Clinical Significance of hMAM mRNA in the Peripheral Blood of Patients with Breast Cancer

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Abstract **Objective** To explored the clinical value of human mammaglobin (hMAM) mRNA in peripheral blood of patients with breast cancer. **Methods** Ninety-seven patients with breast cancer (stage Ⅰ 19 cases, stage Ⅱ 29 cases, stage Ⅲ 31 cases, stage Ⅳ 18 cases) and 15 cases of healthy donors were involved in this study. hMAM mRNA in peripheral blood of these cases was evaluated using nested RT-PCR approach. In addition, serum CEA and CA153 of patients with metastatic breast cancer (stage Ⅳ) were measured. **Results** hMAM mRNA was detected in 22% (21/97) breast cancer patients and 0%(0/15) health donors. The levels of hMAM mRNA were associated with primary tumor size, axillary lymph node status and tumor metastasis(P<0.05). However, they were not associated with tumor cell ER or PR expression or histologic grade of the tumor (P>0.05). For patients with metastatic disease, a combination of hMAM mRNA, CA153 and CEA could improve the accuracy of diagnosis (P<0.05). Chemotherapy could significantly reduce hMAM mRNA levels in patient's peripheral blood. **Conclusion** hMAM mRNA is a very useful molecular marker for the detection of circulating breast cancer cells, and is useful in monitoring of tumor response to chemotherapy.

Key words Breast neoplasms; Circulating tumor cells; Nested RT-PCR; Human Mammaglobin mRNA

Breast cancer is the most frequently diagnosed malignancies in women worldwide. Cancer biology study demonstrated that it usually takes about three years for a breast cancer developing into a 1cm mass in diameter from a single malignant cell, which will allow enough time for cancer cells spreading into the peripheral blood. With the current monitoring criteria, it is impossible to reliably identify those breast cancer patients who will relapse with metastasis disease. Research efforts have therefore been undertaken to identify additional parameters enabling individual risk assessment. In addition to their role as prognostic factors, these parameters may also serve as targets for new therapeutic approaches. Human mammaglobin (hMAM) is a highly specific marker for breast tissue since its expression is restricted to the adult mammary gland and to mammary tumor cell lines and is overexpressed in primary human breast tumors as compared to normal breast tissue [1].

This study is to evaluate the clinical significance of hMAM mRNA positive tumor cells in peripheral blood of breast cancer patients.

MATERIALS AND METHODS

Clinic materials

All samples from patients or healthy volunteers were obtained in accordance with the ethics committee of the Shandong Medical Science Academy upon informed consent. Of the 112 cases included in the study, 97 patients have biopsy confirmed breast cancer. Tumor staging and grading were performed according to AJCC criteria and modified Bloom-Richardson scheme respectively. The patients included 19 cases of stage Ⅰ, 29 cases of stage Ⅱ, 31cases of stage Ⅲ and 18 cases of stage

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Ⅳ (metastasis to supraclavicular lymph nodes, lung, liver or bone). Patient age ranged from 18 to 65 years with a median age of 48 years old. Fifteen healthy volunteers (control group) aged from 20 to 56 years with a median age 44 years old.

Samples collection

Ten (10) ml of peripheral blood was collected from each case. Mononuclear cells were isolated by density centrifugation, and washed twice in phosphate-buffered saline (PBS). The cell pellets were snap-frozen in liquid nitrogen and stored at −70℃ until further analysis. The plasma was used for evaluation of carcinoembryonic antigen (CEA) and carbohydrate antigen 15-3 (CA 15-3)levels. Fifty-eight patients with advanced diseases (StatgeⅢ and Ⅳ) were given 3 cycles chemotherapy according to CAF protocol with a follow-up hMAM mRNA in two weeks.

RNA extraction

Total RNA from frozen cell pellets was extracted using TRIZOl (Gibco-BRL, USA) according to the manufacturer's protocol. To remove potentially contaminating DNA within the RNA preparation, RNA was digested with RNase-free DNase Ⅰ (Life Technologies).

Nested RT-PCR

The nested RT-PCR was carried out using a Thermoscript RT-PCR System (Invitrogen, USA). cDNAs were reverse transcripted from 5μg of total RNA in a 50μl reaction. The sequence of the primers used in hMAM nested RT-PCR were as follows: P1: 5′gaagttgctgatggtcctcatgctggg3′ 63-89; P2: 5′ctcaccatacctgcagttctgtgagc3′ 361-387; P3: 5′ctgccccttattggagaatg 3′ 123-142; P4: 5′agccaaaggtcttgcagaaa3′ 363-382. Reverse transcription was performed with gene-specific oligonucleotides (p2 for hMAM). Primers labeled as the numbers 1(sense) and 2(antisense) were designed for the first PCR, and primers labeled as numbers 3 (sense) and 4 (antisense) were designed for the nested PCR. PCR reactions were performed according to the following standard protocol(initial denaturation at 94℃ for 2 min; 40 cycles: 94℃ for 15 sec, 15 sec at optimized anneal-

Table 1 Breast cancer patient characteristics and detection of hMAMmRNA-positive cells in peripheral blood

Pathology Parameters	Case	Positive case	Positive rate(%)	χ^2	*P*-value
⋆Tumor size					
>2cm	78	18	23.08	8.13	<0.05
⩽2cm	19	3	15.78		
⋆Lymph nodes					
positive	53	15	28.30	12.11	<0.01
negative	44	6	13.64		
⋆Distant metastasis					
yes	18	10	55.56	16.56	<0.01
no	79	11	26.58		
ER					
positive	50	12	24	3.10	>0.05
negative	47	9	19.15		
PR					
positive	48	9	18.75	5.73	>0.05
negative	49	12	24.49		
Histological grade					
Ⅰ	32	9	28.13	2.13	>0.05
Ⅱ and Ⅲ	23	7	30.43		

⋆ $P<0.05$

Table 2 hMAMmRNA versus CA153, CEA of 18 cases patients with metastasis

Marker	Positive case	Sensitivity(%)	Speciality(%)
hMAMmRNA	10	55.56	96.23
CA153	12	66.67	91.21
CEA	10	55.56	90.35
⋆hMAMmRNA+ CA153	16	88.89	88.73
⋆hMAMmRNA+ CEA	14	77.78	87.33
CA153+ CEA	12	66.67	80.25

⋆There´s significant combination of 2 marks than single mark(P<0.05)

Table 3 hMAMmRNA related with tumor size change after chemotherapy

Tumor size reduced	hMAM change to negative		Total (N2)	P−value⋆
	Yes	No		
Yes	10	1	11	0.026
No	3	4	7	
Total (N1)	13	5	18	

P−value⋆: was calculated using Fisher´s Exact test.

ing temperatures, 72℃ for 50 sec; final elongation: 72℃ for 6 min). The integrity of the isolated RNA preparations was checked by amplification of β−actin transcripts. Consequently, in case with failed amplification of β−actin transcripts, the sample was excluded from further analysis. Total RNA preparations isolated from MCF−7 breast cancer cell line were served as positive controls.

Five of 50μl cDNA were subjected to a first hMAM specific amplification step in a 50μl reaction containing 1.5U Taq DNA Polymerase (Life Technologies), 1.5 mM $MgCl_2$, 200pmoles of each dNTP and 12.5pmoles of both oligonucleotide primers (p1 and p2). Five microliters of the amplification products of the first PCR were subjected to a second PCR reaction using the nested primers (p3 and p4) at the same conditions as for the first PCR. All PCR products were size−separated by 1% agarose gel electrophoresis and visualized by ethidium bromide (0.05 mg/ml) staining (Fig. 1).

Detection of CEA and CA15−3

CEA and CA15−3 in plasma samples were detected using an Electric Chemistry Luminescence System. The cut−off values of CEA and CA15−3 were 3.4ng/ml and 24U/ml respectively.

Statistical Analysis

Data were analyzed with SPSS software 10.0 package, differences were analyzed by two−tailed Chi−squared or Fischer´s Exact Test, when appropriate. A P−value of less than 0.05 was considered as significant. Those associations attaining or showing a trend towards statistical significance were subsequently included in a multivariate model, in which hMAM mRNA expression was taken as dependent variable.

RESULTS

hMAM mRNA was detected in the peripheral

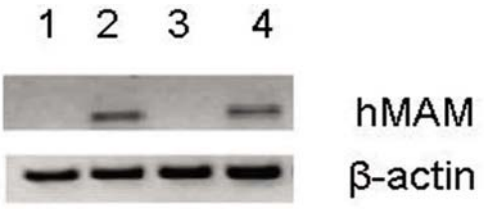

Fig. 1 Nested RT−PCR assay for hMAM mRNA. Lines 1, 3 negative expression. Line 2, MCF−7cell line. Line 4, positive expression.

blood of 21 patients(21/97, 22%) with breast carcinoma. The hMAM mRNA positive rate in patients with tumor more than 2cm in diameter was higher than that in patients with tumor less than 2cm (21.3% versus 15.8%, χ^2=8.13, P<0.05). The hMAM mRNA positive rate in patients with lymph node metastasis was higher than that in patients without metastasis (28.3% versus 13.6%; χ^2=12.11, P<0.05). The positive rate of hMAM mRNA in patients with distant metastasis was higher than that in patients without distant metastasis (55.6% versus 26.6, χ^2=16.56, P<0.01). There was no statistical difference of hMAM mRNA positive rate in carcinomas with or without ER, PR expression (χ^2=3.10, P>0.05); there was no statistical difference of hMAMmRNA positive rate between low and high histological grade carcinoma(Grade Ⅰ versus Grade Ⅱ + Ⅲ, χ^2=2.13, P>0.05) (Table 1).

For patients with distant metastasis, there was no statistical difference in the serum levels of CA153, CEA or the quantitative hMAMmRNA(P>005), but when combined, there was a higher sensitivity than anyone of them(P<0.05), and the specificity of hMAMmRNA had the trend to be elevated (Table2).

After 3 cycles chemotherapy before operation, 13 cases in 18 cases of positive hMAMmRNA turned to be negative, there was statistical difference between before administration of chemotherapy and after that (P< 0.05). The change of circulating cells in peripheral blood after chemotherapy was significantly related to the change of tumor size(r=0.421, P<0.05) (Table3).

DISCUSSION

Tumour markers are often used to evalute clinic stage, detect disease recurrence after primary treatment for cancer and to monitor response to therapy. CEA and CA15－3 are two of the commonly used tumor markers, but they are not specific to breast cancer, and their serum levels do not correlate with tumor stage [2]. Studies by Sutterlin, *et al* showed that 49% of breast cancer patients with positive CEA (>5ng/ml), and 64% with positive CA15－3 (>25u/ml) have metastatic disease, but these makers have very low tumor specificity[3]. The American Society of Clinical Oncology (ASCO) recommends against the routine use of CEA and CA 15－3 to monitor tumor responses to treatment. Therefore, it is clearly a worthwhile goal to find a reliable serum tumour marker with high sensitivity and high specificity for breast cancer. It is now clear that the presence of DTCs correlates with subsequent development of clinically evident bone metastases, and a worse outcome from breast cancer [4]. Studies by Grunewald and colleagues [5] suggested that the presence of hMAM mRNA in the peripheral blood of the breast cancer patient is a promising marker for hematogenous spread of tumor cells.

Our results clearly demonstrated the significant and prognostic value of hMAMmRNA detection by Nested RT－PCR in the peripheral blood of patients with breast cancer, in accordance with very published information which has shown that hMAMmRNA detection in peripheral blood of breast cancer represents a negative prognostic marker in breast cancer[6].

Our results indicated that positive hMAMmRNA expression correlated with primary tumor size, regional lymph node and distant metastasis, respectivel(P<0.05). The serum hMAMmRNA levels were also correlated with tumor response to chemotherapy. However, the expression of hMAM mRNA did not correlate with tumor histological grade or ER/PR status (P>0.05). The detection of hMAMmRNA－positive cells in peripheral blood of breast cancer patients was significantly associated with clinical stage but couldn´t reflect its malignant biology behavior. Moreover, the serum hMAM mRNA levels were well correlated with response to chemotherapy. Our results suggested that patients with elevated CTC later in their treatment course(third cycle or beyond) are very likely to progress or illustrated chemotherapy tolerant and a change of therapy protocol may be needed. The benefit of changing therapy is very early in the course of treatment, needn´t other obvious clinical and/or radiographic signs of progression, especially to those primary lesion was removed before. These observations suggested that serum hMAMmRNA may be a reliable marker of recurrence or therapeutic efficacy.

hMAMmRNA were not detected in all patients with breast cancer (22% in our study). Liotta[7] has pro-

posed that tumor cells may enter peripheral blood in cluster, not continuously. Some patients, including patient with small primary lesion (3 cases of T1 stage) or negative lymph node metastasis (6 cases, N0), had positive hMAM mRNA in their peripheral blood, which suggested tumor cell blood spread in early disease. For these patients, close clinical follow-up and proper triage are neccessary indicated.

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