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PROGNOSTICATING METASTASIS RISK IN EARLY BREAST CANCER WITH EXT1 and WISP1 GENES IN 8q22-24 POSITION

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ABSTRACT

Introduction: Gene expression profile has been suggested for Prognosticating breast cancer. The prognosticating value of 8q22-24 loci is still unknown for early breast cancer. In the present study the gene expression patterns of two genes EXT1 and WIPS1 located in this region in metastatic and non-metastatic breast cancers have evaluated samples.

Methods: In this study 85 women with recurrence or metastasis (15 patients) and without metastasis (70 patients) at the early stages of breast cancer with estrogen receptor and without lymph node involvement who have been undergone mastectomy were investigated between 2001-2005 years who Also, 15 normal tissues breasts were chosen as control group. The women's demographic and clinical characteristics were recorded. The number of mRNA transcripts of EXT1 and WISP1transcripts was measured by Real Time –PCR.

Results: EXT1 and WISP1 gene expressions was observed significantly in metastasis group compared to control group ($P < 0.05$). WISP1 gene was associated with patients' age and tumor size. None of the two studied genes were associated with tumor stage and grade.

Conclusion: It seems that EXT1 and WISP1 genes can be used as probable markers for predicting metastasis risk in early breast cancer. More sample is needed to be analytic for validation of current data.

Keywords: breast cancer; metastasis; gene expression; EXT1; WISP1

INTRODUCTION

Breast cancer is the most common malignancy among women, worldwide [1]; with intrinsically heterogeneous etiology, but similar clinical manifestations in most cases [2]. Many studies have explained that both genetic and environmental factors contribute to breast cancer pathogenesis and progression [3-5]. For example, several proliferation and oncogenic genes have been identified in breast cancer [6]. Therefore, genetic and molecular screening of patients has been proposed to predict disease behavior, response to anti-cancer therapeutics, and patients' survival [7, 8].

A growing bulk of evidence have shown that abnormalities at certain chromosomal positions leads to different tumor behaviors such as; progression, resistance to chemotherapy, and spread to other organs [9-11]. Dellas et al, for instances, suggested that aberrations in chromosomes 11p and18q might be associated with poor prognosis and progression of ductal breast cancer [9]. In addition, Horlings et al. have found a strong correlation between genomic differences and different gene expression signatures causing poor prognosis in breast cancer carcinoma [11].

Moreover, it has been reported that aberrations in chromosome 8q might be associated with resistance to chemotherapy in breast cancer [12].

Association of 8q22-24 position, with breast cancer and other carcinomas has been proposed by many investigators using different molecular approaches such as; genome wide association study (GWAS), array-comparative genomic hybridization (array- CGH) and gene expression profiling methods. However, existing data are controversial. By way of example, WISP1, a member of CCN family, has shown contradictory functions in the context of cancer [13-16].

There are also very few studies on the role of other 8q22-24 genes in the pathogenesis of breast cancer. Based on what mentioned above, herein, we ran an investigation into expression pattern of EXT1, WISP1genes, and compared them between metastatic and non-metastatic breast cancer in order to

examine their potentials as prognostic marker(s) for metastasis risk in human.

METHODS

This retrospective study primarily included 1705 breast tumor samples obtained from bio bank of cancer research center of Shahid Beheshti University of Medical Sciences. Only ER-positive, lymph-node negative tumors with stage of I and II, and tumor size< 5cm were analyzed. The patients were divided Page 4 of 11 into two metastasis and non-metastasis groups based on a 5-year follow-up period after the curative surgery.

Demographic features and clinical data of the patients were collected. Also, 15 matched normal breast tissues were used as control. The study procedure and use of clinical information of the patients was approved by ethical committee located at the center. In addition, identity and personal information of all participants were not disclosed in any stage of the study and afterwards.

RNA Extraction:

Total RNA was extracted from paraffin-embedded tumors and normal breast tissues using RNeasy FFPE kit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions. Briefly, the paraffin was removed from samples by Xylene. Afterwards, sample lysis was performed with proteinase K digestion for 15 minutes, followed by incubation at 80ºC for 15 minutes. Then, the genomic DNA was effectively removed by DNase and DNase Booster Buffer treatment for 15 minutes. Finally, concentrated RNA was purified using RNeasy MinElute spin columns, and eluted in a volume of 20 µl on the QIAcube.

Complementary DNA (cDNA) Synthesis:

The total RNA was directly converted to cDNA using RT2 PreAMP cDNA Synthesis Kit (QIAGEN, Germany), according to the manufacturer's guidelines. Briefly, 1 µl of the total RNA was added to 9 µl of reverse-transcription mix and 10 µl of genomic DNA elimination mix, and the final volume of 20 µl was subjected to reverse transcription PCR at 42°C for 30 minutes. The reaction was immediately stopped by incubating at 95°C for 5 min.

Quantitative Real-time PCR Assay:

The mRNA copy numbers of EXT1, WISP1 genes were measured by SYBR green-based real-time PCR using the respective specificpair of primers (Table 1). All reactions were carried out in duplicates using 7500 Fast Real-Time PCR System (Applied Biosystems®) and Takara Bio SYBR Premix Ex Taq (Tli RNase H Plus) master mix (Takara Bio Inc., Shiga, Japan), according to the manufacturer's recommendations, in a reaction volume of 25 µl. Thermal profile of

the reaction included; hold (initial denaturation) at 95°C for 30 seconds, followed by 40 consecutive two-step cycles of PCR (95°C for 5 seconds and 60 °C for 30 seconds), and termination in a dissociation stage. The cycling threshold (CT) values of the target genes were normalized to that of GAPDH as an internal control, the expression levels were presented as relative units.

Statistical Analysis: For statistical analysis, the Chi-square or Fisher's exact, Mann–Whitney U, and Kruskal–Wallis (followed by post-hoc pair-wise comparisons) tests were performed using SPSS software version 20 (IBM Co., Illinois, USA). Also, association between the variables was examined by parametric Pearson and nonparametric Spearman correlation tests. In addition, the GraphPad Prism 5 for Windows (GraphPad Software, California, USA) was used for development of the graphs. The differences were considered statistically significant wherever P value was less than 0.05. Also, a P value of 0.06 was considered on the borderline of statistical significance.

RESULTS:

Out of 1705 breast cancer registers, a total of 312 patients were identified with tumor stages of I and II, 15 of which had been presented with local recurrence or metastasis during the 5 years following the curative surgery (metastasis group). Therefore, 70 non-metastatic patients (without local recurrence or metastasis) were randomly selected and compared with the metastasis group. Note that one of the patients in

Page 5 of 11 the metastasis group was deceased at the time of the study. As given in Table 2, there were no significant difference between demographic and life-style features of non-metastasis and metastasis groups including; age, marital status, history of pregnancy, childbirth, abortion, type of abortion, smoking, high-fat diet, and family history of breast cancer ($P>0.05$). There were no significant differences between mean ages of control and that of either non-metastasis or metastasis patient groups ($P<0.05$). However, significant differences were observed between these non-metastasis and metastasis groups regarding the number of pregnancies and childbirth each patient had experienced ($P=0.019$ and $P=0.008$, respectively); that is, as depicted in Fig. 1, higher percentage of patients in the non-metastasis group had 3≥ pregnancies and child-birth experience. On the other hand, the mean duration of breastfeeding in the non-metastasis group was lower than that in the metastasis group, and this difference showed a borderline significance ($P= 0.057$).

Clinical Features: With respect to the clinical findings, the non-metastasis and metastasis groups were found to be significantly different, pathological form, and lymphovascular invasion (LVI) wise (P= 0.032 and P= 0.036, respectively); pathologically, invasive lobular (ILC) was more common among the metastasis compared to the non-metastasis patients (31% versus 4%) and the invasive ductal (IDC) appeared vice versa (69% versus 88%).

Surprisingly, higher percentage of the non-metastasis group were LVI positive compared to the metastasis group (52% versus 18%). Also, two groups exhibited a borderline-significance difference regarding the disease stage (P= 0.058), that is higher percentage of the metastasis patients were as stage II compared to the non-metastasis patients (53% versus 28%). However, no significant differences were observed between the patient groups by other tumor features and clinical findings such as; tumor size, grade, ER, PR, HER2, P53, serum vitamin D level, surgery type, etc. (Table 3).

Gene Expression Levels:

For evaluation of mRNA expression of EXT1, WISP1 genes real-time PCR was performed (Fig. 2). Results showed that expressions of EXT1 and WISP1 in metastasis group were significantly decreased compared to both control (P= 0.015 and P= 0.012) and non-metastasis (P= 0.000 and P= 0.000) groups, while no significant difference was found in expressions of these gene between control and non-metastasis groups (P= 0.803 and P= 0.955). There was weak significant correlations between EXT1 and WISP1 (r= 0.293, P= 0.009).

Correlation between Demographic Characteristics:

There was a positive correlation between WISP1 expression and age (r= 0.264, P= 0.026), while no association was observed between WISP1 expression and other demographic characteristics of the patients. Moreover, mRNA expressions of EXT1 showed no association with any of the demographic characteristics of the patients (P<0.05).

Correlation between Gene Expression and Clinical Features:

With respect to the clinical features, mRNA expression of the WISP1 correlated with tumor size (r= -0.242, P= 0.047), vitamin D level (r= -0.220, P= 0.242), surgery type (P= 0.006), and hormone therapy (P= 0.020).Additionally, none of the six studied genes revealed any association with grades and stage of the disease.

Table 1 Oligonucleotide sequences of the primers used in the current study.

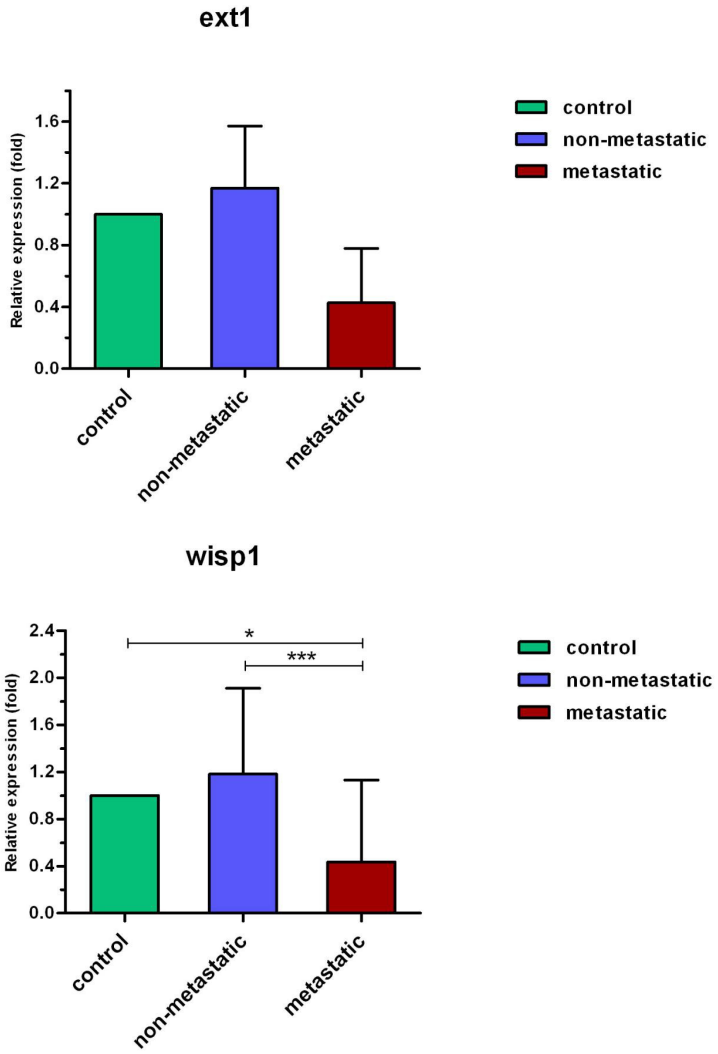
Product length	AT* (°C)	Sequence (5'->3')	Primer
bp 95	55/42	**5'CTTCGTTCCTTGGGATCAAT3'**	**EXT1 Forward**
	57/59	**5'-TGCCTTTGTAGATGCTGGAG-3'**	**EXT1 Reverse**
bp 87	57/59	**5'-CAAGGCTGGATAACAGCTCA-3'**	**WISP1 Forward**
	53/62	**5'-TTCCCAAATTGAGATGCAAA-3**	**WISP1 Reverse**
bp 125	60/29	**5'- ATGGAGAAGGCTGGGGCT-3'**	**GAPDH Forward**
	60/85	**5'ATCTTGAGGCTGTTGTCATACTTCTC3'-**	**GAPDH Reverse**

Table 2 Demographic and life-style features of the breast cancer patients involved in the current study.

P value	Metastasis	Non-metastasis	Variable
0/107	47/8 ± 16/0	53/6 ± 11/6	**Age (Year), Mean ± SD**
0/201	0 : 87 : 13	4 : 94 : 3	**Marital status (single: married: widow) %**
0/561	13 : 87	11 : 89	**Pregnancy (yes: no) %**
0/561	13 : 87	11 : 89	**Childbirth (yes: no) %**
0/378	67 : 33	74 : 26	**Abortion (yes: no) %**
0/545	40 : 60	50 : 50	**Abortion type (medical: criminal) %**
0/057	50/5 ± 51/3	52/8 ± 26/5	**Duration of breastfeeding (week), Mean ± SD**
0/925	79 : 14 : 7	75 : 15 : 10	**Family history (1st degree: 2nd degree: no) %**
0/260	100 : 0	90 : 10	**Smoking (yes: no) %**
0/464	14 : 86	20 : 80	**High-fat diet (yes: no) %**

Table 3: Clinical findings of the breast cancer patients involved in the current study

P value	Metastasis	Non-metastasis	Variable
0/032	0 : 31 : 0 : 0 : 69	2 : 4 :2 : 4 : 88	**Pathology (IDC*: DCIS**: IDC/DCIS: ILC***: IDC/ILC)%**
0/058	53 : 47	28 : 72	**Stage (I: II) %**
0/898	9 : 82 : 9	14 : 76 : 10	**Grade (G1: G2: G3) %**
0/036	82 : 18	48 : 52	**LVI (+: -) %**
1/000	0 :100	0 :100	**ER (+: -) %**
0/557	7 : 93	4 : 96	**PR (+: -) %**
0/589	64 : 36	63 : 37	**HER2 (+: -) %**
0/611	80 : 20	73 : 27	**P53 (+: -) %**
0/106	2/6 ± 1/5	2/1 ± 0/9	**Tumor size (cm), Mean ± SD**
0/174	0 : 50 : 50	18 : 32 : 50	**Surgery type (BCS: MRM: BCS/MRM) %**
0/268	25 : 75	10 : 90	**Chemotherapy (yes: no) %**
0/437	7 : 93	14 : 86	**Radiotherapy (yes: no) %**
0/622	7 : 93	9 :91	**Hormone therapy (yes: no) %**
0/585	87 : 13	88 : 12	**Receiving estrogen- progesterone (yes: no) %**
0/387	36/9 ± 20/0	27/2 ± 25/2	**Vitamin D (20 ng/ml), Mean ± SD**
0/300	100 : 0	91 : 9	**Diabetes (yes: no) %**



Comparison of mRNA expression of *EXT1*, *WISP1* genes between control, metastasis and non-metastasis groups: A) and B) expressions of *EXT1* and *WISP1* were significantly decreased in metastasis group compared to the control and non-metastasis groups

DISCUSSION

Through a large number of investigations, gene expression profiling approach has been established to serve as an appropriate predictor for clinical outcome of human breast cancer [18, 19].

The 8q22-24 position has recently drawn increasingly attractions of many investigator in this field, worldwide. However, most of the respective publications contradict each other, leaving prognostic value of the 8q22-24 position uncertain.

Therefore, in the current study we examined mRNA expression pattern of WISP1, EXT1 genes, located on the 8q22-24 position, in both metastatic and non-metastatic early-stage breast cancers. However, our results contradicted many previous reports, too.

It is strongly emphasized that all the patients included in the current study were lymph-node negative, ER positive and were at the stages I and II of breast cancer, which might be a logical explanation for this controversy. In other words, most of the preceding investigations have included patients with advanced stages of breast cancers, disregarding ER pattern, as well. Moreover, to the best of our knowledge, our study is the first to investigate the target genes in lymph node-negative early stage breast cancers.

WISP1 gene is located on the 8q24.1-8q24.3 region, and encodes WNT1-inducible-signaling pathway protein 1, a microcellular protein that is also called as CCN4, in humans. A growing number of studies indicate that WISP1 might be implicated in development and progression of different types of cancers, suggesting this molecule as a marker for the disease [20]. As regards, conflicting data exist about stimulatory or suppressive role of WISP1 in various cancer development [13-15]. In the current study, we observed that mRNA expression of WISP1 in non-metastasis breast cancer patients was unchanged compared to the normal individuals, while its expression significantly declined in metastasis patients. This finding is in accordance with small number of previous studies, which have reported the WISP1 as a tumor suppressor [15]. For example, Davies and et al. [15], found that mRNA transcripts of WISP1 was decreased in node-positive breast cancer patients who later developed metastasis and died. In line with results of Davies and et al. [15], the decline observed, in the expression of WISP1 gene, herein, seems to be related with aggressive behavior of the tumor in metastatic breast cancer. However, Page 7 of 11 some other preceding evidence contradicts this hypothesis. For instances, in contrast to our findings, Xie and et al.[13] observed that expression level of WISP1 was elevated in primary breast cancer that might have contributed to more advanced features of the disease. Chen et al. [14], also reported that increased expression of WISP1 might be associated with pathogenesis of primary lung cancers.

We also found that the expression of WISP1 was associated with patient's age, serum vitamin D level, tumor size, the surgery type, and having hormone therapy, but it did not have correlations with stage, grade, pathological form and other features of the disease. The negative correlation of WISP1 with patient's age, serum vitamin D level, and tumor size does not support the hypothesis that describes reduced level of WISP1 as a marker for tumor progression or

aggressive feature. However, referring to the literature, we found no evidence regarding the correlation of WISP1 with any of the demographic or pathological features in breast cancer. What's more, Xie et al. [21], have also found no significant association between expression of WISP1 and the pathological features such as tumor grade and stage in primary glioma. Taken together, our findings in the current study may suggest WISP1 as prognostic marker for breast cancer metastasis; though, whether it is a tumor stimulator or suppressor remains unclear, yet.

The EXT1 gene is located on 8q24.11, and encodes exostosin glycosyltransferase 1, primarily known to serve as a tumor suppressor [22]. Howbeit, there is a short line of evidence suggesting a tumor promoting role for EXT1 [23-25]. For example, it has been recently shown that expression of EXT1 gene was amplified following treatment with Heparan sulfate proteoglycans, which indicated that the EXT1 as a glycosylation enzyme participates in Heparan biosynthesis, and probably thereby, contributes to proliferation and invasive potential of breast cancer epithelial cells in ER-negative tumors [23, 24]. Furthermore, increased plasma level of EXT1 have been associated with tumor-genesis in cholangiocarcinoma, a form of malignancy in the biliary duct system [25].

In the current study, the mRNA expression pattern of the EXT1 was similar to that of the WISP1. Besides, it showed positive correlations with WISP1 gene, while unlike these genes, EXT1 was not associated with neither the demographic nor the clinical features of the patients. Based on these findings and owing to further investigations in future, monitoring mRNA level of EXT1 along with that of WISP1 might be helpful in assessing risk of breast cancer metastasis.

The results of the current study showed declines in WISP1, and EXT1 mRNA expression among early-stage ER-positive and lymph node-negative breast cancer patients can be associated with increased risk of metastasis. Therefore, if furthered validated in future by other investigation considering individual genetic background and ethnic variations, the WISP1 and EXT1 genes might serve as a promising indicator of metastasis risk.

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