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Detection of circulating tumor cells in metastatic breast cancer patients

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Objective. The objective of this study was the detection of circulating tumor cells (CTCs) in metastatic breast cancer patients.

Methods. Since only small numbers of circulating tumor cells (CTCs) are found in peripheral blood, at first we performed immunomagnetic separation as a concentration method suitable for selecting circulating tumor cells in peripheral blood. This was followed by analysis of isolated cells with the aid of laser scanning cytometry (LSC). Twenty eight patients with metastatic breast cancer were enrolled in the study and the control group consisted of 19 clinically healthy women. Six milliliters of peripheral blood was drawn for the analyses, but only in two patients the blood has been drawn twice. Blood samples were taken when no chemotherapy was administered, but hormonal therapy has been allowed.

Results. The positivity for CTCs was found in 20 (50.0 %) patients with metastatic breast cancer patients, while in 6 (31.6 %) healthy controls false positive circulating epithelial-like cells were detected. Because we did not use CD45 staining, we could not distinguish these circulating epithelial-like cells from CTCs. In a majority of metastatic breast cancer patients we found a mixed population of HER-2 gene expressing CTCs. We found that HER2+ CTCs in high numbers are CK19 + CTCs, while almost all HER2–CTCs are CK19– CTCs.

Conclusion. The described method was found promising for estimating HER2 status on CTCs from peripheral blood in metastatic breast cancer patients.

Key words: metastatic breast cancer, circulating tumor cells, laser scanning cytometry

Disseminated malignancies are responsible for the majority of cancer related deaths (Jacob et al. 2007). Approximately 25 % of breast cancer patients without lymph node metastases develop systemic relapse. Although the growing body of evidence supports the notion that hematogenous dissemination of breast cancer occurs independently of the lymphatic spread of disease, current clinical practice does not yet involve any routine analyses of circulating tumor cells or disseminated cells (Lang et al. 2007). Actually, during the metastatic process circulating tumor cells (CTCs) are generated and their presence is considered a mandatory step in establishing distant metastases (Jacob et al. 2007). So far, there is no clear interrelation between CTCs from peripheral blood and occult tumour cells (OTCs) in bone marrow. However, the evidence OTCs in bone marrow appeared an independent prognostic factor in patients with the carcinoma of breast (Diel et al. 1966; Wiedsvang et al. 2003), lungs (Pantel et al. 1996), colon and rectum (Lindemann et al. 1992).

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Table 1

Clinical data of 28 metastatic breast cancer patients (IDC = invasive ductal carcinoma; ILC = invasive lobular carcinoma; ME = mastectomy; QE = quadrantectomy; EA = exenteration of axilla; ER = estrogen receptors; PR = progesterone receptors; NA = data not available; CHT = chemotherapy; HT = hormonal therapy; RT = radiation therapy; T = trastuzumab; B = bevacizumab)

No	Age	Histol	Grd	Staging (initial)	ER	PR	HER 2	Ki 67	Surgery	Locality of relapse	Pall treatment
1	37	IDC	NA	Tx N3 M1	+	+	3+	+	no OP	Brain, liver, bone	HT
2	45	ILC	3	T3 Nx M0	+	+	1+	+	no OP	Bone	CHT, RT, T
3	50	IDC	3	T4 N2 M1, IV	-	+	1+	+	no OP	Bone	HT,RT
4	67	IDC	2	T4b N1 M1	+	+	1+	+	no OP	Bone, pleura	CHT
5	60	IDC	2	pT2a N0 M0	-	+	NA	NA	QE+EA	Pleura	CHT,HTRT
6	66	IDC	1	pT2a pN1b Mx	-	+	3+	NA	ME+EA	Lungs	CHT
7	41	IDC	2	pTx pN1p M0	+	+	1+	NA	ME+EA	Liver, lungs, bone	CHT, T
8	56	IDC	2	pT1 pN1p M0	+	+	NA	NA	ME+EA	LAP, liver, lungs, bone, pleura	CHT, RT, B
9	64	IDC	2	T2 N3 M1	+	+	3+	+	ME+EA	Liver, bone	CHT,HT RT, B
10	57	IDC	2	T2 N1 M0	+	+	1+	NA	QE+EA	Liver, bone,	CHT,HT RT, T
11	78	IDC	1	T1 N0 M0	+	+	NA	NA	QE+EA	Lungs, bone	CHT, B
12	80	IDC	2	T2 N1 Mx	+	+	3+	+	ME+EA	Locoregional, axila, lungs	CHT,RT
13	65	IDC	NA	pT3 pN2 Mx	+	+	1+	+	ME+EA	Locoreg., LAP, liver, bone, pleura	HT, RT
14	81	IDC	2	pT2a pN0 pM0	+	+	NA	NA	ME+EA	Pleura	HT
15	80	IDC	NA	Tx N0 MO	+	+	NA	NA	QE+EA	Liver, bone	CHT,HT
16	59	IDC	3	pT2 pN1 pM0	+	+	1+	NA	QE+EA	Axila	HT
17	58	ILC	NA	pT1 pN0 pM0	+	+	1+	+	ME+EA	Liver, bone	HT
18	63	IDC	3	T3 N1 M0	+	-	1+	+	ME+EA	Locoreg., LAP, liver, bone	RT
19	59	IDC	NA	pT1 pN1 pM0	+	+	3+	NA	QE+EA	Lungs	CHT,HT
20	74	IDC	2	pT1 pN1 pM1	+	-	NA	NA	ME+EA	Bone	CHT, RT, B
21	64	IDC	2	pT2 pN2 pM0	+	+	2+	+	QE+EA	Locoregional, LAP, pleura	HT, T
22	63	IDC	2	pT1c pN1pM0	-	-	NA	NA	ME+EA	Locor., LAP, lungs, bone, pleura	CHT,HT RT
23	47	IDC	NA	T2 N1 M0	+	+	NA	NA	ME+EA	Locoreg., liver, bone, pleura	CHT,HT RT
24	40	IDC	2	T4 N1 M1	+	+	1+	NA	No OP	Liver, bone, pleura	CHT
25	76	IDC	3	T2 N0 M0	-	-	1+	+	QE+EA	Locoreg., lungs, pleura	CHT,HT RT
26	46	IDC	2	pT2 pN1 pM0	-	+	NA	NA	ME+EA	LAP, bone, pleura	CHT,HT RT, B
		IDC	*3*	*pT1pN1pM0*	+	+	*1+*	*NA*	*ME+EA*	*Lungs, bone*	*CHT*
27	49	*IDC*	*3*	*pT1pNxpM0*	+	+	*1+*	*NA*	*ME+EA*		
		IDC	*3*	*pT4pN3pM1*	-	-	1+	+	ME+EA	*Liver, bone, lenticular, pleura*	*HT,RT*
28	54	IDC	*3*	*pT1pN1pM1*	-	-	1+	+	ME+EA		

Peripheral blood is considered one of the most important diagnostic specimens and appears an ideal source for monitoring CTCs which can be done in frequent intervals (Lacroix 2006). Prognostic value of CTCs detection in peripheral blood has been demonstrated in patients with metastatic disease (Vincent-Solomon 2008). Several recently published studies have documented that both CTCs within the blood and OTCs in bone marrow can be identified using a variety of techniques (Lang et al. 2007).

The methods for identifying CTCs should distinguish between epithelial and other (mainly hematopoetic) cells. To identify CTCs in peripheral blood, two major approaches are used involving additional antibodies and nucleic acid based techniques. As not all detected cells are also viable cells which can cause metastases, CTCs are very inefficient in causing metastases (Lacroix 2006). Several CTCs are apoptotic cells which are detectable but cannot cause the spread of disease.

Molecular studies of CTCs require some efficient pre-enrichment steps to obtain a pure population of target cells for further characterization (Tveito et al. 2007). Among them, the laser scaning cytometry (LSC) appeared the most suitable method for the visualization of CTCs, as applied after the pre-enrichment. LSC has been used for morphological and cytometric analysis of the cells since the beginning of the nineties (Galbavy et al. 2008). The use of LSC for analyzing cells on membrane made it possible to simplify the preparation of CTCs and to use cytometric analysis of isolated cells (Zabaglo et al. 2003).

The HER-2 gene is amplified by 20-25 % of invasive breast cancers. Survival rates are worse in patients whose tumors carry the HER-2 gene amplification. Moreover HER-2 status is frequently detected in primary breast tumors and this information is used to make therapeutic decisions in the metastatic setting. The concordance between HER-2 expression in primary tumors and distant metastases is ranging between 80 and 94 %. It is suggested that a clinical discordance may result either from the clonal selection or from the upregulation of HER-2 during the metastatic process (Esteva 2008). HER-2 gene amplification can be accurately measured in individual cells (Meng et al. 2004) and this can be used in determining the HER-2 status of circulating tumor cells (Esteva 2008).

The detection of CTCs in patients with metastatic breast cancer, who are about to start a new line of treatment, has been shown to predict the progression-free survival and/or overall survival and treatment benefit. Therefore, the measurement of these cells offers a potential as a surrogate marker for the monitoring of anti-angiogenic treatment and drug activity, and could help to determine the optimal dose of drugs used in medical oncology (Cristofanili 2007).

In this report we describe the analysis of circulating tumor cells suitable for clinical use in patients with metastatic breast cancer and present our first preliminary results in patients with metastatic breast carcinomas.

Patients and Methods

Patients. Twenty-eight metastatic breast cancer patients were enrolled in the study, among them twenty-six patients with unilateral breast cancer and two patients with bilateral breast cancer. All patients received no concurrent chemotherapy or radiotherapy, however, hormonal treatment has been allowed. Clinical data of 28 patients with metastatic breast cancer (average age 59,96 years, min. age 37 and max age 80) was obtained from their medical histories (Table 1). As a control group, blood samples of 19 healthy female volunteers (average age 42.13 years, min. age 27 and max age 67) were included into the assay. Clinical data of the patients is shown in Table 1.

Sample collection. Following informed consent obtained from patients and volunteer controls, six milliliters of peripheral blood were drawn from antecubital into Vacutainer Heparin tubes (Becton Dickinson, San José, CA). The samples were stored at room temperature and were processed within 24 hours post-withdrawal interval.

From 6 ml of blood, two samples of 3 ml each were prepared. The first sample was used for staining the cells with 7AAD, pan-CK-PE and pan-CK-FITC (for these abbreviations see bellow) and the evaluation of this sample has been focused on the detection of circulating epithelial cells with different expression of cytokeratins. The cells from the second sample were stained with 7AAD, pan-CK-PE and HER2-FITC to detect circulating HER2+ epithelial cells.

Preparation of cells for laser scanning cytometry. The samples were centrifuged at 1,500 rpm for 10 min. After centrifugation, plasma was removed and the volume of each sample was restored with saline. The blood was then divided into six portions of 1 ml each and put in Falcon tubes 17×100 mm. To each tube, 10 ml of lysis solution containing ammonium chloride was added. After shaking, the cell suspension was incubated at 4-8 °C for 10 min. The tubes were then centrifuged at 1,500

Table 2

Detected circulating tumor cells in healthy volunteers I

number	events	CV%	CK19± cells	HER2± cells	CV%	CK19- cells	HER2- cells	CV%
1	22690	27,9	1	1	0	1	1	0
2	119020	10,2	7	3	40	7	3	40
3	35205	NA	3	NA	NA	3	NA	NA
4	30028	NA	3	NA	NA	3	NA	NA
5	106657	56,5	11	7	22,2	11	7	22,2
6	51002	12,3	10	2	66,7	9	2	63,6
7	57076	41	6	12	33,3	0	7	100
8	39757	10,2	23	8	48,4	20	8	42,9
9	137503	7,3	23	20	6,9	15	9	25
10	79602	16,2	25	26	2	9	7	25
11	18389	5,9	10	23	39,4	6	17	47,8
12	6223	14,2	2	7	55,5	0	4	100
13	122488	38,9	28	18	21,7	27	18	20
14	38860	34,4	1	4	60	1	4	60
15	104228	34,9	4	15	57,9	4	15	57,9
16	129756	51	16	12	14,3	16	12	14,3
17	17067	36,3	39	4	81,4	39	4	81,4
18	20081	NA	NA	3	NA	NA	3	NA
19	10633	NA	1	NA	NA	1	NA	NA
Median	*39757*	*27,9*	*8,5*	*7,5*	*39,4*	*6,5*	*7*	*42,9*
Min.	*6223*	*5,9*	*1*	*1*	*0*	*0*	*1*	*0*
Max.	*137503*	*56,5*	*39*	*26*	*81,4*	*39*	*18*	*100*

rpm for 10 min and the supernatant was discarded. This step with erythrocyte lysis was then repeated once more. After lysis, 2 ml of phosphate-buffered saline (PBS) and 0.5 % bovine serum albumin (BSA) were added to the tube. Then the samples were centrifuged at 1,500 rpm for 10 min. The supernatant was discarded, the cell pellet was resuspended in 80 µl PBS and 15 µl CD45 MicroBeads were added to each tube, mixed well and incubated at 4-8 °C for 15 min. Subsequently, 500 µl PBS + 0.5 % BSA were added to each tube. LS columns (laser scanning columns; Miltenyi Biotec, Germany) were placed into the magnet equipment. The LS columns were washed with 3 ml PBS + 0.5 % BSA. Three tubes were passed through one LS column (3 ml of whole blood). After passing/washing the last tube, we washed the LS column with 6 ml PBS + 0.5 % BSA. Then the second LS column was placed into the magnet and the washing was repeated with the remaining three tubes. The content that passed through the LS column and was concentrated in the tube was then centrifuged at 1,500 rpm for 10 min. The cell pellet was resuspended in 300 µl PBS, and the cells were spun onto slides using cytospin centrifugation. The cells were fixed in absolute methanol at 4-8 °C for 30 min. After fixation the slides were dried and stored until use.

On the day of LSC analysis the slides were rehydrated with distilled water for 20 min. After hydration they were gently dried and stained using phycoerythrin-conjugated pan-cytokeratin 14, 15, 16 and 19 (pan-CK-PE, BD, USA) (1:10) at room temperature for 15 min.

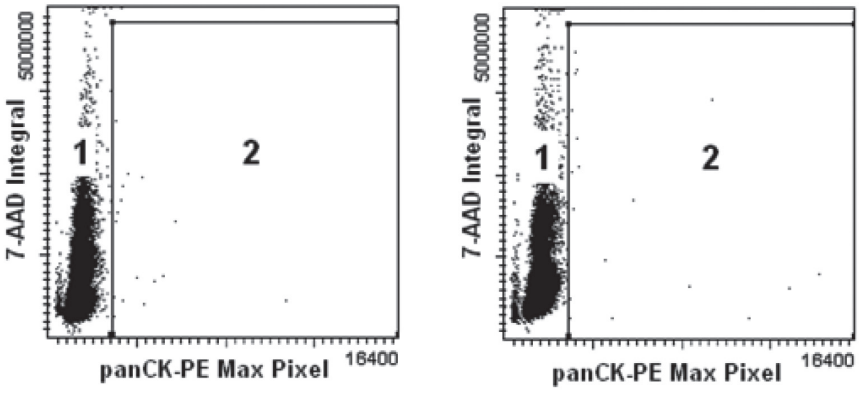

Fig. 1A **Fig. 1B**

Fig. 1. Analysis of the events on a scattergram (7-AAD integral *vs.* pan-CK-PE max pixel).
Sample A, sample B: Area No 1: CK 14, 15, 16, 19 positive cells; Area No 2: CK 14, 15, 16, 19 negative cells.

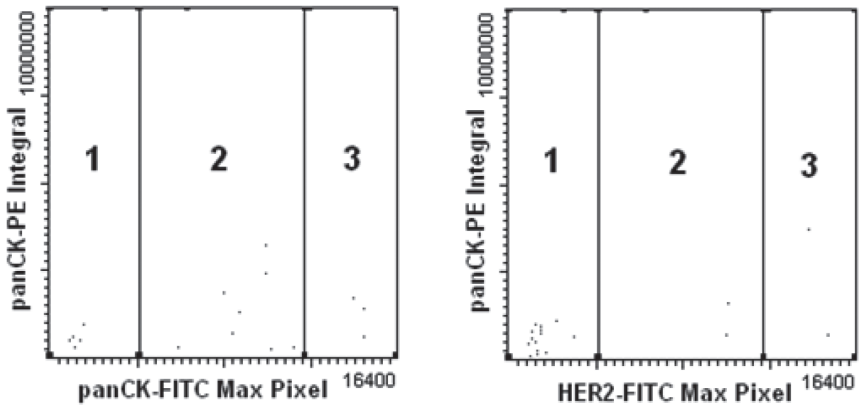

Fig. 2A **Fig. 2B**

Fig. 2. Analysis of the events on a scattergram (pan-CK-PE integral *vs.* pan-CK-FITC max pixel) on the first sample A and analysis of the events on a scattergram (pan-CK-PE integral *vs.* HER2 -FITC max pixel) on the second sample B.
Sample A – expression of different cytokeratins on CTCs
1 – pan-CK 8, 18, 19 negative cells, 2 – low pan-CK 8, 18, 19 expression, 3 – high pan-CK 8, 18, 19 expression.
Sample B – HER2 expression on CTCs
1 – HER2 negative cells, 2 – low HER2 expression, 3 – high HER2 expression.

The samples were washed five times with one ml PBS and were stained with FITC-conjugated pan-cytokeratin (pan-CK-FITC, Miltenyi Biotec, Germany) the staining continued with HER2-FITC (BD, USA) (1:10) at room temperature for 15 min. After second re-staining the samples were washed five times with PBS and stained with 7-aminoactinomycin D (7-AAD, Immunotech, France) (1:10) for 10 min. After the last staining the cells were washed once with PBS and the slides were mounted using glycerine as the mounting medium. The samples were analyzed using laser scanning cytometer (LSC).

LSC analysis. The analysis of membranes was performed in a laser scanning cytometer (CompuCyte Inc., Cambridge, MA) using a WinCyte PC-based software.

Table 3

Detected circulating tumor cells in healthy volunteers II

number	CK19+ cells	CK19low cells	CK19high cells	HER2+ cells	HER2low cells	HER2high cells
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	1	1	0	0	0	0
7	6	3	3	5	4	1
8	3	3	0	0	0	0
9	8	NA	NA	11	8	3
10	16	9	7	12	7	5
11	4	1	3	6	4	2
12	2	2	0	3	1	3
13	1	NA	NA	0	0	0
14	0	0	0	0	0	0
15	0	0	0	0	0	0
16	0	0	0	0	0	0
17	0	0	0	0	0	0
18	NA	NA	NA	0	0	0
19	0	0	**0**	NA	NA	NA
Median	*3,5*	*2,5*	*3*	*6*	*4*	*3*
Min.	*1*	*1*	*3*	*3*	*1*	*1*
Max.	*16*	*9*	*7*	*12*	*8*	*5*

Cell scanning was performed using a 20× objective and argon laser (wavelength 488 nm). The scanned area was defined as a 10×15 mm rectangle which reflected the dimensions of the cytospin window. Cell counting was done through long red/7-AAD. Analysis of the events was performed on an XY scattergram window (7-AAD Integral *vs.* pan-CK-PE Max Pixel). Following LSC analysis, CK-positive cells were relocalized and a fluorescent cell gallery was created.

Statistical evaluation. All samples were measured in duplicates. From the measured events the median and the coefficient of variation (CV) of the events were calculated in CK 19 + / –, HER2 + / –, CK 19 –, and HER-2– cells. To calculate the statistical significance between the compared groups the Student's unpaired t-test was used. The values of $p<0.05$ were considered statistically significant. The correlations were estimated with the aid of Pearson'o coefficient.

Results

The median number of analyzed cells in metastatic breast cancer patients was 23,821.5 cells (min 2,793 cells – max 118,859 cells) with a median CV of 19.4 % (min 2.5 – max 83.5) (Table 4). In control group the median number of analyzed cells was 39,757 cells (min 6,223 cells – max 137,503 cells) with a median CV of 27.9 % (min 5.9 – max 56.5) (Table 2). In the first step, the

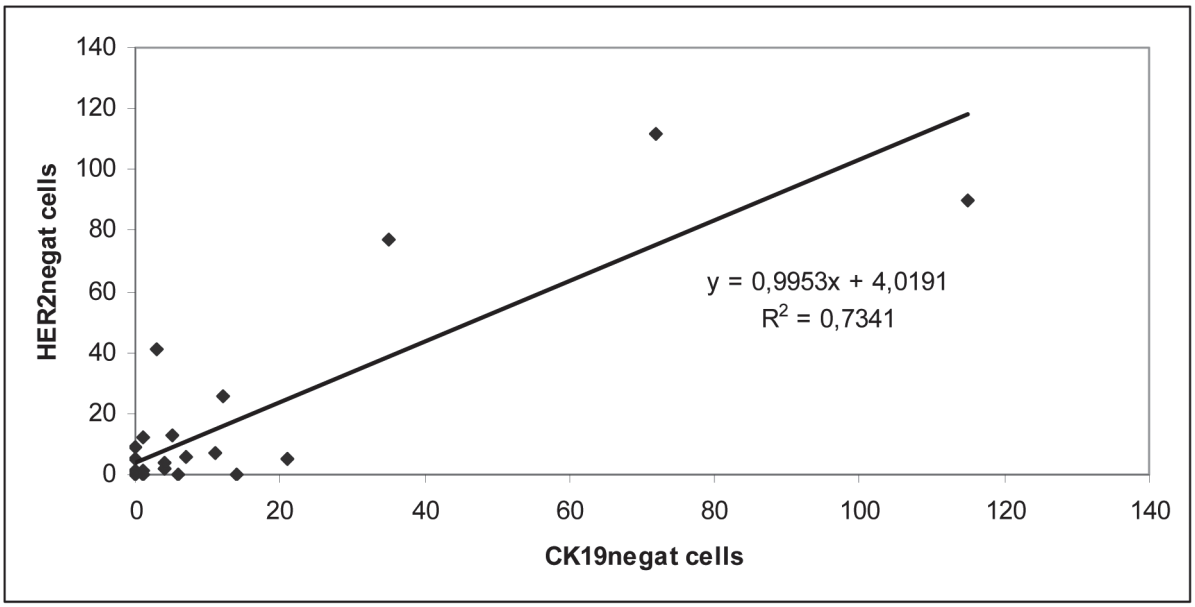

Fig. 3. Correlation between the number of HER2– cells nad CK19–

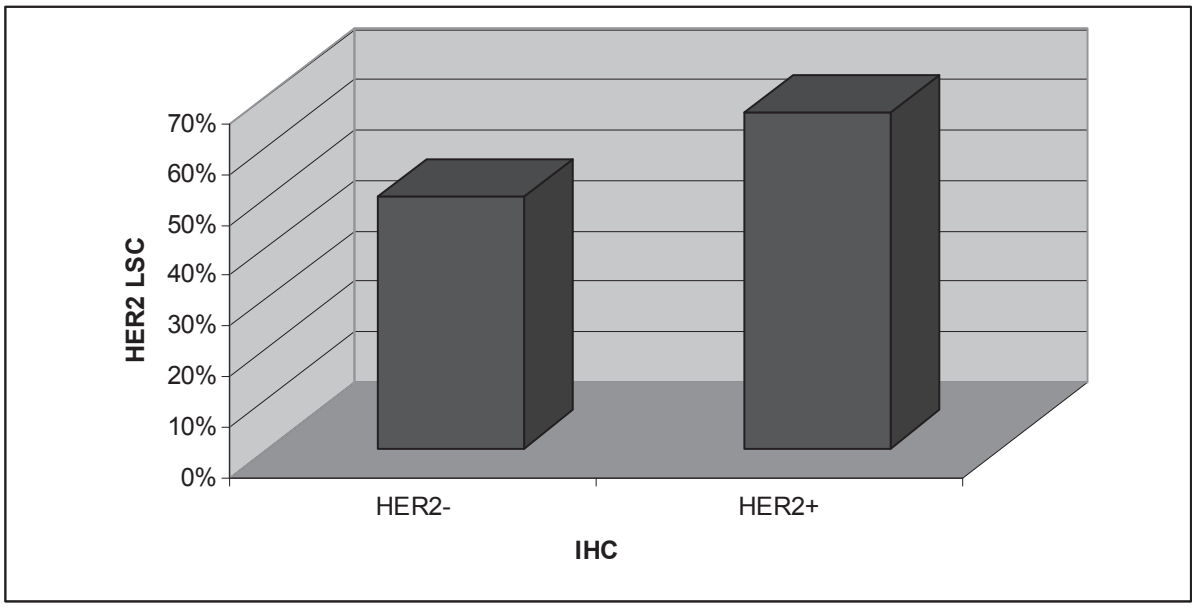

Fig. 4. Interrelation between expression of HER2 on CTCs in peripheral blood and HER2 expression in tissue

cells with positive CK 14, 15, 16 and 19 staining were gated. In the first sample we gated CK 14, 15, 16 and 19 positive cells from CK 14, 15, 16 and 19 negative cells (Fig. 1A). In the second step, CK19+ and CK19– cells were gated on the basis of positive staining for pan-CK 8, 18, and 19 (Fig. 2A). In the second sample, CK 14, 15, 16 and 19 positive cells were gated from CK 14, 15, 16 and 19 negative cells (Fig. 1B). In the second step, CK 14, 15, 16 and 19 positive cells were gated on the basis of positive staining for HER2+ and HER2– cells (Fig. 2B). Following LSC analysis, CK19– cells (Fig. 5) and CK19+ cells (Fig. 6) were relocalized and a fluorescent cell gallery was created.

In general, the numbers of CK19+ cells from the first sample and the numbers of HER2+ cells from the second sample were similar (r=0.82, Pearson) (Fig. 3).

Table 4

Detected circulating tumor cells in metastatic breast cancer patients I

Number	Events	CV%	CK 19± cells	HER2± cells	CV %	CK 19- cells	HER2- cells	CV%
1	57527	46.3	0	10	100	0	9	100
2	18316	16	4	7	27.3	1	1	0
3	6176	48.9	13	39	50	12	26	36.8
4	25958	56.8	36	6	71.4	4	2	33.3
5	37066	50.7	0	0	0	0	0	0
	70645	29.5	2	16	77.8	1	12	84.6
6	107662	9.4	81	127	22.1	72	112	21.7
7	118859	6.8	26	9	48.6	21	5	61.5
8	85095	18.8	35	77	37.5	35	77	37.5
9	81579	11.6	19	10	31	7	6	7.7
10	37857	9.8	8	6	14.3	6	0	100
11	13008	16.4	20	4	66.7	8	4	33.3
12	39185	NA	4	NA	NA	2	NA	NA
13	11715	2.5	198	79	42.9	196	77	43.6
14	50312	NA	75	NA	NA	70	NA	NA
15	102754	6.7	117	93	11.4	115	90	12.1
16	9919	3.2	10	8	11.1	0	5	100
17	66823	83.5	7	5	16.7	4	2	33.3
18	31156	24.5	6	14	40	5	13	44.4
19	2793	69.2	5	49	81.5	3	41	86.4
20	15041	45.2	14	1	86.7	14	0	100
21	10300	2.5	0	5	100	0	5	100
22	8746	20	0	1	100	0	0	0
23	8211	76.9	0	0	0	0	0	0
24	16996	6.5	0	1	100	0	1	100
25	70633	12.2	3	1	50	1	0	100
26	21445	55.3	1	0	100	0	0	0
27	21685	21.1	0	1	100	0	1	100
28	11892	10.3	11	7	22.2	11	7	22.2
	3606	21.1	4	4	0	4	4	0
median	*23821.5*	*19.4*	*6.5*	*6.5*	*45.75*	*4*	*4.5*	*37.15*
min.	*2793*	*2.5*	*0*	*0*	*0*	*0*	*0*	*0*
max.	*118859*	*83.5*	*198*	*127*	*100*	*196*	*112*	*100*

NA – not available

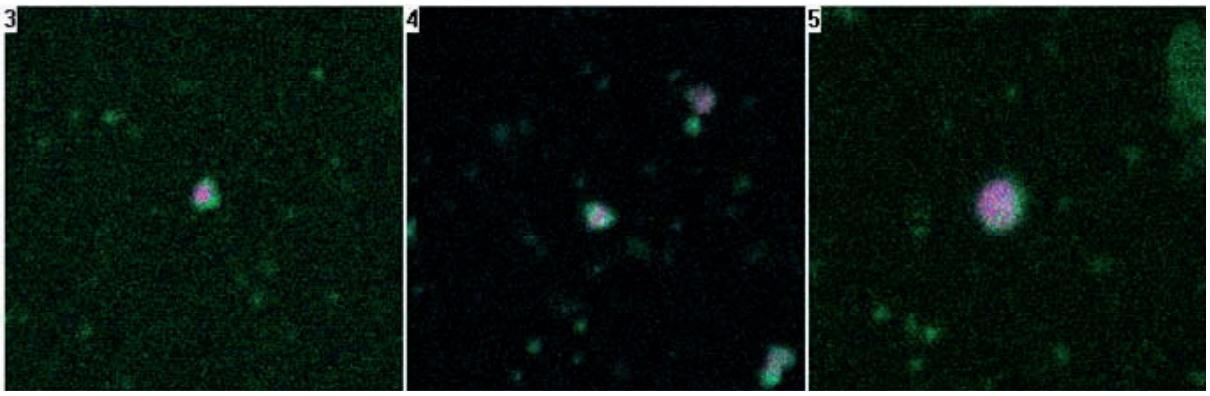

Fig. 5. fluorescent cell gallery of CK19– cells

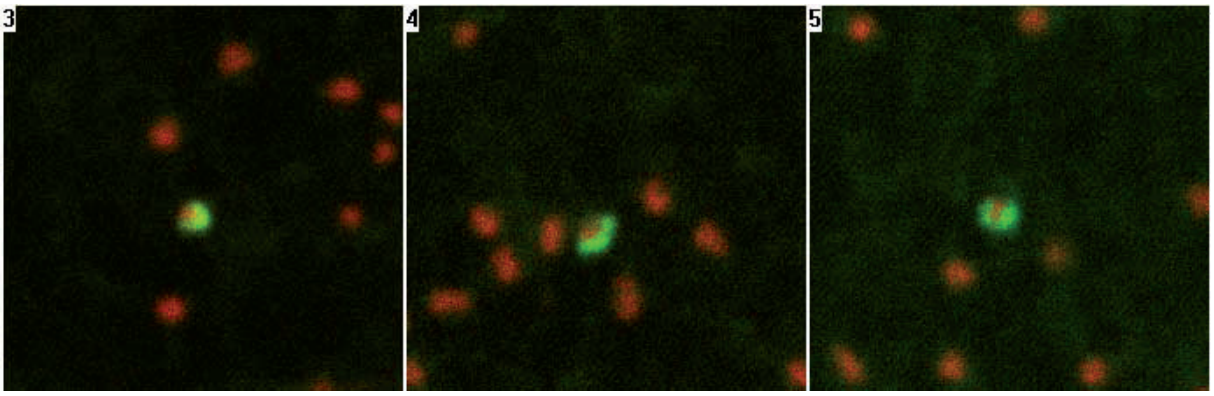

Fig. 6. fluorescent cell gallery of CK19+ cells

This proves a strong significant correlation between both samples in CK19 and HER2 expression. When we correlated other gated cells, we found that the numbers of circulating CK19– epithelial cells significantly correlated with circulating HER-2– epithelial cells (r=0.86, Pearson) (Fig. 4).

In the group of 28 patients the median of 19.4 cells per three ml sample, was found expressing all types of cytokeratins under consideration. The median of CK 8, 18 and 19 non-expressing cells was four cells. More than two cells expressing CK 8, 18 and 19 were found in 14 among 28 patients (50.0 %). In the group of patients with samples containing more than two CTCs we found a median of three cells (minimum two – maximum 32 cells). In the control group we found more than two cells in 6 out of 19 healthy subjects (31.6 %). No statistical correlation was found between CTC positivity in patients with metastatic breast cancer and the control group.

In the group of 14 patients with positive CTCs we were able to analyze HER2 expression in 10 patients (Table 5). HER2 expression was described as into positive and negative expression. HER2 positivity was further subdivided into low-HER2 and high-HER2 expression level. Based on the standard immunohistochemical criteria, the cells with high-HER2 expression have been considered HER2 positive. Out of 10 patients the presence of cells with only high-HER2 expression was observed in two of them, and the presence of cells with only low-HER2 expression was observed in one patient. The other 7 patients had a mixed population of CTCs as to HER2 expression. To classify the patients on the basis of their HER2 status without an unambiguous HER2 expression, we determined the ratio of HER2-high / HER2-all expression level. A HER2-high / HER2-all ratio higher than 50 % was considered as HER2 positive, which was observed in five patients with positive CTCs. In the control group a clear high-HER2 expression for CTCs was not detected in any analyzed subject (Table 3). HER2 positivity for CTCs was found in 6 healthy donors. A HER2-high / HER2-all ratio higher than 50 % was found in one of 6 volunteers (16.7 %).

Table 5

Detected circulating tumor cells in metastatic breast cancer patients II

Number	CK 19+ cells	CK 19 low cells	CK 19 high cells	HER2 + cells	HER2 low cells	HER2 high cells
1	0	0	0	0	0	0
2	3	2	1	6	2	4
3	1	1	0	12	10	2
4	32	16	16	4	1	3
5	1	1	0	4	3	1
	0	0	0	0	0	0
6	9	6	3	15	10	5
7	5	3	2	4	3	1
8	0	0	0	0	0	0
9	12	6	6	6	4	2
10	2	2	0	6	2	4
11	12	2	10	NA	NA	NA
12	2	2	0	NA	NA	NA
13	2	2	0	2	0	2
14	5	3	2	NA	NA	NA
15	2	1	1	3	3	0
16	1	0	1	3	1	2
17	3	1	2	3	0	3
18	1	1	0	1	0	1
19	2	1	1	8	3	5
20	0	0	0	1	0	1
21	0	0	0	0	0	0
22	0	0	0	1	0	1
23	0	0	0	1	1	0
24	0	0	0	0	0	0
25	2	NA	NA	1	NA	NA
26	0	0	0	0	0	0
27	0	0	0	0	0	0
28	0	0	0	0	0	0
	0	0	0	NA	NA	NA
median	*1*	*1*	*0*	*1,5*	*0*	*1*
min.	*0*	*0*	*0*	*0*	*0*	*0*
max.	*32*	*16*	*16*	*15*	*10*	*5*

Correlation between HER2+ in tissues and peripheral blood was performed in 11 patients with metastatic carcinoma. In 8 patients the tissue expression of HER2 was negative (HER2 at 1+ in IHC, and HER2 at 2+ in IHC, and FISH negative) (72.7 % of patients). In three among 11 patients HER2 expression in tissues was positive (HER2 at 2+ in IHC and FISH+, and HER2 at 3+ in IHC) (27.3 %). In peripheral blood we found HER2 positive in 6 out of 11 patients (54.5 %) and HER2 negative in five patients (45.5 %). The correlation of HER2 expression in tissue samples and HER2 positive of CTCs in peripheral blood have shown that even if HER2 expression was negative in tissue samples, then there was 50 % positivity in HER2 expression in peripheral blood. In case of positive HER2 expression in the tissues, 66 % positivity in HER2 expression in peripheral blood was determined (Fig. 5).

Discussion

The sensitivity of our method was 50.0 %, while specificity reached as much as 68.4 %. In a large group of different cancer patients, Allard et al. (2004) reported a lower sensitivity of 37 %, but a very high specificity of 99.7 % by using the CellSearch system (Veridex LLC, U.S.A.) for the detection of circulating tumor cells which consists of a CellPrep system - semiautomated sample preparation system (Kagan et al. 2002a, b), the CellSearch Epithelial Cell Kit, and the CellSpotter Analyzer. The CellSearch Epithelial Cell Kit consists of ferrofluids coated with epithelial cell specific EpCAM antibodies (Momburg et al. 1987) to immunomagnetically enriched epithelial cells; a mixture of two phycoerythrin-conjugated antibodies that bind to cytokeratins (Vincent-Solomon et al. 2008; Momburg et al. 1987; Gomperts et al. 2066) an antibody to CD45 conjugated to allophycocyanin; nuclear dye 4,6-diamidino-2-phenylindole (DAPI) to fluorescently label the cells; and buffers to wash, permeabilize, and resuspend the cells.

We identified increased levels of CTC like cells in 31.6 % of healthy controls. These false positive cells represent circulating epithelial-like cells. Since the CD45 staining was not employed, we cannot distinguish these circulating epithelial-like cells from CTCs. Gomperts et al. (2006) determined a population of CK5+ progenitors in the bone marrow and circulation of mice (CK5 and CK14 are intermediate type filament proteins that are expressed together in basal regenerating cells of complicated epithelia). They found that 5 ± 0.5 % of mononuclear cells isolated from the bone marrow expressed CK5, of which almost 100 % were CD45+. In the circulation, 8 ± 1.5 % of mononuclear cells expressed CK5. Of the CK5+ cells, 76 ± 6 % were also CD45+ (19). Massayo et al. (2000) suggested that the loss or down-regulation of CK13, 14, or 16 is related to the invasive and metastatic ability of cancer. The cytoskeletal system is thus considered to be closely related to the malignant phenotype.

Along with the HER2 positivity in CTCs from tumor survivors, in a small number of healthy control CTC like epithelial cells have been detected. Besides the expression of HER2 on epithelial cells, the expression of HER2 protein was described on hemopoetic cells (myeloid line) and on cord blood cells grown *in vitro*. The HER2 expression was positive in cells entering proliferation (Leone et al. 2003). Also, HER2 expression on activated lymphocytes with PHA and tumor-infiltrating lymphocytes has been determined (Kowalevska et al. 2006). In the majority of metastatic breast cancer patients a mixed population of HER2+ CTCs was present. We found that high number of HER2 positive CTCs are CK19+ CTCs, while HER2– CTCs are CK19– CTCs. Braun et al. (2001) and Zidan et al. (2005) found no correlation between the HER2 staining score of the primary tumor and the presence of HER2-positive micrometastatic tumor cells in bone marrow. We observed a 50 % positivity of HER2 expression on CTCs in metastatic patients being negative in HER2 expression in tissue samples. It has also been reported that a significant percentage of patients with HER2-negative primary tumors develop high concentrations of serum HER2 during tumor progression, supporting the possibility that HER2 gene amplification can be acquired during cancer progression (Meng et al. 2004).

In conclusion, it appeared that the method described might be a perspective used for estimating HER2 status on CTCs from peripheral blood of metastatic breast cancer patients.

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