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COLORECTAL CANCER

VEGF-D expression correlates with colorectal cancer aggressiveness and is downregulated by cetuximab

Markus Moehler, Christian Frings, Annett Mueller, Ines Gockel, Carl C Schimanski, Stefan Biesterfeld, Peter R Galle, Martin H Holtmann

Markus Moehler, Christian Frings, Annett Mueller, Carl C Schimanski, Peter R Galle, Martin H Holtmann, 1st Department of Medicine, Johannes Gutenberg University, Mainz 55101, Germany
Ines Gockel, Department of General and Abdominal Surgery, Johannes Gutenberg University, Mainz 55101, Germany
Stefan Biesterfeld, Institute of Pathology, Johannes Gutenberg University, Mainz 55101, Germany
Author contributions: Moehler M and Frings C contributed equally to this work; Moehler M, Galle PR and Holtmann MH designed research; Frings C, Mueller A, Gockel I, Schimanski CC and Biesterfeld S performed research; Moehler M, Schimanski CC, Biesterfeld S, Galle PR and Holtmann MH contributed new reagents/analytic tools; Moehler M, Frings C and Mueller A analyzed data; Moehler M, Mueller A, Galle PR and Holtmann MH wrote the paper.
Correspondence to: Dr. Markus Moehler, 1st Department of Medicine, Johannes Gutenberg University, Langenbeckstrasse 1, Mainz 55101, Germany. moehler@mail.uni-mainz.de
Telephone: +49-6131-177276 Fax: +49-6131-176410
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Abstract

AIM: To gain mechanistic insights into the role played by epidermal growth factor receptor (EGFR) in the regulation of vascular endothelial growth factors (VEGFs) in colorectal cancer (CRC).
METHODS: The impact of high-level expression of the growth factor receptors EGFR and VEGF receptor (VEGFR)3 and the VEGFR3 ligands VEGF-C and VEGF-D on disease progression and prognosis in human CRC was investigated in 108 patients using immunohistochemistry. Furthermore, the expression of the lymphangiogenic factors in response to the modulation of EGFR signalling by the EGFR-targeted monoclonal antibody cetuximab was investigated at the mRNA and protein level in human SW480 and SW620 CRC cell lines and a mouse xenograft model.
RESULTS: Human CRC specimens and cell lines displayed EGFR, VEGF-C and VEGF-D expression with varying intensities. VEGF-C expression was associated with histological grade. Strong expression of VEGF-D was significantly associated with lymph node metastases and linked to a trend for decreased survival in lymph node-positive patients. EGFR blockade with cetuximab resulted in a significant decrease of VEGF-D expression *in vitro* and *in vivo*.
CONCLUSION: In conclusion, the expression of VEGF-D in colorectal tumours is significantly associated with lymphatic involvement in CRC patients and such expression might be blocked effectively by cetuximab.

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Key words: Human colorectal cancer; Lymphangiogenesis; Vascular endothelial growth factor-C; Vascular endothelial growth factor-D; Epidermal growth factor receptor

Peer reviewer: Peter L Lakatos, MD, PhD, Assistant Professor, 1st Department of Medicine, Semmelweis University, Koranyi S 2A, Budapest H1083, Hungary

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INTRODUCTION

Globally, colorectal cancer (CRC) is one of the three most commonly diagnosed malignancies[1]. For patients with metastatic disease, systemic cytotoxic chemotherapy has been shown to clearly improve survival[2-5]. More recently, the addition of therapeutic antibodies including cetuximab[6-8] and bevacizumab[9,10] to such cytotoxic regimens has been shown to further improve outcomes in first- and second-line settings.

The mode of action of the new therapeutic antibody cetuximab is thought to be based primarily upon perturbation of epidermal growth factor receptor (EGFR)-ligand interactions[11]. Binding of cetuximab blocks EGFR-associated cellular signal transduction cascades, which govern processes such as tumour cell survival, proliferation, invasion and metastasis[12-16]. Since high-level expression of the EGFR gene has been associated with reduced survival in a range of malignancies, targeting growth factor receptor signalling

cascades is a promising anticancer strategy[17-24]. Even more, an additional mode of action of cetuximab has been suggested which relates to tumour cell binding inducing an antibody-dependent cell-mediated cytotoxicity reaction[25,26].

However, even if a number of such molecular tumour characteristics appeared to be associated with the antitumor efficacy of EGFR-targeted agents[27], it remains a matter of debate as to whether the intensity or extent of immunohistochemical detection of EGFR expression in the tumour correlate with prognosis and response to EGFR-targeted agents[28,29].

The hypothesis that growth and spread of tumours are dependent on their vascular and lymphatic systems was proposed several decades ago[30]. Interest in this concept has recently been rekindled following the molecular identification of regulators of (lymph-) angiogenesis such as the vascular endothelial growth factor (VEGF) family, including the ligands VEGF-A, -B, -C and -D, and the VEGF receptors VEGFR1 (FLT1), VEGFR2 (KDR) and VEGFR3 (FLT4)[31] and their clinical utilization by recombinant strategies for targeting angiogenesis, such as the anti-VEGFA antibody, bevacizumab[9] and the decoy receptor, VEGF Trap[32]. Interestingly, VEGF-C and VEGF-D, signalling through VEGFR3, have been identified as key regulators of lymphangiogenesis[33-36]. Data from *in vitro* and murine tumour models further support the key role of VEGF-C and VEGF-D in malignancy. For many tumour types, clinical studies have revealed a correlation between VEGF-C, VEGF-D and VEGFR3 expression and lymphatic spread, tissue invasion or poor prognosis[37-41]. However, in other studies, clear associations were not identified[42,43] or low levels of VEGF-D were correlated with an increased risk of metastasis and reduced survival[44]. Similar data have also been reported for CRC. In one study, VEGF-C and VEGF-D expression correlated with the tumour invasion, lymphatic and venous involvement, lymph node metastasis and liver metastasis, and reduced survival time[45]. A second study also reported that high-grade VEGF-D expression was associated with lymphatic involvement and poor patient survival[46], while a third confirmed that VEGF-D expression correlated with the depth of tumour invasion, lymph node metastasis and reduced survival time[47]. However, in other analyses VEGF-D expression at the mRNA-level was reported to be downregulated in CRCs with lymphatic spread[48] and appeared to be lower at the leading edge of tumours in which lymphatic vessels were present[49].

Given that lymphangiogenesis is increasingly recognized as a critical component of tumourigenesis and that EGFR signalling, a key regulator of tumourigenesis in CRC, possibly acts to some extent through regulation of VEGF-C and VEGF-D expression, we evaluated the co-expression profiles of EGFR, VEGF-C and VEGF-D in human CRC specimens. Results were correlated with the patients' clinicopathological parameters and survival. Furthermore, in order to gain mechanistic insights into the role played by EGFR in the regulation of VEGF-D in colorectal cancer, we analyzed the effect of cetuximab *in vitro* and *in vivo* on the expression of VEGF-D in SW480 and SW620 human colon cancer cell and xenograft models of CRC. We thus showed that expression of VEGF-D is prognostically relevant in CRC and for the first time provided experimental evidence that EGFR-targeted antitumor therapy exerts its effect in part through suppressing lymphangiogenesis by downregulating VEGF-D.

MATERIALS AND METHODS

Tissue samples and patient characteristics

All tissues investigated in this study were obtained from patients (*n* = 108) who underwent colectomy between 1995 and 2003 at the Department of Abdominal Surgery, University Hospital Mainz, Germany. Written informed consent for experimental immunohistochemistry was obtained from all patients before analysis. Expression of EGFR was analyzed in all patients, with assessment of VEGF-C and VEGF-D conducted in 102 cases and 104 cases, respectively, because of limited availability of tumour material.

Patient age at the time of primary surgery ranged from 36.2 years to 83.1 years (63.6 ± 10.45 years). Seven patients were lost to follow up and were therefore censored at the time of last contact (34.86 ± 4.18 mo). Staging and diagnosis of CRC was assessed according to the World Health Organization classification and the TNM classification as set out by the International Union Against Cancer [Union International Contre le Cancer (UICC)]. After resection, patients were followed up every 6 mo. Patients with synchronous or metachronous metastasis underwent additional restaging every 3 mo during chemotherapy.

Immunohistochemical (IHC) staining

Formalin-fixed paraffin-embedded tissues of patients with CRC from the Department of Pathology, University Hospital Mainz, Germany, were used in this study. Tissue sections (4 μm) were cut from these blocks and used for IHC staining. All tissue sections were deparaffinized in xylene and rehydrated in a graded ethanol series.

Staining for EGFR was performed using the commercially available EGFR pharmDx kit (DakoCytomation, Carpinteria, CA, USA), which includes the pharmDx mouse anti- EGFR monoclonal antibody (clone 2-18C9), a negative control reagent (a mouse monoclonal antibody for an enzyme that is not expressed in mammalian tissue), and positive and negative control cancer cell preparations (CAMA-1 breast cancer and HT29 colon cancer cell lines). IHC staining was performed according to the manufacturer's instructions.

Staining for VEGF-C and VEGF-D was carried out following antigen retrieval. Sections were heated in citrate buffer and then cooled for 20 min. Endogenous peroxidase was blocked in 3% hydrogen peroxide in methanol for 15 min. To block nonspecific binding, prior to incubation with the primary antibody, tissue sections were incubated with serum-free DAKO Antigen-Block for 30 min. Primary antibodies specific for VEGF-C

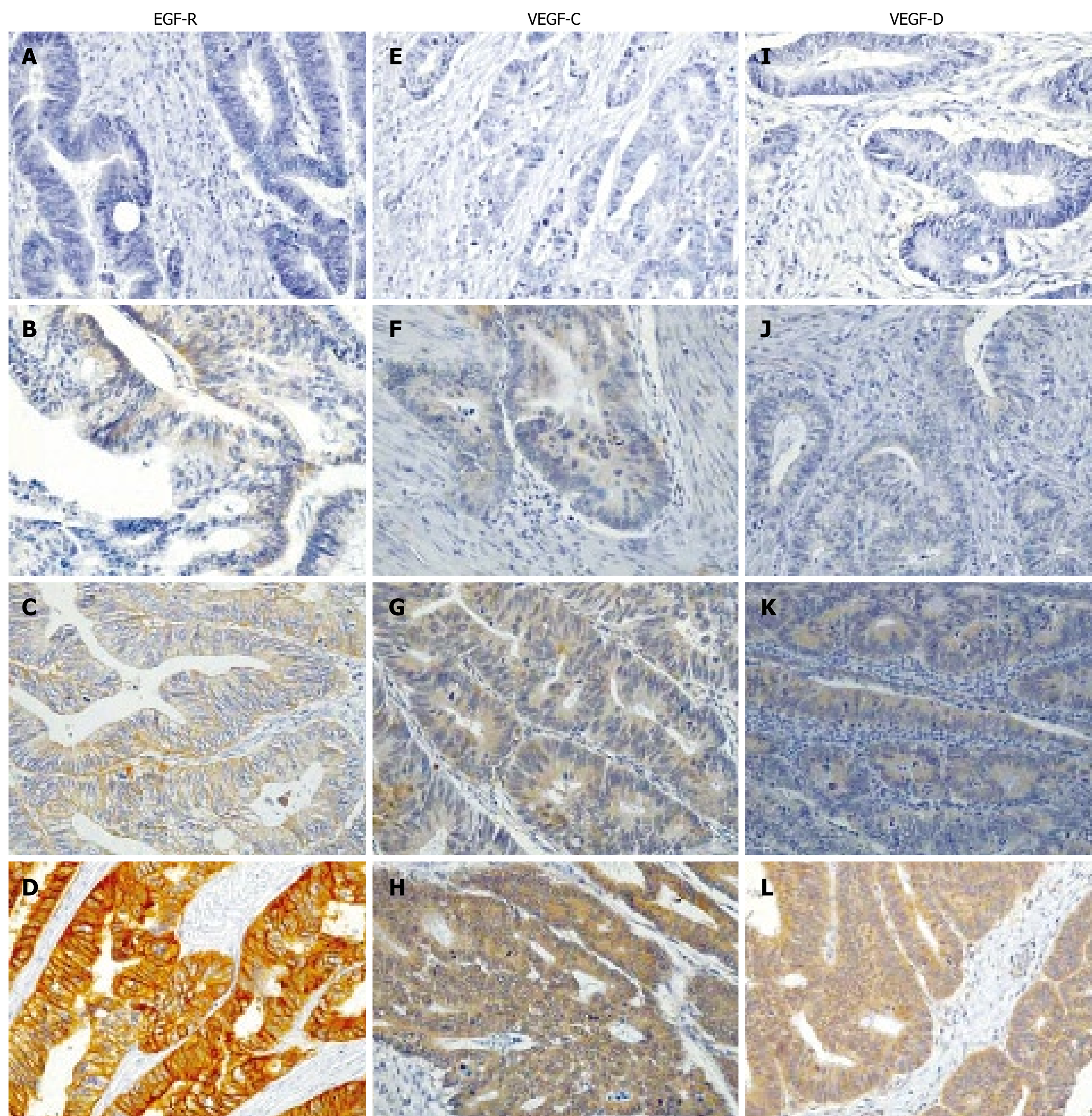

Figure 1 Immunohistochemical analysis of the expression levels of EGFR (**A-D**), VEGF-C (**E-H**) and VEGF-D (**I-L**) in human colorectal cancer sections (× 400). **A**, **E** and **I**: No expression; **B**, **F** and **J**: Weak expression; **C**, **G** and **K**: Moderate expression; **D**, **H** and **L**: Strong expression.

(sc-9047, Santa Cruz Biotechnology Inc. CA, USA) and VEGF-D (sc-13085, Santa Cruz Biotechnology Inc.) and were diluted 1:50 in DAKO ChemMate antibody diluent and sections were incubated for 16 h at 4℃ (VEGF-C) or 2 h at room temperature (VEGF-D). After incubation with an anti-rabbit secondary antibody for 30 min, bound complex was visualized by using diaminobenzidine (ChemMateTM DAKO EnVisonTM Detection Kit). Sections were counterstained with Mayer's hematoxylin and mounted. Between all incubations, sections were washed in phosphate-buffered saline (PBS). Foetal kidney was used as a positive control. Negative controls were prepared by omitting primary antibody from the process (data not shown).

Evaluation of immunostaining

Immunostaining was independently evaluated by 4 authors who were blinded to patient outcome and all clinicopathological findings. EGFR-staining was interpreted according to standard parameters (EGFR pharmDx™ Interpretation Manual, DAKO). The staining intensities (Figure 1) were scored as negative, weak, moderate or strong. To unequivocally categorize cases into two groups, each sample was defined as EGFR-negative or EGFR-positive when < 1% or ≥ 1% of the tumour cells respectively showed an EGFR-immunospecific membranous brown staining.

Staining results for VEGF-C and VEGF-D were similarly classified by estimating both the staining intensity

and the percentage of epithelial cells showing specific immunoreactivity. Staining intensities were not always homogeneous across individual tumour samples (as shown in Figure 1). All results showing more than 25% of tumour cells stained (with weak to strong positivity) were considered to represent biologically relevant levels of expression of these proteins and, for the purposes of statistical analysis, were counted as positive results.

Cell lines

The colon carcinoma cell lines SW480 and SW620 were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640 media (Invitrogen, Carlsbad, CA, USA), supplemented with 10% foetal calf serum (FCS: PAA Laboratories, Pasching, Austria). Cells were cultured at 37℃ in a 5% CO_2 atmosphere and passaged routinely using Trypsin-ethylenediaminetetraacetic acid (PAA Laboratories) treatment.

Cetuximab

Clinical grade anti-EGFR monoclonal antibody cetuximab (Erbitux®) was supplied by Merck KGaA (Darmstadt) at a concentration of 2 mg/mL in a buffer consisting of 10 mmol/L sodium phosphate and 145 mmol/L sodium chloride at pH 7.

Stimulation assay

SW480 and SW620 cells were seeded at 3×10^5 cells/well in a 6-well tissue culture plate with media containing 10% FCS. After 24 h, cells were starved by incubation in media with a reduced FCS level (0.5%) for an additional 24 h. Afterwards, cells were incubated in medium plus 0.5% FCS supplemented either with 5 ng/mL epidermal growth factor (EGF, Sigma Chemical Co., St. Louis, MO, USA) for 20 min followed by 20 μg/mL cetuximab, or alternatively, the same medium supplemented with one or other of these substances. After culturing for another 24 h, monolayers were washed with icecold PBS, centrifuged and pellets were frozen for RNA isolation. The cell culture experiments were independently repeated at least two times.

RNA isolation and quantitative real time RT-PCR

RNA isolation was performed using the RNeasy Kit according to the manufacturer's recommendations (Qiagen, Hilden, Germany). Transcription of the housekeeping gene glycerinaldehyde-3-phosphate-dehydrogenase (GAPDH), VEGF-D and VEGFR3 was analyzed by a one-step reverse transcriptase-polymerase chain reaction (RT-PCR) using a LightCycler 2.0 system (Roche). RT-PCR was performed with 0.1 μg of RNA in a 35 cycle reaction (20 μL total volume; QuantiTect SYBRGreen RT-PCR, Qiagen) according to the recommendations of the manufacturer. All RT-PCR reactions were done in 2 replicates. All PCRs were established with an exponential phase efficiency of 2 to guarantee that the data were comparable. The evaluation of the expression of the target genes was performed relative to the expression of GAPDH. Control and test samples in the EGF/cetuximab and xenograft analyses were compared using the ΔΔCt approach (ΔΔCt=2^[(Ct target gene control - Ct GAPDH control) - (Ct target gene test condition - Ct GAPDH test condition)]. From this formula, for the test compared with the control condition, a ΔΔCt over 1 indicated an increase of a target gene expression and a ΔΔCt of less than 1 indicated a lower expression of the target gene relative to the housekeeping gene GAPDH.

RT-PCR primers used were: GAPDH, forward: 5'-CC CATCACCATCTTCCAGGAGCG-3' and reverse 5'-CA TGCCAGTGAGCTTCCCGTTCA-3' (476 bp product); EGFR, forward: 5'-TCTCAGCAACATGTCGATGGA -3′ and reverse 5'- GCACTGTATGCACTCAGAGTT-3' (92 bp product), VEGF-D, forward: 5'-GTATGGACTCTCGCTCAGCAT-3' and reverse: 5'-AGGCTCTCTTCATTGCAACAG-3' (225 bp product), VEGFR3, forward and reverse; QuantiTect Primer Assay: FLT4 (VEGFR3, 127 bp product, Qiagen). Cycle conditions of the one-step real time LightCycler RT-PCRs were as follows: for reverse transcription, 20 min at 50℃. The subsequent PCR reaction was characterized by: initial denaturation (15 min at 95℃) followed by the respective number of cycles (GAPDH: 25, VEGF-D: 35, VEGFR3: 40, EGFR: 35) of: denaturation (15 s at 94℃), annealing (25 s: GAPDH; 63℃, VEGF-D; 61℃, VEGFR3; 55℃, EGFR; 61℃) and elongation (35 s at 72℃). After the last cycle, a melting curve was plotted to confirm the amplification of a single specific RT-PCR product.

Western blotting

SW480 and SW620 cells were plated at 2.5×10^6 cells/well in 6 wells. Cells were harvested and lysed in RIPA buffer[50]. For EGFR, VEGF-D, VEGFR3 and α-tubulin analysis, 50 μg of cleared lysates were separated on 10.0% sodium dodecyl sulphatepolyacrylamide gels, blotted to nitrocellulose transfer membranes (Schleicher & Schuell) and blocked for 1 h in 5% non-fat dry milk, incubated with a specific primary antibody: antihuman EGFR sc-03, antihuman VEGF-D sc-13085, VEGFR3 sc-321 (Santa Cruz Biotechnology Inc: all antibodies were diluted 1:200 in 5% bovine serum albumin) or anti-α-tubulin (Sigma: diluted 1:10000 in 5% non-fat dry milk). Detection of bound antibody was performed using a peroxidase-conjugated secondary goat anti-rabbit antibody (sc-2030; Santa Cruz Biotechnology Inc.) diluted 1:2000 in 5% non-fat dry milk and an ECL chemiluminescence detection kit (Perkin Elmer).

Animals

Female NOD/SCID -/- mice were purchased from the central animal facility (ZVTE, University of Mainz, Germany). The mice were maintained in a laminar airflow cabinet under pathogen-free conditions and used at 7-10 wk of age. Mice were housed in microisolator cages with laboratory chow.

Treatment of subcutaneous colorectal carcinoma xenografts

Colon carcinoma tumours were established as xenografts

by injecting 1 × 10^7 SW480 cells, mixed in PBS : medium (1:1), subcutaneously into the left flank of eight NOD/SCID -/- mice. Ten days after cell injection, all mice bore a tumour with a minimum diameter of 4 mm. The mice were randomized into two groups of four animals. They were treated with either saline or cetuximab at 1 mg/dose every three days. Cetuximab and the saline placebo were administered intraperitoneally at a constant volume of 0.5 mL/injection. Treated animals were checked daily. After 5 wk of treatment, tumours were isolated and processed with a disperger to enhance subsequent RNA isolation with the RNeasy Kit (Qiagen, Hilden, Germany).

Statistical analysis

The association of staining intensity with clinicopathological patterns was assessed with the χ^2 test and with the unpaired student *t*-test, where appropriate. Differences in migration were evaluated with the unpaired Student's *t*-test. Survival rates were visualized applying the Kaplan-Meier curves and log rank test. *In vitro* and *in vivo* real time gene expression medians were compared using Mann-Whitney *U* test. $P < 0.05$ was considered significant and $P < 0.001$ highly significant in all statistical analyses.

RESULTS

Patient profiles and tumour characteristics

Disease characteristics for the group of 108 patients selected are representative of CRC patient populations in industrialized countries, except for a lower percentage of T3 cancers (Table 1). The mean age of the patient cohort at the time of primary surgical intervention was 63.8 years (SD, 10.45 years). Tumour stage was distributed as follows: UICC stage Ⅰ was found in 14%, stage Ⅲ in approximately a fifth and stages Ⅱ, and Ⅳ, each in approximately a third of all patients (Table 1). The most common histopathological grading was pG2, which was reported for nearly 80% of cases. Approximately half of all patients had positive lymph node status. The UICC stage dependent survival rates after 3 years were 100% for stage Ⅰ, 92% for stage Ⅱ, 78% for stage Ⅲ and 44% for stage Ⅳ, which were similar to those reported for other large CRC population series[51]. Expression was studied in specimens taken from all 108 CRC patients.

Expression of EGFR, VEGF-C and VEGF-D in CRC specimens

The expression of EGFR, which could be assessed by IHC staining for all 108 samples, exhibited a predominantly membranous subcellular localization. In a few specimens, an additional weak cytoplasmic localization was found, which was not scored as indicative of positive staining for EGFR (Figure 1 A-D). EGFR expression in CRC specimens was distributed as follows: in 59 cases (54.6%), the specimen showed no positive staining for EGFR, while in 49 patients (45.4%) the specimen showed a positive membranous staining, including 31 patients (28.7%) where staining was weakly positive and 9 patients (8.3%) each with moderately or strongly positive staining. No statistically significant correlation was identified between EGFR staining and the UICC stage, grading, tumour invasion or lymph node metastasis (Table 2). Comparison with the histopathological grading of the tumour cells however showed a trend towards the expression of EGFR in less well differentiated lesions. Thus, the prognostic value of IHC-determined EGFR expression status in relation to the prediction of poor survival[52-56] could not be confirmed in the current CRC population, again arguing to successfully integrate the anti-EGFR monoclonal antibody cetuximab into therapeutic regimens for patients whose tumours do not appear to express EGFR[57,58].

Table 1 Baseline characteristics of the colorectal cancer patients (n = 108)

	n	%
UICC-stage		
Ⅰ	15	13.9
Ⅱ	36	33.3
Ⅲ	23	21.3
Ⅳ	34	31.5
Grading		
G 1	4	3.7
G 2	85	78.7
G 3	17	15.7
G 4	2	1.9
Lymph node metastasis		
pN 0	53	49.1
pN 1	21	19.4
pN 2	34	31.5

The staining for VEGF-C and VEGF-D was predominantly cytoplasmic (Figure 1 E-H and I-L, respectively). VEGF-C expression could be assessed in 102 specimens, with 74 specimens (68.5%) staining positive for VEGF-C and 28 specimens (25.9%) being negative. Twenty-five percent of the G1 tumours (n = 4) were positive, while 70.6% of G2 tumours, 70.6% of G3 tumours, and 50.0% of G4 graded tumours were positive for VEGF-C. Thus even if statistically significant (Table 2) but only 4 patients were G1, a clinical clear cut comparison of well versus not well differentiated tumours may not be done. However, another finding was the borderline significant correlation between tumour invasion and VEGF-C expression (P = 0.050). Only 11 (55.0%) of the pT1 and pT2 graded tumours were positive for VEGF-C compared with 63 (76.8%) of the tumours with deeper invasion. No statistically significant correlation was apparent with the other clinicopathological parameters including UICC stage and lymph node status (Table 2).

VEGF-D expression was analyzed in 104 available specimens, with 70 (64.8%) staining positive and 34 (31.5%) staining negative. Interestingly, VEGF-D staining was associated with UICC tumour stage (P = 0.048). Nine of 14 (64.3%) specimens from patients with UICC stage Ⅰ tumours stained positive, while 27 (77.1%) of the UICC stage Ⅱ, 9 (42.9%) of the UICC stage Ⅲ and 25 (73.5%) of the UICC stage Ⅳ tumours stained positive for VEGF-D. No significant association

Table 2 Correlation of expression levels of EGFR, VEGF-C and VEGF-D with tumour and patient characteristics

	EGFR				VEGF-C				VEGF-D			
	Total	+ ve	%[1]	*P*	Total	+ ve	%[1]	*P*	Total	+ ve	%[1]	*P*
Stage (UICC)				0.197				0.220				0.048
I	15	6	40.0		14	7	50.0		14	9	64.3	
II	36	14	38.9		34	26	76.5		35	27	77.1	
III	23	15	65.2		20	16	80.0		21	9	42.9	
IV	34	14	41.2		34	25	73.5		34	25	73.5	
Differentiation grading				0.063				0.030				0.066
Well	4	0	0		4	1	25.0		4	1	25.0	
Not well	104	49	47.1		98	73	74.5		100	69	69.0	
Tumour invasion (TNM)				0.797				0.050				0.438
T1/T2	21	9	42.9		20	11	55.0		20	12	60.0	
T3/T4	87	40	46.0		82	63	76.8		84	58	69.0	
Lymph node metastases				0.283				0.600				0.025
1 to ≤ 6	37	20	54.1		35	27	77.1		36	18	50.0	
≥ 7	18	9	50.0		17	13	76.5		17	14	82.4	
Age				0.837				0.813				1.0
≤ 60	35	15	42.9		33	25	75.8		33	22	66.7	
≥ 61	73	34	43.6		69	49	71.0		71	48	67.6	

[1]Percentages relate to number of positive tumours out of total number of cases in the subclass.

was found with several other parameters including grading, tumour invasion and pN status (Table 2). Again, the comparative analyses of the combined VEGF-C and VEGF-D co-expression with well *versus* not well differentiated tumours were statistically significant (P = 0.022), but possibly not clinically clear cut significant due to the low patient number analysed.

However, when VEGF-D staining was analyzed in relation to the number of tumour positive lymph nodes, there was a statistically significant overall association between the number of lymph node metastases and VEGF-D positivity of the primary tumour (P = 0.025). The patient cohort was stratified into patients with no lymph node metastases (n = 51), with up to 6 lymph node metastases (n = 36), and with more than 6 lymph node metastases (n = 17). Among those from patients with up to 6 lymph node metastases, 18 (50.0%) stained positive for VEGF-D, and among the patients with more than 6 lymph node metastases, 14 (82.4%) stained positive for VEGF-D.

When VEGF-D staining was analyzed in relation to the survival of lymph node positive patients, there was a trend (P = 0.067) between the VEGF-D positive and negative groups (Figure 2). The patients with lymph node metastases whose tumours were VEGF-D positive had a 30% lower chance of survival at 36 mo in comparison to the patients with VEGF-D negative tumours. Kaplan Meier analysis also indicated highly statistically significant correlations between the number of lymph node metastases and UICC stage and overall survival (in both cases, log rank test P < 0.001; Figure 2). It was also noteworthy that in several specimens, VEGF-C and VEGF-D were strongly expressed at the tumour invasion front (data not shown). Consistently, the further comparison of VEGF-C and VEGF-D combined staining profiles in relation to survival, double VEGF-C and VEGF-D positive patients had a trend to an unfavorable 5 years prognosis compared to the negative group (log rank test P = 0.2), whereas triple EGFR, VEGF-C and VEGF-D positivity did not influence survival in these two cohorts (data not shown).

EGFR, VEGF-C, VEGF-D and VEGFR3 expression in colorectal carcinoma cell lines

Real time RT-PCR assays and Western blotting were used to measure transcript and protein levels in experimental analyses. Whereas VEGF-D transcripts and protein were expressed at moderate levels in both SW480 and SW620 CRC cell lines, a similar analysis of expression of EGFR and VEGFR3 yielded varying results. EGFR mRNA and protein levels were high in SW480 and markedly lower in SW620 cells. Though mRNA levels of VEGFR3 were high in SW480 and moderate in SW620, the protein levels of VEGFR3 were also higher in SW480 than in SW620 cells (Figure 3).

Effect of cetuximab in CRC cell lines and a xenograft model

In order to analyze the effect of cetuximab on VEGF-D and VEGFR3 transcription, SW480 cells were treated for 24 h with either EGF or cetuximab alone or a combination of these two agents. Incubation of the cells with EGF was associated with a marked relative increase in VEGF-D and VEGFR3 mRNA levels compared with control (0.5% FCS) values. Conversely, incubation with EGF and cetuximab simultaneously resulted in a highly statistically significant (P = 0.004) suppression of this inductive effect, for both genes (Figure 4A). There were similar highly statistically significant (P = 0.004) differences in the levels of VEGF-D and VEFR3 expression in the EGF *versus* cetuximab treated cells. Incubation with cetuximab alone also resulted in a decreased level of VEGF-D mRNA compared to the control. This effect was not seen for VEGFR3. In the EGFR negative cell line SW620 no such effects were detectable (data not shown).

In the *in vivo* xenograft model, treatment with either

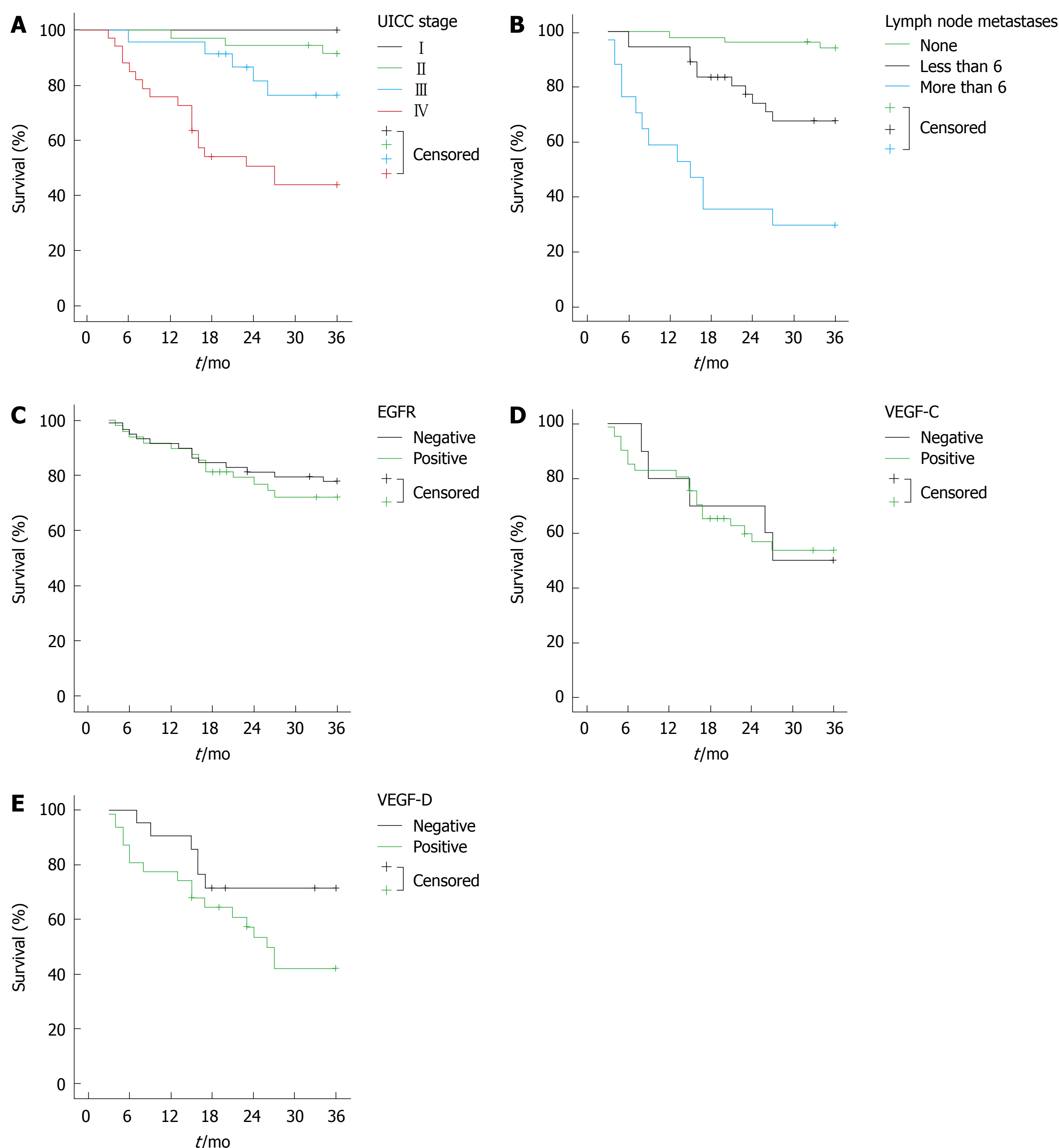

Figure 2 Kaplan Meier survival analyses of colorectal cancer subgroups in relation to baseline disease characteristics and EGFR, VEGF-C or VEGF-D expression. **A-C**: Survival analysis for all patients in relation to UICC stage (*P* < 0.001), the number of metastases (*P* < 0.001) and EGFR staining (*P* = 0.546), respectively; **D** and **E**: The 3 years survival in patients with lymph node metastasis in relation, respectively, to the expression of VEGF-C (*P* = 0.967) and VEGF-D (*P* = 0.067).

cetuximab or saline placebo was administered over a period of 28 d. After this period, the mRNA level of VEGF-D was highly statistically significantly (*P* = 0.004) reduced in the tumour tissue of mice which had received cetuximab compared with those that had received saline. No such effect could be detected for VEGFR3 (*P* = 0.577, Figure 4B).

DISCUSSION

A significant proportion of patients with advanced but non-metastatic CRC which has seemingly been curatively resected experience disease recurrence[52,53]. In order to identify high-risk patients at an early stage, it is important to understand the molecular mechanisms behind the behaviour of these tumour types[54,55]. Herein, activation of EGFR-mediated signalling cascades has been identified in promotion of cell proliferation, malignant transformation, angiogenesis and metastatic dissemination[54,56]. In addition, lymphangiogenesis, mediated through tumour-derived VEGF-C and VEGF-D, gained attention in relation to the facilitation of lymph node metastasis and tumour spread[37,39,40,57]. Recent data from clinical studies suggest that VEGF-C and/or VEGF-D expression in tumour tissue might be prognostic factors for lymphatic spread, tumour invasion and/or poor prognosis in a variety of cancers including gastric, colorectal, endometrial and breast[37,39,40,45,46].

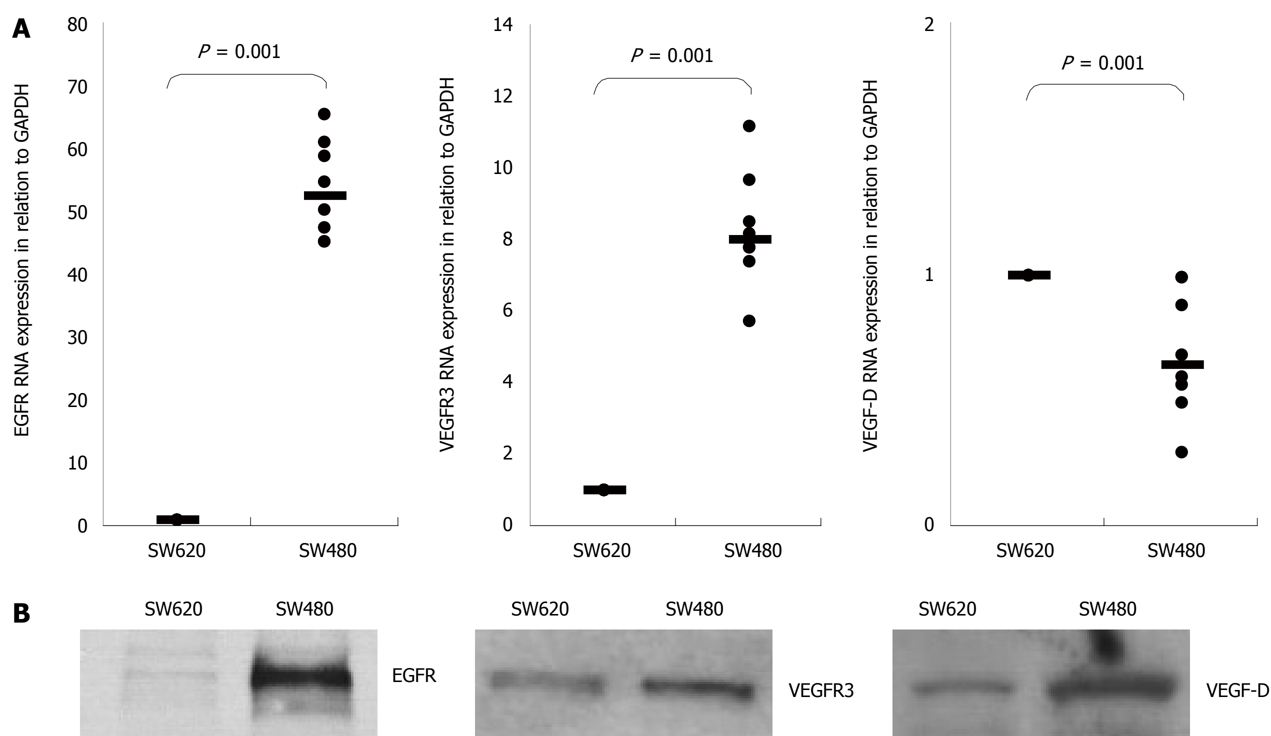

Figure 3 Quantitative EGFR, VEGFR3 and VEGF-D mRNA expression (**A**) and Western blot analysis (**B**) of untreated SW480 and SW620. The α-tubulin signal served as a control to confirm the loading of equivalent amounts of protein per track.

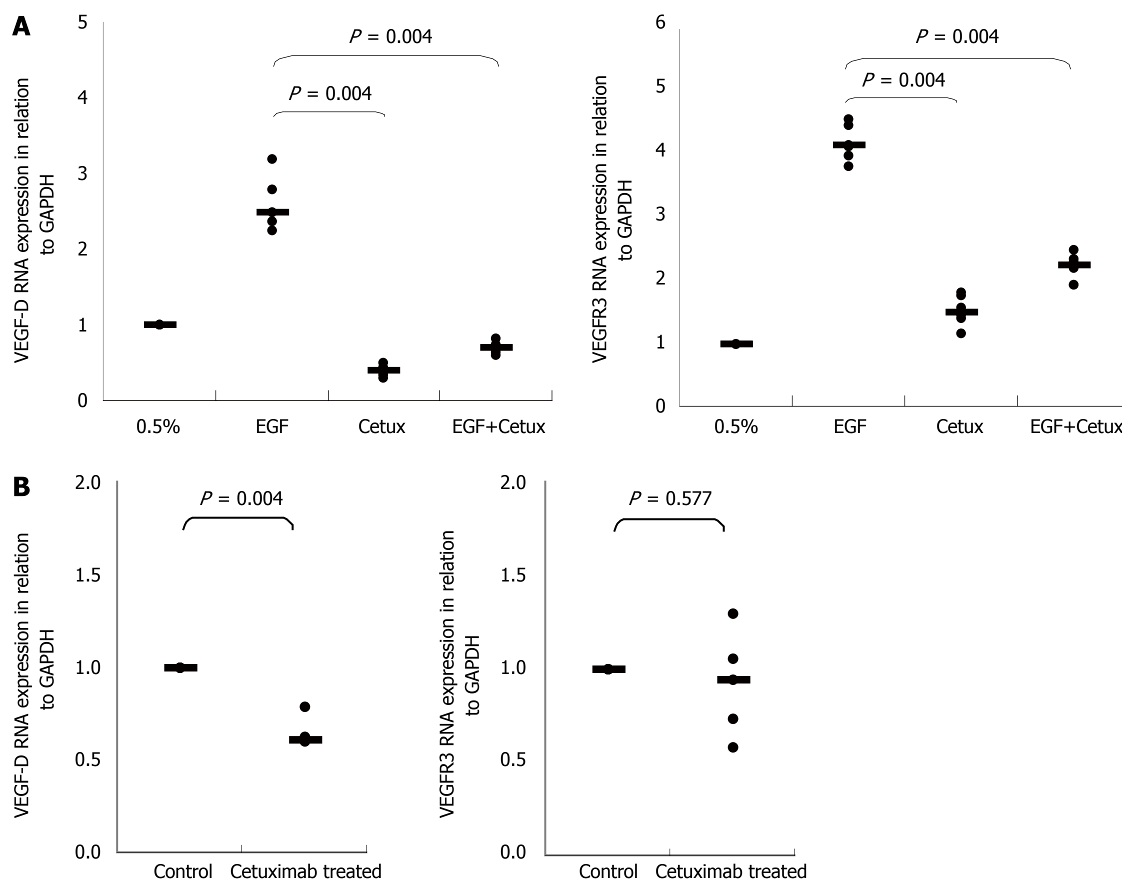

Figure 4 Effect of cetuximab on (**A**) the expression of VEGF-D and VEGFR3 in SW480 cells cultured in the presence and absence of EGF and (**B**) the *in vivo* levels of VEGF-D and VEGFR3 in a NOD/SCID mouse xenograft model.

To our knowledge, this is the first study analyzing concurrently the expression profiles of EGFR and the lymphangiogenic ligands VEGF-C and VEGF-D in a large series of human CRC specimens. With a relatively

low patient numbers used for well differentiated tumors, the statistical correlations of this current study between the histopathological grading and VEGF-C, but not VEGF-D expression levels may only be assumed to hold true for a larger clinical setting. However most interestingly, the comparison of the expression profile of VEGF-D in the colorectal tumour series with clinicopathological parameters revealed a significant association between VEGF-D and the number of lymph node metastasis. Moreover, we show with a clear trend that patients in a pN+ setting and positive VEGF-D expression define a subgroup with shorter survival. These data are consistent with previous analyses in a range of other cancers. Furthermore, they are in agreement with the established clinical observation that lymphatic dissemination in particular the number of metastatic lymph nodes is closely related to the clinical outcome/prognosis of patients with CRC[59].

Signal transduction *via* the ligands VEGF-C and -D and VEGFR3 triggers lymphatic endothelial cell growth and migration[34-36]. Thus, it is noteworthy that in our specimens VEGF-C and VEGF-D were also strongly expressed at the tumour invasion front. We have previously shown that VEGFR3 is expressed in 67% of primary gastric cancers[60]. Data on animal models suggest that VEGF-C/VEGF-D/VEGFR3 signalling can promote tumour lymphangiogenesis and the metastatic spread of tumour cells[35,61-63]. Indeed, it has been suggested that primary tumours may prepare their future metastasis site by producing lymphangiogenic factors that mediate their efficient transport to the sentinel node[64]. Data from Jia *et al* suggested that VEGF-C expression may induce lymphangiogenesis in CRC and as a result, tumour cells could perhaps enter lymphatic vessels more easily[57,65]. These processes could be blocked by inhibition of VEGFR3 signalling by systemic delivery of a soluble VEGFR3-immunoglobulin fusion protein. However, lymph node metastasis was not suppressed if such treatment was started later, after tumour cells had already disseminated, suggesting that tumour cell entry into the lymphatic vessels is a key step during the metastatic process[66]. Consistently with these findings, our double VEGF-C and VEGF-D positive patients had unfavorable survival prognosis compared to the negative groups in the Kaplan-Meier analysis, again arguing for a large analysis of these markers in colorectal cancer patients with chemotherapy and/or cetuximab-based adjuvant therapy.

We further explored whether EGFR blockade influenced the expression of VEGF-D and VEGFR3 using *in vitro* and *in vivo* models. While treatment of the EGFR-expressing CRC cell line SW480 with EGF resulted in an increase in the transcript levels of both genes, there was a highly significant reduction in those induced mRNA levels when cetuximab was added. These data suggest that cetuximab, by blocking EGFR-associated signalling, might act as an inhibitor of VEGF-D expression, and consequently, of lymphangiogenesis. In contrast with an earlier report of another EGFR-specific antibody (ICR62) stimulating VEGF-D[67], cetuximab blocks additionally the production of pro-angiogenic factors such as VEGF and IL-8[12-14,68-70]. Although complete inhibition of VEGF-D expression was not achieved in our model systems the effect of cetuximab was nevertheless dramatic. In a therapeutic context, cetuximab, might also therefore induce a clinically-relevant reduction in tumour lymphangiogenesis. Given that the induction of lymphangiogenesis appears to be one of the early events in the progression of cancer[57], a therapy targeting such processes would have a clear role in (neo-)adjuvant chemopreventive settings. Further preclinical and clinical studies are therefore warranted to explore these issues.

In conclusion, immunohistochemical staining of VEGF-D coupled with the examination of lymph node status may aid in the definition of patient subpopulations with more aggressive tumours. A cetuximab-based adjuvant therapy might then improve overall survival in these patients. Still, the link between prognostic markers and response to a certain treatment remains elusive. However, the new data in the current paper suggest that the inhibition of VEGF-D signalling might contribute to the widely demonstrated clinical activity of cetuximab in the treatment of CRC.

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COMMENTS

Background

Since the use of new biologic agents, such as epidermal growth factor receptor (EGFR)-targeted agents may improve our current therapeutic approaches for advanced human colorectal cancer (CRC) which seeds quite often metastatic tumor cells in the lymphatic glands, is of high interest to analyse prognostic and predictive expression markers for lymphangiogenic tumor spread, particularly in view of the possible modulation of these factors by the EGFR-targeted agents cetuximab and panitumumab.

Research frontiers

Our clinical human colon cancer specimens displayed these markers EGFR, vascular endothelial growth factor (VEGF)-C and VEGF-D expression. Strong expression levels of VEGF-D were associated with lymph node metastasis and linked to decreased survival in lymph node-positive colon cancer patients. In tumor models, the new antibody for EGFR blockade cetuximab resulted in a highly significant decrease of VEGF-D expression.

Innovations and breakthroughs

The lymphangiogenic marker VEGF-D thus associated with lymph node metastasis and is linked to a decreased survival in lymph node-positive patients. The EGFR blockade with cetuximab resulted in a significant decrease of VEGF-D expression, particularly favouring these EGFR-targeted agents as treatment options of lymph node-positive colorectal cancer.

Applications

Patients with advanced lymph node-positive colorectal cancer might better be

selected as well as better be treated in the near future.

Peer review

Immunohistochemical staining of VEGF-D coupled with the examination of lymph node status may define patient subpopulations of with more aggressive colorectal cancers. Since the EGFR blockade with cetuximab resulted in a significant decrease of VEGF-D, EGFR-targeted agents may improve the overall survival, particularly as a new treatment option of lymph node-positive colorectal cancer.

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