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Gene expression profiling of the 8q22-24 position in human breast cancer: *TSPYL5*, *MTDH*, *ATAD2* and *CCNE2* genes are implicated in oncogenesis, while *WISP1 and EXT1* genes may predict a risk of metastasis

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Abstract. Gene expression profiling has been suggested to predict breast cancer outcome. The prognostic value of the 8q22-24 position in breast cancer remains to be elucidated. The present study evaluated expression patterns of the genes located at this position in metastatic and non-metastatic breast cancer. A total of 85 patients with recurrent/metastatic (n=15) and non-metastatic (n=70) early-stage, estrogen receptor-positive and lymph node-negative breast tumors were included. In addition, 15 normal breast tissue samples were used as controls. Demographic and clinical features were recorded. Subsequently, mRNA copy numbers of exostosin glycosyltransferase 1 (*EXT1*), WNT1 inducible signaling pathway protein 1 (*WISP1*), ATPase family, AAA domain containing 2 (*ATAD2*), TSP-like 5 (*TSPYL5*), metadherin (*MTDH*) and cyclin E2 (*CCNE2*) genes were measured by reverse transcription-quantitative polymerase chain reaction assay. The expression of *EXT1* and *WISP1* exhibited a significant decline in the metastatic breast cancer group compared to the control (P=0.015 and P=0.012, respectively). The expression of *TSPYL5*, *MTDH* and *ATAD2* was significantly decreased in the metastatic (P=0.002, P=0.018 and P=0.016, respectively) and non-metastatic (P=0.038, P=0.045 and P=0.000, respectively) breast cancer groups compared with the control. The expression of *CCNE2* in the metastatic and non-metastatic breast cancer groups was significantly increased compared with the control (P=0.002 and P=0.001, respectively). *WISP1* expression demonstrated a correlation with patient age and tumor size, and *TSPYL5* expression was correlated with lymphovascular invasion. None of the genes investigated exhibited any correlation with stage and grade of disease. The *TSPYL5*, *MTDH*, *ATAD2* and *CCNE2* genes may be implicated in the pathogenesis of human breast cancer, while the *WISP1* and *EXT1* genes may have the potential to serve as promising indicators of the risk of metastasis. However, further studies are required to validate these results.

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Introduction

Breast cancer is the most common malignancy among women worldwide (1), with an intrinsically heterogeneous etiology, but similar clinical manifestations in the majority of cases (2). A number of studies have reported that genetic and environmental factors contribute to breast cancer pathogenesis and progression (3-5). For example, several proliferation and oncogenic genes, including breast cancer (*BRCA*) *1*, *BRCA2*, *MYC*, tumor protein 53, retinoblastoma 1, *JUN*, cyclin-dependent kinase inhibitor 2A, human epidermal growth factor receptor 2-neu, cyclin D1 and cyclin E, have been identified in breast cancer (6-8). Therefore, genetic and molecular screening of patients has been proposed as useful to predict disease behavior, response to anti-cancer therapeutics and patient survival (9,10).

A growing body of evidence has revealed that abnormalities at certain chromosomal positions lead to various tumor behaviors, including progression, resistance to chemotherapy and spread to other organs (11-13). Dellas *et al* (11) suggested that aberrations in chromosomes 11p and 18q may be associated with poor prognosis and progression of ductal breast cancer. In addition, Horlings *et al* (13) reported a strong correlation between genomic differences and various gene expression signatures leading to poor prognosis in breast cancer. Furthermore, it has

been reported that aberrations in chromosome 8q may be associated with resistance to chemotherapy in breast cancer (14).

Association of the 8q22-24 position, containing WNT1 inducible signaling pathway protein 1 (*WISP1*), exostosin glycosyltransferase 1 (*EXT1*), ATPase family, AAA domain containing 2 (*ATAD2*), TSP-like 5 (*TSPYL5*), metadherin (*MTDH*) and cyclin E2 (*CCNE2*) genes, with breast cancer and other carcinomas has been proposed by numerous investigators using varied molecular approaches, including genome wide association study, array-comparative genomic hybridization and gene expression profiling methods (13-17). However, existing data are conflicting. By way of example, *WISP1*, a member of the CCN family, has shown contradictory functions in the context of cancer (17-20). Davies *et al* (19) suggested varying prognostic values for the CCN family members, including *WISP1*, *WISP2* and *WISP3*, in human breast cancer; *WISP1* was observed to be a tumor suppressor, *WISP2* was a stimulator of tumor aggressiveness and *WISP3* remained undefined regarding a beneficial or detrimental role. However, in another investigation, contrasting roles were observed for *WISP1* and *WISP2* in human colorectal cancer (20); *WISP1* appeared to be a stimulator of tumor aggressiveness and *WISP2* was characterized as a tumor suppressor.

There have been few studies on the role of 8q22-24 genes in the pathogenesis of breast cancer. Overexpression of *ATAD2* has been reported to drive survival of breast cancer cells, resulting in a poor prognosis (21). Inhibition of *MTDH* has been demonstrated to sensitize breast cancer cells to anti-cancer agents (22,23). The *TSPYL5* gene has been suggested to have a causative role in breast tumorigenesis (24). The *CCNE2* gene has been demonstrated to have a role in the invasiveness of breast cancer cells (25). To the best of our knowledge, there have been no investigations into the role of *EXT1* in the pathogenesis of breast carcinoma.

The present study performed an investigation into the expression patterns of *EXT1*, *WISP1*, *ATAD2*, *TSPYL5*, *MTDH* and *CCNE2* genes, and compared them between metastatic and non-metastatic breast cancer in order to examine their potential as prognostic markers for the risk of metastasis in humans.

Materials and methods

Patients and tumor samples. This retrospective study primarily included 1705 breast tumor samples obtained from the Bio Bank of the Cancer Research Center of Shahid Beheshti University of Medical Sciences (Tehran, Iran). The tissues were taken from breast cancer patients who underwent either breast-conserving surgery or modified radical mastectomy at Khatam-Ol-Anbia Specialty and Subspecialty Hospital of Tehran (Tehran, Iran) between August 2002 and December 2012. Only estrogen receptor (ER)-positive, lymph-node negative tumors with tumors stages I and II and tumor size <5 cm were analyzed. The patients were divided into metastatic and non-metastatic groups based on a 5-year follow-up period following the curative surgery. Demographic features and clinical data of the patients were collected. In addition, 15 matched normal breast tissues, taken from volunteer healthy women who underwent mammoplasty between June 2012 and April 2013, were used as control. Patients had previously signed an informed consent on the prospective use of their specimen for study purposes. The study procedure and use of clinical information of the patients was approved by the ethical committee of Shahid Beheshti University of Medical Sciences. In addition, identity and personal information of all participants were not disclosed at any stage of the study and/or following the study conclusion.

RNA extraction. Total RNA was extracted from already paraffin-embedded tumor samples and normal breast tissues using the RNeasy FFPE kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. Briefly, the paraffin was removed from samples by xylene. Subsequently, sample lysis was performed by proteinase K (Qiagen GmbH) digestion for 15 min, followed by incubation at 80˚C for 15 min. Following incubation, the genomic DNA was effectively removed by DNase and DNase Booster Buffer (Qiagen GmbH) treatment for 15 min. Finally, concentrated RNA was purified using RNeasy MinElute spin columns (Qiagen GmbH) according to the manufacturer's protocol, and eluted in a volume of 20 μl on the QIAcube (Qiagen GmbH).

Complementary DNA (cDNA) synthesis. The total RNA was directly converted to cDNA using the RT2 PreAMP cDNA Synthesis kit (Qiagen GmbH), according to the manufacturer's protocol. Briefly, 1 μl of 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 at 42˚C for 30 min. The reaction was terminated at 95˚C for 5 min followed by a pause at 4˚C (to remove the microtubes), and then samples were preserved at -20˚C until use.

Quantitative polymerase chain reaction (qPCR) assay. The mRNA copy numbers of *EXT1*, *WISP1*, *ATAD2*, *TSPYL5*, *MTDH*, *CCNE2* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) genes were measured by SYBR green-based qPCR using the respective specific pairs of primers (Table I). All reactions were performed in duplicate using the 7500 Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and Takara Bio SYBR Premix Ex Taq (Tli RNase H Plus) master mix (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's protocol, in a total reaction volume of 25 μl. The thermal profile of the reaction was as follows: Initial denaturation at 95˚C for 30 sec, followed by 40 consecutive two-step cycles of PCR (95˚C for 5 sec and 60˚C for 30 sec), and termination in a dissociation stage (increasing the temperature from 65 to 95˚C, rising by 1˚C each step, halting for 90 sec of pre-melt conditioning on the first step and 5 sec for each subsequent step). The cycling threshold values of the target genes were normalized to that of *GAPDH* as an internal control and relative gene expression was calculated by $2^{(-\Delta\Delta Cq)}$ method as follows (26): Relative gene expression=$2^{-\Delta\Delta Cq}$. $\Delta\Delta Cq=\Delta Cq_{case}-\Delta Cq_{control}$. $\Delta Cq=Cq_{target}-Cq_{GAPDH}$.

Statistical analysis. For statistical analysis, the χ^2 or Fisher's exact, Mann-Whitney U or independent *t* test, and analysis of variance or Kruskal-Wallis (followed by *post-hoc* pairwise comparisons) tests were performed using SPSS version 20 (IMB SPSS, Armonk, NY, USA). In addition, associations between the variables were examined by parametric Pearson and non-parametric Spearman's correlation tests. GraphPad Prism 5 for Windows (GraphPad Software, Inc., La Jolla, CA,

Table I. Oligonucleotide sequences of the primers used in the present study.

Primer	Sequence, 5'-3'	Annealing temperature, ˚C	Product length, bp
EXT1			
Forward	5'-CTTCGTTCCTTGGGATCAAT-3'	55.42	95
Reverse	5'-TGCCTTTGTAGATGCTGGAG-3'	57.59	
WISP1			
Forward	5'-CAAGGCTGGATAACAGCTCA-3'	57.59	87
Reverse	5'-TTCCCAAATTGAGATGCAAA-3'	53.62	
ATAD2			
Forward	5'-CCAGACAGCAGGCTGATAAA-3'	57.59	137
Reverse	5'-ACGCACTTCAACATCACCAT-3'	58.10	
TSPYL5			
Forward	5'-TGCACAAGTCTCCCTGCTAC-3'	59.68	87
Reverse	5'-CAGAGGCCAACATGAAGAGA-3'	57.22	
MTDH			
Forward	5'-TGCCGCCAATACTACAAGAG-3'	57.69	105
Reverse	5'-GTTTGGGAGATTCCCAGCTA-3'	56.90	
CCNE2			
Forward	5'-CGGCCTATATATTGGGTTGG-3'	55.44	106
Reverse	5'-ACGGCTACTTCGTCTTGACA-3'	59.04	
GAPDH			
Forward	5'-ATGGAGAAGGCTGGGGCT-3'	60.29	125
Reverse	5'-ATCTTGAGGCTGTTGTCATACTTCTC-3'	60.85	

EXT1, exostosin glycosyltransferase 1; *WISP1*, WNT1 inducible signaling pathway protein 1; *TSPYL5*, TSP-like 5; *MTDH*, metadherin; *ATAD2*, ATPase family, AAA domain containing 2; *CCNE2*, cyclin E2; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

USA) was used for development of the graphs. $P<0.05$ was considered to represent a statistically significant difference.

Results

Demographic characteristics. Out of 1,705 breast cancer patients registered in the Bio Bank of the Cancer Research Center of Shahid Beheshti University of Medical Sciences, a total of 312 patients were identified with tumor stages I and II; only, 85 of them had at least 5 years of follow-up records that were included for analysis. A total of 15 of these patients presented with local recurrence or metastasis during the 5 years following the curative surgery (metastatic group) and 70 had not shown any sign of local recurrence or metastasis (non-metastatic group). Therefore these 70 patients were selected and compared with the metastatic group. One of the patients in the metastatic group was deceased at the time of the study. As shown in Table II, there were no significant differences observed between the non-metastatic and metastatic groups for age (P=0.107), marital status (P=0.201), history of pregnancy (P=0.561), childbirth (P=0.561), abortion (P=0.378), type of abortion (P=0.545), smoking (P=0.260), high-fat diet (P=0.464) and family history of breast cancer (P=0.925). The mean age of the control group was 42.9±9.2 years. There were no significant differences between the mean age of the control group and that of the non-metastatic (P=0.076) or metastatic patient (P=0.654) groups. However, significant differences were observed between the non-metastatic and metastatic groups regarding the number of pregnancies and childbirths each patient had experienced (Fig. 1A and B; P=0.019 and P=0.008, respectively); a markedly higher number of patients in the non-metastatic group had ≥3 pregnancies and/or childbirth events compared with the metastatic group. There was no significant difference between non-metastatic and metastatic groups regarding the number of abortions each patient had experienced (Fig. 1C; P=0.551). The mean duration of breastfeeding in the non-metastatic group was reduced compared with that in the metastatic group, but this difference was not significant (P=0.057).

Clinical features. With respect to the clinical findings, the non-metastatic and metastatic groups were observed to be significantly different with regard to pathology and lymphovascular invasion (LVI; P=0.032 and P=0.036, respectively). Invasive lobular carcinoma was more common among the metastatic patients compared with the non-metastatic patients (31 vs. 4%), and invasive ductal carcinoma was more common in the non-metastatic patients compared with the metastatic patients (69 vs. 88%). Notably, an increased percentage of the non-metastatic group was LVI-positive compared to the metastatic group (52 vs. 18%). In addition, a higher percentage of the metastatic patients exhibited stage II disease compared to the non-metastatic patients (53 vs. 28%). No significant differences were observed between the patient groups for any other tumor features and clinical findings, including tumor size

Table II. Demographic and lifestyle features of healthy control and BC patients in the present study.

Variable	Healthy control	Non-metastatic	Metastatic	P-value		
				Non metastatic vs. metastatic	Non-metastatic vs. control	Metastatic vs. control
Age, years, mean ± SD	42.9±9.2	53.6±11.6	47.8±16.0	0.107[a]	0.076[a]	0.654[a]
Marital status, single: married: widow, %	13:87:0	3:94:3	13:87:0	0.201[b]	0.201[b]	0.701[c]
Pregnancy, yes: no, %	60:40	89:11	87:13	0.561[c]	0.015[c]	0.107[c]
Childbirth, yes: no, %	60:40	89:11	87:13	0.561[c]	0.015[c]	0.107[c]
Abortion, yes: no, %	20:80	26:74	33:67	0.378[c]	0.461[c]	0.341[c]
Abortion type, medical: criminal, %	100:0	50:50	60:40	0.545[c]	0.001[c]	0.053[c]
Duration of breastfeeding, weeks, mean ± SD	43.5±25.1	32.8±26.5	50.5±51.3	0.562[d]	0.094[d]	0.567[d]
Family history of BC, 1st degree: 2nd degree: no, %	20:33:47	10:15:75	7:14:79	0.925[b]	0.102[b]	0.208[b]
Smoking, yes: no, %	33:67	10:90	0:100	0.260[c]	0.035[c]	0.042[c]
High-fat diet, yes: no, %	60:40	80:20	86:14	0.464[c]	0.176[c]	0.215[c]

[a]Tukey's test following analysis of variance; control group is not shown in the table. [b]χ^2 test. [c]Fisher's exact test. [d]Mann-Whitney U test. SD, standard deviation; BC, breast cancer.

(P=0.106), grade (P=0.898), ER status (P=0.100), progesterone receptor status (P=0.557), human epidermal growth factor receptor 2 (HER2) status (P=0.589), P53 status (P=0.611), diabetes (P=0.300), serum vitamin D level (P=0.057), surgery type (P=0.174), and receiving chemotherapy (P=0.268), radiotherapy (P=0.437), estrogen-progesterone (P=0.585) and other hormone therapy (P=0.622; Table III).

Gene expression levels. For evaluation of mRNA expression of *EXT1*, *WISP1*, *ATAD2*, *TSPYL5*, *MTDH* and *CCNE2* genes, qPCR was performed (Fig. 2). The results revealed that expression of *EXT1* and *WISP1* was significantly decreased in the metastatic group compared to the control (P=0.015 and P=0.012, respectively) and non-metastatic (P<0.001 and P<0.001) groups, while no significant difference was observed for the expression of these genes between the control and non-metastatic groups (P=0.803 and P=0.955, respectively). The expression of *TSPYL5*, *MTDH* and *ATAD2* genes in metastatic (P=0.002, P=0.018 and P=0.016, respectively) and non-metastatic (P=0.038, P=0.045 and P=0.000, respectively) groups was significantly decreased compared to the control group. In addition, a significant reduction was observed in the expression of *TSPYL5* in the metastatic group compared to the non-metastatic group (P=0.040), and in that of *ATAD2* in the non-metastatic group compared to the metastatic group (P=0.014). No significant difference was observed in the expression of *MTDH* between the metastatic and non-metastatic groups (P=0.293). The mRNA expression of *CCNE2* in the metastatic and non-metastatic groups was significantly increased compared with the control (P=0.002 and P=0.001), while expression of this gene was not altered in the metastatic group compared to the non-metastatic group (P=0.746).

Correlation between expressions of genes. As depicted in Fig. 3, there was a significant strong positive correlation between expression of *WISP1* and *TSPYL5* (r=0.743; P<0.001), and significant weak positive correlations between *EXT1* and *WISP1* (r=0.293; P=0.009), *EXT1* and *TSPYL5* (r=0.316; P=0.005), and *TSPYL5* and *MTDH* (r=0.395; P<0.001). In addition, non-significant weak positive correlations were observed between expression of *TSPYL5* and *ATAD2* (r=0.244; P=0.102) and *MTDH* and *ATAD2* (r=0.245; P=0.105), and weak non-significant negative correlations were observed between *TSPYL5* and *CCNE2* (r=-0.211; P=0.129) and *MTDH* and *CCNE2* (r=-0.200; P=0.154).

Correlation between gene expression and demographic characteristics. As given in Table IV, expression of *WISP1* was correlated with age (r=0.264; P=0.026) and family history of breast cancer (r=0.209; P=0.088), while no association was observed between *WISP1* expression and other demographic characteristics of the patients (for all, r<0.200; P>0.05). The expression of *TSPYL5* was correlated with abortion (r=0.200; P=0.094), smoking (r=0.243; P=0.044) and family history of breast cancer (r=0.340; P=0.005); *TSPYL5* expression was significantly higher among patients who had a family history of the disease (P=0.002) and those who were smokers (P=0.048). However, no correlation was observed between *TSPYL5* and other demographic features (for all, r<0.200; P>0.05). The *MTDH* expression was also correlated with family history of breast cancer (r=0.203; P=0.100), while it was not correlated with other demographic features (for all, r<0.200; P>0.05).

Table III. Clinical findings of the breast cancer patients in the present study.

Variable	Non-metastatic	Metastatic	P-value
Pathology, IDC: DCIS: IDC/DCIS: ILC: IDC/ILC, %	88:4:2:4:2	69:0:0:31:0	0.032[a]
Stage, I:II, %	72:28	47:53	0.058[b]
Grade, 1:2:3, %	10:76:14	9:82:9	0.898[a]
Lymphovascular invasion, +: -, %	52:48	18:82	0.036[b]
Estrogen receptor, +: -, %	100:0	100:0	1.000[a]
Progesterone receptor, +: -, %	96:4	93:7	0.557[b]
Human epidermal growth factor receptor 2, +: -, %	37:63	36:64	0.589[b]
P53, +: -, %	27:73	20:80	0.611[b]
Tumor size, cm, mean ± SD	2.1±0.9	2.6±1.5	0.424[c]
Surgery type, BCS: MRM: BCS/MRM, %	50:32:18	50:50:0	0.174[a]
Chemotherapy, yes: no, %	90:10	75:25	0.268[b]
Radiotherapy, yes: no, %	86:14	93:7	0.437[b]
Hormone therapy, yes: no, %	91:9	93:7	0.622[b]
Receiving estrogen and progesterone, yes: no, %	12:88	13:87	0.585[b]
Vitamin D, ng/ml, mean ± SD	27.2±25.2	36.9±20.0	0.225[c]
Diabetes, yes: no, %	9:91	0:100	0.300[b]

[a]χ^2 test. [b]Fisher's exact test. [c]Mann-Whitney U test. IDC, invasive ductal carcinoma; DCIS, ductal carcinoma *in situ*; ILC, infiltrating lobular carcinoma; BCS, breast-conserving surgery; MRM, modified radical mastectomy; SD, standard deviation.

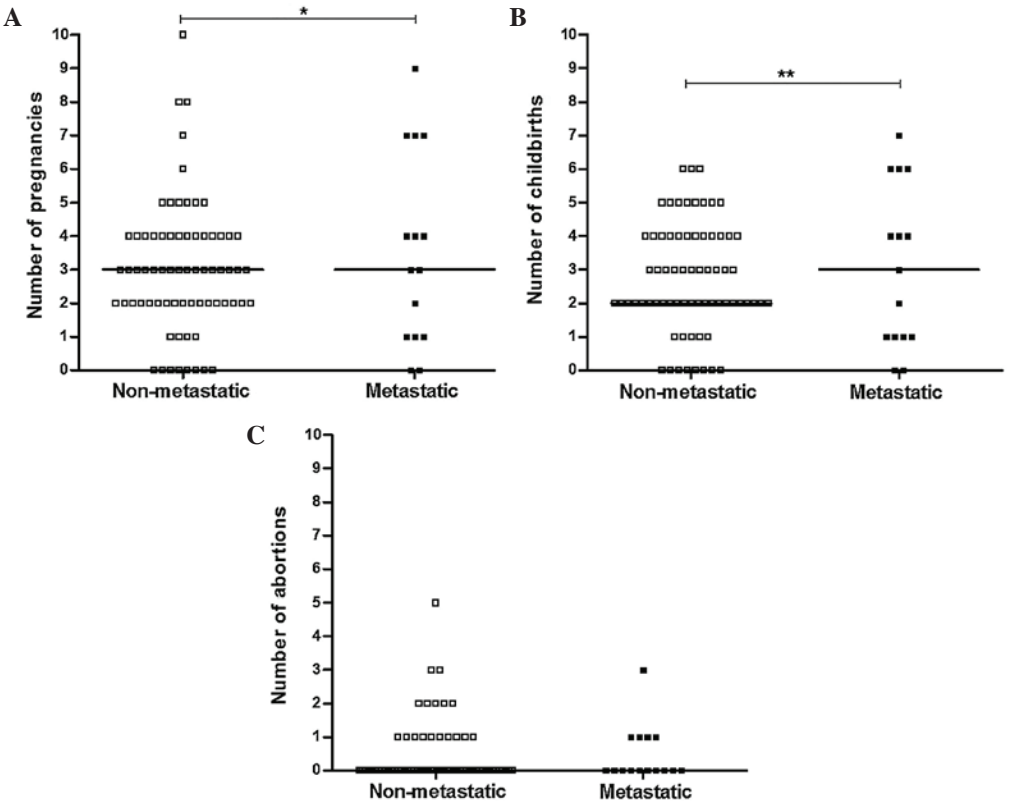

Figure 1. Comparison of number of pregnancies, childbirths and abortions between metastatic and non-metastatic groups. A higher percentage of patients in the non-metastatic group had (A) ≥3 pregnancies and (B) childbirth experiences. (C) However, no significant difference was observed between the number of abortions in the metastatic and non-metastatic groups. * and ** represent P<0.05 and <0.01, respectively.

The expression of *ATAD2* was only correlated with abortion (r=0.207; P=0.205) and smoking (r=0.277; P=0.092). The expressions of *EXT1* and CCNE2 were not correlated with none of the demographic features (for all, r<0.200; P>0.05).

Correlation between gene expression and clinical features. As presented in Table V, expression of *EXT1* demonstrated a significant correlation with hormone therapy (r=0.368; P=0.002), but it did not exhibit any correlation with other

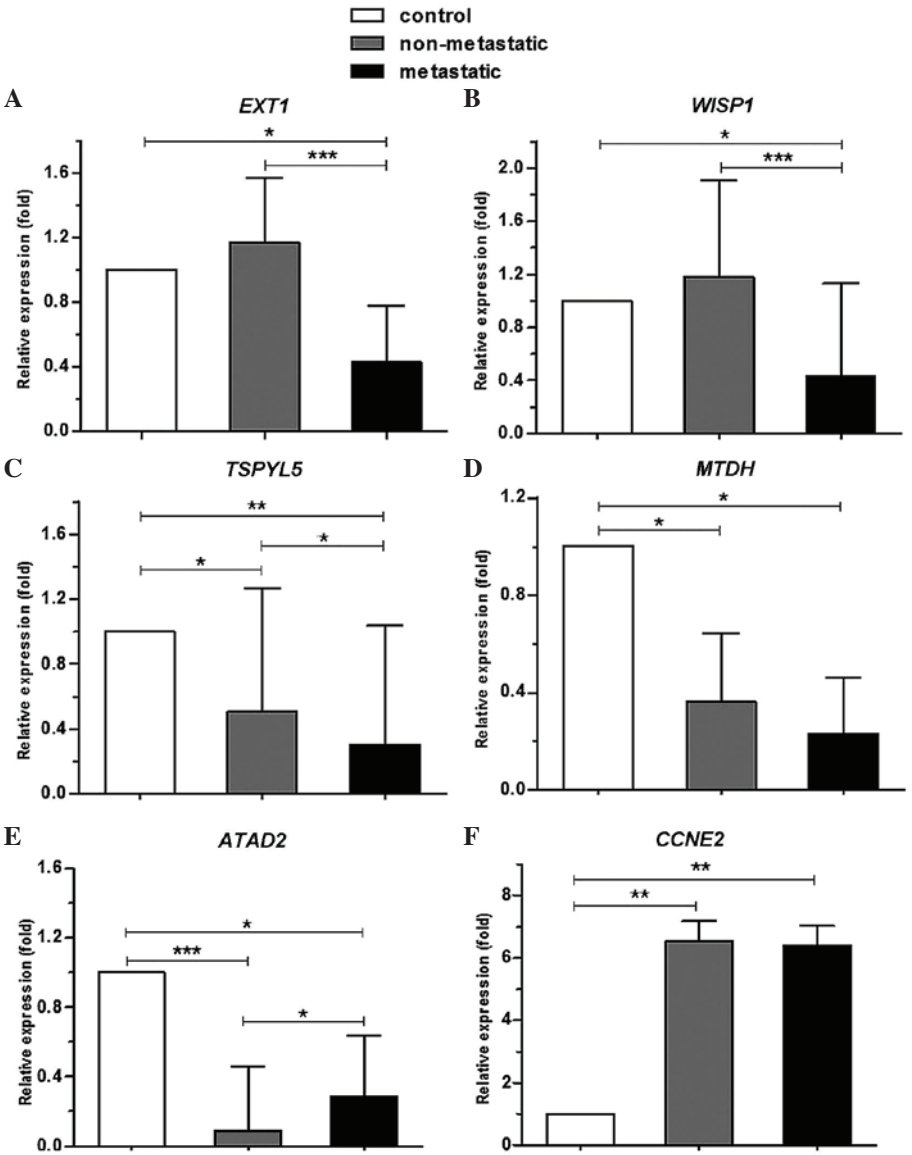

Figure 2. Comparison of mRNA expression of *EXT1*, *WISP1*, *ATAD2*, *TSPYL5*, *MTDH* and *CCNE2* genes between control, metastatic and non-metastatic groups. (A and B) The expression of *EXT1* and *WISP1* was significantly decreased in the metastatic group compared to the control and non-metastatic groups. (C-E) The expression of *TSPYL5*, *MTDH* and *ATAD2* was significantly decreased in metastatic and non-metastatic groups compared to the control. (F) The expression of *CCNE2* was significantly increased in the metastatic and non-metastatic groups compared to the control. *, ** and *** represent P<0.05, <0.01 and <0.001, respectively. *EXT1*, exostosin glycosyltransferase 1; *WISP1*, WNT1 inducible signaling pathway protein 1; *TSPYL5*, TSP-like 5; *MTDH*, metadherin; *ATAD2*, ATPase family, AAA domain containing 2; *CCNE2*, cyclin E2.

clinical features of the patients (for all, r<0.200; P>0.05). *WISP1* expression was correlated with tumor size (r=-0.242; P=0.047) vitamin D level (r=0.220; P=0.242), surgery type (r=0.240; P=0.047) and hormone therapy (r=0.264; P=0.026), whereas no correlation was found between *WISP1* expression and other clinical features (for all, r<0.200; P>0.05). *TSPYL5* demonstrated correlations with LVI (r=0.309; P=0.016), tumor size (r=-0.235; P=0.054), surgery type (r=0.276; P=0.022), radiotherapy (r=0.213; P=0.122) and hormone therapy (r=0.247; P=0.038). However, no correlation was seen between *TSPYL5* and other clinical features (for all, r<0.200; P>0.05). *ATAD2* demonstrated correlations only with LVI (r=0.200; P=0.272), HER2 (r=0.272; P=0.108), vitamin D level (r=0.451; P=0.106) and diabetes (r=0.274; P=0.092). *CCNE2* expression was correlated with pathology (r=0.270; P=0.077), LVI (r=0.223; P=0.185), P53 (r=0.318; P=0.113), surgery type (r=0.319; P=0.033), chemotherapy (r=0.270; P=0.077) and estrogen-progesterone therapy (r=0.310; P=0.062). However, no correlation was observed between *CCNE2* expression and other clinical features of the patients (for all, r<0.200; P>0.05).

Discussion

Following a large number of investigations, the gene expression profiling approach has been established to serve as an appropriate predictor for the clinical outcome of human breast cancer (27,28). The 8q22-24 position has recently drawn the interest of a number of investigators in this field, worldwide (13-17,29). However, to date the majority of relevant publications contradict each other (18-20,30-32), leaving the prognostic value of the 8q22-24 position uncertain. Therefore, in the current study the mRNA expression patterns of *WISP1*,

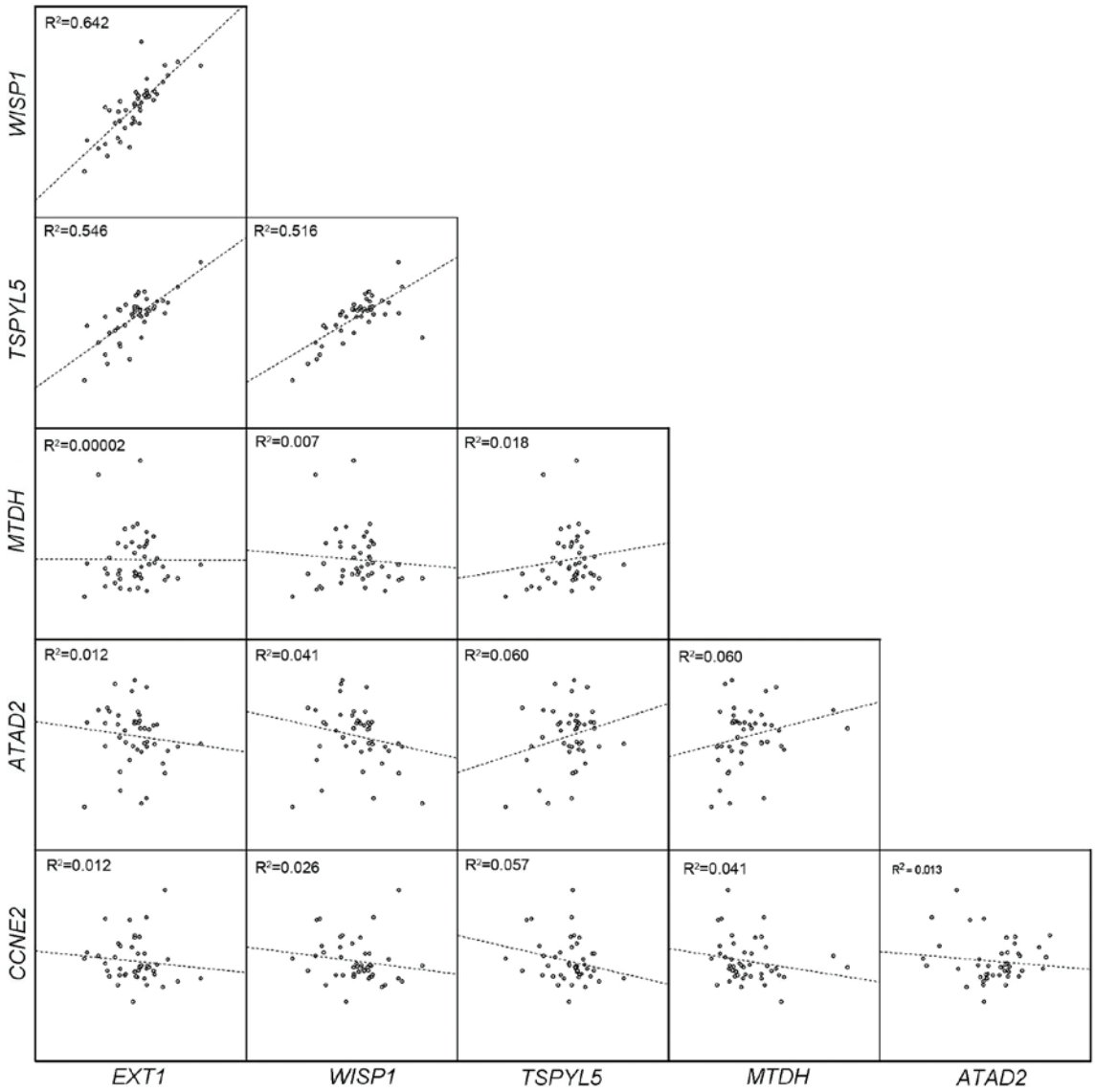

Figure 3. Evaluation of correlations between mRNA expression of *EXT1*, *WISP1*, *ATAD2*, *TSPYL5*, *MTDH* and *CCNE2* genes in the present study. There were marked positive correlations between *EXT1*, *WISP1* and *TSPYL5* expression. *EXT1*, exostosin glycosyltransferase 1; *WISP1*, WNT1 inducible signaling pathway protein 1; *TSPYL5*, TSP-like 5; *MTDH*, metadherin; *ATAD2*, ATPase family, AAA domain containing 2; *CCNE2*, cyclin E2.

EXT1, *ATAD2*, *TSPYL5*, *MTDH* and *CCNE2* genes, located at the 8q22-24 position, were examined in metastatic and non-metastatic early-stage breast cancers. However, the results of the present study contradicted numerous previous reports. All patients included in the present study were lymph-node negative, ER positive and exhibited stage I and II breast cancer, which may be a logical explanation for this observed difference. By contrast, the majority of previous investigations have included patients with advanced stage breast cancers, regardless of ER status. Furthermore, to the best of our knowledge, the present study is the first to investigate target genes in lymph node-negative early stage breast cancers.

The *WISP1* gene is located in the 8q24.1-8q24.3 region, and encodes WNT1-inducible-signaling pathway protein 1, a microcellular protein that is also known as CCN4, in humans (8). An increasing number of studies indicate that WISP1 may be implicated in the development and progression of various types of cancer, suggesting this molecule may be a marker for disease (6,18,26,33). However, conflicting data exist regarding the stimulatory or suppressive role of WISP1 in cancer development (17-19). In the current study, it was observed that the mRNA expression of *WISP1* in non-metastatic breast cancer patients was unchanged compared to normal individuals, while its expression significantly declined in metastatic patients. This finding is in accordance with Davies *et al* (19) who reported *WISP1* as a tumor suppressor gene; it was observed that mRNA transcripts of WISP1 were decreased in node-positive breast cancer patients who subsequently developed metastasis and died. In line with results of Davies *et al* (19), the decline observed in the expression of the *WISP1* gene in the present study appears to be associated with aggressive behavior of the tumor in metastatic breast cancer. However, the results of other previous studies contradict this finding. In contrast to the present study, Xie *et al* (17) observed that expression level of *WISP1* was elevated in primary breast cancer, and this may have contributed to more advanced features of the disease. Chen *et al* (18) also reported that increased expression of *WISP1* may be associated with the pathogenesis of primary lung cancers.

Table IV. Correlation between gene expression and demographic characteristics of breast cancer patients.

	Gene name					
Variable	*EXT1*	*WISP1*	*TSPYL5*	*MTDH*	*ATAD2*	*CCNE2*
Age[a]						
r coefficient	0.074	0.264	0.195	-0.069	-0.179	-0.098
P-value	0.542	0.026	0.103	0.570	0.277	0.516
Marital status[b]						
r coefficient	0.068	0.199	0.067	0.124	0.134	0.060
P-value	0.584	0.103	0.590	0.317	0.415	0.698
Pregnancy or childbirth[b]						
r coefficient	0.150	0.113	0.054	0.029	0.026	0.145
P-value	0.212	0.348	0.653	0.812	0.877	0.337
Abortion[b]						
r coefficient	0.089	0.101	0.200	0.124	0.207	0.048
P-value	0.462	0.403	0.094	0.308	0.205	0.749
Duration of breastfeeding[a]						
r coefficient	-0.094	-0.007	0.088	0.042	0.060	0.059
P-value	0.440	0.955	0.470	0.733	0.718	0.698
Smoking[b]						
r coefficient	0.142	0.142	0.243	0.185	0.277	0.006
P-value	0.244	0.244	0.044	0.131	0.092	0.969
High-fat diet[a]						
r coefficient	0.178	0.186	0.173	0.088	0.165	0.036
P-value	0.160	0.141	0.170	0.491	0.343	0.824
Family history[b]						
r coefficient	0.164	0.209	0.340	0.203	0.059	0.183
P-value	0.183	0.088	0.005	0.100	0.726	0.228

EXT1, exostosin glycosyltransferase 1; *WISP1*, WNT1 inducible signaling pathway protein 1; *TSPYL5*, TSP-like 5; *MTDH*, metadherin; *ATAD2*, ATPase family, AAA domain containing 2; *CCNE2*, cyclin E2. [a]Pearson correlation test. [b]Spearman's ranked correlation test.

The present study also observed that the expression of *WISP1* was associated with a patient's age, serum vitamin D level, tumor size, surgery type and hormone therapy, but showed no association with stage, grade, pathological type and disease features. The negative correlation of *WISP1* with a patient's age, serum vitamin D level and tumor size does not support the hypothesis that a reduced level of *WISP1* is a marker for tumor progression or aggressive features. However, referring to the relevant literature, no evidence regarding the correlation of *WISP1* with any demographic or pathological features in breast cancer was identified. Furthermore, Xie *et al* (34) reported no significant association between expression of *WISP1* and pathological features, including tumor grade and stage, in primary glioma. Taken together, the results of the present study may suggest *WISP1* as a prognostic marker for breast cancer metastasis; though, whether it is a tumor stimulator or suppressor remains to be elucidated.

The *EXT1* gene is located at 8q24.11, and encodes exostosin glycosyltransferase 1, primarily known to serve as a tumor suppressor (30). However, there is evidence suggesting a tumor promoting role for *EXT1* (32,35,36). For example, it has been demonstrated that expression of the *EXT1* gene was amplified following treatment with heparan sulfate proteoglycans, which indicated that, as a glycosylation enzyme, EXT1 participates in heparan biosynthesis, and therefore potentially contributes to the proliferation and invasive potential of breast cancer epithelial cells in ER-negative tumors (32,35). Furthermore, an increased plasma level of EXT1 has been associated with tumorigenesis in cholangiocarcinoma, a form of malignancy in the biliary duct system (36). In the current study, the mRNA expression pattern of *EXT1* was similar to that of *WISP1*. Furthermore, positive correlation was observed between the expression of the *EXT1* and *WISP1* genes, and unlike the other investigated genes, *EXT1* was not associated with the demographic or clinical features of the patients. Based on these findings and following investigations in the future, monitoring mRNA levels of *EXT1* along with *WISP1* may assist with assessing the risk of breast cancer metastasis.

The *TSPYL5* gene is located at 8q22.1, and encodes the testis-specific Y encoded like protein 5 (24). Elevated expression of this gene has been implicated in breast oncogenesis and poor prognosis, via suppression of P53 function (24). Although little is known about the role of *TSPYL5* in the context of cancer, it has been previously suggested to serve as a transcription factor for a number of genes involved in ER-positive breast cancer (37). In

Table V. Correlation between gene expression and clinical features of breast cancer patients.

	Gene name					
Variable	*EXT1*	*WISP1*	*TSPYL5*	*MTDH*	*ATAD2*	*CCNE2*
Pathology[b]						
r coefficient	0.191	0.100	0.058	0.070	0.187	0.270
P-value	0.116	0.414	0.638	0.570	0.266	0.077
Stage[b]						
r coefficient	0.166	0.188	0.155	0.021	0.066	0.061
P-value	0.174	0.122	0.203	0.865	0.693	0.696
Grade[b]						
r coefficient	0.052	0.070	0.110	0.066	0.056	0.009
P-value	0.688	0.584	0.389	0.606	0.761	0.956
Lymphovascular invasion[b]						
r coefficient	0.040	0.182	0.309	0.186	0.200	0.223
P-value	0.761	0.161	0.016	0.152	0.272	0.185
Progesterone receptor[b]						
r coefficient	0.128	0.162	0.109	0.153	0.032	0.018
P-value	0.296	0.184	0.373	0.211	0.848	0.909
Human epidermal growth factor receptor[b]						
r coefficient	0.150	0.075	0.177	0.150	0.272	0.049
P-value	0.243	0.561	0.168	0.243	0.108	0.759
P53[b]						
r coefficient	0.074	0.032	0.111	0.037	0.043	0.318
P-value	0.642	0.839	0.485	0.817	0.856	0.113
Tumor size[a]						
r coefficient	-0.059	-0.242	-0.235	-0.014	-0.084	0.020
P-value	0.630	0.047	0.054	0.911	0.622	0.900
Surgery type[b]						
r coefficient	0.198	0.240	0.276	0.028	0.221	0.319
P-value	0.103	0.047	0.022	0.821	0.183	0.033
Chemotherapy[b]						
r coefficient	0.191	0.100	0.058	0.070	0.187	0.270
P-value	0.116	0.414	0.638	0.570	0.266	0.077
Radiotherapy[b]						
r coefficient	0.082	0.159	0.213	0.009	0.089	0.121
P-value	0.557	0.251	0.122	0.947	0.624	0.477
Hormone therapy[b]						
r coefficient	0.368	0.264	0.247	0.083	0.082	0.092
P-value	0.002	0.026	0.038	0.493	0.621	0.543
Estrogen-progesterone[b]						
r coefficient	0.019	0.052	0.025	0.031	0.171	0.310
P-value	0.883	0.691	0.847	0.811	0.358	0.062
Vitamin D[a]						
r coefficient	0.056	0.220	0.057	0.172	0.451	0.104
P-value	0.767	0.242	0.766	0.362	0.106	0.692
Diabetes[b]						
r coefficient	0.008	0.040	0.035	0.128	0.274	0.120
P-value	0.947	0.739	0.773	0.292	0.092	0.425

EXT1, exostosin glycosyltransferase 1; *WISP1*, WNT1 inducible signaling pathway protein 1; *TSPYL5*, TSP-like 5; *MTDH*, metadherin; *ATAD2*, ATPase family, AAA domain containing 2; *CCNE2*, cyclin E2. [a]Pearson correlation test. [b]Spearman ranked correlation test.

addition, Lyu *et al* (38) demonstrated that regulator of G-protein signaling 2 overexpression in human breast cancer MCF7 cells diminished *TSPYL5* expression, and thereby inhibited growth of the cells. By contrast, in the current study, it was observed that mRNA expression of *TSPYL5* was diminished in metastatic and non-metastatic breast tumors compared with normal tissues, and the expression levels declined even further in the metastatic compared to the non-metastatic tumors. Furthermore, it was observed that mRNA expression of *TSPYL5* was associated with *WISP1* and *EXT1* mRNA expression. *TSPYL5* expression was also significantly associated with family history, smoking, LVI, surgery type and hormone therapy. As the expression of *TSPYL5* was altered in metastatic and non-metastatic breast tumors, this gene cannot be considered as a promising prognostic tool for breast cancer metastasis, and is more likely to be implicated in the pathogenesis of the disease.

The *MTDH* gene is located at 8q22.1, and encodes metadherin, also known as astrocyte elevated gene-1 protein or protein LYRIC (39). Elevated expression of the *MTDH* gene is associated with an increased risk of metastasis of breast cancer to the lungs (31), leading to poor prognosis (40). Hu *et al* (16), suggested that *MTDH* may have a dual role in inducing metastasis and chemoresistance of breast cancer with a poor prognosis. Furthermore, inhibition of *MTDH* has been reported to enhance the sensitivity of breast cancer cells to anti-cancer agents (22,23). However, unlike this previous data, the present study demonstrated that mRNA expression of *MTDH*, similar to that of *TSPYL5*, in metastatic and non-metastatic tumors was lower compared to normal tissues, and its expression in the metastatic tumors was reduced compared with the non-metastatic tumors. Furthermore, the expression of *MTDH* was directly correlated with that of *TSPYL5*. In contrast to previous studies, the results of the present study do not suggest the *MTDH* gene as a prognosticator for metastasis, but rather that it may be implicated in breast cancer development.

The *ATAD2* gene is located at 8q24.13, and encodes an ATPase containing two AAA domains, as well as a bromodomain (15). Previous studies have defined a tumor-driving role for *ATAD2* in breast carcinomas and other malignancies (15,21,41-43). Ciro *et al* (41) suggested that high expression levels of *ATAD2* result in the development of aggressive breast cancer and poor clinical outcomes for patients, potentially via enhancement of transcriptional activity of the MYC oncogene. In addition, Caron *et al* (42) reported that overexpression of *ATAD2* in somatic cells may affect basic features of chromatin, leading to malignant transformation during the development of breast and lung cancer, and resulting in poor prognosis. Additionally, according to Kalashnikova *et al* (21), increased expression of *ATAD2* correlated with poor prognosis in breast cancer patients. Raeder *et al* (15) recently indicated that the amplified expression of the *ATAD2* gene is associated with an aggressive nature in endometrial cancer and a poor outcome for patients. However, the results of the present study contradict those of Kalashnikova *et al* (21), Ciro *et al* (41), Caron *et al* (42) and Raeder *et al* (15). The present study observed that expression of the *ATAD2* gene was significantly decreased in metastatic and non-metastatic patients compared to normal individuals, and this expression was positively correlated with *TSPYL5 and MTDH* expression, and vitamin D level. Taken together, the results of the present study indicate that the *ATAD2* gene, similar to *TSPYL5* and *MTDH*, cannot be considered as a metastasis gene in lymph node-negative breast cancer, but may be involved in the pathogenesis of the disease.

The *CCNE2* gene is located at 8q22.1, and encodes cyclin E2 protein in humans (44). It has been demonstrated to have a significant role in the pathogenesis and invasiveness of breast cancer (25,45). By way of example, Li *et al* (45) reported that the *CCNE2* gene is overexpressed, simultaneously with c-Myc, in human breast cancer, and potentially acts as a promoter for development of the disease. In addition, Caldon *et al* (46) demonstrated that increased expression of *CCNE2* may be associated with drug-resistance in breast cancer cells. Furthermore, Rogers *et al* (47) suggested that overexpression of *CCNE2* is associated with poor prognosis and decreased genomic instability in human breast cancer. In the current study, it was observed that *CCNE2* is overexpressed in metastatic and non-metastatic breast tumors compared with normal tissues. Furthermore, the expression of *CCNE2* was negatively correlated with that of *TSPYL5* and *MTDH*, while it did not exhibit any association with the demographic and clinical features of the patients. The results of the present study are in agreement with those of Li *et al* (45), Caldon *et al* (46) and Rogers *et al* (47), suggesting that amplified expression of *CCNE2* is potentially implicated in breast cancer development; however, it cannot be considered as a biomarker for metastasis risk.

In conclusion, according to the results of the current study, reductions in mRNA expression levels of *TSPYL5*, *ATAD2* and *MTDH* and increases in *CCNE2* mRNA levels may be implicated in the pathogenesis and development of human breast cancer, whereas declines in *WISP1* and *EXT1* mRNA expression among early-stage ER-positive lymph node-negative breast cancer patients may be associated with increased risk of metastasis. Therefore, if validated in future studies considering individual genetic background and ethnic variations, the *WISP1* and *EXT1* genes may serve as a promising indicator of metastasis risk.

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