Bone Marrow and Sentinel Lymph Node Biopsy in Patients with Breast Cancer: from Staging to Ultrastaging?

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Bone marrow (BM) biopsy has been suggested as an independent prognostic tool to improve staging in patients with breast cancer. Twohundred and ten consecutive patients operated for breast cancer from June 2000 to June 2005 who signed an informed consent were enrolled in this protocol. Patients underwent SLN biopsy, and lymph nodes were analysed with serial sections and stained with hematossilin-eosin and immunohistochemistry. At the end of the procedure a BM aspirate from the iliac crest was obtained and 5-10 cc of blood collected. A CEA specific nested reverse transcriptase (RT) polymerase chain reaction (PCR) assay was used to examine BM samples. Results were blinded to both patients and clinicians. The median age of the patients was 56 years (range 34-80), and the median tumor diameter 1,5 cm (range 0.2-4.5). BM aspirates were unsuccessful in ten patients, and RT-PCR was not technically feasible in seventeen women, leaving 183 patients available for analysis of results and follow up. SLN biopsy allowed diagnoses of occult metastases (micrometastases and isolated tumor cells) in 16% of patients (29/183). 25% of T1N0 patients (23/92), 35% of T2N0 patients (6/17), and 44% of N1-2 patients (32/72) were BM+ (p= 0.03). At a median follow up of 35 months 5/122 in the BM- group and 6/61 in the BM+ group have relapsed (p= 0.2), while 1/122 and 4/61 have died of disease (p= 0.04)
In conclusion, ultrastaging of breast cancer patients may identify a substantial subgroup of patients N-/BM- who may not require adjuvant chemotherapy, as well as a subgroup N-/BM+ with a decreased survival who may need more aggressive therapies. Further follow-up is needed to confirm this hypothesis, and several studies are under way.

Key Words:Bone marrow, Breast cancer, Micrometastasis, RT-PCR

Breast cancer staging is essential to stratify prognosis, discuss therapeutic options, direct treatment strategies, and as a whole to review treatment modalities.

Unfortunately, the current TNM classification, although providing a good indication of risk for systemic relapse and survival, is far from being precise. In fact, approximately 40% of node-positive breast cancer patients survive for 10 years or more without recurrence, whereas approximately 30% of axillary negative breast cancer patients develop loco-regional or distant relapse (1,2). This generates confusion as for therapeutic options and follow-up strategies among both clinicians and patients.

Researches have thoroughly analyzed a variety of molecular markers on the primary tumor capable in retrospective studies to serve as prognostic indicators (3,4). However, few of them proved useful, and although a panel of factors may be helpful in delineating the risk of relapse for each single patient, there is no general agreement that they can consistently and powerfully predict prognosis.

The presence of circulating tumor cells has been reported since 1869, and more than 5000 patients have been studied by several groups in the years between 1955 and 1965 (5). However, initial reports have produced conflicting results with poor reproducibility due to low detection rates and to the scarce sensitivity of the technique employed.

The interest in this field has been raised again two decades later with the development of immunohistochemical techniques. Recently, large clinical reviews and clinical reports have consolidated the value of this technique for a variety of solid tumors, and particularly for breast cancer (6). The great majority of the studies published to date have employed an immunohistochemical technique to identify isolated tumor cells in

the bone marrow or in peripheral blood (6).

PCR is an *in vitro* method that allows amplification of a specific DNA sequence, and detection of occult tumor cells is therefore possible through amplification of tumor-specific abnormalities present in the DNA or mRNA of these cells. While standard staining enables to identify 1 cancer cells among 10,000 normal cells, and immunohistochemistry 1 among 100,000, the sensitivity of this technique is 10 times higher (7). To date, only six reports have been published using RT-PCR technique to identify occult tumor cells in the bone marrow or in peripheral blood of breast cancer patients and to assess prognostic significance (8-13)

We report our early experience with bone marrow biopsy analysed with RT-PCR technique in a group of patients operated for primary breast cancer, as well as largest experience with this technique.

Materials and Methods

Consecutive patients with primary breast cancer evaluated to enter the study form June 2000 to June 2005 signed an informed consent approved by the Institutional Review Board. Inclusion criteria were: age between 18 and 80 year, istologic confirmation of a unicentric, T1-T2 cancers without palpable axillary adenopathy, life expectancy >2 years, absence of pregnancy, no previous epithelial malignant neoplasia, and no hemathologic or coagulative deficits. Patients had no radiologic evidence of metastatic disease at the time of surgery, including a negative chest X ray. They were studied with a sentinel lymph node biopsy (SLN) protocol. The technique has been previously reported (14). In brief, SLNs were studied with serial sections at three levels at 100 micron distance each, and each couple of sections was stained with both hematoxylin-eosin and immunohistochemistry (monoclonal antibodies against cytokeratines- pool, MNF 116 (Dako - Denmark).

At the end of surgery, and with the patients under general anesthesia a bone marrow biopsy was obtained from the anterior iliac spine, and 5-10 cc of blood collected.

A CEA specific nested reverse transcriptase (RT) polymerase chain reaction (PCR) assay was used to examine BM samples. Specificity was assessed by examination of a positive control (T47D breast cancer cells) and negative controls (5 healthy bone marrow donors). Results of the RT-PCR assay were blinded to patients and clinicians not to influence the oncologic team with regard to adjuvant therapies and potentially contaminate data on clinical relapses.

Sample preparation

Mononuclear cells were separated by Ficoll-Hypaque density-gradient centrifugation (density, 1.077 g per mole) at 900Xg for 30 mins, then the cells were washed and centrifuged at 150Xg for 5 mins. Total RNA from mononuclear cells was extracted within few hrs, using the RNAqueous kit, following the manufacturer's procedures (AMBION).

Oligonucleotide primers

Oligonucleotides were obtained from Gibco BRL. These primers extend across at least an intron, thus the DNA-derived product was easily distinguished from that expected from amplification of mRNA and, therefore, possible DNA contamination would not pose a significant problem. In Table I the sequences of the primers used for the RT-PCR are reported.

RT-PCR

Reverse transcription was performed using 50 ng of total RNA, and 2.5 units of SuperscriptII reverse transcriptase (Gibco BRL). First strand cDNA was generated with 50 ng of random primers, 10 mMdNTP mix, and 1 unit of RNAsin (Gibco BRL) in 20 µl of final volume. The reaction was incubated at 42°C for 1 hr and at 96°C for 5 mins and then soaked at 4°C. For the first round of PCR a 10 µl aliquot of this reaction was diluted to 50 µl in a mixture with a final concentration of 1X PCR buffer, 0.5 µM of each outer primer for CEA, 1.5mM $MgCl_2$, 0.2 µM DNTPs, and 2 units of Taq polymerase (Invitrogen)) under the following conditions: one cycle of enzyme activation at 94°C for 5 mins, followed by 30 cycles at 94°C for 30 sec (denaturation), 64°C for 1 min (annealing), and 72°C for 1 min (extension), then 72°C for 10 mins (final extension). For the second round of amplification, 30 additional cycles were performed using 5 µl of a 1:100 dilution of the first round product diluted to 50 dilutions, with the same buffer and cycling conditions and using the inner primers for CEA. For PCR product analysis, 30 µl of the second-round product were loaded in a 2% agarose gel, resolved by electrophoresis and visualized under UV light by staining with ethidium bromide (Fig. 1). In each RT-PCR assay, respective controls included a breast cancer cell line RNA (T47D) as reaction-positive control, total human genomic DNA (to detect illegitimate gene marker amplification at the genomic level), PCR reagents and primers without RNA as a reaction-negative control (to reveal contaminations), and amplification control for the housekeeping gene β-actin.

Statistical analysis was performed with a statistical

Table I - Sequences of the primers used for the RT-PCR

Primer sequences		
Gene	5'-3' sequence	Size of PCR product (bp)
CEA outer	TCTGGAACTTCTCCTGGTCTCTCAGCTGG	
CEA outer	TGTAGCTGTTGCAAATGCTTTAAGGAAGAAGC	160
CEA inner	TGTAGCTGTTGCAAATGCTTTAAGGAAGAAGC	
CEA inner	GGGCCACTGTCGGCATCATGATTGG	131
β-actin	CTCTTCCAGCCTTCCTTCCT	
	AGCACTGTGTTGGCGTACAG	116

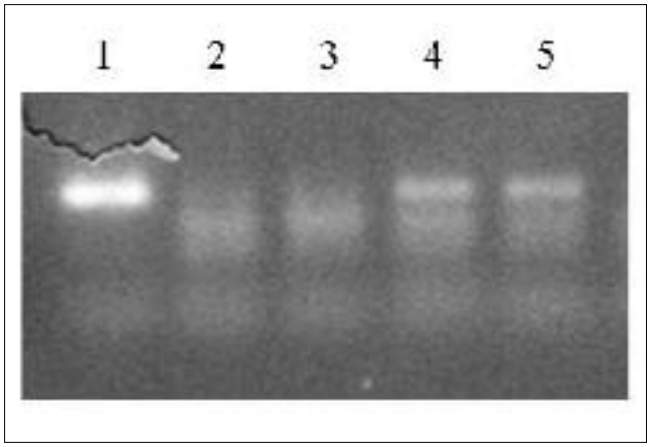

Fig. 1 - Nested RT-PCR for CEA
Lane 1: positive control; lane 2: negative control; lane 3: bone marrow sample negative for CEA expression; lane 4 and 5: two bone marrow samples positive for CEA expression.

package (True Epistat - Richardson, Texas, USA). Chi-square or Fisher exact test were used to compare categorical variables. Significance was defined as 2-tail $p < 0.05$. To date, test results have remained unavailable to patients and clinicians to avoid interference with a possible change of post-operative oncologic strategy, should these data be available.

Follow up was obtained with regular visits every three months for the first three years and quarterly thereafter, and with radiologic and laboratory investigations, including yearly chest-x-ray and liver sonogram, and bone scans. When patients were not available for regular visits, telephone interviews were obtained to check on the disease status. No patient was lost to follow up.

Results

Two hundred and ten patients were enrolled in the study, with a median age of 56 years (range 34-80), and a median tumor diameter of 1,5 cm (range 0.2-4.5). BM aspirates were unsuccessful in ten patients, and RT-PCR was not technically feasible in additional seventeen women, leaving 183 patients available for analysis of results and follow up. The patients underwent a variety of loco-regional procedures, usually under general anesthesia, including wide local excision (n=133; 73%), or mastectomy (n=50; 27%), and axillary node staging (limited to a sentinel lymph node biopsy in 95 patients, 52%).

Results of SLN biopsy have been previously reported in our group of patients (14). In brief, beside being an accurate, mini-invasive and sensitive tool to diagnose axillary lymph node metastases, SLN biopsy allowed the diagnosis of micrometastases (< 2 mm diameter) or isolated tumor cells (< 0,2 mm diameter) in approximately 16% of our patients (15). Most of these low-volume or occult metastases would have not been diagnosed without serial sectioning of the sentinel lymph node or immunohistochemical staining.

Results of bone marrow RT-PCR for CEA are described in Table II. In particular, 61 patients resulted positive (33%), and specifically 25% of T1N0 patients (23/92), 35% of T2N0 patients (6/17), and 44% of N1-2 patients (32/72) were BM+ ($p= 0.03$). Tumor histology (ductal versus non ductal), grade and hormone receptor status were not associated with bone marrow positivity at the univariate analysis (Tab. III).

At a median follow up of 35 months eleven adverse events were reported, including five deaths and six diagnosis of metastatic disease (three bone metastases,

Table II - Results of staging with sentinel lymph node biopsy along with bone marrow RT-PCR for CEA

STAGE	POSITIVES/ N	PERCENT POSITIVE
T1N0	23/92	25%
T2N0	6/17	35.3%
T3N0	0/2	-
T1N1	19/36	52.8%
T1N2	2/6	33.3%
T2N1	7/20	35%
T2N2	2/7	28.6%
T4N2	2/3	66.7%

Table III - Association between tumor characteristics and bone marrow positivity at univariate analysis

CHARACTERISTIC	POSITIVES/ N	PERCENT POSITIVE
GRADE 1	9/29	31%
GRADE 2	21/57	37%
GRADE 3	30/87	34%
RECEPTOR POSITIVE	53/150	35%
RECEPTOR NEGATIVE	9/31	29%
DUCTAL HISTOLOGY	52/155	34%
NON DUCTAL HISTOLOGY	9/28	32%

two liver metastases, and one generalized disease). These occurred in 6/61 patients in the BM+ group (10%) and in 5/122 patients in the BM- group (4%) (p= 0.2). Five patients died of disease, four in the BM + group and 1 in the BM- group (p=0.04).

Discussion

Bone marrow biopsy for systemic micrometastases has been investigated for a variety of cancers other than breast primary, including esophagus, stomach, colon, ovary and lung. This has been correlated with a decreased survival, disease-free survival and early relapse in case of positivity (16-22).

To date, at least 25 studies involving 5747 breast cancer patients have been reported to evaluate the prognostic relevance of minimal bone marrow disease, and a positive finding has been described in 15 of them (6). In addition, a recent pooled analysis of 4703 such patients has been reported (23). Most of these patients had T1-T2 cancers, and the majority were node negative. Micrometastases were detected in 30.6% of the patients, and this finding was correlated with tumor diameter, axillary lymph node status, hormone receptor status, and histologic grade. In the multivariate analysis, bone marrow positivity was an independent predictor of poor outcome, and outperformed the traditional prognostic variables for survival. In patients with metastastic breast cancer, circulating tumor cells have also been recently studied, and one group of researchers found that its number is an independent predictor of progression-free and overall survival (24).

Therefore, bone marrow testing of patients with primary breast cancer has an incredible potential for clinical application, as standard staging is far from being accurate. Today, women with breast cancer, even if potentially cured, suffer a tremendous psychological burden as the risk of relapse, even many years after diagnosis, is real. Consequently, clinicians are frequently faced with the dilemma either to overtreat or risk potential undertreatment. Especially in lymph node negative patients further prognostic factors are urgently needed. Indeed, bone marrow positivity by immunohistochemical technique has been detected in up to 29% of patients in this group, making them a target for more aggressive therapy as well as allowing stratification for adjuvant therapy trials and review of results (22). Moreover, circulating tumor cells may be more appropriate targets for systemic therapies because they should be more readily accessed by them. However, the majority of these micrometastatic tumor cells may be non-proliferative (G_0 phase) (25), and standard cytotoxic chemotherapies aimed at proliferating cells less effective. This might explain, in part, the failure of chemotherapy in some adjuvant settings. Therefore, therapies directed towards both dividing and quiescent cells, such as antibody-based therapies directed against HER-2/neu, gain considerable interest (26).

Another potential application of bone marrow analysis is the evaluation of response, in positive cases, after chemotherapy treatment. The possibility of monitoring in vivo the therapeutic effectiveness after adjuvant therapy has been evaluated by several pilot studies in which the overall prevalence of positive bone marrow findings, before and after chemotherapy, resulted essentially unchanged (27) or diminished but not completely eliminated (28,29). However, three recent studies have recently examined the prognostic value of persistent positivity of circulating tumor cells after chemotherapy

in bone marrow (30,31) or peripheral blood (32). In all of them, this finding was associated with an increased risk of relapse and cancer associated death.

A variety of techniques have been described to analyse the bone marrow aspirate. The majority of studies have employed immunohistochemistry with a pool of monoclonal antibodies against cytokeratines. This has the advantage of simplicity and reproducibility, false positives are rare and generally due to spurious staining of plasmacytoid cells.

However, a molecular approach has also been described using a polymerase chain reaction (PCR)-mediated amplification of tumor cells DNA by reverse transcription of mRNA (RT-PCR). The test proves extremely sensitive, so that current data indicate the possibility to detect 1 tumor cell diluted with 1-10 million normal cells (33). Although standard PCR provides sensitive detection, a critical point is the inability to quantitatively distinguish trace amounts of gene expression from robust expression in metastatic disease. As a result, many investigators have considered PCR technology problematic for clinical investigation, as false positive results secondary to illegitimate transcription or template contamination may occur. Real-time RT-PCR technology now enables to address the issue of quantitation, and many investigators are trying to apply this technology in practice.

The specificity of RNA-based markers is another critical issue due to low-level illegitimate expression of relevant markers in surrounding non-malignant cells and for the fact that distinction between viable and nonviable cells is impossible (34). All these technical problems have raised concerns whether this test, either by immunohistochemistry or RT-PCT, should be included in the standard staging system. Additionally, there are still numerous contradictory findings regarding the prognostic significance of disseminated tumor cells, and the percentage of patients with positive bone marrow findings varies greatly in different studies (35). Finally, despite the fact that many studies report clinical relevance for detection of disseminated tumor cells, the biologic significance of the presence of these cells remains to be substantiated. The present prevailing view is that the metastastic process is inefficient and that only few released tumor cells are able to develop overt clinical metastases over time (36)

To date, only few studies employing the RT-PCR technique have been published in literature to evaluate the role of occult micrometastases in the bone marrow of breast cancer patients. In one such study, 111 patients were evaluated with RT-PCR for mammoglobin, a sensitive molecular marker for breast cancer (13). The authors demonstrated that a positive finding was an independent prognostic predictor along with axillary node and estrogen receptor status.

Our study is the largest report in the literature, so far, using RT-PCR technique to detect tumor cells in the bone marrow of breast cancer patients, and has unique features as our patients have been studied with both sentinel lymph node and bone marrow biopsy.

Sentinel lymph node biopsy is now considered the standard of care for breast cancer patients as it allows identification of minimal metastatic disease in the axilla with minimal morbidity. Therefore, the staging may result enhanced, and recent reports indicate that even minimal sentinel lymph node involvement may have a prognostic significance (37,38). The rationale is that the use of this staging technique, along with bone marrow analysis may render staging, treatment and follow-up strategies more targeted.

RT-PRC technology for CEA mRNA was used because of its sensitiveness and specificity for breast cancer tumors (39,40). We are at present carrying out actually conducting a parallel study comparing RT-PCR CEA versus mammoglobin amplification. We have found a profound correlation between stage and bone marrow positivity. More importantly, the number of events, although limited so far by a relatively short median follow-up, seems to prove effective in case of positive bone marrow on recurrent disease; a significant increase of deaths to disease progression in the BM+ group has also been observed. Furthermore, there is a substantial percentage of T1N0 cases which are BM+. We are carefully following these patients to understand if our test prove useful in predicting distant failure in early breast cancer. One point of potential critique to our study is that a bone marrow biopsy has been performed immediately at the end of surgery, and that, as a consequence, there has been potential for contamination of circulating tumor cells from the operative act. This theoretical risk has not been substantiated in a study in which no statistical influence of surgical manipulation on CK19+ cells was detected (41).

Whether peripheral blood analysis could substitute bone marrow biopsy is an interesting hypothesis since the test would be less invasive and the patients compliance greater. Indeed, two recent studies have shown a correlation between circulating tumor cells and bone marrow micrometastases in patients with breast cancer (42,43). Furthermore, one report has investigated the usefulness of RT-PCR for CK-19 mRNA in the bone marrow and peripheral blood of 148 patients with operable breast cancer (12). In this study, a positive peripheral blood finding was an independent prognostic factor

for disease relapse and death.

We have initiated a parallel study in February 2003 and double samples of peripheral blood and bone marrow for each case are being collected. Preliminary results show a concordance of approximately 80%, so far (data not shown).

It is very likely that diagnoses of occult circulating cells with sophisticated methodologies will not be the sole route of investigation to better characterize risk of relapse in our patients. Gene expression profile of the primary tumors represents today a major field of research, and several groups have shown that it is a powerful outcome predictor. The development of microarray techniques has allowed us to analyse a large number of genes in the same samples; one group has used this technique to identify 70 genes whose expression correctly predicted clinical outcome in 83% of node negative cases (44).

In conclusion, there is evidence that bone marrow biopsy to search for occult metastatic cells may be and independent prognostic factor for survival in breast cancer patients. This may not only better stratify patients' risk of relapse, but may also appropriately direct extended or secondary adjuvant treatments.

One of the major actual concerns regards the lack of standardized techniques: technical problems may produce false positives and prove either time consuming or expensive. Modern molecular approaches may facilitate detection of occult cells, but this has not yet been validated.

We present the largest clinical experience, to date, with bone marrow aspiration and RT-PCR analysis, and confirm encouraging results.

Several national and internations trials, such as ACOSOG-Z0010 and MIMS have been designed to evaluate the prognostic questions regarding occult metastatic disease in the lymph nodes, peripheral blood and bone marrow. These studies are likely to confirm if molecular detection of circulating occult tumor cells will have a profound effect in the management of breast cancer patients.

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