

Epidermal growth factor receptor analyses in colorectal cancer: A comparison of methods

KAREN-LISE GARM SPINDLER, JAN LINDEBJERG, JENS NEDERBY NIELSEN, DORTE AALUND OLSEN, CLAUDIS BISGÅRD, IVAN BRANDSLUND and ANDERS JAKOBSEN

Danish Colorectal Cancer Group South, University of Southern Denmark, Vejle Hospital, Kabbeltoft 25, 7100 Vejle, Denmark

Received May 23, 2006; Accepted July 17, 2006

Abstract. EGFR immunohistochemistry (IHC) status is not a reliable predictive marker for response to EGFR-targeted therapies. The present study compares the EGFR status at DNA, RNA and protein level. Blood samples, corresponding normal colon and colorectal cancer tissue were collected from 199 colorectal cancer (CRC) patients. EGFR status was evaluated by FISH analysis, real-time RT-PCR, ELISA and IHC. A polymorphism in the EGFR promoter was evaluated by PCR analysis. The EGFR levels by different methods were mutually compared. Seventy-eight percent of primary tumours and corresponding lymph nodes had equivalent EGFR status (28/34). There was a tendency to higher median protein level (by ELISA) in IHC positive patients compared to IHC negative patients ($p=0.086$). The median EGFR gene expression level was significantly lower in tumours than in the normal colon with no difference according to IHC status. No tumours had increased gene copy number by FISH. EGFR Sp1-216 polymorphism analysis showed a tendency for different EGFR tumour protein levels and gene expression levels according to the different genotypes. The results show a poor correlation between EGFR status at DNA, RNA and protein level. The predictive value of a combination of methods needs further evaluation in the clinical setting.

Introduction

Colorectal cancer (CRC) is one of the major cancer diseases in the world (1) and holds a poor prognosis. In recent years the development has indicated that the epidermal growth factor receptor (EGFR) is a valuable target for anticancer therapy and new treatment modalities such as monoclonal antibodies (2-4) and small molecules (5,6) targeting EGFR have shown promising results in the clinical setting. However, only a minor fraction of the patients benefit from these new

treatment modalities and predictive markers for outcome are urgently needed.

EGFR is a well-known transmembrane tyrosine kinase receptor and a member of the ErbB family composed of four structurally related transmembrane receptors; EGFR, HER2, HER3 and HER4. The extracellular part of EGFR binds several ligands. Binding of ligand to receptor induces receptor dimerization followed by activation of the tyrosine kinases, which mediates downstream signalling that stimulates the cell cycle pathway and controls cell proliferation. Uncontrolled cell growth, decreased apoptosis, stimulation of angiogenesis and cell proliferation is mediated by dysregulation of the EGFR signalling system. Consequently the EGFR is considered to play a central role in regulation of malignant transformation and tumour growth (7,8). EGFR is present in most epithelial tissue and is overexpressed in various solid tumours among these colorectal cancers (8).

Traditionally the epidermal growth factor receptors have been evaluated by immunohistochemistry (IHC) (9). Overexpression of EGFR has been associated with poor prognosis, shorter survival and increased metastatic ability. In colorectal cancer EGFR overexpression has been reported in 25-82% (9) and has been shown to predict advanced stage and metastatic potential (10), but the impact on survival remains controversial (11).

A major challenge for all targeted therapies is to identify simple and effective predictive markers allowing a rational treatment selection. So far EGFR testing by IHC has not provided clinicians with a reliable method for selection of patients to EGFR-targeted therapies (2,12). Studies have failed to show any relation between EGFR expression level and the clinical efficacy of cetuximab (2,13). Even tumours with undetectable EGFR levels have responded to cetuximab therapy and response rates in EGFR-negative patients were comparable to those in patients with EGFR-positive tumours (14). These discrepancies have raised several explanations including tumour heterogeneity, poor-sensitivity and lack of standardisation of methodology.

Alternative methods for EGFR expression analysis and the underlying mechanism for EGFR regulation are being actively investigated. Dysregulation of EGFR kinase activity may occur as a consequence of EGFR overexpression, gene amplification or through mutations resulting in constitutive activation. EGFR gene copy number can be detected by FISH analysis as described by Moroni *et al* (15). However, the role of gene amplification and gene copy number assessment in

Correspondence to: Dr Karen-Lise Garm Spindler, Department of Oncology, Vejle Hospital, Kabbeltoft 25, DK-7100 Vejle, Denmark
E-mail: kalgsp@vgs.vejleamt.dk

Key words: colorectal cancer, epidermal growth factor receptor, analysis, methods

clinical settings needs further evaluation. Measurement of gene expression levels has been evaluated by real-time RT-PCR (16) and holds promising potential as a predictive marker in cetuximab therapy. EGFR protein quantification is assessable by IHC and enzyme-linked immunosorbent assay (ELISA). Apart from that on IHC, the literature on these methods is scarce regarding CRC.

Recently gene polymorphisms relating to the EGFR signalling system have been described. A functional polymorphism in the Sp1 binding site of the EGFR promoter region has been identified and reported to influence the gene expression level of EGFR in cell lines (17). There are no clinical data on the possible relationship between this Sp1-216 EGFR polymorphism and EGFR gene-expression levels.

The aim of the present study was to compare different methods for EGFR-analysis in CRC. We investigated the receptor status in colorectal adenocarcinoma tissue as well as in normal colon tissue and blood, with the purpose of comparing the EGFR status at DNA, RNA and protein level.

Patients and methods

Patients. The study included 199 patients with CRC during the period of December 2003 to July 2005. All patients underwent surgical resection for adenocarcinomas of the colon or rectum at Department of Surgery, Vejle Hospital, Denmark. Disease extension was classified according to the TNM system. Samples of blood, colorectal tumours and normal colon were collected at surgery after obtaining informed consent from the patients. The study was approved by the Regional Ethics Committee of Vejle and Funen Counties according to Danish law.

Sampling. Fresh tissue from tumour and normal colon was frozen in Tissuetek O.C.TTM compound (SAKRUA) and prepared for protein analysis. Additional tissue samples were stored at -20°C in RNA-later (Qiagen, CA, USA). Further samples and corresponding lymph node metastases were formalin-fixed and paraffin-embedded according to standard procedure.

Immunohistochemistry. Colorectal specimens were immersed in 4% buffered neutral formalin and fixed for 24 h. Paraffin-embedding was performed according to standard procedures. Sections of $4\text{-}\mu\text{m}$ were mounted on coated slides and allowed to dry for 30 min at 60°C and overnight at 37°C . All sections were stained within 24 h of embedding. The slides were deparaffinized in ESTISOL 220 (Esti Chem) and rehydrated in graded alcohol solutions. Endogenous peroxidase was blocked with 3% hydrogen peroxide. Proteolytic antigen retrieval was performed using 0.1% protease at room temperature (RT) for 20 min. Slides were incubated in primary mouse anti-EGFR Mab (clone H-11, Dako Corporation, Carpinteria, USA) for 30 min at RT. Visualisation of the reaction was performed using ENVISION + DAB (Dako Cytomation-DK) followed by counterstaining with haematoxylin. Staining was performed manually.

Evaluation of EGFR IHC. Evaluation was independently performed by two investigators (Karen-Lise Garm Spindler

and Jan Lindebjerg). EGFR positivity was defined according to Dako guidelines, any membrane staining above background level was considered positive. Tumours were graded with regard to intensity and amount of membrane staining. A score of staining intensity was assigned as follows: 1+, weak; 2+, moderate; and 3+, strong membrane staining. The tumour was defined positive if $\geq 1\%$ of the cells had membranous staining for EGFR according to the Dako guidelines. A score was defined according to the percentage of positively stained tumour cells as follows: 0, $<1\%$; 1, 1-10%; 2, 10-25%; 3, 25-50%; 4, $>50\%$.

Quantitative EGFR ELISA

Extraction of proteins. Tissue samples of 10-50 mg were homogenised at 4°C by an ultra-turrax system (Ika, Germany) with 10 Vol (w/v) buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4, 1 mM EDTA, 10% glycerol, protease inhibitor cocktail (cat. no. P8340, Sigma-Aldrich, USA). After homogenisation, Triton X-100 was added to the samples to a final concentration of 1% (v/v). The lysates were mixed and incubated for 30 min at 4°C followed by centrifugation at $16000 \times g$ for 10 min at 4°C . The supernatants were recovered and the protein concentration was determined using the bicinchoninic acid protein assay (Pierce, USA).

EGFR ELISA. A commercially available enzyme-linked immunosorbent assay (Oncogene Science, USA) was used to quantify EGFR in colon cancer tissue and autologous reference tissue. Tissue extracts were adjusted in sample diluent to a final protein concentration of $\sim 50 \mu\text{g/ml}$. Diluted tissue samples along with standards and controls (Oncogene Science) were added to a 96-well microtiter plate coated with a mouse monoclonal anti-(EGFR) antibody and incubated for 1.5 h at 37°C . After this incubation step plates were washed and incubated with an alkaline phosphatase-labelled mouse monoclonal anti-(EGFR) antibody for 30 min at RT. Enzymatic reactions were carried out at RT by adding BluePhos substrate and the reaction was stopped after 60 min by the addition of stop solution. Colour development was measured at 650 nm by using an automated plate reader (Vmax, Molecular Devices, USA) and the EGFR concentration of the unknown samples was estimated from the standard curve. All samples were analysed in duplicate and the average of the two was recorded. The interassay and intraassay coefficients of variation were $<10\%$.

Relative gene expression analysis of EGFR

RNA isolation and cDNA synthesis. Total RNA was isolated using an RNeasy kit from Qiagen according to the manufacturer's instructions. Isolated RNA was quantitated by Spectrophotometry (Ebbendorf, Hamburg, Germany) and cDNA synthesis was performed using M-MLV RT (Invitrogen) as previously described (18).

Real-time PCR quantification of mRNA expression. Following RNA isolation and cDNA synthesis real-time fluorescence PCR was performed for t-EGFR and β -actin using an assay from Applied Biosystems (Hs01076088_m1 4310881E respectively) on an ABI PRISM HT 7900 sequence detection system, TaqMan (Perkin-Elmer Applied Biosystem, Foster

Table I. Primers and probes for Sp1-216 G/T gene polymorphism analysis.

Probes and primers	Sequence	System
EGFR-Sp1G probe	6FAM - AGC AGC CTC CGC C	ABI PRISM 7900 HT
EGFR-Sp1T probe	VIC - AGC AGC CTC CTC C	ABI PRISM 7900 HT
EGFR-Sp1 forward primer	CGT CCG GGC AGC CC	ABI PRISM 7900 HT
EGFR-Sp1 reverse primer	GGC GCT CAC ACC GTG C	ABI PRISM 7900 HT
Forward sequencing primer	GGT CTC CTC CTC CTC CTC GCA	ABI 3100
Reverse sequencing primer	TTG TGG CGT TGG CGG CGA	ABI 3100

City, CA, USA). The housekeeping gene β -actin was used as a denominator for standardization. The PCR mixture and cycling conditions were conducted according to the manufacturer's instructions. The relative gene expression was determined based on the threshold cycles of EGFR and the internal standard β -actin. Quantification was performed as previously published using a standard curve model (19). The line of the EGFR standard curve was $y = -3.3728x + 35.098$ and the linear regression coefficient $R^2 = 0.9948$. The line and regression coefficient of β -actin standard curve were $y = 3.423X + 29.141$ and $R^2 = 0.9992$ respectively. Positive controls (samples of known value) and negative controls (samples without cDNA) were performed in parallel for each PCR experiment ensuring equivalent assay conditions. Quantifications of mRNA were carried out in triplicates.

Sp1-216 G/T EGFR polymorphism analysis. Genomic DNA was isolated from whole blood as previously described (19). Polymerase chain reaction (PCR) analysis of Sp1 gene polymorphism in the EGFR promoter was performed using the ABI PRISM 7900 HT sequence detection system (Perkin-Elmer Applied Biosystem). The results were verified by sequencing on an ABI 3100 sequence detection system. Table I shows primers and probes.

FISH analysis. The tumour samples were paraffin-embedded and formalin-fixed according to standard procedures. FISH was performed on 3- μ m sections using the FISH accessory kit from Dako Cytomation-DK together with a fluorescently labelled DNA probe set, (LSI EGFR SpectrumOrange/CEP 7 SpectrumGreen Probe) from Vysis, Inc. (Downers Grove, IL, USA). Staining procedures were performed according to the manufacturer's instructions. Tumour samples were examined with a Zeiss Axio Imager fluorescence microscope. Evaluation was made by a single trained pathologist (Jan Lindebjerg) who was blinded to patients' data.

Evaluation of FISH analysis. In each tumour sample signals were counted in a total of 100 non-overlapping tumour-cell nuclei. The mean signal number of the EGFR gene as well as CEP7 was calculated and the EGFR gene/CEP7 ratio determined.

Statistical analyses. The correlation between EGFR status and various clinicopathological parameters was determined by the Student's t-test, Fisher's exact test or Wilcoxon rank-sum test for difference in medians when appropriate. The

inter- and intra-observer reproducibility of assessments of the IHC staining was tested by calculating Cohen's κ . The relationship between EGFR immunostaining in primary tumours and metastasis was evaluated by Fisher's exact test. Linear regression analysis was used to describe the correlation between EGFR ELISA protein and gene expression levels in tumour and normal colon. Fisher's exact test was used to evaluate the proportions of IHC positive and negative tumours in the different groups of Sp1-216 genotype. P-values ≤ 0.05 were considered significant and all statistics were carried out using the NCSS statistical software (NCSS Statistical Software, UT 84037, USA).

Results

Patient characteristics are shown in Table II. The median age was 71 years (range 41-91 years). All tumours were histopathologically confirmed adenocarcinomas of the colon or rectum.

IHC. One hundred and ninety-three tumours were available for EGFR IHC staining and 51% (99/193) of tumours were positive. There was no correlation between EGFR score and clinicopathological parameters in terms of age, gender, tumour location and TNM category. Thirty-four patients had corresponding lymph node metastasis available for EGFR staining. One third (5/15) of patients with EGFR-positive tumours had EGFR-negative lymph node metastasis whereas lymph node positive metastasis was found in only 5% (1/19) of patients with negative tumours (Table III). Primary tumours and lymph node metastasis had equivalent EGFR expression in 78% (28/34) of observations.

EGFR protein ELISA. EGFR protein ELISA was performed on 94 paired tumour and normal colon tissue samples. Data are presented in Table IV. There was no difference between the median protein levels of the two groups. Linear regression analysis showed a weak correlation between protein levels in normal tissue and tumours (0.22, $R^2 = 0.05$). The median EGFR protein level in the IHC negative group was 13 ng/mg compared to 15.3 ng/mg in patients with IHC positive tumours. The difference was not significant ($p = 0.086$). EGFR protein level did not correlate with IHC score or intensity (data not shown).

EGFR gene expression analysis. EGFR gene expression analysis was performed on 82 corresponding primary tumours

Table II. Additional clinicopathological parameters (NS, not significant).

Parameter	Number N=199 (%)	Correlation to IHC
Gender		NS
Male	107 (54)	
Female	92 (46)	
Topography		NS
Right colon	51 (26)	
Left colon	65 (33)	
Rectum	79 (40)	
Right and left colon	4 (2)	
T category		NS
1	2 (1)	
2	25 (13)	
3	137 (69)	
4	34 (17)	
Other	1 (0.5)	
N category		NS
0	106 (53)	
1	34 (17)	
2	36 (18)	
3	22 (11)	
Unknown	1 (0.5)	
M category		NS
0	145 (73)	
1	39 (20)	
Unknown	15 (7)	
Available for IHC of primary tumour	193	
EGFR-positive	94 (49)	
EGFR-negative	99 (51)	
Available for IHC of meta-stasis	34	
EGFR-positive	11 (32)	
EGFR-negative	23 (68)	
Available for gene-expression analysis of primary tumours and normal colon tissue	82	NS
Available for protein ELISA analysis of primary tumour and normal colon tissue	94	NS
Available for FISH analysis of primary tumours	60	NS
Available for Sp1 analysis	79	NS
GG	36 (46)	
GT	36 (46)	
TT	7 (9)	

Table III. EGFR IHC status in primary tumours and corresponding lymph node metastasis ($p=0.00015$, Fisher's exact test).

	Metastasis EGFR-positive	Metastasis EGFR-negative	Total
Primary tumour EGFR-positive	10	5	15
Primary tumour EGFR-negative	1	18	19
Total	11	23	34

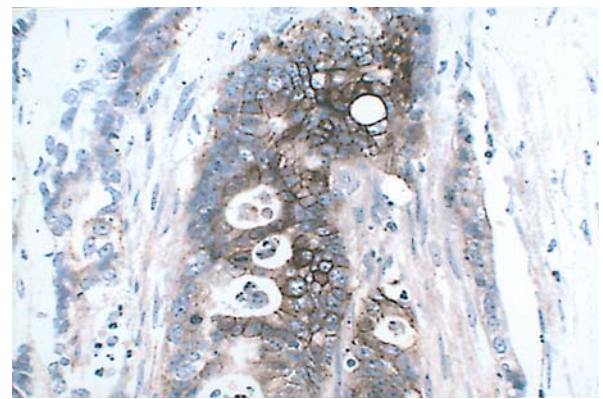


Figure 1. Immunohistochemical staining of EGFR in colorectal adenocarcinoma with intensity score 3+.

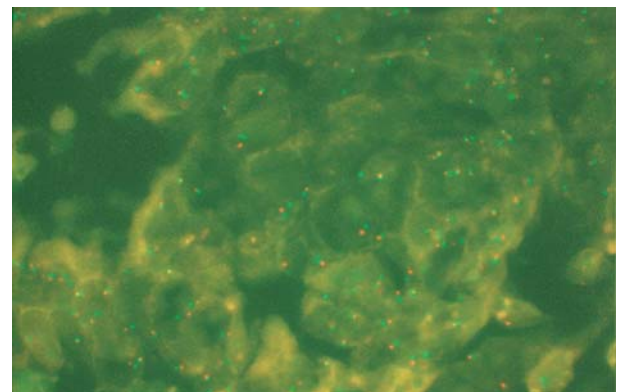


Figure 2. FISH analysis of EGFR in CRC. Red dots, EGFR gene; green dots, CEP7.

and normal colon tissue. The median EGFR level was 1.05 (0.98-1.23, 95% CI, range 0.49-2.2) in the normal colon compared to 0.7 (0.63-0.85, 95% CI, range 0.18-1.49) in the colorectal tumours (Fig. 3). The difference was significant ($p=10^{-6}$). There were no significant differences in EGFR gene expression levels between IHC positive and IHC negative tumours. Data are presented in Table IV. EGFR protein analysis by ELISA did not reflect the gene expression level of EGFR with no correlation between tumour protein level and

Table IV. Quantitative analysis of EGFR in tumour tissue and normal colon.

Method	Range	Median	95% CL	p-value
EGFR gene-expression	(qEGFR/qBA)	(qEGFR/qBA)		
Normal colon	0.49-2.20	1.05	0.98-1.23	10 ⁻⁶
Tumour overall	0.18-1.49	0.70	0.63-0.85	
IHC positive	0.26-1.49	0.70	0.63-0.85	0.92
IHC negative	0.20-1.45	0.71	0.53-0.89	
EGFR protein ELISA	(ng/mg)	(ng/mg)		
Normal colon	2.5-36.3	14.35	12.4-15.9	
Tumour overall	2.9-49.6	13.65	12.2-15.6	0.98
IHC positive	5.6-49.6	15.30	12.4-17.9	
IHC negative	2.9-39.9	13.20	9.5-15.9	0.086
EGFR gene-copy number by FISH				
Tumour overall	127-198	151	145-154	
IHC positive		151	143-157	
IHC negative		150	142-154	NS
EGFR gene/CEP7 ratio				
Tumour overall	0.928-1.151	0.9935	0.9833-1.007	
IHC positive		0.9833	0.60-1.007	
IHC negative		1.0	0.988-1.026	NS

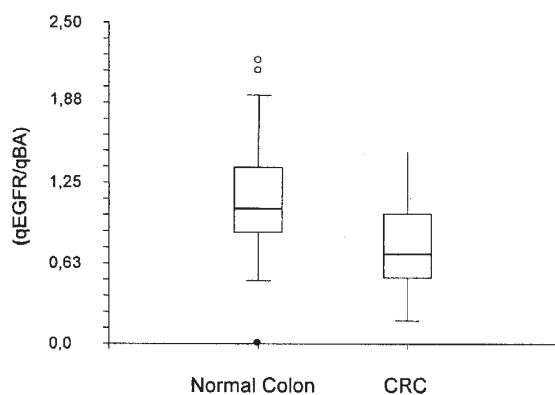


Figure 3. Boxplot of EGFR gene expression levels in normal colon and tumour. Vertical, EGFR gene expression level (qEGFR/qBA); horizontal, normal colon tissue and adenocarcinomas.

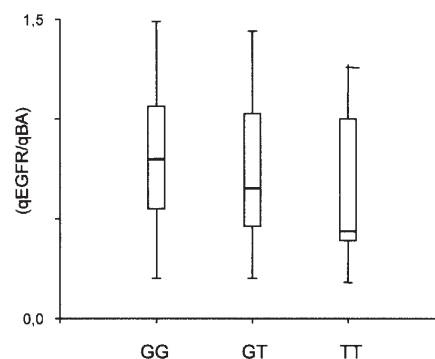


Figure 4. EGFR gene expression (qEGFR/qBA) according to EGFR Sp1-216. Vertical, EGFR gene expression level (qEGFR/qBA); horizontal, EGFR Sp1-216 genotype.

EGFR gene expression. Furthermore EGFR gene expression was not correlated to clinicopathological parameters or IHC score.

Sp1 polymorphism. Seventy-nine patients were available for Sp1 analysis. Forty-six percent (36/79) of the patients had the GG genotype, 46% (36/79) and 9% (7/79) were GT and TT respectively. There were no significant differences in median EGFR tumour gene expression levels according to Sp1-216 EGFR genotype but there was a tendency of TT genotype corresponding to a lower level than the GG groups ($p=0.067$) (Fig. 4) and that T containing variants had lower EGFR

tumour protein levels than GG variants ($p=0.08$). There were no differences between the Sp1 groups according to IHC score or clinicopathological parameters.

EGFR gene copy number. Sixty tumours were evaluated by FISH analysis. The median EGFR gene copy number was 151 (range 127-198) with no difference between IHC positive and IHC negative tumours (150 and 151 respectively). Results are shown in Table IV. The median EGFR gene/CEP7 ratio was 0.99 with a range between 0.929 and 1.15, indicating no gene amplification. When using the definition for balanced ratio as described by Sauer *et al* (20) (EGFR gene/CEP7 ratio between 0.8 and 1.2) all tumours in this study were balanced.

Discussion

There is no consensus with regard to the clinical importance of EGFR evaluation by immunohistochemistry (21,22). Concerning prognosis the current results are contradictory. Furthermore there is no clear indication that EGFR testing by immunohistochemistry can be used to predict the response to anti-EGFR therapies (2,13,14) as described previously.

The apparent discrepancies concerning EGFR evaluation by IHC in CRC have raised several hypotheses. Methodological problems are of major importance. Inconsistent interpretation and use of different antibodies contribute to the problem as discussed by Goldstein and Armin (23). The presence of both high- and low-affinity EGF receptors might be important and Atkins and colleagues have shown that the commonly used EGFRpharmDx kit depends on fixative type and storage time and it is not suitable for testing archival tissue in CRC (24). In addition multiple different scoring systems have been used according to the different studies. In the present study we used anti-EGFR antibodies from Dako and tissues sections were cut and stained within the appropriate time. A standardized scoring system according to the manufacturer's guideline was used.

The EGFR IHC expression rate shows considerable variability in the literature. Scartozzi *et al* (12) found that 53% of the primary tumours were positive when defining cut-off for EGFR positivity by $\geq 1\%$, which is similar to data presented here. On the other hand this is in disagreement with other studies (11). The present data showed that IHC score did not correlate to EGFR levels as measured by any of the methods presented here. These data point out the variability of EGFR detection by IHC.

Most studies address EGFR in primary tumours, which might be biologically different from the metastatic lesions. Scartozzi *et al* found that 36% of EGFR-positive tumours had EGFR-negative metastases and 15% of the EGFR-negative tumours had EGFR-positive metastases (12). McKay and colleagues (10) showed that only 40.5% of paired samples of tumour and metastases had equivalent EGFR expression and Bralet *et al* found that EGFR expression was positive in 73% of corresponding primary tumours and metastasis. In the present study we analysed 34 corresponding primary tumours and lymph node metastasis and found equivalent EGFR expression in 78% of the paired samples (Table III). These data support the idea that IHC staining of primary tumours for treatment selection to metastatic disease might be insufficient. The lack of correlation between EGFR status in primary tumour and metastatic sites could be related to possible genetic changes, tumour heterogeneity as well as methodological problems. Still if a standardised reproducible assay was developed, the question is whether it would provide the clinicians with reliable tools for treatment selections in these settings.

The diverging results of IHC have led to the consequence that several alternative methods have been proposed for evaluation of the biological activity of EGFR. The present study evaluated the relationship between EGFR at the DNA, RNA and protein levels in tumour and normal tissue. The results of this study underline the complexity of the EGFR signalling system.

EGFR can be addressed at the DNA level in several ways of which gene copy number assessment by FISH analysis has shown promising but inconsistent results. Recently Ooi *et al* found that 58% of IHC positive colorectal cancers were amplified, and showed a correlation between IHC and FISH, but not between primary tumour and metastasis (25). On the contrary Sauer and colleagues showed that all tumours with gene copy loss were IHC positive suggesting that EGFR gene copy loss is a surrogate marker for EGFR mutation/deletion (20). Moroni *et al* report that increased EGFR gene copy number may be used as predictor for response to cetuximab treatment (15). The study included 31 patients receiving either cetuximab monotherapy (n=12), cetuximab + irinotecan (n=9) or panitumumab (n=10). Eight out of 9 responders had an increased gene copy number compared to 1/20 of the non-responders. Consequently assessment of gene copy number was suggested as a potential predictive marker for response in this setting. In the present study all tumours were balanced with respect to EGFR gene copy/CEP7 ratio with a very narrow range, which is in disagreement with the above mentioned literature. The data presented here indicate that gene amplification in CRC is rare and its application as a predictive marker is dubious.

Gene expression analysis by real-time RT-PCR has been evaluated as a molecular determinant of cetuximab efficacy in colorectal cancer by Vallböhmer and colleagues (16). Thirty-nine patients were included in the study. There was no association between IHC and gene expression of EGFR, which is in concordance with the data presented here. Furthermore Vallböhmer *et al* reported no association between mRNA EGFR and response but found that a low mRNA EGFR level was associated with longer survival compared to patients with high mRNA levels. We did not find any association between protein levels, Sp1-216 polymorphism nor gene copy number and gene expression levels, underlining the complexity of EGFR testing. The present data showed a significantly higher EGFR expression in normal colon tissue compared to colon tumours. These findings are supported by data from EGFR gene expression analysis in prostate cancer patients (26) and challenge the common opinion of EGFR overexpression as a main contributor to EGFR dysregulation. Obviously the issue needs further evaluation in prospective trials.

EGFR protein level was evaluated by traditional IHC and ELISA on fresh frozen tissue. The literature on EGFR ELISA on fresh frozen tissue is limited. It was our aim to investigate if EGFR quantification by ELISA correlated to the IHC score. EGFR ELISA protein level did not reflect IHC scoring intensity and did not correlate to gene expression levels. The role of ELISA quantification of EGFR as a supplement to other methods needs further investigation.

The present study is the first evaluation of the possible relationship between EGFR Sp1-216 polymorphism and EGFR gene expression in CRC. Forty-seven percent of patients had GG genotype, 43% and 9% were GT and TT respectively. This distribution of genotype in the population is in concordance with the data produced by Liu *et al* who furthermore reported that cell lines with the TT variant had low gene expression levels of EGFR (17). The present study showed a tendency of the TT genotype corresponding to a lower level than the GG

group. Furthermore T containing variants showed a tendency of lower EGFR ELISA protein levels than GG variants. Obviously the low number of observations in the TT group should be considered when interpreting these data. Liu and colleagues suggested that -216 G/T polymorphisms might contribute to the inter-individual variability in EGFR expression and response to EGFR targeted therapies. Analysis of EGFR Sp1-216 G/T polymorphism is a reliable and easy method. Therefore it could be a good candidate for a predictive marker but more data from clinical trials are needed.

In conclusion the results of the present study show a poor correlation between EGFR status as measured by different methods at DNA, RNA and protein level. Our results underline the complexity of the EGFR regulation. Therefore a comparison of different methods should be taken with caution. Prediction of treatment effect can probably not rely on a single method. A further step forward would be to investigate the possible value of a combination of different methods in clinically well-characterised patient populations.

Acknowledgements

We thank Lone Frischknecht, Birgit Roed Sørensen and Anette Buhl for their technical assistance and Kirsten Birgitte Hjermitslev for proofreading. The study was supported by grants from Vejle County.

References

- Parkin DM, Bray F, Ferlay J and Pisani P: Estimating the world cancer burden: Globocan 2000. *Int J Cancer* 94: 153-156, 2001.
- Cunningham D, Humblet Y, Siena S, *et al*: Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N Engl J Med* 351: 337-345, 2004.
- Tyagi P: Recent results and ongoing trials with panitumumab (ABX-EGF), a fully human anti-epidermal growth factor receptor antibody, in metastatic colorectal cancer. *Clin Colorectal Cancer* 5: 21-23, 2005.
- Calvo E and Rowinsky EK: Clinical experience with monoclonal antibodies to epidermal growth factor receptor. *Curr Oncol Rep* 7: 96-103, 2005.
- Blanke CD: Gefitinib in colorectal cancer: if wishes were horses. *J Clin Oncol* 23: 5446-5449, 2005.
- Shepherd FA, Rodrigues PJ, Ciuleanu T, *et al*: Erlotinib in previously treated non-small-cell lung cancer. *N Engl J Med* 353: 123-132, 2005.
- Marmor MD, Skaria KB and Yarden Y: Signal transduction and oncogenesis by ErbB/HER receptors. *Int J Radiat Oncol Biol Phys* 58: 903-913, 2004.
- Yarden Y and Sliwkowski MX: Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2: 127-137, 2001.
- Spaulding DC and Spaulding BO: Epidermal growth factor receptor expression and measurement in solid tumors. *Semin Oncol* 29: 45-54, 2002.
- McKay JA, Murray LJ, Curran S, *et al*: Evaluation of the epidermal growth factor receptor (EGFR) in colorectal tumours and lymph node metastases. *Eur J Cancer* 38: 2258-2264, 2002.
- Spano JP, Lagorce C, Atlan D, *et al*: Impact of EGFR expression on colorectal cancer patient prognosis and survival. *Ann Oncol* 16: 102-108, 2005.
- Scartozzi M, Bearzi I, Berardi R, Mandolesi A, Fabris G and Cascinu S: Epidermal growth factor receptor (EGFR) status in primary colorectal tumors does not correlate with EGFR expression in related metastatic sites: implications for treatment with EGFR-targeted monoclonal antibodies. *J Clin Oncol* 22: 4772-4778, 2004.
- Saltz LB, Meropol NJ, Loehrer PJ Sr, Needle MN, Kopit J and Mayer RJ: Phase II trial of cetuximab in patients with refractory colorectal cancer that expresses the epidermal growth factor receptor. *J Clin Oncol* 22: 1201-1208, 2004.
- Chung KY, Shia J, Kemeny NE, *et al*: Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. *J Clin Oncol* 23: 1803-1810, 2005.
- Moroni M, Veronese S, Benvenuti S, *et al*: Gene copy number for epidermal growth factor receptor (EGFR) and clinical response to anti-EGFR treatment in colorectal cancer: a cohort study. *Lancet Oncol* 6: 279-286, 2005.
- Vallböhmer D, Zhang W, Gordon M, *et al*: Molecular determinants of cetuximab efficacy. *J Clin Oncol* 23: 3536-3544, 2005.
- Liu W, Innocenti F, Wu MH, *et al*: A functional common polymorphism in a Sp1 recognition site of the epidermal growth factor receptor gene promoter. *Cancer Res* 65: 46-53, 2005.
- Lindebjerg J, Nielsen JN, Hoeffding LD, Bisgaard C, Brandslund I and Jakobsen A: Expression of thymidylate synthase in primary colorectal adenocarcinoma. *Appl Immunohistochem Mol Morphol* 14: 37-41, 2006.
- Jakobsen A, Nielsen JN, Gyldenkerne N and Lindeberg J: Thymidylate synthase and methylenetetrahydrofolate reductase gene polymorphism in normal tissue as predictors of fluorouracil sensitivity. *J Clin Oncol* 23: 1365-1369, 2005.
- Sauer T, Guren MG, Noren T and Dueland S: Demonstration of EGFR gene copy loss in colorectal carcinomas by fluorescence *in situ* hybridization (FISH): a surrogate marker for sensitivity to specific anti-EGFR therapy? *Histopathology* 47: 560-564, 2005.
- Nicholson RI, Gee JM and Harper ME: EGFR and cancer prognosis. *Eur J Cancer* 37: 9-15, 2001.
- Steele RJ, Kelly P, Ellul B and Eremin O: Epidermal growth factor receptor expression in colorectal cancer. *Br J Surg* 77: 1352-1354, 1990.
- Goldstein NS and Armin M: Epidermal growth factor receptor immunohistochemical reactivity in patients with American Joint Committee on Cancer Stage IV colon adenocarcinoma: implications for a standardized scoring system. *Cancer* 92: 1331-1346, 2001.
- Atkins D, Reiffen KA, Tegtmeier CL, *et al*: Immunohistochemical detection of EGFR in paraffin-embedded tumor tissues: variation in staining intensity due to choice of fixative and storage time of tissue sections. *J Histochem Cytochem* 52: 893-901, 2004.
- Ooi A, Takehana T, Li X, *et al*: Protein overexpression and gene amplification of HER-2 and EGFR in colorectal cancers: an immunohistochemical and fluorescent *in situ* hybridization study. *Mod Pathol* 17: 895-904, 2004.
- Turkeri LN, Sakr WA, Wykes SM, *et al*: Comparative analysis of epidermal growth factor receptor gene expression and protein product in benign, premalignant, and malignant prostate tissue. *Prostate* 25: 199-205, 1994.