JBUON 2016; 21(1): 35-41
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

Human mammaglobin: A specific marker for breast cancer prognosis

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Summary

Purpose: *In this study, we examined the expression of human mammaglobin (hMAM) mRNA and the protein levels in patients with breast cancer and their relationship with prognostic clinicopathological parameters.*

Methods: *hMAM mRNA expression in leucocytes from peripheral blood samples from patients diagnosed with primary invasive breast cancer (IC), carcinoma in situ (CIS), or benign breast diseases was analyzed using RT-PCR. The hMAM protein levels and expression patterns in tissue from 3 patient groups were evaluated by immunohistochemical staining, and several non-breast neoplasms were selected as negative controls, undergoing the same examination.*

Results: *The expression of hMAM mRNA was significantly higher in patients with IC or CIS compared to those with benign tumors (both<0.01). Immunohistochemical staining revealed similar results, where patients with IC or CIS had higher levels of hMAM protein ($p<0.01$ and $p<0.01$, respectively), while none of the negative controls expressed hMAM. Further analyses showed a strong correlation between hMAM protein/mRNA expression and clinicopathological factors, such as histological grade, clinical stage, and lymph node status, in patients with IC.*

Conclusion: *The hMAM mRNA and protein expression profiles validate the potential of hMAM as a specific marker for breast cancer diagnosis and target treatment delivery.*

Key words: *breast cancer, mammaglobin, prognostic factor*

Introduction

Breast cancer, known for its morphological diversity and unpredictable clinical behavior, is the most common malignancy in females worldwide [1]. Although management strategies have improved over the past decades, distant recurrence can still be detected 5 years after initial verification of node-negative disease [2]. To date, several breast biomarkers have been postulated and applied in both cancer diagnosis and prognosis, such as estrogen and progesterone receptors (ER and PR, respectively), and gross cystic disease fluid protein-15 (GCDFP-15) [3-5]; however, there are obvious limitations to the current approaches for malignancy assessment. Despite their underlying potential, the above-mentioned biomarkers are compromised due to unsatisfactory sensitivity and specificity. Additionally, the lack of tissue specificity further prevents their application.

hMAM is classified as a member of the secretoglobin-uteroglobin family, and its corresponding gene locates at chromosome 11q12.3-13.1, with a length of 503bp [6]. The expression of hMAM protein and its related mRNA has been proposed as a novel marker for breast cancer diagnosis and prognosis [7-10]; nevertheless, controversial results have led to arguments against

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Received: 04/08/2015; Accepted: 25/08/2015

the use of *hMAM* mRNA levels as a predictor of recurrence in patients with non-metastatic breast cancer [11]. In addition, only a few studies have examined the difference between the two patterns of hMAM protein expression, i.e., cytoplasmic and membranous [12-14].

In this study, we investigated the relationship between hMAM protein and mRNA expression and clinicopathological parameters in breast cancer, and discussed the potential of hMAM as a valuable molecular biomarker for breast cancer prognosis. In addition, we sought to reveal the difference in hMAM expression patterns in tumor cells from patients with breast cancer, and evaluated the potential of a specific pattern as a molecular target for the future development of therapeutic tools or drug delivery methods.

Methods

Patients and tissues

This prospective study comprised 172 patients with breast disease treated at Anhui Provincial Hospital from 2012 to 2014. All 80 primary IC cases were chosen stochastically, and histological confirmation was made by an experienced pathologist according to World Health Organization (WHO) criteria [1]. The demographic and clinicopathological data of the 80 patients are summarized in Table 1. We also selected 39 cases of CIS, representing early cancerous lesions, which comprised 35 cases of ductal CIS (DCIS) and 4 cases of lobular CIS (LCIS). Fifty-three cases of benign breast diseases, including mammary gland hyperplasia and fibroadenoma, were enrolled in the control group. Tissue microarray (US Biomax, Inc., Rockville, MD, USA) was carried out in 121 cases of diverse non-breast neoplasms and used as negative control.

All peripheral blood samples (2 mL per patient) were collected before treatment (chemotherapy for patients with IC; surgery for patients with CIS/benign breast diseases) according to standard protocols and stored at −70°C for final analysis. All formalin-fixed, paraffin-embedded (FFPE) human tissue blocks were obtained from the Tissue Bank of the Anhui Provincial Hospital, Department of Pathology. This study was approved by the ethics committee of the Anhui Provincial Hospital, and written informed consent was obtained from the enrolled patients.

RNA extraction and reverse transcription–polymerase chain reaction (RT-PCR) analysis

The hMAM primer was designed and purchased from Shanghai ShengGong Bioengineering (Shanghai,China). The forward and reverse primers were 5'-TGCCACCCGCGACTGAACAC-3' and 5'-GCAGCCAGAGCCTGCGTAGC-3', respectively; the amplification product length was 105 bp. The forward and reverse primers of the internal reference glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5'-TGACGCTGGGGCTGGCATTG-3' and 5'-GCTCTTGCTGGGGCTGGTGG-3', respectively (Sigma-Aldrich, St Louis, MO, USA); the amplification product length was 154 bp. All primers were desalted when purified.

Total RNA was extracted from the leucocytes of peripheral blood samples from the enrolled patients using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and subjected to quantitative analysis with spectrophotometry and qualitative evaluation with electrophoresis. Total RNA was reverse-transcribed into complementary DNA (cDNA) in 20-μL reactions consisting of 4 μL total mRNA, 4 μL 5×buffer, 0.5 μL oligodT, 0.5 μL dNTP, 1 μL Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase, and 10 μL distilled water (ultrapure, DNase- and RNase-free). The reverse transcription reaction parameters were reverse-transcribed for 1 h at 37°C and holding at 95°C for 5 min to inactivate the M-MLV reverse transcriptase.

PCR amplification of the obtained cDNA was performed in standard 50-μL reactions containing 0.5 μL

Table 1. Expression of h*MAM* mRNA and protein in different breast diseases

	hMAM mRNA		*Positive (%)*	*p*	*hMAM protein*		*Positive (%)*	*p*
	+	-			+	-		
Benign breast disease	0	53	0.0	<0.01**	2	51	3.8	<0.01**
Carcinoma *in situ*	20	19	51.3	<0.01*	8	31	20.5	<0.01*
Invasive breast cancer	60	20	75.0	0.10***	58	22	72.5	<0.01***

Benign breast disease vs carcinoma in situ, p<0.01; **benign breast disease vs breast cancer, p<0.01;***carcinoma in situ vs breast cancer, p=0.10

forward primer and 0.5 μL reverse primer, 2 μL cDNA template, 32.5 μL SYBR Green Mix, and 14.5 μL ultrapure water. The reaction conditions have been described previously [9]. In brief, the above reaction system was subjected to the following cycle 35 times, with final elongation at 72°C for 5 min: 95°C denaturation for 5 min, 94°C melting for 45 sec, 56°C annealing for 1 sec, 72°C extension for 1 sec.

The PCR products were separated by electrophoresis and visualized in ethidium bromide–stained 2% agarose gels. As an internal control, *GAPDH* was amplified in the same tube as h*MAM*, and subsequent qualitative analysis of the detected h*MAM* mRNA was conducted through comparison with the *GAPDH* mRNA expression level. Positive expression of h*MAM* mRNA was defined as the signal intensity of an indicated sample matching baseline GAPDH expression, and a negative result was where signal intensity was below the baseline.

Immunohistochemistry

EnVision immunohistochemistry (Zhongshan Biotechnology, Beijing, China) was used to detect hMAM protein expression in the breast disease tissues. The FFPE tissues were sectioned into 4-μm slices, deparaffinized, and subjected to antigen retrieval and endogenous peroxidase blocking. After pretreatment, the slides were incubated with anti-hMAM monoclonal antibody (1:500, rabbit anti-human; clone: EP249, ZSGB-BIO, Beijing, China) at 4°C overnight, incubated with a secondary antibody (PV6000, Zhongshan Biotechnology, China), and visualized using an enhanced diaminobenzidine (DAB) kit (Zhongshan Biotechnology, China). The immunostained slides were counterstained with hematoxylin and were evaluated using an Olympus microscope with a digital camera. GCDFP-15 expression was evaluated only in breast cancer tissues using the same protocol as above, using anti–GCDFP-15 antibody (rabbit anti-human; clone: EP95, ZSGB-BIO, China). Positive and negative controls were performed alongside the samples. Phosphate-buffered saline (PBS) substitution for primary antibody served as the negative control, and slides incubated with only secondary antibody were used as the blank control. The positive control was performed according to the instructions in the applied kit.

Immunohistochemical analysis criteria

Immunohistochemical staining was evaluated by an experienced pathologist. Ten high-power fields were stochastically selected and at least 500 cells were counted for each slice. Staining intensity and the proportion of the positive cell population were aggregated for evaluation as a whole. The cut-off value was 10% with respect to the ratio of positive cells to overall cells. The immunohistochemical staining results were documented as negative (-) or positive (+).

Statistics

SPSS 13.0 software (SPSS, Chicago, Ill, USA) was used for all data analyses. Differences between different groups and of positive and negative rates of various clinicopathological parameters were assessed using the Pearson's chi-square test. Results with $p<0.05$ were considered statistically significant.

Results

hMAM mRNA expression in the peripheral blood of patients with breast diseases

The expression of h*MAM* mRNA was not detected in any patient with benign breast disease. In contrast, positive results were observed in 20 of the 39 patients (51.28%) with CIS (DCIS and LCIS) and 60 of the 80 patients with IC (75.00%, Table 1). The differences between patients with benign disease and patients with CIS or IC were statistically significant ($p<0.01$), while no difference was observed between patients with CIS and IC ($p=0.10$).

hMAM protein expression in tissues from diverse breast diseases

The expression of hMAM protein was found in 3.77% (2/53) of patients with benign breast disease, 20.51% (8/39) of patients with CIS, and 72.50% (58/80) of patients with IC (Table 1, Figure 1). There was a significant difference between the patients with benign lesions and patients with CIS or IC ($p<0.01$ and $p<0.01$, respectively).

Expression of hMAM protein in non-breast neoplasms

The immunoreactivity profile of hMAM in 121 cases of non-breast neoplasms revealed no hMAM protein expression in all selected tissues (Table 2).

Expression pattern of hMAM in breast cancer tissues

Two hMAM immunostaining patterns were observed in the pathological analyses: 86.21% (50/58) of positive hMAM staining presented a cytoplasmic pattern. However, hMAM protein expression was detected in both the membrane and cytoplasm in some patients with IC (Figure 1D).

Relationship between hMAM protein expression and clinicopathological characteristics in patients with breast cancer

The expression of hMAM protein in IC tis-

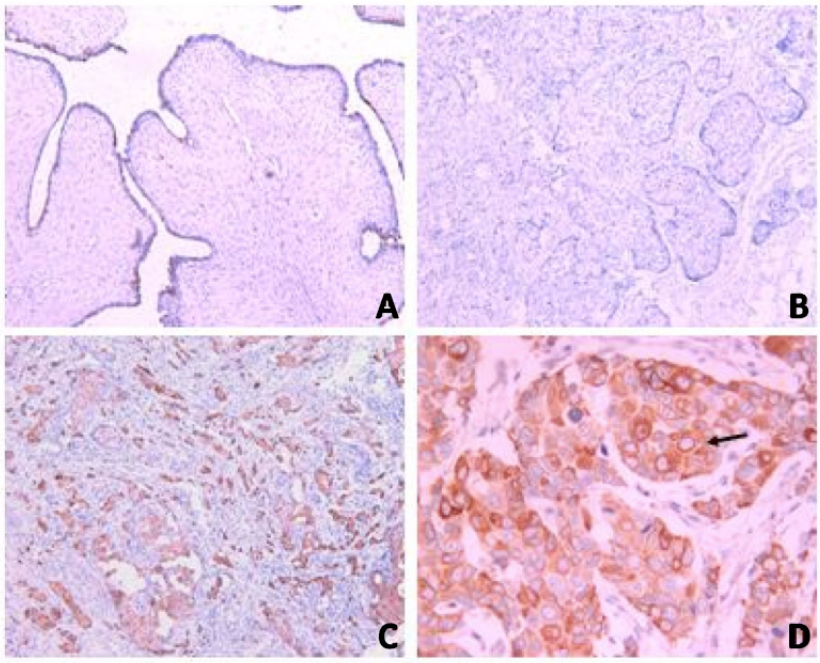

Figure 1. hMAM protein expression in tumor tissues of diverse breast disease. **A:** Breast fibroadenoma; **B:** Carcinoma *in situ*; **C**: Breast invasive carcinoma; **D:** Arrow indicates membranous pattern of hMAM expression on the surface of some tumor cells in some invasive breast cancer patients, although the cytoplasmic pattern is also present (x400).

Table 2. Expression profile of hMAM in non-breast neoplasms

Neoplastic tissue type	*Positive/Total*
Gastric cancer	0/22
Colon cancer	0/18
Hepatocellular cancer	0/11
Esophageal cancer	0/7
Lung cancer	0/7
Pancreatic cancer	0/5
Prostate cancer	0/13
Cervical cancer	0/5
Ovarian cancer	0/5
Renal cancer	0/5
Bladder cancer	0/4
Thyroid cancer	0/10
Ewing's sarcoma	0/3
Melanoma	0/3
Mesothelioma	0/3

sues was correlated with clinical stage (p=0.02). Similar results were obtained between patients with lymph node metastasis and patients without metastasis (92.50 vs 52.50%, p<0.01). GCDFP-15 expression was consistent with hMAM expression in patients with IC (Table 3).

Relationship between hMAM mRNA expression and clinicopathological characteristics in patients with breast cancer

The expression of h*MAM* mRNA in IC tissues was correlated with lymph node metastasis (p<0.01). However, no association was observed between h*MAM* mRNA expression and histological grade or clinical stage (Table 4).

Discussion

In this study, we found that the expression of h*MAM* mRNA in peripheral blood samples and hMAM protein in the original tumor tissues were significantly higher in patients with CIS or IC. Various previous studies have attempted to detect the gene expression levels of h*MAM* in different samples, including the primary tumor and bone marrow [9,10,15,16]; however, investigations of metastasis in the bloodstream are limited [11]. Circulating tumor cells (CTC), originating from a primary tumor and travelling to a distant location to facilitate malignant colonization at a second site [17], have proven to be an essential prognostic factor in metastatic breast cancer [18]. Nonetheless, accurate detection of CTC remains controversial, and the procedure is relatively expensive. In

Table 3. Relationship between hMAM protein expression and clinicopathological parameters in patients with primary breast invasive carcinoma

Parameters	*N*	*hMAM protein expression*		*Positive (%)*	*p value*
		+	-		
Histological grade					0.33
1	24	19	5	79.2	
2	33	21	12	63.6	
3	23	18	5	78.3	
Clinical stage					0.02
I	9	5	4	55.6	
II	50	33	17	66.0	
III	21	20	1	95.2	
Lymph node metastasis					0.01
Positive	40	37	3	92.5	
Negative	40	21	19	52.5	
GCDFP-15 expression					0.025
Positive	74	56	18	75.7	
Negative	6	2	4	33.3	

Table 4. Relationship between hMAM mRNA expression and clinicopathological parameters in patients with primary invasive breast carcinoma

Parameters	*N*	*hMAM mRNA expression*		*Positive (%)*	*p value*
		+	-		
Histological grade					0.99
1	24	18	6	75.00	
2	33	25	8	75.76	
3	23	17	6	73.91	
Clinical stage	80				0.28
I	9	6	3	66.67	
II	50	34	14	68.00	
III	21	20	3	95.24	
Lymph node metastasis					<0.01
Positive	40	38	2	95.00	
Negative	40	22	18	55.00	

this work, we performed an aggregate study to detect h*MAM* mRNA expression in leucocytes from peripheral blood as a surrogate for CTC, as it has been revealed that hMAM is a specific CTC marker [7] and that h*MAM* mRNA cannot be amplified from lesions with benign diseases, as indicated in our study. Higher expression of h*MAM* mRNA from leucocytes indicates increased CTC, which allows for advanced disease migration. This hypothesis correlates with our present study findings in that the overexpression of h*MAM* mRNA was consistent with lymph node metastasis. We discovered that h*MAM* mRNA levels in IC and CIS were remarkably higher; furthermore, no amplification was detected in blood samples from patients with benign breast lesions. This result correlates with prior evidence that h*MAM* mRNA is a potential gene marker of metastasis of breast cancer and has superior prognostic significance [8,9]. Not only did we assess hMAM mRNA expression

in blood cells, immunohistochemistry testing was also performed to evaluate hMAM protein levels in various tumor tissues. As expected, hMAM protein levels were noticeably high in patients with CIS, IC, and metastatic disease. Additionally, the absence of hMAM protein from the 121 cases of non-breast neoplasms indicated that its expression is restricted to breast carcinoma.

Recent studies have reported that GCDFP-15 is a potential protein marker in the diagnosis of carcinoma of breast origin [3,5]; however, controversial results have indicated that hMAM is more valuable than GCDFP-15 as a protein marker [15,19]. Our data revealed a significant correlation between hMAM and GCDFP-15 protein levels and indicated specific expression of hMAM protein in breast malignancy, validating the value of hMAM as a diagnostic protein marker. That hMAM protein and mRNA are expressed specifically in patients with breast cancer justifies the use of hMAM as a diagnostic marker for carcinoma of breast origin.

Locoregional and distant metastasis have long been regarded as indicators of poor prognosis in patients with breast IC [20,21]. The current idea is that poor prognosis is associated with lymphatic metastasis and advanced clinical stage. Moreover, identifying lymphatic metastasis is essential in tumor staging and is critical with respect to the choice of postsurgical management. Therefore, in the present study, we evaluated the correlation between hMAM protein expression and both clinical stage and lymphatic metastasis status. We found that hMAM protein expression in IC samples detected by immunohistochemistry was remarkably higher in patients with advanced disease. Furthermore, there was a significant correlation between hMAM protein expression and lymphatic metastasis. Thus, we postulate that hMAM protein is a valuable marker implicated in the presence of lymphatic metastasis and advanced tumor stage. It can serve as a parameter for clinical evaluation and regimen arrangement in patients with breast cancer.

Two hMAM expression patterns were detected in this study. The membranous pattern was observed in patients with certain breast cancers, although the cytoplasmic pattern was present in the majority of the cases. A previous publication investigated the secretory nature of hMAM and found that hMAM protein was present in the medium of cultured MAM-positive breast cancer cells [12]. Even though a study that used currently available anti-MAM antibodies revealed that the hMAM-positive staining was predominantly confined to the cytoplasm [13], and only one study has provided robust evidence proving that some hMAM proteins are associated with the membrane [14], our finding of the membranous pattern in some breast cancer tissues illustrates the possible existence of membrane-associated MAM proteins. In fact, the membranous pattern could be prevalent; however, simple immunostaining might not be an efficient means of detecting it. Based on the finding that hMAM possibly exists on the surface of breast cancer cells, we suggest that the presence of membrane-associated MAM could be a valuable molecular target for future development of therapeutic tools. Nonetheless, the presence and interpretation of the membranous pattern should be further examined, and a well-designed study should be carried out to evaluate the prognostic potential of this marker.

In conclusion, the expression profiles of h*MAM* mRNA and protein in the peripheral blood and tumor tissues demonstrated in this study support the potential of hMAM as a specific marker of breast cancer prognosis and the determination of malignancy of breast origin. Furthermore, the secretory pattern of hMAM protein provides a potential clue for the future development of target therapy.

Acknownledgements

This work was supported by the Supporting Project for Youth of Health Department, Anhui Province (09B120).

Authors' contributions

LCY carried out the molecular genetic studies, participated in the sequence alignment, carried out the immunoassays and drafted the manuscript. ZTG conceived the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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