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# Combination of Immunomagnetic Enrichment with Multiplex RT-PCR Analysis for the Detection of Disseminated Tumor Cells

VEIT ZIEGLSCHMID[1], CHRISTIANE HOLLMANN[1], BERTHA GUTIERREZ[1], WINFIRED ALBERTI[1], DARK STROTHOFF[2], EBERHARD GROSS[2] and OLIVER BÖCHER[1]

[1]*AdnaGen AG, Ostpassage 7, D-30853 Langenhagen;*
[2]*Allgemeines Krankenhaus Barmbek, Rübenkamp 148, D-22291 Hamburg, Germany*

**Abstract.** *Background: A highly specific and sensitive tumor cell detection assay is reported, which combines immunomagnetic enrichment with multiplex RT-PCR analysis. Materials and Methods: The effect on the recovery rate of breast, testicular and colorectal cancer cells using single antibodies and combinations of them for IMS was examined by fluorescence microscopy and multiplex RT-PCR. The clinical utility of a tumor cell detection assay using IMS with multiplex RT-PCR was tested by examination of colorectal cancer blood samples and by comparing the results with CEA serum protein levels. Results: A combination of antibodies for IMS and multiplex RT-PCR analysis proved to be the most sensitive approach for detection of tumor cells in peripheral blood with a detection limit of two tumor cells. The examination of blood of colorectal cancer patients by using a multiplex RT-PCR assay in comparison with CEA serum protein levels indicated a distinct advantage of the former over the latter with respect to a more reliable prediction of an ongoing metastatic process. Conclusion: The results indicate that a combination of antibodies for immunomagnetic enrichment with multiplex RT-PCR analysis detects disseminated tumor cells with high sensitivity and specificity, thus indicating a metastatic process several months earlier compared to CEA serum protein level measurements. This assay might be valuable for prognosis in cancer.*

Metastases are the major cause of death in patients suffering from solid neoplasms like breast, colorectal or testicular cancer. The dissemination of malignant epithelial cells from the primary tumor to distant parts of the body *via* lymph or blood is an essential step in cancer progression. In particular, the hematogeneous spreading of tumor cells can be regarded as the main route to the formation of clinical manifest metastases (1, 2). Although it has been demonstrated in animal models that the metastatic process is inefficient, *i.e.* the majority of tumor cells entering the blood circulation are killed by mechanical forces or by individual immune response, it is assumed that considerable numbers of tumor cells survive and leave the circulation successfully (3-5). The detection of disseminated tumor cells in the blood could thus be regarded as a promising tool to reliably predict the potential formation of clinical manifest metastases and to obtain direct evidence of a high possibility of recurrence in individual cases after surgical resection of the primary tumor or in the course of therapy (6).

Detection modalities could be divided into antibody-mediated tumor cell detection techniques, such as immunocytochemistry and immunohistochemistry (IHC) and molecular tumor cell detection techniques, such as PCR or RT-PCR. Although methods such as IHC staining continue to play a central role in the diagnosis and characterization of cancer, it is likely that newer techniques, such as RT-PCR and microarray analyses, will eventually play a central role in cancer diagnosis (7). Because disseminated tumor cells are only present in low amounts (1-10 cells per milliliter blood), the applied detection method must provide sufficient sensitivity (8). So far, only RT-PCR has been shown to provide the sensitivity and practicability necessary to detect the low number of cancer cells in blood (9).

On the other hand, the high sensitivity of RT-PCR could result in the detection of "illegitimate transcripts". The phenomenon of "illegitimate transcription" is caused by the leakiness of promoters, *i.e.* it can be expected that any promoter could be activated by ubiquitous transcription factors, which leads to an estimated expression level of one tumor-associated transcript in 500-1000 non-tumor cells

*Correspondence to:* Dr. rer. nat. Veit Zieglschmid, AdnaGen AG, Ostpassage 7, 30853 Hannover - Langenhagen, Germany. Tel: +49(0)511-72 59 50-50, Fax: +49(0)511-72 59 50-40, e-mail: vz@adnagen.com

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(10). Highly sensitive methods can detect these minute quantities of mRNA, thus emphasizing the need for enrichment procedures to decrease background levels. Tumor cell enrichment techniques such as immunomagnetic cell isolation make use of antibodies coupled to small magnetic particles, which bind to surface antigens on disseminated tumor cells. The cells can then easily be isolated with a magnetic device and finally analyzed by fluorescence microscopy or RT-PCR.

In the present study, single antibodies and antibody combinations were used for IMS, and the resulting effect on the recovery rate of breast, testicular and colorectal cancer cells spiked into blood of healthy donors was examined using fluorescence microscopy and multiplex RT-PCR. Tumor-associated transcripts were chosen because of known expression or overexpression in testicular, breast and colorectal cancer, respectively.

Once the specificity and sensitivity of the multiplex RT-PCR assay for the detection of colorectal cancer cells had been evaluated, the existence of disseminated tumor cells in peripheral blood of colorectal cancer patients was examined. Tumor cell detection was compared with CEA levels.

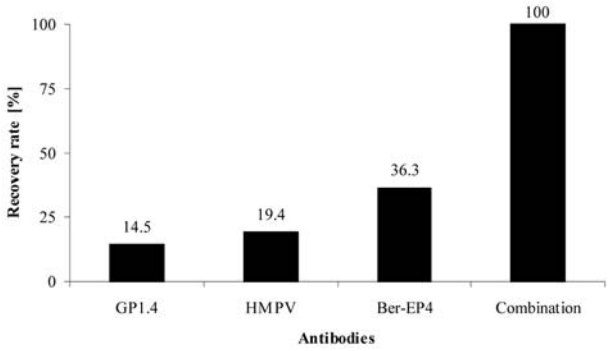



Figure 1. *Recovery rate of tumor cells after immunomagnetic cell separation with single antibodies and a combination of antibodies. Recovery rate of SKBR3 cells after immunomagnetic cell separation with the antibodies GP1.4, HMPV and Ber-EP4 as well as with a combination of them. The recovery rate obtained with the combination of antibodies is set as 100%. Antibodies were coupled to Dynabeads® and used for selection of SKBR3 cells previously spiked into blood of healthy donors. After selection, the cells were detached from the beads, stained and analyzed by fluorescence microscopy.*

## Materials and Methods

Patients with histologically confirmed colorectal carcinoma were investigated. Peripheral, venous blood samples (5 mL) were collected in EDTA-tubes and processed within four hours, as described below.

The effect on the recovery rate of breast, testicular and colorectal tumor cells using monoclonal antibodies and combinations of them for IMS was examined in spiking experiments. Blood samples (5 mL) of healthy donors served as negative controls, and the breast cancer cell line SKBR3, the testicular cell line Tera1 and the colorectal carcinoma cell line T84 served as positive controls. The determination of the lower detection limit of IMS combined with multiplex RT-PCR was done by spiking blood of healthy donors with defined numbers of carcinoma cells. Cell numbers up to 100 cells were serial diluted, whereas 10, 5 and 2 cells were manually picked under microscopic control to avoid statistical uncertainties observed in serial dilution experiments with small cell numbers.

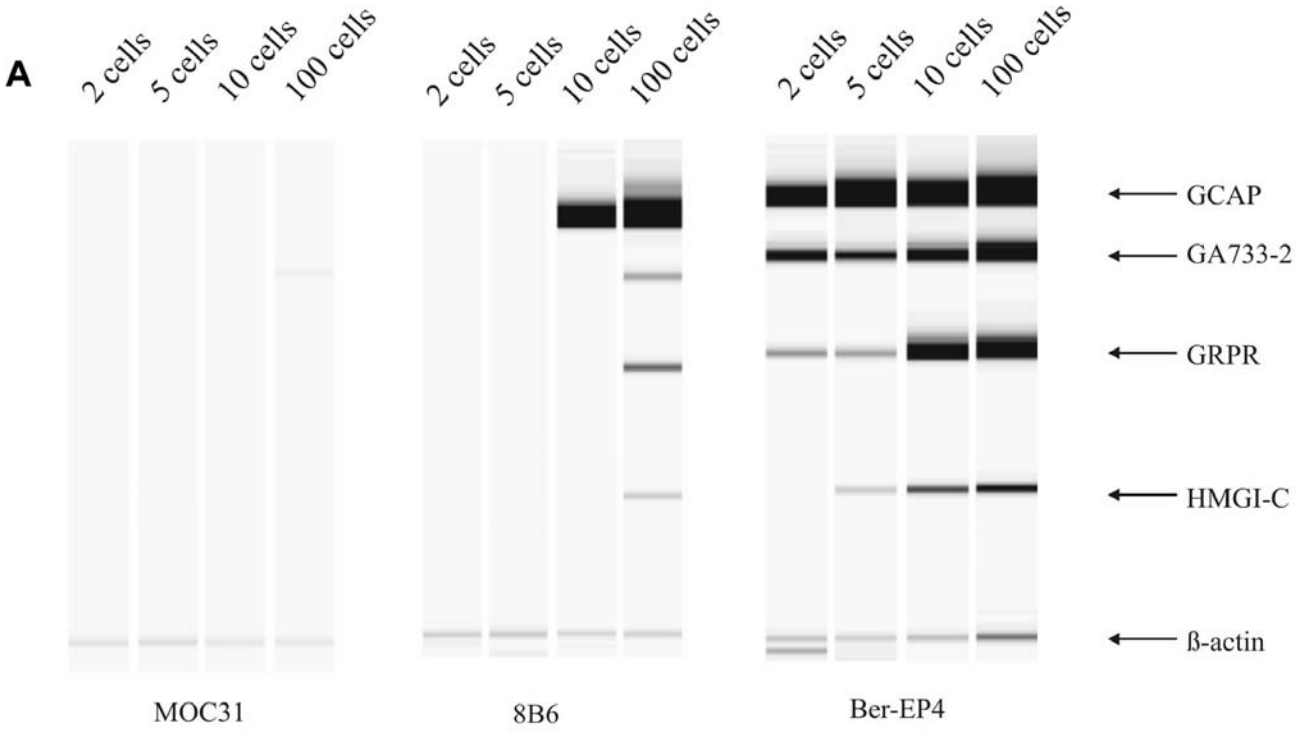
Tumor cell enrichment was done by IMS using monoclonal antibodies directed against tumor cell surface antigens coupled to magnetic beads (Dynabeads® M-450 Pan Mouse IgG) (Dynal, Oslo, Norway). The following antibodies were used for different tumor cell selection systems: Ber-EP4 (DAKO, Hamburg, Germany), HMPV (BD, San Diego, USA), GP1.4 (Neomarkers, Fremont, USA), 8B6 (Cymbus, Chilworth, UK), MOC31 (Neomarkers, USA). Monoclonal antibodies were coupled to Dynabeads® (Dynal) according to the manufacturer's instructions.

Patient blood samples (5 mL), positive and negative controls were mixed with 4 x $10^7$ Dynabeads®, pre-washed with phosphate-buffered saline (Invitrogen, Carlsbad, USA) and incubated for two hours at room temperature on a tube rotator. Magnetic separation and washing were performed according to the protocol (Dynal) after the incubation.

Determination of tumor cell recovery rate was done by detaching tumor cells from the magnetic beads, staining and analyzing them by fluorescence microscopy. For this purpose, tumor cells were selected with Dynabeads® (CELLection™ Pan Mouse IgG) (Dynal), coated with antiobodies *via* DNA-linker instead of Dynabeads® M-450 Pan Mouse IgG, detached by DNase treatment and recovered in the supernatant after bead separation. Selected tumor cells were stained with Hoechst 33342 and secondary goat anti-mouse antibody labelled with Alexa 488 (Molecular Probes, Eugene, USA). Stained cells were spun on slides and analyzed by fluorescence microscopy.

The standard components of the Dynabeads mRNA DIRECT Micro Kit (Dynal) were used for the mRNA isolation, according to the protocol (Dynal). The total mRNA/bead mixture (29.5 μL) was reverse transcribed in 0.5 μL RNase inhibitor (40 U/μL; Promega, Mannheim, Germany), 4 μL RT buffer, 4 μL dNTP's, and 2 μL Sensiscript reverse transcriptase (Qiagen, Hilden, Germany). Reverse transcription was performed in a one-step reaction for 60 min at 37˚C followed by 5 min at 93˚C. The cDNA was immediately chilled on ice or stored at –20˚C.

The analysis of tumor-associated transcripts was performed by multiplex PCR. The primer mixture for analyzing testicular cancer cells contains ten specific primer pairs to amplify four tumor-associated transcripts (germ cell alkaline phosphatase (GCAP), gastrointestinal tumor-associated antigen (GA733-2), gastrin-releasing peptide receptor (GRPR) and high-mobility group (non-histone chromosomal) protein isoform I-C (HMGI-C)) and one internal control (β-actin). The primer mixture for analyzing colorectal cancer cells contains eight specific primer pairs to amplify three tumor-associated transcripts (GA733-2, carcinoembryonic antigen (CEA) and epidermal growth factor receptor (EGFR)) and one internal control (β-actin). The primers were designed according to previously published sequences in the NCBI database. All PCR reactions were performed in a final volume of 50 μL PCR mixture



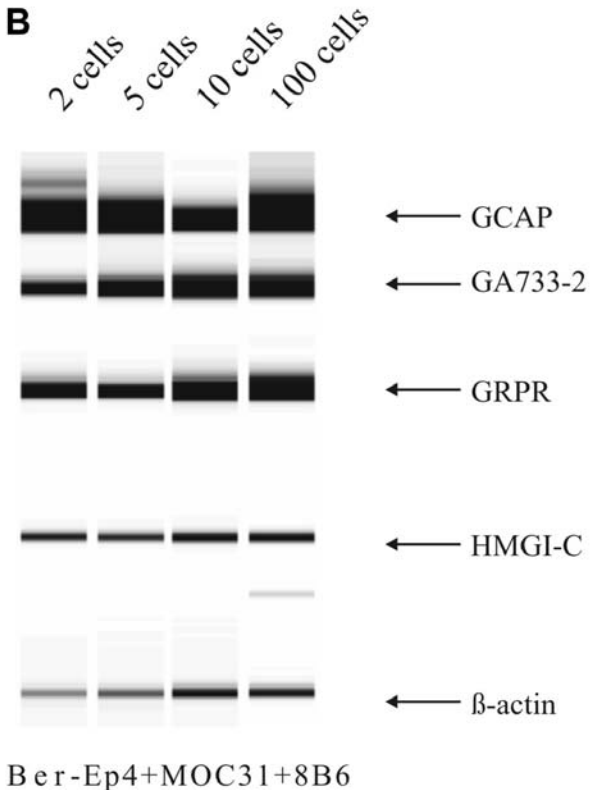




Figure 2A. *Detection of testicular cancer cells by multiplex RT-PCR after immunomagnetic enrichment: single antibodies. Detection of Tera1 cells by multiplex RT-PCR after immunomagnetic enrichment with the antibodies MOC31, 8B6 and Ber-EP4. Amplified DNA fragments of the tumor-associated transcripts GCAP (440 bp), GA733-2 (395 bp), GRPR (308 bp), HMGI-C (213 bp) and the internal control β-actin (118 bp) are shown. DNA fragments were analyzed by capillary electrophoresis with the Bioanalyzer 2100 (Agilent Technologies).*

Figure 2B. *Detection of testicular cancer cells by multiplex RT-PCR after immunomagnetic enrichment: mixture of antibodies. Detection of Tera1 cells by multiplex RT-PCR after immunomagnetic enrichment with a combination of the antibodies MOC31, 8B6 and Ber-EP4. Amplified DNA fragments of the tumor-associated transcripts GCAP (440 bp), GA733-2 (395 bp), GRPR (308 bp), HMGI-C (213 bp) and the internal control β-actin (118 bp) are shown. DNA fragments were analyzed by capillary electrophoresis with the Bioanalyzer 2100 (Agilent Technologies).*

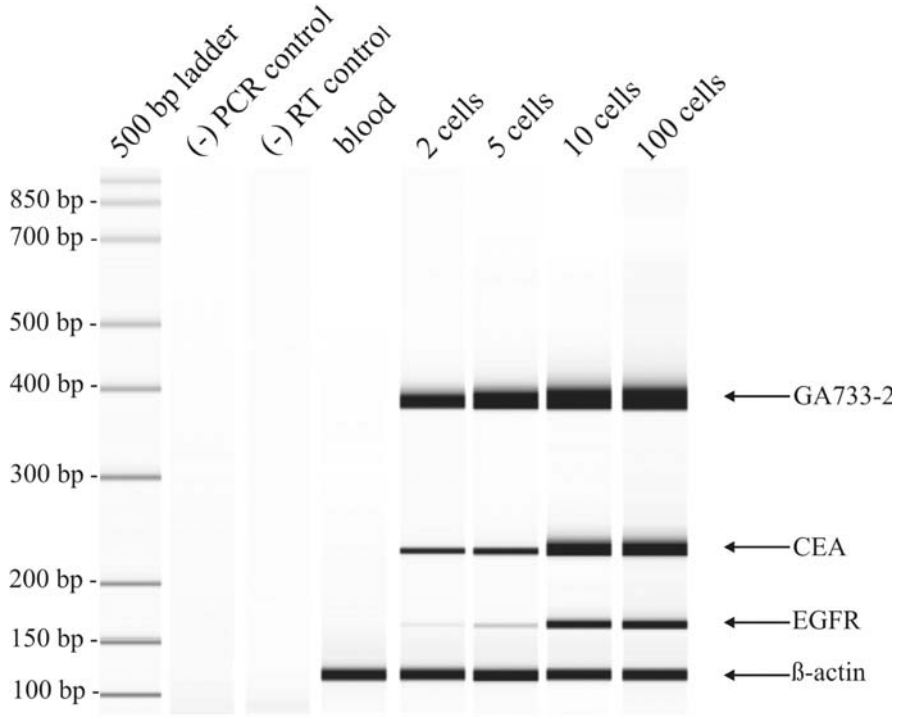



Figure 3. *Detection of colorectal cancer cells by multiplex RT-PCR after immunomagnetic enrichment. Detection of T84 cells by multiplex RT-PCR after immunomagnetic enrichment with a combination of the antibodies MOC31 and Ber-EP4. Amplified DNA fragments of the tumor-associated transcripts GA733-2 (384 bp), CEA (231 bp), EGFR (163 bp) and the internal control β-actin (118 bp) are shown. DNA fragments were analyzed by capillary electrophoresis with the Bioanalyzer 2100 (Agilent Technologies).*

containing 8 μL cDNA, 3.6 μL primer mixture for analyzing testicular cancer cells (AdnaGen, Langenhagen, Germany) or 4.4 μL primer mixture for analyzing colorectal cancer cells (AdnaGen), 25 μL HotStarTaq Master Mix (Qiagen), and 13.6 μL water. The PCR consisted of: pre-denaturation at 95˚C for 15 min, followed by 40 cycles of denaturation at 94˚C for 1 min, annealing at 58˚C for 1 min, extension at 72˚C for 1 min, and a final extension step at 72˚C for 10 min. Control samples were run in parallel with each experiment to exclude contamination, in which mRNA and cDNA were replaced by water in the reverse transcription and the polymerase chain reaction, respectively. PCR products were analyzed by capillary electrophoresis with the Bioanalyzer 2100 (Agilent, Waldbronn, Germany).

## Results

The influence on the recovery rate of tumor cells using different single monoclonal antibodies and a combination of them for IMS was determined.

Monoclonal antibodies were coupled to magnetic beads and used subsequently to select tumor cells from blood. After IMS, the recovery rate of tumor cell selection was assessed by counting tumor cells previously spiked into blood of healthy donors. Selected tumor cells were detached from the magnetic beads, stained and analyzed by fluorescence microscopy. Cells of large size, high nucleus-to-plasma ratio and green fluorescence were regarded as tumor cells. The recovery rate of tumor cells was determined after enrichment with single antibodies and a combination of antibodies. The recovery rate obtained with a combination of antibodies was set as 100% and compared with the recovery rates obtained with single antibodies. The selection of tumor cells with the antibodies GP1.4, HMPV and Ber-EP4 resulted in a recovery rate of 14.5%, 19.4% and 36.3%, respectively (Figure 1). Surprisingly, the combination of antibodies resulted in a higher total recovery rate of tumor cells than would have been expected by addition of the recovery rates obtained with single antibodies (70.2% total), indicating a synergistic effect. This high recovery rate can be considered as a prerequisite to provide the necessary sensitivity to detect rare tumor cells in peripheral blood.

The effect on the sensitivity of tumor cell detection using single antibodies and combinations of them for IMS was examined by multiplex RT-PCR analysis. The rationale of a multiplex RT-PCR analysis is based on the assumption that tumor cells exhibit *in vivo* a high degree of heterogeneity

Table I. *Comparison of tumor cell detection in peripheral blood with elevated CEA serum levels in three colorectal cancer patients. Comparison of tumor cell detection by multiplex RT-PCR after immunomagnetic enrichment with CEA serum levels in the follow-up monitoring of three patients with colorectal cancer. After immunomagnetic enrichment with a combination of the antibodies MOC31 and Ber-EP4, tumor cells were analyzed by multiplex RT-PCR using the tumor-associated transcripts GA733-2, CEA, EGFR and the internal control β-actin. A blood sample was considered positive if at least one tumor-associated transcript was detected and marked with +. A CEA concentration above 5 ng/ml serum was considered to be elevated and marked with +. Indication of multiple liver metastases is marked by an asterisk.*

| | | prior surgery | 1.5 months | 3 months | 4.5 months | 6 months | 9 months | 12 months | 18 months |
|---|---|---|---|---|---|---|---|---|---|
| Patient 1 | ELISA | + | - | - | - | - | - | - | - |
| | PCR | + | - | - | - | + | + | + | + |
| Patient 2 | ELISA | - | - | - | - | - | - | - | - |
| | PCR | - | - | - | - | - | + | + | + |
| Patient 3 | ELISA | - | - | - | 0 | 0 | 0 | +* | |
| | PCR | + | + | + | 0 | 0 | 0 | +* | |

with respect to gene expression, thus rendering a multiplex RT-PCR assay more reliable in detecting disseminated tumor cells than a singleplex RT-PCR assay.

Tumor cell detection by a multiplex RT-PCR assay was shown by spiking variable numbers of testicular cancer cells into 5 mL blood of healthy donors and analyzing them by IMS and multiplex RT-PCR (Figure 2A). For the IMS procedure, three monoclonal antibodies (MOC31, 8B6, Ber-EP4) were coupled to magnetic beads. IMS was carried out with single antibodies and an antibody mixture. The multiplex RT-PCR used the molecular markers GCAP, GA733-2, GRPR, HMGI-C and β-actin as an internal control.

After IMS of Tera1 cells with single antibodies and subsequent multiplex RT-PCR analysis, variable numbers of tumor cells were detected (Figure 2 A). After IMS with the antibody MOC31, only the expression of the tumor-associated transcript GA733-2 was detected by multiplex RT-PCR when 100 Tera1 cells were spiked into 5 mL blood, resulting in a low detection sensitivity of 100 tumor cells. After IMS with the single antibody 8B6, the expression of all four tumor-associated transcripts was detected only when 100 tumor cells were present and the expression of GCAP when 10 tumor cells were spiked, resulting in a detection sensitivity of 10 tumor cells. IMS with the antibody Ber-EP4 eventually led to the detection of the expression of all four tumor-associated transcripts in 5, 10 and 100 tumor cells. The expression of three tumor-associated transcripts (GCAP, GA733-2 and GRPR) was detected in samples with two spiked tumor cells. Ber-EP4 thus proved to be the most efficient single antibody in selecting tumor cells, resulting in a sensitivity of two tumor cells. However, considering the high degree of tumor cell heterogeneity *in vivo*, low amounts of tumor cells expressing only HMGI-C might be missed when only Ber-EP4 is used. After IMS with a mixture of the antibodies MOC31, 8B6 and Ber-EP4, the expression of all tumor-associated transcripts were detected in two tumor cells spiked into 5 mL blood (Figure 2B). Additionally, in contrast to single antibodies, the antibody mixture rendered detection of two tumor cells more reliable (data not shown). Taken together, the mixture of antibodies for IMS combined with multiplex RT-PCR analysis provided a tumor cell detection assay with high sensitivity down to the limit of two tumor cells.

The combination of a mixture of monoclonal antibodies for IMS with the combination of multiplex RT-PCR analysis was used to detect colorectal carcinoma cells in peripheral blood. The sensitivity and specificity of tumor cell detection were assessed by analyzing colorectal carcinoma cells (T84) spiked into blood of healthy donors by IMS with multiplex RT-PCR. A mixture of antibodies (Ber-EP4 and MOC31) was used for IMS, and three tumor-associated transcripts (GA733-2, CEA, EGFR), as well as an internal control (β-actin), were used for multiplex RT-PCR.

After IMS and multiplex RT-PCR analysis, the expression of all three tumor-associated transcripts was detected when two T84 cells were spiked into 5 mL blood (Figure 3). In blood without spiked tumor cells, no tumor-associated transcripts, but only the internal control β-actin, were detected, revealing a high degree of specificity.

It can be concluded that spiked colorectal carcinoma cells were detected in blood with high sensitivity and specificity, using the combination of different antibodies for IMS with different tumor-associated transcripts for multiplex RT-PCR.

The clinical usefulness of the multiplex RT-PCR assay was determined by analyzing peripheral blood of colorectal cancer patients. Tumor cell detection was compared with CEA serum protein levels. Peripheral blood (5 mL) of patients with colorectal cancer was collected prior to surgery and at regular time intervals (1.5, 3, 4.5, 6, 12 and 18 months)

after surgery and analyzed by IMS with multiplex RT-PCR. A blood sample was considered positive if at least one tumor-associated transcript was detected. Determination of CEA levels was carried out in the same blood sample used for tumor cell detection. A CEA concentration above 5 ng/ml serum was considered to be elevated and clinically indicative of tumor relapse.

The comparison between tumor cell detection by the multiplex RT-PCR assay with an elevated CEA level over 6 months showed no coherence, as exemplified in Table I. Patient 1 showed disseminated tumor cells and an elevated CEA level prior to surgery. Shortly after surgery, the CEA concentration dropped below 5 ng/ml and no tumor cells were detected, indicating a successful removal of the primary tumor. Six months after surgery, disseminated tumor cells reappeared in the peripheral blood while the CEA level was still below 5 ng/ml, giving an indication of potential tumor relapse. Similar results were observed in the follow-up monitoring of patients 2 and 3. Again, disseminated tumor cells were detected prior to the elevation of CEA in serum. In Patient 3, disseminated tumor cells were detected from the beginning of monitoring while the CEA levels remained below 5 ng/ml. After 12 months of follow-up, patient 3 was diagnosed with multiple liver metastases.

The results indicate that the multiplex RT-PCR assay might serve as a more reliable tool for the prediction of potential formation of clinical metastases than determination of the serum tumor marker CEA, since the multiplex RT-PCR assay gives a positive result earlier.

## Discussion

The effect on the recovery rate of breast, testicular and colorectal cancer cells using single antibodies and combinations of them for IMS was examined by fluorescence microscopy and multiplex RT-PCR. The results demonstrated that a tumor cell detection limit of two tumor cells is possible by a combination of antibodies for IMS with a multiplex RT-PCR analysis. The examination of blood of colorectal cancer patients with this assay in comparison with CEA level determination indicated a distinct advantage of the former with respect to a more reliable prediction of an ongoing metastatic process.

Hematogeneous metastasis is the most important factor affecting prognosis in carcinoma patients (11). Based on this assumption, detection of disseminated tumor cells in blood of cancer patients could be of prognostic value (1). One difficulty in detecting disseminated tumor cells relies in their rarity (8, 12). Therefore, detection techniques must provide enough sensitivity to make the detection of heterogeneous disseminated tumor cells reliable and feasible. However, sensitivity must not compromise specificity, which would result in the detection of "illegitimate transcription", *i.e.* the ectopic expression of a gene transcript in a tissue where it would not usually be expressed (13). Moreover, disseminated cancer cells are characterized by a high degree of heterogeneity with respect to surface antigens, mutation frequency and gene expression (14-19). The heterogeneous nature of disseminated tumor cells must therefore be taken into account when high sensitivity is required. By applying an immunomagnetic enrichment procedure using combinations of antibodies against epithelial surface and tissue-specific antigens, we could enhance sensitivity of tumor cell detection while maintaining specificity. The results are in-line with others who have used IMS with antibodies against epithelial cell surface antigens to enhance the sensitivity of tumor cell detection (20-23).

In contrast, the effect on the recovery rate of tumor cells using single and combinations of antibodies for IMS were evaluated by others without convincing results regarding the beneficial effect of antibody combinations for improvement of tumor cell detection sensitivity (24, 25).

The reported results reflect the difficulties in proving the value of a combination of antibodies for IMS when homogeneously expressing cell lines are used for *in vitro* experiments. However, it is expected that disseminated tumor cells in patient samples exhibit a much more heterogeneous expression of surface antigens. Hence, it could be assumed that a combination of antibodies for IMS in the routine analysis of patient samples will lead to enhanced sensitivity. Sensitive detection of disseminated tumor cells by using a combination of antibodies for IMS has already been performed successfully by others (26, 27).

Tumor progression can involve a period in which metastatic cells with different gene expression profiles exist (28). Detection of those metastatic cells requires the use of more than one tumor-associated transcript by RT-PCR analysis. It could, therefore, be expected that tumor cell detection assays could lead to false-negative results and poor detection rates when only one tumor-associated transcript is used. Based on this assumption, multiplex RT-PCR assays using several molecular markers were developed to enhance the sensitivity of tumor cell detection, which was assessed in spiking experiments to be two tumor cells. All chosen tumor-associated transcripts were shown to be predominantly expressed or overexpressed in the tissue of tumor origin.

Heterogeneity of gene expression was shown in disseminated tumor cells in peripheral blood, leading to enhanced detection sensitivity when multiple tumor-associated transcripts were used (28, 29). These results confirm our hypothesis that a multiplex RT-PCR assay enhances sensitivity of tumor cell detection when patient samples are examined.

Once the sensitivity and specificity of our assay for detection of colorectal cancer cells using a combination of

two antibodies for IMS and a combination of three tumor-associated transcripts for multiplex RT-PCR was successfully evaluated *in vitro*, we examined blood samples of colorectal cancer patients. Our aim was two-fold: on the one hand, we wanted to prove the clinical utility of the assay to detect disseminated colorectal cancer cells in peripheral blood and, on the other, with respect to improved prediction of metastases formation in the clinical setting, we wanted to show a potential advantage of tumor cell detection by using our assay over the standard clinical parameter used for monitoring of colorectal cancer patients, *i.e.* the elevation of CEA serum protein levels.

We were able to show the clinical usefulness of the multiplex RT-PCR assay by detecting disseminated tumor cells in peripheral blood samples of colorectal carcinoma patients at the time of primary diagnosis and in follow-ups over a period of 18 months. Moreover, it was shown that the detection of disseminated tumor cells in most cases preceded the elevation of CEA levels in peripheral blood. In one case, disseminated tumor cells could be detected 12 months prior to the development of multiple liver metastases, while the CEA concentration remained below the level of significance. Based on this case report, it could be proposed that the existence of tumor cells in the blood prior to surgery and possibly the reappearance of tumor cells in the follow-up of the disease is indicative of an ongoing manifestation of metastases. Hence, a prognostic value of this assay could be assumed.

## Conclusion

Our findings show that a combination of antibodies for immunomagnetic tumor cell enrichment and multiplex RT-PCR analysis is a suitable tool for the detection of disseminated tumor cells in carcinoma patients. Moreover, our results indicate that the multiplex RT-PCR assay may overcome the problem of tumor heterogeneity and enhance the sensitivity of tumor cell detection. The results provide evidence for a prognostic factor by using our assay to predict an ongoing metastatic process earlier and more precisely than the CEA levels.

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