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Mammaglobin and maspin transcripts in blood may reflect disease progression and the effect of therapy in breast cancer

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ABSTRACT. Detection of residual tumor cells in the circulation can provide prognostic as well as therapeutic information and help in identifying patients at high risk for developing metastases. Maspin and mammaglobin are two molecules that are specifically associated with breast cancer. We looked for mammaglobin and maspin transcripts in the peripheral blood of patients with breast cancer and evaluated their utility as a marker of the response to therapy. Maspin and mammaglobin transcripts were analyzed in 85 breast-cancer patients by nested RT-PCR, prior to and after treatment. Before therapy, 10 patients were found positive for mammaglobin and 20 patients were positive for maspin. In four patients, both transcripts were de-

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tected. Immediately following treatment, only one patient was still positive for mammaglobin while maspin transcripts persisted in three patients. Disease progression was observed mainly in patients in whom maspin transcripts were not detectable. Molecular detection of circulating tumor cells during therapy based on analysis for mammaglobin and maspin transcripts is an easy and practical method that can be applied to follow-up patients. We suggest that detection of mammaglobin mRNA is useful to determine the effect of therapy while maspin transcripts may indicate more aggressive disease.

Key words: Mammaglobin; Maspin; Breast cancer

INTRODUCTION

Breast carcinoma is the most frequent cancer among women, and early detection is one of the most crucial factors for a favorable outcome (Ballestrero et al., 2005). Currently, few diagnostic tools are available to identify the patients at risk, and there is a need for relevant independent prognostic and predictive factors with proven clinical utility to assess an individual patient's risk of disease recurrence (Stojadinovic et al., 2007; Gonzalez-Mancha et al., 2008). Early occult dissemination of tumor cells is one of the major causes of relapse after local treatment. Tumor cells in peripheral blood are capable of clonogenic growth *in vitro*, thus possibly participating in relapse (Sabbatini et al., 2000). Thus, the identification of circulating tumor cells in the peripheral blood of breast cancer patients can potentially provide important prognostic information and may have therapeutic implications. The development of sensitive approaches for the detection of residual tumor cells would also provide a valuable surveillance tool to evaluate the response to the therapy. In recent years, several highly sensitive methods have been developed to detect circulating cancer cells in the blood of patients with different types of malignancies. Reverse transcription-polymerase chain reaction (RT-PCR) provides a high analytical sensitivity to detect up to a single cancer cell among 10^7 normal cells (Raj et al., 1998). Various molecular markers, including CEA, CK-19, Muc1, or CK-20, have been proposed to detect circulating breast cancer cells (Krismann et al., 1995; Bostick et al., 1998). However, most of these are poorly specific for tumor cells, and illegimate transcripts are occasionally detected in the blood, lymph nodes and bone marrow of healthy volunteers (Bae et al., 2000; Silva et al., 2001). Some earlier studies reporting the detection of circulating tumor cells have later been refuted, when it was shown that these molecular markers are also expressed by normal cells. Thus, these markers are not specific enough to be used for the detection of occult breast cancer cells (Corradini et al., 2001).

Moreover, breast tumors are composed of heterogenous cells with different levels of individual gene expression. Gene expression analysis provides a potentially easy and noninvasive approach, more accurate than existing markers and with prognostic relevance (Potemski et al., 2009). It has been suggested that the combination of different markers may eliminate some of the inherent problems associated with tumor heterogeneity. However, increasing the number of molecular markers may not necessarily increase clinical or diagnostic utility. Concordantly, it has been shown that multiple marker tests are not superior in sensitivity and that two-marker tests are a suitable approach to this

issue (Fabisiewicz et al., 2004).

The mammaglobin gene (hMAM) is located on chromosome 11q13 and encodes a glycoprotein (Watson and Fleming, 1996). hMAM expression has been reported in 70-80% of primary and metastatic breast tumors and has been associated with unfavorable prognosis (Min et al., 1998; Leygue et al., 1999). Expression of hMAM is restricted to breast epithelial cells. Due to this exclusive expression in breast tissue hMAM is a promising marker of interest in breast cancer.

Maspin is a 42-kDa cytoplasmic protein belonging to the serpin family of serine protease inhibitors (Zhang, 2004). Numerous studies have implicated maspin function in cancer progression and angiogenesis (Zou et al., 1994). It has been shown to act as a tumor suppressor, and it is able to inhibit breast cancer cell motility, invasion and metastasis (Sheng et al., 1996). Surprisingly, the expression of maspin has been frequently observed in carcinoma cells with an aggressive phenotype (Yoshihisa et al., 2002), and it has been suggested that maspin plays a role in apoptosis (Khalkhali-Ellis and Hendrix, 2003) and breast cancer metastasis (Shi et al., 2003). Tamoxifen has also been shown to induce maspin promoter activity, a new mechanism associated with the clinical efficacy of the drug (Khalkhali-Ellis et al., 2004). Thus, detection of maspin may provide a putative molecular predictor of hormone responsiveness (Bieche et al., 2003). In contrast to other tissue-specific markers, mammaglobin and maspin are not detected in lymph nodes or blood samples from healthy donors (Luppi et al., 1996).

There are only a few studies in the literature investigating mammaglobin and maspin transcripts in the peripheral blood of patients. Their utility in predicting the efficiency of therapy has not been addressed yet. There is only a single study in the literature (Sabbatini et al., 2000) on the modulation of maspin expression in a small group of patients under treatment. Association of circulating mammaglobin transcripts with therapy has not been analyzed at all. In this study, we aimed to investigate the detection of mammaglobin and maspin transcripts in peripheral blood of breast cancer patients and to evaluate their potential as markers for the prediction of response to therapy.

MATERIAL AND METHODS

Peripheral blood samples from 85 patients with histologically confirmed breast cancer (mean age: 50.7 ± 10.1 years) were analyzed before and after chemotherapy for hMAM and maspin transcripts by nested RT-PCR. All patients had undergone surgery for tumor resection. Blood samples from 28 healthy women (mean age: 51.2 ± 11.4 years) were used as the control group. The clinical characteristics of the patients are shown in Table 1. Twenty-nine patients received 4 cycles of AC (600 mg/m^2 cyclophosphamide, 60 mg/m^2 Adriamycin) and 56 patients with higher risk of recurrence received 6 cycles of FAC (500 mg/m^2 cyclophosphamide, 50 mg/m^2 Adriamycin, 500 mg/m^2 5-fluorouracil). The median follow-up period after blood sampling was 48 (range 12-74) months.

Mononuclear cells were isolated by Ficoll-Histopaque (Sigma Chemicals Inc., St. Louis, USA) density gradient centrifugation and were homogenized in 1 mL Tritidy G reagent (Applichem, Darmstadt, Germany). Total cellular RNA was extracted according to manufacturer instructions. RNA quality was examined electrophoretically on a 1.5% agarose gel.

Table 1. Clinicopathological features of patients with breast cancer.

Clinical data	No. of patients (%)
Age (years)	
<50	28 (32.95%)
50-60	42 (49.4%)
>60	15 (17.65%)
Histology	
Invasive ductal cancer	67 (78.8%)
Invasive lobular cancer	9 (10.6%)
Ductal/lobular cancer	4 (4.7%)
Other	5 (5.9%)
T stage	
T1	31 (36.5%)
T2	43 (50.6%)
T3	9 (10.6%)
T4	2 (2.3%)
Number of positive lymph nodes	
0	28 (32.95%)
1-3	34 (50.6%)
4-10	18 (10.6%)
>10	5 (5.95%)
Grade	
1	3 (3.5%)
2	37 (43.5%)
3	43 (50.6%)
Unknown	2 (2.4%)

cDNA was synthesized from 1 µg RNA using the Improm II Reverse Transcription System (Promega, Madison, USA) in a reaction mixture containing 5X reaction buffer, 2 mM $MgCl_2$, 0.5 mM deoxynucleotide triphosphate (dNTP), 0.5 µg/µL random primer, 20 U ribonuclease inhibitor and 1 µL reverse trancriptase. The mixture was incubated for 5 min at 25ºC for annealing, extension was performed for 1 h at 42ºC, and termination was at 4ºC for 5 min. The quality of RNA and cDNA synthesis and absence of inhibitors was ascertained by amplification of the G6PDH gene as internal control.

The primer sequences used in the study are listed in Table 2. PCR for mammaglobin was carried out in 50 µL final volume using 3 µL cDNA, 5X PCR buffer, 1.5 mM $MgCl_2$, 0.5 mM of each dNTP, 20 ng of each of the outer primers and 1 U Taq DNA polymerase. The reaction was begun by incubation for 5 min at 95ºC, followed by 35 cycles of denaturation at 95ºC for 40 s, annealing at 55ºC for 40 s, and extension at 72ºC for 40 s. For the second round of PCR, 1 µL of the first round PCR product was used in combination with 20 ng hMAM-IF and hMAM-IR inner primers in 50 µL final volume. The cycling and reaction conditions were the same. The outer and inner primer pairs for the mammaglobin transcripts yielded products of 402 and 367 bp, respectively.

Table 2. The inner (I) and outer (O) primer sequences and product sizes of the mammaglobin (hMAM) and maspin (Mas) transcripts.

Primer	Sequence	Product size (bp)
hMAM-OF	5'-CAG CGG CTT CCT TGA TCC TTG-3'	402 bp
hMAM-OR	5'-ATA AGA AAG AGA AGG TGT GG-3'	
hMAM-IF	5'-TGA ACA CCG ACA GCA GCA G-3'	367 bp
hMAM-IR	5'-TCC GTA GTT GGT TTC TCA CC-3'	
Mas-OF	5'-TCA AGC GGC TCT ACG TAG AC-3'	447 bp
Mas-OR	5'-CCT CCA CAT CCT TGG GTA GT-3'	
Mas-IF	5'-GAT CTC ACA GAT GGC CAC TT-3'	175 bp
Mas-IR	5'-GCA CTG GTT TGG TGT CTG TC-3'	

To investigate the maspin (Mas) transcripts, 3 µL cDNA was added to 50 µL reaction mixture as described above. Denaturation at 95ºC for 5 min was followed by 35 cycles of denaturation at 95ºC for 40 s, annealing at 58ºC for 40 s, and extension at 72ºC for 40 s. For the second round PCR, 1 µL of the PCR product was used in combination with 20 ng Mas-IF and Mas-IR primers in 50 µL final volume. The cycling and reaction conditions were the same. The primer pairs for the maspin transcripts yielded products of 447 and 175 bp.

The PCR products were electrophoresed on 2% agarose gels and evaluated using a gel documentation system (Vilber-Lourmat, Marné a Vallée, France) after staining with ethidium bromide. The sensitivity of the RT-PCR assay was controlled by dilution of the RNA samples. In 10 representative tumor samples, the presence of mammaglobin and maspin RNA transcripts was successfully and reproducibly detected up to a dilution of 10^{-6} (10^{-7} in four samples).

Statistical calculations were performed using the chi-square test. Kaplan-Meier and log rank tests were used for survival analysis.

RESULTS

We investigated mammaglobin and maspin mRNAs as markers for the detection of carcinoma cells by RT-PCR and for the effects of systemic chemotherapy, in the peripheral blood of breast cancer patients. Representative examples of hMAM and maspin products detected in sera are shown in Figures 1 and 2, respectively. All healthy controls were negative for hMAM and maspin mRNAs. Prior to therapy, 10 patients were positive for hMAM (proportion: 11.76; 95%CI: 5.79-20.57) and 20 patients were positive for maspin mRNA (proportion: 23.5; 95%CI: 15.0-33.97) (Table 3). In 4 patients (4.7%), both transcripts were detected.

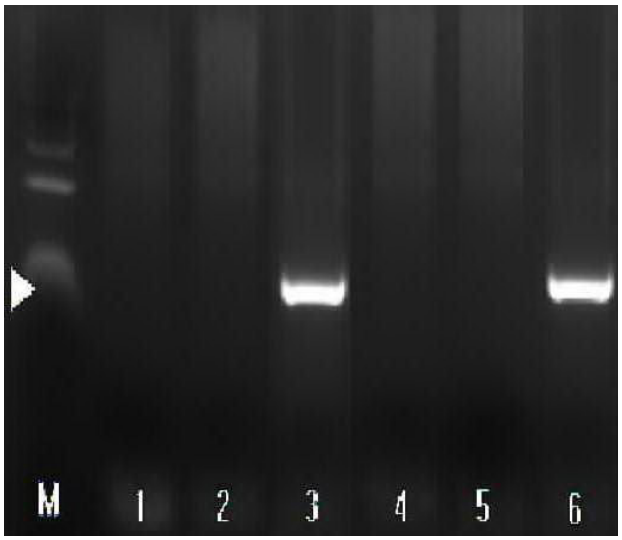

Figure 1. Ethidium bromide staining of an agarose gel showing nested RT-PCR analysis of mammaglobin mRNA expression in peripheral blood of breast cancer patients. *M*: Molecular weight marker (QX174/*Hae*III); *lanes 3*, *6*: mammaglobin-positive samples; *lanes 1*, *2*, *4*: mammaglobin-negative samples; *lane 5*: negative control.

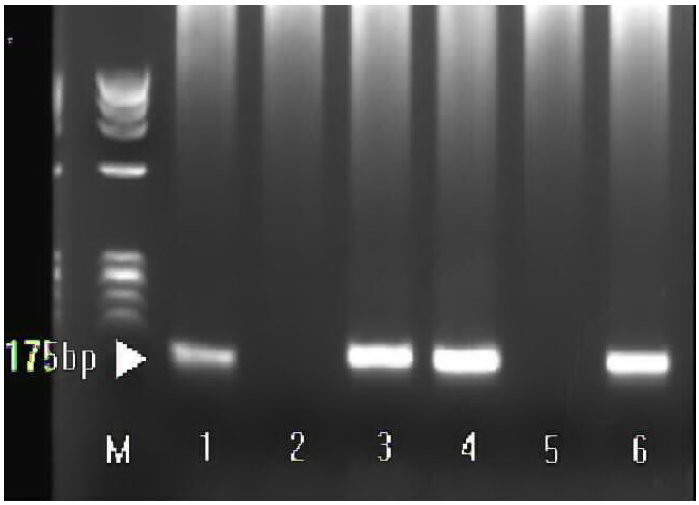

Figure 2. Analysis of maspin mRNA in peripheral blood of breast cancer patients. *M*: Molecular weight marker (QX174/*Hae*III); *lanes 1*, *3*, *4*, *6*: maspin-positive samples; *lane 2*: maspin-negative sample; *lane 5*: negative control.

Table 3. Mammaglobin and maspin transcripts in the patients.

	Before treatment	After treatment
Mammaglobin	10 (11.7%)	1 (1.2%)
Maspin	20 (23.5%)	3 (8.2%)

Data are reported as number of patients with percent in parentheses.

Following treatment, only one patient remained positive for hMAM (proportion: 1.18; 95%CI: 0.03-6.38) and 3 patients remained positive for maspin mRNAs (proportion: 8.24; 95%CI: 3.38-16.23). The cumulative probability of positivity before and after treatment was 30.5% for mammaglobin and 21.2% for maspin. Following therapy, mammaglobin transcripts were observed in 9 patients and three of these developed metastases. However, the difference was not significant. Among the patients who had tested negative before chemotherapy, mammaglobin transcripts were detected in 9 patients, and maspin transcripts were detected in 7 patients, following treatment. No association was observed between mammaglobin or maspin expression in the two different chemotherapy regimens.

After a median follow-up of 48 months, disease progression was observed in 18 patients. In these patients, maspin transcripts were either completely absent at any stage or had been positive before therapy but disappeared during treatment. Progressive disease was observed in 11 patients in whom maspin transcrips were initially present but disappeared after therapy (Table 4). This difference was highly significant ($P < 0.004$). No statistically significant correlation between maspin or mammaglobin positivity and clinical parameters were found including age, stage of disease, histological type, estrogen and progesterone receptor status, presence of metastasis on admission and location of the tumor. The survival analysis revealed no association with PCR positivity for either transcript.

Table 4. Distribution of mammaglobin- and maspin-positive patients with disease progression.

	Before treatment	After treatment
Mammaglobin	3 (16.6%)	4 (22.2%)
Maspin	11 (61.1%)	0 -

Data are reported as number of patients with percent in parentheses.

DISCUSSION

The detection of tumor-associated circulating markers in peripheral blood may be useful for prognostic and therapeutic implications. Association of these with possible dissemination and the therapeutic response remains an important area of research. A blood test for circulating tumor cells provides a promising approach for the early prediction of therapeutic efficacy.

There are a number of markers widely used to identify circulating tumor cells; however, most of these molecules are also detected in the peripheral blood of healthy volunteers. Specificity of the RT-PCR techniques is abrogated when the mRNA marker is expressed by normal hematopoietic cells (Corradini et al., 2001). Furthermore, during chemotherapy, normal proliferating cells are also damaged by cytotoxic drugs and may contribute to the release of these molecules. Therefore, any marker to be used to evaluate the therapeutic effect should be highly specific for tumor cells. Mammaglobin and maspin are two putative breast tissue-specific markers frequently used for detection of occult tumor cells in peripheral blood, bone marrow and lymph nodes of breast cancer patients (Ballestrero et al., 2005). There are no studies in the literature investigating their utility in evaluating response to therapy. The present study was undertaken to investigate the detection of mammaglobin and maspin transcripts in peripheral blood of patients with breast cancer and to evaluate the changes in response to chemotherapy.

Transcripts of both genes were detected by RT-PCR in the peripheral blood of breast cancer patients but not of healthy volunteers. We achieved a much higher sensitivity than in earlier reports using conventional RT-PCR (Sabbatini et al., 2000; Corradini et al., 2001; Stathopoulou et al., 2003). The specifity of mammaglobin and maspin for circulating tumor cells was very high (100%) due to the preselection and enrichment by the two-step nested approach used; however, the sensitivity of hMAM was considerably lower (11.8%) when compared to maspin (23.5%). These data are in agreement with a very recent report in which similar rates of positivity at comparable specificity were found for mammaglobin (7%) and maspin (24%) transcripts in untreated breast cancer (Mercatali et al., 2006). We observed no difference for mammaglobin or maspin expression in response to the addition of 5-FU to the chemothrerapy regimen. This issue has not been investigated so far, and to date no information is available on a possible effect of the chemotherapeutic drugs on mammaglobin or maspin expression other than a probably indirect apoptosis-sensitizing effect of maspin in head and neck cancer (Marioni et al., 2008) and ovarian carcinoma (Surowiak et al., 2006) patients treated with cisplatin-containing regimes. In these studies, elevated maspin expression has been associated with cisplatin resistance. Neither maspin nor mammaglobin has been associated with any other chemotherapeutic drug.

Lack of correlation between mammaglobin and maspin expression and clinical parameters is also consistent with previous findings (Suchy et al., 2000) as well as recent reports

on breast (Marques et al., 2009), ovarian (Surowiak et al., 2006; Klasa-Mazurkiewicz et al., 2009) and laryngeal cancer (Marioni et al., 2008).

In our study, mammaglobin transcripts were observed in 11% of the patients. The positivity rate for mammaglobin is lower than that reported by two previous studies (Zach et al., 1999; Bossolasco et al., 2002).

This variation is in accordance with a report in which mammaglobin mRNA has been found to be the most specific molecular marker for hematogenous spread of breast cancer cells (Ntoulia et al., 2006) and can be explained by the small number of metastatic patients in our study population. hMAM transcripts have been detected in the peripheral blood of up to 25% of breast cancer patients when metastasis is present (Suchy et al., 2000; Bossolasco et al., 2002). While only 7% of the patients in our group had metastatic disease on admission, metastasis was present in 44 and 23.7% in the cohort of the above studies. The detection rate in non-metastatic patients at diagnosis and during follow-up was comparable to our data in the same reports (11.2 and 9%, respectively). In our study, 33% of the patients with detectable mammaglobin transcripts after therapy developed metastases.

Maspin transcripts were observed in 23.5% of patients and in none of the controls. This rate is higher than in earlier reports (Sabbatini et al., 2000; Stathopoulou et al., 2003) but in accordance with recent data (Mercatali et al., 2006). The positivity rate for maspin expression is also in concordance with maspin expression in tumor tissue (Yoshihisa et al., 2002).

Following therapy, all patients initially positive for the mammaglobin transcript turned negative. This probably reflects the effects of systemic therapy on circulating tumor cells. Concordantly, the detection of late transcripts in patients who were negative in the beginning may be associated with disease progression. In accordance with this finding, a high incidence of relapses has been reported in the group of patients who displayed mammaglobin mRNA-positive cells in their blood (Ntoulia et al., 2006). An association between circulating CEA mRNA and higher risk of metastasis has also been reported recently for colon cancer patients (Wang et al., 2006).

Association between loss of maspin expression and progressive disease is in line with the role of maspin as a tumor suppressor and has been recently reported in malignant melanoma (Denk et al., 2007), head and neck cancer (Marioni et al., 2009) and ovarian cancer (Surowiak et al., 2006; Secord et al., 2006). In colorectal cancer patients, non-detectable maspin expression has been shown to increase the risk of disease progression and death 2-fold (Boltze et al., 2005). In seven patients who were negative on admission, maspin transcripts were detected after treatment. This may be due to the mobilization of tumor cells in response to cytotoxic treatment, as suggested by a recent study (Sabbatini et al., 2000). However, this report should be interpreted with caution due to the very small number of patients where only half were available for evaluation after therapy. Alternatively, exposure to specific cytokines may also induce maspin expression (Ballestrero et al., 2005). Recently, it has been reported that tamoxifen can also exert an inductive effect on the maspin promoter (Khalkhali-Ellis et al., 2004). However, none of the patients in our study received tamoxifen during the sampling period.

We have shown that this molecular assay can identify circulating tumor cells and may have predictive implications in evaluating the efficacy of treatment. Our data indicate that accurate molecular detection of circulating tumor cells based on the analysis of breast-specific mammaglobin and maspin genes in peripheral blood may provide a practical and rational approach for the surveillance of breast cancer patients during therapy. Although mammaglobin would not be useful as a classical tumor marker, monitoring mammaglobin mRNA levels may reflect the efficacy of the therapy. Disappearance of the maspin transcripts with chemotherapy

might be associated with a higher risk of progression and possibly aggressive tumors. Further studies with larger series of patients and longer follow-up are needed to validate these results and to determine whether this assay will be useful in guiding clinical decisions.

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