[Frontiers in Bioscience, Landmark, 18, 394-421, January 1, 2013]

Prognostic significance and targeting of HER family in colorectal cancer

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1. ABSTRACT

Colorectal cancer is one of the leading causes of cancer deaths. At present, anti-epidermal growth factor receptor (EGFR) monoclonal antibodies (mAbs) cetuximab and panitumumab and anti-vascular endothelial growth factor (VEGF) mAb bevacizumab have been incorporated into treatment paradigms for patients with refractory metastatic colorectal cancer. However, many patients simply do not respond to these treatments or eventually acquire resistance following a short course therapy. In this article, we review the literature for studies on the expression patterns, prognostic significance and predictive value of HER (also called erbB) family members and other factors for response to therapy with the HER inhibitors in colorectal cancer. We discuss some of the advances, challenges as well as future opportunities for more effective targeting of the HER receptors using a cocktail of HER inhibitors (e.g. dual and pan HER TKIs, monospecific or bispecific antibodies) in combination with other therapeutic interventions.

2. INTRODUCTION

Despite major advances in the diagnosis, treatment and management of cancer patients, colorectal cancer is still a major health problem worldwide. In 2008, colorectal cancer was estimated to be the third most commonly diagnosed cancer (1.23 million) and the fourth leading cause of cancer deaths (608,000) worldwide (1). However, there are worldwide variations for both the incidence rates and mortality rates of colorectal cancer. The highest incidence rates for colorectal cancer are located in North America and Europe and the lowest rates in countries in Asia, Africa and South America (2). In 2012, colorectal cancer is estimated to be the fourth most commonly diagnosed cancer (143,460) but the second leading cause of cancer deaths (51,690) after lung cancer in the USA (3) . Although colorectal cancer diagnosed at an early stage has 5 year survival of about 90%, the majority of colorectal cancer patients are diagnosed with locally advanced or metastatic disease and have a poor response to conventional forms of therapy with a 5 year survival rates of 68% and 10% respectively (4, 5). Therefore, it is of prime importance to discover more specific biological and molecular markers that could be used not only for the early detection of colorectal cancer, but also to investigate their importance as prognostic indicators, predictive factors and therapeutic targets.

In the past forty years, metastatic colorectal cancer has been treated with fluoropyrimidine-based chemotherapy and more recently, drugs such as irinotecan and oxaliplatin have been introduced to this already established chemotherapeutic regimen. Although these treatments have increased time to progression and improved overall survival, they have certain drawbacks and adverse effects (6, 7). For example, one of the major limitation of these regimens is the non-specificity of the treatment which ultimately may cause various haematological disorders and increase the risk of infections (8). In the past twenty years, due to a better understanding of tumour biology and following demonstration of the aberrant expression and activation of growth factor receptor system in a variety of human tumours, there have been major initiatives in targeted therapy of human cancers using monoclonal antibody (mAb) based products and small molecule tyrosine kinase inhibitors (TKIs). Of several monoclonal antibody based drugs, anti-epidermal growth factor receptor (EGFR) mAbs cetuximab and panitumumab and anti-vascular endothelial growth factor (VEGF) mAb bevacizumab have been approved for the treatment of patients with refractory metastatic colorectal cancer (9-12). In this article, we will review the literature for studies on the expression patterns, prognostic significance and predictive value of the HER family members for response to therapy with HER inhibitors in colorectal cancer. We will discuss some of the advances and challenges together with future opportunities for the identification of a more specific population of colorectal cancer patients who are most likely to benefit from therapy with the HER inhibitors in combination with other therapeutic interventions.

3. THE TYPE I GROWTH FACTOR RECEPTOR AND THEIR LIGANDS

The type I growth factor receptor family of tyrosine kinases, also known as the ErbB or HER family, consists of four members namely EGFR (HER-1, ErbB-1), HER-2 (neu, ErbB-2), HER-3 (ErbB-3) and HER-4 (ErbB-4) (13-15). Of these, EGFR and HER-2 are the most extensively studied and best understood receptors in this family. The EGFR can be activated following the binding of several ligands such as epidermal growth factor (EGF), transforming growth factor alpha (TGFα), amphiregulin (AR), Heparin-binding betacellulin (BTC), and epiregulin (EPI) to its extracellular domain (16, 17). The binding of the cognate ligands to the external domain of the receptor induces the formation of homo- or hetero-dimers between different members of the HER family causing autophosphorylation of numerous tyrosine residues in its intracellular domain and ultimately the activation of the downstream signalling molecules such as the Ras, Raf, mitogen activated protein kinase (MAPK) cell proliferation pathways and the phosphatidylinositol 3 kinase (PI-3K)/Akt cell survival pathway (18) (Figure 1). Although MAPK and PI-3K/Akt pathways are the two major and most studied pathways activated by the EGFR, other pathways such as janus kinase and signal transducer and activator of transcription (JAK/STAT) and phospholipase C (PLC-γ)/Ca^{2+}/calmodulin-dependant kinases are also activated by EGFR and other members of the HER family (14). In the past few years, the role of the MAPK pathway in colorectal cancer has been studied extensively and in most studies K-RAS and B-RAF mutations have been associated with poor response to therapy with anti-EGFR mAbs and shorter survival and shorter progression free survival in patients with colorectal cancer (19). The biological consequences of EGFR activation include increased cell proliferation, reduced apoptosis, increased angiogenesis, migration, invasion and resistance to chemotherapy and radiotherapy which are the hallmarks of human cancer (20-26).

In the past 40 years, the aberrant expression and activation of different members of the HER family have been reported in a variety of human tumours and in some studies have been associated with resistance to the conventional forms of therapy and a poorer prognosis (27, 28) (also see Eccles *et al.* and Ioannou *et al.* in this issue). The aberrant activation of HER family members can be due to several mechanisms including mutations in the intracellular or extracellular domains of the receptor, overexpression of the wild-type receptor, overproduction of autocrine and paracrine ligands, homodimerisation and heterodimerisation between different members of the HER family or activation by a heterologus receptor such as IGF-IR (14, 29-36). In addition to the traditional pathways of HER signalling, several studies have reported the presence of intact HER family members such as EGFR, as a transcription factor, in the nucleus of proliferating normal and cancer cells. For example, nuclear EGFR has been detected in a number of human cancers including breast, bladder, pancreatic and colorectal, and nuclear expression of the EGFR has been associated with acquired resistance

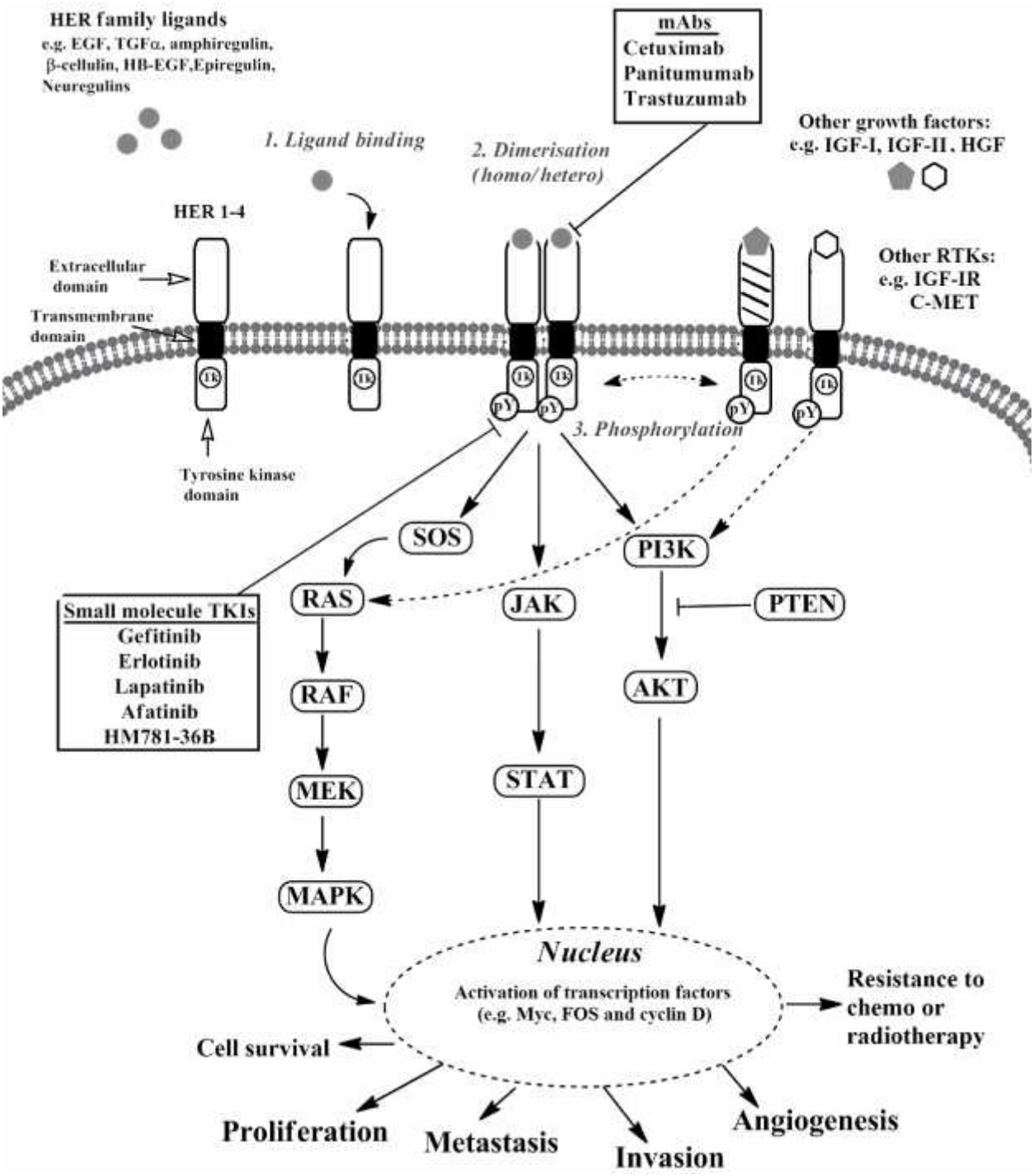

Figure 1. HER signal transduction pathway and various agents targeting different steps of the signalling pathway. Binding of growth factor ligands to HER results in the formation of homo- or heterodimers; leading to phosphorylation and activation of tyrosine kinase activity in the C-terminal domain of the receptors. This activation of downstream signalling pathways promotes cell proliferation and survival, differentiation, inhibition of apoptosis, angiogenesis, maturation, adhesion and invasion. TK, Tyrosine kinase domain; pY, phosphorylated tyrosine; HGF, hepatocyte growth factor.

to therapy, tumour aggressiveness and poor survival (37-43). This demonstrates the complex mechanisms by which HER signalling may be activated and the need for further studies to unravel the role of nuclear HER family members in the proliferation and progression of tumour cells as wells as its potential as a prognostic marker, predictive biomarker and therapeutic target in colorectal cancer patients (39, 40, 44-47).

Table 1. Studies investigating the expression pattern and prognostic significance of EGF receptor protein and gene amplification and its ligands in colorectal cancer

Study	Number of patients	Tumour type	Method of assessment (Marker)	Percentage expression (%)	Summary
(63)	92	Dukes' A-D	IHC (EGFR)	16.3	No significant correlation was found between EGFR expression and prognosis
(58)	32	Dukes' A-D	IHC (EGFR)	44	EGFR expression was found to be significantly higher in more advanced stage
(51)	82	Dukes' A-D	IHC (EGFR)	97.6	Strong EGFR staining intensity correlated with poor survival
(216)	102	Stage IV	IHC (EGFR)	75.5	EGFR expression correlates with disease relapse and death
(65)	249	Dukes' C and D	IHC (EGFR)	72.7	No significant correlation between EGFR expression in paired colorectal cancer tumours and lymph node metastasis
(89)	125	Dukes' A-D	IHC (EGFR)	53	Overexpression of EGFR was not significantly associated with shortened survival
(61)	99	Primary/met	IHC (EGFR)	53	No correlation between EGFR expression in primary tumour and related metastases
(48)	244	Stage 0-IV	IHC (EGFR)	8	Overexpression was frequently accompanied with gene amplification
(217)	80	Stage IV	IHC (EGFR)	80	Significant correlation between EGFR status and paired primary tumours and distant metastatic sites
(49)	150	Primary/met	IHC (EGFR)	97	EGFR was overexpressed and significantly associated with T3.
(218)	48	Primary	FISH (EGFR)	15	Loss of EGFR gene copy might be a surrogate marker for EGFR mutation
(99)	30	Metastatic	FISH (EGFR)	31	Significant number of patients with increased GCN showed a greater response rate.
(54)	158	Primary/met	IHC (EGFR) CISH (EGFR)	85 (primary) 79 (met)	Small fraction of EGFR positive tumours detected by IHC are associated with gene amplification
				12 (primary) 8 (met)	
(69)	87	Dukes' C	IHC (EGFR)	76	No significant association between EGFR expression and overall survival
(219)	32	Stage IV	IHC (EGFR)	84	EGFR expression in tissues sections from primary colorectal cancer and their related metastases were similar and frequent
(59)	154	Dukes' A-D	IHC (EGFR)	35.6	EGFR related to disease recurrence and worse prognosis in both univariate and multivariate analysis
(50)	27	Metastatic	IHC (EGFR) FISH (EGFR)	100 30	No significant correlation was found between EGFR protein expression and gene amplification
(100)	58	Metastatic	FISH (EGFR)	34.5	metastatic colorectal cancer patients with EGFR gene amplification are less likely to respond to treatment with panitumumab
(170)	130	Stage I-IIB	IHC (EGFR)	73	EGFR status variable between different metastatic sites
(66)	106	Primary/met	IHC (EGFR) IHC (AR)	12.3 54.7	Amphiregulin expression in primary lesion of colorectal cancer is an important predictive biomarker of liver metastases
(83)	124	Stage I-IV	IHC (EGFR)	60	EGFR expression did not correlate with stage of the disease or tumour differentiation
(97)	85	Metastatic	FISH (EGFR)	50.6	KRAS mutations are associated with cetuximab failure in EGFR FISH positive metastatic colorectal cancer, even if it does not preclude response
(220)	164	Stage I-IV	IHC (EGFR)	43.9	EGFR was found to be overexpressed and significantly associated with advanced T stage
(64)	109	Stage IIA-IIIC	IHC (EGFR)	57.8	No correlation between EGFR expression and disease relapse and overall survival
(55)	755	Metastatic	IHC (EGFR) FISH (EGFR)	61.7 15.3	EGFR GCN has no predictive value for response to treatment
(53)	101	Metastatic	IHC (EGFR) FISH (EGFR)	89 59	No correlation between the intensity of EGFR IHC and EGFR GCN amplification. Increase EGFR GCN was significantly associated with better clinical outcome, irrespective of KRAS status
(67)	120	Dukes' A-C	RT-PCR (EGFR) (AR) (EPI)	12.3 0.16 (median) 0.03 (median)	AR and EPI expression was associated with decreased survival. It might be a useful prognostic marker in KRAS wild-type patients who never received anti-EGFR therapy.

Abbreviations: IHC Immunohistochemistry, FISH fluorescence in situ hybridisation, CISH chromogenic in situ hybridisation, RT-PCR real-time polymerase chain reaction, AR amphiregulin, EPI epiregulin, met metastatic

3.1. Expression pattern and prognostic significance of EGFR and its ligands in colorectal cancer

The expression of EGFR has been reported in patients with colorectal cancer but ranges from 8 to 100% of cases examined (48-51) (Table 1). In one of the earliest studies Mayer *et al.* determined EGFR expression using immunohistochemistry (IHC) in 82 Dukes' A-D colorectal cancer patients and found a positive EGFR expression in 97.6% of the cases examined (51). Similarly, in another key study, Spano and colleagues determined EGFR expression by IHC in 150 colorectal cancer patients and found EGFR reactivity and EGFR over-expression in 97% and 80% of the cases respectively (49). In another study, Frattini and colleagues investigated EGFR over-expression by IHC and fluorescence *in situ* hybridisation (FISH) in 27 metastatic colorectal cancer patients and they found a positive EGFR expression in 100% (IHC) and 30% (FISH) of the cases examined (50) (Table 1). On the other hand, other studies have found the expression of EGFR in colorectal cancer to be much lower. Ooi and colleagues examined EGFR expression in a large cohort of 244 stage 0-IV colorectal cancer patients using IHC but found EGFR positive tumours in only 8% of the cases examined (48). In other studies, the EGFR status of the tumour was determined by measuring the EGFR gene copy number (GCN) and/or IHC. The EGFR GCN was determined using FISH,

chromogenic *in situ* hybridisation (CISH) and polymerase chain reaction (PCR). The expression of EGFR GCN for colorectal cancer also exhibits wide variation, ranging from 6 to 59% of the cases examined (52, 53) (Table 1). In one study, tumour specimens from 147 primary and metastatic colorectal cancer patients were examined for the expression of both EGFR protein and EGFR GCN. EGFR protein positive tumour was found in 85% and 79% of primary and metastatic colorectal cancer cases examined but EGFR gene amplification was detected in 12% of primary and 8% of metastatic cancer cases respectively. Interestingly, all tumours that were negative for EGFR protein were also negative for EGFR gene amplification (54) (Table 1). Moreover, in one of the largest and most recent study's to date, Jolien and colleagues investigated EGFR expression in 755 metastatic colorectal cancer patients using PharmDx kit and FISH. Tumours were considered EGFR positive when the membranous EGFR staining was present in more than 1% of tumour cells. They found 61.7% and 15.3% of the cases to be EGFR positive and to have EGFR gene amplification respectively (55). In contrast, the results of another study involving 101 metastatic colorectal cancer patients showed a higher percentage of the patients with both EGFR GCN amplification (59%) and expression of the EGFR protein (89%) (53) (Table 1). Some of the contributing factors for such a wide variation in the percentage of EGFR positive colorectal cancer cases could be the use of different anti-EGFR antibodies, different scoring system, different patient subpopulations and the small number of patients in some studies (33, 56, 57) (Table 1).

In some studies, the EGFR expression or increased gene copy number have been associated with a poor prognosis and other established prognostic indicators in colorectal cancer (49, 51, 58, 59) (Table 1). For example, in one study, Kluftinger and colleagues investigated the prognostic significance of EGFR in 32 colorectal cancer patients and found 44% of the cases to be EGFR positive and EGFR expression was associated with more advanced tumours and lympho-vascular invasion ($p < 0.05$) (58). In another study, Galizia *et al.* determined EGFR expression in 154 Dukes A-D colorectal cancer patients and found a significant association between EGFR expression and tumour node metastases ($p = 0.003$), nodal status ($p = 0.025$), presence of distant metastases ($p = 0.004$) and Dukes' stage ($p = 0.001$). They also found that colon cancer cells which had metastasized to a distant site expressed up to five times more EGFR mRNA when compared to Dukes' A tumour cells (59). More recently, in another study involving 386 stage I-IV colorectal cancer patients the variation in EGFR expression within tumours was investigated, comparing central parts to the invasive margin (60). The study found positive EGFR immunostaining in 46% of the central part compared with 58% in the invasive margins of the primary tumours. The increased score at the invasive margin compared to central parts of the tumours was found in 25% of the cases examined and was associated with a more aggressive behaviour and led to a survival disadvantage (60). In contrast, other studies did not find any association between the EGFR expression and prognosis (61-64). For example, McKay and colleagues examined tumour specimens from 249 Dukes' C and D colorectal cancer patients. While tumour specimens from 72.7% of the patients were EGFR positive, they did not find any significant association between the EGFR expression and overall survival. In addition, EGFR expression in primary and metastatic tumours was not significantly different (65). In another study, Yamada and colleagues determined the EGFR and AR expression in tumour specimens from 106 primary colorectal cancers and 16 metastatic liver lesions. They found 12.3% and 54.7% of the primary lesions to be AR and EGFR positive respectively. Interestingly, 81.6% of metastatic liver cancer cases were AR positive and AR expression in the primary colorectal cancer was found to be a predictive biomarker of liver metastasis (66). More recently, Kuramochi and colleagues examined AR and EPI mRNA expression level in tumour specimens from 120 colorectal cancer patients with liver metastasis (100 with synchronous metastasis, 20 with metachronous). They found a modest correlation between the AR and EPI expression in the primary tumour and liver metastasis but no significant survival difference between patients with low or high AR and EPI expression. However, in multivariate analysis, low EPI expression was found to be significantly associated with better overall survival in patients who never received anti-EGFR therapy (67) (Table 1).

Despite such a wide variation in the percentage of EGFR positive cases reported in the literature, the extracellular domain of EGFR is an ideal therapeutic target for anti-EGFR antibodies and IHC is the most commonly used method for the evaluation of the EGFR expression level and its cellular location. However, the lack of a uniform scoring system, the use of different antibodies, different patient populations and, as has already been noted, the small number of patients in some studies are considered to be some of the contributory factors for the wide variation reported for the expression pattern and percentage of EGFR positive cases in patients with colorectal cancer and the conflicting data on its prognostic significance (33) (Table 1). Therefore, in order to resolve the conflicting data on the expression pattern and prognostic significance of EGFR in patients with colorectal cancer, it is essential to conduct more detailed studies on the expression level of the EGFR ligands, various forms of EGFR (e.g. wild-type, mutated, soluble, phosphorylated EGFR) and their cellular locations (e.g. cytoplasmic, membranous or nuclear) and such studies should be conducted on a larger group of patients using a standard scoring system (39, 40, 44, 56, 68-71).

3.2. Expression pattern and prognostic significance of HER-2 in colorectal cancer

The human epidermal growth factor receptor-2 (HER-2), also known as *neu* or *c-erb-2*, is the second most extensively studied and understood member of the HER family. Many studies have shown the aberrant expression of HER-2 in a variety of human tumours including breast cancer and the anti-HER-2 antibody trastuzumab (Herceptin) has already been approved for the treatment of patients with HER-2 overexpressing early stage and metastatic breast cancer as well as in patients with metastatic stomach cancer (72, 73).

Table 2. Studies investigating the expression levels and prognostic significance of HER-2 in colorectal cancer

Study	Number of patients	Tumour type	Method of assessment	HER-2 expression (%)	Summary
(58)	32	Dukes' A-D	IHC	38	HER-2 positivity did not correlate with advanced stage, poor differentiation, vascular and lymphatic invasion
(88)	35	Stage I-IV	IHC	83	HER-2 and HER-3 may contribute to tumour growth and disease progression. Co-expression of HER-2 and HER-3 may suggest heterodimerisation
(89)	125	Dukes' A-D	IHC	35	HER-2 and HER-4 co-expression was significant associated with late stage
(221)	169	Stage I-IV	IHC FISH	3.6 2.4	Low prevalence of HER-2 gene amplification and protein overexpression suggests that this oncogenes plays an infrequent role in the development and progression of colon cancer
(81)	170	Dukes' B-C	IHC	87 (cyto) 54 (mem)	Significant association was observed between positive cytoplasmic expression and overall survival in Dukes' C colorectal cancer patients
(48)	244	Stage 0-IV	IHC	3	Overexpression was frequently accompanied with gene amplification
(222)	138	Metastatic	IHC	8	Low overexpression rate of HER-2 in advanced colorectal cancer limits the potential for further investigation of regimens involving trastuzumab
(62)	87	Dukes' C	IHC	89	No significant association between HER-2 expression and overall survival
(82)	77	Dukes' A-D	IHC	30	HER-2 expression was observed in colon cancer but rarely in therapeutic range. HER-2 is unlikely to play a major role in the therapeutic management of colorectal cancer
(76)	137	Stage I-IV	IHC FISH	47.4 1.45	HER-2 overexpression may constitute an independent prognostic factor in colorectal cancer patients
(83)	124	Stage I-IV	IHC	27.4	HER-2 overexpression was correlated with shorter survival
(79)	132	Dukes' A-D	IHC	13.6	Overexpression of HER-2 is not a predictor of outcome.
(64)	109	Stage IIA-IIIC	IHC	8.3	No correlation between HER-2 expression and disease relapse and overall survival
(223)	186	Dukes' A-D	FISH	26.3	HER-2 amplification could be one of the genes to be considered in the therapeutic management of colorectal cancer patients
(55)	755	Metastatic	FISH	11.5	HER2 GCN have no predictive value for response to treatment with cetuximab
(78)	202	Primary	IHC	66 (cyto) 27 (mem)	No significant correlation between HER-2 expression and survival
(80)	60	Primary node positive	IHC	1.8	HER-2 expression appears to be very rare in colorectal cancer

Abbreviations: IHC Immunohistochemistry, FISH fluorescence in situ hybridisation, CISH chromogenic in situ hybridisation, RT-PCR real-time polymerase chain reaction, cyto cytoplasmic, mem membranous

Several studies have reported the overexpression of HER-2 in colorectal cancer (74-76) (Table 2). However, like the expression pattern of EGFR described above, there is also a wide variation in the expression level of HER-2 reported in patients with colorectal cancer. For example, in one study involving 146 Dukes' B patients, HER-2 expression was demonstrated in 70% of the colorectal cancer cases (77). Similarly, Kruszewski *et al.* determined the expression levels of HER-2 in 202 colorectal cancer patients with 66% (cytoplasmic) and 27% (membranous) of the cases being HER-2 positive (78). In another study, Kavanagh and colleagues examined tumour specimens from 132 Dukes' A-D colorectal cancer patients and tumour specimens from 13.6% of the patients were HER-2 positive (79). More recently however, Wei and colleagues examined tumour specimens from 60 primary node positive colorectal cancer cases and found HER-2 expression to be rare (1.8%) in such patients (80) Table 2).

In the past two decades, several studies have also investigated the prognostic significance of HER-2 in patients with colorectal cancer (76, 78, 79, 81, 82). In one study, Essapen and colleagues examined 170 archival specimens of Dukes' B and C colorectal cancer using IHC and 87% of the cases showed cytoplasmic staining, but only 2% showed membranous staining for HER-2. Positive cytoplasmic immunostaining was found to be associated with a significantly better overall survival (HR 0.46, CI95 0.24-0.87) in the Dukes C cancers, but no survival benefit was seen in the Dukes' B cancers (81). In another study, Antonacopoulou *et al.* examined 124 stage I-IV colorectal cancer patients for the expression of HER-2. Tumours were considered HER-2 positive when there was only membranous reactivity. Based on this criteria, they found 27.4% of the cases to be HER-2 positive and HER-2 expression was associated with shorter survival (83). In contrast, other studies did not find any association between HER-2 expression and survival (64, 79) (Table 2). For example, in one study involving 132 Dukes' A-D colorectal cancer patients, there were no significant associations between HER-2 positivity and gender, age, grade, Dukes' stage, TNM stage, time to recurrence and 5 year survival. Moreover, HER-2 gene amplification did not correlate with the established prognostic indicators (79). In addition, in the study by Kruszewski and colleagues described above, they also did not find any correlation between either membranous or cytoplasmic expression of HER-2 and colorectal cancer patients survival (78) (Table 2).

3.3. Expression pattern and prognostic significance of HER-3 and HER-4 in colorectal cancer

The third and fourth members of the type I growth factor receptor family, HER-3 and HER-4 are found in many organs such as the stomach, lung, pancreas, and breast and they are known to play a key role in various pathological processes including cancer (84-87). However, in the past 2 decades, there have been very few studies on the expression level of HER-3 and HER-4 in colorectal cancer. As with the results from the studies noted above on the expression of EGFR and HER-2, the expression of HER-3 reported in colorectal cancer also exhibits wide variation, ranging from 16 to 89% of the cases examined (80, 88-91) (Table 3). In one study, Maurer *et al.* examined tumour specimens from 35 colorectal cancer patients and

Table 3. Studies investigating the expression levels of and prognostic significance of HER-3 and HER-4 and all members of HER family in colorectal cancer

Study	Number of patients	Tumour type	Markers assessed	Percentage Expression (%)	Summary
(88)	35	Stage I-IV	HER-2 HER-3	83 89	HER-2 and HER-3 may contribute to tumour growth and disease progression. Co-expression of HER-2 and HER-3 may suggest heterodimerisation
(91)	55	Dukes' A-C	HER-3	78	Absence of HER-3 expression was significantly associated with longer survival
(89)	125	Dukes' A-D	EGFR HER-2 HER-3 HER-4	52 35 36 22	HER-3 expression was common in early stage. Heterodimerisation between HER-2 and HER-4 may play a role in the late stages of carcinogenesis
(85)	106	Dukes' B-D	HER-3 HER-4	17 (mem), 28.3 (cyto) 18.9 (mem), 30.2 (cyto)	HER-4 membranous protein expression was found to predict for lymph nodes Positivity. HER-4 expression status may identify tumours with aggressive biological behaviour and increased metastatic potential
(92)	64	Stage I-III	EGFR HER-2 HER-3 HER-4	76 54 67 81	No significant difference between HER-3, and HER-4 expression in primary tumours and metastases. A significant increase of HER-3/HER-4 co-expression in late stage tumours.
(64)	109	Stage IIA-IIIC	EGFR HER-2 HER-3 HER-4	57.8 8.3 69.7 11 (mem), 19.3 (cyto)	Membranous positive HER-4 expression was an independent prognostic factor for recurrence. HER-3 negative expression was an independent prognostic factor for recurrence and survival in the multivariate analysis.
(80)	60	Primary/met	EGFR HER-2 HER-3	29 1.8 16	HER-3 expression was observed with a good concordance in primary tumour and lymph node metastasis and liver metastasis.

Abbreviations: mem membranous, cyto cytoplasmic, met metastatic

found 89% of the cases to be HER-3 positive (88). In contrast, other studies have found a lower percentage of HER-3 positive colorectal cancer cases. For example, Wei and colleagues determined HER-3 expression by IHC in 60 colorectal cancer patients and found only 16% of the primary tumours to be HER-3 positive (80).

The expression of HER-4 in colorectal cancer ranges from 11 to 81% of the cases examined (64, 85, 89, 92) (Table 3). However, there are a limited number of studies on the prognostic significance of HER-3 and HER-4 or co-expression of all members of the HER family in colorectal cancer (64, 85, 89, 91, 92) (Table 3). Kapitanović and colleagues examined HER3 expression in 55 Dukes A-C cancer patients and found patients whose tumours were HER-3 negative had a significantly longer survival (Kapitanović *et al.*, 2000). In contrast, Baiocchi *et al.* found an association between negative HER-3 expression and lymphovascular invasion and a poorer overall survival (64). There are also conflicting data on the expression of HER-3 at different stages of colorectal cancer and, as noted above for the other HER family members, it is suggested that such differences could be due to the usage of different antibodies, different scoring systems as well as differences in the number of patients at different stages of the disease (89, 92).

In the case of HER-4, there have only been four studies on its expression pattern and prognostic significance in patients with colorectal cancer to date (64, 85, 89, 92) (Table 3). In addition, only three studies have examined the expression of all members of the HER family in colorectal cancer (64, 89, 92) (Table 3). In 2002, Lee and colleagues compared the expression levels of all HER family members in 125 Dukes' A-D colorectal cancer cases and did not find overexpression of any of the HER family members alone to be significantly associated with shorter overall survival, but simultaneous overexpression of HER-2 and HER-4 was found to be significantly associated with a poorer overall survival (89). Baiocchi *et al.* examined 109 stage II-III colorectal cancer patients for the expression levels of HER-1 to HER-4. Interestingly, their study found membranous positive HER-4 expression as an independent prognostic factor for recurrence, while HER-3 negative expression was an independent prognostic factor for recurrence and poorer survival in the multivariate analysis(64). In addition, Ljuslinder and colleagues investigated the difference between the expression of HER1 to HER4 in primary and metastatic sites of 64 stage I-III colorectal cancer patients. They did not find any significant differences between HER-2, HER-3 and HER-4 expressions in primary and metastatic sites. Interestingly, while HER-3 and HER-4 co-expression was found to be significantly higher in late stage tumours, EGFR expression was lost in metastatic sites (92). More recently, we determined the expression level of all HER family members in a large panel of human colorectal tumour cell lines and found significant association between the co-expression of EGFR, HER-2 and HER-3 and response to treatment with an irreversible pan-HER blocker, afatinib ($P = 0.021$) (93).

The wide variation in the expression level of the HER family members reported in the literature in patients with colorectal cancer, as well as the limited number of studies on the expression level of all members of HER family and their ligands, warrant further investigations on the expression patterns and prognostic significance of all forms of HER family members and their ligands in both primary and metastatic lesions and such studies should ideally be conducted on a larger group of colorectal cancer patients.

Table 4. Summary table of EGFR family and IGF-IR inhibitors under preclinical and clinical investigation in colorectal cancer

Agents	Properties	Status	Reference/clinical trial
Antibodies			
Cetuximab/C225	Chimeric anti-EGFR (IgG1)	Approved for colorectal cancer, head and neck cancer	(104)
Panitumumab/ABX-EGF	Fully human anti-EGFR (IgG2)	Approved in 2006 for colorectal cancer	(111)
Nimotuzumab/h-R3	Humanised anti-EGFR (IgG1)	Phase II in colorectal cancer	NCT00972465
Necitumumab/IMC-11F8	Fully human anti-EGFR (IgG1)	Phase II in colorectal cancer	NCT00835185
ICR62	Rat anti- EGFR (IgG2b)	Preclinical/phase I in solid tumours	(93, 224, 225)
Trastuzumab/Herceptin	Humanised anti-HER-2 (IgG1)	Approved for breast cancer Phase II in metastatic colorectal cancer	(146)
Pertuzumab/2C4	Humanised anti-EGFR and HER-2	Phase I/II in colorectal cancer	NCT00551421
MM-121	Fully human anti-HER-3	Phase I in solid tumours	NCT01451632
MM-111	Bispecific anti-HER-2 and HER-3	Phase I in solid tumours	NCT00911898
EI-04	Bispecific anti-EGFR and IGF-IR	Preclinical in colorectal	(215)
Figitumumab/CP-751,871	Fully human anti-IGF-IR	Phase I in colorectal cancer	(226)
Dalotuzumab/MK-0646	Humanised anti-IGF-IR	Phase II in colorectal cancer	(227)
Ganitumab/AMG479	Fully human anti-IGF-IR (IgG1)	Phase II in colorectal cancer	NCT00819169, NCT00813605
Cixutumumab/IMC-A12	Fully human anti-IGF-IR (IgG1)	Phase II in colorectal cancer	(228)
h7C10	Humanised Anti-IGF-IR	Preclinical in	(214)
Small Molecules			
Gefitinib/Iressa	Reversible anti-EGFR	Phase II in colorectal cancer Approved for NSCL cancer	(122)
Erlotinib/OSI-774	Reversible anti-EGFR	Phase II in colorectal cancer Approved for NSCL cancer and pancreatic cancer	(123)
Afatinib/BIBW-2992	Irreversible ErbB family blocker	Phase II in colorectal cancer	(155), NCT01152437
Canertinib/CI-1033	Pan HER inhibitor	Preclinical in colorectal cancer	(229)
HM781-36B	Pan HER inhibitor	Phase I solid tumours	NCT01455584, NCT01455571
Lapatinib/GW572016	Reversible anti-EGFR and anti-HER-2	Approved for Breast Cancer Phase II in colorectal cancer	(152)
BMS-599626	Pan HER inhibitor	Phase I in solid tumours	(162), NCT00095537
EKB-569	Irreversible anti-EGFR	Phase I/II in colorectal cancer	NCT00072748
NVP-AEW541	anti-IGF-IR	Preclinical in colorectal cancer	(230)
OSI-906	anti-IGF-IR and IR	Preclinical in advanced solid tumours	NCT01154335, NCT01016860
BMS-754807	anti-IGF-IR and IR	Phase I/II in colorectal cancer	NCT00908024
BMS-536924	anti-IGF-IR and IR	Preclinical in solid tumours	(213)

4. TARGETING OF HER FAMILY MEMBERS IN COLORECTAL CANCER

4.1. EGFR targeted therapy In the past two decades, there has been a rapid increase in the targeted therapy of human cancers. In particular, aberrant expression and activation of EGFR in a variety of tumours has stimulated the development of several EGFR inhibitors for use in cancer therapy. Of these, anti-EGFR mAbs cetuximab and panitumumab have already gained the approval of the US Food and Drugs Administration (FDA) for the treatment of patients with metastatic colorectal cancer in combination with chemotherapy (9, 10, 50, 52, 94-100). Small molecule TKIs have been extensively studied in colorectal cancer and in addition have been approved for treatment of patients with non-small cell lung cancer (gefitinib and erlotinib) and pancreatic cancer (erlotinib) (101-103). There are many other types of EGFR inhibitors including the dual EGFR/HER-2 TKI and pan HER TKIs that are at different stages of preclinical and clinical evaluations and some of these will be discussed here (Table 4).

4.1.1. Anti-EGFR mAbs

4.1.1.1. Cetuximab (Erbitux®)

Cetuximab is an IgG1 chimeric monoclonal antibody that binds selectively to the external domain of EGFR and inhibits binding of the ligands to the receptors (9, 104), (also see Fan *et al.* in this issue). This in turn prevents the activation of the receptor's tyrosine kinase activity and downstream cell signalling pathways (9). Since cetuximab is an IgG1 antibody, it can also induce anti-tumour activity *in vivo* by mediating antibody dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) (also see Fan *et al.* in this issue).

The pivotal BOND trial conducted by Cunningham and colleagues highlighted the benefit of cetuximab in chemo-refractory disease (94). This study included 329 randomly selected patients with disease progression during or within 3 months after treatment with irinotecan. The patients were subsequently randomised into two groups of 218 patients receiving combination therapy with cetuximab and irinotecan and 111 patients for cetuximab monotherapy. The study found the overall response rate in the intention-to-treat population to be 22.9% in combination therapy versus 10.8% found in the monotherapy group (P=0.007). The median time to progression was also noted to be longer in patients receiving the combination therapy of both drugs at 4.1 months compared to 1.5 months for those receiving cetuximab alone. However, there was no significant difference in the median overall survival rate (8.6 months versus 6.9 months, P=0.48). There were no significant adverse reactions to the combination therapy reported in this study. However, in 4 of the 329 patients severe anaphylactic reactions to cetuximab were noted and the treatment was stopped. In December 2004, as a result of the BOND data, cetuximab gained approval by the FDA for use in metastatic colorectal cancer patients whose tumours were refractory to irinotecan based therapy (9). Recent studies have shown that the effectiveness of cetuximab is limited to those patients whose tumours express wild-type k-RAS. In approximately 40% of colorectal cancer cases

KRAS is found to have a mutation which is associated with poor prognosis (105).

The addition of cetuximab to standard chemotherapy in patients with k-RAS wild type metastatic liver disease secondary to colorectal cancer, can lead to a significantly increased response rate, leading to an increased liver resection rate. The CELIM study (106) was a randomised phase II study which compared tumour response and resectability rates in patients with initial unresectable liver metastases, treated with standard chemotherapy with or without cetuximab. The response rate for tumours was significantly higher with the addition of cetuximab in the k-RAS wild-type group versus the mutant k-RAS group (70% vs 41%, p=0.008). This translated into a higher resection rate of liver metastases from 32% at baseline to 60% after chemotherapy ($p<0.0001$). This study confirmed the increased response rates, reported by other investigators, with the addition of cetuximab to either FOLFOX or FOLFIRI standard chemotherapy. The CRYSTAL trial (107) randomised 1217 patients to FOLFIRI with or without cetuximab as first line treatment for metastatic colorectal cancer. This group confirm the findings of previous studies (94) of significantly improved median progression free survival with the addition of cetuximab (8.9 months versus 8 months for FOLFIRI alone). Furthermore, the study also noted a higher objective response rate (47% versus 39%) in patients treated with cetuximab in combination to FOLFIRI compared to FOLFIRI alone. However, the benefit was observed in patients with liver only metastases. Moreover, further studies such as the EPIC trial conducted by Sobrero *et al.* (108) evaluated the role of cetuximab in patients previously untreated with irinotecan and further in the first line metastatic setting. The study randomised 1298 EGFR-positive patients failing an oxaliplatin based first line regimen to either irinotecan or cetuximab combined with irinotecan. As a result, study found significant improvement in progression free survival with the combination compared to the single agent therapy of irinotecan (3.9 months versus 2.56 months). In addition, response rates were also noted to be significantly higher at 16% compared to 4% in patients treated with irinotecan alone.

In August 2009, NICE (UK) agreed to the use of cetuximab in addition to either FOLFOX or FOLFIRI chemotherapy in patients with liver metastases only, whose tumours are k-RAS wild-type, and in whom the metastases cannot be surgically removed, without significant down staging with primary chemotherapy.

4.1.1.2. Panitumumab (Vectibix®)

Unlike the chimeric IgG1 anti-EGFR mAb cetuximab, panitumumab is a fully human anti-EGFR mAb of IgG2 subclass (109, 110) (Table 2). As a result of a large, multinational, randomised, open label study conducted by Van Cutsem *et al.* panitumumab was approved by the US FDA for the treatment of metastatic colorectal cancer that is refractory to 5-Flouraracil, oxaliplatin, and irinotecan based regimens. This phase III study included 463 patients, treated with panitumumab plus best supportive care (BSC) (231 patients) or BSC alone (232 patients). The study reported a 46% reduction in the relative progression rate of the disease compared to the patients receiving only BSC. In addition, mean progression free survival time was higher in those receiving panitumumab than BSC alone (13.8 weeks versus 8.5 weeks), while no significant difference in overall survival between the groups were observed (111).

In 2007, Hecht *et al.* conducted a multicenter, non- randomised phase II study. The study included 148 patients with metastatic colorectal cancer refractory to fluoropyrimidines, oxaliplatin, and/or irinotecan. The patients were stratified into two groups according to staining intensity of high and low EGFR positivity (105 and 43 respectively). The study reported overall response of 9%, and stable disease was observed in 29% of patients, while progression free survival was 14 weeks and median overall survival being 9 months (112). In addition, several studies have evaluated the role of panitumumab in combination with other classic chemotherapy regimens as first-line treatment in metastatic colorectal cancer. However, the data available is limited as most of these studies are still ongoing. The PRIME study is one such study conducted by Siena *et al.* (113). It is the first phase III study to investigate the role of panitumumab in combination with FOLFOX4 as first-line treatment in metastatic colorectal cancer. The study enrolled 1,150 untreated patients randomised into two groups to receive panitumumab plus FOLFOX4 or FOLFOX4 alone. Progression free survival was the study's primary endpoint. In a pooled safety analysis, 903 patients were treated, with neutropenia (28%) and diarrhoea (11%) being the two most common adverse effects of the treatment. In addition, 93 patients (10%) reported a grade 3 skin reaction and only 3 patients experienced a grade 4 skin reaction. Similarly, in another ongoing multicenter phase III study the role of panitumumab in addition to FOLFIRI as a second-line treatment is being investigated (114). The study included 1,100 patients previously treated with only one fluoropyrimidine based chemotherapy regimen for metastatic colorectal cancer randomised into two groups receiving panitumumab plus FOLFIRI or FOLFIRI alone. The findings of the preliminary safety yet again identified neutropenia (15%) and diarrhoea (9%) to be the two most common adverse effects. Furthermore, 12% of the patients reported a grade 3 skin reaction while grade 4 skin toxicities were seen in <1% of the patients.

4.1.1.3. Necitumumab (IMC-11F8)

IMC-11F8 is another fully human anti-EGFR mAb. The mechanism of action of IMC-11F8 is similar to that of cetuximab. Interestingly, while both cetuximab and IMC-11F8 bind to the same domain with the same binding affinity, IMC-11F8 has been found to have similar anti-tumour potency but fewer side effects in comparison (115, 116). IMC-11F8 has been shown to block the phosphorylation of EGFR and MAPK in several tumour cell lines. The activity and efficacy of IMC-11F8 has been evaluated in various *in vivo* models including colon human tumour xenografts in athymic mice. Treatment with IMC-11F8 caused a dose dependent growth inhibition of tumours

in mice models. In addition, combination of IMC-11F8 with irinotecan was found to have a superior anti-tumour effect in colon xenograft models (DLD-1, GEO, HT29) (117). More recently, a phase I study of sixty patients with advanced solid malignancies treated with IMC-11F8 found the antibody to be well tolerated and a partial response and stable disease was noted in 2 and 16 patients respectively (116). It is currently in phase II clinical trials in patients with metastatic colorectal cancer patients (NCT00835185).

4.1.1.4 Other anti-EGFR mAbs

Several other anti-EGFR mAbs (e.g. nimotuzumab, ICR62) have also been produced which are at different stages of pre-clinical and clinical evaluations (Table 4). ICR62 is a rat IgG2b anti-EGFR mAb and has been raised against the extracellular domain of the EGFR on a breast carcinoma cell line. MAb ICR62 was shown to be very effective in 1) inhibiting the binding of EGFR ligands (e.g. EGF, TGFα, HB-EGF, betacellulin, epiregulin) to the receptor, 2) blocking the ligand-induced tyrosine phosphorylation of the EGFR, 3) inhibiting the growth of several EGFR-overexpressing human tumour cell lines [e.g. colorectal (DiFi), head and neck (HN5)] both *in vitro* and *in vivo* by inducing G1 cell cycle arrest, apoptosis and terminal differentiation and 4) localizes efficiently to metastatic lesions in cancer patients (93, 118-120). By inducing antibody dependent cellular cytotoxicity (ADCC), ICR62 was also found to be very effective in the inhibiting the metastasis to the lung of cells containing EGFR mutation (i.e. EGFRvIII) in athymic mice (121) and the production of a humanised version of this antibody is currently planned (Table 4).

4.1.2. EGFR Tyrosine kinase inhibitors (TKIs)

Another class of antigen specific drugs with potential for the treatment of human cancer are the small molecule TKIs. TKIs are directed against the intracellular ATP-binding site of the EGFR tyrosine kinase domain and upon binding, they may inhibit the tyrosine phosphorylation of such receptors through reversible or irreversible mechanisms (Figure 1). EGFR TKIs have been shown to be effective in various tumour types, including lung and pancreatic cancer (122, 123). Furthermore, these agents are being extensively studied in colorectal cancer.

4.1.2.1. Gefitinib (Iressa)

Gefitinib is the first orally active, selective inhibitor of EGF that has gained approval of the US FDA for the treatment of advanced non-small cell lung cancer (122) (Table 4). Preclinical studies in colorectal cancer have demonstrated that gefitinib not only inhibited the cell growth of human colorectal tumour cells but also induced down regulation of EGFR, reduction in the basal levels of pEGFR, pMAPK, and pAKT (118). In the past decade, several clinical studies have investigated the efficacy of gefitinib against colorectal cancer (124-132). In one of the earliest phase I/II clinical studies, gefitinib in combination with oxaliplatin was evaluated in 14 patients with advanced colorectal cancer and was found to be inactive in such patients (133). In another randomised phase II trial Rothenberg *et al.* studied the effect of two different doses of gefitinib (250 or 500mg orally once a day) in 110 patients with recurrent colorectal adenocarcinoma. They found gefitinib to be inactive as a single agent in patients with previously treated colorectal cancers (134). Similar results were reported in several other phase I/II clinical trials (135-138). More recently, a randomised phase II study conducted by Vietez and colleagues investigated the efficacy of raltitrexed, a quinazoline antifolate inhibitor of thymidylate synthase, approved for the treatment of colorectal cancer in the EU, in combination with gefitinib as a second line therapy in 76 metastatic colorectal cancer patients. The study found no significant difference in progression free survival and overall survival (124). In contrast, two phase II studies investigated the efficacy of gefitinib in combination with 5-FU, leucovorin, and oxaliplatin in colorectal cancer patients. In the first study, of the 35 metastatic colorectal cancer patients who received treatment, complete response was seen in 40% and disease stability in 80% with a median survival time of 21.9 months (127). The second study enrolled 45 metastatic colorectal cancer patients. Based on the RECIST criteria 72% of the cases were shown to have either complete or partial response to treatment with median overall survival time of 20.5 months (126). To our knowledge, there are currently no ongoing clinical trials with gefitinib in patients with colorectal cancer.

4.1.2.2. Erlotinib (Tarceva™)

Erlotinib is a reversible inhibitor of the EGFR tyrosine kinase domain, currently approved by the US FDA for the treatment in non-small cell lung carcinoma and pancreatic cancer and is at an advance stages of clinical development in a wide range of cancers including colorectal cancer (123, 139) (Table 4) .

The role of erlotinib in colorectal cancer has been investigated in a large number of clinical studies. Unlike gefitinib, various studies have reported some clinical activity of erlotinib as a single agent (140, 141). Several other clinical studies have been conducted to evaluate erlotinib in combination with standard chemotherapy regimens in colorectal cancer. The efficacy of erlotinib in combination with capecitabine and oxaliplatin was determined in a phase II study in 32 patients who were previously treated for metastatic colorectal cancer. They observed partial response in 25% and stable disease in 44% suggesting the therapeutic potential of such combination in metastatic colorectal cancer patients who had received prior chemotherapy (142). In another phase II trial Meyerhardt and colleagues investigated the effect of erlotinib in combination with FOLFOX and the anti-VEGF mAb bevacizumab as first line therapy in 35 patients with metastatic colorectal cancer. However, due to higher level of toxicity, the study could not reach conclusions regarding the efficacy of such combination (103). In another phase I trial, Messersmith and colleagues investigated the MTD of erlotinib in combination with FOLFOX4 and bevacizumab 15 advanced colorectal adenocarcinoma patients. They found a response rate of 78% with median progression free survival of 9.5 months and median overall survival of 30 months (143). The MTD of erlotinib with FOLFOX4 with or without bevacizumab was found to be 100mg daily and despite increased toxicity it did show antitumour activity.

More recently, Weickhardt and colleagues evaluated the efficacy of the dual targeting of EGFR using the combination of cetuximab and erlotinib in both preclinical and the Phase II DUX study in patients with chemo-refractory advanced colorectal cancer. Interestingly, the combination of cetuximab and erlotinib had resulted in synergistic growth inhibition of colon cancer cell lines as a result of enhanced inhibition of the EGFR pathway and differential effects on STAT3. Of 48 patients evaluated, the overall response rate was 31% with a median progression free survival of 4.6 months and mainly in patients with wild type KRAS (144). In addition, the study reported far fewer adverse effects in comparison to some of the previous studies which investigated the combination of erlotinib with standard chemotherapeutic agents (144). Several clinical trials with erlotinib in combination with anti-EGFR mAbs, bevacizumab and cytotoxic drugs are currently underway (clinicaltrials.org NCT00940316, NCT01229813, NCT01416688).

4.2. HER-2 and HER-3 targeted therapy

As noted earlier, overexpression of HER-2 and HER-3 has been reported in a variety of solid tumours including colorectal cancer (table 2 & 3). Such receptors are ideal targets for ttherapy with onoclonal antibodies, small molecule tyrosine kinase inhibitors in combination with otherapeutic interventions.

4.2.1. Trastuzumab (Herceptin)

Trastuzumab is a humanised monoclonal antibody that binds to the extracellular domain of the HER-2 receptor. Currently, it is approved for the treatment of patients with HER-2 overexpressing breast and stomach cancers (73). In colorectal cancer, a number of preclinical studies have investigated the anti-tumour activity of trastuzumab. However, very few studies have been conducted in a clinical setting with colorectal cancer patients. In one of the earliest preclinical studies, a panel of human colorectal tumour cell lines were treated with trastuzumab (mAb 4D5) and cetuximab as single agent and in combination (145). While the relative levels of HER-2, EGFR, HER-3 and HER-4 were not predictive of responsiveness to anti-HER-2 mAb 4D5, treatment with the HER-2 antibody caused a decrease in HER-2 protein levels in all of the colon cancer cell lines and also significantly decreased EGFR levels but only in the EGFR-dependent cell lines. In addition, treatment with a combination of HER-2 and EGFR antibodies caused large areas of necrosis in EGFR-dependent colon cancer xenografts supporting the superior therapeutic potential of such a combination (145). More recently in another study trastuzumab has been used in the treatment of metastatic colorectal cancer patients harbouring HER-2 gene amplification. The study found a marked radiographic response to the trastuzumab (146). However, the full therapeutic potential of trastuzumab in colorectal cancer has not yet been fully explored and warrants further investigations.

4.2.2. MM-121

Due to very weak tyrosine kinase activity, HER-3 was previously not considered as an important therapeutic target as the EGFR and HER-2. However, recent studies have demonstrated a key role of HER-3 in the activation of PI3K/AKT signalling pathway in EGFR, HER2, and the hepatocyte growth factor receptor, MET, addicted tumours and in particular resistance to HER inhibitors (90, 147-149).

MM-121 is a fully human monoclonal antibody that has entered clinical development as the first selective HER-3 antagonist (Table 4). In a recent study, MM-121 was found to effectively ligand-dependent activation of HER-3 induced by either EGFR, HER2, or MET and to abrogate resistance to anti-EGFR therapies by preventing reactivation of ErbB3 (150). These results suggest that the therapeutic potential of MM-121 in cancers with ligand-dependent activation of HER-3 (150). In another study, MM-121 was also shown to inhibit the growth *in vivo* of ovarian tumours (151). Currently, several clinical studies of MM-121 are underway in a range of solid tumours including colorectal cancer (clinicaltrials.gov NCT01451632).

4.3. Dual and Pan-HER Inhibitors of EGFR family members

The development of resistance to HER targeted monotherapy and the relatively small population of patients whose tumours are sensitive to treatment with a single HER blocker, as well as the co-expression of multiple members of the HER family in a varity of human cancers has stimulated research on the development of dual and pan inhibitors of HER family members and these are discussed in the following sections (Table 4).

4.3.1. Lapatinib

Lapatinib is an orally administered reversible dual receptor tyrosine kinase inhibitor of EGFR and HER-2/neu. Currently, lapatinib has been approved for the treatment of HER-2 over-expressing chemorefractory breast cancer patients (152). However, it is also under clinical investigation in other solid tumours including colorectal cancer (Table 4). Several studies have reported the therapeutic potential of lapatinib as a single agent and in combination with other chemotherapeutic agents. For example, lapatinib was found to potently inhibit the growth of a HER-2 over-expressing gastric cancer cell line and showed moderate activity in colon cancer. In addition, in combination with SN-38, the active metabolite of irinotecan, lapatinib produced synergistic growth inhibition of all human colon and gastric cancer cell lines (153). Furthermore, lapatinib was found to downregulate thymidylate synthase, which is frequently over-expressed in fluoropyrimidine resistant cancer cells, through inhibition of nuclear translocation of EGFR and HER-2 and ultimately re-sensitising such cancer cells to fluoropyrimidines (154). Most recently, a phase I/II study of lapatinib in combination with oxaliplatin and capecitabine were conducted in a small group of 12 advanced colorectal cancer patients. The full analysis is appending (clinicaltrials.gov NCT00536809).

4.3.2. Afatinib (BIBW-2992)

Currently under clinical development in solid tumours including colorectal cancer, Afatinib is an example

of an irreversible pan-HER tyrosine kinase inhibitor (155) . In experimental models, afatinib was shown to be an effective and selective irreversible inhibitor of EGFR and HER-2/neu (156). In addition, afatinib was found to inhibit the total tyrosine phosphorylation of the EGFR and HER-2/neu and tumour cell proliferation *in vivo* (157).

More recently, we examined the effect of afatinib, as a single agent or in combination with cytotoxic drugs (5-fluorouracil, irinotecan and oxaliplatin) or anti-EGFR mAb ICR62 on the proliferation of a panel of human colorectal tumour cell lines. Afatinib inhibited the growth of EGFR-overexpressing DiFi cells (with an IC50 value of 45 nM) and other tumour cell lines with IC50 values which ranged from 0.33 μM (CCL-221) to 1.62 μM (HCT-116). There was a significant association between the co-expression of EGFR, HER-2 and HER-3 and response to treatment with afatinib (R=0.915, P=0.021) (93). In a phase II clinical trial involving 46 metastatic colorectal cancer patients afatinib produced disease-stability in 43.5% of the patients with an acceptable safety profile (158). In another recent phase I trial 53 patients received afatinib at 10 to 50mg/day. The recommended phase II dose was found to be 50mg/d and confirmed partial response was seen in 3 out of 53 patients and this was sustained for up to 34 months. A further 7/53 (13.2%) patients had disease stabilisation lasting ≥ 6 months (159). Currently, afatinib is under clinical investigation for treatment of a number of cancers including colorectal cancer (clinicaltrials.gov NCT01152437, NCT00906698)

4.3.3. HM781-36B

HM781-36B is a quinazoline-based irreversible pan-HER inhibitor and one of the latest agents to enter preclinical and clinical development for a range of human cancers. Results of the preclinical studies investigating the efficacy of HM781-36B appear to be very promising. In a recent study, the anti tumour activity of HM781-36B in combination with cytotoxic drugs was investigated in gastric cells *in vitro* and *in vivo*. HM781-36B was found to inhibit phosphorylation of all HER family members and downstream molecules accompanied with apoptosis and G1 arrest. In addition, treatment with HM781-36B in combination with chemotherapy agents was found to be superior to treatment with the single agent (160). More recently, HM781-36B was shown to be highly effective in inhibition the growth *in vitro* of the erlotinib resistant non-small cell lung carcinoma cells as well as other EGFR dependent cancer cells (161). Currently there are two ongoing phase I clinical studies of HM781-36B in advanced solid malignancies including colorectal cancer (clinicaltrials.gov, NCT01455584, NCT01455571).

4.3.4. Other anti-HER inhibitors

In addition to the therapeutic agents discussed above, several other mAbs and TKIs are currently at different stages of development in colorectal cancer. These agents include irreversible anti-EGFR TKIs such as EKB-569, pan TKIs such as BMS-599626 (AC480) (Table 4). For example, BMS-599626 was found to inhibit the growth of a panel of human tumour cell lines and tumour xenografts by inhibiting the formation of homodimers and heterodimers between EGFR and HER-2 (162). In another phase I trial, EKB-569 in combination with capecitabine in patients with advanced colorectal cancer, disease stability was noted in 48% of the patients (163). Furthermore, other inhibitors such as bi-specific mAb MM-111, which targets HER-2 and HER-3, is currently in phase I clinical studies for HER-2 amplified solid tumours (164)(clinicaltrials.gov, NCT00911898). These new generations of pan-HER inhibitors or bispecific antibodies should therefore have potential in the treatment of colorectal cancer patients whose tumour co-expresses different members of the HER family (Table 3 and 4). Several clinical trials are currently ongoing or have just been completed, but the results have not yet been published (clinicaltrials.gov, NCT00072748, NCT00095537).

5. PREDICTIVE MARKERS FOR RESPONSE OR RESISTANCE TO THERAPY WITH ERBB INHIBITORS IN COLORECTAL CANCER

The EGFR is an established therapeutic target in patients with colorectal cancer and the anti-EGFR mAbs cetuximab and panitumumab have been found to improve the overall survival in patients with metastatic colorectal cancer. However, many patients simply do not respond to these targeted treatments or eventually acquire resistance after a short period of time. In the following sections, we will discuss some of the predictive markers and mechanisms of resistance to EGFR targeted therapy in colorectal cancer (33, 165).

5.1. Predictive value of EGFR protein expression measured by IHC

As discussed above (section 3.1), EGFR protein expression has been reported in a large majority of studies in colorectal cancer. However, to date, there has been no clear association between EGFR protein expression and response to therapy (94, 111, 112, 166, 167). It is considered that a wide range of factors may contribute to the conflicting data on the EGFR expression and its prognostic significance in colorectal cancer, thus influencing its predictive value. Of these, the use of a particular primary antibody and various scoring systems are known to play a crucial role behind the wide variation in the EGFR protein expression and lack of correlation with response to therapy. For example, the primary antibodies (e.g. Dako PharmDx Kit) which have been routinely used in the IHC determination of EGFR and its predictive value for response to therapy with anti-EGFR inhibitors has been shown to bind both wild-type EGFR and the mutated form of this receptor (i.e. EGFRvIII) (56, 168). In addition, the majority of these studies have adopted a scoring system in which tumours with >1% of membranous staining were considered EGFR positive (i.e. nearly 100% of the cases to be EGFR positive) and hence contributing to the lack of association between EGFR expression and response to therapy (94, 166, 167, 169). Furthermore, there has been conflicting data on EGFR expression in the primary colorectal tumour and their related metastatic lesions (33, 61, 92, 169, 170)) Therefore, further studies of tumour specimens from the primary colorectal tumours and their related metastatic sites as well as tumour endothelial cells with anti-EGFR primary antibodies which are specific for

Table 5. Predictive biomarkers of response to HER inhibitors

Markers of Sensitivity	**Target antigen**	**Inhibitor**	**References**
L858R	EGFR	Gefitinib/Erlotinib	(173, 231)
Amphiregulin	EGFR	Cetuximab	(231)
Epiregulin	EGFR	Cetuximab	(52, 182, 231)
PTEN	EGFR	Cetuximab/Erlotinib	(203, 232)
IGF-IR	EGFR	Gefitinib	(210)
EGFR gene amplification	EGFR	Cetuximab	(180, 233)
Markers of resistance	**Target antigen**	**Inhibitor**	**Reference**
T790M	EGFR	Gefitinib/Erlotinib	(174, 234)
KRAS	EGFR	Cetuximab/Panitumumab	(165, 188, 235, 236)
S492R	EGFR	Cetuximab	(177)
BRAF	EGFR	Cetuximab	(19, 203)
PIK3CA	EGFR, HER-2	Cetuximab/Trastuzumab/ Lapatinib	(197, 237)
IGF-IR	EGFR	Cetuximab	(209)
HER-3	EGFR	Cetuximab	(147)
NF-κβ	EGFR	Cetuximab	(147)
HER-3	HER-2	Trastuzumab	(238)

the wild type EGFR and a uniform scoring system are warranted and should unravel the true potential of the EGFR protein expression as a predictive marker of response to therapy with anti-EGFR inhibitors in patients with colorectal cancer (56, 171).

5.2. Predictive value of EGFR mutations and EGFR gene amplification

Mutations of HER family members, in particular mutations in the extracellular (e.g. EGFRvIII) and intracellular (i.e. tyrosine kinase) domains of the EGFR, have been reported in a wide range of solid tumours and in some studies have been associated with response or resistance to therapy with the small molecule EGFR tyrosine kinase inhibitors. For example, treatment of non-small cell lung cancer patients containing exon 19 deletion or L858R EGFR mutations with the EGFR TKIs erlotinib and gefitinib erlotinib resulted in a significantly longer progression free survival compared to patients with wild type EGFR (p= 0.02) (172, 173) (Table 5). In contrast, a secondary mutation in the EGFR gene (T790M) was associated with acquired resistance to gefitinib and erlotinib (174). However, the mutation of the EGFR tyrosine kinase domain is rare in patients with colorectal cancer (175, 176) (Table 5). In a retrospective study of 87 archival specimens from node positive (Dukes' C) colorectal cancer patients, Cunningham and colleagues found co-expression of EGFR and EGFRvIII in 34% of the cases examined (69). Interestingly, in a more recent study, Montagut and colleagues found that an acquired mutation in the EGFR extracellular domain (S492R) prevents cetuximab binding to the human colorectal cell line DiFi and confers resistance to cetuximab. However, DiFi cells with such a mutation retained binding to and were growth inhibited by panitumumab (177). The same mutation was also detected in 2 of 10 patients with disease progression after cetuximab treatment (177). These findings suggest EGFR mutation may be a responsible factor for the development of resistance to a particular type of EGFR inhibitor, the presence of such mutations does not necessarily indicate insensitivity to other EGFR inhibitors. Therefore, current therapies for patients who develop resistance to one particular anti-EGFR therapy should still include an alternative EGFR inhibitor.

Interestingly, several studies have investigated EGFR gene amplification as a predictive biomarker for response to therapy with anti-EGFR inhibitors (53, 100, 178, 179) (Table 5). In one of the most recent studies, Algars and colleagues assessed EGFR gene copy number (GCN) from areas with the highest EGFR expression as a predictive indicator to response to anti-EGFR therapy in 80 metastatic colorectal cancer patients and found clinical benefit in 73% of high EGFR GCN patients compared to 20% of low EGFR GCN patients who showed response to therapy with cetuximab or panitumumab (180). While the findings of these studies suggest EGFR gene amplification could serve as an additional predictive marker for response to therapy with anti-EGFR inhibitors, more reliable methods of assessing EGFR gene amplification are needed and require validation in larger groups of patients.

5.3. Predictive value of EGFR ligands

In colorectal cancer, several studies have investigated the predictive value of amphiregulin and epiregulin expression in primary tumours for response to therapy with anti-EGFR mAbs in patients with colorectal cancer (Table 5). For example, Khambata *et al.* determined expression of epiregulin and amphiregulin in 110 metastatic colorectal cancer samples and found tumours with high expression levels of either ligands were more likely to have disease control with cetuximab (52). In another study involving 220 chemorefractory metastatic colorectal cancer patients, a significant association was found between the expression of both ligands and response to therapy with cetuximab (181). Similarly, in a more recent study Saridaki *et al.* also investigated expression of amphiregulin and epiregulin in 112 metastatic colorectal cancer patients and found expression of amphiregulin and epiregulin in 45% and 49% of the cases respectively. In addition, the study found significant association between the expression of epiregulin and response to cetuximab (182). Further studies involving a larger group of patients should unravel the full potential of all seven EGFR ligands as predictive biomarkers for the identification of patients who are most likely to benefit from therapy with the anti-EGFR antibodies and other EGFR inhibitors.

5.4. Predictive value of downstream cell signalling pathways

As described above, PI3K/AKT and Ras/Raf/MAPK pathways are the main signalling cascades

activated by aberrant expression and activation of HER family members. In several studies, the constitutive activation of these downstream pathways by *de novo* mutations have been associated with acquired resistance to the EGFR inhibitors in colorectal cancer patients and some of these will be discussed in the following sections (183).

5.4.1. KRAS

KRAS is a proto-oncogene activated by EGFR signalling. Mutations within KRAS lead to a permanently activated state resulting in the activation of RAS/RAF signalling. In approximately 40% of colorectal cancer cases, KRAS is found to have a mutation (105). Many studies have found KRAS mutations to have a negative outcome on the overall survival of the patient (184, 185), whereas other studies did not find such significant associations (186) (Table 5).

In addition several studies have demonstrated KRAS mutations as a biomarker of resistance to anti-EGFR targeted therapy in colorectal cancer. In one of the first studies evaluating the clinical relevance of KRAS mutation as a marker of resistance to EGFR inhibitors; Lievre and colleagues retrospectively studied 30 patients with metastatic colorectal cancer treated with cetuximab alone or in combination with irinotecan and/or 5-fluorouracil (5-FU). The results of the study significantly associated KRAS mutations with the lack of response to cetuximab in all 11 patients (P=0.0003) (187). These initial results were reinforced by a randomised phase III study of panitumumab monotherapy versus best supportive care in 463 metastatic colorectal cancer patients. The study found KRAS mutations in 43% of the cases. While none of the patients with KRAS mutation responded to panitumumab, only 17% of patients with wild type KRAS showed response to treatment with panitumumab (188). In 2009, the US FDA approved a change for the usage of cetuximab and panitumumab to be limited to colorectal cancer patients without KRAS mutations (189). Shortly after, the results of CAIRO II phase III trials were published. The study involved 755 metastatic colorectal cancer patients who were treated with capecitabine, oxaliplatin and bevacizumab and randomised to receive cetuximab or the three drug combination. The study found the response rate to cetuximab in patients with KRAS mutations to be lower than that in patients with wild-type KRAS (46% vs 61.4% respectively) (190). However, not all patients with wild type KRAS would gain benefit from therapy with anti-EGFR mAbs. In addition, objective responses were reported in some colorectal cancer patients with KRAS mutations. For example, Stintzing and colleagues published results from the subgroup of 100 metastatic colorectal cancer patients with KRAS mutation who were treated with cetuximab and found objective response in 44% of patients (191). These studies suggest that not all tumours with KRAS mutation are resistant to therapy with anti-EGFR mAbs such as cetuximab and there is a need for the identification of more reliable predictive markers for response to the EGFR inhibitors.

5.4.2. B-RAF

Another candidate for predicting response to therapy with anti-EGFR monoclonal antibodies such cetuximab could be BRAF (Table 5). BRAF is also a member of the RAS-RAF-MAPK pathway (192). A number of studies have evaluated the prognostic value of BRAF in colorectal cancer (19, 186, 192). The predictive role for BRAF in colorectal cancer therapy was evaluated by Di Nicolantonio *et al.* (192). They retrospectively analysed tumour tissue from 113 patients, who had undergone previous treatment with either cetuximab or panitumumab and found 10% of the patients to have a BRAF mutation but none of them responded to therapy with anti-EGFR mAbs. In another study by Laurent-Puig and colleagues none of the 5 patients with mutated BRAF responded to treatment with cetuximab (19). There is growing evidence for the predictive value of BRAF mutation (186, 193), but these studies suffer from the small number of patients in such studies and therefore would require further validations.

5.4.3. P-I3K

HER family receptors also activate the P-I3K signalling pathway (Figure 1). Mutations in this pathway occur in around 30% of colorectal cancer patients and the majority of these occur in the exon 9 or exon 20 of *PIK3CA* gene which encodes the p110α isoform (194, 195). Mutant *PIK3CA* stimulates the AKT pathway and as a result promotes cellular growth and anti-apoptosis in a variety of human malignancies including colorectal cancer. Mutations of *PIK3CA* have been associated with poor response in patients with colorectal cancer in some studies (Table 5). For example, in an earlier study Sartor-Bianchi *et al.* investigated the association of *PIK3CA* mutations in 110 metastatic colorectal cancer patients with response to therapy with EGFR mAbs. The study found *PIK3CA* mutations in 13.6% of the cases examined and these were significantly associated with clinical resistance with panitumumab or cetuximab. Interestingly, a stronger significance between PIK3CA mutation and clinical resistance to either mAbs was observed when only KRAS wild-type patients were analysed (196). In another large study of 649 chemo-refractory metastatic colorectal cancer patients the effects of *PIK3CA* mutations on the efficacy of cetuximab plus chemotherapy was analysed. The study found 14.5% of the patient tumours harboured *PIK3CA* mutations and found a significant association between *PIK3CA* mutations and low response rate (197). In contrast, in another study which examined tumour specimens from 200 patients with chemo-refractory metastatic colorectal cancer found *PIK3CA* mutations in 12% of the cases examined and found no significant association between *PIK3CA* mutations and response to cetuximab. Interestingly however, the study found that patients harbouring the KRAS and PIK3CA wild-type gene responded less (30%) compared to those with PIK3CA mutations (35%) to treatment with cetuximab (198). Similarly, in another study involving 559 metastatic colorectal patients, *PIK3CA* mutations were found in 9.9% of the cases examined and no significant associations were found between *PIK3CA* mutations and response to therapy with cetuximab (55).

5.4.4. PTEN

The tumour suppressor phosphatase and tensin (PTEN) gene/protein has also been investigated in several

studies as a potential predictive marker for anti-EGFR mAb response. PTEN is involved in inhibiting a PI3K initiated signalling and therefore any deregulation in the activity of PTEN could have a major impact on the progression of various cancers including colorectal cancer (199) (Table 5). Various mechanisms are responsible for the loss of activity of PTEN these include mutations, allelic losses and hypermethylation. Of these, the allelic loss of chromosome 10 is the most common cause of loss of function of PTEN in colorectal cancer (200). Several studies have investigated the loss PTEN in colorectal cancer and its association with resistance to anti-EGFR targeted therapy (19, 50, 196, 201). In a study Negri and colleagues determined PTEN status and its association to response to treatment with cetuximab in advanced colorectal cancer. The study evaluated 67 primary (n=43) and metastatic colorectal cancer (n=24) patients and found the loss of PTEN expression in the metastatic sites was negatively associated with response. Interestingly, the study also noted 25% discordance between the expression levels of PTEN in primary versus metastatic tumours (202). More recently, in another study involving 75 metastatic colorectal cancer patients, positive PTEN expression was found in 50.7% of the cases examined. The study found positive PTEN expression to be significantly associated with a favourable clinical outcome following treatment with cetuximab (203). While all these studies suggest the loss of PTEN expression is associated with clinical resistance in patients with colorectal cancer, further studies are warranted to unravel the full potential of PTEN as a predictive biomarker to response to anti-EGFR targeted therapy.

5.5. Predictive value of IGF-IR and other heterologus receptors

Another mechanism which plays a crucial role in driving resistance to anti-EGFR targeted therapy is the expression of other receptor family members such as VEGFR, PDGFR and IGF-IR and c-MET (204, 205) (Table 5) (see also Corvaia *et al.* and Goetschl *et al.,* in this issue). For example, the aberrant expression of the IGF-IR has been reported in a range of human malignancies, including lung, breast, pancreas and colorectal cancer (62, 206-208). In addition, in several experimental models, IGF-IR expression has been associated with resistance to therapy with anti-EGFR and anti-HER-2 based therapies (209) (210). In one study, Cunningham and co-workers examined co-expression of IGF-IR, EGFR and HER-2 in 87 Dukes' C colorectal cancer patients using immunohistochemistry and found 93%, 83% and 89% of the cases expressed IGF-IR, EGFR, and HER-2 respectively (62). In addition, co-expression of IGF-IR, EGFR and HER-2 was common and present in 75% of the cases examined suggesting the need for further investigation of the co-targeting of such receptors using a cocktail of IGF-IR and HER inhibitors (211).

Currently, several anti-IGF-IR antibodies and small molecules IGF-IR TKIs are at different stages of clinical development in solid tumours, including patients with colorectal cancer and the results are awaited (clinicaltrials.gov, NCT01154335, NCT01016860, NCT00819169, NCT00813605, NCT00908024 (212-215) (see also Corvaia *et al.* in this issue) (Table 4).

While there are a limited number of studies investigating the role of IGF-IR as a potential target in colorectal cancer, the current findings appear to be very encouraging and support the inclusion of such therapies into the current therapeutic regimen for colorectal cancer patients and development of a new generation of inhibitors such as the bispecific anti-EGFR and IGF-IR antibody EI-04 (215) which could be an ideal inhibitor for use in future studies involving colorectal cancer patients with tumours co-expressing EGFR and IGF-IR (62).

6. PERSPECTIVES

It is clearly evident that the type I growth factor receptor family members and their signalling pathways play a fundamental role in tumour progression in patients with colorectal cancer. In addition, the anti-EGFR mAbs cetuximab and panitumumab have been incorporated into treatment paradigms for patients with refractory metastatic colorectal cancer. While EGFR-targeted therapies have transformed the standard care of various human cancers including colorectal cancer, only a fraction of patients will actually gain benefit from these targeted therapies and many patients simply do not respond or acquire resistance after a short period of time. In addition, there is currently no reliable predictive marker for response to therapy with HER inhibitors. In particular, there are a limited number of studies on the co-expression of all members of the HER family in patients with colorectal cancer and no studies on the expression level, prognostic significance and predictive value of all HER ligands in patients with colorectal cancer. To improve the response rate and circumvent resistance to therapy with anti-EGFR mAbs, it is considered to be essential to conduct detailed studies of the expression profiles of the various forms of the HER family members (e.g. wild type, mutated, phosphorylated), their cellular locations (e.g. nuclear, membranous, soluble) and their prognostic significance and predictive value for response to therapy with the HER inhibitors. In addition, such studies should involve the examination of both primary and metastatic lesions, as well as tumour endothelial cells on a larger group of patients. Furthermore, it is essential to determine the expression level of all the HER ligands and other receptors (e.g. IGF-IR, c-MET, PDGF-R) in patients with colorectal cancer and to determine the therapeutic benefit of anti-HER inhibitors when used in combination with agents targeting other molecular pathways and other standard therapeutic interventions. Such investigations could ultimately lead to the selection of a more specific population of colorectal cancer patients whose tumour are HER dependent and therefore are most likely to gain long term benefit from therapy with the HER inhibitors.

7. ACKNOWLEDGEMENT

This work is supported by a grant from the BRIGHT charity, UK.

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Key Words: EGFR, HER-2, HER-3, HER-4, Small Molecule Tyrosine Kinsase, Monoclonal Antibody, Colorectal Cancer, Review

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