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MDM2, E-cadherin, Survivin and Her2 mRNA Status in Peripheral Blood of Patients with Breast Cancer

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Abstract

Background: MDM2, E-cadherin, survivin and Her2 genes are involved in the regulation of normal cell growth. However, any crucial change in their expression levels can convert a normal cell into a cancer cell. Numerous studies have identified alterations of these gene expression levels in various cancers, particularly in breast cancer. Thus, they may be used as diagnostic biomarkers. In this experiment, we aim to evaluate these gene transcripts in patients' peripheral blood and compare the results with healthy individuals.

Methods: RNA was extracted from peripheral blood cells of 52 breast cancer patients and 52 healthy volunteers. Then, cDNA was synthesized and assessed for MDM2, E-cadherin, and survivin and Her2 gene transcriptions in peripheral blood samples by quantitative real-time polymerase chain reaction.

Results: There were no considerable differences in the expression of MDM2, E-cadherin, survivin and Her2 in cancer patients compared to healthy individuals. However, there were significantly correlation between E-cadherin, survivin and Her2 expression and some of the clinicopathological characteristics of patients studied. Also, survivin transcripts expression was positively correlated with Her2, E-cadherin, and MDM2 gene expressions.

Conclusion: These results indicated no variations in MDM2, E-cadherin, Her2 and survivin gene expressions in patients compared to controls. We might not consider the examined biomarkers as valuable prognosis factors in primary breast cancer. However, additional research should be undertaken to assess these four genes in a larger sample size.

Keywords: Breast cancer, MDM2, E-cadherin, survivin, Her2

Abbreviations: Quantitative real-time polymerase chain reaction (qRT-PCR), Murine double minute 2 (MDM2), Human epidermal growth factor receptor 2 (Her2).

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Introduction

Breast cancer is the most common malignancy among women worldwide. Despite considerable progression over recent years, the increasing incidence rates of cancer have revealed that current detection and treatment modalities are insufficient.[1] Still more than 30% of women will either be diagnosed with metastatic breast cancer or develop metastases and eventually die from their illness.[2] Therefore, there is a compelling need to diagnose breast cancer at earlier stages and locate more effective treatment methods. The fundamental step is to discover novel biomarkers for early diagnosis.[3] An improvement in biomarker detection strategies is necessary, as only a few of the newly discovered markers have been clinically approved for breast cancer.[4]

There are significant alterations in the expression levels of murine double minute 2 (MDM2), E-cadherin, survivin and human epidermal growth factor receptor 2 (Her2) in the majority of tumors, including breast cancer. MDM2 has been shown to play an important role in a variety of physiological and pathological processes. Overexpression of the human homologue of MDM2, referred to as HDM2, occurs in diverse human malignancies such as soft tissue sarcomas and brain, breast, ovary, cervix, lung, colon, and prostate cancers.[5] MDM2 overexpression in tumors correlates with a poor prognosis for those patients.[6] Several studies have shown that amplification of the MDM2 gene occurs more frequently in metastatic and recurrent cancers than in primary tumors.[7]

It should be considered that the loss of epithelial differentiation in carcinomas is accompanied by increased motility and invasiveness of the tumor cells, often as a consequence of reduced intercellular adhesion.[8,9] The downregulation of cell-cell adherent junctions is a hallmark of the epithelial-to-mesenchymal transition, which involves the loss of functional E-cadherin protein by either transcriptional repression or silencing mutations of its gene.[9-11] Interestingly, endocytosis of E-cadherin has recently been shown to be an important process in the regulation of cadherin function in remodeling adhesive contacts, although the precise mechanism has not been fully elucidated.[12] Evidence to support a novel role for MDM2 in regulating cell adhesions by a mechanism that involves degrading and downregulating the expression of E-cadherin via an endosome pathway has been provided.[13]

Survivin plays multiple roles in malignancy, including the inhibition of apoptosis, the stimulation of proliferation and the promotion of angiogenesis. High levels of survivin are detected in many human cancer types, including 70% of breast cancers.[5,6] Many studies have found that survivin expression is related to an unfavorable prognosis in patients with various solid tumors.[7] Survivin is currently investigated as a target for new anticancer treatments and as a new tumor marker.[8]

Her2/neu proto-oncogene, a major biomarker, encodes a 185-kDa transmembrane receptor with tyrosine kinase activity.[9] Most studies have shown that amplification of Her2 gene or overexpression of the protein correlates with a poor prognosis for breast cancer patients. Overexpression of Her2 might influence a number of aspects of the breast cancer phenotype.[10] These include reduced endocrine responsiveness, increased metastatic ability and drug resistance.[11] Detection of these indicated biomarkers in circulating tumor cells is one of the current hot spots in breast cancer research. Therefore, in this study we aim to evaluate the gene transcripts of MDM2, E-cadherin, survivin and Her2 in peripheral blood of breast cancer patients and healthy individuals.

Materials and Methods

Breast cancer patients and healthy individuals

There were 52 patients who participated in this study. Patients' diagnoses of breast cancer were confirmed by histological studies. During 2011, we received the patients' samples with their informed consent from the Breast Clinic at Shiraz University of Medical Sciences, Iran. Peripheral venous blood samples (2 mL) were collected by venipuncture before any intervention and EDTA was used as the anticoagulant. Prior to sampling,

Table 1. The used primer sequences for each target gene.

Primer	Forward	Reverse
Beta actin	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG
E-cadherin	TGCCCAGAAAATGAAAAAGG	GTGTATGTGGCAATGCGTTC
MDM2	GTGATCTTGGCTCACTGCAA	ACGAGGTCAGGAGATCGAGA
Her2	AGTACCTGGGTCTGGACGTG	CTGGGAACTCAAGCAGGAAG
Survivin	GCCTTTCCTTAAAGGCCATC	AACCCTTCCCAGACTCCACT

patients did not undergo any immunotherapy, chemotherapy or radiotherapy. As a control group, we obtained blood samples from 52 healthy volunteers who had no malignancies or autoimmune disorders. The healthy group's age ranged from 32-57 years, whereas patients' age ranged from 30 to 78 years. This study approved by Ethics committee at Shiraz University of Medical Sciences.

RNA extraction and cDNA synthesis

Total RNA was prepared from blood cells after lysis with ammonium chloride and TRizol reagent (Invitrogen, Paisley, UK). For cDNA synthesis, RNA was treated with DNase I (Invitrogen-Gibco, Paisley, UK) to avoid DNA contamination, then cDNA was synthesized from 5 µg of total RNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentase, Vilnius, Lithuania).

Quantitative real-time polymerase chain reaction (qRT-PCR)

MDM2, E-cadherin, survivin and Her2 gene expressions and their quantities were verified using a Bio-Rad system (Chromo4 Real-time PCR Detector, Bio-Rad, Foster City, CA, USA) with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). β-actin was used as a housekeeping gene. Each PCR reaction was performed in absolute 25µL volume that consisted of 100 nM of each primer, 0.5 µg of the cDNA product, and 1× PCR master SYBRGreen I (Applied Biosystems, USA) that contain SYBRGreen I, dNTPs, reaction buffer and FastStart gold DNA polymerase. The primer sequences are listed in Table 1. Primers were designed using Primer 3 open source software (Sourceforge, USA). For gene transcriptions, thermal cycling was performed at 95°C for 10 min for denaturation, in 40 cycles (at 95°C denaturation in 15 s, 30 s annealing step at 60°C and at last the extension process for 34 s at 60°C while fluorescence appeared). The products of quantitative real-time polymerase chain reaction (qRT-PCR) amplification were assessed by melting curve analysis and 1% agar gel electrophoresis (Data not shown).

Statistical calculations

MDM2, E-cadherin, survivin and Her2 gene expressions in peripheral blood were defined to the control samples equal values by the nonparametric Mann-Whitney test with SPSS software v.[15] (SPSS, Chicago, IL, USA). The relative amounts of MDM2, E-cadherin, survivin, and Her2 transcripts were determined using the $2^{-\Delta Ct}$ formula. Target-to-reference gene ratios were calculated by the Pfaffl method.[14] We used GraphPad Prism 5 (GraphPad Software, Inc. La Jolla, CA, USA) to plot and evaluate relative expression. Spearman's rank parallel tests were applied to study the correlations between different values. *P* value less than 0.05 with 95% confidence interval (CI) was considered significant for the entire statistical analyses.

Results

Pathological and clinical characteristics

Clinical and pathological information was obtained by histopathological and clinical examination of the 52 patients. Table 2 shows tumor size, grade, necrosis, Estrogen Receptor (ER), Progesterone Receptor (PR), Her2 expression, lymph node involvement and clinical stage for all patients.

MDM2 Expression

MDM2 gene was expressed 1.4 fold higher in breast cancer patients compared to control group, but this change was not confirmed by statistical analysis (P>0.05) (Figure 1A). MDM2 gene expression did not correlate with other histopathological information (P>0.05).

E-cadherin Expression

E-cadherin transcript levels were compared to healthy individuals and show no significant difference in peripheral blood samples (Figure 1B). However, we have found significant decrease in E-cadherin transcripts in the patients with tumor size less than 2 cm (P<0.001), low grade (P<0.001), Her2 negative (P<0.001), ER negative (P=0.004), PR negative (P=0.004), peritumor vessel involvement (P=0.015), tumor necrosis positive (P=0.018) and without lymph node involvement (P=0.005) in comparison with controls. It was also revealed that patients with tumor size more than 2 cm, high grade, and Her2 positive express increased amount of E-cadherin mRNA compared to counterpart patients (P=0.006, P=0.009 and P=0.049, respectively).

Survivin Expression

The level of survivin mRNA in the peripheral blood of breast cancer patient was similar to control individuals (Figure 1C). However, patients with tumor size more than 2 cm and Her2 positive showed more amount of survivin transcripts compared to apposite group (P=0.003 and P=0.021, correspondingly). Also, survivin transcripts expression were positively correlated with Her2 (CI 0.35-0.74, P<0.001), E-cadherin (CI 0.28-0.74, P<0.001), and MDM2 (CI 0.04-0.57, P=0.023) gene expression (Figure 2).

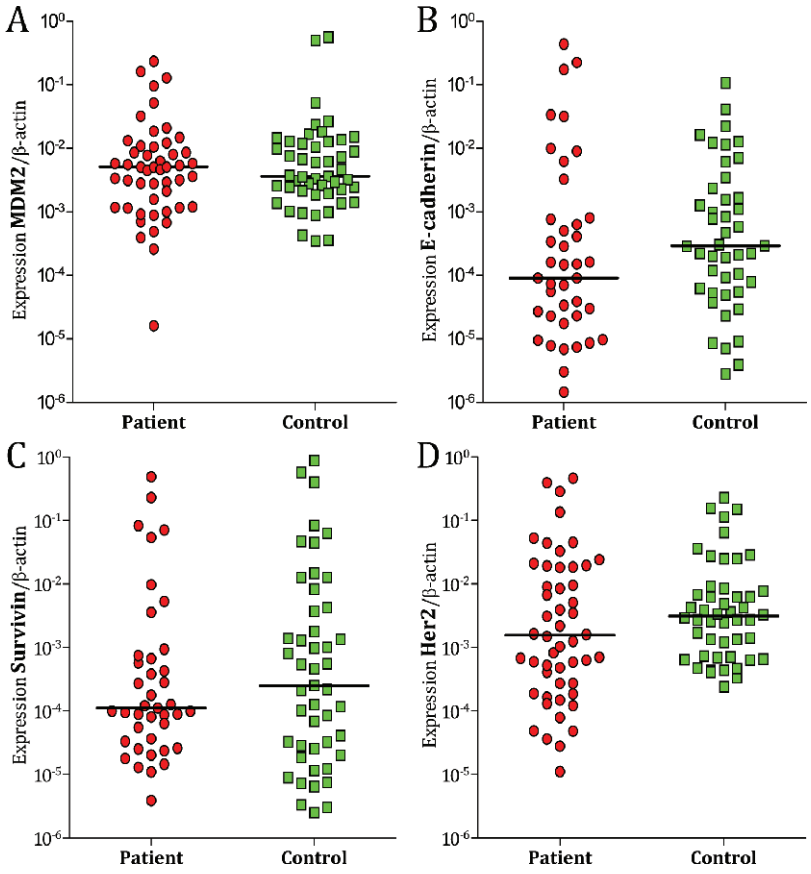

Figure 1. Expression MDM2, E-cadherin, survivin and Her2 in periphery of breast cancer patients The expression level of each mRNA related to β-actin as housekeeping gene was detected by qReal Time PCR. As figure shows, the expression levels of MDM2 (A), E-cadherin (B), surviving (C) and Her2 (D) in peripheral blood samples of breast cancer patients were similar to healthy individuals. The solid line represents the median of $2^{-\Delta Ct}$ of each gene expression.

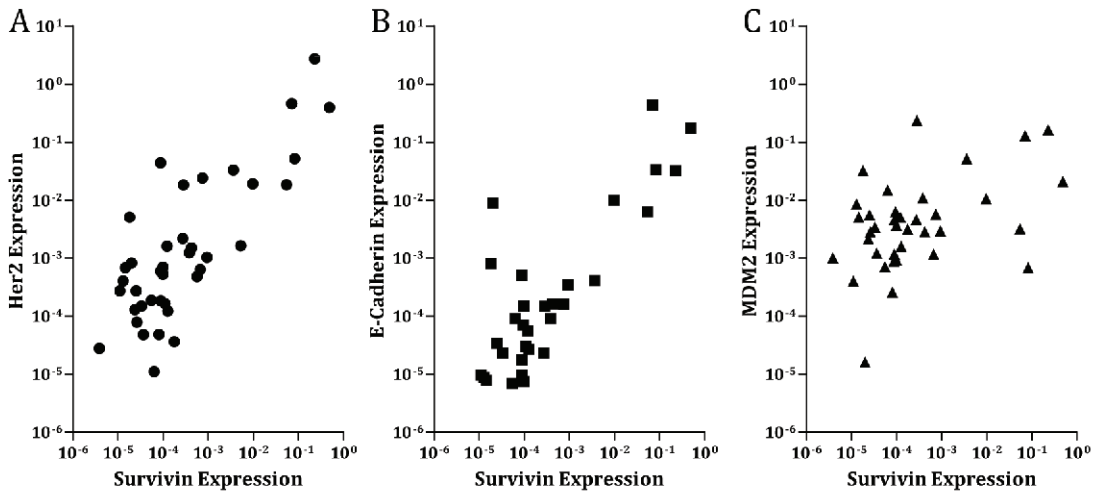

Figure 2. Positive association between transcripts levels of Her2, E-cadherin and MDM2 with survivin mRNA in breast cancer patients As figure shows significant positive correlation between transcripts expressions of (A) Her2 and survivin (CI 0.35-0.74, $P<0.001$), (B) E-cadherin and survivin (CI 0.28-0.74, $P<0.001$), (C) MDM2 and Survivin (CI 0.04-0.57, $P=0.023$).

Her2 Expression

The expression of Her2 gene was explored in the periphery of breast cancer patients. Our data revealed that Her2 expression was not changed significantly in the breast cancer patients in compared to control group (Figure 1D). However, Her2 negative breast cancer patients showed lower expression of Her2 transcripts compared to Her2 positive and control individuals ($P=0.026$ and $P<0.001$, respectively). We have found similar results in patients with tumor size smaller than 2 cm compared to patients with tumor size more than 2 cm and control group ($P<0.001$). Interestingly, the patients with no lymph node involvement expressed lower amount of Her2 gene in comparison with control group ($P=0.001$).

Discussion

Previous studies investigated the roles of MDM2, E-cadherin, survivin and Her2 genes by different techniques in breast cancer patients. The results varied and the biomarker reliability for typing breast cancer was controversial.[15,16] In this study, we have tried to find the possible correlation of these genes transcripts with cancer prognosis.

MDM2 regulates cell cycle and E-cadherin expression. Thus, it may have a critical role in initiation, growth and further metastasis of breast cancer. Yang et al. have reported the overexpression of MDM2 in tissue specimens and its correlation with downregulation of the E-cadherin gene in metastatic breast cancer patients.[13] Resetkova et al. have demonstrated overexpression of MDM2 which leads to stimulation of p53 degradation in tissue biopsies of breast cancer.[17] However, according to Hao et al. MDM2 expression remained unchanged in metastatic tumor cells.[18] Our results showed no significant difference in MDM2 gene transcripts between breast cancer patients and control group in peripheral blood samples.

E-cadherin has a critical role in epithelial cell adhesion. In invasive breast cancer, reduction or loss of E-cadherin expression might correlate with lymph node metastases. It has lower expression in lymph node metastasis in diverse tumors, particularly in invasive breast cancer tissue specimens.[19-22] Dissimilarities in E-cadherin expressions in ductal and lobular breast carcinomas have been reported by Berx et al. and Acs et al.[23,24] However, some studies reported that E-cadherin expression was not correlated with lymph node involvement in breast cancer patients.[25,26] In our study, it was found no difference in E-cadherin gene transcripts in peripheral blood samples of breast cancer patients

and healthy individuals. However, we have found significant reduce in E-cadherin expression in the initial phase of cancer progress and in the patients with Her2, ER, and PR negative in comparison with controls. We also revealed that E-cadherin mRNA expression was associated with tumor size, grade and Her2 expression.

Survivin as another critical studied marker acts as an inhibitor of apoptosis, proliferation inducer and angiogenesis stimulator. It is highly expressed in primary breast tumor tissue biopsies and this overexpression correlates with upregulated Her2 expression.[27,28] It has been reported that survivin levels remained unchanged in the patients with early stage breast cancer compared with normal individuals.[29] However, another study reported that the patients with positive lymph nodes had increased levels of serum survivin.[30] In our study, we have found no difference in survivin expression in periphery of breast cancer patients compared with controls, but it was correlated with tumor size, Her2, E-cadherin, and MDM2 gene expressions.

Her2 is another biomarker which regulates proliferation and differentiation of cells. Overexpression of Her2 has been shown to play a vital role in stimulating breast cancer growth and malignancy. Significant amplification and overexpression of Her2 was reported in tissue arrays of breast cancer patients with a poor survival rate.[31,32] The result of the current study showed no significant difference in Her2 gene transcripts in breast cancer and control blood samples. However, we found significant decline in Her2 transcripts in peripheral blood samples of patients with no lymph node involvement, Her2 negative, and small tumor size.

In conclusion, our results presented no significant variations in MDM2, E-cadherin, Her2 and survivin gene expressions in patient blood samples. Despite some pathological reports showed no correlation between expressions of these markers and cancer progression, we have found that E-cadherin and Her2 mRNA blood levels could be correlated with cancer progression. However, additional research is necessary before

Table 2. The pathological characters of breast cancer patients.

Criteria	Prevalence (%)	Number
Tumor Size		
T1	23	12
T2	44	23
T3	4	2
Unknown	29	15
Lymph node Involvement		
N0	36.5	19
N1	17	9
N2	11.5	6
N3	4	2
Unknown	31	16
Metastasis		
M0	65	34
M1	4	2
Unknown	31	16
Stage		
Stage I	13	7
Stage II	37	19
Stage III	15	8
Stage IV	4	2
Unknown	31	16
Grade		
Grade I	17	9
Grade II	36.5	19
Grade III	8	4
Unknown	38.5	20
Tumor Side		
Right	38.5	20
Left	36.5	19
Both	2	1
Unknown	23	12
Vascular Invasion		
Positive	61.5	32
Negative	11.5	6
Unknown	27	14
Tumor Necrosis		
Positive	33	17
Negative	36	19
Unknown	31	16
ER		
Positive	16	8
Negative	42	22
Unknown	42	22
PR		
Positive	18	9
Negative	42	22
Unknown	40	21
HER2		
HER2-	21	11
HER21+	8	4
HER22+	6	3
HER23+	15	8
Unknown	50	26

these genes can be considered as breast cancer prognosis biomarkers.

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