A new comprehensive gene expression panel to study tumor micrometastasis in patients with high-risk breast cancer

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Abstract. The incidence and prognostic relevance of bone marrow (BM) and leukapheresis (PBPC) tumor cell contamination (TCC) in breast cancer patients is still to be circumstantiated. We developed a new comprehensive gene expression panel to study cytokeratins (CK), maspin (MAS) and mammaglobin (MAM) as possible predictors of prognosis. Forty-eight patients undergoing high dose chemotherapy (HDCT) and PBPC support were enrolled and analyzed for TCC on 116 PBPC apheresis and 96 BM obtained at basal conditions. All of the patients were evaluated by reverse transcriptase nested PCR (RT-PCR) for MAM and MAS gene expression and by immunocytochemistry (ICC) and nested RT-PCR to evaluate CK expression. PBPC and BM frequency of CK-positive (+) cells was 12-13% by ICC and 71-73% by RT-PCR respectively. Sixty-seven percent of CK ICC$^+$ samples were MAM RT-PCR$^+$ and 89% of them were MAS RT-PCR$^+$. PBPC and BM frequency of MAM$^+$ cells was 21% and 31% respectively, while for MAS$^+$ cells it was 48% and 52% respectively by RT-PCR. After 71 mo median FU, 16 patients (33%) relapsed and 14 (88%) had BM/PBPC TCC. No marker had an impact on overall survival (OS) but MAS expression on BM and MAM expression on PBPC correlated with a statistically significant improved (p=0.05) and worsened RFS (p=0.06) respectively. These data confirm the activity of MAM as a negative prognostic factor and show for the first time that MAS could work as a tumor suppressor gene even in a clinical setting, since it protects from recurrence.

Introduction

The reinfusion of mobilized PBSC has become the standard procedure in autologous transplantation after HDCT for different neoplastic diseases. This is supported by a high number of phase II-III clinical trials, showing that patients treated with this stem cell source engraft more rapidly than those treated with BM (1). HDCT with hematopoietic support produces high response rates and some long-term disease-free survivors, in patients with metastatic breast carcinoma (2). Moreover, HDCT treatment in patients with high-risk breast cancer appears to improve relapse-free survival (RFS) and overall survival (OS) if compared to standard CT at least in a randomized trial (3), but it is still a matter of debate.

As many questions still remain unanswered regarding the best methods to detect the presence of tumor cells in peripheral blood, BM and/or apheresis products, the impact of BM and leukapheresis tumor cell contamination (TCC) on patient prognosis and therapeutic outcome must be circumstantiated. However, TCC is common in PBSC samples, raising the issue of whether current techniques are able to identify, reduce, remove or separate them from the stem samples.

Between 4 and 50% of women with primary breast cancer have tumor cells contaminating their bone marrow at the time of diagnosis, as determined by sensitive immunocyto-chemical techniques (4,5). Some investigators correlated micrometastatic disease in the bone marrow with other prognostic factors and determined micrometastatic disease in the bone marrow to be an independent predictor of relapse (6-8).

Other authors suggest that tumor cells in nodes and/or in bone marrow may represent different populations of metastatic clones (7,9). On the other hand, there is now some evidence that TCC of stem cell products may be related to disease relapse, since Brenner and colleagues, demonstrated that previously gene-marked tumor cells, after reinfusion contributed to neoplastic recurrence (10).

Many studies show a correlation between BM TCC and OS (Moss, *et al*, Proc ASCO 16: abs. 90a, 1997; 11-18), but meta-analyses on 20 studies regarding a total of 2,494 patients, failed to clearly demonstrate the impact of BM micrometastatic disease on prognosis. In particular 5 out of 11 studies indicated the presence of epithelial cells in the BM as an independent predictor of short disease-free survival (DFS) and only 2 out of 12 showed a correlation with OS using multivariate analysis. In conclusion meta-analyses confirm the need for further studies using standardized protocols before micrometastasis detection can be used as a prognostic tool for use in the TNM classification (19).

More specific markers are needed to better understand the real impact of TCC on prognosis of breast cancer patients:

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Table I. Characteristics of the patients enrolled in the study.

Patients	Start	Stage	>20 LN+	Schedule	Relapse	Site	Follow-up
1	10/08/98	IIIA	no	T-ICE	18/12/00	bone	14/05/02
2	12/08/98	IIB	no	EC	13/06/01	bone	03/07/02
3	25/08/98	IIB	no	T-EC	no		14/02/01
4	31/08/98	IIB	no	T-EC	no		16/04/02
5	15/09/98	IIB	no	EC	no		12/06/02
6	28/09/98	IIB	no	T-ICE	12/05/99	breast	dead
7	28/09/98	IIIA	yes	T-EC	08/09/99	liver	20/05/02
8	05/10/98	IIA	no	EC	16/11/00	lung and liver	05/04/02
9	12/10/98	IIB	no	T-ICE	01/12/99	brain	dead
10	02/11/98	IIIA	no	T-ICE	22/12/98	abdomen	dead
11	11/11/98	IIA	no	T-EC	no		20/02/02
12	16/11/98	IIB	no	T-EC	16/09/99	brain	dead
13	16/11/98	IIB	no	T-EC	no		19/04/02
14	17/11/98	IB	no	EC	10/05/99	cervical LN	07/06/01
15	17/11/98	IIIA	yes	T-EC	no		15/04/02
16	23/11/98	IIIA	yes	T-EC	no		08/07/02
17	23/11/98	IIB	no	T-EC	01/11/99	bone	dead
18	25/11/98	IIB	no	T-EC	no		05/06/02
19	10/12/98	IIB	no	T-ICE	20/05/99	skin	dead
20	14/12/98	IIB	no	EC	no		10/04/02
21	14/12/98	IIB	no	T-EC	no		16/06/02
22	13/01/99	IIIA	no	ICE	01/02/00	lung and brain	dead
23	18/01/99	IIB	no	T-EC	26/04/02	lung and LN SC	11/07/02
24	25/01/99	IIIA	yes	EC	no		17/04/02
25	04/02/99	IIB	no	EC	no		19/04/02
26	04/02/99	IIB	no	T-EC	no		22/05/02
27	22/02/99	IIB	no	T-EC	16/02/01	liver	10/05/02
28	01/03/99	IIA	yes	T-EC	no		30/01/02
29	01/03/99	IIB	yes	T-EC	01/03/02	bone	14/06/02
30	01/03/99	IIIA	yes	T-EC	28/12/99	liver	dead
31	01/03/99	IIIA	yes	ICE	10/10/01	LN clavear	11/03/02
32	03/03/99	II	no	EC	01/02/02	liver	dead
33	08/03/99	IIB	no	EC	no		10/06/02
34	15/03/99	IIIB	no	ICE	28/09/99	liver and breast	dead
35	15/03/99	IIIA	yes	T-EC	30/01/01	brain	dead
36	18/03/99	IIB	no	T-EC	no		31/01/02
37	06/04/99	IIB	no	T-EC	12/11/01	skin	06/03/02
38	17/05/99	IIB	no	EC	no		16/01/02
39	28/06/99	IIB	no	T-EC	no		18/05/00
40	05/07/99	IIB	no	EC	no		09/05/02
41	06/07/99	IIB	no	T-EC	no		15/05/02
42	03/08/99	IIIA	no	ICE	no		18/03/02
43	09/08/99	IIB	no	T-EC	31/01/02	brain	23/05/02
44	10/08/99	IIB	no	T-EC	no		18/04/02
45	16/08/99	IIIA	no	T-EC	no		17/06/02
46	25/08/99	IIIA	no	ICE	no		02/08/01
47	06/09/99	IIA	no	EC	no		19/06/02
48	07/09/99	IIIA	no	T-EC	05/05/00	lung and liver	dead

Patients received 3 cycles of myeloablative chemotherapy followed by a reinfusion of autologous hematopoietic progenitors. TEC =Taxotere + Epirubicin + Cyclofosphamide; T-ICE = Taxotere + Ifosphamide + Carboplatin + Etoposide, EC = Epirubicin + Cyclofosphamide; ICE = Ifosphamide + Carboplatin +Etoposide.

maspin and mammaglobin might represent good candidates to type this disease.

Maspin is a protein related to the serpin family of protease inhibitors and is widely expressed in epithelial tissues. *In vitro* it shows tumor suppressor activity, acts directly on endothelial cells to stop their migration towards b-fibroblastic growth factor (b-FGF) and vascular endothelial growth factor (VEGF) treatment and limits mitogenesis and tube cellular formation. In a xenograft mouse model of human prostate cancer, maspin blocks tumor growth and reduces tumor-associated microvessel density working as an anti-angiogenesis modulator (20,21). Moreover, Xia and colleagues demonstrated that maspin expression is linked with improved survival of patients with oral squamous cell carcinoma being associated with reduced lymph-nodal involvement and favorable prognosis (22). Shi and colleagues demonstrated in a syngeneic breast cancer mouse model that overexpression of the maspin gene is able to block tumor growth, invasion and metastasis, supporting the concept of a protein with a strong protective role against tumor progression (23).

Mammaglobin, a mammary-specific member of the uteroglobin gene family is a glicoprotein overexpressed in human breast cancer (24). The expression seems to be related to progression from localized to locally advanced and metastatic disease (25).

The present study was designed to answer the pending questions about the significance of TCD in the BM and its correlation with TCC of the apheresis using different standard and innovative markers.

Patients and methods

Patient characteristics. Forty-eight patients were enrolled in the study from September 1998 to September 1999. Informed consent was obtained according to the Helsinki Declaration. Patients' ages ranged between 16 and 65 years. Each had a performance status of 0-1 (ECOG scale), histologically proven breast cancer and underwent surgery followed by adjuvant HDCT with PBPC support.

The primary tumor was classified as T1-T3, N1-2, or M0, (UICC 1993) and the patients entered at least one of the following categories: a) ≥10 involved axillary nodes; b) ≥5 involved axillary nodes and ER-negative primary tumor; c) ≥5 involved axillary nodes and T3 primary tumor. Characteristics of the patients are reported in Table I.

Sample harvesting and processing. Forty-eight patients were analyzed for TCC on PBPC apheresis and BM. BM aspiration was performed under local anesthesia from posterior iliac crest puncture before chemotherapy delivery. Five to ten milliliters of aspirate was collected and lysed by NH_4Cl to remove red cells, resuspended in buffered saline, washed twice and counted. Part of the cell suspension (12-16x10^6 cells) was cytospinned on slides and the rest stored with guanidine isothiocyanate at -80˚C for further RNA analysis.

Immunocytochemical staining. Slides were stained with a commercially available alkaline phosphatase anti-alkaline phosphatase (APAAP) based kit for CK^+ breast cancer cell detection. The identification of epithelial cells is based on the reactivity of murine monoclonal antibody A45-B/B3 with the epithelial cell cytoskeleton, which is mainly composed of cytokeratins. Cells from peripheral blood (PB), PBPC or BM are of mesenchymal origin and their cytoskeleton is mainly composed of vimentin and thus it does not react with the antibody. A range of 9-12x10^6 cells per sample was analyzed by microscopy for the enumeration of breast cancer cells. Peripheral blood nucleated cells from healthy donors were contaminated with 5% breast cancer cell line MCF-7 and used as the positive control of contamination.

Reverse transcriptase-polymerase chain reactions. Total cellular RNA was extracted from cells by QIAamp RNA kit (Qiagen, Hilden, Germany) and treated with a reverse transcriptase enzyme (Super Script II, Gibco, Gaithersburg, MD). The obtained cDNA was amplified for CK, MAM and MAS expression according to Mochinsky *et al* (26), Zach *et al* (27) and Luppi *et al* (28), respectively. Single round RT-PCR using B-actin specific primers confirmed the presence of intact RNA, an adequate cDNA synthesis as well as the absence of inhibitors. Amplified products for CK, MAM and MAS were revealed by single bands of 108-201-175 base pairs respectively on ethidium bromide-stained 2% agarose gel. To reduce false positives for CK transcript we introduced a hybridization step with an internal radiolabelled probe as previously described (41).

Statistical analysis. Progression-free time was calculated from the start of therapy to the date of disease progression. Patients who did not have any progression were censored at their last follow-up time. The progression-free survival was estimated by the Kaplan and Meier method and the log-rank test was used to test for differences between groups of patients.

Results

Specificity and sensitivity

CK hybridization with an internal probe to avoid false positive results. Specificity was tested on 12 cell lines, 13 BM of patients affected by non-epithelial malignancies, 12 BM and PB of healthy donors (Table II). Specificity of the CK-amplified samples was confirmed by hybridization with a specific and ^{32}P-labeled internal probe, showing that not all the RT-PCR amplified products were indeed CK19 transcripts. In fact about 30% of them did not anneal to the probe and did not reveal themselves by autoradiography (41). To determine the RT-PCR sensitivity of the three markers, PBMCs obtained from donors were mixed to decreasing numbers of MCF7 and CG-5 breast cancer cell lines. Sensitivity of CK, MAM and MAS methods was 1x10^{-7}, 1x10^{-6}, and 1x10^{-6} cells respectively.

No false positive results were detected in cell lines, apart for the K-562 leukemic cell line, which expressed MAS-RNA. Moreover, no false positives were found in BM and PB samples used as controls of specificity, in any of the amplification procedures for CK, MAM and MAS as reported in Table II.

CK expression revealed by ICC and nested RT-PCR. By ICC, cells were easily distinguishable from background mononuclear

Table II. Controls of specificity for CK, MAM and MAS expression.

Controls of specificity	No.	CK (RT-PCR)	MAM (RT-PCR)	MAS (RT-PCR)
Healthy donors BMs and PB	12	Negative	Negative	Negative
Non-epithelial malignancies BMs	13	Negative	Negative	Negative
Cell lines	12	Negative	Negative	K-562 positive

Controls of specificity for CK, MAM and MAS expression on 12 healthy donor bone marrow (BM) and peripheral blood samples (PB), 13 bone marrow samples of patients affected by on-epithelial malignancies and 12 cell lines (TF1, CEM, Namalwa, Molt, Huvec, K-562, Sultan Neo, Rap1, Dohh2, Karpass 299, Bonna 12, Jurcatt).

Table III. Number of patients with positive samples for each method and sample is reported in total numbers and percentage respectively.

Samples	CK (IHC)	CK (RT-PCR)	MAS (RT-PCR)	MAM (RT-PCR)
BM	5/48 (12%)	35/48 (73%)	25/48 (52%)	15/48 (31%)
PBPC	6/48 (13%)	34/48 (71%)	23/48 (48%)	10/48 (21%)

cells by bright red staining of cell membrane and cytoplasm. On 116 PBPC and 96 BM specimens, CK^+ cells were detected in 6/48 patients (13%) and in 5/48 patients (12%) respectively (Table III).

Using a nested RT-PCR assay for CK19 followed by specific probe hybridization and autoradiography, a single 175-bp band was identifiable in a much higher percentage of cases, without loosing specificity. Results showed CK19 amplification on PBPC specimens of 34/48 patients (71%), and on BM of 35/48 patients (73%) (Table III).

Southern blotting with a radiolabelled CK-specific probe after PCR amplification, reduced false positive results by 30% (41), demonstrating that pseudogene expression and/ or illegitimate transcription represent a real but avoidable problem.

MAM expression and its relationship with CK expression. Results are reported in Table III and show 10/48 positive samples (21%) on PBPC and 15/48 (31%) on BM. We found a good correlation as expressed by Pearson's coefficent between ICC evaluation for CK and RT-PCR for MAM, since all the ICC CK^+ specimens resulted in an overexpression of the MAM transcript, showing a high reliability of the two methods. No correlation existed when the comparison involved the MAS gene evaluation.

MAS expression and its relationship with CK expression. Forty-eight percent of the patients (23/48) were found to be MAS positive when evaluated on PBPC samples, while 52% of them (25/48) expressed the MAS product on BM, with an inverse relationship when compared to disease relapses (Table III).

Results obtained with the amplification of the MAS gene in the chosen high-risk breast cancer patient group did not show a good correlation with CK amplification or immuno-staining and even with MAM expression as already reported by Lopez-Guerrero *et al* (29).

RFS and OS on the basis of tumor cell detection in BM and/or PBPC

MAS expression protects from disease relapse, while MAM is a marker of poor prognosis. Studying our results on PBPC collections and BM, we tested all of the possible combinations, matching the expression of the 3 markers, CK, MAM and MAS with patient relapses in order to score a possible effect on prognosis. Not one case was found to have a statistically significant correlation between OS in those patients who had tumor cell detection in BM and/or PBSC versus those who do not, independently for the marker or the method used. When RFS was analyzed, MAS expression on BM specimens and MAM expression on PBPC seemed to impact on the probability of relapse (Figs. 1 and 2).

Sixteen out of 48 (33%) patients relapsed in the subset of PBPC and/or BM samples: 4/16 (25%) had positive CK ICC staining, 14/16 (87.5%) had positive CK PCR amplification, 8/16 (50%) had positive MAM PCR amplification and 8/16 (50%) had positive MAS PCR amplification respectively (Table III).

After a median follow-up of 71 months, differences between RFS and OS curves were not statistically significant for CK as a marker of occult micrometastasis either in the bone marrow or in PBPC collections when evaluated either by PCR or ICC (Fig. 1).

Starting at two years, PBPC MAM-positive vs -negative curves on RFS showed a clear trend of diversion, which became nearly statistically significant ($p=0.06$) with longer follow-up confirming our previously reported data (41). On the other hand, MAS expression identified a subset of patients who showed a statistically significant lower relapse rate and thus a better RFS ($p=0.05$). Significance was reached only when we evaluated RFS on BM but neither on PBPC alone or in combination with BM, nor for OS probably due to the low numbers of events reported (Fig. 2).

Interestingly, among the patients found positive for CK and MAM expression in at least one sample, which were considered micrometastatic, the contemporary expression of MAS protects from relapse and patients have a statistically significant advantage concerning DFS (data not shown).

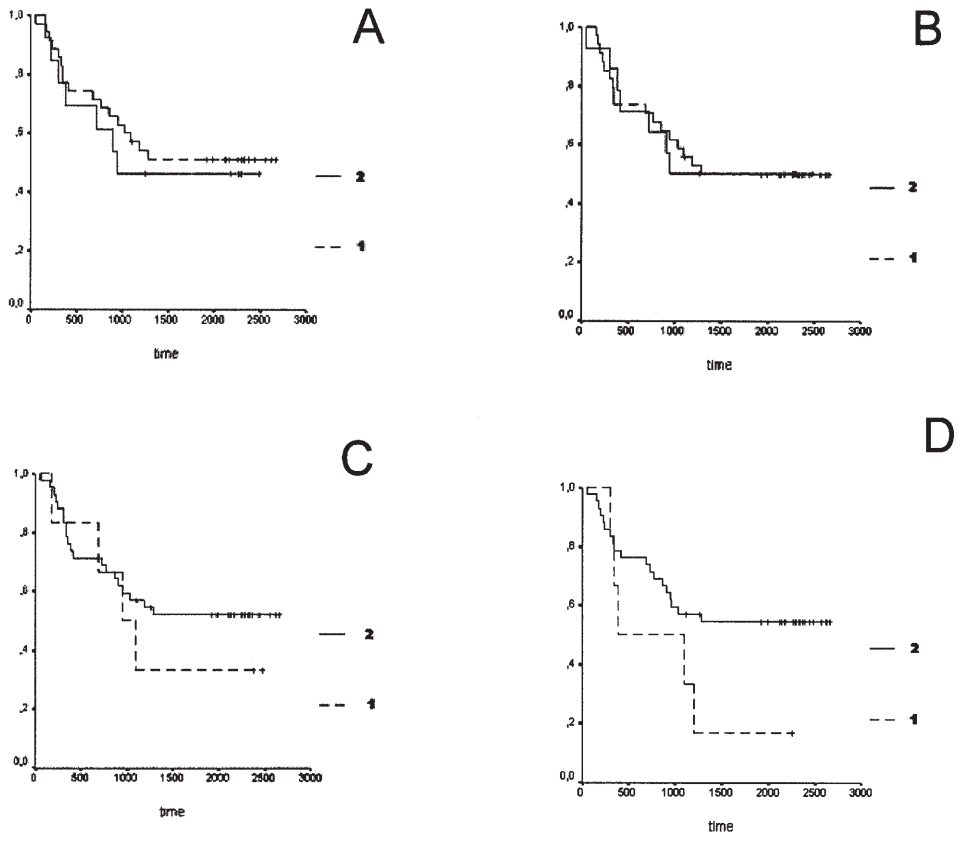

Figure 1. RFS curves for patients expressing CK evaluated by RT-PCR in BM (A), by RT-PCR in PBPC (B), by ICC in BM (C) and by ICC in PBPC (D) respectively. Curve 1: CK positive patients; curve 2: CK negative patients.

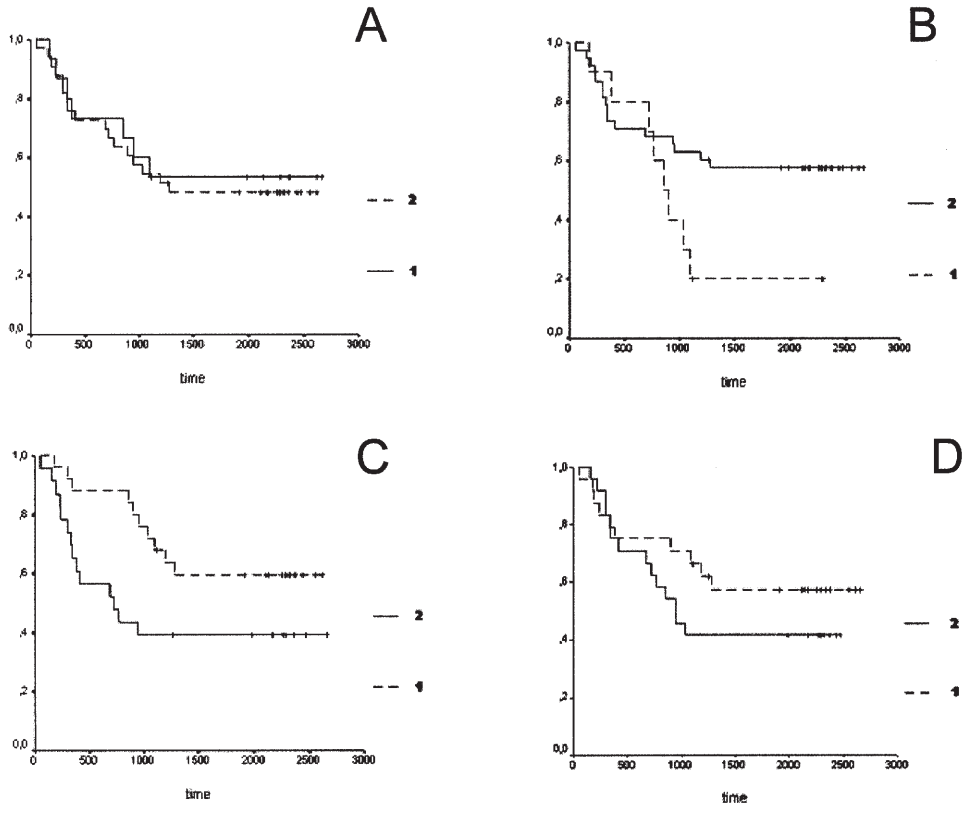

Figure 2. RFS evaluated by RT-PCR in BM of patients MAM$^+$ and MAM$^-$ (A, curve 1 and 2); MAS$^+$ and MAS$^-$ (C, curve 1 and 2). RFS evaluated by RT-PCR in PBPC of patients MAM$^+$ and MAM$^-$ (B, curve 1 and 2); MAS$^+$ and MAS$^-$ (D, curve 1 and 2).

Discussion

The TNM classification offers some fairly good chances to evaluate the probability of relapse in patients with large breast tumors and positive nodes. Expression of estrogen/progesterone receptors, age, and overexpression of oncogenes give other parameters in the decision making after surgery (31).

Unfortunately more than 20% of low-risk breast cancer patients relapse at 5 years and almost 70% of high-risk patients develop a metastatic disease within 10 years despite adjuvant chemotherapy. The presence of bone marrow micro-metastatic disease at diagnosis could partially explain this data.

Since 1981, when immunocytochemical techniques for tumoral cell detection were developed, various groups studying different diseases have demonstrated the presence of occult metastasis in the bone marrow and related it with a worse survival (32-37). These metastases were described as tumoral cells expressing epithelial markers and presenting themselves as single or grouped, the latter having the worst prognostic relevance. They can remain long term in a state of dormancy and be difficult to erase by chemotherapy (38). Better results could be reached using monoclonal antibodies, since they continue to express superficial antigens, which could be targeted by immunotherapeutic agents (38). Moreover, BM micrometastatic cells are believed to belong to different clones from those colonizing nodes as they express different superficial molecules as demonstrated by co-immunostaining experiments (39).

Further characterization of these cells will offer a better understanding of their metastatic potential, but it seems possible to explain the failure of the TNM system, at least in part, by the presence at diagnosis of occult tumoral cells in different sites of the body, other than breasts and lymph nodes.

Identification of tumoral cells in the BM is not yet accepted as a standard procedure to be used in order to decide on adjuvant treatments, but for many scientists it represents a valid prognostic tool to be further circumstantiated.

The lack of standardization of the different techniques and the wide numbers of markers that have been used over the years, are two of the major problems explaining the differences in terms of results and reproducibility observed and reported in the past. In particular the use of immunocytochemical techniques leaves a strong subjective bias in identifying different cell sub-populations and scoring the epithelial from the non-epithelial ones. RT-PCR allows a higher percentage of sample positivity, but increases the number of false positives due to pseudo-gene amplification or illegitimate transcription.

Studies on breast cancer have shown that approximately 20-25% of patients with disseminated disease will have contamination of the PBSC product using routine immunocytochemical analysis (13; Moss TJ, *et al*, Proc ASCO 16: abs. 90a, 1997). Moss *et al* performed immunochemistry (ICC) on 133 PBSC apheresis taken from 48 patients to determine the frequency of circulating carcinoma cells: TCC was found in 10% of cases from approximately 19% of women (Moss TJ, *et al*, Proc ASCO 16: abs. 90a, 1997).

The extent of the disease and its sites of metastases seem to correlate with TCC of the hematopoietic support as well as with the number of tumor cells detected (14).

A still unclear number of patients with histologically negative BM have tumor cells detected in the autologous BM or PBSCs used for transplantation (10,11,16).

As reported by the Duke University BM Transplant Program for high-risk primary breast cancer patients, TCC of the BM transplants and even the TC number were associated with a shorter DFS and OS (17,18). In this study, there was no relationship between the TCC of the BM supports and the sites of relapse, suggesting that TCC could be a marker of residual disease prior to HDCT and not directly contributing to relapse.

Moss *et al* in similar studies on metastatic breast cancer confirmed the data on the poor prognostic value for TCC-positive BM transplants, but failed to obtain significant information about PBSC transplants (Moss TJ, *et al*, Proc ASCO 16: abs. 90a, 1997).

We introduce a novel approach in evaluating the micrometastatic disease in breast cancer patients, using a contemporary evaluation of a multiple panel of genes, including MAS and MAM and a further step on CK RT-PCR amplification with hybridization of an internal probe to enhance specificity without affecting sensitivity.

Our data confirm that a great proportion of high-risk breast cancer patients present BM micrometastatic disease at diagnosis and many of them, undergoing leukapheresis, present TCC in their autografts. Unfortunately some of such patients have breast cancer cells in their apheresis without histologically evident micrometastases in their bone marrow. We failed to demonstrate any direct relationship between the presence of bone marrow tumoral infiltration and TCC of the apheresis products (data not shown). This could be due to the sensitivity of the methods used, or the limitations of the marrow aspiration procedure, which is limited to a single or double space harvest. Moreover no data are available about the possible mobilization of occult tumoral cells from other sites than BM.

Previously published data showed that tumoral epithelial cells, which are present in the apheresis, are clonogenic and potentially able to cause a relapse (16), but we found no evidence that reinfusing tumoral cells could worsen prognosis. We speculate that a cell, when reinfused in the blood, needs to find an idoneal microenvironment in order to settle and begin to proliferate, and it also needs a permissive immunological status of the recipient.

Unfortunately, we failed to reach statistical significance in the evaluation of prognostic relevance of CK expression as a marker of micrometastatic disease and therefore we could not confirm previously published data. In particular we observed a low level of CK positivity by immunocytochemistry when compared to Braun and colleagues, even though each slide was reviewed by at least two experienced and blinded individuals.

When CK was evaluated by PCR the sensitivity reached very high levels without loosing specificity. In fact by using our hybridization strategy, we were able to eliminate up to 30% of false positive results depending on erroneing pseudo-gene amplification or illegitimate transcription. However, CK^+ vs CK^- curves show no difference in RFS advantage probably due to low numbers of events encountered.

MAM seems to be a good marker for specificity, since it is tightly linked to the identification of mammary derived

cells, but did not for sensitivity, probably because the method used was not able to discriminate between different levels of expression in each sample. Recently, Leone *et al* showed by real-time PCR that high levels of expression are necessary to obtain an effect on prognosis. In fact, MAM-overexpressing patients showed a statistically significant augmented probability to relapse when compared to low expressing ones (40-41). We suppose to reach that level on leukapheresis products where, after a long follow-up and more events, we demonstrated a direct relationship between MAM expression and relapse.

MAS showed a protective effect on relapse from breast cancer either independently or when plotted against CK. In fact if we consider micrometastatic all those patients who have been found positive for at least one marker in at least one evaluation, those who are positive also for maspin expression have less probability to relapse (data not shown). However the reduced relapse rate appears evident even when MAS is the only positive marker reaching statistical significance (p=0.05).

Three studies support the hypothesis that MAS works as a protecting factor to epithelial cancer development and metastasis. The first one reports that patients affected by head and neck cancer overexpressing MAS protein experienced a lower incidence of locoregional lymph nodal involvement and had a better prognosis (22). The second shows, *in vitro*, that recombinant MAS inhibits tumor cell migration and invasion. Additionally, MAS acts as an angiogenesis inhibitor in a rat cornea model and in a xenograft tumor model (20,21). The third study confirms our clinical data on an animal breast cancer model showing that MAS-transfected tumors tended to have tumor encapsulation and less necrosis, which were associated with better prognosis and lower invasiveness (23).

Among metastatic cells, those which express the MAS gene do not acquire neo-angiogenic ability and/or migration capacity. On the other hand MAS down-regulation has been demonstrated to be associated with breast cancer development (42-45). The reverse is also true, since increased levels of MAS expression *in vivo* protect against tumor progression as demonstrated in two studies using different transgenic mouse models (46,47).

The mechanism of action of MAS seems to be a direct apoptotic effect on breast carcinoma cells since it has been demonstrated that its endogenous expression increases Caspase expression and induces poly-polymerases (PARP) proteolytic inactivation showing an reverse correlation with mutant p53 (45,48).

We could even speculate that MAS protein is in reality produced by other cells than the metastatic ones and patients who have MAS positivity are not metastatic, but protected from the metastatic event.

In conclusion we failed to show a prognostic relevance of all the different markers we used for breast cancer micro-metastatic disease evaluation, but the multi-gene panel we created is a possible solution for research into the maximum possible sensitivity and specificity. MAS is supposed to serve as a metastasis-protecting factor with some possible future applications in cancer therapy, since systemic delivery of the MAS gene in a syngeneic tumor model inhibits breast tumor progression (49).

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