Abstract. Background: Epidermal growth factor receptor (EGFR) analysis by traditional immunohistochemistry does not provide clinicians with a reliable tool for the selection of patients to EGFR-targeted treatment in colorectal cancer (CRC). Alternative methods and further understanding of the EGFR signaling network are being investigated and mutations in the EGFR gene have been identified. The type III epidermal growth factor receptor, a tumour-specific, ligand independent, constitutively activated form of EGFR, might contribute to the malignant phenotype in CRC and may be a potential target for anticancer therapy. The aim of the present study was to investigate the presence of EGFRvIII in CRC by PCR and protein analysis. Materials and Methods: The study included 79 colorectal cancer patients for PCR analysis and 50 patients for protein analysis by Western blots, in two different laboratories. Results: No type III mutations were detected in our material. Conclusion: The EGFRvIII mutations are rare in colorectal adenocarcinomas and overall probability does not appear to contribute to the malignant phenotype of this disease.

Colorectal cancer (CRC) is one of the major cancer diseases in the world and, regardless of the substantial development in new treatment modalities, the overall prognosis is still poor (1). Recently, therapies targeting the epidermal growth factor receptor (EGFR) have been added to the panel of cytotoxic drugs for this disease. Targeting the EGFR has shown promising potential in metastatic CRC, but no reliable predictive markers have been found regarding EGFR-targeted therapies in this disease (2-4).

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The EGFR is a 170 kDa transmembrane glycoprotein that belongs to the receptor tyrosine kinase family of growth factor receptors. EGFR is present in most epithelial-derived tissues and is overexpressed in many solid tumours (5). The receptor is comprised of an extracellular ligand binding domain, a transmembrane and an intracellular domain, which contain the intrinsic tyrosine kinase sites. Ligands bind to the extracellular domain of EGFR, and consequently initiate conformational changes and dimerisation of the receptor. Hetero- or homo-dimerisation of receptors results in receptor autophosphorylation and activation of the intrinsic tyrosine kinases. A complex intracellular downstream signaling cascade is hereby activated, initiating changes in gene transcription. The EGFR is known to influence cell proliferation and growth (6) and dysregulation of the EGFR has been associated with tumour development and growth, rendering the receptor an attractive target for anti-cancer therapies (7, 8). The EGFR can contribute to malignant transformation in various ways, e.g. receptor overexpression, autocrine loops (9), gene amplification (10) and/or mutations.

Several mutations in the EGFR gene have been identified. The exons 16 to 22 encode the intracellular tyrosine kinase domain of the EGFR and mutations in these locations have been associated with changed sensitivity to the anti-EGFR tyrosine kinase inhibitor gefitinib in NSCLC, but are not frequent in colorectal cancer (11-13). Deletions in the extracellular domain often occur in a number of human tumours (14).

The most common mutation of EGFR is the type III variant (also named EGFRvIII, de2-7 EGFR or delta EGFR), which lacks amino acids 6-273 of the extracellular domain, a 145-kDa range compared to the 170-kDa range of the EGFRwt receptor (15). The mutation arises from genomic deletion or alternative splicing. The deletion is reported to be associated with gene amplification in some tumours (14, 16).

Although activation of the wild-type receptor depends on the ligand binding, the EGFRvIII lacks the ligand binding sites of the extracellular domain. Instead, conformational changes have rendered the type III mutation ligand-independent and constitutively active. Furthermore, EGFRvIII activates the cell survival pathway (17).
was reported to enhance tumorigenicity of tumour cells in vitro and especially in vivo and is capable of transforming non-tumorigenic cell lines into a malignant phenotype (18, 19).

EGFRvIII was investigated in normal tissue of breast, ovary, lung, skin, kidney, brain, prostate glands and colon and was not detected (14, 20). On the other hand, a high expression rate was reported in various tumours, including glioblastomas, NSCLC, breast and ovarian carcinomas (15). Clinical data suggest that EGFRvIII expression is associated with poor prognosis in some tumours (21). Thus, not detected in normal tissue and frequently expressed in various tumours, the EGFRvIII mutation is a promising target for cancer-specific therapy. Several strategies for targeting the receptor have been developed: toxic antibodies, modified viruses, mRNA-specific ribozymes, small molecule inhibitors or the provocation of an immunological response against the EGFRvIII were previously investigated (15).

The literature on EGFRvIII in colorectal cancer is scarce. Recently, Cunningham et al. reported the presence of EGFRvIII in 34% of colorectal tumours, detected by immunohistochemistry with an EGFRvIII-specific antibody. Cytoplasmatic staining was correlated with longer survival in patients, previously treated by radiation therapy (22).

With a promising potential as an anticancer target and considering the positive results of EGFRwt inhibitors in colorectal cancer patients, investigations of the present and predictive value of the mutated receptor seem relevant in this disease. The aim of the present study was to investigate the presence of EGFRvIII in CRC by PCR and protein analysis.

Materials and Methods

Seventy-nine patients were included during the period between December 2003 and July 2005. All patients underwent surgical resection for adenocarcinomas of the colon or rectum at the Department of Surgery, Vejle Hospital, Denmark. Dukes’ and TNM classifications were used for evaluation of disease extension. Samples of normal colon and colorectal tumours were collected at surgery after informed consent and fresh tissue from each tumour was frozen in Tissuetek O.C.T.™ compound (Sakura) and prepared for protein analysis. Additional tissue samples were stored at −20°C in RNA-later (Qiagen, CA, USA) and prepared for RNA isolation. The study was approved by the Regional Ethics Committee of Vejle and Funen Counties, according to Danish law.

**Extraction of tissues.** Tissue samples of 10 to 50 mg were homogenised at 4°C by an ultra-turrax system (Ika, Germany) with 10 volumes (w/v) buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4, 1 mM EDTA, 10% glycerol, protease inhibitor cocktail, cat. No. P3340, 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 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).

**Page and Western blotting, laboratory A.** One-dimensional sodium dodecyl sulphate (SDS)-PAGE was performed in 7.5% uniform gels (Biorad) using a Tris-glycine buffer system. Equal amounts of protein (60 µg) from tissue samples along with 5 µg of control lysate (EGFRvIII-positive control: NR6M cell line, kindly provided by Dr. Darell Bigner, Duke University Medical Center, Durham North Carolina, USA and EGFRwt positive control: A431 cell line, (Upstate)) were subjected to PAGE in the presence of 2% SDS and 30 mM dithiothreitol (DTT). In order to define the positive detection limit of the assay, decreasing amounts of protein from the EGFRvIII-positive cell line were loaded onto the gel. For subsequent analysis by western blotting, proteins were electrophoretically transferred for 45 min at 100V in 25 mM Tris-Amine, 192 mM Glycine, pH 8.3, 10% methanol, 0.037% SDS to a 0.45 µm Polyvinylidene difluoride membrane. The membranes were blocked with 20 mM Tris-HCl, 137 mM NaCl, pH 7.6 (TBS) containing 5% (w/v) BSA.

EGFRwt and EGFRvIII were subsequently detected using a monoclonal anti-human EGFR antibody (specific for both the EGFR wild-type and the truncated type III variant: clone 31G7, 1 µg/ml, Zymed) and HRP-conjugated rabbit anti-mouse IgG (Dako, Glostrup, Denmark). All incubations were done in TBS containing 0.1% (v/v) Tween 20. Immunoreactive bands were detected with an Immun-Star HRP Chemiluminescent detection kit from Biorad.

**Extraction of RNA.** Total RNA was isolated using the RNeasy kit from Qiagen according to manufacturer’s instructions. The isolated RNA was quantitated by Spectrophotometry (Eppendorf, Hamburg, Germany) and cDNA synthesis was performed using M-MLV RT (Qiagen). Total RNA isolation and cDNA synthesis, real-time fluorescence PCR was performed on an ABI prism HT 7900 Sequence detection system, TaqMan. (Perkin-Elmer, Applied Biosystem, Foster City, USA). For detection of EGFRvIII, primers and probe were designed using a Primer express programme. For detection of EGFR and beta-actin, pre-developed assays (PDAR) from Applied Biosystems
(Hs01076088_m1) were used. The housekeeping gene β-actin was used as denominator for standardisation. The PCR mixture and cycling conditions were conducted according to manufacturer’s instructions. The relative gene-expression was determined based on the threshold cycles of EGFRvIII and the internal standard β-actin. Quantification was performed as previously published using a standard curve model (23). The standard curve was obtained from total RNA isolated from EFGRvIII positive cell line N6M. The line of the EGFRvIII standard curve was $y = -3.2738x + 29.098$ and the linear regression coefficient $R^2 = 0.9997$. The line and regression coefficient of the 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 triplicate. EGFRvIII primer sequences: EGFRvIII forward: 5′- ggctctggaggaaaagaaaggtaatt 3′ and EGFRvIII reverse: 5′- ccgtcttcctccatctcatagc 3′. Probe sequence: EGFRvIII Probe: 5-FAM-tgacagatcacggctc-MGB.

**Results**

The clinicopathological parameters are shown in Table I. Fifty percent of tumours were Dukes’ C (38/79).

**EGFRwt and EGFRvIII gene expression.** EGFR wild-type (EGFRwt) was expressed at varying levels in the samples tested (data not shown). The median qEGFRwt/qBA in tumour samples was 0.7 (range 0.18-1.49) compared to 1.04 (range 0.001-2.2) in normal colon and was detected by Western blot. EGFRvIII was not found in any of the 79 tumour samples tested. (0/79: 97.5% CI 0.00-4.56).

**EGFRvIII analysis by Western Blot.** The positive controls of the NRM6 cell line and human EGFRvIII-positive brain tumour showed positive bands at 145 kDa, as shown in Figures 1 and 2.
A positive detection limit of 0.25 µg protein in NR6M cell line was found (Figure 3), corresponding to approximately 300 cells or 6x10⁷ receptors. (The average amount of cells were counted in a sample and found to be 1200 cells/µg protein.)

Fifty tumours and 44 corresponding normal colon samples were available for Western blotting in laboratory A and 44 of the tumour samples were analysed in laboratory B with an EGFRvIII-specific antibody. None of the samples were positive for the EGFRvIII protein (Figures 1 and 2). (Laboratory B, 0/44 and laboratory A, 0/50: 97.5% CI: 0.00-8.04 and 0.00-7.10, respectively). A total of 38 tumours lacking EGFRvIII were Dukes’ C: 0/38; 97.5% CI 0.00-9.3. When comparing the proportion of positive tumours in the present study (0/38) with the proportion found by Cunningham et al. (30/87) by Fishers exact test, the p-value was less than 0.0001.

**Discussion**

The EGFRvIII mutation is promising as a target for anticancer therapy, since the type III variant is selective for cancer cells. The role of EGFRvIII as a predictive and prognostic marker is yet to be evaluated in larger clinical settings. EGFR inhibitor mAb cetuximab showed promising potential in metastatic CRC (4), but immunohistochemistry of the EGFRwt failed as a predictive marker in this setting. Even tumours with IHC-negative staining responded to cetuximab, which mainly targets the extracellular domain of the receptor. Response to treatment and IHC status might be influenced by the presence of mutated receptors, as seen in NCSLC during treatment with gefitinib, which has been intensively investigated (13). Evaluation of EGFRvIII in CRC could provide additional knowledge concerning the complex issue of EGFR signaling in CRC. This study however, is only the second study addressing EGFRvIII mutations in CRC, which might be due to publication bias.

We did not detect any tumours positive for EGFRvIII. However, Cunningham et al. recently reported that 30 out of 87 Dukes C colorectal tumours were positive for EGFRvIII, using immunohistochemistry (22). The data showed that membranous staining was not associated with survival, but that cytoplasmatic staining was correlated with longer survival, in patients previously treated by radiation therapy. Furthermore, the EGFRvIII was detected in the normal mucosa of several specimens. Immunohistochemistry of the truncated receptor is, however, complex. The EGFRvIII-specific antibody used by Cunningham et al. is no longer commercially available and a comparison of the two studies is therefore not possible. Instead, we approached the subject by quantitative PCR and traditional Western blotting, for which we used two different antibodies. The positive controls were a well known reliable cell line with high expression of EGFRvIII (and no expression of the wild-type receptor) and an EGFRvIII-positive human brain tumour.

The absence of EGFRvIII mutations in the present study could obviously be the result of a low sensitivity method. Validation of the present methods for page and Western blotting included using the NR6M cell line for titration of decreasing amounts of protein. The results showed a positive assay, with as little as 0.25 µg protein in a sample. Only clinically non-significant amounts of protein would be missed by this assay.

The present study has the obvious limitation of a rather small number of observations. However considering the EGFRvIII expression rate of 34% as presented by Cunningham et al., the present lack of positive observations are not statistically likely to be the result of a small sample size. Even if only considering the Dukes’ C tumours, the confidence interval did not include 34%, as reported by Cunningham et al.

Studies of different tumours, e.g. glioblastoma multiforme, have shown that EGFRvIII mutation is associated with gene-amplification (16). However, in colorectal cancer, the data on EGFR gene copy number show inconsistent results. Only a few studies indicated increased gene copy number (24, 25), in disagreement with other studies, where increased gene-copy number or amplifications were not detected (26). The low amplification rate seen in colorectal cancer might be reflected by the absence of EGFRvIII mutations.

In conclusion, the present study clearly indicates that EGFRvIII is rare in CRC. EGFRvIII does not appear to contribute to the malignant phenotype in this disease and consequently, targeting the EGFRvIII for treatment in CRC is questionable.

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**References**

Spindler et al: Lack of EGFRvIII in CRC


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