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Evaluation of the epidermal growth factor receptor (EGFR) in colorectal tumours and lymph node metastases

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Abstract

Overexpression of the epidermal growth factor receptor (EGFR) often correlates with an aggressive tumour phenotype and poor prognosis. To examine the relevance of EGFR in colorectal cancer, we determined the expression of EGFR protein in 249 colorectal adenocarcinomas and 42 lymph node metastases using immunohistochemistry. Moreover, we investigated a $(CA)_n$ dinucleotide repeat polymorphism of the *EGFR* gene in a subset of 114 tumours. High levels of EGFR protein were observed in 123/249 (49.4%) samples. EGFR expression in colorectal carcinomas correlated with differentiation grade ($P=0.014$). However, there were no associations with Dukes' stage, site, patient age or gender. EGFR protein expression did not influence survival in this colorectal cancer patient cohort ($P \geqslant 0.05$). Expression was not identical in paired colorectal tumours and lymph node metastases, with only 17/42 (40.5%) samples showing equivalent EGFR levels ($P > 0.05$). The distribution of the $(CA)_n$ dinucleotide repeat alleles in colorectal adenocarcinomas was not associated with EGFR protein expression ($P > 0.05$). These results indicate that while EGFR overexpression is a common event in colorectal carcinogenesis, it does not influence patient prognosis.

Keywords: Colorectal cancer; Epidermal growth factor receptor; Prognosis; Polymorphism; Immunohistochemistry; Lymph node metastases

1. Introduction

Colorectal cancer is the second most common malignancy in the developed world, which accounts for over 124 000 deaths per annum in Europe [1]. At present, patient prognosis is determined primarily by spread of the tumour at presentation. However, patients with an identical pathological disease stage can display widely differing outcomes in terms of survival and response to chemotherapy [2]. It is therefore fundamentally important to identify molecular markers of more aggressive colorectal tumour phenotypes in order to adjust patient treatment accordingly.

The epidermal growth factor receptor (EGFR, c-erbB-1) is the first identified member of the type I receptor tyrosine kinase family and is a major regulator of several distinct, diverse cellular pathways [3]. The ability to activate multiple pathways results from the numerous activating ligands which can bind EGFR and also from the various fellow family members with which EGFR can form dimer complexes [4]. Activated EGFR has been shown to cause transformation *in vitro* [5] and a wide range of human tumours have been shown to overexpress EGFR *in vivo*, including breast [6], lung [7], and bladder [8] carcinomas. While EGFR has been related to more aggressive behaviour in a number of tumour types [9], the clinical relevance of EGFR overexpression in colorectal cancer has not been comprehensively investigated.

Knowledge of EGFR levels within colorectal adenocarcinomas is also important in terms of therapeutic intervention. EGFR is the focus of various anticancer agents currently under investigation. Modulators of EGFR activity, for example anti-EGFR monoclonal antibodies and low molecular weight inhibitors of EGFR [10–12], have been shown to inhibit tumour

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proliferation *in vitro* and in certain cases promote apoptosis. Clinical trials are currently underway to assess the toxicities and anti-tumour activities of such compounds *in vivo* [10–12].

While overexpression of EGFR can be attributed to gene amplification, this is more often not the case for solid tumours [13]. Although mutations in the *EGFR* gene are rarely observed, a polymorphic $(CA)_n$ dinucleotide repeat in intron 1 of the *EGFR* gene has been reported [14] which is associated with gene expression [15]. In a model system, transcription was shown to have a stepwise decrease with increasing numbers of $(CA)_n$ repeats ($n=14$ to $n=21$; [15]). In addition, further study has shown that the $(CA)_n$ dinucleotide repeat length modulates intratumoral EGFR content in breast tumours *in vivo* [16]. However, to date there have been no studies examining the $(CA)_n$ repeat in colorectal tumours.

This study has evaluated EGFR protein expression in a series of 249 primary colorectal adenocarcinomas and 42 corresponding lymph node metastases using immunohistochemistry, to investigate the influence of EGFR on colorectal cancer. In addition, we have ascertained the *EGFR* gene $(CA)_n$ dinucleotide repeat polymorphism length in a subset of 114 primary colorectal tumours, to determine if it plays a role in this disease.

2. Patients and methods

2.1. Patients and tumour samples

Archived tumour samples were available from 249 patients who had undergone elective surgery for colorectal cancer at Grampian University Hospitals NHS Trust, Scotland, UK, between 1994 and 1998. Lymph node metastases from a sub-group of 42 Dukes' C and D patients were also available. Samples were collected at the Department of Pathology, University of Aberdeen, under the auspices of the Aberdeen Colorectal Initiative. Tumours were routinely fixed in 10% neutral buffered formalin for 24 h, then embedded in paraffin wax. An experienced gastro-intestinal pathologist reviewed all cases to confirm the diagnosis of adenocarcinoma. Detailed clinicopathological data (Dukes' stage, differentiation grade, site of primary tumour, patient gender and age) was available for each sample, as previously described in Ref. [17]. Cases of perioperative death were excluded from the survival analysis to avoid events that were not associated with disease ($n=5$). 154 of the 244 eligible patients were alive at the most recent assessment (May 2001), with a median follow-up of 48 months (range 31–86 months). A subset of 114 colorectal adenocarcinomas was used for the molecular analyses. These tumours were randomly selected and were representative of the tumour cohort as a whole. Relevant approval from local ethical committees was obtained for this study.

2.2. Immunohistochemistry

The levels of EGFR protein expression in colorectal adenocarcinomas and lymph node metastases were determined using immunohistochemistry and an avidin/biotin/horseradish peroxidase development system as previously described in Refs. [18,19]. Briefly, 5 μm formalin-fixed wax embedded sections were dewaxed and rehydrated, and endogenous peroxidase activity was blocked with 3% H_2O_2/methanol. Sections were microwaved for 20 min in 10 mM citrate buffer pH 6.0 to enhance antigen retrieval, following which endogenous biotin activity was blocked using a biotin blocking kit (Vector Laboratories Ltd, Peterborough, UK), to prevent non-specific background staining. Sections were then sequentially incubated with a mouse monoclonal anti-EGFR antibody (clone EGFR.113, 1 in 20 dilution; Novocastra Laboratories Ltd, Peterborough, UK), biotinylated rabbit anti-mouse immunoglobulin (Dako A/S Ltd, Glostrup, Denmark) and streptavidin/biotin/horseradish peroxidase complex (Dako A/S Ltd). Sites of bound antibody were then visualised using liquid 3′-diaminobenzidine (DAB) Plus (1:150) (Dako A/S Ltd), and the slides were counterstained with Mayer's haematoxylin. Sections of human placenta were used as positive controls, and slides were incubated with tris buffered saline (TBS) in place of primary antibody for negative controls.

2.3. Scoring system

Sections were analysed using light microscopy by two independent observers who were unaware of the clinicopathological details. Interobserver discrepancies were resolved by simultaneous dual re-evaluation. EGFR expression in colorectal tumours was assessed according to both the distribution of immunostaining (i.e. $\leqslant 5\%$, 6–25%, 26–50%, 51–75%, $\geqslant 76\%$), and the localisation of immunoreactivity (i.e. membranous and/or cytoplasmic staining). Localisation of EGFR protein was recorded in order to assess the impact of differential compartmentalisation on clinicopathological parameters. Staining was evaluated over the entire tumour section. Statistical analyses of EGFR distribution were performed both using these criteria, and by using dichotimised EGFR expression (i.e. 5, 25, 50 and 75% cut-off points [20]).

2.4. DNA extraction

Genomic DNA was extracted from whole blood samples (5 ml) using a sodium perchlorate-chloroform extraction method (Nucleon II kit; Scotlab, Coatbridge, UK). The extracted DNA was resuspended in 1 ml TE

buffer (10 mM Tris–HCl, 1 mM ethylenediamine tetraacetic acid, pH 8.0), and stored at 4 °C. Paraffin sections containing formalin-fixed normal colon tissue were also used for genomic DNA extraction. Dewaxed and rehydrated material was microdissected into a microfuge tube, and digested with 0.5 mg/ml proteinase K at 55 °C for 4 h. The digests were then heated to 95 °C for 3 min to inactivate the proteinase K, and spun briefly to pellet any cell debris. The resulting supernatant was used directly in the polymerase chain reaction (PCR) analysis.

2.5. $(CA)_n$ dinucleotide repeat length analysis

Analysis was carried out essentially as described previously in Refs. [14,15]. Each 50-μl PCR reaction consisted of 1 μl genomic DNA, 10 mM Tris–HCl, pH 8.3, 50 mM KCl (Applied Biosystems, Warrington, UK), 2 mM $MgCl_2$ (Applied Biosystems), 200 μM each of deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanine triphosphate (dGTP) and deoxycytidine triphosphate (dCTP) (Promega, Southampton, UK), 1.25 U AmpliTaq Gold (Applied Biosystems) and 25 pmol of both the forward and reverse primers (Oswel, Southampton, UK). Primers used for the determination of the $(CA)_n$ dinucleotide repeat length in genomic DNA were 5′ GTT TGA AGA ATT TGA GCC AAC C (forward primer), and 5′ TTC TTC TGC ACA CTT GGC AC (reverse primer). The reverse primer was labelled with HEX fluorochrome at the 5′-end. PCR was carried out in a Techne Unit Genius thermocycler (Techne, Cambridge, UK), and consisted of an initial heating at 95 °C for 12 min to activate the enzyme, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 60 s and extension at 72 °C for 60 s, with a final extension at 72 °C for 3 min. PCR products ranging from 114 to 128 bp were obtained, corresponding to 14 to 21 CA repeats. DNA was omitted from negative control samples, whilst genomic DNA from a healthy volunteer was used as a positive control for the reaction.

Following PCR, 1 μl of product and 0.5 μl of Genescan 500 TAMRA molecular weight standard (Applied Biosystems) were denatured in 12.5 μl of formamide at 95 °C for 5 min. Separation was carried out using an Applied Biosystems Prism 310 genetic analyser (Applied Biosystems) with POP4 polymer, and the fragment lengths were determined.

2.6. Direct sequencing of $(CA)_n$ dinucleotide repeats

Direct sequencing of PCR products was carried out on a number of samples in order to confirm the $(CA)_n$ dinucleotide repeat lengths. For each of three fragment lengths (i.e. 118, 122 and 126 bp), two homozygous samples were sequenced using both the forward and reverse primers.

PCR was carried out as described in Section 2.5, except that the reverse primer was not fluorescently labelled. Following purification, PCR products were directly sequenced using the BigDye terminator dideoxy chain termination method and an ABI 377 automated sequencer (Applied Biosystems), and analysed using SeqEd version 1.03 for Windows 95 (Applied Biosystems).

2.7. Statistics

The ability of EGFR protein expression in primary colorectal tumours to predict expression in lymph node metastases was evaluated using the kappa test. In addition, a paired samples test (Chi-square test) was used to assess the correlation between the expression in the primary and metastatic tumours. Expression of EGFR protein in colorectal tumours was assessed with respect to Dukes' stage, differentiation grade, localisation, site, patient gender, and $(CA)_n$ repeat length using the Chi-square test, while patient age was examined using the Mann–Whitney U test. The effect of EGFR on survival was tested using Kaplan–Meier survival plots and analysed using the log rank test. Significance levels were set at $P < 0.05$, and all statistical analyses were carried out using the Statistical Package for the Social Services (SPSS) for Windows 95 version 9.0 (SPSS UK Ltd, Woking, Surrey, UK).

3. Results

3.1. Immunohistochemistry—primary colorectal tumours

The expression of the EGFR protein was examined in 249 primary colorectal tumours, and the levels of expression are detailed in Table 1. One hundred and eighty-one tumours (72.7%) were found to express EGFR, with almost half of the tumours (123/249, 49.4%) displaying high levels of this protein (>50% of tumour cells positive). Of the 181 immunoreactive tumours, 73 displayed membranous EGFR expression, with a further 94 showing both membranous and cytoplasmic immunostaining. A small number of tumours

Table 1
Distribution of EGFR protein expression in colorectal cancer ($n = 249$)

Percentage of positive tumours cells (%)	n (%)	Localisation of immunostaining	n (%)
⩽5	68 (27.3)	Negative	68 (27.3)
6–25	30 (12.0)	Membrane	73 (29.3)
26–50	28 (11.2)	Membrane and cytoplasm	94 (37.8)
51–75	38 (15.3)	Cytoplasm	14 (5.6)
⩾76	85 (34.1)		

EGFR, epidermal growth factor receptor.

(14/249, 5.6%) displayed only cytoplasmic immunoreactivity towards EGFR (Table 1).

3.2. Immunohistochemistry—lymph node metastases

Expression of EGFR was also assessed in lymph node metastases from a subgroup of 42 patients with Dukes' stage C or D tumours. EGFR protein was evident in 35/42 (83.3%) metastatic tumours. However, only 11/42 (26.2%) lymph node metastases were classified as having EGFR expression in $>50\%$ of tumour cells. Of the evaluated paired samples, 17/42 (40.5%) displayed equivalent EGFR expression in both the primary and the metastatic tumours. Of the remaining 25 cases, 21 (50.0%) had higher EGFR expression in the primary colorectal tumour, while 4 (9.5%) had higher EGFR protein levels in the lymph node metastasis. Using the kappa test, which is a measure of concordance between paired samples, only a poor level of agreement was observed ($\kappa = -0.027$, $P = 0.80$; Table 2). Using the Chi-square test, no associations were apparent in EGFR expression in the primary and metastatic colorectal tumours ($P > 0.05$ in each case).

3.3. EGFR protein and clinicopathological information

The expression of EGFR protein was evaluated with respect to patient clinicopathological data, and the results detailed in Table 3. EGFR expression, using a 50% cut-off point, was associated with the differentiation grade of the tumour, with the frequency of expression of high levels of EGFR protein decreasing from well to moderate to poor differentiation ($P = 0.014$). There were no correlations between EGFR protein expression and patient gender, site of the primary tumour, Dukes' stage, or patient age ($P > 0.05$ in each case). In addition, the localisation of EGFR protein within the tumour cells had no impact on the clinicopathological parameters examined ($P > 0.05$ in each case).

3.4. EGFR and colorectal cancer survival

The effect of EGFR protein expression on patient survival was examined. Log rank analysis was carried out to evaluate the effect of the level of EGFR protein (i.e. using 5, 25, 50 and 75% expression as cut-off points) and its localisation (i.e. membranous and/or cytoplasmic) on patient outcome. No significant effect on patient outcome was apparent due to either EGFR protein expression level or localisation ($P > 0.5$ in each case). Fig. 1 demonstrates this lack of effect seen using a 50% cut-off level for high and low EGFR protein expression.

Table 2
Comparison of EGFR protein expression in primary colorectal tumours and paired lymph node metastases ($n = 42$)

	Lymph node metastases		
	EGFR ($<50\%$)	EGFR ($>50\%$)	
	n (%)	n (%)	P[a] value
Colorectal tumour			
EGFR ($\leqslant 50\%$)	10 (23.8)	4 (9.5)	
EGFR ($>50\%$)	21 (50.0)	7 (16.7)	0.80

[a] Kappa test.

3.5. $(CA)_n$ dinucleotide repeat length in colorectal tumours

The length of a $(CA)_n$ dinucleotide repeat polymorphism within intron 1 of the *EGFR* gene was investigated in a subset of 114 colorectal tumours. Alleles corresponding to each of the previously reported repeat lengths were observed within this sample group (i.e. $n = 14$ to $n = 21$), and the frequencies of each repeat length are documented in Table 4. Heterozygosity was 71.0% for this series of colorectal tumours. In 78 of the 114 patients (68.4%), the smaller allele consisted of 16 CA repeats, while the most common genotype was found to be heterozygous for 16 and 20 CA repeats.

EGFR protein expression, as assessed by immunohistochemistry, was not associated with the $(CA)_n$

Table 3
EGFR protein expression and patient clinicopathological data

Characteristic	EGFR ($\leqslant 50\%$) n (%)	EGFR ($>50\%$) n (%)	P value
Sex			
Male	61 (45.5)	73 (54.5)	
Female	65 (56.5)	50 (43.5)	NS[c]
Site[a]			
Proximal colon	54 (60.0)	36 (40.0)	
Distal colon	45 (45.5)	54 (54.5)	
Rectum	27 (45.0)	33 (55.0)	NS
Differentiation grade			
Well	1 (12.5)	7 (87.5)	
Moderate	101 (49.3)	104 (50.7)	
Poor	24 (66.7)	12 (33.3)	0.014
Dukes' stage[b]			
A	13 (40.6)	19 (59.4)	
B	68 (55.3)	55 (44.7)	
C	43 (47.8)	47 (52.2)	
D	2 (50.0)	2 (50.0)	NS
Age			
Range	32–91	33–89	
Mean	69	66	
Median	70	68	NS

[a] Proximal colon tumours arose proximal to the splenic flexure (i.e. caecum, ascending colon, transverse colon); distal colon tumours arose distal to this point (i.e. the descending colon and sigmoid colon).

[b] Colorectal cancers were classified as Dukes' stage D when histologically confirmed liver metastases were present at the time of surgery.

[c] NS, non significant.

dinucleotide repeat, in terms of individual genotypes, the most common allele length (i.e. 16 repeats versus other), the most prevalent genotype (i.e. $n=16$ and 20 repeats versus other), or whether the tumour was homo- or heterozygous for the repeat ($P>0.05$ in each case). Furthermore, repeat length was not associated with patient survival, as assessed by log rank analysis ($P>0.05$ in each case).

4. Discussion

Overexpression of EGFR *in vivo* has been demonstrated in a number of human tumour types, including breast, lung, head/neck and bladder carcinomas [6–8], and has been related to a more aggressive clinical behaviour in a number of reports. While experimental data have demonstrated a direct relationship between the expression of EGFR by colorectal cancer cells and their ability to produce hepatic metastases [21], the role of EGFR overexpression in colorectal tumours *in vivo* has not been extensively investigated [20,22]. This study was performed to evaluate the levels of EGFR protein expression in a large series of colorectal tumours and in a subgroup of lymph node metastases, and to correlate these findings with clinicopathological information, including patient survival.

We found that EGFR is commonly expressed in colorectal adenocarcinomas (72.7% of samples displayed this protein), with almost half of the examined tumours exhibiting high levels of this protein. This is the largest single study of EGFR protein expression in invasive colorectal tumours. However, previous investigations on smaller numbers of tumours of mixed stages have found a similar frequency (74.5–97.6%) of EGFR expression in colorectal cancer [20,22]. Such consistent expression of EGFR in colorectal adenocarcinomas suggests that it may be of use as a target of chemotherapy in this disease. Several strategies aimed at inhibiting the multiple growth-promoting activities of EGFR are currently under investigation [10–12], and the results of this study indicate that colorectal tumours may represent a valid model for clinical trials with anti-EGFR therapy.

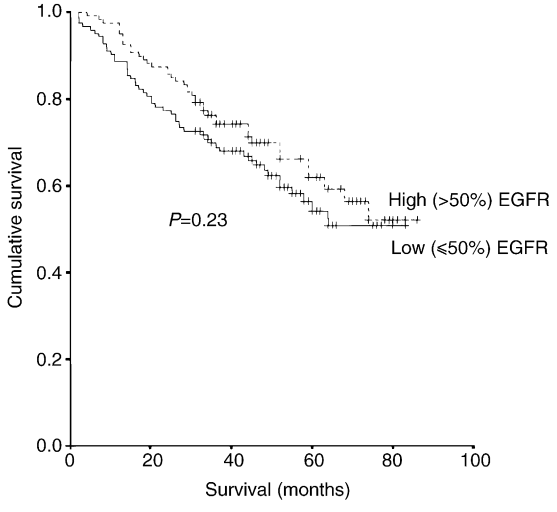

Fig. 1. Log rank analysis of a Kaplan–Meier plot demonstrates that the level of EGFR protein within a tumour does not affect the survival of colorectal cancer patients ($P>0.05$).

However, therapy against advanced colorectal cancer is generally targeted towards metastatic lesions, and it has been shown that such metastatic tumours are often genetically distinct from their progenitor cells [18,23]. By examining expression in a subset of metastatic lymph node tumours corresponding to primary colorectal tumours in our series, we have ascertained that EGFR protein levels frequently differ between primary and metastatic lesions. In the majority of non-concordant cases, absence of EGFR protein was observed in the metastatic tumour while the corresponding primary tumour displayed a high level expression. These results indicate that EGFR expression in primary colorectal tumours does not reflect the situation in lymph node metastases. Although the molecular basis for this disparity is not known, this has important clinical implications. With the development of anti-EGFR therapy, tumour profiling for EGFR status has been proposed to guide patient selection. This has proved useful in the case of trastuzumab treatment for HER-2-positive breast cancer patients [24]. However, the data from this study shows that primary tumour, the easiest tissue to obtain for patient studies, is unlikely to give a clear indication of the EGFR status. This problem is not easily overcome, as biopsy of metastatic tumour is difficult to obtain for both technical and ethical reasons.

While EGFR may be of use as a target of chemotherapeutic intervention in colorectal adenocarcinoma, given that approximately half of tumours have high levels of EGFR, the present study has shown that it does not have prognostic power in this disease. While an earlier study found a direct relationship between high EGFR protein levels and poor outcome in 84 colorectal cancer patients [20], no such correlation was apparent in

Table 4
Allele frequencies of the $(CA)_n$ dinucleotide repeat in intron 1 of the *EGFR* gene in colorectal cancer patients (n = number of alleles; 114 patients/228 alleles)

Allele length (bp)	(CA) repeats	n	Allele frequency
114	14	1	0.004
116	15	2	0.009
118	16	104	0.456
120	17	6	0.026
122	18	42	0.184
124	19	2	0.009
126	20	59	0.259
128	21	12	0.053

our larger series of tumours. In addition, EGFR was not shown to be an independent prognostic factor in a study of 82 colorectal cancer patients [20]. The discrepancies between these studies, which may be due to procedural differences or to the effects of varying chemotherapy regimens used to treat this disease, suggest that the immunohistochemical evaluation of EGFR in colorectal adenocarcinomas will be of little clinical relevance in this situation.

In addition to being unrelated to prognosis, EGFR expression was not correlated to the Dukes' stage of the colorectal tumours in this series, suggesting no major role in tumour aggressiveness. However, a clear association between EGFR and the differentiation grade of the tumour was noted ($P=0.014$), with well and moderately differentiated tumours more frequently displaying EGFR protein than poorly differentiated lesions. This is in agreement with earlier studies using human colon cancer cell lines [25], where all eight moderately and well differentiated cell lines were found to express high levels of the receptor, whereas none of the five poorly differentiated cell lines examined displayed EGFR protein. It is unclear why such an association should exist; however, it may be that anti-EGFR therapies would be more suited to treating differentiated colorectal tumours.

This study also examined a $(CA)_n$ dinucleotide repeat length polymorphism in intron 1 of the *EGFR* gene. This polymorphism has previously been related to *EGFR* gene expression both *in vitro* and in human breast cancer [15,16]. We found no association between the repeat length and EGFR protein expression, as determined by immunohistochemistry, in our series of colorectal tumours. This is in contrast to breast tumours, where heterozygous samples displayed lower levels of EGFR than homozygous cases and an inverse relationship existed between the length of the smaller allele and intratumoral EGFR content [16]. Such differences may indicate tissue-specific regulation of EGFR expression, or may be simply due to technical variations between studies (i.e. EGFR expression assessed by immunohistochemistry versus enzyme-linked immunosorbent assay (ELISA). It is possible that the immunohistochemistry technique used in this study is too subtle to detect the impact of differing repeat lengths. We also found that the distribution of allele lengths in colorectal cancer was not significantly different from that observed in a Caucasian reference pedigree [11], which suggests that this polymorphism is not likely to be related to the risk of developing colorectal cancer.

In conclusion, we have found that high levels of EGFR protein are frequently observed in colorectal tumours, which may represent a useful target of therapy for this disease. However, equivalent expression is not displayed by metastatic tumours, which may limit the effectiveness of anti-EGFR treatment of advanced disease. In addition, EGFR protein expression was not found to be a prognostic marker in colorectal cancer. Finally, we have determined that the polymorphic allele length of a dinucleotide repeat in the *EGFR* gene is not associated with the likelihood of developing colorectal cancer.

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