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ORIGINAL ARTICLES

Detection Of Minimal Residual Disease In Breast Cancer Patients Undergoing Autologous Pbsct Using Flow Cytometry And Rt-Pcr

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ABSTRACT

Carcinoma of the breast is the most prevalent cancer among Egyptian women. Hematogenous distant metastasis is the leading cause of cancer-related deaths in breast cancer and other solid epithelial tumors. The present study was planned to evaluate CK-19 mRNA detection by nested RT-PCR assay as a detection marker of circulating breast cancer cells in the peripheral blood and disseminated tumor cells in bone marrow of patients with operable breast cancer before the initiation of any adjuvant therapy. Another aim was detection of minimal residual cancer cells contaminating the peripheral blood stem cell (PBSC) apheresis products of patients with advanced breast cancer undergoing high-dose chemotherapy (HDCT) and autologous PBSC transplantation, and to determine the impact of minimal residual disease (MRD) detection on the outcome of long-term follow up of these patients after transplantation. This present study included 64 breast cancer patients. Molecular and cellular approaches have been used in the detection of the occult tumor cells. RT-PCR targeting tissue-specific mRNA and flow cytometry using monoclonal antibodies against cytokeratin were applied on 14 bone marrow samples, 30 peripheral blood samples as well as 20 PBSC samples from high-risk primary and metastatic breast cancer patients who underwent high-dose chemotherapy and autologous transplantation. Flow cytometry detected minimal residual disease in 7 patients (35.0%) of group I (PBSC), while in group II bone marrow micrometastasis was detected in 3 (21.4%) patients. The nested RT-PCR assay for CK-19 mRNA detected minimal residual disease in 11(55.0%) patients of group I (PBSC) and 5 (36.0%) of group II (bone marrow micrometastasis) patients as well as 8 (27.3%) patients of group III (CTC). The concordance of flow cytometry and RT-PCR was 82.4%. There was a significant association between tumor stage and CK-19 mRNA by RT-PCR assay (p-value = 0.017) in group I (PBSC), while larger primary tumors > 2 cm were significantly associated with a higher incidence in micrometastasis patients (group II) than were tumors that were 2 cm or less in diameter ($p<0.001$). The presence of CK-19 mRNA positive tumor cells in the PBSC was significantly associated with a decreased DFS ($p=0.04$). Patients with bone marrow micrometastasis had a higher risk of relapse ($p=0.007$) and death ($p=0.01$) than patients without bone marrow micrometastasis. Measuring CTC counts can facilitate the early prediction of treatment response and thereby avoid unnecessary therapy. CTCs may also be a useful biomarker for molecular targeted agents, enabling the identification of patients most likely to respond to a given treatment and facilitating treatment selection.

Key words: CK-19, DTC, CTC, Cancer breast, RT-PCR, Flowcytometry

Introduction

Breast cancer remains an important public health problem. It is the most common malignancy among women and represents the second leading cause of cancer death; the overall incidence of breast cancer in all Egyptian women is approximately 29% of all cancers identified at National Cancer Institute in Cairo (Omar *et al*., 2003).

Hematogenous distant metastasis is the leading cause of cancer-related deaths in breast cancer and other solid epithelial tumors. By applying sensitive immunocytochemical and molecular assays, disseminated tumor cells in bone marrow can be detected in 20-40% of primary breast cancer patients without any clinical or even histopathological signs of metastasis (Pantel et at, 2004).

Minimal residual disease (MRD) or micrometastatic cells in the bone marrow may be the source of potentially fatal overt distant metastasis in bone and other organs even years after primary treatment (Janni et at, 2005).

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Circulating tumor cells (CTC) in peripheral blood represent an important biologic link in the hematogenous spread of breast cancer from primary to metastatic diseases. CTC are strong predictors of prognosis and clinical outcome in patients with metastatic breast cancer . In early breast cancer, the use of CTC detection for identification of patients at risk for future relapse is still controversial (Pierga et at, 2004).

The emergence of highly sensitive and sophisticated reverse transcriptase-polyrnerase chain reaction (RT-PCR) assays, combining a preanalytical enrichment step with the assessment of multiple molecular tumor markers expressed in circulating tumor cells, provides a powerful tool in detecting CTC with high sensitivity and specificity (Zieglschmid *et al*, 2005).

RT-PCR assay now enables specific detection of occult breast tumor cells in peripheral blood, BM and peripheral blood stem cell (PBSC) apheresis. It detects cytokeratin 19 mRNA (CK-19), thus indicating the expression of a specific gene. Many studies have used CK-19 gene as a marker Ag which is expressed in epithelial tumor cells and is not expressed in hematopoeitic cells. Subsequently, the detection of CK-19 mRNA within the blood of a patient with breast cancer would indicate the presence of circulating epithelial cells and potentially tumor cells.

The aim of this work is to evaluate the clinical significance of bone marrow micrometastasis and circulating tumor cells in peripheral blood detected with nested reverse transcriptase-polymerase chain reaction for cytokeratin-19 mRNA in patients/with operable breast cancer before the initiation of any adjuvant therapy. As well as the significance of minimal residual disease in the peripheral blood stem cell (PBSC) apheresis in advanced breast cancer patients.

As well as to assess the clinical outcome of disseminated tumor cells in the bone marrow of operable breast cancer patients detected by nested RT-PCR and flow cytometry techniques, Also to determine the impact of minimal residual disease detection on the clinical outcome during the follow-up period of patients who underwent high-dose chemotherapy and autologous transplantation.

Patients And Methods

This present study was carried out on 64 female patients with breast cancer, age ranged from 30 to 65 years with (mean 50±10 years).Ten age-matched healthy female blood donors were enrolled in our work as negative control cases for cytokeratin 19 (CK-19) expression. The studied patients were categorized into the following groups:

1- The Transplanted breast cancer patients (group I):

Twenty patients who had received high-dose chemotherapy (HDCT) and autologous peripheral blood stem cell (PBSC) transplant. These patients were selected from the BMT & clinical oncology unit of AL Manial Specialized Hospital, Cairo University, during the period between January 1998 and September 2000. These patients were eligible for entrance the protocol of HDCT and autologous PBSC transplant.

2-The operable breast cancer patients (groups II and III):

Group II: Bone marrow samples were obtained from 14 patients with newly diagnosed operable breast cancer after surgery and before any therapy. They were selected from centre of Radiation Oncology and Nuclear Medicine (NEMROCK), Cairo University, during the period from 2001 and 2002. Bone marrow samples were obtained from upper anterior iliac crest after the resection of the primary tumor and before the initiation of adjuvant therapy.

Group III: Peripheral blood samples were obtained from 30 patients of newly diagnosed operable (stages I and II) breast cancer patients after surgery and before adjuvant chemotherapy were included in this study.

3-Control cases:

Peripheral blood samples were withdrawn from 10 healthy female blood donors and bone marrow aspirates from 5 patients with hematological malignancies. All samples were dispensed into sterile EDTA vacutainer tubes. The control cases acted as negative control with age and sex matched to the study group.

Follow-up:

Patients' follow-up consisted of pertinent medical history and physical examination, with laboratory and imaging studies restricted as indicated, every three months for the first two years, every six months for the next

three years and yearly thereafter. The median follow-up time for the transplanted patients (group I) was 63 months while for the newly diagnosed operable patients (group II) was 38 months.

Sample handling and processing:

Bone marrow samples were obtained from fourteen patients with operable breast cancer after the removal of the primary tumor and before the initiation of adjuvant chemotherapy (group II). Bone marrow (2 ml) was aspirated from the upper iliac crest under local anesthesia into vacutainer tubes containing EDTA as anticoagulant. Peripheral blood (10 ml in EDTA) was obtained from another thirty patients with operable breast cancer (group III). To avoid contamination with epithelial skin cells, all blood samples were obtained at the middle of vein puncture after the first 5 mL of blood was discarded.

Peripheral blood and bone marrow samples were diluted with phosphate-buffered saline (PBS). Peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells were isolated by density- gradient centrifugation through Ficoll-Hypaque for 30 minutes and washed twice with PBS. After final centrifugation, the cell pellets were resuspended in a guanidine- containing buffer and stored at- 80^{o}C until RNA extraction.

Cryopreserved stem cell apheresis samples from twenty patients with high-risk primary and metastatic breast cancer were studied. The PBSC samples were collected as follows:

** Mobilization of peripheral blood stem cells:*

PBSC was mobilized by administration of the chemotherapy and Granulocyte colony stimulating factor (G-CSF). Two mobilization regimens were used: Cyclophosphamide 2 gm /m^2/day + G-CSF were given to 9/20 (45%) of patients. Taxol 175 mg /m^2/day + G-CSF (5µg/kg/day) were given to 11/20 (55%) of patients.

Complete blood count was done on daily basis starting on D_5. Immunophenotyping for CD_{34}^+ cells was performed daily starting from D_7 permitting a standardized and rapid assessment of PBSC. Cells for immunophenotyping were stained using conjugated monoclonal antibodies for CD_{34}. Two-color or three- color analysis of subsets was carried out using a FAC Scan flow cytometer (Becton Dickinson Immunocytometry systems, BD, San Jose, CA). A sequential gating was employed to identify the CD_{34}^+ population**.**

**Collection and cryopreservation of PBSC:*

Apheresis was performed following the administration of chemotherapy and G-CSF, apheresis was initiated when the white blood count started to rise following the nadir (starting from 1 x 10^9/L) or /and when the CD_{34}^+ cells count in the circulation started to rise by monitoring it with the flow-cytometry.

Leukapheresis was performed daily until a target cell dose of $\geq$ 2.5 x 10^6 CD_{34}^+ cells /kg/patient had been collected, using a continuous flow centrifuge cell separator (COBE BCT Spectra). PBSC harvested cells were cryopreserved in 10% DMSO, which is used as a cryoprotectant to maintain cell integrity.

An aliquot was taken from the entire collection of PBSC on the day of apheresis for determination of CD_{34}^+ cells content and for RT-PCR and flow- cytometry assays. After sampling, the PBSC harvest was stored in liquid nitrogen.

**High-dose chemotherapy and hematopoietic stem cell transplant:*

Pretransplant conditioning chemotherapy protocol received by the patients was according to their clinical status and according to the mobilization regimen used. Some patients received Ifosphamide, Carboplatin and Etoposide (ICE). Other patients received Carboplatin, Mitoxantrone and Taxol (CMT).

On day 0 cryopreserved PBSC were thawed and infused through a central venous access. **Table (1A)** shows the engraftment data and CD_{34} x10^6/Kg doses infused to the patients. The mean CD_{34} x10^6/Kg doses was 5.7$\pm$1.66, median 5.8 with range of (3.7-8.3). Patients were discharged from hospital when the neutrophil count was above 1.5 x 10^9/L. Patients were followed-up on regular basis in the outpatient clinic after discharge.

Table 1A: The engraftment data and CD_{34} x 10^6/kg dose received by the 20 patients who under went the high-dose chemotherapy and PBSC

	Mean	Std. Deviation	Median	Range
Hemoglobin g/dl	8.2	$\pm$ 0.84	8.1	(7.1 – 10)
Absolute neutrophilic count ANC>500 in (days)	11.3	$\pm$ 0.82	12	(10 – 12)
Platelets > 20x10^3/µl	44.5	$\pm$ 10.34	30	(30 – 65)
CD_{34} dose x 10^6/kg	5.7	$\pm$ 1.66	5.8	3.7-8.3

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Detection of Cytokeratin-19 by RT-PCR:

This assay relies on the detection of CK-19 mRNA-positive cells by nested primer RT-PCR, which is capable of detecting up to one breast cancer cell in 10^6 normal marrow cells.

"Nested" sets of primers for cytokeratin 19 were used to enhance specificity. Nested sets of primers involved one set of cycles of repeated synthesis with a pair of primers and a second set of cycles with primer sequences located between the primers used in the previous round.

RNA was extracted from bone marrow and peripheral blood stem cell sample using QIAGEN RNA extraction mini kit Qiagen (Catalog No 52304). All RNA preparation and handling steps took place in a laminar flow hood, under sterilized conditions. Reverse transcription of RNA into cDNA was performed using proSTAR RT- PCR STRATAGENE kit (Catalog No. #200420)

cDNA was synthesized according to the manufacturer's instructions. Two different PCR reactions were performed with each sample in order to amplify fragments of CK-19. The oligonucleotide primers were designed with the aid of Primer Express Software (Applied Biosystem).The sequences of primers used (synthesized by Biometra ,Batch no: # 32338 – 32341) were as follows: for CK 19 outer nest ; GCCGACAAATTGTTGTAG (downstream); ATGCGAAGCCAATATGAG (Upstream), for CK 19 inner nest; CATCACTATCAGCTCGCACATC (downstream); GCACACTGGCAGAAACGGAG (Upstream). The corresponding sizes of PCR products were 745 base pairs. The conditions for CK-19 PCR were 35 cycles composed of denaturation at 94oC for 1 minute, annealing at 65oC for 1 min (optimal for the CK-19 primers) and extension step at 72oC for 2 minutes. A final extension at 72oC for 10 minutes completed the amplification followed by cooling to 4oC.

Following the completion of the amplification, 10 µl of the PCR product was electrophoresid on 2% agarose gel, stained with ethidium bromide and visualized by ultraviolet transilluminator. Electrophoresis analysis was done using appropriate molecular weight DNA markers for the presence of CK-19. Positive amplification resulted in sharp clonal band of approximately 745bp product. CK-19mRNA positive cells indicate the presence of micrometastatic breast cancer cells in the sample (bone marrow, peripheral blood or PBSC).

Detection of Cytokeratin-19 by Flow cytometry:

Identification of minimal residual disease (MRD) in peripheral blood stem cell harvests or bone marrow aspiration samples was accomplished using flow-cytometric analysis. This method employed monoclonal antibodies; Anti- cytokeratin (CAM 5.2) FITC Catalog No. 347653 (Becton Dickinson, San Jose, CA), which identifies cytokeratin (CK) peptides 8, 18 and 19.

Human white blood cells were isolated from adult peripheral blood using RBC lysis buffer as follows: 50 µl of blood and 2 ml Lysing Solution (Catalog No. 349202) were mixed with vortex gently and incubated for 10 minutes at room temperature, then centrifuged at 1,200 rpm for 5 minutes. The supernatant was aspirated and added to 500 µl of Permeabilizing Solution (Catalog No. 340457). Vortex followed and incubation for 10 minutes at room temperature in the dark was done and then followed by twice rinse with PBS. Twenty microliters of fluorescent-conjugated Anti-Cytokeratin antibodies were added to the supernatant. The samples with monoclonal antibodies were incubated for 30 minutes at room temperature in the dark. After fixation with 500 µl of 1% paraformaldehyde for 30 minutes at room temperature, samples were analyzed by flow cytometer FACSCalibur (Becton Dickinson).

Statistical Analysis:

SPSS (Statistical Package for Social Sciences) version 12.0 was used for data analysis. Mean and standard deviation are descriptive values for quantitative data. Student t test was used for comparing means of two independent groups and Kruskal-Wallis ANOVA (analysis of variance) for comparing means of more than 2 independents groups. Chi-square measured the degree of concordance of RT-PCR results and flow cytometry regarding CK19 positivity. When Kappa = 0, it indicates no agreement and when equal one it indicates complete concordance. The Kaplan- Meier method estimated probability of overall and disease free survival. Log rank test was used for comparing survival curves. P value is significant at 0.05 levels.

Results:

Patient characteristics:

A total of 64 female patients with breast cancer with diagnosis of breast cancer selected from the BMT & clinical oncology unit of AL Manial Specialized Hospital, Cairo University, during the period between January

1998 and September 2000 and from centre of Radiation Oncology and Nuclear Medicine (NEMROCK), Cairo University, during the period from 2001 and 2002. The studied patients were categorized into the following groups. Group I included 20 patients with advanced breast cancer who had received high-dose chemotherapy (HDCT) followed by autologous peripheral blood stem cell (PBSC) transplant. Group II included 14 patients with newly diagnosed primary breast cancer (stages I, II and III). Group III included 30 patients with operable breast cancer (stages I and II). Patients' characteristics are summarized in **Table 1B**.

Detection Of Occult Tumor Cells In Pbcs, Bm And Peripheral Blood By Rt-Pcr And Flow Cytometry:

Peripheral blood stem cell (PBSC) aphaeresis products were obtained from twenty patients (group I), bone marrow aspirates were obtained from 14 patients (group II) and 30 peripheral blood samples from (group III). Peripheral blood samples were obtained from 10 normal blood donors and bone marrow aspirates from 5 patients with hematological malignancies were used as control cases.

Samples were analyzed for the detection of occult tumor cells by two different techniques. Molecular detection of cytokeratin 19 (CK-19) mRNA positive cells was carried out to all groups using nested reverse transcriptase-polymerase chain reaction (RT-PCR) **(Fig 1)**. Flow cytometry (FCM) technique was performed to group I and group II using anti-cytokeratin monoclonal antibodies against cytokeratins which are specific markers of epithelial cancer cells in solid tumors **(Fig 2).**

Flow cytometry detected minimal residual disease in the hematopoietic PBSC of 7 patients (35.0%) in the group I, while in group II bone marrow micrometastasis was detected in 3 (21.4%) patients **(Table 2)**. The nested RT-PCR assay for CK-19 mRNA detected minimal residual disease in 11(55.0%) patients of group I and bone marrow micrometastasis was detected in 5 (36.0%) patients of group II as well as circulating tumor cells (CTC) were detected in peripheral blood of 8 (27.3%) patients of group III **(Table 3;Fig 1)**. CK-19mRNA positive tumor cell was detected in none of the control cases.

The concordance of flow cytometry and RT-PCR was shown in **(Table 4**). The results of 82.4% of the cases were typically the same (either positive or negative) by the two methods, as 100% of the positive cases by flowcytometer were positive by RT-PCR technique and 53% of the positive cases by RT-PCR were also positive by flowcytometer. It demonstrated that RT-PCR was more sensitive than flow cytometry in detecting CK-19 positive cells; *p* value was significant **(*p*-value 0.005)** but flowcytometry is more specific test because there are no false positive results as 25% of the cases with negative flowcytometer were positive by PCR. It has false negative results.

Comparison Between The Presence And Absence Of Ck-19 Mrna As Regards The Clinico-Pathological Prognostic Parameters:

In patients of group I as shown in **(Table 5)**, minimal residual disease was detected in 1 patient (33.0%) with stage II disease, 2 patients (33.3%) with stage III and 8 of 11 patients (72.7%) with stage IV; there was a significant association between tumor stage and CK-19 mRNA by RT-PCR assay **(*p*-value = 0.017**). However there was no statistically significant association between the detection of CK-19 mRNA in the PBSC and the patients' menopausal status, tumor size, histological grade of the tumor and number of involved lymph nodes and hormone-receptor status.

In group II as shown in **(Table 5)**, most patients 8(58%) had primary tumors $\leq$ 2 cm. Larger primary tumors > 2 cm were significantly associated with a higher incidence of micrometastasis than were tumors that were 2 cm or less in diameter (***p*<0.001**). However there was no statistically significant association between the detection of BM micrometastasis (CK-19+ve) and the menopausal status, histological grade of the tumor, lymph node metastasis or hormone-receptor status.

In group III as shown in **(Table 5)**, 30 patients with operable breast cancer were prospectively studied by RT-PCR to evaluate the clinical significance of the presence of circulating tumor cells (CK-19 mRNA +ve cells) in peripheral blood. CK-19 mRNA positive cells were detected in the peripheral blood of 8(27.3%) patients. There was no statistically significant association between the detection of circulating tumor cells in peripheral blood and the menopausal status, stage of the disease, size and histological grade of the tumor, lymph node metastasis or estrogen or progesterone receptor positivity.

Correlation Between Detection Of Minimal Residual Disease In Pbsc And Clinical Outcome:

In group I patients (*n*=20), during the follow-up period of 63 months (range, 15 to 107), 15 patients had developed relapse, 11 out of the 15 patients (73.3%) had CK-19 mRNA positive cells in the PBSC. Furthermore, 11 patients died of breast cancer related causes, 10 out of 11 patients (90.9%) showed CK-19 mRNA positivity **(Table 6)**. The presence of minimal residual disease in the PBSC harvests was significantly associated with increased relapse rates and high risk of death.

The Kaplan-Meier estimates curve of the cumulative disease-free survival (DFS) for the patients with CK-19 mRNA positive and CK-19 mRNA negative cells in the PBCS was shown in **(Fig. 3)**.The presence of CK-19 mRNA positive tumor cells in the PBSC was significantly associated with a decreased DFS **(*p*=0.04)**.

The cumulative overall survival of the patients with CK-19 mRNA positive and negative cells was shown in **(Fig. 4)**. There was no statistically significant difference in overall survival between patients with occult residual cells in PBSC and patients without occult residual cells **(*p*=0.07).**

Correlation Between Bone Marrow Micrometastasis And Clinical Outcome:

In group II patients (*n*=14), during the follow-up period of 28 months (range, 7 to 62 months), four out of 14 patients (29%) relapsed, 3of them (75%) had CK-19 mRNA positive cells in the bone marrow **(Table 6)**. On the other hand, 3/14 patients (21%) died, 2 of them (67%) had CK-19 positive cells in the bone marrow. Patients with bone marrow micrometastasis had a higher risk of relapse **(*p*=0.007)** and death **(*p*=0.01)** than patients without bone marrow micrometastasis.

The Kaplan-Meier estimates curves of the cumulative disease-free survival (DFS) and overall survival (OS) for the CK-19 mRNA positive and negative patients were shown in **(Fig. 5** and **6)** respectively. The presence of disseminated tumor cells in the bone marrow of patients was significantly associated with a decreased DFS **(*p*=0.007)** and poor overall survival rate **(*p*=0.01).**

Table 1B: the Clinico-pathological Characteristics of the Patients in the Studied Groups

	Group I		Group II		Group III	
	No.	%	No.	%	No.	%
Patients enrolled	20		14		30	
Age (years) Mean ± SD Range	47.1± 6.98 (35 - 62)		48 ± 9.8 (30 – 65)		50 ± 10 (30 – 65)	
Menopausal status Premenopausal Postmenopausal	 12 8	 60 40	 3 11	 23 77	 12 18	 41 59
Stage I II III IV (metastatic)	 - 3 6 11	 - 15 30 55	 7 4 3 -	 50 29.1 20.9 -	 10 20 - -	 31.7 68.3 - -
Tumor size T1: ≤2 T2: 2-5 T3: > 5 T4:Infiltration of skin or chest wall	 6 12 1 1	 30 60 5 5	 8 5 1 -	 58 34 8 -	 5 17 8 -	 16.7 56.7 26.6 -
Histological grade 1 2 3	 2 8 10	 10 40 50	 7 6 1	 51.4 41.9 6.7	 14 12 4	 46.7 40 13.3
Lymph nodes involvement 0 1-3 4-9 ≥10	 - 3 9 8	 - 15 45 40	 8 2 4 -	 54.5 18.8 26.7 -	 10 9 11 -	 33 30.4 36.6 -
Receptor status ER $^{+}$ ER $^{-}$ unknown PR $^{+}$ PR $^{-}$ Unknown	 12 8 - 11 9 -	 60 40 - 55 45 -	 9 5 - 4 10 -	 60.8 39.2 - 32 68 -	 17 11 2 8 20 2	 57.1 37.9 5 28.6 65.8 5

ER, estrogen receptor; PR, Progesterone receptor; SD, standard deviation

Table 2: Results of CK- 19 detected by flow cytometry

	Group I *N*=20	Group II *n*=14

	No.	%	No.	%
Flow cytometry + ve	7	35.0	3	21.4
- ve	13	65.0	11	78.6
Total No.	20	100.0	14	100.0

Table 3: Results of CK-19mRNA detected by RT-PCR

	Group I *n*=20		Group II *n*=14		Group III *n*=30	
	No.	%	No.	%	No.	%
RT-PCR + ve	11	55.0	5	36.0	8	27.3
- ve	9	45.0	9	64.0	22	72.7
Total No.	20	100.0	14	100.0	30	100.0

Table 4: Concordance of RT-PCR and flow cytometry results

			RT - PCR		Total
			+ve	-ve	
Flow cytometry	+ve	Count %	10 100.0%		10 100.0%
	-ve	Count %	6 25.0%	18 75.0%	24 100.0%
Total*		Count %	16 47.0%	18 53.0%	34 100.0%

* *p* value = 0.005(Highly Significant)

Table 5: Clinico-pathological prognostic characteristics and frequency of CK-19 mRNA-positive cells in the studied groups.

	Group I							Group II							Group III						
	All patients	CK-19 mRNA$^+$		CK-19 mRNA$^-$		*P value	Sig.	All patients	CK-19 mRNA$^+$		CK-19 mRNA$^-$		*P value	Sig.	All patients	CK-19 mRNA$^+$		CK-19 mRNA$^-$		*P value	sig.
	No.	No.	%	No.	%			No.	No.	%	No.	%			No.	No.	%	No.			
Patients enrolled	20	11	55.0	9	45.0			14	5	36	9	64			30	8	27.3	2	2.7		
Menopausal status Premenopausal Postmenopausal	12 8	8 3	66.7 37.5	4 5	33.3 62.5	0.510	NS	3 11	1 4	38.0 35.0	2 7	62.0 65.0	0.953	NS	12 18	3 5	27.3 27.4	3	2.7 2.6	.989	S
Stage I II III IV (metastatic)	- 3 6 11	- 1 2 8	- 33.0 33.3 72.7	- 2 4 3	- 67.0 66.7 27.3	0.017	S	7 4 3 -	2 1 2 -	29.0 34.9 60.0 -	5 3 1 -	71.0 65.1 40.0 -	0.380	NS	10 20 - -	3 5 - -	29.4 26.4 - -	5	0.6 3.6	.686	S
Tumor size T1: ≤2 T2: 2-5 T3: > 5 T4:Infiltration of skin or chest wall	6 12 1 1	3 7 - 1	50.0 58.0 0 100.0	3 5 1 -	50.0 42.0 100.0 -	0.080	NS	8 5 1 -	2 2 1 -	25.0 40.0 100.0 -	6 3 0 -	75.0 60.0 0.0 -	<0.001	HS	5 17 8 -	2 4 2 -	40 23.5 25.0 -	3	0.0 6.5 5.0	.994	S
Histological grade 1 2 3	2 8 10	1 3 7	50.0 38.0 70.0	1 5 3	50.0 62.0 30.0	0.660	NS	7 6 1	2 3 1	29.0 50.0 100.0	5 3 0	71.0 50.0 0.0	0.534	NS	14 12 4	4 3 1	29.3 26.1 20.0	0	0.7 3.9 0.0	.664	S
Lymph nodes involvement 0 1-3 4-9 ≥10	- 3 9 8	- 2 4 5	- 67.0 44.0 63.0	- 1 5 3	- 33.0 56.0 37.0	0.190	NS	8 2 4 -	3 1 1 -	38.0 50.0 25.0 -	5 1 3 -	62.0 50.0 75.0 -	0.130	NS	10 9 11 -	3 2 3 -	28.3 26.5 27.1 -		1.7 3.5 2.9	.979	S
Receptor status ER$^+$ ER$^-$ Unknown	12 8	8 3	66.7 37.5	4 5	33.3 62.5	0.370	NS	9 5	3 2	33.0 40.0	6 3	67.0 60.0	0.275	NS	17 11 2	4 4 0	26.1 32.8 0.0	3	3.9 7.2 00.0	.370	S
PR$^+$	11	7	63	4	36.			4	1	25.	3	75.0			8	3	39				

PR-unknown	9	4	.6 44 .4	5	4 55. 6	0. 66 4	NS	10	4	0 40. 0	6	60.0	0.42 2	N S	20 2	5 0	.1 24 .5 0. 0	5	0.9 5.2 00.0	.0 68	S

Abbreviations: ER, estrogen receptor; PR, progesterone receptor, p values for the comparison of numbers of patients were calculated by the Chi- Square, p value <0.05 means significant, p value <0.001 in highly significant (HS), Sig. for significant, NS for non significant.

Table 6: Incidence of disease recurrence and deaths according to the detection of CK-19 mRNA positive cells

	Group I							Group II						
Clinical outcome	No.	CK-19 mRNA+		CK-19 mRNA-		*P value	Sig.	No.	CK-19 mRNA+		CK-19 mRNA-		*P value	Sig.
		No.	%	No.	%				No.	%	No.	%		
	20	11	55.0	9	45.0			14	5	36.0	9	64.0		
Relapse of disease Yes No														
No	15 5	11 0	73.3 0.0	4 5	26.7 100.0	0.04	S	4 10	3 2	75.0 27.0	1 8	25.0 73.0	0.007	S
Breast cancer death Yes														
No	11 9	10 1	90.9 11.0	1 8	9.1 89.0	0.07	NS	3 11	2 3	67.0 27.0	1 8	33.0 73.0	0.01	S

* *p* values for the comparison of numbers of patients were calculated by the Chi- Square.

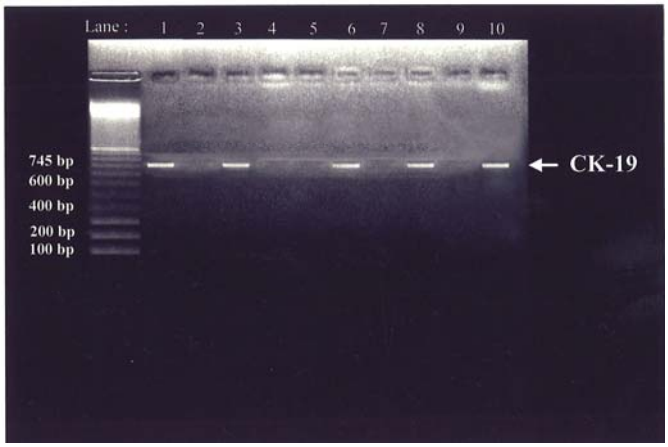

Fig. 1: Gel electrophoresis showing CK-19 positive cells in bone marrow aspirates Detection of occult breast cancer cells in bone marrow aspirates of stage I, II, III breast cancer patients. A detectable band (745 bp) is seen for patients' no.1, 3, 6, 8 & 10

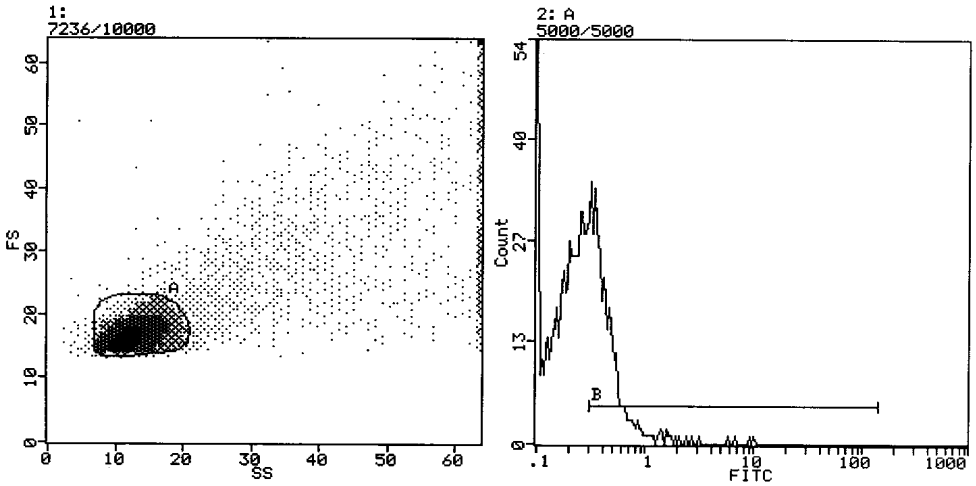

Fig. 2: Flow cytometric run for detection of CK in a studied patient (positive sample).

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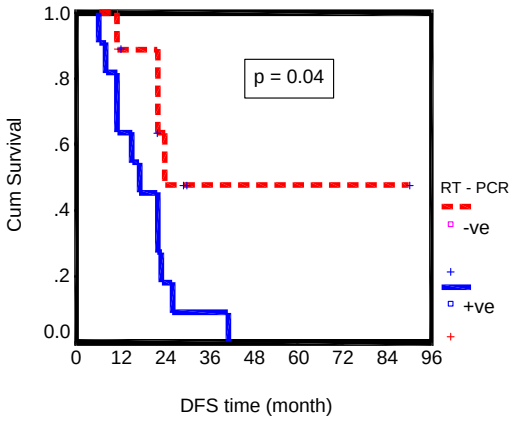

Fig. 3: Disease free survival according to PBSC positivity for CK19 in breast cancer patients.

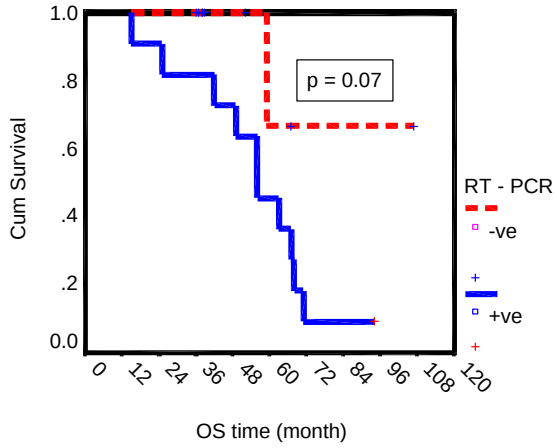

Fig. 4: Overall survival according to PBSC positivity for CK19 in breast cancer patients.

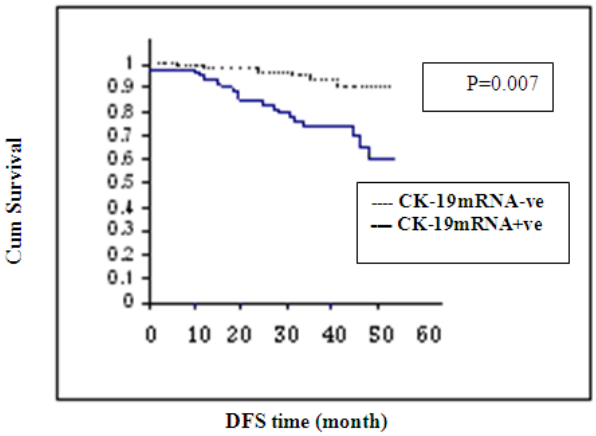

Fig. 5: Kaplan–Meier estimates of the cumulative disease free survival for CK-19mRNA detection in the bone marrow.

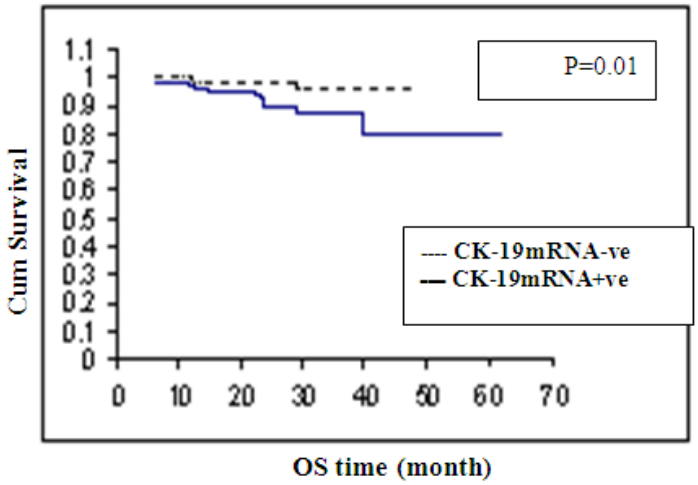

Fig. 6: Kaplan–Meier estimates of the cumulative overall survival for CK-19mRNA detection in the bone marrow

Discussion:

Breast cancer is considered a systemic disease because early tumor cell dissemination may occur even in patients with small tumors. The established prognostic factors of tumor size and axillary lymph node status contained in the tumor-node-metastasis classification of breast cancer are incapable of identifying a subgroup of women who, although they present with early-stage disease, may be at high risk of relapse and death. This is due to the early dissemination of malignant cells from the original tumor through hematogenous and/or lymphatic pathways and the failure of the adjuvant treatment to eliminate them **(Pantel and Brakenhoff, 2004)**.

In this study, CK-19 mRNA-positive cells have been detected by RT-PCR in the peripheral blood of 8 patients (27.3%) of group III. However, in these patients there was no statistically significant correlation between the detection of CK-19 mRNA-positive cells in the peripheral blood and important parameters of tumor burden, such as stage of disease, size and histological grade of the tumor, and number of involved axillary lymph nodes.

There have been few studies regarding CTCs and early breast cancer, the reported CTC positivity rate has ranged from 9.4% to 48.6% (Riethdorf *et al*., 2010, Apostolaki *et al*., 2009, Ferro *et al*., 2010, Graves & Czerniecki, 2011). Moreover, Chen *et al* 2010., reported that the detection rate of CTCs in the blood of patients with early breast cancer was 54.0%, significantly higher than in patients with benign breast disease and healthy blood donors (p=0.002,p= 0.000 respectively). Also, it is interesting to note that only 10% of 1767 patients with early disease had more than one CTC per 23 ml of peripheral blood when tested with the automated Cell Search System (Rack *et al*.,2008)

The results of the present study of patients in group III, demonstrated that occult cancer cells were detected in the peripheral blood of 28.3% of the patients with histological negative axillary lymph nodes (N0) using nested RT-PCR assay. The detection of CK-19 mRNA positive cells in the PB of these patients was the only indication of dissemination of tumor cells. This is in agreement with clinical data indicating that 20-30% of patients with N0 breast cancer may develop local or distant metastases, suggesting that these patients have occult micrometastatic disease and were under-staged (Stathopoulos *et al*., 2005).

In a study by Wiedswang *et al*., (2006), they compared the clinical significance of an increased sensitivity method (negative immunomagnetic isolation) for detection of CTC with a standard DTC analysis. PB and BM were prospectively collected from 341 patients with early breast cancer. CTC were present in 10% and DTC in 14% of the patients, indicating that PB contains tumor cells with malignant potential at reduced frequency when compared to BM. Both CTC and DTC were significantly associated with reduced disease free survival (DFS) and breast cancer specific survival (BCSS). On the other hand, in metastatic breast cancer, a strong association between circulating tumor cells, as detected by a higher volume (7.5 ml) peripheral blood analysis and clinical outcome was reported by Cristofanilli *et al*., (2005).

In this work, BM samples were analyzed for micrometastasis by RT-PCR and flow cytometry. CK-19 mRNA positive tumor cells were detected in BM of 36% of patients using RT-PCR while cytokeratin-positive cells were detected in 21.4% of patients by flow cytometry. Also in our study in group II, it was found that larger primary tumors were highly significantly associated with a higher incidence of BM micrometastasis than were tumors of 2 cm or less in diameter ($p<0.001$). Although histological involvement of ALNs is the

standard risk factor used for prognostic evaluation, we found there was no significant difference in the incidence of BM micrometastasis in patients with lymph node metastasis and those without it. In addition, there was no statistically significant association with the menopausal status, histological grade of the tumor and hormone-receptor positivity.

Similar to our results, there was no significant correlation between the occurrence of CTCs or DTCs with tumor classification, tumor histologic grade, positive ER status, positive PR status, or positive HER2status, and axillary LN status (Krishnamurthy *et al*., 2010). It was reported earlier that the detection of CTCs before the initiation of adjuvant chemotherapy in patients with operable breast cancer, irrespective of the presence of axillary lymph node involvement, or an estrogen/progesterone or HER2 receptor expression (Daskalaki *et al*., 2009)

On the other hand, Braun *et al*., (2005)found a significant correlation between bone marrow positivity and tumor size, nodal status, tumor grading and hormone receptor-negative tumors of the studied patients ($p<0.001$ for all variables).

In this study during the follow-up period of 28 months, patients with BM micrometastasis had a higher risk of relapse ($p=0.007$) and death ($p=0.01$) than patients without BM micrometastasis. These results clearly demonstrate the significant and independent prognostic value of the presence of CK-19 mRNA in the bone marrow of patients with early stages breast cancer. Our results were consistent with a survey on a total of more than 3500 stage I-III breast cancer patients, the incidence of DTC in bone marrow detected by immunocytochemistry ranged from 13 to 43% and presented an independent prognostic factor for reduced disease free survival or poor overall survival (Braun and Naume, 2005). This is in agreement with Molloy *et al* 2011who reported that CTC and DTC in early caner is also an independent factor associated with decreased DFS and OS. Similar results were shown in many studies (Dasklaki *et al*, 2009; Saloustros *et al*, 2011; Pierga *et al*, 2012). However, the survival analysis of large cohorts of breast cancer patients and further immunophenotyping of DTCs has indicated that only a subset of DTCs might be relevant for metastatic relapse. Thus, the characterization of the biologic properties of DTCs is of utmost importance (Panabières *et al*, 2009). In addition, 28.4 and 32.2% of patients without detectable CTCs or DTCs, before chemotherapy, respectively, presented with CK-19 mRNA-positive cells after the completion of adjuvant chemotherapy. This observation suggests that chemoresistant cells in patients identified as DTC(−) or/and CTC(−) because of a low tumor load that is undetectable by the assay could proliferate during the administration of adjuvant chemotherapy and reach the detection limit after the end of treatment (Daskalaki *et al*, 2009).

The above observations point to the presence of subpopulations of CTCs or/and DTCs that bear an inherent resistance to the chemotherapy regimen used as adjuvant treatment. The presence of chemoresistant CTCs or DTCs has been previously described (Xenidis *et al*, 2009) and attributed to the low proliferative capacity of these cells (Muller *et al*, 2005). Therefore, it could be hypothesised that persistence of occult tumor cells in the bone marrow (Wiedswang *et al*, 2004, Janni *et al*, 2011) or peripheral blood (Xenidis *et al*.,2006, 2007) after adjuvant chemotherapy or during follow-up is associated with an adverse clinical outcome and could be used as a surrogate marker for the efficacy of the adjuvant treatment used.

However, even though occult tumor dissemination may occur early, not all patients with detectable CTCs/DTCs will develop overt metastases. Meng *et al* looked at 36 breast cancer patients 7 to 22 years after mastectomy and found that 36% had evidence of CTCs with no evidence of clinical disease (Meng *et al*, 2004). Similarly, in a large pooled analysis by Braun *et al*., only half of DTC-positive breast cancer patients relapsed over a ten-year period (Braun *et al*, 2005). These CTCs/DTCs may be in a state of dormancy and the exact mechanism of transition to overt metastases is unclear. Likely factors involved in this transition include host microenvironment, host immune response, and genetic changes in the tumor cell. It was also reported that the detection of CTCs and/or DTCs after chemotherapy was not associated with increased risk for relapse or death (Daskalaki *et al*, 2009).

However, in patients with early breast cancer, who are asymptomatic, repeated and frequent bone marrow aspirations for the detection of DTCs may not be easily acceptable. Conversely, the use of blood for detecting CTCs is more convenient and could be easily acceptable by patients, thus representing a valuable alternative solution. Although some previous studies (Ismail *et al*, 2004; Pierga *et al*, 2004; Muller *et al*, 2005; Benoy *et al*, 2006; Wiedswang *et al*, 2006) have shown a correlation between the detection of DTCs and CTCs, this fact has not been unanimously accepted. This could be due to the fact that in most studies, the comparison of the detection of CTCs and DTCs was carried out using immunocytochemical assays, which, in general, have a lower sensitivity compared with molecular assays (Daskalaki *et al*, 2009). The lack of a complete overlap between the presence of CK-19 mRNA-positive CTCs and DTCs either before (Ismail *et al*, 2004; Pierga *et al*, 2004; Muller *et al*, 2005; Wiedswang *et al*, 2006) or after chemotherapy (Daskalaki *et al*, 2009) could be related to the fact that occult tumor cells are rare events and therefore their evaluation is greatly influenced by sampling variability. In addition, it has been suggested that blood represents a temporary compartment for disseminated cells (Muller *et al*, 2005), whereas only a subpopulation of CTCs can settle in distant organs such as the bone marrow (Muller *et al*, 2005).

Although the prognostic value of DTC detection in BM has been in several studies and other reports reported evaluating the prognostic significance of disseminated tumor cells in blood and bone marrow concluded that the detection of CTCs is prognostically inferior to the detection of DTCs (Benoy *et al*, 2006; Wiedswang *et al*, 2006), the test has not come into routine clinical practice. However, for convenience, the detection of CTCs could be used as an alternative to bone marrow for the identification of occult breast cancer cells and for monitoring minimal residual disease. This is of particular interest in the light of the results of recent trials, indicating that sequential treatment during the disease-free period may improve OS in breast cancer (Coombes *et al*, 2007). Conceivably, secondary adjuvant treatment could be administered in patients selected on the basis of persisting CTCs. However, this should be tested in prospective clinical trials (Dasklaki *et al*, 2009).

In the present study, we analyzed retrospectively cyopreserved PBSC apharesis samples of 20 patients (group I) with metastatic breast cancer (MBC) and high risk primary breast cancer (HRPBC). Minimal residual disease (MRD) was detected in 55% of patients using RT-PCR, while in 35% of patients by flow cytometry methods as observed. The incidence of MRD increased significantly with advanced stage of disease, with 33% for stage II-III and 72.7% for stage IV patients (P=0.017).After follow up period of 63 months 15/20 had developed relapse, 11/15 (73.3%) had CK-19 mRNA positive cells in PBSC. Furthermore, 11/20 patients died of breast cancer related causes, 10/11 patients (90.9%) showed CK-19 mRNA positive cells. It was concluded that the presence of CK-19 mRNA positive cells in PBSC was significantly associated with a decreased DFS (P=0.04). There was no significant difference in overall survival between patients with MRC in PBSC and patients without occult residual cells (P=0.07).

In fact, according to multivariate analysis results, marrow involvement was the only significant predictor for blood stem cell product contamination. Patients without marrow involvement who had fewer apharesis procedures were also observed to have a significantly lower incidence rate of blood stem cell contamination than patients who had more procedures (P < or =0.008), and patients who received combined chemotherapy and cytokine mobilization therapy had less contamination than patients who received cytokine alone (P = .0001). Patients with negative blood stem cell products had significantly longer progression-free survival (PFS) and overall survival (OS) than did patients with positive blood stem cell products (Pecora *et al*, 2002). This is in agreement with Arpaci *et al* who reported that tumor cells in apharesis decrease DFS in stage III (Arpaci *et al*, 2009).

On the other hand, there was no significant correlation between the presence of detectable tumor cells in the graft product and outcome in early breast cancer (Reed *et al*, 2003). Also, it was reported that contamination of apharesis products remains a rare event, which does not seem to affect clinical evolution, even when reinfused into the patient (Viret *et al*, 2003).

Syme *et al* mentioned in his study on 83 cancer patients that 11 patients had more than three cancer cells detectable in their APs (apharesis products) and 72 patients were shown to have less than three cells detectable. When patients with more than three cells were compared to patients with 0-3, we found statistically significant differences in progression-free survival. We also found a significant difference in overall survival (OS) between the two groups. No difference was observed in OS since the time of diagnosis. He concluded that patients with more than three contaminating cells in their APs have micrometastases and represent a poor prognosis group (Syme *et al*, 2003).

In a study done by Nieto *et al*, he reported that, in HRPBC patients, at a median follow-up of 7 years, the presence of OTCs correlated with worse event-free survival (P = .007) and overall survival (P = .002). In the MBC group, OTCs correlated with worse event-free survival (P = .04), but not overall survival (P = .2). In multivariate analyses, the presence of OTCs had an independent adverse effect on outcome in HRPBC, but not MBC. Our observations imply a direct role of OTCs in post transplantation relapse in HRPBC (Nieto *et al*, 2004).

However, the clinical significance of CTCs remains to be established because of the extremely small number of CTCs in peripheral blood as compared with the number of blood cells. Technical problems (e.g. reproducibility and reliability) in the detection of CTCs also remain to be solved.

CTCs have been confirmed to be a useful prognostic factor. This system was also suggested to be useful for monitoring treatment response in patients with metastatic breast cancer and was approved by the United States Food and Drug Administration in 2004. Measuring CTC counts can facilitate the early prediction of treatment response and thereby avoid unnecessary therapy. CTCs may also be a useful biomarker for molecular targeted agents, enabling the identification of patients most likely to respond to a given treatment and facilitating treatment selection. However, the widespread use of CTC monitoring as a routine examination requires a further improvement in measurement sensitivity, the establishment of criteria for quantitative and qualitative evaluations, and additional clear-cut evidence supporting the clinical significance of CTCs. We expect that CTCs will be established to be a new diagnostic and therapeutic index for breast cancer.

Further prospective studies including a larger number of patients are needed to clarify which is the most relevant time point for the evaluation of minimal residual disease before or after adjuvant chemotherapy.

Moreover, the reproducibility and stability of the assays used should also be evaluated in a routine clinical setting.

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