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Maspin Gene Expression in Invasive Ductal Carcinoma of Breast

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KEY WORDS

Breast cancer
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Real-time PCR
Iran

ABSTRACT

Background: The breast cancer is the most prevalent cancer among women, on the other hand absence of myoepithelial cells play a pivotal role in pathogenesis of this cancer. Thus we aimed to investigate the possible abilities of the molecular assay technique to find a relationship between mammary serine protease inhibitor (Maspin) gene expression possibly secreted by myoepithelial cells, grade of breast cancer and other prognostics factors (ER, PR, and c-erb-B2).

Methods: Paraffin embedded blocks of 31 breast cancer patients together with two normal breast tissues were used for IHC staining and Maspin gene RNA detection uses the real-time PCR method. Applying QIAGEN kit, we were able to measure Maspin RNA and Extract the cDNA of different samples for evaluating the Maspin RNA level.

Results: We found that the RNA level was considerably lower in these cancer samples compared with normal samples. In addition, different grades of breast cancer in the obtained results adopt some distinguishable values. The Maspin expression in samples with grades II and III is much lower than the ones in normal group ($P < 0.05$) which could be considered as a promising way in diagnosing of this disease. The results showed no considerable differences in Maspin gene expression of the c-erb-B2 scores in the tumor group except the samples having score 0. The other observation of this research study confirmed that Maspin gene expression couldn't show any differences between the values of both ER and PR in different scores of the tumor group. On the other hand, the cDNA of these patients showed lower values compared with normal samples.

Conclusion: Maspin expression was reduced in samples with grade II & III of invasive ductal carcinoma. Based on expression of Maspin Inc-erb-B2, it seems that more expression happened in normal group comparing with different scores of it. We could suggest that there was a reverse relationship between tumor formation and Maspin gene expression. These results showed possible role of Maspin as prognostic factor

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Introduction

After cardiovascular disease, cancer is the most prevalent cause of death in humans. Among

various types of cancer, breast carcinoma is more common malignant tumor in women (1, 2). One out of eight women would suffer from breast cancer in their lifetime. In Iran, 24% of women with debilitating disease in age specific rate (ASR) have cancer, in which 23.65% of them have breast cancer. The prevalence and incidence of the breast cancer in Iran is respectively approximated 120 in 100000 persons and 22 in 100000 women (3, 4).

In contrast other deadly malignant tumors, if breast cancer could be diagnosed in its early stage then it would be curable (5-7). Early diagnosis of this disease which leads to an easier treatment process is a challenge for clinicians (4) since it is silent and usually diagnosed late with poor prognosis (8).

For many years, presence of myoepithelial cells "was and is" the hallmark of "H&E and even IHC staining" for diagnosis of: benign ductal hyperplasia, atypical ductal hyperplasia, ductal carcinoma in situ (DCIS) comparing with invasive ductal carcinoma (IDC). From other point of view, up to now some prognostic markers have been presented in breast cancer including estrogen receptor, progesterone receptor and c-erb-B2 (9-11). Although these markers have been influential in treating breast cancer, the recently introduced molecular techniques pave the way for pathologists to find more accurate prognostic factors (12-15).

Breast cancer has a complex phenotype with variable genetic disorder. Recent studies in molecular technology show good progression in molecular taxonomy of this common cancer (16). Maspin (mammary serine protease inhibitor) gene, is part of serine protease inhibitor/non-inhibitor lineage. This gene produces Maspin protein seen in myoepithelial cells and has a correlation with intra and extracellular protein in cell adhesion, motility, apoptosis and angiogenesis (1, 17-19).

Maspin gene expression decreases in some types of cancers such as gastric, prostate, and melanoma and increases in pancreatic,

gallbladder, colorectal, and thyroid cancers (20-22).

The difference in Maspin gene expression gives us a hint to investigate role in various cancers in order to find its various functions in different cell types. Therefore, we aimed to investigate the possible abilities of the molecular assay technique to find a relationship between Maspin gene expression, grade of breast cancer and other prognostics factors.

Material and Methods

Study Design and Sample Preparation

Maspin gene expressions in different grades of breast cancer were evaluated using relative quantification Real time PCR with one-step method and cDNA method, prepared at first from RNA and then amplified in the same reaction.

The study was approved by the Ethics Committee of Kerman University of Medical Sciences, Kerman, Iran.

Thirty three different samples were evaluated and categorized into two groups. In the first group, 31 FFPE (fixed formalin paraffin embedded) were obtained from breast cancer patients of Afzalipoor & Shahid Bahonar pathology wards. The second group contained of two normal breast tissues which obtained from mammoplasty samples paraffin blocks.

FFPE samples were prepared for H&E and IHC staining. H&E slides were reviewed by two pathologists double-blindly.

IHC Staining

Dehydrated, deparaffinized sections along with retrieval buffer were microwaved for 20 min (3 min at 850 watts; 17 min at 180 watts), then endogenous peroxidase blocked for 10 min with 0.5% H₂O₂. The sections were incubated for one hour at room temperature with monoclonal antibodies, in this way:

HER2-neu (1:100; DAKO); PR (1:100; DAKO, Clone PgR 636); ER (1:50; DAKO, Clone 1D5): Ready to use. Slides were rinsed with wash buffer for 5 min; this step was repeated twice between all stages. Envision polymer (30 min) was added using 3, 3'-diaminobenzidine (DAB) as the chromogen (10 min) after these steps hematoxylin staining for 2 min, dehydration and mounting the slides.

Then, the obtained slides were scored by two pathologists according to the standard scores for ER, PR, and c-erb-B2as defined by WHO.

Extraction of RNA from the Samples

For simultaneous RNA extraction, All Prep® DNA/RNA FFPE (QIAGEN® Germany) kits were applied. This kit has the ability to purify genomic RNA of formalin-fixed and paraffin-embedded tissues. According to the protocol of this kit, two 20 µm sections of each sample were prepared, then deparaffinization solution (cat. No 190093, QIAGEN) was employed. Then, the RNA extraction was investigated by the step by step procedures according to manufacture instructions.

First of all, in order to destruct tissues, PKD buffer with proteinase k were added. Then, the purification process of RNA was done based on their specific columns. Moreover, DNase was respectively applied for purification process of RNA. Finally, RNA was eluted with 20 and 50 µL of ATE buffer respectively. To prevent damage of the obtained RNA, part of the purified RNA was immediately put on ice at -20 °C to be used in the one step method and the other part of the RNA was changed into cDNA.

cDNA Synthesis

Five µg of extracted RNA was employed for cDNA synthesis and amplification with QuantiTect® Reverse Transcription (QIAGEN® Germany) kit.

Design Primers & Probe of Maspin and GAPDH Genes

To properly design Maspin gene primers and probe, sequence of reference RNA NM_002639 was pulled out from the gene bank and by running an examination on exon 2. The primers and probe were evaluated by AleleID 6 software. The results of the addressed procedure are as follows: Forward primer 5' ATG GAT GCC CTG CAA CTA GC 3' Reverse primer 5' GAG AGA CAG ATT GGA GAG AAG AGG 3' Probe Fam 5' CCC AGT GGC TCC TTT TCA CAT AGT TGT 3' TAMRA

Their specificities were evaluated by BLAST software in NCBI. For normalization of real-time PCR housekeeping gene, GAPDH as endogenous control and reference gene, were considered (23). As a consequence, the sequence of primer and probe of this gene were as follows: Sense 5' CCC ATG TTC GTC ATG GGT GT 3' Antisense 5' TGG TCA TGA GTC CTT CCA CGA TA 3' Probe Fam 5' CTG CAC CAC CAA CTG CTT AGC ACC C 3' TAMRA

Quantitative Real-Time PCR

To perform the real-time PCR, the QuantiFast® Probe PCR kit was used. Based on the protocol of this kit, we aimed to reach a reaction with the final volume of 25 microliter. Therefore, 12.5 µL master mix, 2 µL (1 mM) forward and reverse primers and also 1.5 µL (0.5 mM) probe were mixed together. The rest of the volume of this mixture was DEPC water. In addition, based on the instruction for one-step method, 5 microliter of extracted RNA and 0.25 microliter QuantiFast RT Mix was used in this reaction. In cDNA method, 5 µL cDNA was added to the mixture.

Termocycles of these studies was considered as follows:

As initial activation, 5 min 95 °C then for PCR; Denaturation 10 sec, 95 °C; annealing 30 sec, 60 °C, 40 cycles were considered. In the one-step method before starting the explained

procedure, the reverse transcription was done at 50 °C for 10 min.

Statistical Analysis

Maspin gene expression in real-time PCR was evaluated using $\Delta\Delta CT$. In this study, two normal samples as calibrator and also GAPDH expression as reference gene were selected. The $\Delta\Delta CT$ of each sample was calculated using the equation presented below:

$$\Delta\Delta CT = (Ct, \text{Target Gene for Test} - Ct, \text{Reference Gene for Test}) - (Ct, \text{Target Gene for Calibrator} - Ct, \text{Reference Gene for Calibrator})$$

All statistical analyses were done using SPSS software, version 16 (Chicago, IL, USA). The preliminary analysis on the data indicated a non-normal distribution behavior. As a result, the mean of the normal samples were chosen as some test reference values. For comparison, t-test and ANOVA or its equal nonparametric test (Kruskal-Wallis) was used. $P < 0.05$ was considered as statistically significant. It should be notified that the mean values of data in this analysis were expressed as SE (standard error).

Results

The main pathologic data of these samples are shown in Table 1. More than 21% of the patients were under 40 yr of age, 60% were 40-60 yr old,

and the rest were older than 60. We found that 24.24% of examined samples had grade I tumor, 51.5% grade II, 18.18% grade III, and the rest were normal.

• Maspin Gene Expression

Maspin gene expression in breast cancer was evaluated using cDNA and one-step methods to evaluate the cycle of threshold (CT) in these samples. No significant differences were observed in the mean of $\Delta\Delta CT$ by applying these two methods.

The mean of Maspin gene expressions were also compared in tumor and normal samples. We found that by using the cDNA method, the Maspin gene expression in tumor samples was 469 times lower than normal ones ($P < 0.005$).

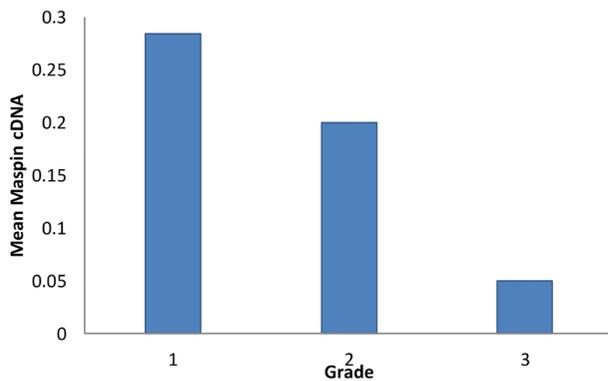
• Correlation between Breast Cancer Grades and Maspin RNA Expression

We also compared tumor samples with normal ones considering the cDNA approach. Based on this comparison, the samples were defined as grade II and grade III revealed lower Maspin expression compared with the normal ones ($P < 0.05$). In contrast, we found no significant difference between normal samples and those defined as grade I. However, using the direct method, a noticeable decrease was observed when comparing tumor samples of various grades with the normal ones ($P < 0.05$) (Fig. 1).

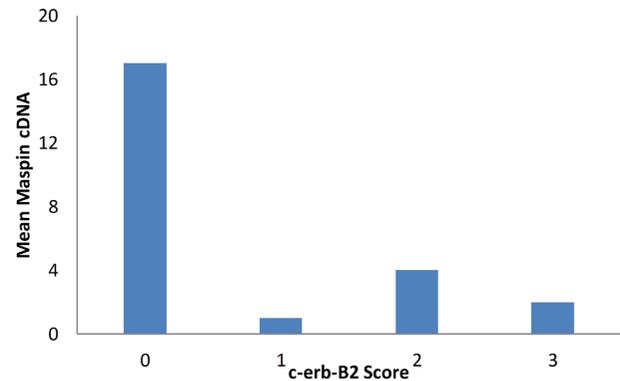
• Correlation between c-erb-B2 and Maspin DNA Expression

Table 1
Pathologic Data of the studied Samples

Samples Pathologic Data											
Number	1	2	3	4	5	6	7	8	9	10	11
Age (yr)	66	35	67	42	44	42	51	50	44	35	44
Grade	2	2	2	2	1	3	1	1	2	2	2
Samples Pathologic Data (continued)											
Number	12	13	14	15	16	17	18	19	20	21	22
Age (yr)	47	44	67	45	43	66	38	51	47	68	45
Grade	3	2	2	3	1	3	2	2	2	1	1
Samples Pathologic Data (continued)											
Number	23	24	25	26	27	28	29	30	31	32	33
Age (yr)	51	50	33	35	61	45	21	22	45	41	45
Grade	1	2	1	3	2	2	Normal	Normal	2	2	3

**Fig. 1**

The Mean cDNA Maspin expression values with respect to different tumor grades ($P < 0.005$)

**Fig. 2**

Correlation between c-erb-B2 and Maspin DNA expression ($P < 0.005$)

We evaluated Maspin gene expression in various c-erb-B2 groups. From 31 samples in tumoral group c-erb-B2 scores were as follows: 14 (45.16%) samples had score 0; 9 (29.04%) samples had score 1; 3 (9.68%) samples had score 2, and 5 (16.12%) samples had score 3. Although Maspin expression decreased in tumoral group in comparison to normal group but we did not find any meaningful difference among groups with different c-erb-B2 scores except samples in group with score 0, this group had meaningful difference in contrast the other c-erb-B2 scores in Maspin expression ($P < 0.005$) (Fig. 2).

• Correlation among Estrogen Receptor, Progesterone Receptor and Maspin RNA Expression

We also examined the correlation of ER and PR in Maspin RNA expression. In tumoral group 14 (45.16%) of samples were ER and PR positive (score 1) and 17 (54.84%) samples were ER and PR negative (score 0). Maspin gene expression was unable to make any difference between the values of both ER and PR IHC staining scores (0-1). This results could be experienced applying for both of cDNA and one-step method.

Discussion

Breast cancer is remarkably heterogenous at the genomic level. Among the various genes

associated with breast cancer, Maspin may play a key role in breast cancer prognosis. To shed light on various prognostic aspects of breast cancer, we aimed to examine the differences that may be seen in Maspin gene expression for various grades of breast cancer. Moreover, we compared Maspin gene expression in different prognostic factors associated with breast cancer including ER, PR, and c-erb-B2. The obtained results support the fact that Maspin gene expression in tumoral samples is lower than the samples obtained in the normal population; although statistical differences in Maspin gene expression of various scores associated with the c-erb-B2 could be seen only in score 0. We also found decreased levels of Maspin gene expression in samples related to tumoral grades II and III. The obtained results also confirmed that there was no relation between the other prognostic factors, i.e. ER and PR with different scores (0 and 1) and Maspin gene expression.

Maspin gene expression was down regulated in breast cancer (20). In contrast, with 92 (invasive breast cancer), 145 (Ductal Carcinoma in Situ), 27 (atypical hyperplasia), and 94 (usual hyperplasia), Maspin gene expression was frequently detected in invasive ductal carcinoma with high histologic grade (24, 25). Therefore, it was an indicator of poor prognosis (26). Researchers examined Maspin and p53 expression in patients

with invasive ductal carcinoma and found that tumor size, high histologic grade, positive p53, negative ER, or PR status correlated with Maspin expression (26, 27).

Consistently, Maspin expression shows a decrement in primary and metastatic tumors (28, 29). The decrease in Maspin gene expression was associated with the transition of cancers from insitu to invasive. Researchers also found lack of expression in cases of highly metastatic carcinoma (30-32). To shed light on the role of Maspin gene expression in breast cancer, we found that Maspin gene expression decreased in samples placed in grade II and III.

There was an inverse correlation between Maspin and c-erb-B2 expression in breast cancer cells (33). The c-erb-B2 scores differed significantly in Maspin expression only in those with a score of 0. These differences could be because of small sample size and/or not specifying type of carcinoma of our samples.

Maspin nuclear staining was significantly related with good prognostic factors, while cytoplasmic staining was related with poor prognostic markers. These data offered that the presence of Maspin in two different compartments of the cell may have different rules (34).

In this study, we tried to find a correlation among Maspin expression and invasive ductal carcinoma grades and IHC markers. The results showed that it is better to conduct this on different subtypes of invasive ductal carcinoma and other prognostic factors used for comparison with this gene expression.

Conclusion

Maspin gene expression decreased in high grade (II&III) invasive ductal carcinoma. The comparison between IHC markers was used in this study and Maspin expression showed higher expression in samples with c-erb-B2 score 0 in tumoral group but did not have any correlation with other IHC markers that we used (ER&PR).

It showed that we can use possible Maspin expression as a prognostic factor.

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Conflict of interest

The authors declare that there is no conflict of interests.

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