressed biomarkers were knocked down using siRNA in the respective resistant cell lines for 72 hours. *HBEGF* was knocked down by 79.4% in SNUC1, *EGR1* was knocked down by 85.6% in SW48 and *AKT3* was knocked down by 95.3% in COLO-320DM.

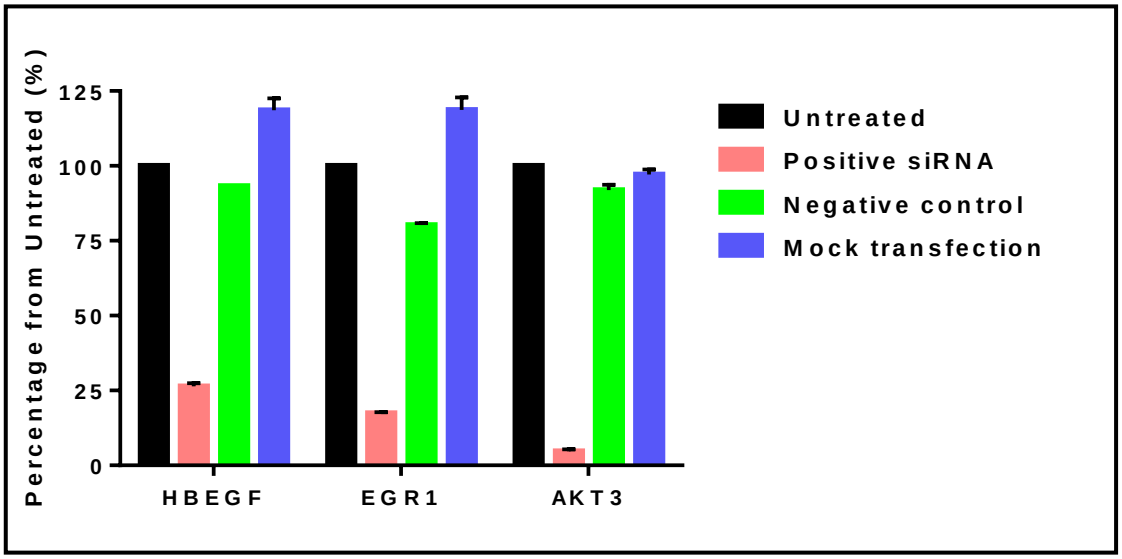

Figure 2: After 72 hours siRNA treatment, 79.4% *HBEGF* knockdown was achieved SNU-C1, 85.6% *EGR1* knockdown was achieved in SW48 and 95.3% *AKT3* knockdown was achieved in COLO-320DM.

4.3.5 siRNA knockdown validated by western blots

Protein extracts (30 µg) were resolved by SDS-PAGE; transferred to PVDF membrane; and immunoblotted with *HBEGF*, *EGR1* and *AKT3* antibodies. Actin was used as a loading control. There was reduction in the specific protein in the extracts derived from *HBEGF*-, *EGR1*- and *AKT3*-knocked down cells, compared to the negative scrambled control and mock transfected cells. Representative western blots of *HBEGF* expression in SNU-C1 (Figure 3), *EGR1* expression in SW48 (Figure 4) and *AKT3* expression in COLO-320DM (Figure 5) are shown below. Actin was used as a loading control.

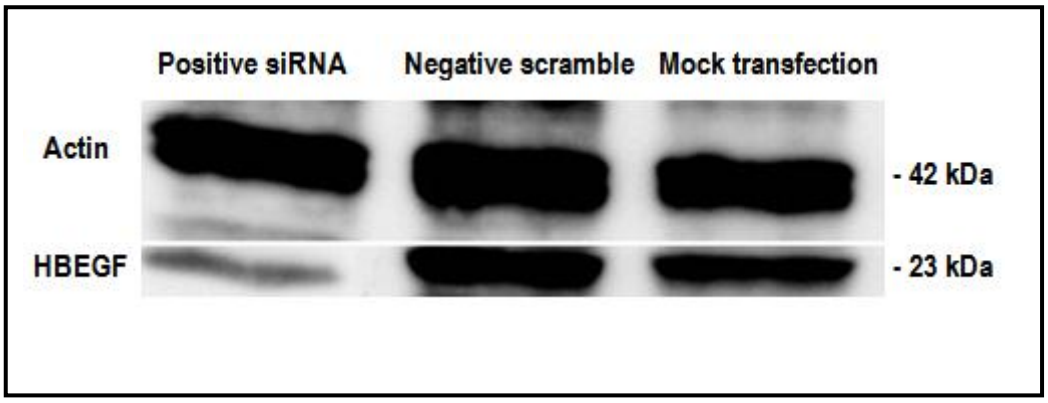

Figure 3: Western blot showing *HBEGF* protein reduction in *HBEGF*-knocked down cells (positive siRNA panel).

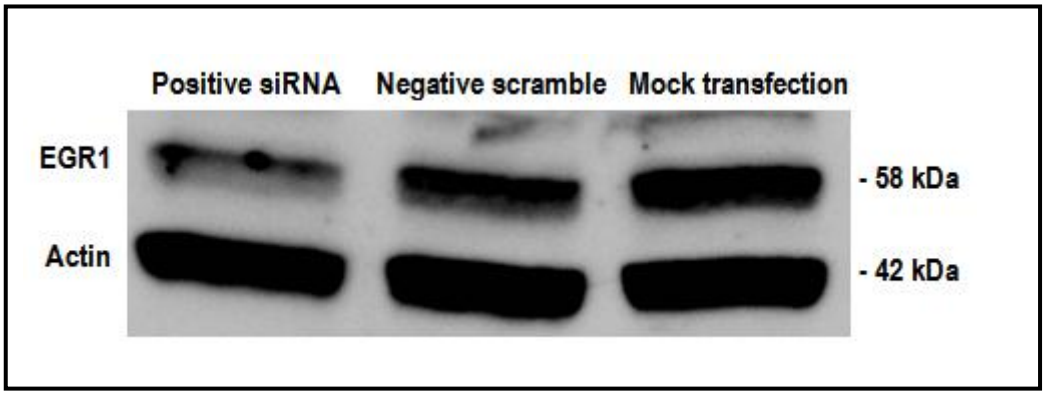

Figure 4: Western blot showing *EGR1* protein reduction in *EGR1*-knocked down cells (positive siRNA panel).

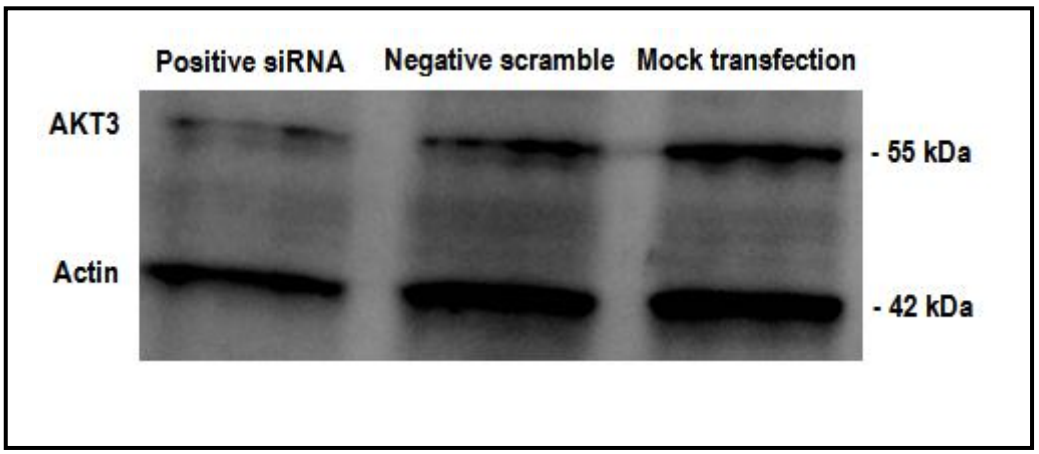

Figure 5: Western blot showing *AKT3* protein reduction in *AKT3*-knocked down cells (positive siRNA panel).

4.3.6 Proliferation assay – sensitivity to anti-*EGFR* restored after siRNA treatment

After 24 hours treatment with anti-*EGFR*, proliferation rate for *HBEGF*-knocked down cells was 46.9% compared to cells without knockdown at 103.2% (Figure 6). Proliferation rate for *EGR1*-knocked down cells was 49.1% compared to cells without knockdown at 99.8% (Figure 7). Proliferation rate for *AKT3*-knocked down cells was 64.1% compared to cells without knockdown at 92.2% (Figure 8).

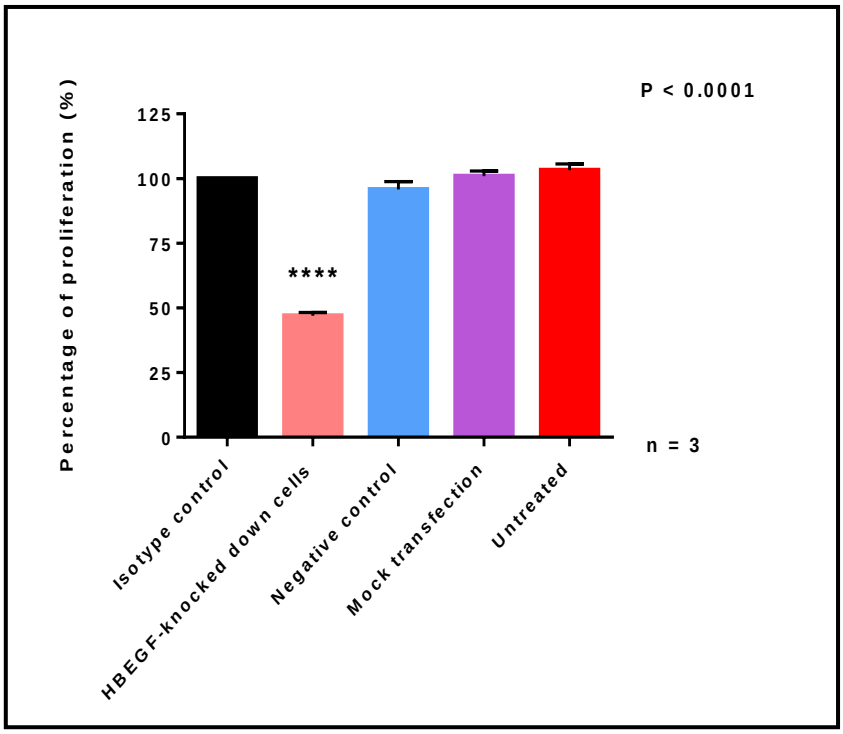

Figure 6: Proliferation rate for *HBEGF*-knocked down cells was 46.9% after 24-hour treatment with anti-*EGFR* compared to cells that were not knocked down. The proliferation rate difference was statistically significant at p< 0.0001 (ANOVA).

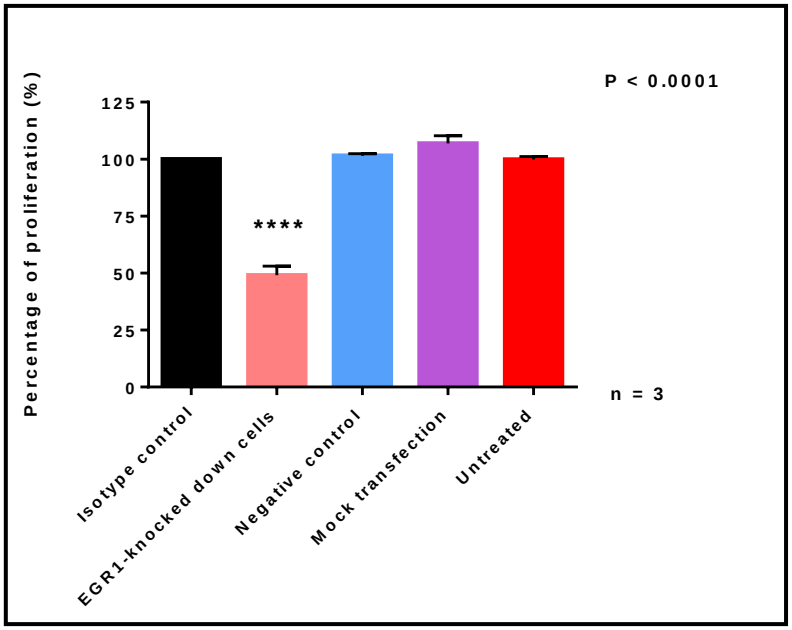

Figure 7: Proliferation rate for *EGR1*-knocked down cells was 49.1% after 24-hour treatment with anti-*EGFR* compared to cells that were not knocked down, p < 0.0001 (ANOVA).

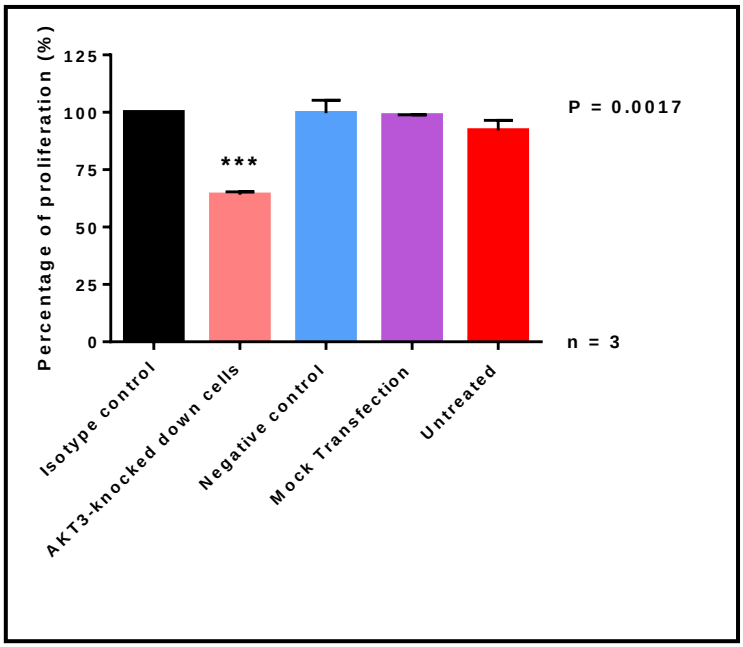

Figure 6: Proliferation rate for *AKT3*-knocked down cells was 64.1% after 24-hour treatment with anti-*EGFR* compared to cells that were not knocked down, p = 0.0017 (ANOVA).

4.3.7 Impact of biomarkers' expression values on the outcome of salvage cetuximab therapy

Figure 7, 8 and 9 show examples of overexpression of each marker matched with their normal slides. Table 3 summarises the impact of overexpression of biomarkers on the outcome of cetuximab monotherapy. 1 non-responder patient (10%) recorded overexpression of all 3 markers, 3 non-responder patients (30%) recorded overexpression of *AKT3* and *EGR1* and 1 non-responder patient (10%) recorded overexpression of *HBEGF* and *EGR1*. 5 out of total of 7 (71.4%) non-responder patients recorded overexpression of 2 or more of these markers.

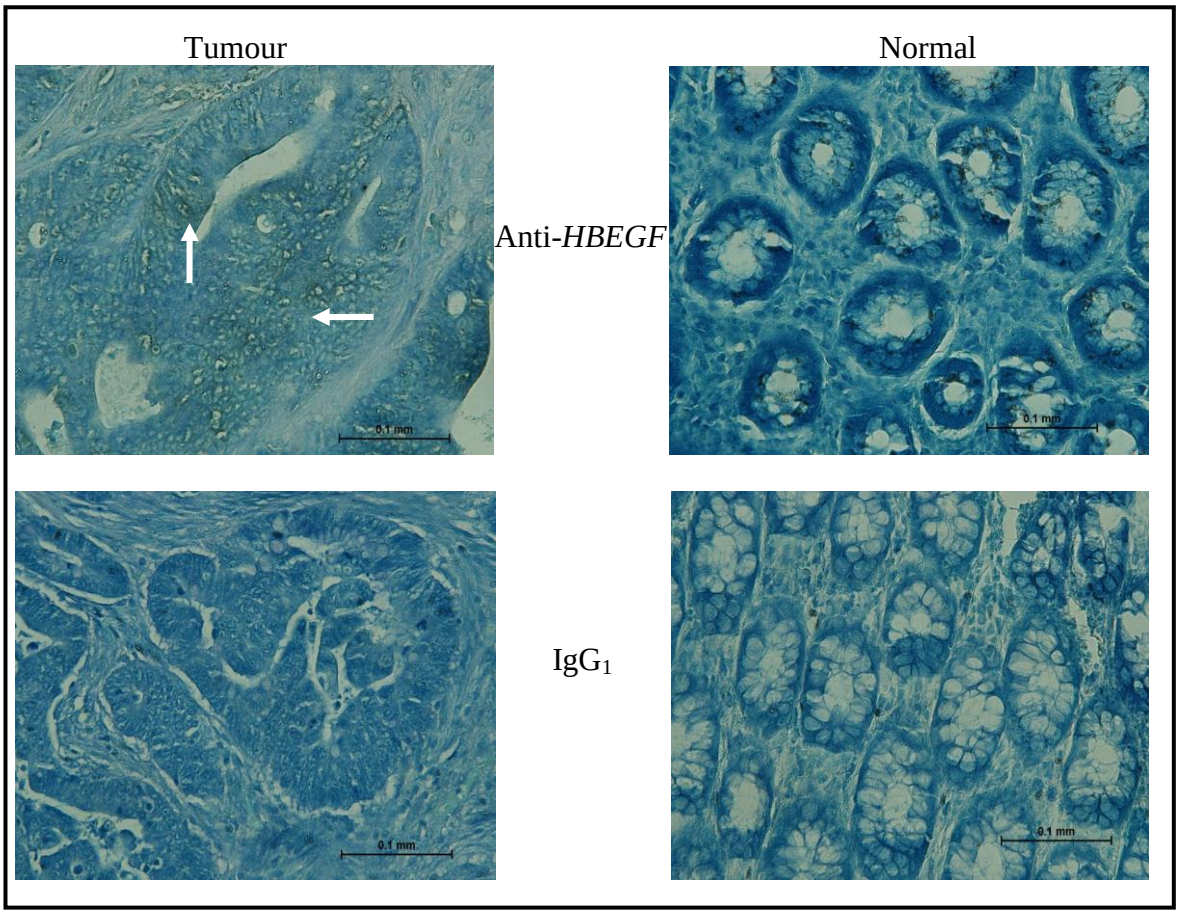

Figure 7: Panel shows expression of *HBEGF* and its nonspecific immunoglobulin as negative control (all at 200X magnification) for tumour samples and its matched normal margin of a mCRC patient who received cetuximab monotherapy. Tumour sample shows positive expression of *HBEGF* (brown stain on tumour cells as shown by white arrows in the top left image) compared to its normal margin which was negative. Both tumour and normal slides were negative for isotype control expression.

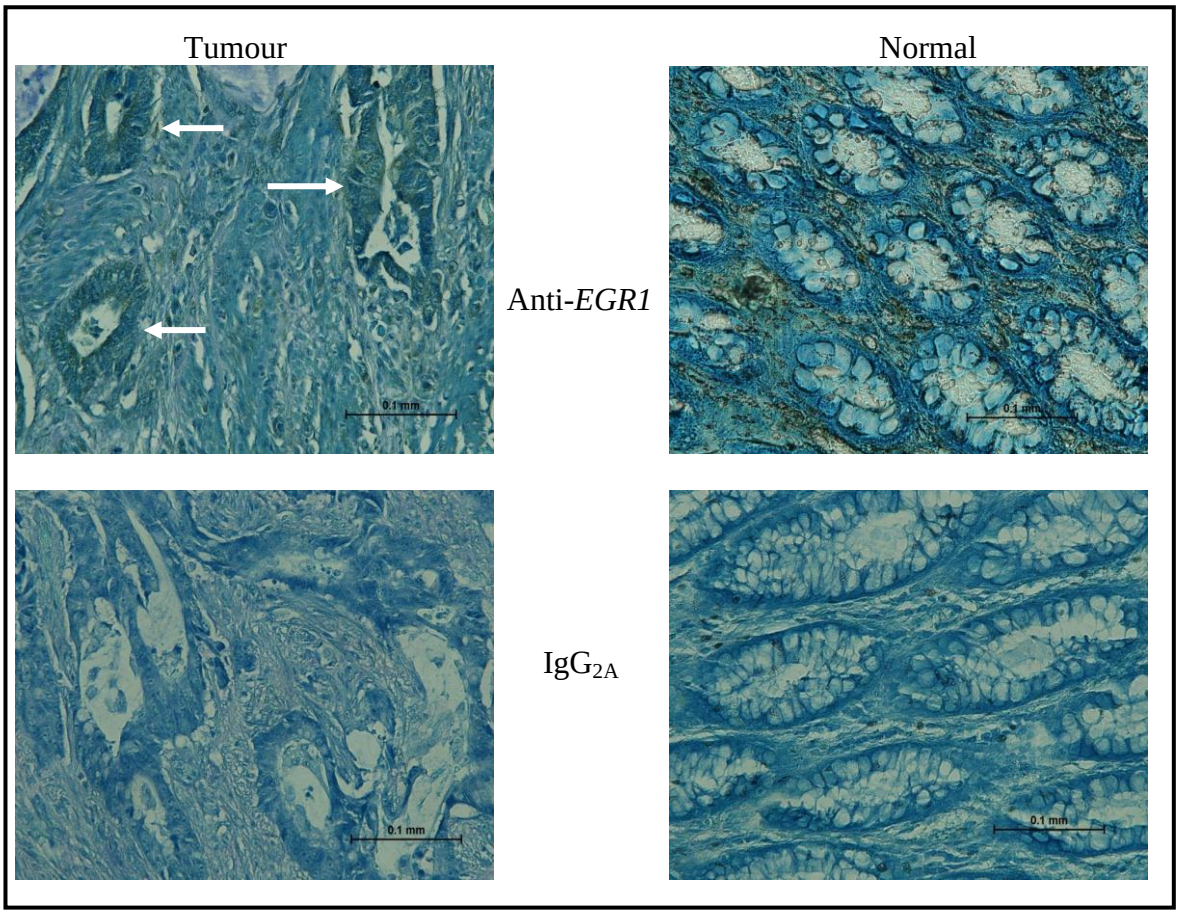

Figure 8: Panel shows expression of *EGR1* and its nonspecific immunoglobulin as negative control (all at 200 x magnification) for tumour samples and its matched normal margin of a mCRC patient who received cetuximab monotherapy. Tumour sample shows positive expression of *EGR1* (brown stain on tumour cells as shown by white arrows in the top left image) compared to its normal margin which was negative. Both tumour and normal slides were negative for isotype control expression.

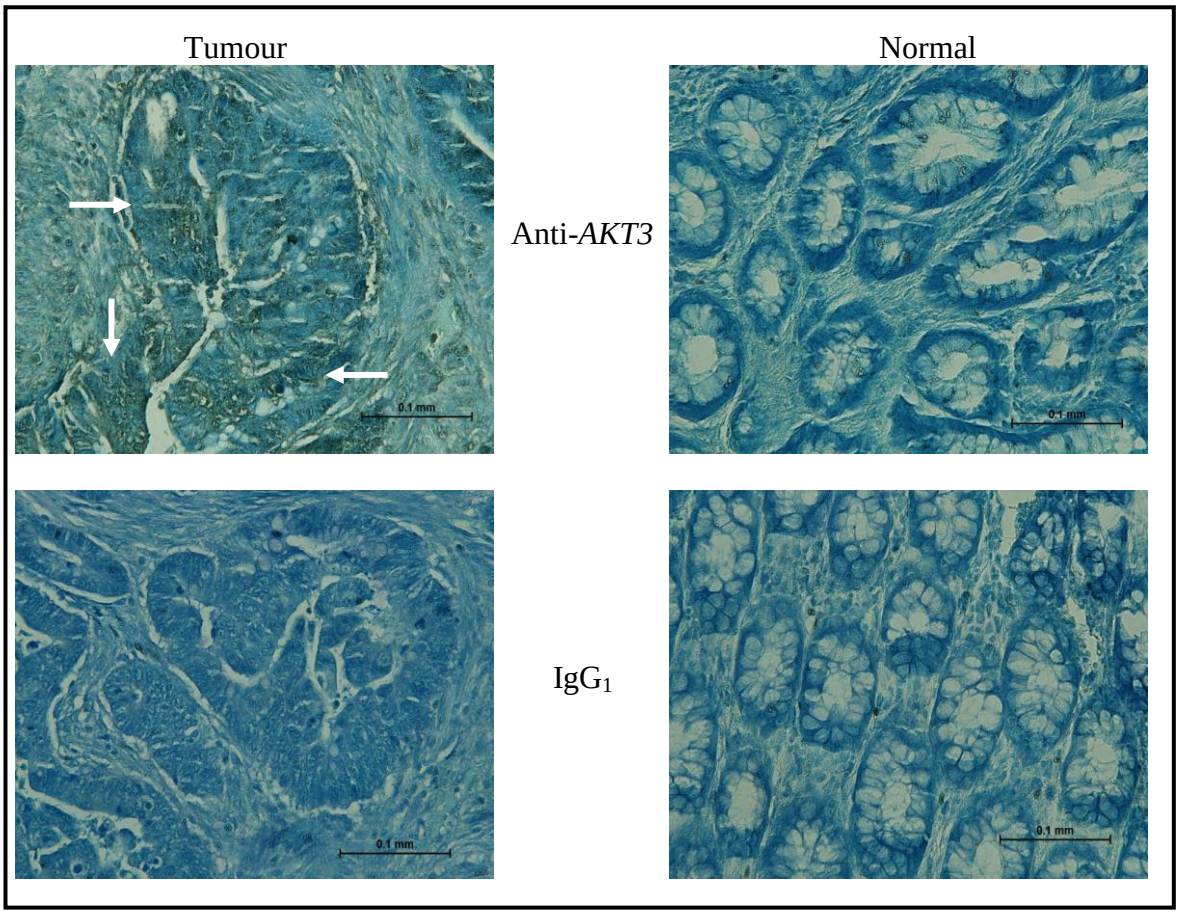

Figure 9: Panel shows expression of *AKT3* and its nonspecific immunoglobulin as negative control (all at 200 x magnification) for tumour samples and its matched normal margin of a mCRC patient that received cetuximab monotherapy. Tumour sample shows positive expression of *AKT3* (brown stain on tumour cells as shown by white arrows in the top left image) compared to its normal margin which was negative. Both tumour and normal slides were negative for isotype control expression.

Table 3: Scores of biomarkers with their respective isotype controls amongst mCRC patients that received cetuximab monotherapy.

Patients	Biomarkers	Tumour (T) or Normal (N)	Intensity (0, 1, 2, 3)	Percentage of positive (%)	Score (≥4 is positive)	Response to cetuximab
Patient 1	*HBEGF*	T	2	20%	3 (-)	Responder
		N	0	0	0	
	EGR1	T	1	50%	3 (-)	
		N	0	0	0	
	AKT3	T	1	30%	2 (-)	
		N	0	0	0	
	IgG_1	T	0	0	0	
		N	0	0	0	
	IgG_{2A}	T	0	0	0	
		N	0	0	0	
Patient 2	*HBEGF*	T	2	80%	6 (+)	Non-responder
		N	0	0	0	
	EGR1	T	1	90%	4 (+)	
		N	0	0	0	
	AKT3	T	3	100%	6 (+)	
		N	0	0	0	
	IgG_1	T	0	0	0	

		N	0	0	0	
	IgG_{2A}	T	0	0	0	
		N	0	0	0	
Patient 3	*HBEGF*	T	0	0	0	Responder
		N	0	0	0	
	EGR1	T	1	40%	3 (-)	
		N	0	0	0	
	AKT3	T	1	70%	4 (+)	
		N	0	0	0	
	IgG_1	T	0	0	0	
		N	0	0	0	
	IgG_{2A}	T	0	0	0	
		N	0	0	0	
Patient 4	*HBEGF*	T	0	0	0	Non-responder
		N	0	0	0	
	EGR1	T	1	70%	4 (+)	
		N	0	0	0	
	AKT3	T	1	80%	4 (+)	
		N	0	0	0	

	IgG_1	T	0	0	0	
		N	0	0	0	
	IgG_{2A}	T	0	0	0	
		N	0	0	0	
Patient 5	*HBEGF*	T	0	0	0	Responder
		N	0	0	0	
	EGR1	T	1	90%	4 (+)	
		N	0	0	0	
	AKT3	T	2	100%	5 (+)	
		N	0	0	0	
	IgG_1	T	0	0	0	
		N	0	0	0	
	IgG_{2A}	T	0	0	0	
		N	0	0	0	
Patient 6	*HBEGF*	T	2	90%	5 (+)	Non-responder
		N	1	10%	2 (-)	
	EGR1	T	3	80%	6 (+)	
		N	0	0	0	
	AKT3	T	1	20%	2 (-)	

		N	2	10%	3 (-)	
	IgG_1	T	0	0	0	
		N	0	0	0	
	IgG_{2A}	T	0	0	0	
		N	0	0	0	
Patient 7	*HBEGF*	T	0	0	0	Non-responder
		N	2	10%	3 (-)	
	EGR1	T	1	10%	2 (-)	
		N	1	30%	3 (-)	
	AKT3	T	1	30%	3 (-)	
		N	3	80%	6 (+)	
	IgG_1	T	0	0	0	
		N	0	0	0	
	IgG_{2A}	T	0	0	0	
		N	0	0	0	
Patient 8	*HBEGF*	T	1	30%	3 (-)	Non-responder
		N	0	0	0	
	EGR1	T	2	90%	5 (+)	
		N	0	0	0	

	AKT3	T	1	80%	4 (+)	
		N	1	30%	3 (-)	
	IgG_1	T	0	0	0	
		N	0	0	0	
	IgG_{2A}	T	0	0	0	
		N	0	0	0	
Patient 9	*HBEGF*	T	0	0	0	Non-responder
		N	1	10%	2 (-)	
	EGR1	T	2	40%	4 (+)	
		N	0	0	0	
	AKT3	T	2	90%	5 (+)	
		N	2	30%	4 (+)	
	IgG_1	T	0	0	0	
		N	0	0	0	
	IgG_{2A}	T	0	0	0	
		N	0	0	0	
	HBEGF	T	0	0	0	
		N	0	0	0	
	EGR1	T	1	30%	3 (-)	

Patient 10		N	0	0	0	Non-responder
	AKT3	T	0	0	0	
		N	0	0	0	
	IgG_1	T	0	0	0	
		N	0	0	0	
	IgG_{2A}	T	0	0	0	
		N	0	0	0	

4.4 DISCUSSION AND CONCLUSION

EGFR and its downstream intracellular targets regulate key cellular events that drive the progression of CRC. Cetuximab and panitumumab, two monoclonal antibodies targetting *EGFR*, are now part of standard care for extended *RAS* WT CRC therapy. Clinical data has reported a correlation between the severity of rash (a side effect of these antibodies) and response to the therapy (Segaert, Chiritescu et al. 2009). This information alone does not aid clinicians to optimise the selection of patients to receive anti-*EGFR* therapies. The small responsive patient population for *EGFR* inhibitors, coupled with high costs have prompted clinicians to investigate other predictive biomarkers. It has been reported that tumour growth can be driven by constitutive activation of signalling pathways downstream of the *EGFR*, such as the *RAS-MAPK* and *PI3K* pathways (Scaltriti and Baselga 2006). The oncogenic activation of components in these intracellular pathways can circumvent the *EGFR*-driven signalling cascade and impair the clinical efficacy of anti-*EGFR* monoclonal antibodies. Such activation does occur via mutations in oncogenes such as *KRAS* and *BRAF* in one axis of the *EGFR*-mediated pathway and by *PIK3CA* mutations and loss of tumour suppressor genes, such as *PTEN* in another axis.. The study by Lièvre et al. (Lievre, Bachet et al. 2006) was the first to report an association between *KRAS* mutations and the lack of response to *EGFR*-targeted monoclonal antibodies. The data stemming from a number of large randomised phase II and III trials have provided convincing evidence that activated mutations of *KRAS*, which account for 35%–55% of sporadic CRC, can predict lack of response to anti-*EGFR* treatment, regardless whether combined with FOLFOX (folinic acid, fluorouracil, and oxaliplatin) or FOLFIRI (folinic acid, fluorouracil, and irinotecan) chemotherapy as a single agent (Karapetis, Khambata-Ford et al. 2008; Bokemeyer, Bondarenko et al. 2009). Given the complex landscape of the

EGFR signalling system, no one biomarker can be identified to reveal the subpopulation of CRC patients that will benefit from anti-*EGFR* targeted therapy in practice. It is more likely that predictive algorithms or a panel that involve several molecular biomarkers will be developed for mCRC as predictive markers for response to therapy.

To identify potential biomarkers of resistance other than *KRAS* and *BRAF*, we treated 6 WT *KRAS* exon 2/*BRAF* CRC cell lines to anti-*EGFR* to determine the response (sensitive or resistant) to the treatment. The cDNA of these cell lines was applied to RT Profiler Array Human *EGF/PDGF* pathway arrays (Qiagen, USA) to determine differentially overexpressed biomarkers in the resistance CRC cell lines. In the proliferation assay, SW48, SNU-C1 and COLO-320DM were resistant to anti-*EGFR* treatment compared to LIM1215, CaCo2 and SW948 which were sensitive to the treatment (P=0.0001). All of these cell lines were wild type for *KRAS* and *BRAF*.

The study by Jhawer *et al.* (Jhawer, Goel et al. 2008) showed a link between the proliferative response of CRC cell lines to anti-*EGFR* treatment and their mutational status that explained the sensitivity of some cell lines to the treatment. Separation of CRC cell lines according to *KRAS* and/or *BRAF* mutation status did not stratify the cell lines according to the response to cetuximab, although it showed a trend for *KRAS*/*BRAF* mutant lines to be more resistant to cetuximab (P = 0.11) (Jhawer, Goel et al. 2008). The study also observed that two *KRAS* mutant cell lines GEO and SW403 showed a significant response to cetuximab. While it remained to be clarified whether *KRAS*/*BRAF* mutational status alone is sufficient to stratify mCRC patients for cetuximab treatment, that study observed that the 6 most resistant cell lines to cetuximab harboured mutations or loss of expression in both the *KRAS*/*BRAF* and *PIK3CA*/*PTEN* pathways respectively (Jhawer, Goel et al. 2008). In the same study, some CRC cell lines that were WT for *KRAS*/*BRAF* were reported to be more sensitive

to cetuximab compared with *KRAS*/*BRAF* mutant cell lines, although this effect was not statistically significant (22.6 ± 5.4% versus 40% ± 9.4% growth inhibition for *KRAS*/*BRAF* mutant versus WT cell lines respectively, P=0.11) (Jhawer, Goel et al. 2008). Several studies (Lievre, Bachet et al. 2006; Benvenuti, Sartore-Bianchi et al. 2007; Di Fiore, Blanchard et al. 2007; Khambata-Ford, Garrett et al. 2007) that have investigated the correlation of *KRAS* mutation status and cetuximab response have shown that WT *KRAS* and *BRAF* tumour show improved response to cetuximab. In some of these studies, patients harbouring mutant *RAS* tumours showing clinical response to cetuximab treatment were also identified (Benvenuti, Sartore-Bianchi et al. 2007). In a retrospective study, *KRAS* mutations were detected in 3 of 27 (11%) mCRC patients that responded to cetuximab therapy and in 27 of 53 (51%) non-responders (Khambata-Ford, Garrett et al. 2007). This data suggest that the presence of *KRAS* mutation correlated with a lack of response to cetuximab therapy (P=0.0003). This study shows that patients that do not harbour *KRAS* mutations have a higher disease control rate (48%) than patients with *KRAS* mutations (10%), suggesting that the presence of *KRAS* mutations might circumvent cetuximab's inhibitory activity (Khambata-Ford, Garrett et al. 2007). Interestingly, *BRAF* mutations which are predominantly mutually exclusive of *KRAS* mutations have also been associated with resistance to anti-*EGFR* targeted therapies in CRC (Di Nicolantonio, Martini et al. 2008; Vaughn, Zobell et al. 2011). *BRAF* mutation correlated with poor prognosis and worse outcomes when compared to tumours with WT *BRAF*, suggesting tumours that do not harbour this mutation would elicit a better response to anti-*EGFR* targeted therapies (Van Cutsem, Kohne et al. 2009; Van Cutsem, Kohne et al. 2011; Arrington, Heinrich et al. 2012).

The resistance of CRC cell lines to anti-*EGFR* treatment shown in our study mirrors the finding of Moroni *et al.* (Moroni, Veronese et al. 2005), where the study assessed the effect of cetuximab on several CRC cell lines including SW48. SW48 in their study was deemed to be resistant at 60% proliferation rate while all of the other cell lines were sensitive to cetuximab (Moroni, Veronese et al. 2005). Another study observed that the lack of clinical response to *EGFR*-targeted antibodies is due to the oncogenic activation of other biomarkers that are involved in the intracellular pathways that could bypass *EGFR* signalling cascade, thus conferring primary resistance to cetuximab and panitumumab (Bardelli and Siena 2010). The alterations in other key elements of the *EGFR* signalling cascade such as *AKT1* or *MAPK*, and the presence of genetic alterations in the tyrosine kinase receptors other than *EGFR* provide an alternate pathway of survival and proliferation (Bardelli and Siena 2010). The concomitant analysis of multiple genetic and epigenetic events that are involved in the cellular events activated by the *EGFR* will aid in tailoring cetuximab and panitumumab therapies to patients that will benefit the most.

This project has led to the discovery of three differentially upregulated biomarkers in the resistant CRC cell lines using RT Profiler Array Human *EGF/PDGF* pathway kit (Qiagen): *HBEGF* (Heparin-binding epidermal growth factor-like growth receptor) and *EGR1* (early growth response protein 1) and *AKT3* (v-*AKT* murine thymoma viral oncogene homolog 3). These three markers may be conferring resistance to anti-*EGFR* treatment by circumventing the *EGFR* and activating downstream signalling. One of the resistant cell lines SNU-C1 was reported to harbour *HRASLS* and *HRASLS5* gene (both tumour suppressors), modulates the *HRAS*-mediated signalling pathway that is found in the *EGFR* signalling cascade. However, there have been no

studies reporting the *HRASLS* and *HRASLS5* mutations contributing to tumour formation or progression, or involved in anti-*EGFR* resistance.

Our immunohistochemistry study showed overexpression of two or more of these proposed resistance markers in 5/7 mCRC patients that did not respond to cetuximab monotherapy, suggesting a synergistic activity and cross-talk between the intracellular pathways in the *EGFR* signalling cascade. The overexpression of these markers correlated with the lack of response to cetuximab with an exception of two patients that had overexpression of one or more markers but showed response, suggesting other markers might be playing a role in conferring resistance to the anti-*EGFR* targeted therapy. One of the limitations of the immunohistochemistry study was the small sample size of the acquired patient tissues to validate the cell line study, though it yielded interesting preliminary observations. Another limitation of immunohistochemistry method is that it is semi-quantitative and it lacks a standardised scoring system. It is also largely subject to variations amongst the observers. The specificity of the antibodies was confirmed by the datasheet from the manufacturer and other studies that have used the antibodies, anti-*HBEGF* (Tang, Deng et al. 2012), anti-*EGR1* (Jafferji, Bain et al. 2009) and anti-*AKT3* (Shin, Wall et al. 2010). Concentrations used for the immunohistochemistry study were recommended by the manufacturer (Abcam, USA)

Presently, biomarkers have been often assessed retrospectively as a single event. Some of these events often display an overlapping pattern of occurrence which further complicates their analysis. Thus, an integrated analysis of the entire oncogenic pathway triggered by the *EGFR* is likely to enhance the prediction ability of the markers used individually as shown by a study by Peeters et al. (Peeters, Oliner et al. 2013). That study reported some of genes involved in the *EGFR* signalling cascade limited the

efficacy of panitumumab in some mCRC patients, by employing the usage of massively parallel multigene sequencing techniques (next-generation sequencing) to analyse 3 types of alterations simultaneously: additional *RAS*-activating mutations such as *KRAS* codon 61, *NRAS* codons 12/13/61, other *EGFR* signalling pathway genes that were known to be mutated in mCRC such as *EGFR*, *BRAF*, *PTEN*, *PIK3CA*, and *AKT*, and some of the genes that are known to play a role in CRC tumourigenesis and progression such as *TP53* and *CTNNB1* (Peeters, Oliner et al. 2013).

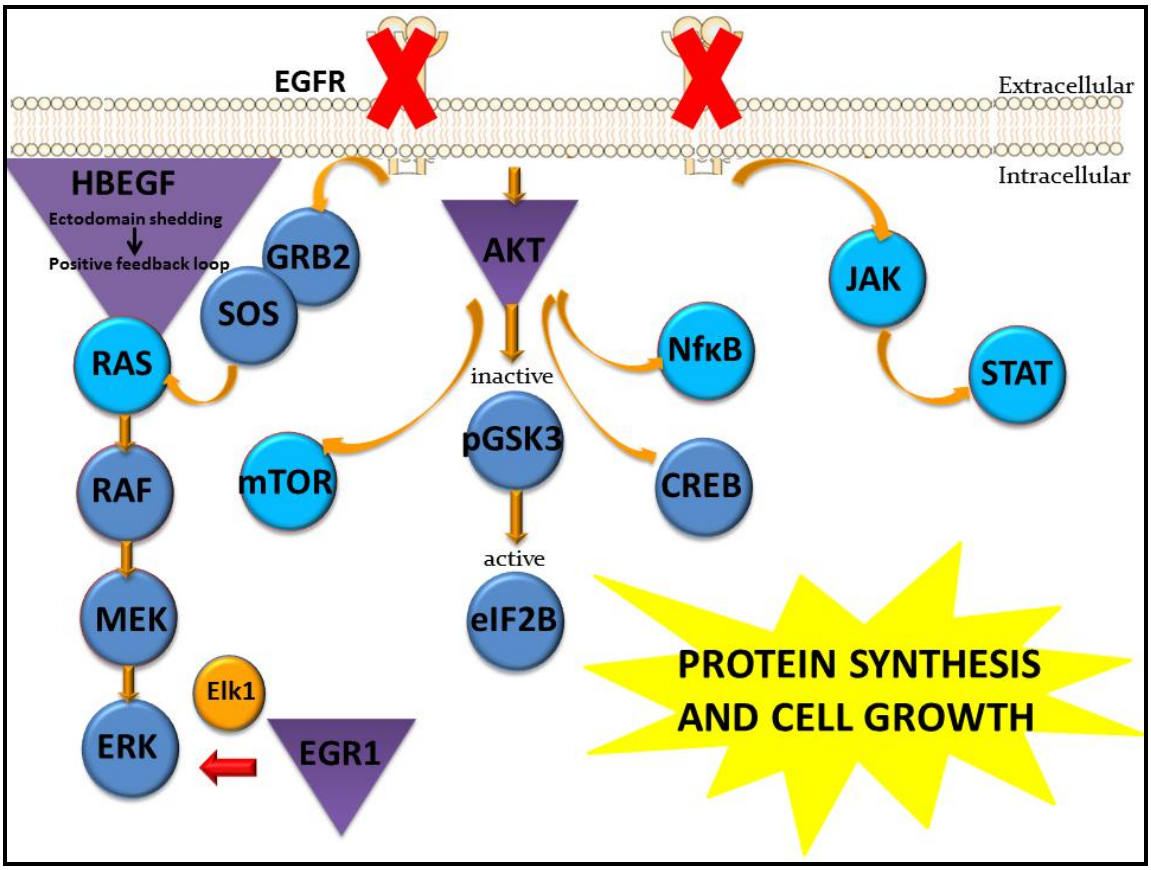

Figure 10: Schematic of the *EGFR* signalling pathway to summarise the roles of these overexpressed biomarkers and how they may be conferring resistance to anti-*EGFR* treatment (treatment represented as red Xs). *HBEGF* is cleaved at the cell surface by a mechanism called ectodomain shedding that yields a soluble form of *HBEGF* that activates *EGFR*. This leads to a continuous positive feedback loop that activates the *ERK/MAPK* pathway, which leads to cancer progression. *EGF* regulates *ERK* and *ERK* in turn is the major regulatory mechanism for *EGR1* (the elevation of *EGF* in colon cancer has been reported to lead to disease progression). A downstream target of *ERK*, *Elk1*, confers its transcriptional activity upon *EGR1*, which leads to its overexpression and consequently leading to cell growth and proliferation. For *AKT3*, the activation of *AKT* inactivates the *GSK3* protein, and this activates the eukaryotic translation initiation factor 2b. This factor recruits ribosomes during the initial phase of translation, leading to protein synthesis. So these biomarkers may emerge as biomarkers of resistance and be predictive of response to anti-*EGFR* therapy.

Heparin-binding epidermal growth factor-like growth factor (*HBEGF*), a heparin-binding member of the EGF family (Raab and Klagsbrun 1997), was first identified in the conditional medium of human macrophages (Higashiyama, Abraham et al. 1991). It serves as a potent mitogen as well as a chemotactic factor for fibroblasts and smooth muscle cells (Dluz, Higashiyama et al. 1993; Fukuda, Inui et al. 1995). *HBEGF* is reported to bind to *EGFR* and HER4, and activate several signal transduction cascades that are involved in diverse functions including development, proliferation, differentiation and migration (Nishi and Klagsbrun 2004). *HBEGF* has also been shown to stimulate the growth of a variety of cells in an autocrine or paracrine manner, and it is reported to be involved in stromal proliferation. *HBEGF* is initially synthesized as a transmembrane protein of 208 amino acids (Nishi and Klagsbrun 2004). The membrane-anchored form of *HBEGF* (pro-*HBEGF*) is cleaved on the cell surface to yield a soluble growth factor of 75–86 amino acids and a considerable amount of pro-*HBEGF* still remains uncleaved on the cell surface (Nishi and Klagsbrun 2004). One study demonstrated that *HBEGF* binds to a novel 140-kDa receptor identified as *N*-arginine dibasic convertase, a metalloendopeptidase of the M16 family, and that binding to *N*-arginine dibasic convertase is highly specific for *HBEGF* among *EGF* family members (Hashimoto, Higashiyama et al. 1994). This specific binding modulates *HBEGF*-induced cell migration via *EGFR* (Hashimoto, Higashiyama et al. 1994). Several laboratories have described *HBEGF* as being upregulated in response to oncogenes and in oncogene-transformed cells. It has also been identified as an immediate-early response gene that can be activated by the RAS-RAF signalling pathway. In non-transformed human mammary epithelial cells, *HBEGF* expression is induced by *EGF* and *RAS* overexpression (Martinez-Lacaci, De Santis et al. 2001), strongly implying that *HBEGF* is a direct target of mitogen-activated protein kinase (MAPK). Previous

observation by Onguhasa and colleagues (Ongusaha, Kwak et al. 2004) demonstrated that *HBEGF* is induced in response to tumour suppressor *p53*, as well as DNA damage. *HBEGF* induction antagonises apoptosis mediated by genotoxic stress through the activation of the *RAS-RAF-MAPK* cascade and the *AKT* pathway, suggesting a pro-survival function for *HBEGF*. *HBEGF* expression has been implicated in tumour initiation, progression, metastasis and it has been reported to be overexpressed in many tumours including colon, pancreatic, hepatocellular, breast and bladder cancers (Sloss, Wang et al. 2010). In vitro cell line and human tumour specimen studies have identified the involvement of *HBEGF* in resistance to a wide variety of clinically used molecular agents (Wang, Liu et al. 2007). This shows that *HBEGF* is not only a potent inducer of tumour growth, but it is also a survival factor involved in response to cellular stress (Sloss, Wang et al. 2010). The significance of *HBEGF* overexpression in tumourigenesis is implied by the identification of *HBEGF* as a *p53* downstream target gene and *p53* induction of *HBEGF* could activate cell survival signalling, including the *AKT* and *MAPK* cascades (Miyoshi, Higashiyama et al. 1997). Inducible expression of oncogenic *RAF* in normal epithelial cells has been demonstrated to strongly induce autocrine expression of *HBEGF*, transforming growth factor alpha (*TGF-α*) and amphiregulin (an *EGFR* ligand), all of which were directly implicated in the sustained stimulation of *RAF-MAPK* pathway to protect cells from apoptosis (Gangarosa, Sizemore et al. 1997). *HBEGF* has been reported to enhance transformed phenotypes and is associated with the stimulation of *MMP-9*, *MMP-3* and *cyclin-D* activation which promotes tumorigenesis and angiogenesis. Given its elevated expression in human cancers compared to normal tissues, and along with its contribution to enhanced transformed phenotypes (Ongusaha, Kwak et al. 2004), *HBEGF* may behave as an oncogene and could have importance as a therapeutic target. There are no known studies

elucidating the anti-tumour effects of *HBEGF* inhibition in mCRC. One study investigated the effect of a nontoxic mutant of diphtheria toxin (*CRM197*), a specific inhibitor of *HBEGF* in metastatic oral cancer. *CRM197* were injected intra-peritoneally into tumour-bearing mice, and tumour volume was measured over time. *HBEGF* expression in HSC3 and SAS cells treated with *CRM197* was significantly reduced and cell proliferation was inhibited. In addition the invasiveness of *CRM197*-treated cells was relatively low. The co-administration of anti-cancer compound cis-diamminedichloroplatinum (II) (cisplatin) and *CRM197* at 1 mg/kg/day completely inhibited tumour formation (Dateoka, Ohnishi et al. 2012). These results suggest that *HBEGF* is an attractive therapeutic target for malignancies.

Early growth response 1 or *EGR1* (also known as NGFI-A, zif268, krox24 and Tis8) is a zinc-finger transcriptional factor belonging to a group of early response genes together with *EGR2*, *EGR3*, *EGR4*, *EGR-α* and the tumour suppressor WT1 (Wilms' tumour gene product). *EGR1* is an important transcriptional regulator and has been described as a "master switch" linking extracellular stimuli to diverse long-term responses including cell growth, proliferation, and apoptosis (Yan, Fujita et al. 2000; Thiel and Cibelli 2002). *EGR1* targets include the tumour suppressors *PTEN* and *p53* and the pro-apoptotic factor *c-Jun* (Virolle, Adamson et al. 2001; Levkovitz and Baraban 2002; Krones-Herzig, Mittal et al. 2005). In some instances, the absence of *EGR1* keeps cell proliferation and growth signals in check by allowing the growth suppressors to act unopposed (Gregg and Fraizer 2011). One candidate for regulation of *EGR1* in the colon is *EGF*. Both *EGF* and its receptor have been shown to be elevated in many malignancies including colon, prostate and breast tumours (Soulitzis, Karyotis et al. 2006; Gregg and Fraizer 2011) and are associated with disease progression (Di Lorenzo, Tortora et al. 2002; Bartlett, Brawley et al. 2005). *EGF* transmits its signal

through the extracellular regulated kinase (*ERK*) pathway. A well-described downstream target of *ERK* is the *Elk-1* transcription factor (Janknecht, Ernst et al. 1993; Gille, Kortenjann et al. 1995). *Elk-1* is characterised by its *Ets*-binding domain that recognises the canonical sequence 5′-GGA(A/T)-3′, and also its ability to form a ternary complex with the serum response factor (*SRF*) on target promoters at the serum response element (*SRE*) (Sharrocks 2002). The *Elk-1* protein structure provides docking sites for *ERK* and other MAPKs (Whitmarsh, Yang et al. 1997; Cruzalegui, Cano et al. 1999) and also contains a phosphorylation site that confers *Elk-1* transcriptional activity onto target genes (Gille, Kortenjann et al. 1995; Yang, Whitmarsh et al. 1998). Some of the best-described targets of *Elk-1* are the immediate early genes (*IEG*s), *c-fos* (Gille, Sharrocks et al. 1992; Hill, Marais et al. 1993), *EGR1* (Mora-Garcia and Sakamoto 2000), and JunB (Hodge, Liao et al. 1998), whose expression is often rapid and transient after stimulation (Adamson and Mercola 2002). Functional studies using antisense *EGR1* RNA has been shown to reduce cell proliferation, colony formation, and growth in soft agar in prostate cancer cells, suggesting that *EGR1* is necessary for transformed tumour phenotype (Baron, De Gregorio et al. 2003). *EGR1* has been implicated in the control of cell growth, survival and transformation (Thiel and Cibelli 2002; Ahmed 2004). *EGR1* has also been associated with the development of human cancers, playing a role in multistage carcinogenesis of the skin (Riggs, Rho et al. 2000). High levels of constitutive *EGR1* expression have been observed in most human prostate cancers and is also found to be correlated with more advanced stages of malignancy and poor prognosis (Eid, Kumar et al. 1998). It was reported that tumour progression in transgenic mouse models of prostate cancer was significantly impaired when *EGR1* was not expressed (Abdulkadir, Qu et al. 2001). *EGR1* basal expression was also found to be much higher in gastric cancer tissues compared to in normal gastric mucosa and high

EGR1 mRNA expression correlated with metastasis to lymph nodes and remote organs (Mahalingam, Natoni et al. 2010). *EGR1* is implicated in tumour progression and apoptosis in response to diverse stimuli mediated by *EGR1* target genes such as *TNF-α*, *p53*, *Rb* and *BAX* (Baron, Adamson et al. 2006; Zagurovskaya, Shareef et al. 2009). Other studies have reported that *EGR1* is constitutively expressed at a relatively high level in colon carcinoma cell lines and is upregulated at the mRNA level in early-onset colorectal cancers (Hong, Ho et al. 2007; Mahalingam, Natoni et al. 2010). The study done by Song et al. (Song, Liu et al. 2012) demonstrated that *EGR1* was highly expressed in 90% of advanced colon cancer tissues, as compared with para-cancer tissues. The data suggest that *EGR1* contributes to proliferation of colon cancer cells in part by promoting anion exchanger 2 (*AE2*) expression, which is a downstream target of the *ERK* pathway. Clinicopathological analysis done in that study demonstrated that *AE2* expression is correlated with bigger tumour size, poor tumour differentiation state and shortened patient survival time (Song, Liu et al. 2012). The role of *EGR1* in the *EGFR* pathway in controlling cell proliferation and growth makes it an attractive therapeutic target for various malignancies, including colon cancer. There are no known studies that have elucidated the anti-tumour effects of *EGR1* inhibition. A recent study reported that *EGR1* is crucial in the development of various carcinomas with opposing biofunctions, describing *EGR1* as a tumour repressor that directly or indirectly upregulates multiple tumour suppressors, including *PTEN*, *TP53*, *BCL-2*, and *TGF-β1*, to inhibit cell growth, proliferation, and metastasis, as well as induce apoptosis. In that study, it was elucidated that *EGR1* assisted in the inhibition of non-small-cell lung carcinoma (NSCLC) development functioning as a tumour suppressor (Zhang, Chen et al. 2014).

The *AKT* protein kinase family is comprised of three isoforms: *AKT1* (also known as *PKB*α), *AKT2* (*PKBβ*), and *AKT3* (*PKBγ*). These three *AKT* isoforms belong to the class of AGC kinases. They are encoded on three distinct chromosomes, share a considerable homology, and contain three common structures: the N-terminal pleckstrin homology domain (PH), the catalytic kinase domain (KD), and the C-terminal regulatory hydrophobic region (Gonzalez and McGraw 2009; Beaulieu, Del'guidice et al. 2011; Karar and Maity 2011; Schultze, Hemmings et al. 2012). Both the regulatory and catalytic domains are critical for the biological actions mediated by *AKT* protein kinases and exhibit the maximum degree of homology among the three *AKT* isoforms (Noguchi, Ropars et al. 2007; Krzeslak, Pomorski et al. 2011). One of the hallmarks of the maintenance of a transformed cell phenotype is the constitutive enhanced activity of the *AKT*-related pathway, as reported by several studies (Agarwal, Brattain et al. 2013; Carpenter and Jiang 2013; Dobbin and Landen 2013). This ensures equilibrium between the activities of cellular tumour suppressor factors and proto-oncogenes within the *AKT* pathway (Gaikwad and Ray 2012; Slomovitz and Coleman 2012; Zagouri, Sergentanis et al. 2012). If the equilibrium is disturbed, it will cause overexpression of *AKT*-related factors, which may contribute to the development of the malignant cell phenotype (Almhanna, Strosberg et al. 2011; Bennani-Baiti 2011; Madhunapantula, Mosca et al. 2011). *AKT*-related pathway is also known to confer resistance to malignant cells against cisplatin treatment through an off-target resistance mechanism which takes place in two stages. *PI3K-AKT* signalling system is maintained at a baseline activity initially and there is an increase of cyclin-dependent kinase inhibitor 1A (*CDKN1A*) expression levels within the cell nucleus (Mitsuuchi, Johnson et al. 2000). The cisplatin-injured malignant cell may take advantage of a temporary *CDKN1A* induced cell cycle arrest during this period to start repairing the damaged genomic DNA (Mitsuuchi, Johnson et

al. 2000). In a second stage, the surviving malignant cells must continue the proliferation process (Galluzzi, Senovilla et al. 2012). This continued proliferation usually occurs through a subsequent increment of *PI3K-AKT* activity, which in turn leads to the nuclear rejection of *CDKN1A* (Mitsuuchi, Johnson et al. 2000; Ikeguchi and Kaibara 2001; Galluzzi, Senovilla et al. 2012). Once *CDKN1A* is outside the cell nucleus, it is not able to impose cell cycle arrest and therefore malignant cells resume proliferation (Galluzzi, Senovilla et al. 2012).

PI3K-AKT signalling system that is hyperactive is also responsible for the development of cancer cells with increased resistance to a wide spectrum of chemotherapeutic drugs (Gottesman 2002; Caracciolo, Laurenti et al. 2012) and radiotherapy (Fedrigo, Grivicich et al. 2011; Isebaert, Swinnen et al. 2011). Some of the anti-cancer drugs that become clinically ineffective comprise paclitaxel (Gagnon, Van Themsche et al. 2008; Levallet, Bergot et al. 2012), doxorubicin (Liu, Yan et al. 2013; Maxwell and Mousavi-Fard 2013; van Oosterwijk, van Ruler et al. 2013), gefitinib (Bodzin, Wei et al. 2012; Wang, Wang et al. 2012; Li, Zhou et al. 2013), imatinib (Isoyama, Dan et al. 2012; Puissant, Dufies et al. 2012; Wohrle, Halbach et al. 2013), and flavopiridol (Gomez, de Las Pozas et al. 2006; Caracciolo, Laurenti et al. 2012). *EGFR* signalling activates the *PI3K–AKT* pathway and promotes cell growth and survival by several mechanisms. *AKT* promotes cell survival by inhibiting pro-apoptotic B cell lymphoma 2 (*Bcl-2*) family members *Bcl-2*-associated death promoter (*BAD*) and *Bcl-2*-associated X protein (*BAX*) (Cantley 2002; Engelman, Luo et al. 2006). *AKT* also inhibits negative regulation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (*NF-κB*), leading to increased transcription of anti-apoptotic and pro-survival genes (Hresko and Mueckler 2005). Phosphorylation of human oncoprotein Mouse double minute 2 homolog (Mdm2) by *AKT* antagonises

tumour suppressor *p53*-mediated apoptosis, and *AKT* negatively regulates forkhead transcription factors, leading to the reduction of cell death-promoting proteins production (Duronio 2008). *AKT* also phosphorylates tuberous sclerosis protein 2 (*TSC2*), a tumour suppressor, thereby inhibiting the rheb trimeric G proteinase (*GTP*ase) activity of the *TSC1/TSC2* dimer. The activated rheb stimulates the mammalian target of rapamycin (*mTOR*)–containing protein complex *mTORC1,* leading to increased *p70* S6 kinase activity (Engelman, Luo et al. 2006). Activation of *mTOR*C1 results in increased protein synthesis by phosphorylation of eukaryotic initiation factor 4E and the ribosomal S6 protein (Engelman, Luo et al. 2006). Second *mTOR* complex, *mTORC2,* contributes to complete *AKT* activation by phosphorylating *AKT* on serine 473 (Hresko and Mueckler 2005; Sarbassov, Ali et al. 2006), leading to uncontrollable cell growth, proliferation and survival. One study has reported that the downregulation of *AKT3* significantly inhibits the growth of triple negative breast cancer cell lines in three-dimensional (3D) spheroid cultures and in mouse xenograft models, whereas loss of *AKT1* or *AKT2* have more modest effects (Chin, Yoshida et al. 2014). There are no known studies investigating the effect of *AKT3* inhibition in mCRCs. Inhibition of *PI3K-AKT* signalling in mCRC can diminish cell proliferation, and promote cell death. Consequently, components of this pathway present attractive targets for cancer therapeutics.

The results of this study suggests that other biomarkers within the *EGFR* signalling pathway seem to confer the same predictive value for lack of response to anti-*EGFR* antibody therapy that specific mutations in the *RAS* family genes (*KRAS* and *NRAS*) and some targets in the *PI3K-AKT* family seem to confer. The growing interest in discovering a panel of biomarkers of resistance to anti-*EGFR* treatment reinforces that this pathway has shown potential to add another class of therapeutic agents to the

management of mCRC in the near future. Currently, on-going research continues to unravel the complexities of the *EGFR* pathway, including its interaction with the *VEGF* pathway. The management of CRC is ever evolving, and has become more dynamic as increasing numbers of predictive biomarkers are identified. A select population of CRC patients stand to benefit from the panel of biomarkers of resistance as in the future, anti-cancer therapy will be increasingly tailored to specific tumour profiles, ensuring maximum effectiveness of the given treatment, and at the same time minimising any toxicities that may occur and lessen economic burden for the patient and healthcare system.

CHAPTER 5:
FINAL DISCUSSION

5.1 FINAL DISCUSSION

EGFR signalling is a complex pathway that is involved in the proliferation and survival of normal cells and alterations within this signalling pathway, such as gene amplification, gene mutations and aberrant protein expression, promote colorectal carcinogenesis. Identifying and defining the subpopulation of patients for *EGFR*-targeted therapy based on the molecular predictors of tumour response should be the strategy for future treatment decisions. *RAS* testing as a validated diagnostic tool used for selection of patients for *EGFR*-targeted treatment, is one of the most important recent advances and based on the current evidence, new definition of '*RAS* MT' should be adopted to include testing for mutations in both *KRAS* exons 2–4 and *NRAS* exons 2–4. Ultimately, ongoing research and subsequently a better understanding of the functional interactions within the *EGFR* signalling cascade will be essential, as it will not only provide biomarkers to select appropriate patients for therapy, but also provide additional therapeutic targets. Although *RAS* mutation status has been found to be a highly specific negative biomarker for response to anti-*EGFR* monoclonal antibodies, identification of WT status is unable to fully predict responsiveness, since not all WT *KRAS* patients derive a benefit from cetuximab or panitumumab. This study looked at 6 WT *KRAS*/WT *BRAF* CRC cell lines and identified biomarkers differentially expressed in the *EGFR*-resistant cell lines, and analysed the expression of these markers in 10 mCRC patients from clinical trials of cetuximab therapy. With increased patient sample size from clinical trials and the usage of high-throughput approaches such as Next Generation Sequencing platforms and RNA Seq analysis, more predictive biomarkers of resistance can be identified and validated. In addition, these platforms have good reproducibility, high sensitivity, and quantitative accuracy over a wide range. Antibody

and protein arrays are complementary and in some aspects preferable to separation-based and mass spectrometry-based technologies as the reproducibility and throughput can be higher, and the identities of the measured proteins are known or can be readily characterised (Ahn, Lee et al. 2014).

To explore the functionality of these biomarkers of resistance as therapeutic targets, siRNA technique was used to knockdown the overexpressed genes in the resistant CRC cell lines, validated them using qRT-PCR and western blot, and upon performing proliferation assay, the knocked down cells were more sensitive to the anti-*EGFR* treatment compared with the cells that were not knocked down. The applications of RNA interference can be mediated through two types of molecules; the chemically synthesised double-stranded small interfering RNA (siRNA) as we have used in this project or vector based short hairpin RNA (shRNA). siRNA-mediated RNA interference activity is reported to peak 24-72 hours post-delivery and diminishes within 96-120 hours which makes conducting functional assays such as proliferation, invasion, migration, angiogenesis and apoptosis assays challenging. shRNAs, as opposed to siRNAs, are synthesised in the nucleus of cells, further processed and transported to the cytoplasm, and then incorporated into the RNA-induced silencing complex (RISC) for activity (Cullen 2005). One limitation of using the siRNA technique is that it provides a transient gene knockdown that diminishes after 4 days, whereas shRNA can be continuously synthesised by the host cell and therefore its effect is much more durable especially when simultaneous knockdown of all 3 biomarkers are done and would be more conducive to conduct functional assays and animal studies. For effective knockdown, concentrations necessary usually in the low nM range for most siRNAs, while less than 5 copies of shRNA integrated in the host genome is sufficient to provide continual gene knockdown effect (Rao, Vorhies et al. 2009). Though shRNA seems

ideal for cancer-related therapeutic development as evidenced by studies that have used this technology in ovarian cancer (Marcotte, Brown et al. 2012), cervical and prostate cancer (Beheshti Zavareh, Sukhai et al. 2012), and breast cancer (Deng, Liu et al. 2014), future studies will benefit from new technology such as bi-functional RNA interference that may provide an even greater opportunity for enhancement in potency as well as heightening safety thereby increasing the opportunities for multiple target therapy. Following these, the establishment of animal models as evidenced by the study that reported downregulation of *AKT3* leads to significant inhibition of triple negative breast cancer in mouse xenograft models, (Chin, Yoshida et al. 2014), would also shed more light on the roles of these predictive biomarkers of resistance and their mechanisms of circumventing anti-*EGFR* treatment and conferring resistance.

In conclusion, the studies presented in this thesis have demonstrated that components of *EGFR* signalling cascade have emerged as important biomarkers of resistance for anti-*EGFR* targeted therapies. The roles in this pathway, their interaction and their synergistic activity or effect should be further investigated in pre-clinical and clinical settings, particularly in cancers that exhibit resistance to anti-*EGFR* monoclonal antibodies. Further assessment of the molecular mechanisms that dictate this resistance and identification of other specific biomarkers for these agents will provide valuable information to identify the most effective therapy for primary and mCRC patients.

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