Elevation in peripheral blood circulating tumor cell number correlates with macroscopic progression in UICC stage IV colorectal cancer patients

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Abstract. *Aims:* Cytokeratin(CK) based real-time RT-PCR assays (QRT-PCR) are now available for peripheral blood circulating tumor cell (CTC) evaluations in colorectal cancer(CRC) patients. Results are non-existent for the application of these techniques to the determination of progression and therapy response in Dukes stage D CRC patients.

Patients and methods: Each month 30 ml peripheral blood of 30 Dukes D patients (17 with progression) were drawn. CEA, CA19-9, CA72-A and TPA-M determinations were made. CK20, thymidilate synthase(TS) QRT-PCR was performed, as well. Buffy coat was used for immunmagnetic cancer cell isolation and CTC counting. Correlation between elevated CTC and macroscopic progression within 3 months was determined by Chi² test.

Results: Microscopic CTC single cell, doublet, cluster number were found in correlation with CK20 QRT-PCR results ($p < 0.01$). There was a significant increase in microscopic CTC number, CK20 and decrease in TS QRT-PCR levels ($p < 0.05$) in the peripheral blood of the non-responder as compared to responder patients. Elevation of the CTC was in significant correlation with macroscopic progression of the disease in 3 months ($p < 0.01$).

Conclusions: CTC number reflects the chemotherapeutic sensitivity of CRC patients. Elevation of circulating tumor cell number in peripheral blood is in correlation with macroscopic progression.

Keywords: Circulating tumor cells, colorectal cancer, CK-20 RT-PCR, microscopic counting, progression

1. Introduction

The presence of circulating tumor cells in colorectal cancer patients proved to be a significant prognostic factor [1] in bone marrow specimens. Paget [2] first described these malignant cells in the blood more than 100 years ago. In the 1970's, correlation was shown between the presence of circulating tumor cell clusters and the development of metastasis in animal experiments [3].

Recently, in addition to routine cytology [4], immunocytochemistry of cytocentrifuged specimens and flow cytometry was applied for the evaluation of fluorescent labeled cells [5]. Our group reported the presence of circulating tumor cells and cell clusters in the peripheral blood of CRC patients using immunomagnetic enrichment and immunocytochemical labelling [6]. Automated microscopy based methods were also developed for the analysis of cytocentrifuged specimens [7].

The development of RT-PCR methods supported the detection of RNA targets from circulating tumor cells in the peripheral blood cell compartment. Beside CEA [8] and telomerase [9], tissue specific markers like cytokeratins (CK) were evaluated. However, some of

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these markers like the guanylin cyclase [10] and the cytokeratins [11] were also shown to be expressed by hematopoietic cells.

CK20 RT-PCR was successfully used in CRC patients for CTC detection [12]. It was shown in a recent mRNA expression study of circulating tumor cells that CK20 is differentially expressed together with CK19, CEA, MGB1, MGB2, PIP and DCC in the circulating tumor cell compartment [13].

The specificity of the RT-PCR assays could be enhanced by immunobead RT-PCR [8] as it was also confirmed by our group [14]. The CK20 RT-PCR reactions' specificity could be increased by the application of the quantitative PCR technique [14]. This method could support the determination of a cut off value for RT-PCR [15] as in flow cytometry [5], and provide correlation between the circulating cell numbers and the quantitative RT-PCR signals. We showed in a recent report [14] that correlation exists between the quantitative RT-PCR signals and the number of HT29 tumor cells added artificially to healthy blood samples. In an international collaborative study we also demonstrated that CK20 QRT-PCR is a reliable, reproducible method for the quantitative determination of CK20 mRNA's presence in peripheral blood [16].

Correlation between microscopic CTC, CTC cluster number and CK20 QRT-PCR data has not been shown in a clinical setting. A positive correlation would be advantageous for the QRT-PCR technique due to the higher throughput, lower cost and better automation possibility.

Considering the clinical application of follow-up CTC determinations, only limited data is available. In animal gastric cancer experiments [17] and in human breast cancer studies peripheral blood was successfully used for the determination of circulating tumor cells and the therapy responder status [18].

Until now the functional evaluation of CTCs for predicting the therapy response in CRC patients has not been reported. Recently a new assay was developed for the quantitative determination of thymidylate synthase, the target of the commonly used 5-fluorouracil therapy. In primary colorectal cancer the intratumoral expression of TS was found to be an independent prognostic factor [19]. Ehrnrooth could measure peripheral blood leukocyte TS mRNA quantitatively by a competitive RT-PCR [20].

Little is known about the correlation between the immunomagnetic cancer cell isolation, QRT-PCR results and routine serum tumor markers like CA19-9, CEA, TPA, CA72-4.

In this study we evaluated the results of prospective quantitative CK20 QRT-PCR determinations in 30 UICC stage IV patients and compared those to immunomagnetically isolated cell number, TS QRT-PCR, serum marker data and clinical, radiological progression. We were interested in finding out whether the responder status of the UICC stage IV colorectal cancer patients to chemotherapy is reflected in these serum mRNA, cancer cell and protein parameters.

2. Materials and methods

2.1. Patients

30 patients commencing systemic therapy over a given period were included in this study. All patients had UICC stage IV colorectal cancer disease (Table 1). Inclusion criteria were: histologically proven metastatic disease, clinically or radiologically assessable disease.

The patients underwent examinations for tumor progression by abdominal ultrasound every month, by CT and bone scintigraphy every quarter year.

The study was reviewed and approved by the local ethics review board and all patients gave informed consent to the study before blood samples were taken.

Assessment of treatment response was made by clinicians that were blind to the assay results according to the UICC criteria. Those undertaking QRT-PCR and serum tumor marker determinations were blind to the clinical status and the identity of the patients.

2.2. Treatment dosage and schedules

Patients received chemotherapy according to the Mayo protocol as follows: 20 mg/m² folinic acid iv., 425 mg/m² 5-Fluorouracil iv. Treatments were continued according to clinical response and toxicity. Patients with complete or partial response continued full courses of treatment. In case of progression, therapy was altered for 500 mg/m² folinic acid inf. (30 min), 2600 mg/m² 5-Fluorouracil inf. (24 h) (AIO regimen) or oral tegafur/uracil, depending on the patient's ECOG score.

2.3. Determination of responder status and progression

Classification into responder and non-responder group was made on the basis of the standard UICC criteria [21] as follows: complete response was defined as

Table 1
Patient characteristics

	Nr. of patients (responder/non-responder)	%
Sex	Male 19, Female 11	63.3%, 36.6%
Age		
Median	57	
Range	37–71	
Localisation of the primary tumor		
Rectum	7	23.3%
Sigma	7	23.3%
Colon desc.	4	13.3%
Colon trans.	4	13.3%
Colon asc. and coecum	8	26.6%
Mean follow-up time:	7.2 ± 4.6	
Site of metastases		
Liver	24 (11/13)	80.0%
Peritoneum	8 (4/4)	26.6%
Lung	7 (3/4)	23.3%
Bone	6 (2/4)	20.0%

complete disappearance of all clinical disease by physical or radiological examination for a period of at least 4 weeks. Partial response was defined as a greater than 50% reduction of tumor size as determined by two measurements not less than 4 weeks apart. Stable disease was defined as less than 25% progression or less than 50 % tumor reduction. Responder patients (13) were those with complete (0), partial response (5 patients) or stable disease (8 patients). Progressive disease (non-responders, 17 patients) was defined as greater than 25% increase in tumor measurements.

2.4. Cell and mRNA isolation

For the evaluation of clinical samples mononuclear cells were isolated from EDTA uncoagulated blood with standard density gradient centrifugation (Histopack 1.077, Sigma Aldrich, USA) as described in our previous papers [14,16]. The isolated cell pellet was immediately lysed in “RNA/DNA stabilization reagent for blood and bone marrow” (Roche Diagnostics GmbH, Mannheim, Germany) in 1 to 50 dilutions. mRNA was isolated from the lysed cell solution with the mRNA Isolation Kit for Blood and Bone Marrow (Roche Diagnostics GmbH, Mannheim, Germany). The final volume of the isolated mRNA was 200 μ l and stored at -80°C .

2.5. CK20 CTC and TS QRT-PCR on RNA from the density gradient centrifuged samples

CK20 RT-PCR evaluation was performed on a Lightcycler Instrument using CK20 Quantification kit and Relative Quantification Software (Roche Diagnostics

GmbH, Mannheim Germany) using 10 μ l of the isolated mRNA. A detailed description of this approach can be found in a recently appeared article by Soong et al. [15] and by our group [14,16]. CK20 expression was quantified (normalized ratio) as a relative ratio (RR) of CK20 to the housekeeping gene porphobilinogen deaminase (PBGD) in a test sample, relative to the CK20/PBGD ratio on a calibrator that includes RNA from HT29 cells.

Cut off values for CK20 were taken from literature and from our own experience (cutoff = 100 Norm. Ratio) [14–16].

The thymidilate synthase content of the peripheral blood samples was determined in a two-step procedure: first, cDNA was reverse transcribed using AMV reverse transcriptase and random hexamer priming. Second, a 111 bp fragment of TS mRNA was amplified from the cDNA in the Light Cycler using specific primers.

Using the same cDNA preparation, but a separate PCR reaction, a 123 bp fragment of glucose-6-phosphate dehydrogenase (G6PDH) was amplified as a reference gene. The reaction product served as a control for RNA integrity and in addition, as a reference for relative quantification. The calibrator RNA included the total RNA from immortalized cell lines expressing continuous TS.

The calibration of the data was performed in a way that the amount of target (TS) is calculated as ratio of target gene copies to reference gene copies (G6PDH). In the second step the ratio of target to reference copies (T:R) of the samples was divided by the T:R ratio of the calibrator RNA that was run in parallel with each sample. In this kit the calibrator ratio of T:R was set to 1.

Table 2
Correlation between CK 20 QRT-PCR results and immunomagnetic CTC number determination

	CK20	Single cells	Doublets	No. of clusters	No. of cells in the biggest cluster	No. of epithelial cells in all of the clusters	No. of lymphocytes in all of the clusters
CK20	1.00	0.96*	0.94*	0.72*	0.83*	0.87*	0.85*
Single cells		1.00	0.99*	0.78*	0.41	0.77*	0.85
Doublets			1.00	0.86*	0.67	0.84*	0.80*
No. of Cluster				1.00	0.84*	0.94*	0.93*
No. of cells in the biggest cluster					1.00	0.79*	0.73*
No. of epithelial cells in all of the clusters						1.00	0.92*
No. of lymphocytes in all of the clusters							1.00

*Significant correlation $p < 0.01$.

The number of single cells are in correlation with the number of circulating cell clusters. Note that the number of circulating cell clusters are in correlation with the number of cells in the clusters.

2.6. Immunomagnetic separation, cytochemical labeling and microscopic counting

In 7 patients (4 non-responders, 3 responders) immunomagnetic cell isolation with consecutive immunocytochemical labeling and light microscopic counting was performed as previously described [6] in parallel with the CK20 QRT-PCR.

2.7. CA 19-9, CEA, CA 72-4, TPA tumor marker determinations

CA19-9, CEA, and CA72-4 determinations were performed using the Elecsys System and reagents (Roche Diagnostics GmbH, Mannheim, Germany). In each case 2 ml serum was evaluated according to the manufacturers' protocol. The TPA was determined using the TPA Liaison TPA-M reagent on a LIA III analyser (DiaSorin Spa, Saluggia, Italy). The normal value with a 99.5% confidence interval was 4.3 ng/ml for the CEA, 9.8 U/ml for the CA72-4, 39 U/ml for the CA19-9. Concerning the TPA 80 U/ml was the cut off value. TPA is a mixture of cytokeratin 19 (44%), cytokeratin 8 (30%) and cytokeratin 18 (36%).

3. Statistical analysis

Statistical analysis was performed with the Statistica program package (Statsoft, V.6.0, Tulsa, OK, USA). Unpaired T-test was used for the determination of significant differences in the parameters between the responder and non-responder group.

Multiple regression analysis using a logistical regression model was performed to provide insight into the clinical relevance of the QRT-PCR and other markers.

Chi-Square test was performed for the determination of qualitative (elevated/normal) in-group correlation.

Spearmann-R coefficient was calculated in the quantitative in-group correlation analysis.

The correlation between the elevated CTC number and the macroscopic progression, within 3 months from the time of elevation, was determined by the Chi-square test.

4. Results

4.1. Correlation between CK20 QRT-PCR and the immunomagnetic CTC number

In the present study we have found the same, three different types of cells as we had previously described [6]: single CK+ cells, doublets containing CK+ and negative cells and cell clusters with a mixture of different number of cytokeratin positive and negative cells. Clusters were present only in correlation with increasing number of single cells.

The CK20 quantitative RT-PCR was in correlation with the single cell number, doublet number and cluster number (Table 2). The number of single cancer cells was in correlation with the doublets, the cluster numbers and the number of cancer cells in the clusters. The number of clusters was in correlation with the cell numbers in the largest cluster.

4.2. Response to therapy and changes in the CTC, CK20, TS QRT-PCR values and in serum tumor markers

Significantly increased number of single cells, doublets and clusters were observed in the non-responder group as compared to the responder group (Table 3). Especially the numbers of epithelial cancer cells in the clusters were significantly higher in the non-responder group.

Table 3
Differences in the CTC number between the responder and non-responder group

	Single Cells*	Doublets	No. of clusters*	No. of cells in the biggest cluster*	No. of epithelial cells in all of the clusters*	No. of lymphocytes in all of the clusters*
Responder	2.1 ± 3.1	0.25 ± 0.8	0.12 ± 0.42	0.31 ± 1.25	0.4 ± 1.2	0.31 ± 1.33
Non-Responder	19.9 ± 34.1	9.5 ± 27.3	2.6 ± 3.91	2.75 ± 3.8	10.4 ± 9.8	5.75 ± 8.01

* $p < 0.05$.

Note the significant difference in the CK20 norm. ratio. single cell number. The number of circulating clusters and the number of epithelial cells in the cluster have also increased significantly.

Table 4
Differences between the measured parameters in relation to the responder status

	CK20* Norm. Ratio Mean ± SD	TS+ Norm. Ratio Mean ± SD	CEA+ ng/ml Mean ± SD	CA19-9* ng/ml Mean ± SD	CA72-4* U/ml Mean ± SD	TPA+ U/ml Mean ± SD
Responder	1250.3 ± 199.5	1.73 ± 3.1	37.5 ± 34.1	10.82 ± 18.2	1.9 ± 1.2	149.3 ± 293.1
Non-responder	37823.4 ± 1427.6	0.47 ± 1.9	214.7 ± 172.1	328.2 ± 284.1	18.2 ± 14.3	525 ± 442.1

*Significant difference ($p < 0.01$).

+Significant difference ($p < 0.05$).

In the non-responder group there was a significant increase in CK20, CEA, CA72-4, CA19-9 and TPA values as compared to the responder one. However, the TS was significantly increased in the responder patients ($p < 0.05$) (Table 4).

The CK values were in the elevated range both in the responder and the non-responder group. The ratio between the two group's (non-responder/responder) mean was 64,3x. In the tumor markers we found that the mean of CA19-9 was in the normal range. CEA, CA72-4, TPA were in the pathologic range with a ratio of 8 to 30x of the normal value.

We could detect progressions in the malignant disease in 17 out of 30 patients. In 7 of the 17 progressive patients all of the measuring points showed significantly increased CK20, CEA CA72.4, TPA and decreased TS values. In all of these cases death of the patient occurred at the end of the follow-up period (Fig. 1E). The changes in the serum tumor marker levels were continuously increasing during our observations. In the first half (2–4 months) of the observation time the CK20 values showed high variation. In the second half, continuously increasing CK20 values were observed.

Significant elevations in the circulating tumor cell number were detected at different points in time in the peripheral blood in 8 of the remaining 10 patients (Fig. 1 B,C and D). New metastases appeared in these cases within 3 months after these circulating tumor cells had been shed into the blood ($p < 0.01$).

No circulating tumor cells were detected in two patients with progression, who were without new metastases.

Beyond the 8 patients with stable disease, minor regressions were detected during the follow-up in 5 patients (Fig. 1A). Their CK20 and serum parameters were in the normal range. Interestingly, the TS values showed high variance.

Multiple logistic regression analysis ($R = 0.64$; $R^2 = 0.41$, adjusted $R^2 = 0.36$; $p < 0.01$, Std. Error of estimate 0.39) proved independent significant correlation between CK20 RT-PCR values and the responder status ($p < 0.01$). CA72-4 was also found to be an independent prognostic factor ($p < 0.01$).

4.3. Correlation analysis between CK20 QRT-PCR and serum tumor marker parameters

First we performed the correlation analysis on a qualitative basis (elevated / normal value). TS was not involved in this evaluation as no cut off value was available for this parameter during our study. Elevated CK20 QRT-PCR was in significant correlation with elevated CA19-9, CA72-4 and TPA but not with CEA ($p < 0.01$).

The CK20 QRT-PCR values were in significant correlation with the serum TPA values in the clinically non-responding group in the quantitative correlation analysis. CEA correlated to the CA19-9, but all of the parameters were in significant correlation to CA72-4 ($p < 0.05$) (Table 5). The increase of peripheral tumor cell load was not followed by the serum tumor markers in these patients, as it is shown in Fig. 1B and D. If the tumor markers increased, they did almost linearly as the follow-up time went on. Only the CK20 showed discontinuous changes (by magnitudes of 10 to 100x).

Results of a responding patient with no change in the clinical status

Fig. 1. A, B, C, D, E: Results of one therapy-responding (A) and 4 non-responding patients (B, C, D, E). Patient E died at the end of the observation period.

Results of non-responding patients with macroscopic progression

Fig. 1B.

CK20 had no correlation to any parameters in the responder group (Table 6). However TS showed significant positive correlation to CA19-9 and CEA. TPA correlated to CA19-9 and CA72-4 ($p < 0.01$ and 0.05 respectively).

5. Discussion

In our previous work we were able to detect circulating tumor cells and cell clusters in the blood of colorectal cancer patients [6]. In earlier animal studies

Results of non-responding patients with macroscopic progression

Fig. 1C.

Results of non-responding patients with macroscopic progression

Fig. 1D.

significant correlation was shown between the tumor cell load and the development of metastases. In animal experiments not only the tumor cell number, but their characteristics have also influenced the frequency of metastasis [3], namely more complex and larger tumor cell clumps lead to more frequent metastasis. In our

study we have found that the presence of clusters depends on the overall number of circulating tumor cells in the peripheral blood.

The results of the present study show, that the higher the absolute number of single circulating tumor cells, the higher is the rate and the number of epithelial cells

Results of non-responding patients with macroscopic progression and lethal end

Fig. 1E.

Table 5
Correlation between the measured parameters in the non-responder group

No. of Patients	TS QRT-PCR	CEA	CA19-9	CA72-4	TPA
CK20 QRT-PCR	-0.08	0.12	0.29	-0.2	0.69*
TS QRT-PCR		-0.19	-0.19	-0.12	-0.1
CEA			0.64*	0.06	0.34
CA19-9				0.12	0.45*
CA72-4					0.23

*Marked correlations are significant by ($p < 0.05$).

Table 6
Correlation between the measured parameters in the responder group

No. of Patients	TSQRT-PCR	CEA	CA19-9	CA72-4	TPA
CK20 QRT-PCR	-0.19	-0.24	-0.05	0.00	-0.21
TS QRT-PCR		0.24	0.51*	0.15	0.67*
CEA			0.23	0.08	0.24
CA19-9				0.86*	0.92*
CA72-4					0.74

*Marked correlations are significant by ($p < 0.05$).

in the CTC clusters. Our results showed significant correlation between the CK20 QRT-PCR and the immunomagnetic enrichment and microscopic counting. This very important result paves the way for the widespread use of the QRT-PCR assays. The application of QRT-PCR for the detection of CRC in peripheral blood proved to be a faster, more automated, quantitative procedure over the immunomagnetic, immunocytochemical CTC determination method from the practical point of view.

Although real-time PCR is one of the most sensitive techniques to detect circulating tumor cells, its specificity may be influenced by illegitimate transcription of tumor-associated/epithelial – specific genes in hematopoietic cells [10,11]. This is not only essential in healthy subjects but it can be even more important in patients with inflammatory diseases.

Micrometastatic bone marrow tumor cells were successfully evaluated for the control of systemic therapy in breast cancer [18] as well as in recent colon cancer study [22]. A recent human breast cancer study showed

that circulating tumor cells are significant independent prognostic factors [23]. This study supports our findings that increased colorectal CTC number is in correlation with progression in the macroscopic status. With the enhanced detection and characterization of circulating tumor cells in blood a dedicated therapy could be developed and introduced for leukemia [24]. Similar attempts could be observed for a bone marrow based monitoring of chemotherapy in breast cancer [25,26]. In CRC clinically important results were found in two very recent papers [27,28] using quantitative realtime PCR for CK20 determinations. Dandachi et al. showed elevated CK20 levels in peripheral blood of CRC patients as compared to healthy volunteers. Iinuma found elevation of CK20 frequency in the blood of postoperative patients as compared to preoperative and healthy ones. He could show that CK20 elevation is also in negative correlation with disease-free survival.

Our data showed that tumor cells appear in the peripheral blood in two different patterns: there are cases with a non-continuous, random elevation type and there are others with continuously increasing values. A correlation was shown between the random, significant CTC elevation and a consecutive macroscopic progression detectable in 3 months. The continuously increasing pattern leads to a near fatal end of the disease with therapeutic resistance.

TS QRT-PCR is proven to be a useful marker for the determination of 5-FU chemosensitivity and responder status in the multivariate model. The source of the TS mRNA in the peripheral blood has not been determined.

Qualitative correlation between elevated CK20 normalized ratio to elevated serum tumor markers was shown by Weihrauch [29] too. However, the quantitative miscorrelation of peripheral blood CTCs with the serum protein based tumor markers found in our study shows that these protein markers must originate mostly from the primary or metastatic tumor and not from the circulating tumor cell component. Our results concerning discorrelation between the CRC peripheral blood CTC numbers and the tumor markers, are in accordance with a recent study [18].

CK20 QRT-PCR in addition to serum tumor marker determinations is a preferable method for quantitative determination of peripheral tumor cell load, chemosensitivity and can be an early marker of tumor progression.

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