Tumour-associated transcripts and EGFR deletion variants in colorectal cancer in primary tumour, metastases and circulating tumour cells

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Abstract. The clinical relevance of circulating tumour cells (CTC) in peripheral blood of patients with colorectal cancer (CRC) has been described as an independent prognostic factor useful to monitor drug effects and clinical status. The aim of the present study was to compare the epidermal growth factor receptor (EGFR) status of primary tumour, related metastases and CTC of patients with CRC. Therefore, in addition to EGFR, the tumour-associated transcripts gastrointestinal tumour-associated antigen 733-2 (GA733-2) and carcinoembryonic antigen (CEA) were analyzed in a multiplex RT-PCR to characterize CTC. 55% patients were positive for CTC. EGFR expression was detected in 18% of these patients. EGFR was expressed more frequently in metastatic and primary tumour tissues as revealed by immunohistochemistry. Besides, detailed expression profiling of EGFR variants in various colorectal and glioma cell lines has been performed to generate positive controls, resulting in the discovery of two new transcript deletion variations (cEX12_15del, cEX12_14del) located on the extracellular domain of the EGFR.

Keywords: Circulating tumour cells (CTC), colorectal cancer, RT-PCR, immunomagnetic enrichment, EGFR, EGFR variants

1. Introduction

The biological properties of metastases may be quite different from those of the primary tumour as reviewed in [3]. During tumour progression tumour cells are able to detach from the primary tumour and to enter the blood circulation or lymphatic system. Once established at their secondary site, these tumour cells can undergo further phenotypic changes depending on their location within the growing metastasis. The presence of CTC in the peripheral blood of patients with colorectal cancer has already been noted [6,18,31] and their clinical relevance has extensively been described

as an independent prognostic marker for disease-free survival [14,28]. Recent efforts to determine CTC have used reverse transcriptase polymerase chain reaction (RT-PCR) to identify tumour-associated transcripts like GA733-2, CEA, cytokeratin 20 (CK-20) or EGFR combined with different separation methods like immunomagnetic enrichment, density gradient centrifugation or lyses of erythrocytes [14,29,30]. With these methods the comparison of the gene expression of the primary tumour, CTC and metastases can be undertaken to show similarities and to describe differences.

CTC can be used as surrogate markers to monitor drug effects and the clinical status as well. One relevant tumour-associated marker and therapeutic target regarding CRC patients is the EGFR. The EGFR status in the primary tumour is not always identical to the status in the corresponding metastatic site [4,12,25]. This could be one reason for the lack of clinical benefit in some patients regarding therapies targeted to EGFR. In

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addition, a number of genetic alterations of the EGFR have been described in gliomas, astrocytomas, NSCLC and colorectal carcinomas on genomic, transcript and protein levels that lead to simultaneous expression of both wild-type and mutant receptors [16]. Until now, the occurrence of variants in the extracellular domain of EGFR has not been studied except for EGFR vIII (EX2_7del) but may be important for antibody-based therapies [1,5,7,11,19,23,26].

One aim of our study was to compare EGFR expression in primary tumours, their liver metastases and CTC in order to find out whether assessing the EGFR status of CTC is more related to metastases than to the primary tumour. In addition, expression profiling concerning different extracellular variants of the EGFR in CRC patients was undertaken.

2. Materials and methods

2.1. Patients

In this pilot study 20 patients with metastatic adenocarcinoma of the colon or rectum and resectable liver metastases were enrolled at the Department of

Gastroenterology, Hepatology and Endocrinology of the Hannover Medical School, Germany (Table 1). Informed consent was obtained from all patients; the Ethics Committee of the Hannover Medical School approved the study protocol that conformed to the Declaration of Helsinki. Peripheral blood obtained from 22 healthy donors was examined as negative control.

The following parameters were recorded: age, sex, diagnosis, TNM classification, immunohistological results for EGFR expression at the time of surgery of the primary tumour and at surgery of the metastatic lesions, treatment within 6 months prior to surgery of metastatic lesions, RT-PCR results for EGFR, CEA and GA733-2 expression on CTC.

2.2. Study design

Peripheral blood was obtained from patients with metastatic adenocarcinoma of the colon or rectum prior to surgery of metastatic lesions. The blood specimens were processed in duplicates for the specific enrichment of CTC and for a subsequent expression analysis of EGFR, CEA and GA733-2. The obtained cDNA was also used for the detection of different extracellular EGFR variants.

Table 1
Patient characteristics

Patient ID	Sex	Age at diagnosis	Initial tumour stage	Age at surgery of the metastases	Treatment within 6 months prior to surgery of the metastases
1	M	56	T3N1M0	56	5-FU/FA/oxaliplatin
2	F	57	T2N0M1	58	–
3	M	77	T4N2M0	78	–
4	M	63	T3N2M1[†]	64	5-FU/FA/oxaliplatin
5	M	66	T2N0M0	69	–
6	F	39	T4N1M1[†]	40	–
7	M	76	T3N0M1	78	–
8	M	56	T2N0M0[†]	64	–
9	M	67	T2N1M0[†]	71	–
10	M	41	T4N2M1	44	5-FU/FA/oxaliplatin
11	M	54	T3N2M1[†]	56	5-FU/FA/oxaliplatin/CPT 11
12	M	81	T3N2M0	83	–
13	M	66	T3N1M0[†]	68	–
14	M	62	T3N1M1	62	5-FU/FA/oxaliplatin
15	M	65	T4N0M0[†]	67	–
16	M	52	T2N0M0[†]	55	5-FU/FA/oxaliplatin
17	F	39	T3N0M1	42	5-FU/FA/oxaliplatin/CTP 11/cetuximab
18	M	69	T3N2M0	70	–
19	F	68	T2N1M1[†]	68	–
20	M	68	T3N0M1	68	–

[†]Adenocarcinoma of the rectum.

M: male; F: female; 5-FU: 5-fluorouracil; FA: folinic acid; CTP 11: irinotecan-HCl.

Tissue samples of the primary tumours and resected liver metastases were examined by immunohistochemistry (IHC) for the expression of EGFR.

2.3. Tumour cell enrichment and multiplex RT-PCR of CTC

Peripheral blood (10 ml) was collected in EDTA tubes (Sarstedt AG & Co., Nümbrecht, Germany) and processed within 4 hours. The samples were processed in duplicate for the enrichment of CTC and subsequent expression profiling. Additional 5 ml were required for the examination of EGFR variants. The *AdnaTest ColonCancerSelect* and the *AdnaTest Colon-CancerDetect* (AdnaGen AG, Langenhagen, Germany) were employed according to the manufacturer's protocol for the detection of CTC and EGFR transcripts. The combination of immunomagnetic tumour cell enrichment and the analysis of tumour-associated transcripts EGFR, CEA and GA733-2 by multiplex RT-PCR were previously described [30]. Actin was amplified as an internal PCR control. The PCR products were analyzed with DNA 1000 assays on an Agilent 2100 Bioanalyzer (Analysis Software 2100 expert, version B.02.03.SI307, Agilent Technologies, Böblingen, Germany). Signals <0.1 ng/μl were defined as negative.

2.4. IHC of primary tumours and resected metastases

Tumours were fixed in buffered formalin, embedded in paraffin and stained with hemalaun and eosin. EGFR expression was evaluated immunohistochemically on 3 μm thick tissue sections obtained form paraffin-embedded specimens fixed in 10% (v/v) neutral buffered formalin using mouse anti-EGFR antibody (clone 31G7, Novocastra, Newcastle Upon Tyne, United Kingdom). EGFR expression was detected as membranous and/or cytoplasmic staining of neoplastic cells with various intensities. Positive expression for EGFR was defined as any membrane staining above background level. The amount of EGFR reactivity was scored as follows: 0 = negative; 1 = 1% positive cells; 2 = 1–20% positive cells; 3 = more than 20% positive cells.

2.5. mRNA isolation and cDNA synthesis from cancer cell lines

The following human CRC cell lines were screened for the presence of extracellular EGFR variants to generate positive controls: COLO 205, HCC-2998, HCT-116 (NCI/NIH, National Cancer Institute/National Institutes of Health, Bethesda, USA), LoVo, WiDr (Nippon Roche, Kamakura, Japan); CACO-2, HT-29, SW-480 (DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany); T84 (ATCC, American Type Culture Collection, Manassas, USA); DLD-1, SW-948, SW-1116 (provided by Dr. Christian Röder, Christian-Albrechts-University, Kiel, Germany). Especially in glioma cell lines the ability to find EGFR variants is high, therefore human glioma cell lines were examined also to generate positive controls: U251, SNB-78, SF-295, SNB-75 (NCI/NIH, Bethesda, USA) and U-87 MG (ATCC, Manassas, USA).

The isolation of mRNA obtained from 1×10^4 cells each of human CRC and glioma cell lines was performed using Dynabeads mRNA Direct™ Micro Kit (Dynal Biotech GmbH, Hamburg, Germany) according to the manufacturer's protocol. 10 μl mRNA was used in a reverse transcription reaction. The RT reaction was set at 60 min at 37°C and 5 min at 93°C; cDNA was stored at -20°C.

2.6. PCR amplification of EGFR variants from cancer cell lines and CTC

The PCR for the detection of EGFR variants was optimized to detect the variants in a background expression of EGFR wild-type receptor [21] and, afterwards, performed under the following conditions: 1.0 μM of each primer and 25 μl of HotStarTaq Mix (Qiagen GmbH, Hilden, Germany) were used. The PCR programme was set for 15 min at 95°C, followed by 45 cycles at 94°C for 30 s, at 60°C for 30 s and at 72°C for 1 min, followed by a final step of 72°C for 5 min. The analyses of the PCR fragments have been performed with DNA 1000 or DNA 7500 assays on the Agilent 2100 Bioanalyzer (Analysis Software 2100 expert, version B.02.03.SI307, Agilent Technologies, Böblingen, Germany). The primer sequences were designed to cover most parts of the N-terminus for the detection of extracellular EGFR variants. One primer pair spans exons 9–16 with the following sequences: EGFR P1 5′-AAACTGCACCTCCATCAGTG-3′, EGFR P2 5′-ATTCGTTGGACAGCCTTCAAG-3′. A second primer pair spans exons 1–8 with the following sequences: EGFR P3 5′-GTCCAGTATTGATCGGG-AGAGC-3′, EGFR P4 5′-GAGCCGTGATCTGTCA-CCAC-3′. The PCR with primer pair P3/P4 was performed under the same conditions with the exception

of an annealing temperature of 63°C and an elongation time of 2 min. The specificity of the primers was verified with BlastN 2.2.10 (http://www.ncbi.nlm.nih.gov/BLAST/). Optimized PCR protocols were used with cDNA of CTC isolated as described before.

2.7. Cloning of PCR fragments and sequencing

Amplified PCR fragments were separated on a 2% MetaPhor agarose gel (Biozym Diagnostik GmbH, Hessisch Oldendorf, Germany). Excised fragments were isolated with the QIAquick Gel Extraction Kit according to the manufacturer's protocol (Qiagen GmbH, Hilden, Germany). Cloning of the fragments was done with the TOPO TA Cloning® Kit (Invitrogen GmbH, Karlsruhe, Germany) and the isolation of plasmid DNA was done with the QIAprep Spin Miniprep Kit (Qiagen GmbH, Hilden, Germany). Positive clones were sequenced on both strands by Agowa GmbH, Berlin, Germany with standard procedures. The analysis of sequences was done with DNASTAR 5.0 (GATC Biotech AG, Konstanz, Germany).

3. Results

3.1. Detection of CTC in blood of CRC patients with liver metastases

Blood samples of 20 CRC patients with liver metastases were analyzed for CTC using RT-PCR and subsequent DNA fragment analysis. Exemplarily, a typical analysis of CTC patients is shown in Fig. 1(a). 11/20 (55%) patients had CTC. Within the CTC positive

samples the tumour-associated transcript CEA was expressed most frequently 10/11 (91%). 6/11 (55%) of the CTC expressed GA733-2 and 2/11 (18%) showed an expression of EGFR (Fig. 1(b)).

3.2. EGFR expression in CTC, primary tumour and metastases

As described before EGFR wild-type expression on CTC was rare. Therefore, the biopsies from resected liver metastasis and, if available, the primary tumour tissue of the CRC patients were analyzed for EGFR expression by IHC. Results are summarized in Table 2

Metastatic tumour tissue of 19 patients was analyzed for EGFR expression by IHC, 10/19 (53%) patients were positive for EGFR (score 1–3). In 7/10 (70%) patients positive for EGFR by IHC, CTC were found using multiplex PCR. 9/19 (47%) specimens did not show EGFR expression by IHC (score 0), in 5/9 (56%) corresponding EGFR-negative blood samples CTC could not be detected either.

The primary tumour tissue of 13 patients was analyzed by IHC resulting in 7/13 (54%) samples that were EGFR positive. In 3/7 (43%) patients positive for EGFR, CTC were detected in the corresponding blood samples. 6/13 (46%) primary tumour tissue samples did not reveal EGFR expression and in 3/6 (50%) corresponding EGFR-negative blood samples no CTC were detected. In total, EGFR expression on CTC was detected in 2/20 blood samples. In one patient the corresponding tumour tissue and metastasis biopsy were negative for EGFR expression by IHC. The second patient with detectable EGFR expression in CTC also
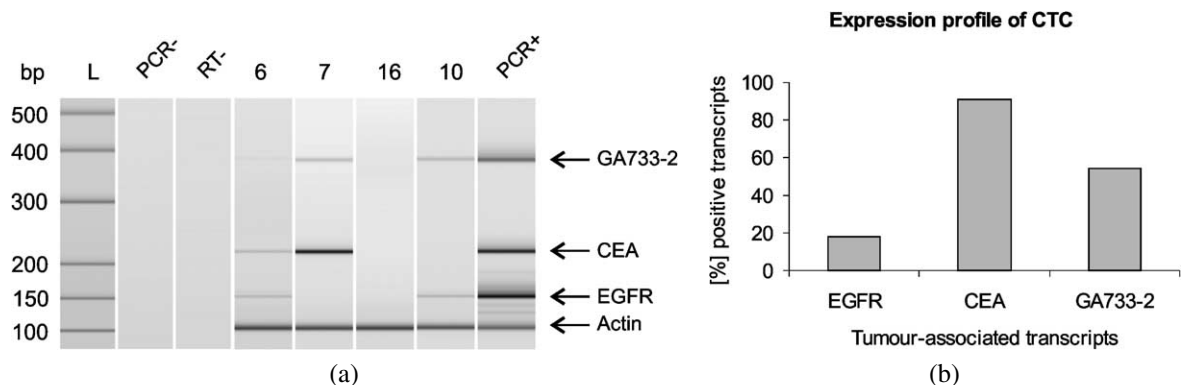

Fig. 1. Detection and expression profiling of CTC in blood of CRC patients by multiplex RT-PCR after immunomagnetic tumour cell enrichment. (a) Amplified cDNA fragments of tumour-associated transcripts GA733-2, CEA and EGFR and the internal control actin are shown. Fragments were analyzed by capillary electrophoresis with the Bioanalyzer 2100 (Agilent Technologies, Böblingen, Germany). bp: Base pairs; L: ladder; (−): negative control for PCR or RT; (+): positive control for PCR, numbers are patient IDs. (b) Percentage of the expression of tumour-associated transcripts in CTC positive patients.

Table 2

Analysis of CTC and EGFR expression in primary tumour and metastases by immunohistochemistry

Patient ID	CTC	EGFR IHC score	
		Primary tumour	Metastasis
1	+	0	0
2	+	n.a.	1
3	+	2	3
4	+	n.a.	1
5	−	0	0
6	+[†]	n.a.	2
7	+	1	1
8	−	0	0
9	−	n.a.	0
10	+[†]	0	0
11	+	0	0
12	+	3	2
13	+	n.a.	0
14	+	n.a.	1
15	−	1	1
16	−	0	0
17	−	1	0
18	−	1	n.a.
19	−	1	3
20	−	n.a.	1

	Patients [n]		
	20	13	19
CTC	11(+)/9(−)	6(+)/7(−)	11(+)/8(−)
EGFR	2(+)/18(−)	7(+)/6(−)	10(+)/9(−)

+: positive; −: negative; [†]positive for EGFR; n.a.: not available; IHC score: 0 = negative, 1 = 1% positive cells, 2 = 1–20% positive cells, 3 ⩾ 20% cells.

showed EGFR expression in the corresponding liver metastasis. Direct comparison of CTC and primary tumour samples or metastases demonstrated that a number of CTC tests were negative for EGFR while IHC analysis of biopsies were positive for EGFR expression.

The multiplex PCR was not appropriate to detect different variants of the EGFR. Therefore, we developed additional PCRs for the detection of extracellular variants of the EGFR.

3.3. Expression of EGFR variants in glioma and colorectal cancer cell lines

Since wild-type EGFR seems to be rare on CTC in blood of metastatic CRC patients, the patients' cDNA was examined for N-terminal EGFR variants. There-

fore, mRNA of 12 colorectal cancer and 5 glioma cell lines were screened for N-terminal EGFR variants to initially establish positive controls. A typical PCR fragment analysis for the glioma cell lines U-87 MG and U251 using primer pair P1/P2 is shown in Fig. 2(a). An EGFR wild-type fragment was detected in the examined glioma cell lines at 837 bp, but the expected fragment for variant vII (c.EX14_15del) [8] could not be found. Only U-87 MG showed a fragment at 504 bp for variant vIII/Δ12–13 (c.EX12_13del). Unexpectedly, two further fragments could be detected. Sequencing of the cDNA fragments revealed two new deletion variants of EGFR: (1) deletion of exons 12–15 (c.EX12_15del) with a fragment length of 256 bp (GenBank accession number: EF210211) and (2) deletion of exons 12–14 (c.EX12_14del) with a fragment length of 414 bp as shown in Fig. 2(b) (GenBank accession number: EF210210).

Afterwards, the optimized PCR setting was applied to colorectal carcinoma cell lines to determine the possibility to detect these variants in CRC patients as well. The results are summarized in Table 3. EGFR wild-type and variant c.EX12_13del as well as the two new deletion variants were found in different amounts and combinations in various cell lines.

Comparable experiments were done with primer pair P3/P4. Except for the wild-type EGFR, no variants concerning exons 2–7 could be detected (Table 3).

3.4. Detection of EGFR variants in blood of healthy donors and CRC patients

The optimized PCR protocols for the detection of EGFR variants were performed with cDNA isolated from blood of 22 healthy donors and 20 CRC patients. Neither EGFR variants nor wild-type EGFR could be detected in the blood of the healthy donors (data not shown). In contrast to glioma and CRC cell lines EGFR variants could not be detected in CRC patients; however, two patients showed an expression of wild-type EGFR.

4. Discussion

Detection of CTC in peripheral blood from patients at risk for cancer or tumour relapse has become a valuable diagnostic tool [9]. Using a combined antibody based enrichment method followed by a multiplex RT-PCR, peripheral blood samples from patients undergoing resection of colorectal liver metas-
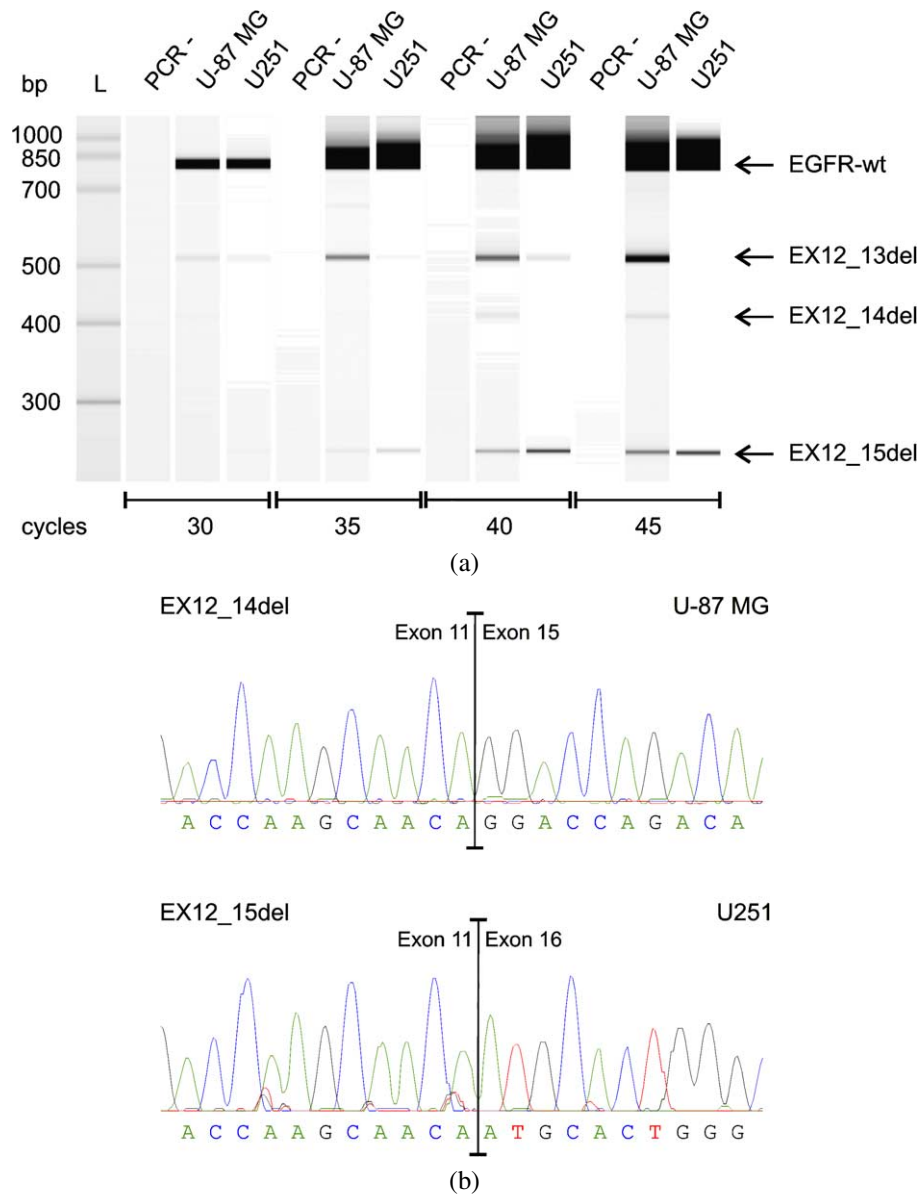
Fig. 2. Detection of EGFR variants in glioma cell lines. (a) Amplified cDNA fragments of EGFR wild-type and different variants. Fragments were analyzed by capillary electrophoresis with the Bioanalyzer 2100 (Agilent Technologies, Böblingen, Germany). bp: Base pairs, L: ladder, PCR−: negative control for PCR. (b) Partial cDNA sequences of the EGFR deletion variants in glioma cell lines showing the fusion points of different exons.

tases were analyzed. CTC could be detected in the majority of samples analyzed (55%). In agreement with our previous study [31], CEA expression was found in 91% of CTC positive metastasised patients and EGFR expression was only detected in 18% of CTC positive blood samples, although 52% of the examined metastatic tumour tissues showed EGFR expression by IHC. In contrast to patients with metastasis, the expression of EGFR on CTC was found more frequently in early stages of CRC patients, whereas the expression of CEA was decreased [31]. CEA is described as an epithelial marker as well as an oncofetal marker. Therefore, in future experiments it may be meaningful to focus not only on the over-expression of epithelial markers but on oncofetal markers in advanced colorectal cancers like α-fetoprotein (AFP), human chorionic gonadotropin (hCG) or cancer antigen 125 (CA 125) although these markers were only relevant for gyneco-

Table 3
EGFR expression analysis of colorectal and glioma cell lines

Cell lines	EGFR-WT	c.EX2_7del	c.EX2_7dup	c.EX12_13del	c.EX12_14del	c.EX12_15del	c.EX14_15del
Colorectal							
CACO-2	+++	−	−	+	+	+	−
COLO 205	+++	−	−	+	−	−	−
DLD-1	+++	−	−	−	−	+	−
HCC-2998	−	−	−	−	−	−	−
HCT-116	+++	−	−	+	+	++	−
HT-29	+++	−	−	−	−	+	−
LoVo	+++	−	−	+	++	−	
SW-480	+++	−	−	−	−	−	−
SW-948	+++	−	−	−	+	+	−
SW-1116	+++	−	−	+	+	+	−
T84	+++	−	−	+	+	+	−
WiDr	+++	−	−	−	−	−	−
Glioma							
SNB-75	+++	−	−	−	−	−	−
SNB-78	+++	−	−	+	−	+	−
SF-295	+++	−	−	+	+	+	−
U-87 MG	+++	−	−	++	+	++	−
U251	+++	−	−	+	−	++	−

−: no transcript; +: 0.06–1.00 ng/μl; ++: 1.01–3.00 ng/μl; +++: >23.00 ng/μl.

logical and germ-cell cancers until now [24]. In other groups, EGFR expression was found in about 82% primary tumours and metastases of CRC patients [7,23]. This is a very high percentage in contrast to the present data where only about 53% of the patients were positive for EGFR in the primary tumour tissue or 52% in the metastases respectively. Analysis of intravascular tumour cells or lymph node metastasis might result in an even higher number of EGFR positive primary tumour samples. In addition, there was no direct correlation between the detection of EGFR expression in CTC and EGFR expression in metastases. EGFR over-expression in both the liver metastasis and in CTC was only found in one patient. It cannot be excluded that this discrepancy may be the result of the different methods used for the analysis of mRNA and protein. Another reason might be a phenomenon called epithelial–mesenchymal transition (EMT) that will be discussed later.

In order to exclude that EGFR mutations in CTC might be the reason for the failure to detect wild-type EGFR on CTC, a search for EGFR mutants was made. Expression of some EGFR mutants has been reported to be associated with a poor prognosis [10] or as predictive factor for receptor tyrosine kinase therapeutics. In addition, some mutants are capable of oncogenic transformation in the absence of a ligand [2,20].

Previous studies have reported a co-expression of the wild-type EGFR if mutants were detected in biopsies. Particularly in glioblastoma the detection of deletion mutations is coupled with an over-expression of the wild-type EGFR [27]. These findings are comparable to our results concerning the glioma and colorectal cell lines. None of the cell lines expresses a mutated EGFR only (Table 3). Two new deletions variants were found in most of the colorectal and glioma cell lines in addition to the known deletion mutations of the N-terminal domain.

A parallel deletion of exons 12–13 and exons 2–7 as it is described for variant vIII/Δ12–13 [15] was not detected in the glioma or colorectal cell lines. Thus, it seems that in some of the analyzed cell lines a new variant of EGFR resulting in the deletion of exons 12–13 is expressed. It is tempting to speculate that the break point at exon 11/12 is a hot spot for mRNA splicing. In contrast, no variants of the EGFR were present in the blood from 20 analyzed CRC patients. For EGFR variant vIII this finding is in agreement with other studies [1,26]. Ongoing work is focused on showing that the new variants are not cloning artefacts of cell lines but rare events in CRC patients.

In conclusion, parallel analyses of CTC and liver metastases from patients with CRC demonstrated that CTC could be detected in the majority of patients us-

ing combined immunomagnetic tumour cell enrichment and multiplex RT-PCR technique. In contrast to the EGFR expression in approximately half of the patients with liver metastases shown by IHC, no EGFR expression was detected in most CTC-positive patients. EGFR expression might be down-regulated during detachment from the tumour and during entering the blood circulation like it is described for EpCAM (epithelial cell adhesion molecule), another tumour-associated epithelial marker encoded by the GA733-2 gene [22]. Further explanations may be that most tumour cells entering the blood circulation do not express EGFR until they disseminate to different organs or that cells not expressing EGFR have a higher propensity to disseminate. One reason might be the epithelial-mesenchymal transition (EMT) that occurs during the development of metastases. Tumour cells have to detach from the epithelial cell structure before they invade the blood circulation. One possibility to do that is that the tumour cell or the CTC respectively changes its expression profile to a more mesenchymal phenotype. The procedure often shows a down regulation of epithelial cell markers e.g. E-cadherin and an up regulation of mesenchymal cell markers e.g. N-cadherin [13]. Once the target organ is reached the CTC reverses the expression profile via mesenchymal-epithelial transition (MET) and evades the circulation. EGFR is a tumour-associated epithelial cell marker and may be another protein that underlies such changes in expression. Therefore, in future experiments it may be meaningful to focus not only on the over-expression of epithelial or oncofetal markers but on mesenchymal cell markers on CTC as well. Interestingly, there were contradictory findings concerning HER2 (human epidermal growth factor receptor 2) expression in breast cancer patients [17]. Hence, it seems that not all tumour-associated epithelial markers are down regulated during the circulation.

This pilot study demonstrates the usefulness of the analysis of CTC with a simple and time saving technique to identify the expression of potential therapeutic targets as well as their variants.

Acknowledgements

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