Lack of correlation between expression of human mammaglobin mRNA in peripheral blood and known prognostic factors for breast cancer patients

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Human mammaglobin (hMAM) mRNA is considered to be a promising candidate for a sensitive molecular marker for breast cancer. In this study, we attempted to relate the presence of hMAM mRNA in the peripheral blood with certain established clinicopathological features of breast cancer in order to validate its clinical utility. A total of 139 subjects including 79 with localized cancer, 33 with metastatic disease, and a control group of 27 individuals were studied. hMAM mRNA expression was assessed by reverse transcriptase polymerase chain reaction on cells from peripheral blood. The expression of hMAM mRNA was found in 0 of the 27 control subjects, 1 of the 8 stage 0 (12.5%) patients, 4 of the 16 stage I (25%) patients, 13 of the 40 stage II (32.5%) patients, 5 of the 15 stage III (33.3%) patients, and 18 of the 33 (54%) cases of metastatic disease. There was a statistically significant (P=0.045) difference in frequency between patients with localized disease (29%) and those with metastatic disease. Although trends of increasing frequency of hMAM mRNA expression existed in patients with unfavorable prognostic factors, including primary tumor size, T stage, N stage, overall stage, presence of angiolymphatic permeation, negative estrogen receptor, high S-phase fraction (>7%), and aneuploid DNA index, none of the differences was significant. In conclusion, the clinical utility of hMAM mRNA may not be in screening or staging of breast cancer, but in following patients after surgery to detect recurrence. Further evaluation of hMAM mRNA in combination with other molecular markers to follow post-operative breast cancer patient is warranted. (Cancer Sci 2003; 94: 99–102)

Breast cancer is one of the most common cancers in women, and its incidence and death rate are rapidly increasing in Taiwan. Although three-quarters of breast cancer patients only have clinical stage I or stage II disease, nearly 30% of patients eventually die of the disease.[1)] The fact that small numbers of cancer cells can be detected in the peripheral blood, marrow or lymph node may be useful in diagnosing or monitoring disease for early intervention. Previous studies have examined immunohistochemical staining to detect cancer cells in bone marrow by using epithelial cell-associated antigen. The sensitivity of this method is generally reported to be adequate to detect one tumor cell in 10^5 nucleated cells.[2)] Some studies have identified the presence of bone marrow micrometastases as a prognostic factor for distant metastases or death.[3–5)] However, this method yields some false-positives.[6)]

The expression of mRNA differs among different types of cells. For example, epithelial genes are expressed only in epithelial cells and not in hematopoietic cells.[7)] Therefore, the reverse transcriptase polymerase chain reaction (RT-PCR) technique is potentially a very sensitive technique for detecting small amounts of specific mRNA species in large mRNA samples. Several authors have developed assays based on the RT-PCR technique, using epithelial gene markers as amplifications targets.[8–17)] This technique provides a high sensitivity (up to one cell in 10^7 nucleated cells).[18)] Various molecular markers have been proposed for detecting circulating breast cancer cells, including carcinoembryonic antigen (CEA), cytokeratin-19, β-hCG and Muc-1.[8, 9, 12–17, 19)] However, the sensitivity of these markers is influenced by tumor differentiation and the markers may also be expressed in non-breast tissue, including hematopoietic cells.[20, 21)] Consequently, their diagnostic value is limited.

Human mammaglobin (*hMAM*) gene was cloned in 1996, and is a member of the uteroglobin family. hMAM encodes a glycoprotein, but the cellular function of the gene product remains uncertain. The expression of hMAM was initially believed to be restricted to the adult mammary gland and breast cancer cell lines. Additionally, hMAM expression is high in human breast cell lines and primary breast cancer, but low in normal breast tissues.[22)] Zach *et al.* reported that hMAM is over-expressed in 23% of primary breast tumors. Expression of hMAM mRNA among breast tumors did not correlate with histological type, tumor grade, tumor stage or hormone receptor status.[23)] Based on its breast cancer-associated expression, and breast tissue-restricted distribution, hMAM appears to be a promising candidate as a breast tumor marker. Since the presence of circulating cancer cells may to some extent reflect the relative tumor load and possibly the capacity of tumor dissemination, hMAM mRNA expression in the peripheral blood could be a good prognostic factor and should be correlated to certain established clinicopathological features for breast cancer. Therefore, in this study, we attempted to determine whether hMAM mRNA expression in the peripheral blood can be used as a marker to predict the prognosis for breast cancer.

Materials and Methods

Patient selection and tumor characteristics. From December 2000 to June 2001, 139 study subjects, including early breast cancer (stages I, II, III) patients, metastatic breast cancer patients, and patients with breast disease other than mammary gland malignancy, as well as healthy volunteers, were enrolled for study after having given informed consent. Blood samples were taken prior to operation in the early-stage patients, and patients who had excision biopsy prior to their primary surgery were excluded to avoid possible cancer cell contamination after the biopsy. Meanwhile, patients with metastatic disease were required to have had no cytotoxic chemotherapy during the 2 months prior to the study to avoid the effect of chemotherapy on circulating cancer cells. The tumor staging was determined by a qualified pathologist (S. H.) from the pathological specimens. The pathologist also reviewed the histological features,

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including Scarff-Bloom-Richardson (SBR) grading (only applicable for ductal carcinoma), angiolymphatic permeation of cancer cells, and immunohistochemical findings of estrogen receptor (ER), progesterone receptor (PR) and Her-2 oncoprotein. Fresh tumor specimens from patients whose primary tumor was excised at our hospital were sent to a clinical pathology laboratory for DNA flow cytometry studies and the fraction of S-phase and DNA ploidy were retrieved from the medical records.

Breast cancer cell line. The breast cancer cell lines MDA MB-453, and MDA MB-415, as well as human HL 60 cells, were cultured in Dulbecco's modified Eagle's medium (DMEM) with the addition of 10% fetal calf serum, ampicillin and streptomycin. The adherent cell lines were passaged by trypsin digestion every 3 to 4 days. These cells served as the positive control for measuring mRNA marker expression or as a negative control.

Blood sampling for RT-PCR. Ten milliliters of blood was collected in sodium citrate-treated tubes. The peripheral mononuclear cells were then fractionated using the Ficoll Hypaque gradient method according to the manufacturer's instructions (Amersham Pharmacia Biotech, Uppsala, Sweden). Nucleated cells were washed twice with phosphate-buffered saline following isolation of the RNA with a RNeasy Mini kit (Qiagen, Hilden, Germany). The amount of each RNA sample was measured by ultraviolet spectrophotometry at 260 nm. The integrity of samples was verified by RT-PCR with a control gene (*GADPH*) followed by ethidium bromide gel electrophoresis. Samples were then stored at −80°C until required.

RT-PCR. Each RNA sample was converted to cDNA by Moloney murine leukemia virus reverse transcriptase. Total RNA (0.5–1 μg) was subjected to RT using 0.5 μg oligo-dT priming, 3 m*M* $MgCl_2$, 0.5 m*M* each of deoxynucleotide triphosphate, 1× PCR buffer II (75 m*M* KCl, 50 m*M* Tris-HCl, pH 8.3), 1 unit/μl RNAase inhibitor, and 200 units of Moloney murine leukemia virus RT in a total volume of 20 μl according to the manufacturer's instructions (Life Technologies, Inc., Rockville, MD). The mixture was incubated for 10 min at room temperature, followed by 50 min at 42°C (reverse transcription reaction), and 15 min at 70°C (denaturation), and then maintained at 5°C for up to 2 h before proceeding with the PCR reaction. All RT reactions were performed with oligo-dT priming to avoid transcription of spurious non-polyadenylated mRNA. The first step PCR reaction was performed by diluting the 20 μl RT reaction mixture to a total volume of 50 μl with $MgCl_2$ (1.5 m*M*), PCR buffer II (1×), Ampli*Taq* DNA polymerase (5 units/μl) (Perkin Elmer, Boston, MA), and primers (0.5 μ*M* each). The PCR conditions for GADPH were 94°C denaturation for 2 min, followed by 35 cycles of denaturation at 94°C for 45 s, then annealing at 60°C for 45 s and extension at 72°C for 2 min, and a final extension step at 72°C for 10 min. The PCR conditions for hMAM were 94°C for 10 min, followed by 25 cycles of 94°C for 40 s, 59°C for 40 s, 72°C for 1 min, and then a final extension step at 72°C for 7 min with *Taq* DNA polymerase (HT Biotechnology, Limited, Cambridge, UK). Nested PCR was performed by adding 5 μl of the secondary PCR product mixture to 95 μl of a nested reaction mix, which is identical to that used in the first step PCR, except that 0.8 μ*M* of each nested primer was added. Nested PCR conditions were the same as in the first step. The primer sequences for the mRNA markers and the size of the RT-PCR products were derived from published data.[22] Aliquots of 20 μl of the PCR products were electrophoresed on 2% agarose gels containing ethidium bromide for 1 to 1.5 h, then visualized on an ultraviolet transilluminator and photographed. A 100-bp DNA ladder (Life Technologies, Inc.) was used as a marker. In each experiment, RT-PCR mixtures without mRNA were used as negative controls. MDA MB-435, a breast cancer-derived cell line which expresses hMAM, was used as a positive control. All samples were tested at least twice.

Statistical analysis. All data analyses were performed using the χ^2 or Student's *t* test with SPSS 9.0 software (SPSS, Inc., Chicago, IL). Differences were considered statistically significant if the *P* value was less than 0.05.

Table 1. Characteristics of patients with localized disease (*N*=79)

Characteristics	Number
Age	
Median	47 years
Range	32–85
Histology	
Ductal carcinoma *in situ*	8
Invasive ductal carcinoma	59
Invasive lobular carcinoma	5
Mucinous carcinoma	5
Tubular carcinoma	1
Medullary carcinoma	1
T stage	
0	8
1	22
2	38
3	5
4	4
Unknown	2
N stage	
0	44
1	22
2	11
Unknown	2

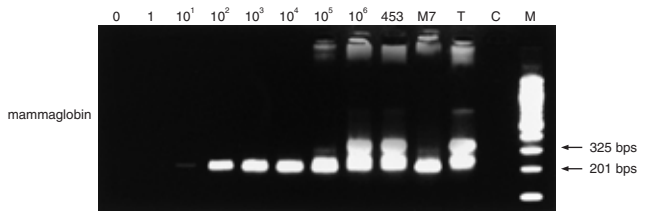

Fig. 1. Sensitivity test of hMAM mRNA RT-PCR. From left to right: from no MDA MB-453 cancer cells to 10^6 cancer cells in 10^7 HL 60 cells. 453, MDA MB-453 cell line; M7, MCF-7 cell line; T, a tumor specimen from a patient; C, a control without cells; M, DNA markers. The 325 base pair product is the 1st PCR product, while the 201 base pair product is the final product.

Results

Samples. The 139 subjects included 8 stage 0, 16 stage I, 40 stage II and 15 stage III early breast cancer patients, as well as 33 stage IV breast cancer patients. Table 1 lists the demographics of the early-stage patients. Among the patients with metastatic disease, only 2 patients had local recurrence on the chest wall, while the remaining patients had multiple distant metastases. The sample also contained 24 healthy volunteers, 1 with gastric cancer, 1 with breast lymphoma and 1 with breast phylloides tumor.

RT-PCR of hMAM mRNA. The RT-PCR of hMAM was first examined with various breast cancer cell lines and fresh samples from primary breast carcinoma. hMAM mRNA expression was observed in MDA MB-453, and MCF-7, as well as in tumor specimens, but not in the HL 60 leukemia cell line. To determine the sensitivity of RT-PCR assays, 10^7 HL 60 cells were

mixed with decreasing numbers of MDA MB-453 cells, with a final number of 10 cells in 10^7 HL 60 cells. The RT-PCR could detect 1 to 10 cells in 10^6 HL 60 cells (Fig. 1).

Patients with breast cancer were grouped into those with localized disease (stages I, II, III) and metastatic disease (including local or regional recurrence). In patients with localized breast cancer, hMAM mRNA expression in the peripheral blood was found in 1 of the 8 stage 0 patients (12.5%), 4 of the 16 stage I patients (25%), 13 of the 40 stage II patients (32.5%), and 5 of the 15 stage III patients (33.3%). Although frequency of hMAM expression was increased in advanced-stage cancers, the increase was not statistically significant. hMAM mRNA expression was detected in the peripheral blood of 18 (54%) of the 33 patients with metastatic disease. Compared to those with localized disease (29%), the relatively high frequency of hMAM mRNA expression in the patients with metastatic disease is statistically significant (P=0.045). hMAM mRNA expression was observed in patients with distant metastasis, but not in those with local recurrent disease.

None of the subjects in the non-breast cancer group was positive for hMAM mRNA expression, including the normal volunteers and patients with cancers other than breast carcinoma.

Comparison of hMAM mRNA expression with clinicopathological factors. Further analysis revealed that hMAM mRNA expression frequency was increased in patients with unfavorable prognostic factors, including primary tumor size, T stage, N stage, overall stage, presence of angiolymphatic permeation, negative estrogen receptor, high S-phase fraction (>7%), and aneuploid DNA index, but none of the differences was significant (Table 2).

Discussion

Because of the specific expression of hMAM mRNA in the breast tissue, the application of RT-PCR of hMAM mRNA to detect small volumes of breast cancer cells has been studied using lymph node, marrow or peripheral blood of breast cancer patients. A recent study of multiple molecular markers to compare their sensitivity has found that hMAM mRNA was the most sensitive marker for breast cancer.[24, 25] Although most of the study results were preliminary, RT-PCR of hMAM mRNA has been suggested as a potential tool for detecting micrometastases in the axillary lymph node, and for detecting micrometastases in the autologous marrow of breast cancer patients.[26, 27] However, the detection of minimal numbers of breast cancer cells from the peripheral blood of breast cancer patients remains controversial, and a pilot study found that the use of RT-PCR of hMAM mRNA in the peripheral blood has low sensitivity, particularly in patients with early breast cancer.[28] Furthermore, expression of hMAM mRNA did not correlate with tumor grade, size or stage in some studies, though an increasing positive trend was found when comparing early-stage cancer with metastatic disease. The findings of this study were in agreement with this previous work. In the present study, we used several established clinicopathological features of localized breast cancer[29] to establish whether the presence of hMAM mRNA in the peripheral blood could be useful as a marker to predict prognosis. However, this study was unable to find any correlation between the presence of hMAM mRNA in the peripheral blood and known prognostic factors for breast cancer.

Since the presence of hMAM mRNA did not correlate with other known prognostic factors, its role as an independent prognostic factor remains elusive and may require longer follow-up of those patients to observe recurrence or death. The effectiveness of the use of immunohistochemistry to detect micrometastases in the bone marrow as a prognostic factor for breast cancer remains similarly uncertain. A meta-analysis of 20 published studies concluded that this assay lacked prognostic significance.[30] The study hypothesized that the presence of micrometastatic cancer cells can be problematic due to the varied sensitivity and specificity of detection methods. For example, necrotic cells shed from a tumor may be detectable yet nonviable, and thus not be predictors of metastases. Indeed, circulating cancer cells are not always viable and may lack the capacity to survive distantly. Developing a method of characterizing the shed cells to confirm their viability may enhance the ability of this molecular marker to predict tumor recurrence. Furthermore, perhaps the most significant prognostic factor for localized breast cancer is the number of lymph node metastases.[29] However, a discrepancy existed between the hMAM RT-PCR results and the status of lymph node metastases in this study. A further study should analyze the prognosis of patients with positive hMAM expression stratified by nodal status to elucidate the relationship between hMAM and lymph node metastases. Furthermore, the presence of circulating tumor cells in localized cancer may not depend on tumor stage, or differentiation, but rather on the biological characteristics of the tumor,

Table 2. Correlation of hMAM and prognostic factors

	Total number	hMAM			P value
		Negative	Positive	% positive	
Tumor size					
≥3 cm	24	15	9	37.5%	
<3 cm	55	41	14	25.5%	0.294
Tumor stage					
0	8	7	1	12.5%	
1	22	16	6	27.8%	
2	38	25	13	34.2%	
3	5	4	1	20%	
4	4	2	2	50%	0.054
Node stage					
0	44	33	11	25%	
1	22	14	8	36.4%	
2	11	7	4	36.7%	0.559
Overall stage					
0	8	7	1	12.5%	
1	16	12	4	25%	
2	40	27	13	32.5%	
3	16	10	5	31.3%	0.670
SBR grade					
1	17	9	8	47.1%	
2	29	21	8	27.6%	
3	20	15	5	25%	0.288
Angiolymphatic permeation					
Positive	27	17	10	37%	
Negative	42	31	11	26.2%	0.424
ER receptor					
Positive	42	32	10	23.8%	
Negative	33	22	11	33.3%	0.440
PR receptor					
Positive	23	17	6	26.1%	
Negative	52	37	15	28.9%	0.806
Her-2 over-expression					
Positive	25	18	7	28%	
Negative	48	35	13	27.1%	0.943
S-phase fraction					
≤7%	37	27	10	27%	
>7%	25	16	9	36%	0.452
DNA ploidy					
Diploid	31	23	8	25.8%	
Aneuploid	31	20	11	35.6%	0.409

particularly the molecules involved in the development of metastases (such as metalloproteinase), and angiogenic factors (such as vascular endothelial growth factors). The possible correlation of these biological markers with circulating cancer cells remains an interesting area for exploration. Additionally, the function of hMAM in breast cancer is not known, and it is unclear whether it is associated with the prognosis or metastatic potential of breast cancer.

A study by Zach *et al.* revealed a 49% positive rate of hMAM mRNA expression in patients with metastatic disease.[23] This finding is comparable to the rate of 54% for hMAM mRNA expression in metastatic breast cancer found in this study. This rate is statistically significantly higher than that of hMAM mRNA expression of patients with localized disease. This may indicate that hMAM mRNA expression in peripheral blood is related to tumor volume to some degree. Additionally, this study suggested that RT-PCR of hMAM mRNA may be an important tool for detecting recurrent or metastatic breast cancer. However, whether hMAM mRNA can detect micrometastases before the development of clinical disease is uncertain, and a prospective study is required to examine this question.

hMAM mRNA is one of the most sensitive molecular markers for breast cancer, but it may not be clinically useful for the screening or staging of breast cancer owing to its lack of correlation with other clinicopathological features. The potential role of this technique could be in following patients after surgery, and detecting recurrence or metastasis. Further evaluation is warranted of the combination of hMAM mRNA with other molecular markers to follow post-operative breast cancer patients.

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