The Clinical Significance of Circulating Tumour Cells in Breast Cancer and Colorectal Cancer Patients

SIEGFRIED HAUCH[1], SILKE ZIMMERMANN[1], SILKE LANKIEWICZ[1], VEIT ZIEGLSCHMID[1], OLIVER BÖCHER[2] and WINFRIED HANS ALBERT[1]

[1]*AdnaGen AG, D-30853 Langenhagen, Germany;*
[2]*Prionics AG, CH-8952 Schlieren, Switzerland*

Abstract. *Background: Circulating tumour cells (CTC) in the blood of cancer patients indicate disease progression. Their presence reflects a relapse or metastasising process since CTC survive only a short time in the circulation. Materials and Methods: Test systems developed by AdnaGen have been used for the sensitive and specific analysis of CTC. Results: Case reports of 2 breast cancer patients demonstrate the successful detection of CTC for therapy monitoring purposes. The disappearance of CTC reflects therapy success. The patient that responded towards therapy was characterized by the disappearance of CTC from the first therapeutic unit (TU) onwards. In contrast, CTC remained detectable in the other patient during the whole therapy pointing to only limited therapeutic efficacy and a progressive disease. Furthermore, systematic changes in the expression profile of CTC in colorectal patients at different stages of disease could be observed. Whereas EGFR was expressed in 90% of the patients with CTC during primary disease the expression level decreased to 15% in CTC of metastatic patients. On the other hand the expression of CEA was low in CTC found after primary surgery (15%) and dominant in CTC of metastatic patients (80%). Conclusion: The analysis of CTC is a useful tool for therapy monitoring of breast cancer and colorectal cancer patients in the adjuvant and palliative situation. The molecular profiling of CTC may be used to identify therapeutic targets such as HER2 or EGFR for personalised treatment that is likely to have an important impact on the therapeutic efficacy of drugs like Herceptin® or Erbitux®.*

Correspondence to: Winfried Hans Albert, AdnaGen AG, D-30853 Langenhagen, Germany. Tel: +49 51172595050, Fax: +49 51172595040, e-mail: wa@adnagen.com

Key Words: Circulating tumour cells in blood (CTC), disseminated tumor cells in bone marrow (DTC), immunomagnetic enrichment, clinical significance, breast cancer, colorectal cancer, peripheral blood.

The presence of disseminated tumour cells in bone marrow (DTC) has been widely confirmed as an independent prognostic factor (1). DTC can have a very long survival time because they are not always destroyed by the body's defence mechanisms. Therefore, they are not useful for obtaining a detailed account of the time course of the disease or the success of a therapeutic intervention (2).

The clinical significance of circulating tumor cells in the peripheral blood (CTC) of cancer patients has also been proven [reviewed in (3)]. These cells survive only for up to 24 hours in the patient's blood (4). The accidental detection of CTC seems unlikely unless there is a permanent new influx of tumour cells. CTC are of special importance because their detection in patients' blood mirrors a dynamic process, whereas tumour cells located in bone marrow might be the result of a process that became static at some time after onset of the disease. CTC can most likely be detected during tumour progression offering a prognostic value and an independent use for the evaluation of therapeutic efficacy. This has been confirmed through several clinical studies during the past four years (5-8).

The detection of CTC in peripheral blood of cancer patients is difficult for two reasons. One reason is the illegitimate transcription of tumour-associated mRNAs by nucleated blood cells (*e.g.* thrombocytes) (9). This can lead to a strong background that varies between persons and may complicate the distinction between healthy donors and patients. A lowering of this background level can only be achieved by an immunomagnetic enrichment of tumour cells, by the removal of other nucleated cells or by the loss of sensitivity of the detection method. Another reason for the difficulty in detecting CTC is the extremely high variability of surface antigen expression. Only an optimized combination of different antibodies binding to different epitopes expressed on tumour cells prevents the loss of analytical sensitivity. Combining the immunomagnetic enrichment of CTC with the analysis of tumour-associated transcripts results in high analytical sensitivity whilst maintaining a high specificity as has been described (10, 11).

The detection of a dynamic process like tumour cell spread into the blood circulation offers diagnostic potential for the surveillance of the adjuvant and palliative situation. To date therapy strategies have only been established based on parameters obtained from the primary tumour. The aim of our experiments was to show the clinical significance of CTC regarding therapy monitoring in breast and colorectal cancer patients. The technology developed by AdnaGen enables an effective therapy monitoring. Variations in the expression profile of CTC during adjuvant and palliative therapy at several time points of blood withdrawal from different patients were detected.

Materials and Methods

Five ml blood samples were collected from breast cancer patients and colorectal cancer patients with an S-Monovette® (Sarstedt AG & Co., Nümbrecht, Germany) with EDTA and were stored at 4˚C until further examination. The samples had to be processed within 4 h. The cell enrichment method employed is described elsewhere (10, 11). In brief, the blood samples were incubated with a ready-to-use antibody mixture commercialized as AdnaTest BreastCancerSelect in the case of breast cancer patients and as AdnaTest ColonCancerSelect in the case of colon cancer patients according to the manufacturer's (AdnaGen AG, Langenhagen, Germany) instructions. mRNA isolation from lysed, enriched cells was performed with the Dynabeads mRNA DIRECT™ Micro Kit (Invitrogen GmbH, Karlsruhe, Germany) that is included in the AdnaTest BreastCancerDetect or AdnaTest ColonCancerDetect according to the manufacturer's (AdnaGen AG, Langenhagen, Germany) instructions. Sensiscript® Reverse Transcriptase (QIAGEN GmbH, Hilden, Germany) was used for the reverse transcription because of its high sensitivity (recommended for amounts of <50 ng RNA) in combination with oligo(dT) coupled Dynabeads of the mRNA DIRECT™ Micro Kit (Invitrogen GmbH, Karlsruhe, Germany) according to the manufacturer's instructions (12).

The analysis of tumour-associated mRNA isolated from circulating tumour cells was performed in a multiplex PCR for 3 tumour-associated transcripts: human epidermal growth factor receptor 2 (HER2), Mucin 1 (MUC1) and epithelial glycoprotein GA733-2 in the case of breast cancer patients (11), or epidermal growth factor receptor (EGFR), carcinoembryonic antigen (CEA) and GA733-2 in the case of colon cancer patients. PCR was performed with the HotStarTaq Master Mix (QIAGEN GmbH, Hilden, Germany). Actin was used as internal PCR positive control. Visualization of the PCR fragments was carried out with a 2100 Bioanalyzer using the DNA 1000 assay (Agilent Technologies, Waldbronn, Germany).

Results

Palliative therapy monitoring of breast cancer patients. Efficacy of therapy can be immediately assessed through the detection of tumour cells as shown in Figure 1 for metastasized breast cancer patients. Patient 1 suffered from multiple lung (PUL) and bone (OSS) metastases and was negative for estrogen receptor (ER), progesteron receptor (PR) and HER2 as prognostic factors. Patient 2 had liver (HEP), lung (PUL) and bone (OSS) metastases and was positive for all prognostic factors mentioned above. Patient 1 received four units of taxane therapy (Paclitaxel), while patient 2 was treated with five units of an anthracycline therapy. Prior to therapy, patient 1 showed an expression of GA733-2 and MUC1 and, therefore, was positive for CTC. After the onset of the therapy, CTC immediately disappeared. In the course of the treatment the patient was only positive again for CTC on one occasion. Patient 2 showed an expression of three tumour-associated transcripts prior to therapy as well as during the subsequent treatment. Only after therapy unit 4 (TU), where the dose of anthracycline was doubled, did the transcript HER2 disappear and the signal strength of GA733-2 and MUC1 decreased slightly. In patient 1 the disappearance of CTC was related to clinical response, whereas in patient 2 progressive disease under therapy was related with positive findings of CTC.

Adjuvant therapy monitoring of breast cancer patients. The breast cancer patient whose data is shown in Figure 2 was monitored over a period of approximately 2.5 years. The primary tumour was classified as pT1c, N2a, M0, the prognostic factors ER, PR and HER2 were negative. The patient was treated with epirubicin/cyclophosphamid (EC), radiation therapy and trastuzumab (Herceptin®). Prior to surgery the patient showed an expression of MUC1 and therefore was positive for CTC. After surgery and several units of chemotherapy the patient remained positive for CTC. The chemotherapy did not affect the presence of tumour cells in the circulation. After radiation CTC disappeared but immediately re-occurred with the beginning of a trastuzumab treatment. Finally, a computer tomography (CT) examination revealed small lesions in the lung although the patient had no clinical symptoms at that point in time.

Expression profiling of CTC of colorectal cancer patients. The expression of the tumour-associated genes CEA, EGFR and GA733-2 was assessed and compared in patients with primary or metastatic disease. In Figure 3, characteristic data of 5 patients of each group are shown. These data derive from a study including 76 patients.

All patients with primary disease showed expression of EGFR on CTC whereas only patient 4 was positive for CEA and GA733-2. In contrast, all patients with metastatic disease expressed CEA but only patient 6 was positive for EGFR on CTC. The expression pattern of patients with non-metastatic primary disease compared to patients with metastatic disease changes from an expression of EGFR to CEA.

Discussion

The disappearance of CTC indicates therapeutic efficacy in metastasised patients. Exemplary this was shown in Figure 1. CTC that remained detectable during the entire therapy point

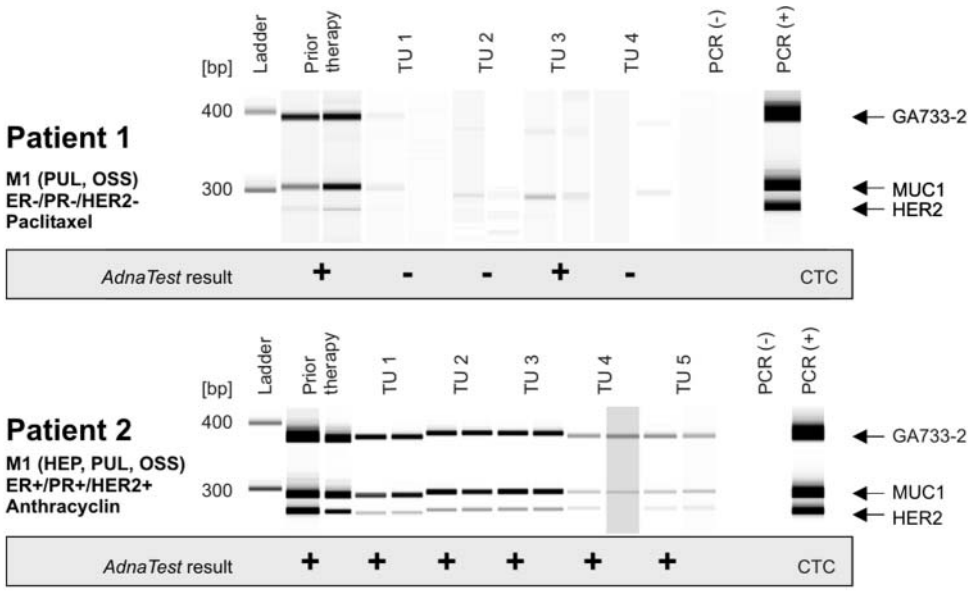

Figure 1. *Monitoring of palliative therapy of breast cancer patients. In each therapy unit (TU) blood samples were analysed in duplicates. Data of the internal PCR control are not shown. PCR (–): negative control; PCR (+): positive control.*

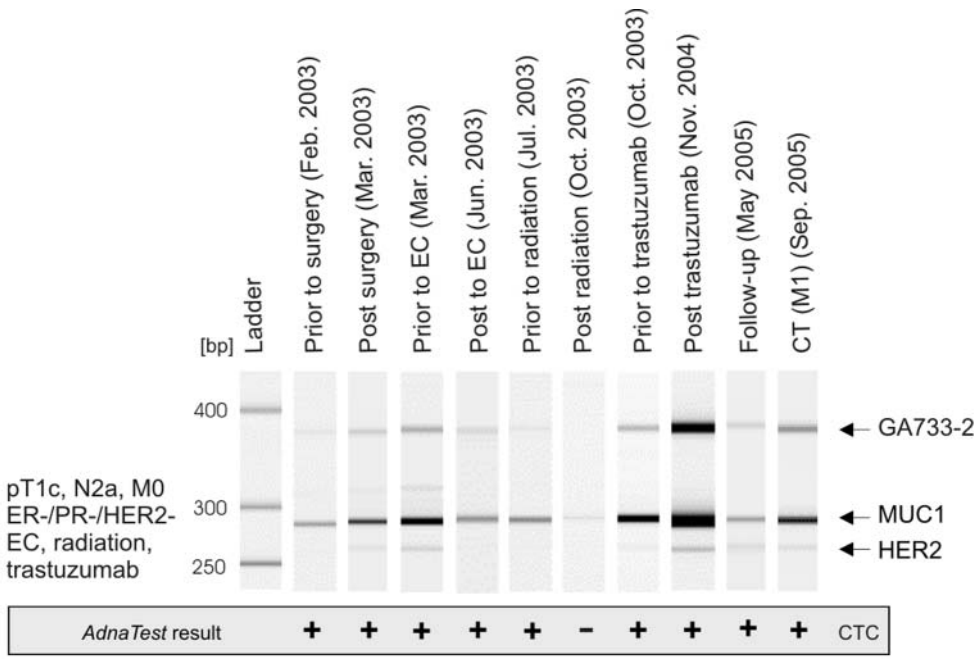

Figure 2. *Monitoring of adjuvant therapy of a breast cancer patient. Data of the internal PCR control, negative and positive controls are not shown. T: tumor size; N: lymph nodes; M: metastases.*

to limited therapy efficacy. During chemotherapy or antibiotic therapy, respectively, the continuous appearance of CTC in blood may only occur if there is a proliferation process. Inhibition of tumour growth is reflected by the disappearance of CTC, resulting in stable disease or remission.

However, not only therapy monitoring in metastasized patients but also an assessment of the adjuvant situation is an urgent need in cancer management. Using the AdnaTest BreastCancer it could be shown that the detection of CTC might provide the key to solving this problem. In the case

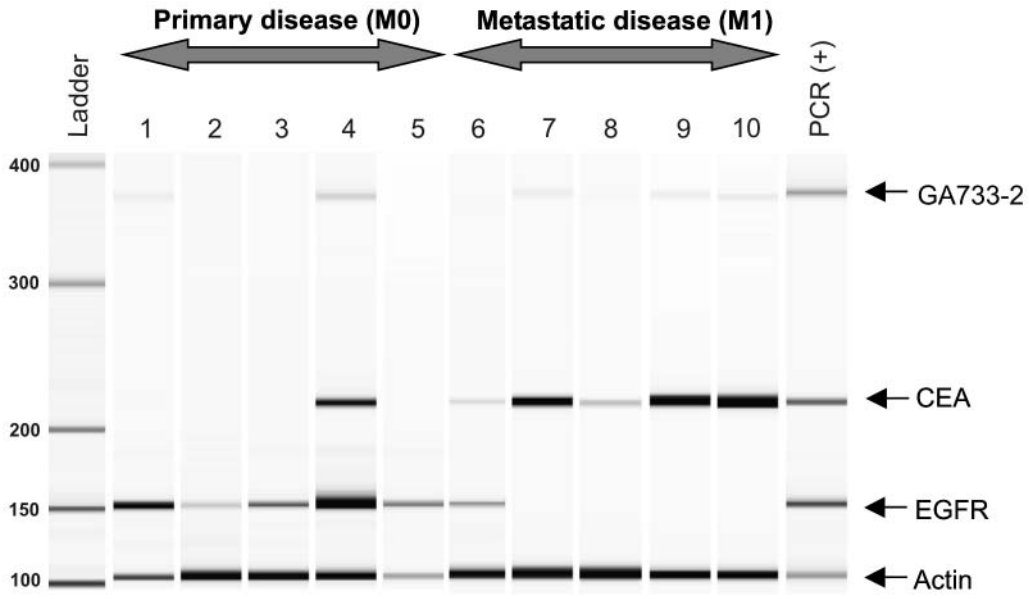

Figure 3. *Expression profile of CTC of colorectal cancer patients. The tumor-associated transcipts CEA, EGFR and GA733-2 were analysed. Patients 1-5: primary disease; patients 6-10: metastatic disease; PCR (+): positive control; actin: internal PCR control.*

described in Figure 2 tumor cells remained detectable in the blood independent of the treatment during the whole course of the adjuvant therapy. This might indicate a progressive behaviour of a remaining tumor load, that is not affected by the therapy regimens used. Even if radiotherapy caused a short disappearance of CTC, their immediate reappearance can lead to the assumption, that the tumor progression was not affected.

Investigating colon cancer patients with primary *versus* metastatic disease it could be furthermore shown that the detection of CTC enabled the assessment of specific changes in expression profiles. EGFR as a potential therapeutic target was predominantly expressed in the primary tumor rather than in metastasis. Such findings might be useful for targeting anti-EGFR therapy more precisely.

In summary, the detection and analysis of CTC prior to surgery and in the course of adjuvant and palliative treatment is a useful tool for therapy monitoring. Analysing the expression profiles of CTC might furthermore be useful for therapy guidance.

Conclusion

After some controversial findings in the beginning of the research on CTC it was clearly shown in recent years that the CTC detection and analysis is a valid new parameter in the monitoring of disease progression and optimisation of therapy. In spite of this, recent publications should be interpreted with caution, since lack of sensitivity using immunocytochemistry or specificity using RT-PCR without cell enrichment cannot be ruled out (13). Therefore the transfer of statistical data to individual patient situations is complicated. By combining two independent technologies (immunomagnetic enrichment and RT-PCR), possible sources of false results have been successfully excluded, enabling the valid diagnostic use of CTC detection (10).

The presence of CTC in breast and colorectal cancer patients showed metastatic potential and, moreover, a therapy failure, which is in accordance with the findings of several other investigators (14-16). Furthermore, the molecular profiles of CTC can be helpful for the identification of drug targets present during the adjuvant and palliative situation. Changes in the expression of HER2 or EGFR in the course of primary disease *versus* metastatic disease may have a crucial influence on the efficacy of drugs, as has been previously described for HER2 in breast cancer and EGFR in colorectal cancer (17, 18). Therefore, these target molecules should be analysed before and during treatment.

References

1 Solomayer EF, Becker S, Pergola-Becker G, Bachmann R, Kramer B, Vogel U, Neubauer H, Wallwiener D, Huober J and Fehm TN: Comparison of HER2 status between primary tumor and disseminated tumor cells in primary breast cancer patients. Breast Cancer Res Treat *98*: 179-184, 2006.

2 Braun S, Kentenich C, Janni W, Hepp F, de Waal J, Willgeroth F, Sommer H and Pantel K: Lack of effect of adjuvant chemotherapy on the elimination of single dormant tumor cells in bone marrow of high-risk breast cancer patients. J Clin Oncol *18*: 80-86, 2000.

3 Smerage JB and Hayes DF: The measurement and therapeutic implications of circulating tumour cells in breast cancer. Br J Cancer *94*: 8-12, 2006.
4 Patel H, Le Marer N, Wharton RQ, Khan ZA, Araia R, Glover C, Henry MM and Allen-Mersh TG: Clearance of circulating tumor cells after excision of primary colorectal cancer. Ann Surg *235*: 226-231, 2002.
5 Stathopoulou A, Vlachonikolis I, Mavroudis D, Perraki M, Kouroussis C, Apostolaki S, Malamos N, Kakolyris S, Kotsakis A, Xenidis N, Reppa D and Georgoulias V: Molecular detection of cytokeratin-19-positive cells in the peripheral blood of patients with operable breast cancer: evaluation of their prognostic significance. J Clin Oncol *20*: 3404-3412, 2002.
6 Xenidis N, Vlachonikolis I, Mavroudis D, Perraki M, Stathopoulou A, Malamos N, Kouroussis C, Kakolyris S, Apostolaki S, Vardakis N, Lianidou E and Georgoulias V: Peripheral blood circulating cytokeratin-19 mRNA-positive cells after the completion of adjuvant chemotherapy in patients with operable breast cancer. Ann Oncol *14*: 849-855, 2003.
7 Giatromanolaki A, Koukourakis MI, Kakolyris S, Mavroudis D, Kouroussis C, Mavroudi C, Perraki M, Sivridis E and Georgoulias V: Assessment of highly angiogenic and disseminated in the peripheral blood disease in breast cancer patients predicts for resistance to adjuvant chemotherapy and early relapse. Int J Cancer *108*: 620-627, 2004.
8 Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, Reuben JM, Doyle GV, Allard WJ, Terstappen LW and Hayes DF: Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med *351*: 781-791, 2004.
9 Chelly J, Concordet JP, Kaplan JC and Kahn A: Illegitimate transcription: transcription of any gene in any cell type. Proc Natl Acad Sci USA *86*: 2617-2621, 1989.
10 Zieglschmid V, Hollmann C, Gutierrez B, Albert W, Strothoff D, Gross E and Böcher O: Combination of immunomagnetic enrichment with multiplex RT-PCR analysis for the detection of disseminated tumor cells. Anticancer Res *25*: 1803-1810, 2005.
11 Demel U, Tilz GP, Foeldes-Papp Z, Gutierrez B, Albert WH and Böcher O: Detection of tumour cells in the peripheral blood of patients with breast cancer. Development of a new sensitive and specific immunomolecular assay. J Exp Clin Cancer Res *23*: 465-468, 2004.
12 Lankiewicz S, Gutierrez Rivero B and Böcher O: Quantitative real-time RT-PCR of disseminated tumor cells in combination with immunomagnetic cell enrichment. Mol Biotechnol *34*: 15-28, 2006.
13 Perey L, Benhattar J, Peters R, Jaunin P and Leyvraz S: High tumour contamination of leukaphereses in patients with small cell carcinoma of the lung: a comparison of immunocytochemistry and RT-PCR. Br J Cancer *85*: 1713-1721, 2001.
14 Meng S, Tripathy D, Shete S, Ashfaq R, Haley B, Perkins S, Beitsch P, Khan A, Euhus D, Osborne C, Frenkel E, Hoover S, Leitch M, Clifford E, Vitetta E, Morrison L, Herlyn D, Terstappen LW, Fleming T, Fehm T, Tucker T, Lane N, Wang J and Uhr J: HER-2 gene amplification can be acquired as breast cancer progresses. Proc Natl Acad Sci USA *101*: 9393-9398, 2004.
15 O'Hara SM, Moreno JG, Zweitzig DR, Gross S, Gomella LG and Terstappen LW: Multigene reverse transcription-PCR profiling of circulating tumor cells in hormone-refractory prostate cancer. Clin Chem *50*: 826-835, 2004.
16 Hayes DF, Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Miller MC, Matera J, Allard WJ, Doyle GV and Terstappen LW: Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. Clin Cancer Res *12*: 4218-4224, 2006.
17 Hayes DF, Walker TM, Singh B, Vitetta ES, Uhr JW, Gross S, Rao C, Doyle GV and Terstappen LW: Monitoring expression of HER-2 on circulating epithelial cells in patients with advanced breast cancer. Int J Oncol *21*: 1111-1117, 2002.
18 Italiano A, Saint-Paul MC, Caroli-Bosc FX, Francois E, Bourgeon A, Benchimol D, Gugenheim J and Michiels JF: Epidermal growth factor receptor (EGFR) status in primary colorectal tumors correlates with EGFR expression in related metastatic sites: biological and clinical implications. Ann Oncol *16*: 1503-1507, 2005.

Received December 19, 2006
Revised March 20, 2007
Accepted March 21, 2007