



**HAL**  
open science

## Evaluation of a RT-PCR based routine screening tool for the detection of disseminated epithelial cells in the bone marrow of breast cancer patients

Sven Becker, Graziella Becker-Pergola, Malgorzata Banys, Natalia Krawczyk, Diethelm Wallwiener, Erich Solomayer, Christina Schuetz, Tanja Fehm

### ► To cite this version:

Sven Becker, Graziella Becker-Pergola, Malgorzata Banys, Natalia Krawczyk, Diethelm Wallwiener, et al.. Evaluation of a RT-PCR based routine screening tool for the detection of disseminated epithelial cells in the bone marrow of breast cancer patients. *Breast Cancer Research and Treatment*, 2008, 117 (2), pp.227-233. 10.1007/s10549-008-0174-3 . hal-00478263

**HAL Id: hal-00478263**

**<https://hal.science/hal-00478263>**

Submitted on 30 Apr 2010

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# Evaluation of a RT-PCR based routine screening tool for the detection of disseminated epithelial cells in the bone marrow of breast cancer patients

Sven Becker · Graziella Becker-Pergola · Malgorzata Banys ·  
Natalia Krawczyk · Diethelm Wallwiener · Erich Solomayer ·  
Christina Schuetz · Tanja Fehm

Received: 28 May 2008 / Accepted: 25 August 2008 / Published online: 14 September 2008  
© Springer Science+Business Media, LLC. 2008

**Abstract** *Purpose* Numerous studies have shown that the presence of clinically occult disseminated tumor cells (DTC's) in the bone marrow (BM) of breast cancer patients is associated with an unfavourable clinical outcome. Immunocytochemistry (ICC) remains the gold standard for their detection. While assays based on RT-PCR are available, they have not been used for routine detection of DTC's. *Methods* To assess the quality of the assay, we performed a direct comparison of DTC detection rates in a large cohort of 385 patients using both standardized ICC and real-time RT-PCR protocols. Correlation rates were assessed, and results were compared with clinical data. *Results* A significant correlation between ICC and RT-PCR was observed ( $P < 0.01$ ). Positivity rates were similar (both 35%) and the results of both methods agreed in 73% of cases (280/385). *Conclusions* We describe a real-time RT-PCR based protocol for DTC-detection that has been specifically designed for routine clinical laboratory use. As such, RT-PCR has the potential to become an alternative testing method for BM evaluation in breast cancer patients.

**Keywords** Disseminated tumor cells · Immunocytochemistry · Minimal residual disease · Bone marrow · Breast cancer · RT-PCR

## Introduction

Treatment decisions for breast cancer patients are mostly based on TNM-status, tumor grade, hormone receptor status and HER2-status [1]. Despite complete surgical resection and optimal systemic therapy a subset of patients still suffers from relapse. Patients who are at high-risk for relapse can be identified by the presence of clinically occult disseminated tumour cells (DTCs). Numerous studies, including a large pooled analysis, provide level I evidence that the presence of such cells in the bone marrow (BM) is associated with an unfavourable clinical outcome [2–5]. Currently, immunocytochemistry using pancytokeratin antibodies is the gold standard for the detection of DTCs [6]. A consensus has been published for the standardised detection of DTC's as well as the clinical implications of their detection [7]. Recently, RT-PCR based methods have been established as an alternative to ICC. Various markers were evaluated including mammaglobin and cytokeratin 19 (CK19) [8–13]. CK19 is an intermediate filament cytokeratin and stably expressed in epithelial tissues but not in mesenchymal haemopoietic cells. We have developed an assay for the detection of DTCs targeting the CK19 transcript with a real-time RT-PCR using the Light Cycler system. Our focus was the possibility of routine clinical application. 385 patients were screened over a 12 month period using both methods. Endpoint of our study was to compare ICC and RT-PCR-positivity rates.

## Materials and methods

This study was approved by the University of Tübingen Ethics Committee in compliance with the Declaration of Helsinki ethical principles for medical research involving

S. Becker (✉) · G. Becker-Pergola · M. Banys ·  
N. Krawczyk · D. Wallwiener · E. Solomayer ·  
C. Schuetz · T. Fehm

Department of Gynaecology and Obstetrics, University Hospital  
Tuebingen, Calwerstrasse 7, 72076 Tuebingen, Germany  
e-mail: sven.becker@med.uni-tuebingen.de

human subjects. After written informed consent, BM samples were obtained from 385 breast cancer patients who were treated at the Tuebingen University Breast Center (Tuebingen University Hospital, Tuebingen, Germany) over a 12 month period. 15–20 ml of BM were aspirated from the anterior iliac crest into syringes containing heparin under local or general anesthesia using the Jamshidi technique [14]. Time of aspiration was noted on the test tube and samples arrived in our in-house laboratory within 30 min. Samples were divided into two parts: (a) 7–10 ml were transferred into a new tube and immediately processed for RT-PCR detection and (b) 7–10 ml were set aside for subsequent ICC processing. RT-PCR-Samples were processed immediately and mixed with 50 ml RNA/DNA Stabilization Reagent for Blood and BM (Roche Diagnostics), causing cell lysis and stabilization of nucleic acids (guanidinium thiocyanate effect). Samples were then immediately frozen at minus 20°C until further processing. Subsequent analysis was performed in batches of 5–10 samples.

#### RT-PCR assay

MRNA was extracted using the mRNA Isolation Kit for Blood and BM (Roche Diagnostics). The system uses a magnetic glass particle technology in a two step procedure and yields purified mRNA. MRNA isolation was performed according to the manufacturer's instructions and eluted in 10 µl DEPC treated H<sub>2</sub>O. Light Cycler One-Step RT-PCR for CK19 using the RNAMaster Hybridization Probes Kit (Roche Diagnostics) was performed and yielded a product of 309 bp. 5 µl of mRNA were subjected to RT-PCR with a Mn[OAc]<sub>2</sub> concentration of 3 mM, 0.5 µM of each primer (primer A: AGATCCTGAGTGACATGCGAA; primer B: CCCAGCTGGGCTTCAATAC) and 0.125 µM of each probe (probe A: CCCTTCAGGGGTCTTGAGATGAGCx, probe B: LCRedGCAGTCACAGCTGAGCATGAAAGCp) were used. Design and synthesis of primers and probes are specific for the CK19 gene (Tibmolbio) and avoid amplification of the CK19 pseudogene [15]. Reverse Transcription

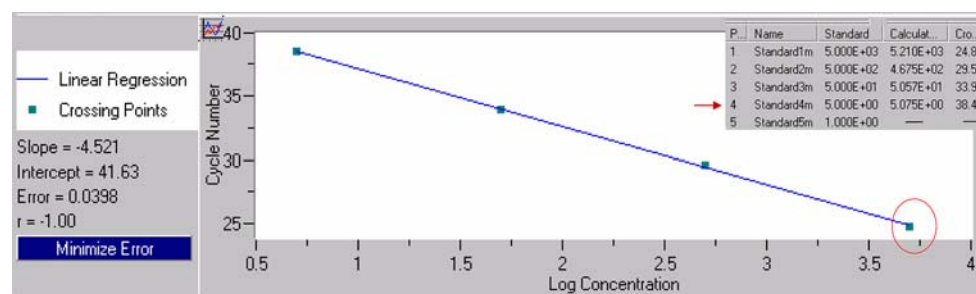
was performed at 60°C for 20 min immediately preceding the PCR (Hotstart at 95°C for 30 s and 50 cycles: 95°C for 1 s, 55°C for 15 s, 72°C for 13 s). PCR products were then analysed using the Fit Points Method of the Light Cycler software. Specificity was ensured by a negative control of 5 ml blood from a healthy volunteer that was included in the experiment. In addition one H<sub>2</sub>O sample was run in parallel to check for possible contamination of RT-PCR reagents and equipment. One positive control, consisting of preparations of MCF-7 cells spiked with 5 ml blood from a healthy volunteer was included with each batch. Data analysis was performed with the LC software version 3.5 Fit Points method and results were compared to a previously established standard curve of positive control samples with a detection limit of 5 MCF-7 cells mixed with 5 ml blood containing  $3 \times 10^6$  PBMCs. The housekeeping gene GAPDH was amplified with specific primers (Tibmolbio) to check for RNA integrity.

#### Preliminary testing

RNA stability assays were performed before patient samples were analysed using direct RNA stabilisation after various time intervals. These tests showed highly time sensitive RNA degradation starting immediately after BM sampling [16]. Preceding the analysis of clinical specimens, a dilution series of tissue cultured breast cancer cells (MCF-7) cells helped to define the sensitivity of the assay of 5 tumor cell in  $3 \times 10^6$  PBMCs and is shown in Fig. 1. One positive control consisting of RNA from 5 isolated tumor cells mixed with  $3 \times 10^6$  PBMCs from a healthy individual was included with each patient batch in order to verify specific amplification of the CK19 gene and ensure adequate sensitivity.

#### Detection of CK positive cells by ICC

Tumor cell isolation and ICC detection was performed according to recommendations for the standardized detection of DTC's by the Collaborative BM Micrometastases



**Fig. 1** Determination of sensitivity of the RT-PCR assay by Fit Points method of the Cycler Software package. A 10-fold dilution series of MCF-7 breast cancer cells ( $5 \times 10^3$ ,  $5 \times 10^2$ ,  $5 \times 10^1$ ,  $5 \times 10^0$ ; squares) mixed with  $3 \times 10^6$  PBMCs determined the

sensitivity of the assay by positive amplification of the  $5 \times 10^0$  sample (circle & arrow). The y-axis indicates the exponential phase (cycle number) of the PCR. Slope 4.521 indicates optimal amplification of the 10-fold dilution series

Group [7]. BM was diluted with Phosphate Buffered Saline (PBS) and mononucleated cells were obtained by density gradient centrifugation over Biocoll (density 1077 g/ml, Biochrom). Cells were washed with PBS and—if necessary—red blood cells were lysed with Lysis buffer. Cytospins were prepared with  $10^6$  cells using a cytocentrifuge (Hettich). Fixation of cells occurred in a 0.5% neutral buffered formalin solution for 10 min. Automated staining was performed on the Autostainer (DAKO Cytomation) using the A45B-B3 monoclonal antibody for pancytokeratin (Mikromet) at a dilution of 1:400 and AP-AAP detection (Dako Cytomation). The A45B-B3 antibody is directed against common cytokeratin epitopes including the CK heterodimers 8/18 and 8/19. A positive control consisting of preparations of MCF-7 cells mixed with PBMC's was included with each batch of samples, as well as a negative control, consisting of PBMC's from healthy volunteers. In order to check for unspecific staining, isotype matched myeloma protein was included as a staining control (Sigma). For each patient  $2 \times 10^6$  cells were analyzed on two slides. Analysis was performed on the Automated Cell Imaging System ACIS<sup>®</sup> (Chromavision). Criteria for DTC-detection were based on recommendations of the European ISHAGE Working group for standardization of tumor cell detection [6].

## Statistics

Chi-squared test was used to examine the relationship between categorical variables. Statistical analysis was performed using SPSS (version 11.5). *P*-values less than 0.05 were considered statistically significant.

## Results

### Patient characteristics

BM samples from 385 breast cancer patients were analyzed. 305 samples were obtained at the time of surgery for

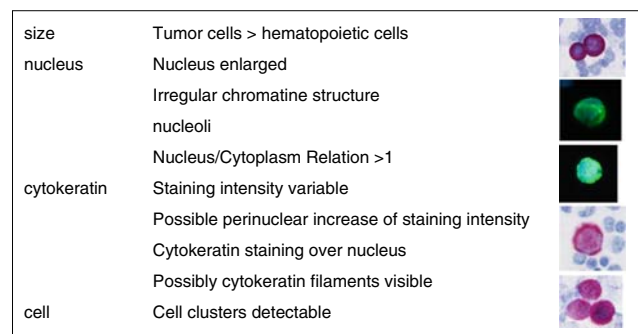
primary breast cancer. Sixty-three patients had a history of breast cancer and underwent secondary BM aspiration during follow-up at our outpatient clinic. The remaining seventeen patients were diagnosed with recurrent breast cancer. Clinical data of patients are shown in Table 1.

### BM analysis by ICC

135 of 385 patients (35%) had DTC's in their BM. The number of cells ranged from 1 to 8 cells per  $10^6$  MNC's. Figure 2 demonstrates examples of CK positive cells in the BM of a primary breast cancer patient. The highest positivity rates were seen in primary breast cancer patients who underwent primary systemic chemotherapy. 15 of 63 (24%) follow up samples of breast cancer patients with no evidence of disease were DTC positive.

### BM analysis by RT-PCR

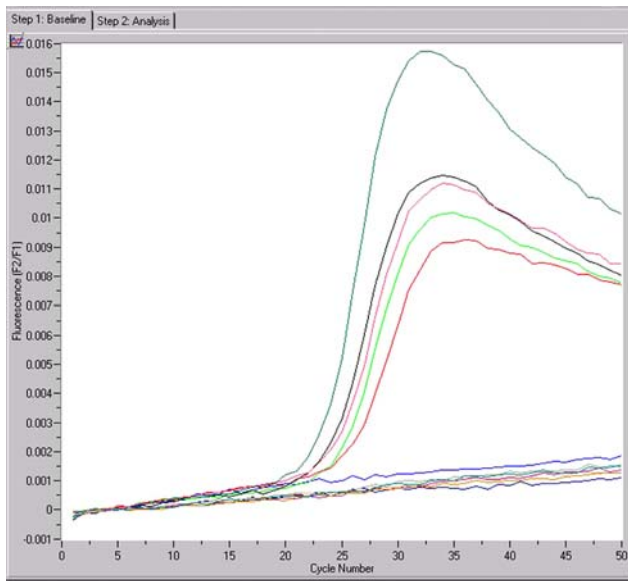
The CK19 detection rate by RT-PCR was 35%. Figure 3 demonstrates a typical result of CK19 positive samples obtained with the Fit Points Method of the Light Cycler version 3.5 (Roche Diagnostics). In primary breast cancer, 117 of 305 patients (38%) were DTC positive. After



**Fig. 2** Criteria for evaluation of positive DTC's in bone marrow and examples from primary breast cancer patients

**Table 1** Patient characteristics

Patients enrolled	<i>n</i> = 385	ICC positive	RT-PCR positive
Primary breast cancer	305	111 (36%)	117 (38%)
Prior surgery/no neoadjuvant chemotherapy	277	95 (34%)	103 (37%)
Stage I	164	54 (33%)	64 (39%)
Stage II	73	25 (34%)	24 (33%)
Stage III	30	13 (43%)	12 (40%)
Stage IV	10	3 (30%)	3 (30%)
After primary systemic therapy	28	16 (57%)	14 (50%)
Recurrences	17	9 (53%)	5 (29%)
Secondary bone marrow aspiration	63	15 (24%)	12 (19%)
Total	385	135 (35%)	134 (35%)



**Fig. 3** Amplification curves obtained by the Light Cycler version 3.5 software package. The x-axis shows the cycle number of the PCR where amplification started. The y-axis shows fluorescence intensity. Five of 11 samples amplified the CK19 gene

completion of surgery and chemotherapy 12 of 63 follow-up patients (19%) had DTCs detected by RT-PCR.

#### Correlation between ICC and RT-PCR

All 385 BM samples were analyzed by ICC and RT-PCR. The positivity rates of both methods were 35%. 49% of the BM samples (187 of 385) were positive by at least one method. 14% of the samples were positive only by RT-PCR and another 14% only by ICC. Both methods showed a statically significant correlation ( $P < 0.01$ ).

In 73% of the patients (280 of 385 cases) identical results were obtained. 198 (51%) patients were BM negative by both methods. Positive results by both methods were obtained in 82 (21%) patients (Table 2a). In order to rule out that discordant results obtained by the two methods was the result of inferior sensitivity of the RT-PCR, we

compared the number of CK+ cells detected by ICC with PCR positivity. Table 2b demonstrates that the number of detected tumor cells on ICC did not correlate with RT-PCR results ( $P = 0.365$ ). Thus, RT-PCR correctly evaluates the BM-status for presence or absence of CK-positive cells, irrespective of their number, which would correspond to the clinical significance of the “positive” or “negative” BM-status as established by ICC.

#### Discussion

Over the past 15 years, the standard method of detection of disseminated tumor cells (DTCs) in the BM of breast cancer patients has been immunocytochemistry (ICC). This method has shown that DTCs are correlated independently with early disease relapse and decreased survival [17, 18]. DTC's have the potential to become targets for new therapeutic modalities, which would make routine evaluation of the BM-status an important aspect of breast cancer care. Recent efforts to standardize the ICC-approach underline its growing clinical relevance and the need for an accurate and generally accepted assessment of BM-status [7]. While ICC has emerged as the method of choice for DTC-detection, it has not been without criticism. Differences of detection rates between research groups using ICC have been attributed to the use of different cell preparation techniques, different antibodies as well as different cytomorphologic diagnostic criteria. The most consistent results have been obtained with the A45B-B3 antibody. Recent consensus meetings have recommended using this antibody and introduced standardized technical and diagnostic criteria [7]. Another problem has been the subjective aspect of cytologic evaluation. Furthermore the implementation of BM-ICC into routine clinical practice is labor intensive and costly. Efforts continue to provide an alternative, both from a methodological as well as from a logistic point of view. The focus of interest has been RT-PCR. Different research groups have established RT-PCR-protocols for the detection of DTCs [19–25].

**Table 2** Positivity rates obtained by ICC and RT-PCR

	RT-PCR negative	RT-PCR positive	Total	
<i>(a)</i>				
ICC Negative	198 (51%)	55 (14%)	253 (65%)	
ICC Positive	53 (14%)	82 (21%)	135 (35%)	
Total	251 (65%)	134 (35%)	385 (100%)	
<i>(b)</i>				
1 CK+ Cell ICC	24 (46%)	28 (54%)	52 (100%)	
2 CK+ Cells ICC	12 (32%)	26 (68%)	38 (100%)	
≥3 CK+ Cells ICC	17 (38%)	28 (62%)	45 (100%)	
Total positive ICC	53 (39%)	82 (61%)	135 (100%)	$P = 0.365$

**Table 3** Comparison of RT-PCR and ICC

	Smith et al. [26]	Benoy et al. [8]	Schroder et al. [27]	Schoenfeld et al. [28]	Becker et al. [16]
Total <i>n</i> both tests performed	133	25	31	65	385
Blood/bone marrow	Blood	Bone marrow	Blood	Bone marrow	Bone marrow
M status patients	M+	M+	M0	M0	M0
Positivity rate RT-PCR CK19	50%	80%	50%	35%	35%
Positivity rate ICC pCK	42%	63% <sup>a</sup>	0% <sup>b</sup>	22%	35%
Same result both methods	71%	75%	67%	74%	73%
Correlation ICC/PCR	kappa 0.43	$P < 0.0001$	n.a.	n.a.	$P < 0.001$
Sensitivity RT-PCR assay	1 in $10^7$ cells	5 in $10^7$ cells	1 in $10^6$ cells	1 in $10^6$ cells	5 in $3 \times 10^6$ cells
Sensitivity ICC assay	n.a.	1 in $10^6$ cells	1 in $2 \times 10^6$ cells	>1 in $10^5$ cells	1 in $10^6$ cells

<sup>a</sup> Total ( $n = 24$ ) samples analysed

<sup>b</sup> Total ( $n = 149$ ) samples analysed

Only few have compared their results to ICC as shown in Table 3 [8, 26–28]. RT-PCR based assays have the advantage of being highly sensitive and reproducible. They offer an objective, observer-independent way of assessing the presence of cytokeratin-bearing cells in the BM. However, due to key methodological difficulties such as the inherent instability of the CK-related RNA, its implementation remains a challenge. Our group has established a one-step real-time RT-PCR based protocol using the Light Cycler system. Our focus was the establishment of a protocol suited for clinical routine and comparable to ICC with regard to sensitivity, specificity and reproducibility. Based on our findings and those of other research groups regarding RNA-degradation we did not use any pre-enrichment strategy [16, 29, 30]. RNA degradation starts immediately after BM is obtained and pre-enrichment uses up additional time. Because RNA-levels of CK19-positive cells within a background of hematopoietic cells are at the threshold of detection, even small levels of degradation can lead to false negative results. Without pre-enrichment,

rapid RNA stabilization (<30 min after the sample was obtained) was essential. After the addition of the RNA-stabilizer, samples were stored at minus 20°C until further analysis, allowing sample batching, which is important for routine clinical use. The intermediate filament CK19 has emerged as the PCR-marker of choice for microdisseminated breast cancer cells [8, 31–35]. CK19 is stably expressed in epithelial tissues and tumors but not usually in mesenchymal and/or haematopoietic cells [11, 12]. Alternative markers tested by different groups have been Muc-1, urokinase type plasminogen activator receptor (uPAR), EpCAM and mammaglobin each originally considered breast cancer specific [36–38]. Some of these markers, however, have been detected in peripheral blood of a control group of healthy patients [19, 22] and data regarding their clinical significance remains scarce. Based on the available evidence regarding the significance of CK-positive cells in the BM, it is clear that RT-PCR would have to look at established CK-markers, too. Table 4 compares the test-specific characteristics of RT-PCR and

**Table 4** Comparison of ICC and RT-PCR protocols considering aspects for routine application of protocols

	One-step RT-PCR	ICC
Marker/Antigen	CK19	CK8, CK18 and CK19
Sensitivity	5 MCF-7 cells/ $3 \times 10^6$ PBMCs	1 MCF-7 cell/ $10^6$ PBMC's
Enrichment strategy	None <sup>a</sup>	Density gradient
Specificity <sup>b</sup>	Primers CK19	Monoclonal PanCK antibody
Time needed for assay	200 min	300 min
Time for entire procedure	1 day	2 days <sup>c</sup>
Cost (material only)	€ 35,-/assay	€ 13,-/assay
Subjective element	No	Yes
Stability of target	RNA, instable molecule	Protein, stable
Morphological evaluation	No	Yes

<sup>a</sup> Advantage: (a) RNA stabilization feasible within minutes, (b) saves time

<sup>b</sup> Evaluation of—negative controls (blood) were obtained from healthy blood donors—positive controls (cells from breast cancer cell line spiked with PBMCs of healthy donors)—Isotype control (MOPC21 antibody to check for unspecific staining)

<sup>c</sup> Cytospins dry overnight

ICC. Having established an easy to use CK19-RT-PCR assay, we prospectively compared the results of our RT-PCR assay with routine ICC assessment of the BM of 385 breast cancer patients. The results show a significant correlation between the RT-PCR based method and ICC, confirming previous results seen in a smaller cohort of patients [8]. Both methods rely on the detection of epithelial specific markers as indicators of epithelial derived cancer cells usually not present in the BM. While the ICC technique specifically looks for the presence of multiple proteins (CK8, 18 and 19), RT-PCR detects elevated gene expression of only CK19. CK19 is the most frequently used marker for RT-PCR detection of DTC in breast cancer patients, both in the BM and in sentinel-lymphnodes. While CK18/8 are part of the A45B-B3 antibody they have only rarely been used for DTC detection individually or as part of RT-PCR assays. The expression patterns of these epithelial cytokines are very similar to that of CK19 and they are not expected to provide more specificity than detection of the single marker CK19. In fact, the A45B-B3 antibody was initially used for the detection of non-breast cancer epithelial tumor cells. CK 18, for example, has been widely used as a marker for DTC-detection in colon cancer, but has been found to be less relevant in breast cancer.

Significant problems remain. It has been shown that haematopoietic cells potentially (though rarely) express CK19 at very low levels [39, 40]. If sensitivity is too high, “illegitimately” expressed mRNA transcripts would be detected and specificity suffers. One way to solve this problem is to use quantitative RT-PCR, which requires a defined cut-off, above which expression of a gene is considered tumor cell specific. However, due to the heterogeneity of tumor cells, they too express CK19 at very different levels, so it has been difficult to define an exact cut off value that does not arbitrarily exclude tumor cells, reducing the value of the test overall.

So far, only one study has looked at the follow-up of patients with RT-PCR positive BM: the presence of CK19-mRNA in BM was correlated with a significantly reduced overall survival [41]. Our data provides good evidence that RT-PCR might play an important part in the detection of DTC's. The test correlates well with the established standard and could serve to accumulate clinical data which then needs to be compared to clinical follow up as well. Survival data of patients recruited for this study will be available within the next 3–5 years. In summary, looking at the largest patient group to date, we report on a pragmatic and easy to use RT-PCR based assay for DTC-detection in the BM of breast cancer patients. Our results show good correlation with the existing standardized ICC. If the BM-status of breast cancer patients becomes more important for their management, new test-options need to be explored. The RT-PCR approach is fast, can be performed in batches

and requires no cytologist-evaluation. Being largely automated it can easily be implemented in established laboratory routine.

**Acknowledgements** This work was supported by the University of Tübingen AKF Foundation (Angewandte Klinische Forschung).

## References

1. Goldhirsch A, Glick JH, Gelber RD, Coates AS, Thürlimann B, Senn HJ et al (2005) International expert consensus on the primary therapy of early breast cancer. *Ann Oncol* 6:1569–1583. doi:[10.1093/annonc/mdi326](https://doi.org/10.1093/annonc/mdi326)
2. Braun S, Vogl FD, Naume B, Janni W, Osborne MP, Coombes RC et al (2005) A pooled analysis of bone marrow micrometastasis in breast cancer. *N Engl J Med* 353:793–802. doi:[10.1056/NEJMoa050434](https://doi.org/10.1056/NEJMoa050434)
3. Braun S, Pantel K, Muller P, Janni W, Hepp F, Kantenich CR et al (2000) Cytokeratin-positive cells in the bone marrow and survival of patients with stage I, II, or III breast cancer. *N Engl J Med* 342:525–533. doi:[10.1056/NEJM200002243420801](https://doi.org/10.1056/NEJM200002243420801)
4. Diel IJ, Kaufmann M, Costa SD, Holle R, von Minckwitz G, Solomayer EF et al (1996) Micrometastatic breast cancer cells in bone marrow at primary surgery: prognostic value in comparison with nodal status. *J Natl Cancer Inst* 88:1652–1658. doi:[10.1093/jnci/88.22.1652](https://doi.org/10.1093/jnci/88.22.1652)
5. Solomayer EF, Diel IJ, Salanti G, Hahn M, Gollan C, Schutz F et al (2001) Time independence of the prognostic impact of tumor cell detection in the bone marrow of primary breast cancer patients. *Clin Cancer Res* 7:4102–4108
6. Borgen E, Naume B, Nesland J, Kvalheim G, Beiske K, Fodstad O et al (1999) Standardization of the immunocytochemical detection of cancer cells in BM and Blood: I. Establishment of objective criteria for the evaluation of immunostained cells. *Cytotherapy* 1:377–388. doi:[10.1080/0032472031000141283](https://doi.org/10.1080/0032472031000141283)
7. Fehm T, Braun S, Muller V, Janni W, Gebauer G, Marth C et al (2006) A concept for the standardized detection of disseminated tumor cells in bone marrow from patients with primary breast cancer and its clinical implementation. *Cancer* 107:885–892. doi:[10.1002/cncr.22076](https://doi.org/10.1002/cncr.22076)
8. Benoy IH, Elst H, Van der Auwera I, Van Laere S, van Dam P, Van Marck E et al (2004) Real-time RT-PCR correlates with immunocytochemistry for the detection of disseminated epithelial cells in bone marrow aspirates of patients with breast cancer. *Br J Cancer* 91:1813–1820. doi:[10.1038/sj.bjc.6602189](https://doi.org/10.1038/sj.bjc.6602189)
9. Datta YH, Adams PT, Drobyski WR, Ethier SP, Terry VH, Roth MS (1994) Sensitive detection of occult breast cancer by the reverse-transcriptase polymerase chain reaction. *J Clin Oncol* 12:475–482
10. Reinholz MM, Nibbe A, Jonart LM, Kitzmann K, Suman VJ, Ingle JN et al (2005) Evaluation of a panel of tumor markers for molecular detection of circulating tumor cells in women with suspected breast cancer. *Clin Cancer Res* 11:3722–3732. doi:[10.1158/1078-0432.CCR-04-1483](https://doi.org/10.1158/1078-0432.CCR-04-1483)
11. Bosma AJ, Weigelt B, Lambrechts AC, Verhagen OJ, Pruntel R, Hart AA et al (2002) Detection of circulating breast tumor cells by differential expression of marker genes. *Clin Cancer Res* 8:1871–1877
12. Gradilone A, Gazzaniga P, Silvestri I, Gandini O, Trasatti L, Lauro S et al (2003) Detection of CK19, CK20 and EGFR mRNAs in peripheral blood of carcinoma patients: correlation with clinical stage of disease. *Oncol Rep* 10:217–222

13. Eaton MC, Hardingham JE, Kotasek D, Dobrovic A (1997) Immunobead RT-PCR: a sensitive method for detection of circulating tumor cells. *Biotechniques* 22:100–105
14. Jamshidi K, Swaim WR (1971) Bone marrow biopsy with unaltered architecture: a new biopsy device. *J Lab Clin Med* 77:335–342
15. Ruud P, Fodstad O, Hovig E (1999) Identification of a novel cytokeratin 19 pseudogene that may interfere with reverse transcriptase polymerase chain reaction assays used to detect micrometastatic tumor cells. *Int J Cancer* 80:119–125. doi:10.1002/(SICI)1097-0215(19990105)80:1<119::AID-IJC22>3.0.CO;2-X
16. Becker S, Becker-Pergola G, Fehm T, Wallwiener D, Solomayer EF (2004) Time is an important factor when processing samples for the detection of disseminated tumor cells in blood/bone marrow by reverse transcription-PCR. *Clin Chem* 50:785–786. doi:10.1373/clinchem.2003.025510
17. Wiedswang G, Borgen E, Karesen R, Kvalheim G, Nesland JM, Qvist H et al (2003) Detection of isolated tumor cells in bone marrow is an independent prognostic factor in breast cancer. *J Clin Oncol* 21:3469–3478. doi:10.1200/JCO.2003.02.009
18. Janni W, Rjosk D, Braun S (2000) Clinical relevance of occult metastatic cells in the bone marrow of patients with different stages of breast cancer. *Clin Breast Cancer* 1:217–225
19. Xenidis N, Perraki M, Kafousi M, Apostolaki S, Bolonaki I, Stathopoulou A et al (2006) Predictive and prognostic value of peripheral blood cytokeratin-19 mRNA-positive cells detected by real-time polymerase chain reaction in node-negative breast cancer patients. *J Clin Oncol* 24:3756–3762. doi:10.1200/JCO.2005.04.5948
20. Gilbey A, Burnett D, Coleman R, Holen I (2004) The detection of circulating breast cancer cells. *J Clin Pathol* 57:903–911. doi:10.1136/jcp.2003.013755
21. Ring A, Smith I, Dowsett M (2004) Circulating tumour cells in breast cancer. *Lancet Oncol* 5:79–88. doi:10.1016/S1470-2045(04)01381-6
22. Silva JM, Dominguez G, Silva J, Garcia JM, Sanchez A, Rodriguez O et al (2001) Detection of epithelial messenger RNA in the plasma of breast cancer patients is associated with poor prognosis tumor characteristics. *Clin Cancer Res* 7:2821–2825
23. Kahn HJ, Yang LY, Blondal J, Lickley L, Holloway C, Hanna W et al (2000) RT-PCR amplification of CK19 mRNA in the blood of breast cancer patients: correlation with established prognostic parameters. *Breast Cancer Res Treat* 60:143–151. doi:10.1023/A:1006350913243
24. Aerts J, Wynendaele W, Paridaens R, Christiaens MR, van den Bogaert W, van Oosterom AT et al (2001) A real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) to detect breast carcinoma cells in peripheral blood. *Ann Oncol* 12:39–46. doi:10.1023/A:1008317512253
25. Berois N, Varangot M, Aizen B, Estrugo R, Zarantonelli L, Fernandez P et al (2000) Molecular detection of cancer cells in bone marrow and peripheral blood of patients with operable breast cancer: comparison of CK19, MUC1 and CEA using RT-PCR. *Eur J Cancer* 36:717–723. doi:10.1016/S0959-8049(99)00338-X
26. Smith BM, Slade MJ, English J, Graham H, Luchtenborg M, Sinnett HD et al (2000) Response of circulating tumor cells to systemic therapy in patients with metastatic breast cancer: comparison of quantitative polymerase chain reaction and immunocytochemical techniques. *J Clin Oncol* 18:1432–1439
27. Schroeder CP, Ruiters MH, de Jong S, Tiebosch AT, Wesseling J, Veenstra R et al (2003) Detection of micrometastatic breast cancer by means of real time quantitative RT-PCR and immunostaining in perioperative blood samples and sentinel nodes. *Int J Cancer* 106:611–618. doi:10.1002/ijc.11295
28. Schoenfeld A, Kruger KH, Gomm J, Sinnett HD, Gazet JC, Sacks N (1997) The detection of micrometastases in the peripheral blood and bone marrow of patients with breast cancer using immunohistochemistry and reverse transcriptase polymerase chain reaction for keratin 19. *Eur J Cancer* 33:854–861. doi:10.1016/S0959-8049(97)00014-2
29. Naume B, Borgen E, Nesland JM, Beiske K, Gilen E, Renolen A et al (1998) Increased sensitivity for detection of micrometastases in bone marrow/peripheral blood stem-cell products from breast cancer patients by negative immunomagnetic separation. *Int J Cancer* 78:556–560. doi:10.1002/(SICI)1097-0215(19981123)78:5<556::AID-IJC5>3.0.CO;2-G
30. Benez A, Geiselhart A, Handgretinger R, Schiebel U, Fierlbeck G (1999) Detection of circulating melanoma cells by immunomagnetic cell sorting. *J Clin Lab Anal* 13:229–233. doi:10.1002/(SICI)1098-2825(1999)13:5<229::AID-JCLA7>3.0.CO;2-Y
31. Stathopoulou A, Gizi A, Perraki M, Apostolaki S, Malamos N, Mavroudis D et al (2003) Real-time quantification of CK-19 mRNA-positive cells in peripheral blood of breast cancer patients using the lightcycler system. *Clin Cancer Res* 9:5145–5151
32. Ring AE, Zabaglo L, Ormerod MG, Smith IE, Dowsett M (2005) Detection of circulating epithelial cells in the blood of patients with breast cancer: comparison of three techniques. *Br J Cancer* 92:906–912. doi:10.1038/sj.bjc.6602418
33. Slade MJ, Smith BM, Sinnett HD, Cross NC, Coombes RC (1999) Quantitative polymerase chain reaction for the detection of micrometastases in patients with breast cancer. *J Clin Oncol* 17:870–879
34. Kruger W, Krzizanowski C, Holweg M, Stockschrader M, Kroger N, Jung R et al (1996) Reverse transcriptase/polymerase chain reaction detection of cytokeratin-19 mRNA in bone marrow and blood of breast cancer patients. *J Cancer Res Clin Oncol* 122:679–686. doi:10.1007/BF01209032
35. Gradilone A, Gazzaniga P, Silvestri I, Gandini O, Trasatti L, Lauro S et al (2003) Detection of CK19, CK20 and EGFR mRNAs in peripheral blood of carcinoma patients: correlation with clinical stage of disease. *Oncol Rep* 10:217–222
36. Pierga JY, Bonneton C, Magdelénat H, Vincent-Salomon A, Nos C, Boudou E et al (2005) Real-time quantitative PCR determination of urokinase-type plasminogen activator receptor (uPAR) expression of isolated micrometastatic cells from bone marrow of breast cancer patients. *Int J Cancer* 114:291–298. doi:10.1002/ijc.20698
37. de Cremoux P, Extra JM, Denis MG, Pierga JY, Bourstyn E, Nos C et al (2000) Detection of MUC1-expressing mammary carcinoma cells in the peripheral blood of breast cancer patients by real-time polymerase chain reaction. *Clin Cancer Res* 6:3117–3122
38. Ferrucci PF, Rabascio C, Gigli F, Corsini C, Giordano G, Bertolini F et al (2007) New comprehensive gene expression panel to study tumor micrometastasis in patients with high-risk breast cancer. *Int J Oncol* 30:955–962
39. Ko Y, Grunewald E, Totzke G, Klinz M, Fronhoffs S, Gouni-Berthold I et al (2000) High percentage of false-positive results of cytokeratin 19 RT-PCR in blood: a model for the analysis of illegitimate gene expression. *Oncology* 59:81–88. doi:10.1159/000012126
40. Bostick PJ, Chatterjee S, Chi DD, Huynh KT, Giuliano AE, Cote R et al (1998) Limitations of specific reverse-transcriptase polymerase chain reaction markers in the detection of metastases in the lymph nodes and blood of breast cancer patients. *J Clin Oncol* 16:2632–2640
41. Benoy IH, Elst H, Philips M, Wuyts H, Van Dam P, Scharpe S et al (2006) Real-time RT-PCR detection of disseminated tumour cells in bone marrow has superior prognostic significance in comparison with circulating tumour cells in patients with breast cancer. *Br J Cancer* 94:672–680