Mammaglobin A, a novel marker of minimal residual disease in early stages breast cancer*

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Mammaglobin A, in contrast to other factors, is a breast specific member of uteroglobin gene family. Expression is restricted to normal and neoplastic breast epithelium. A highly homologous mammaglobin B is not specific to breast tissue. In this pilot feasibility study we examined expression of both markers for minimal residual disease in the bone marrow of patients with breast cancer.

We obtained bone marrow aspirates of 34 patients with stage I (41%), II (56%) and III (3%) breast cancer who underwent either immediate complete resection of the tumor or neoadjuvant therapy with subsequent curative surgery. mRNA was isolated using QIAamp RNA blood mini kit (Qiagen®). Subsequently two-step nested RT-PCR for the expression of mammaglobin A and mammaglobin B was performed.

Mammaglobin A was detected in samples from 4 (12%) out of 34 patients. None of the specimens was positive for mammaglobin B. With a median follow-up of 21 month we observed only 2 recurrences, one in patient with mammaglobin A positive bone marrow.

RT-PCR assay for mammaglobin A may be a useful tool for detection of occult breast cancer cells in the bone marrow. Clinical and prognostic relevance of minimal residual disease should be further investigated.

Key words: breast cancer, bone marrow, mammaglobin A, minimal residual disease

Breast cancer ranks first among cancer related death in women in developed countries. Annually more than 5000 women are newly diagnosed in the Czech Republic [13]. The early occult hematogenous spread of isolated tumor cells may be involved in subsequent formation of metastases, yet is usually missed by conventional tumor staging. More than 95% patients with breast carcinoma have no evidence of metastatic disease according to clinical, radiological, and biochemical examination [7]. Approximately half of patients with operable breast cancer would develop distant metastases within 5 years after primary surgery. In the group of patients even without metastatic involvement of ipsilateral axilary lymph nodes about one third relaps [9].

The presence of occult tumor cells in bone marrow has been correlated with early recurrence and shorter overall survival. Immunocytochemical methods have been used to detect micrometastases, the occurrence of which is related to other prognostic features of the primary carcinoma (tumor size, presence of vascular invasion, lymph node involvement) and predicts possible early recurrence [6, 8, 10, 12, 14]. Immunocytochemical methods have been estimated to be capable of detecting approximately one cancer cell per 10^4 to 10^5 normal bone marrow cells [11, 17], whereas measurement of epithelial cell-specific gene transcripts such as cytokeratin 19 (*CK-19*) by reverse transcriptase polymerase chain reaction (RT-PCR) has been reported by some groups as being capable of detecting one cancer cell per 10^6 normal bone marrow cells [19]. RT-PCR in this context has proven to be controversial, because the specificity with which malignant cells can be detected depends on the number of amplification cycles and the design of the primers. The absence of reliable quantification by RT-PCR has

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meant that results are generally expressed as either positive or negative, which makes it difficult to relate the level of RT-PCR detectable disease to the micrometastatic load as judged by immunocytochemistry. Cytokeratins are epithelial markers which are not specific for breast epithelium [23]. It may lead to decreased specificity when using sensitive methods such as RT-PCR. Thus, new breast specific markers need to be defined in order to improve accuracy of RT-PCR detection. One of such novel markers is mammaglobin. The mammaglobin gene encodes a novel secreted protein whose corresponding mRNA is frequently upregulated in human breast cancer [25]. In non-malignant tissues, expression is also strictly limited to mammary epithelium. Mammaglobin belongs to the uteroglobin gene family [27]. Breast specific mammaglobin is also called mammaglobin A. In contrary, highly homologous mammaglobin B is not specific for mammary tissue [1, 4]. In primary tumor and infiltrated lymph nodes, expression of mammaglobin A was detected in 80–90% of cases with advantage of 100% specificity [26]. Once the sensitivity and specificity of this marker was established we assessed mammaglobin A and mammaglobin B expression in bone marrow samples collected from patients with early stages breast cancer.

Patients and methods

Patients. Samples of bone marrow from sternum were obtained from patients with primary breast cancer treated in the Department of Oncology First Faculty of Medicine. All patients enrolled had to be either after primary surgery with no evidence of disease or prior to neoadjuvant therapy in cases of locally advanced disease. Patients were diagnosed between March 2001 and March 2003. Selection criteria included presentation with primary breast cancer stage I, II, and III according to AJCC [2], completion of appropriate curative surgical procedure, or planned neoadjuvant therapy with curative intent. A total of 34 patients who fulfilled these criteria were chosen: 14 (41%) with stage I, 6 (18%) with stage IIA, 13 (38%) with stage IIB, and 1 (3%) with stage IIIB. The detailed patients' characteristics are listed in Table 1. All patients received adjuvant therapy according to St. Gallen consensus from 2001 [24]. Adjuvant chemotherapy with antracyclin, non-antracyclin, or antracyclin-taxane based regimen was delivered to 26 (76%) patients. All 29 (85%) patients with expression of hormonal receptors received adjuvant tamoxifen. Six premenopausal patients with persistent ovarian function underwent ovarian ablation either with goserelin or surgery in addition to tamoxifen. Patients were treated with radiation of the breast when breast conserving surgery had been performed. Patients with more than 3 infiltrated axillary lymph nodes underwent radiation of axilla. Follow-up of all patients was performed at regular time intervals.

Table 1. Patients' characteristics (n=34)

Characteristics		Patients No.	Patients %
Age			
Median	51		
Range	34–76		
Premenopausal		12	35
Postmenopausal		22	65
Stage			
I		14	41
IIA		6	18
IIB		13	38
IIIB		1	3
N+ disease		15	44
HR+		29	85
HER2/neu+		13	38
Histology			
invasive ductal carcinoma		31	94
invasive lobular carcinoma		3	6
Chemotherapy			
Neoadjuvant (F)AC*		3	6
Adjuvant			
(F)AC*		16	47
CMF**		4	12
antracylines-taxanes		6	17
Tamoxifen		29	85

*(F)AC – (fluorouracil), doxorubicin, cyclophosphamide, **CMF – cyclophosphamide, methotrexate, fluorouracil.

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethical Review Board. All patients provided written, informed consent.

Bone marrow aspiration. To avoid epithelial contamination of bone marrow aspirates, the skin incision was performed before starting the aspiration. Between 0.5 and 4.0 ml of bone marrow was aspirated from sternum using disposable 15-gauge (1.8 mm) marrow-gauge bone marrow aspirate needles (Allegiance Healthcare Corporation, McGaw Park, IL, USA) into syringes primed with EDTA. The samples were immediately processed as described in further section. Bone marrow aspiration from sternum yielded sufficient amount of mRNA for subsequent procedures.

Preparations of bone marrow samples. Total RNA was extracted from bone marrow aspirates using commercial kit (QIAamp RNA Blood Kit, Qiagen, Valencia, USA) according to the manufacturer's protocol for fresh blood samples preparations. Purified RNA was quantified and purity was assessed using UV spectrophotometer.

RT-PCR. Complementary DNA (cDNA) was prepared using M-MuLV Expand Reverse Transcriptase (Roche) according to manufacturer's protocol. Briefly, 1 μg of RNA with 20 pmoles of random hexanucleotides was incubated 10 minutes at 65 $^{\circ}$C. The RT reaction was performed by

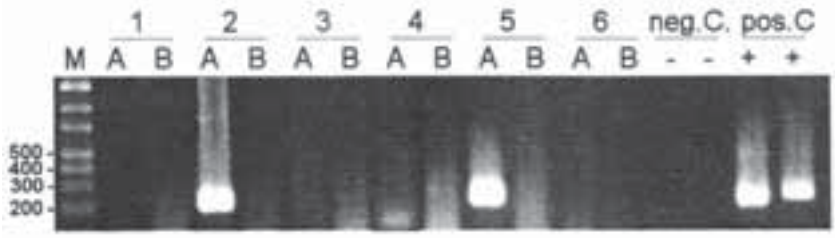

Figure 1. RT-PCR analysis of mammaglobin A, and B expression in breast patient's bone marrow samples. Result of agarose electrophoresis following the second PCR is shown. Patient's samples number 2 and 5 represented expression of mammaglobin A only in bone marrow aspirates. M – DNA ladder (numbers in base pairs; bp), A – nested PCR of mammaglobin A (219 bp), B – nested PCR of mammaglobin B (245 bp), neg.C. – negative control (no cDNA added), pos.C – positive control (RNA from tumor used for cDNA synthesis).

Table 2. Amplification primers used for RT-PCR analysis

Primer	Sequence	PCR conditions T_A	cycles
Mammaglobin A (accession #:NM_002411)			
MA01f	5'-CTTATTGGAGAATGTGATTTCC	53 °C	30
MA02r	5'-TTCTCACCATACCCTGCAGTTC		
MA03f	5'-CCAAGACAATCAATCCACAAG	61 °C	30
MA04r	5'-TGTGAGCCAAAGGTCTTGCAG		
Mammaglobin B (accession #: NM_002407)			
MB01f	5'-AACTCCTGGAGGACATGGTTG	53 °C	30
MB02r	5'-TGGCCATAGTCTGTAGCCCTC		
MB03f	5'-ACTCCTGGAGGACATGGTTGA	55 °C	30
MB04r	5'-TCTGAGCCAAACGCCTTGGGT		

adding of 4 μl of Expand reverse transcriptase buffer, 2 μl 100 mM DTT, 50U Expand Reverse Transcriptase, and 2 μl 10 mM dNTP's. Reaction mix was incubated 10 minutes in 30 °C followed by 45 minutes in 42 °C, and placed on ice. PCR analyses were performed using nested PCR protocol. First round PCR was performed in 10-μl volume containing 2 μl of cDNA template, 0.25 U LA Taq Polymerase (TaKaRa), 1 μl 10x PCR buffer, 1 μl 25 mM $MgCl_2$ 12 pmoles of each primer, and 1.6 μl 2.5 mM dNTP's mixture. Nested PCR amplification was performed in 20-μl reaction volume containing 1 μl of first round PCR, 15 pmoles of each primer, 0.25 μl 50x dNTP's (Invitek), 0.25 U DynaZyme II Polymerase (Finnzymes) and 2 μl 10x PCR buffer with $MgCl_2$ (Finnzymes). PCR cycling (30 cycles) 1 min at 94 °C, 1 min at T_A, 1 min at 72 °C, followed by 10 minutes at 72 °C was performed in Dyad PCR machine (MJ Research) under optimized conditions listed in Table 2. The integrity of all RNA samples was controlled by amplification of β-globine mRNA by RT-PCR as described previously. Five microlitre of PCR product was used for electrophoresis on 2% agarose gel at 1x TAE end visualized with ethidium bromide staining.

Primers. Amplification primers used in the study are listed in Table 2. Accession number depicts the sequences retrieved from NCBI database.

Statistical analysis. Statistical analysis was performed using software Statistica version 6 (StatSoft © 2003). We assessed correlation between minimal residual disease and other prognostic variables. Furthermore, we performed univariate analysis of minimal residual disease and disease free survival.

Results

Bone marrow aspirates from 34 patients were collected prior to administration of any systemic therapy. Mammaglobin A mRNA transcripts were detectable in bone marrow of 4 (12%) patients. All patients had stage II disease: two stage IIA, and two stage IIB. In addition three of them had N+ disease (involvement of axillary lymph nodes), but correlation did not reach statistical significance (r=0.44; p>0.05). When compared to other prognostic features there was no correlation with tumor grade, hormonal receptor status and HER2/neu expression (respectively: r=0; r=0.18; r=0.24; p>0.05 in all cases). There was much more frequent lobular histology (in 2 out of 4 cases) among patients with mammaglobin A transcripts in the bone marrow when compared to the group of patients without minimal residual disease (1 out of 30 patients). This finding was statistically significant (r=0.53; p<0.05). Mammaglobin B transcripts were not detected in any of the samples examined.

The median follow-up period at the time of analysis was 21 months (range, 7 to 30 months). Only in 2 patients (6%) disease recurrence occured thus far, but one of them had occult tumor cells in the bone marrow aspirates with mammaglobin A transcripts detected. At the time of analysis 2 (6%) patients had died. None of these deaths was tumor related (car accident, and pulmonary embolism). Both patients had no mammaglobin A in bone marrow aspirates. There was a trend towards shorter disease free survival in those with mammaglobin A in the bone marrow. However, it did not reach statistical significance (p=0.063).

Discussion

The detection of tumor cells in the bone marrow of patients with early stages breast cancer predicts disease relapse and shorter overall survival [5]. The lack of cancer specific markers led to use of cytokeratins. They have been thoroughly studied especially in breast cancer. Use of cytokeratins is limited with relatively low specificity [21] due to low level of illegitimate RNA transcripts [15, 20] and amplification of the processed CK-19 pseudogene from contam-

inating genomic DNA is virtually identical to the CK-19 cDNA sequence [3]. In contrast the detection of human mammaglobin A mRNA is both specific and sensitive [26].

Overall in this study 12% (4 of 34) of the bone marrow aspirates collected from patients with early breast cancer were positive for human mammaglobin A transcripts. Relatively small proportion of positive results in comparison to the other series [22, 23] correlates with small recurrence rate. In 21 month of median follow up we observed only two recurrences. Two deaths observed in the study group were not cancer related. It is too early to determine prognostic impact of presence of occult tumor cells detected with RT-PCR for mammaglobin A mRNA, but one of 2 relapsed patients had mammaglobin A proven in the bone marrow aspirates. We also observed statistically significant higher incidence of mammaglobin A positivity in the group of patients with lobular histology. There was a trend, however not statistically significant, toward higher incidence of axillary lymph nodes involvement among patients with detected mammaglobin A. We have not identified in any sample mammaglobin B transcripts.

Our pilot study showed that it might possible to use mammaglobin A as a marker of minimal residual disease in the bone marrow of patients with early stages breast cancer. Highly homologous mammaglobin B failed to prove the same utility. These findings are in concordance with previously published data [1, 4].

The practical implications of this study are two-fold: First, it may be possible to use the assay to monitor patients with disseminated occult tumor cells at the time of diagnosis of primary tumor. At present, it seems that conventional immunocytochemistry is unlikely to be sensitive enough and is subject to a greater degree of sampling errors [16]. Second, it may be possible to further investigate this type of assay for monitoring the effect of adjuvant therapy where other biochemical and radiological techniques cannot be used [18]. The major disadvantage of RT-PCR is that this technique is quite expensive and technically demanding. Further studies with full follow-up will be needed to clarify this issue and to consider reliability of value of RT-PCR in relation to other tests for determining the prognosis of primary breast cancer.

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