Molecular Detection of the Mammaglobin Expression in the Peripheral Blood of Thai Breast Cancer Patients

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Abstract : **Mammaglobin (*hMAM*) is expressed exclusively in the mammary glands of adult women and mammary tumour cell lines. Thus, we examined *hMAM* expression as a marker for the detection of carcinoma cells in the peripheral blood of patients with breast cancer in Thailand. In addition, we studied the correlation between *hMAM* expression in circulatory mammary carcinoma cells and clinicopathological prognostic factors of breast cancer. Blood samples obtained from two hundred breast cancer patients at various stages of their disease and from sixty females without breast cancer (thirty healthy individuals and thirty patients with various malignancies other than breast cancer) were screened for *hMAM* mRNA by a nested reverse transcriptase polymerase chain reaction (RT-PCR) assay. We found significant differences between patients with breast cancer and those with other malignancies or healthy controls. None of the samples from the peripheral blood of sixty females without breast cancer was positive, whereas sixty four (32%) of the two hundred samples from breast cancer patients tested positive for *hMAM* mRNA. While our *hMAM* nested RT-PCR approach has 100% specificity, its sensitivity is only 32%. The presence or absence of *hMAM* expression in these breast cancer patients was not associated with clinicopathological prognostic factors including stage, oestrogen and progesterone receptors status, lymph node metastases, histological type, tumour size, differentiation, lymphatic invasion, vascular invasion, menopausal status or age. We summarised that the *hMAM* nested RT-PCR assay may be an effective tool for the detection of circulating mammary carcinoma cells of breast cancer patients. Nevertheless, the clinical relevance of *hMAM* RT-PCR based tumour cell detections should be further evaluated in prospective studies.**

Key words : Breast Cancer/ Circulating Cancer Cell/ Mammaglobin/ Nested RT-PCR

เรื่องย่อ : **การตรวจหาแมมมาโกลบินในกระแสเลือดของผู้ป่วยมะเร็งเต้านมในประเทศไทย**

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การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาความเป็นไปได้ที่จะใช้ความจำเพาะของการแสดงออก

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ของยีนแมมมาโกลบินต่อเซลล์ของต่อมสร้างน้ำนมและเซลล์มะเร็งเต้านม เป็นตัวบ่งชี้ในการตรวจหาเซลล์มะเร็งเต้านมในกระแสเลือดของผู้ป่วยมะเร็งเต้านมในประเทศไทย โดยทำการตรวจหาการแสดงออกของยีนแมมมาโกลบินในกระแสเลือดของผู้ป่วยมะเร็งเต้านม และศึกษาความสัมพันธ์ระหว่างการตรวจพบเซลล์มะเร็งเต้านมในกระแสเลือดของผู้ป่วย กับปัจจัยทางด้านพยาธิกำเนิดของโรค

จากการตรวจหายีนแมมมาโกลบินในกระแสเลือดใน 3 กลุ่มตัวอย่าง คือ กลุ่มผู้ป่วยมะเร็งเต้านมเพศหญิงในระยะต่างๆ จำนวน 200 คน, กลุ่มผู้ป่วยหญิงที่เป็นมะเร็งชนิดอื่นๆ จำนวน 30 คน และกลุ่มอาสาสมัครหญิงที่มีสุขภาพดี จำนวน 30 คน ด้วยวิธี nested reverse transcriptase polymerase chain reaction (RT-PCR) พบการแสดงออกของยีนนี้ในกระแสเลือดของผู้ป่วยมะเร็งเต้านม 64 คน จาก 200 คน (32 เปอร์เซ็นต์) แต่ไม่พบการแสดงออกของยีนนี้ในกระแสเลือดของคนปกติและผู้ป่วยที่เป็นมะเร็งชนิดอื่นๆ การตรวจพบเซลล์มะเร็งเต้านมในกระแสเลือดของผู้ป่วยไม่มีความสัมพันธ์ทางสถิติกับปัจจัยทางด้านพยาธิกำเนิดของโรค ที่ระดับความเชื่อมั่น $p < 0.05$

จากการวิจัยครั้งนี้มีข้อสรุปว่า การตรวจหายีนแมมมาโกลบิน โดยวิธี nested RT-PCR อาจเป็นวิธีที่เป็นประโยชน์สำหรับการตรวจหาเซลล์มะเร็งในกระแสเลือดของผู้ป่วยมะเร็งเต้านม แต่อย่างไรก็ตาม ยังต้องมีการศึกษาเพิ่มเติมในแง่ของประโยชน์ในทางคลินิกของการตรวจหาเซลล์มะเร็งในกระแสเลือด โดยวิธี nested RT-PCR นี้ ต่อไปในอนาคต

INTRODUCTION

Breast cancer is among the most common human cancers, representing 32 percent of all incident cancers and 19 percent of cancer-related deaths in women. Currently, more than 470 women in Siriraj Hospital and almost 1 million women worldwide are diagnosed with breast cancer every year. Thus, breast cancer is one of the biggest challenges for clinical and basic oncological research.

Metastasis of cells from the primary tumour is the hallmark event in cancer progression and could potentially become an important prognostic factor for survival. In breast cancer, the haematogenous spread of metastatic cancer cells greatly influence the outcome of the disease for most patients[1]. The presence of micrometastatic breast cancer cells in bone marrow, lung, liver and pleural of patients with primary breast cancer has been reported to confer a worse prognosis. Hence, the detection of circulating tumour cells may have important prognostic and therapeutic implications. Several methods were used to identify circulating tumour cells. Cytologic staining methods have low sensitivity and immunocytologic tests have higher sensitivity but with some degree of false positivity[1-3]. Recently, nested reverse transcriptase polymerase chain reaction (nested RT-PCR) was shown to be superior to conventional techniques in detecting circulating tumour cells by amplification of tissue-specific mRNA[2,3]. This technique allows the identification of 1 malignant cell mixed with 1 to 10 million normal cells; this advent has led to rapid advances in the detection of cancer cells in the peripheral blood based on specific genetic markers[4,5]. The detection of mRNA for certain genes, e.g., carcinoembryonic antigen (CEA)[5,6], cytokeratins[7,8] and Muc-1 as tumor markers for the presence of tumour cells in the peripheral blood of breast cancer patients, has been proposed. However, it has been shown to exhibit limited value because the frequency of expression of these markers is not always confined to breast tissue. These genes are of low specificity for tumour cells since a substantial number of false

positives were seen in patients with other cancers and healthy volunteers[5,6].

Because of the magnitude of the public health problem, the desire to reduce the impact of this disease on women, and the suitability of breast cancer as a model for the study of the molecular basis of cancer, an increasing number of investigators have focused on this disease in recent years. In 1996, the human mammaglobin gene (hMAM), a novel breast cancer-associated gene, was first identified[9]. Its amino acid sequence is very similar to that of several secreted epithelial proteins of the uteroglobin gene family. However, the functional significance of this protein in the process of mammary tumourigenesis is currently unclear. The hMAM gene is localised by fluorescent in situ hybridisation to chromosome 11q13, a genomic region frequently amplified in breast neoplasia[10]. To the extent that it is known, the expression of hMAM is restricted to the adult mammary gland and to mammary tumour cell lines and is overexpressed in 23% of primary human breast tumours compared with normal breast tissue[9].

Based on its breast cancer-associated and breast-specific pattern of expression, we believe that hMAM is an excellent candidate for a novel and clinically useful breast tumour marker. Because metastatic breast tumour cells frequently express hMAM, and hMAM cannot be detected in normal lymphoid populations, we employed the sensitive nested RT-PCR assay for mammaglobin mRNA to detect circulating breast tumour cells. Moreover, the correlation between the presentation of circulating breast tumour cells and the clinicopathological prognostic factors were also investigated. Theoretically, the circulating cancer cells represent cancer spreading and can be correlated well with the prognostic factors for poor survival. For our systematic correlation study, most of the clinicopathologic factors shown to be meaningful were included, for example, stage, oestrogen and progesterone receptors status, lymph node metastases, histological types, tumour size, differentiation, lymphatic invasion, vascular invasion, menopausal status and age.

MATERIALS AND METHODS

Patients and Cell Line

Blood samples from 30 healthy volunteers, 30 patients with various malignancies other than breast cancer (as controls) and 200 breast cancer patients were collected. Stage, oestrogen and progesterone receptors status, lymph node metastases, histological type, tumour size, differentiation, lymphatic invasion, vascular invasion, menopausal status and age were taken from patient records. The SKBR3 human mammary carcinoma cell line was a generous gift of Prof. Neelobol Neungton, obtained originally from the American Type Culture collection.

RNA Extraction and Nested RT-PCR

Total cellular RNA from 5 ml blood and breast cancer cell line was obtained by the acid guanidium thiocyanate phenol chloroform extraction procedure (AGPC)[11]. For RT-PCR analysis, 1 µg of indicated total RNA was reversely transcribed with random hexamer primers and superscript reverse transcriptase in a volume of 20 µl containing 1 × RT-buffer [50 mM TrisHCl (pH 8.3), 75 mM KCl, 3 mM MgCl2] 10 mM DTT, 200 µM dNTPs, 100 ng random hexamer primers, and 100 units reverse transcriptase. Reactions were performed at 42°C for 1 hour.

PCR reaction mixture consists of one-tenth of each RT reaction, 10 × concentrated reaction buffer, 200 µmol/L of each nucleotide, 0.5 µmol/L of mammaglobin specific primers and 1.5 units of Amplitag DNA polymerase. Primer sequences for the *hMAM* nested RT-PCR assay are MG-1 (5' GAA GTT GCT GAT GGT CCT CAT GCT GGC 3'), MG-2 (5' CTC ACC ATA CCC TGC AGT TCT GTG AGC3'), MG-3 (5' CTC CCA GCA CTG CTA CGC AGG CTC 3') and MG-4 (5' CAC CTC AAC ATT GCT CAG AGT TTC ATC ATC CG 3') (12). Primer pair MG-1 and MG-2 was used for the first amplification and MG-3 and MG-4 was used for the second. The nested primers for *hMAM* were designed to generate a PCR product spanning the whole translated region of its mRNA which is 201 base pairs. The cycle conditions were as follows: 2 minutes at 95°C, 35 cycles of 15 seconds at 95°C, 15 seconds at 62°C, 20

Table 1. Detection of circulating mammary carcinoma cells by hMAM nested RT-PCR

	Number	*hMAM*-positive	*hMAM*-negative	P-value
Breast cancer	200	64	136	
Female patients with other malignancies*	30	0	30	<0.001
Healthy female volunteers	30	0	30	<0.001

*Seven patients with hepatocellular carcinoma, seven with cholangiocarcinoma, six with colon carcinoma, five with gastric carcinoma, and five with pancreatic carcinoma.

seconds at 72°C, and, lastly, 7 minutes at 72°C. Two µl of the first amplification product were subjected to a second amplification using the second primer pair and the same cycle conditions as described above. In all PCR assays, cDNA from the breast cancer cell line SKBR3 and from healthy volunteers were used as positive and negative controls, respectively.

The presence of intact RNA and adequate cDNA synthesis was confirmed by a single-round RT-PCR using β_2-microtubulin genes specific primers[13]. Each sample was subjected to electrophoresis with 2% agarose gels stained with ethidium bromide.

Statistical Analysis

The χ^2 test (or Student's test for continuous variables) and Fisher's exact test were used to compare *hMAM* expressions in breast cancer patients and in the control group and assessed the statistical correla-

Table 2. Sensitivity and specificity values

	Value 95% confidence interval	Percent (%)
Sensitivity	0.320	32
Specificity	1.000	100

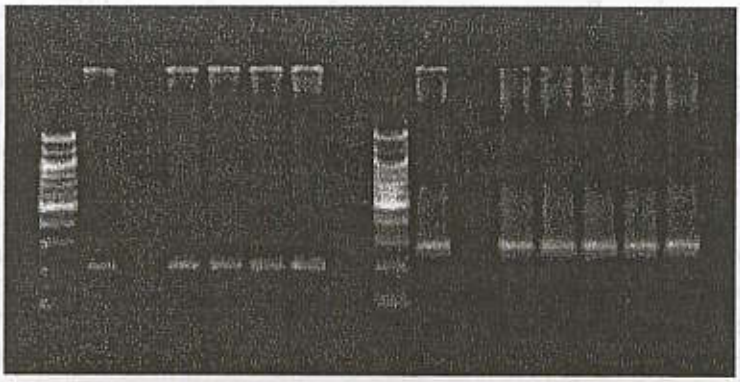

Figure 1. Ethidium bromide-stained 2% agarose gel of amplified β_2-microtubulin and *hMAM* PCR products. Two RNA preparations were made from each patient, and each preparation was subjected to β_2-microtubulin and *hMAM* nested RT-PCR setups. Samples were derived from breast cancer patients (lanes 1 to 5). Lane M, 100-bp DNA ladder; lane P, mammary carcinoma cell line SKBR3; lane N, water (blank control). Samples in lane 1 to 4 were positive for *hMAM* mRNA, whereas the sample in lane 5 was negative. The length of PCR products was 201 bp for *hMAM* and 268 bp for β_2-microtubulin.

Table 3. Characteristics of 200 breast cancer patients and tumours according to the results of nested RT-PCR

Factors	Nested RT-PCR		P
	hMAM (+) (n=64)	*hMAM* (-) (n=136)	
Patient age (yrs)			
Mean ± SD	49.56±10.2	51.54±11.1	0.2300[a]
Median			
≤ 50 yrs	39	66	
> 50 yrs	25	70	0.1370[b]
Menopausal status			
Premenopause	36	62	
Postmenopause	28	74	0.2090[b]
Tumour size (cm)			
≤ 2	25	46	
> 2 and ≤ 5	25	61	
> 5	14	29	0.6768[b]
Stage (n=186)			
I	15	32	
II,III and IV	46	93	0.9753[b]
Regional lymph nodes (n=186)			
Positive	34	59	
Negative	27	66	0.3487[b]
Histological type			
Noninvasive ductal carcinoma	3	11	
Invasive ductal carcinoma	61	125	0.5550[c]
Oestrogen receptor status (n=128)			
Positive	27	50	
Negative	19	32	0.9484[b]
Progesterone receptor status (n=122)			
Positive	22	30	
Negative	21	49	0.2241[b]
Histological grade (n=181)			
Well	6	8	
Moderately	35	70	
Poorly	18	44	0.3281[b]
Lymphatic invasion			
Positive	4	11	
Negative	60	125	0.7780[c]
Vascular invasion			
Positive	5	10	
Negative	59	125	1.0000[c]

[a]Student's t test
[b]χ^2 test
[c]Fisher exact test

tion between various clinicopathological risk factors and *hMAM* expression in peripheral blood. P values less than 0.05 were considered statistically significant. All tests were two-sided and computations were carried out using SPSS version 11.

RESULTS

Detection of *hMAM* Transcripts in Peripheral Blood

In order to identify circulating mammary carcinoma cells of breast cancer patients, 200 cases that included both lobular and ductal tumours were selected. All cases had been histologically proven to represent cases of breast cancer. These samples had previously been concentration-matched by their optical density readings. A 201-bp nested RT-PCR product, which corresponded to *hMAM* $mRNA_s$ was ampified in 64 of 200 (32%) breast cancer patients (Table 1). Amplified products of the nested RT-PCR assay for *hMAM* of five breast cancer patients are shown in Figure 1. Meanwhile, RNA samples prepared from peripheral blood of all 30 healthy female individuals and 30 female patients with malignancies other than breast cancer were negative for *hMAM* transcripts. An amplified *hMAM* product of the SKBR3 mammary carcinoma cell is shown in Figure 1 as a positive control (lane P). We used the detection of β_2-microtubulin mRNA in all reactions to prove that the absence of *hMAM* expression was not due to degraded RNA. The PCR band of β_2- microtubulin was detected in all samples. We found significant differences between patients with breast cancer and those with other malignancies or healthy controls (Table 1). After all, our *hMAM* nested RT-PCR approach has 100% specificity whereas its sensitivity is only 32% (Table 2).

Correlation between hMAM Transcripts in Peripheral Blood and Clinicopathological Prognostic Factors of Breast Cancer

To investigate whether the presence of *hMAM*-expressing breast cancer cells in blood was preoperatively correlated with a clinical prognosis, we performed a statistical analysis including almost all of the related prognostic factors of breast cancer. Although *hMAM* mRNA was found in breast cancer patients rather than in healthy controls and those with other cancers, the presence of these cells was not associated with any clinicopathological prognostic factors (Table 3). No significant difference in age distribution, proportion of menopausal patients, or tumour characteristics was detected. To avoid a potential false-positive result due to the age difference, these patients were divided into two age groups: ≤ 50 years of age, and ≥ 50. Again, we found no difference in *hMAM* transcripts among the groups. The difference in *hMAM* transcripts detected between stage I and II, and III and IV, was also not significant (24.7% vs. 8.1%, P = 0.5890). The level of positive results for the moderately differentiated group seems higher than for the well-differentiated and poorly-differentiated groups, but the number of patients with well differentiation was too small to make an adequate comparison. Similarly, a comparison between poor differentiation and non-poor differentiation (well and moderately differentiated) also showed no statistical significance (9.9% vs. 22.5%, P = 0.5220). The prognostic factors representing tumour extension and metastasis (i.e., lymphatic invasion, vascular invasion, and lymph node metastasis) also were not related to *hMAM* positive transcripts.

DISCUSSION

Detection of a malignant spread at its early stage is considered necessary because this may have important prognostic and therapeutic implications. Tumor metastasis is currently diagnosed by clinical manifestations and imaging studies along with serum marker assays. These techniques are only advantageous in later stages of tumour spread because of the necessity of a critical minimal tumour volume[14]. Some immunocytologic tests, sensitive to the detection of single tumour cells in the peripheral blood of cancer patients, were then exploited. Nevertheless, the antibodies used had some degree of false positivity, and this, as a result, limited their prognostic value[15]. Nested RT-PCR allows the specific detection of tu-

mour cells at the mRNA level in secondary sites like peripheral blood or bone marrow with an analytical sensitivity of one tumour cell measurable in the background of 10^6 normal cells without the need of tumour cell enrichment or purification prior to analysis. In contrast to other tumours, specific marker genes for the detection of disseminated cancer cells in the blood of breast cancer patients are not yet available. Most tumour-specific genes (e.g. CEA) or epithelium-specific genes (e.g. Cytokeratin 19) exhibited a limited diagnostic value because of either ectopic expression or limited sensitivity and specificity[13,16-19]. Thus, tissue and/or tumour specific marker genes are needed for the detection of metastases in the peripheral blood of breast cancer patients.

Mammaglobin seems to be a promising candidate for a breast cancer specific marker with regard to tumour specificity and sensitivity, allowing the detection of tumour cell dissemination in axillary lymph nodes and blood[12]. As the characterisation of tumour cell dissemination is the main focus of our study, we established a nested RTPCR test for the detection of *hMAM* expression in peripheral blood of Thai breast cancer patients. The established test proved to be a sensitive and robust method in detecting mRNA expression.

In peripheral blood of 200 breast cancer patients, a significant expression of *hMAM* mRNA was detectable in 64/200 (32%) of samples. Zach et al.[12] also reported that 25% of breast cancer patients exhibited *hMAM* expression in peripheral blood. With respect to specificity, *hMAM* expression was only detectable in the blood of breast cancer patients, but not in the control group of apparently healthy females. Furthermore, no *hMAM* mRNA expression was detected in all peripheral blood samples from patients with malignancies other than breast cancer. This indicated a high specificity of *hMAM* as a marker gene for cells derived from mammary glands. The high specificity of our assay was compatible with other reports using RT-PCR to detect the mRNA of epithelial markers[20]. However, there are enough studies that report high false-positive rates in healthy controls to question this method's use for clinical diagnosis. The sensitivity of RT-PCR commonly varies and may be caused by a lack of standardisation, including criteria for patient selection, sample collection, RNA extraction, PCR conditions, and primers designed[21]. The high specificity of our assay seems to be related to our careful protocol. To confirm pure RNA preparation, the samples were checked periodically for genomic DNA contamination using RNA as the template for PCR assay.

Although node-negative breast cancer patients have a favourable prognosis, recurrent disease occurs in approximately 20% of patients within 10 years after surgery[22]. Therefore, considerable efforts have been made to find markers conclusively associated with the risk of relapse. As clinical outcomes in breast cancer often require a prolonged follow-up, which is still in progress for the current study, *hMAM* expression was evaluated against known prognostic clinicopathological factors for correlation.

RNA-based assays by epithelial markers of circulating tumour cells have shown preliminary clinical significance[5,21,23-26]. According to a CEA mRNA assay, the level of positive circulating breast cancer cells was associated with the increased stage, suggesting a concept of molecular pathology[5]. It appears that analysing blood for tumour cells is a promising prognostic tool. However, there are only a few similar studies on breast cancer patients.

In our study, there was no statistical significance of the potential correlations between *hMAM* mRNA in circulating cells and prognostic clinicopathological factors. *hMAM* mRNA expression was not related to tumour size, stage, histological type, differentiation status oestrogen receptor and progesterone receptor status, lymph node metastases, lymphatic invasion, vascular invasion, menopausal status or patient age. Paradis et al. and Yuan et al.[27,28] similarly reported poor correlations between circulating tumour cells, as determined by RT-PCR of biomarker mRNA, and prognosis in prostate cancer and cervical cancer patients, respectively. Therefore, the utility of this assay has not yet been confirmed.

It seems sensible to study these patients continually to clarify the long-term effect of the positive assays on prognosis, possibly for 5-10 years. Adding patients with advanced breast cancers would be more

informative for this study, and observing these patients during chemotherapy to see the *hMAM* mRNA expression after each course of chemotherapy in comparison to prechemotherapy would add significant understanding to their possible clinical-pathologic importance.

In summary, our results show that mammaglobin nested RT-PCR exhibited a specific method in detecting circulating breast cancer cells. While it detected one breast cancer cell in 10 to 10 million white blood cells, none of the samples from the peripheral blood of healthy individuals were positive, whereas 32% of samples from breast cancer patients were positive for *hMAM* mRNA. The detection of circulating cancer cells in breast cancer patients may indicate an early phase of haematogenous spreading that possibly will develop further into metastasis or recurrence. Although the long-term effect of this phenomenon has not yet been clarified, especially regarding survival, this assay provides a model for monitoring early dissemination of cancer cells, which may ultimately allow us to prevent breast cancer from metastasising or recurring.

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