E-cadherin, Snail, ZEB-1, DNMT1, DNMT3A and DNMT3B expression in normal and breast cancer tissues*

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Objective: Breast cancer is known as one of very important cancers among females, given that a variety of external (i.e., environmental risk factors) and internal factors (i.e., genetics, and epigenetics) are related to the emergence and progression of breast cancer. Among genetic and epigenetic factors, DNA methyltransferase and EMT related genes have critical roles in breast cancer pathogenesis. In the study presented here, we investigated expression of DNA methyltransferases (e.g., DNMT1, DNMT3A and DNMT3B) and EMT-related genes (e.g., E-cadherin, Snail, ZEB-1). Methods and Materials: Tissue samples were collected from 18 cancer and 24 normal breast tissues. We evaluated the expression levels of DNA methyltransferases and EMT related genes using Quantitative real-time PCR (qRT-PCR). Results: Our results indicated that the expression levels of ZEB-1, Snail, and DNMT3B were increased in breast cancer subjects in comparison to the control group. On the other hand, there was a significant decrease in E-cadherin expression in breast cancer tissues in comparison to the normal tissues. Moreover, there were no significant changes for DNMT1 and DNMT3A expression in breast cancer tissues when compared to the normal tissues. Conclusion: Taken together, our finding show that up regulation of ZEB-1 and Snail could be associated with down regulation of E-cadherin and results in promotion of cancer cell invasion. Moreover, down regulation of E-cadherin may be related to deterioration of DNMT3B in patients with breast cancer.

Key words: breast cancer, DNA methyltransferases, E-cadherin, Snail, ZEB-1, DNMT

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Abbreviations: C, threshold cycle; EMT, epithelial-mesenchymal transition; E-cadherin, Snail, ZEB-1, EMT-related genes; DNMT1, DNMT3A, DNMT3B; DNA methyltransferases related genes; qRT-PCR, quantitative real-time PCR

INTRODUCTION

Breast cancer is one of the main cancers among women, given that more than 25 000 new breast cancer cases were diagnosed in the United States in 2017 (Waks & Winer, 2019). Increasing evidences indicate that breast cancer is a complex disease with a sequence of internal and external factors involved in its pathogenesis. Besides external factors, genetic and epigenetic factors are very important players in the initiation and progression of this cancer. Hence, deregulation of key genes has a central role in breast cancer pathogenesis (Mirzaei et al., 2018; Dyrstad et al., 2015; Bruckaert et al., 2017; Tyrer et al., 2004; Faghihloo et al., 2014; Vaezjalali et al., 2013). Several studies revealed that deregulation of metastasis-related genes is one of the major steps in the development of breast cancer (Kotiyal et al., 2014). Metastasis is a vital biological process which helps cancer cells to migrate to new sites in the body. Epithelial-mesenchymal transition (EMT) is a conserved program which has critical function in carcinogenesis and emergence of metastatic properties of cancer cells via promoting invasion, mobility, and resistance to apoptotic stimuli. Furthermore, EMT-derived tumor cells acquire stem cell properties and exhibit a marked therapeutic resistance. Therefore, better understanding of EMT-related genes’ expression could introduce new therapeutic platforms and inhibitors for metastasis (Mittal et al., 2018). Multiple lines of evidence confirmed that various genes, such as E-cadherin, Snail, and ZEB-1 are known as EMT-related genes. It has been shown that deregulation of these genes is associated with metastasis and other cancer-related processes (Tavakolian et al., 2019; Brabletz et al., 2018). In a study, Chen et al., indicated that expression of E-cadherin is associated with the metastasized lymph node (Chen et al., 2015).

Besides genetic alterations, epigenetic modifications are important players in breast cancer pathogenesis. Methylation of different genes are associated with cancerous conditions. In this regard, DNA methyltransferases have a pivotal function in expression of various genes, such as cancer related genes (Mirzaei et al., 2018; Karsli-Ceppiglou et al., 2014).

Methylation of genes can induce a noticeable change in gene transcription and may have an effect on the chromosome structure; therefore, it can be related to activation, or silencing of oncogenes (Diego & Richard, 2014). Methyltransferases are a group of enzymes which have a regulatory role in methylation of cancer-related genes, such as E-cadherin. Given that there are several subtypes of these enzymes, DNMT1, DNMT3B and DNMT3B are the most common DNA methyltransferases which are conserved with the same amino acid sequence in mammals (Michalak & Visvader, 2016). These enzymes have an N-terminal domain designated for binding to nucleic acids, or nucleoproteins, and also a C-terminal domain which accounts for the methylation activity (Estève et al., 2005). DNMT1 is required during replication, and contribute in the methylation pattern procedure in daughter cells and their parents (Schermelleh et al., 2007). DNMT3A and DNMT3B are expressed in germ cells during embryogenesis. There is a down-ex-
pression of DNMT3A and DNMT3B in differentiated cells (Chen et al., 2002; Okano et al., 1999). Dereulation of these genes is involved in breast cancer pathogenesis (Mirza et al., 2013), hence, assessment of these genes’ expression could be used for monitoring breast cancer patients. Moreover, better understanding of behaviors of cancer-related genes could contribute to designing and developing new therapeutic platforms (Subramaniam et al., 2013).

In the study presented here, we investigated the expression of DNA methyltransferases (e.g., DNMT1, DNMT3A and DNMT3B) and EMT related genes (e.g., E-cadherin, Snail, ZEB-1).

MATERIALS AND METHODS

Sample information. This study was approved by the Shahid Beheshti University of Medical Sciences (IR. SBMU.MSP.REC.1397.552, Grant No14315). We collected 18 cancer and 24 normal breast tissues from Taleghany hospital in Tehran between 2017 and 2018. All tissues were stored in RNAlater solution (Qiagen GmbH, Hilden, Germany) at –20°C. Two pathologists collected 18 cancer and 24 normal breast tissues from Taleghany hospital in Tehran between 2017 and 2018. All tissues were stored in RNAlater solution (Qiagen GmbH, Hilden, Germany) at –20°C. Two pathologists have confirmed the stage of tissues. All sample information was recorded and is summarized in Table 1. The inclusive criteria in this study included having no history of chemotherapy or any kind of cancers.

RNA extraction. All tissues were digested with the use of a homogenizer and 1 ml RNX-plus solution (Cinna-gen, Tehran, Iran). After adding chloroform, RNA was precipitated with isopropanol and washed with 70% ethanol. RNA was diluted with DEPC-treated water and its purity was evaluated with Nanodrop.

cDNA synthesis. RNA from cancer and normal tissues was converted into cDNA by combining 10 µl of reverse transcriptase (cDNA kit, BioFACT, Daejeon, South Korea) and 10 µl of RNA; the samples were first incubated at 95°C for 5 minutes, and cDNA was synthesized by incubation at 50°C for 40 minutes. The process was performed with thermo cycler (Bio Intellectica PCR). We then added 20 µl of sterile water to the samples and used them as template DNA.

Quantitative real-time PCR. Real-time PCR primers were used were: ZEB-1 F, ZEB-1 R, Snail 1-F, Snail 1-R, E-cadherin-F, E-cadherin-R, DNMT1-F, DNMT1-R, DNMT3A F, DNMT3A R, DNMT3B F, DNMT3B R, GAPDH F, and GAPDH R were used for control. All primer sequences are listed in Table 2.

Quantitative real-time PCR was performed in a final volume of 20 µl, with the use of Rotor-gene 6000 (Corbett life sciences, Sydney,Australia), with 36-well Gene Discs. We used 10 µl of BIOFACT™ 2X real-time PCR master mix (for SYBR Green 1; BIOFACT, South Korea), 1 µl of forward primer (10 pmol), 1 µl of reverse primer (10 pmol), 2 µl of 1/2 diluted cDNA and 6 µl of sterile water. All samples were run simultaneously in triplicate in order to confirm our results.

To analyze the level of gene expression, we compared all genes with GAPDH as an internal control at 95°C for 10 minutes; 40 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds.

To analyze genes expression, threshold cycle (Ct) was measured. The Ct shows the cycle number. To normalize data, all gene threshold cycles were compared with Ct of the housekeeping gene (GAPDH) by the 2^-ΔΔCt method.

Statistical analysis. Graph-Pad Prism software and ANOVA test were used for analyzing all data. The unpaired, two-tailed student’s t-test was done to analyze the statistical differences between groups using Graph-Pad Prism software. P-value less than <0.05 (P<0.05) was taken as a statistically significant difference.

Table 1. Clinical characteristics of 18 cancer and 24 normal breast tissues.

| Sample Information | 18 Cancer | 24 Normal |
|-------------------|-----------|-----------|
| Age >60           | 67%       |           |
| Tumor localization| (Left: 45%) – (right: 55%) |           |
| Family history    | (Absent: 88.1%) – (Present: 11.9%) |           |
| Lymph node metastasis | (Negative: 48%) – (positive: 52%) |           |
| Tumor size        | (I-II : 45%) – (I-III : 55%) |           |
| Estrogen receptor (ER) status | (Negative: 54.3%) – (positive: 45.7%) |           |
| Progestrone receptor (PR) status | (Negative: 46.6%) – (positive: 53.4%) |           |

Table 2. Primers used for Real-time PCR

| Gene name | Real-time PCR primer |
|-----------|---------------------|
| ZEB-1-F   | GATGATGAATGCGATCGATGC |
| ZEB-1-R   | CTGGCTCCTTCAGGTGCC    |
| Snail-1-F | CACACCCACTCAGATCTCA   |
| Snail-1-R | CTGGTCCTCTTCAGGTGCC    |
| E-cadherin-F | AGGGTTGAAGCACAACAGCA   |
| E-cadherin-R | GTATTGGGGCATCGACCCGAT |
| DNMT1-F   | CTTTAGAGACGCCTGTCAT    |
| DNMT1-R   | CTAGGCTCTCTTCATCCGACT  |
| DNMT3A-F  | CTTTTGCTGGAAGTGCTGG    |
| DNMT3A-R  | GCGAGCTTTGCCAGTCTCC    |
| DNMT3B-F  | AGGAGTGTGAAGCAAGGAGC   |
| DNMT3B-R  | CGAGCTTGGCAGTCTCC      |
| GAPD-H-F  | GGGCTAAGCAGTTGGTGTG    |
| GAPDH-R   | GGGCTAAGCAGTTGGTGGT    |

Figure 1. Evaluating the mRNA expression level of E-cadherin in normal and breast cancer tissue. The results of RT-PCR analysis indicated that there was a decrease in E-cadherin expression in breast cancer tissues when compared to the normal ones. Values are given as mean ± standard deviation. *P<0.05, **P<0.01, revealed significant changes in comparison to normal breast tissues.
The expression of E-cadherin, Snail, ZEB-1, DNMT1, DNMT3A and DNMT3B in breast cancer

RESULTS

Decrease in E-cadherin expression

One of the EMT genes in our study was E-cadherin which is involved in adhesion between various cells. Our results indicated that there was a significant down regulation of E-cadherin expression in the breast cancer tissue when compared to the normal one (**P<0.01; Fig. 1).
are the most important genes of this group that could be associated with invasion properties of cells (Faghihloo et al., 2016; Montserrat et al., 2011).

Our results indicated that there was a significant down regulation in E-cadherin expression in the breast cancer tissue when compared to the normal tissue. Moreover, there was a significant up-regulation of ZEB-1 and Snail expression in the cancer tissues when compared to the normal tissues. Many studies have investigated EMT-related genes, such as E-cadherin. Singhai and others (Singhai et al., 2011) had found that E-cadherin can be down-regulated in the breast invasive cancer tissues. Another study had suggested that down-regulation of E-cadherin could be used as a tumor marker in breast cancer tissues (Younis et al., 2007), while Horne and colleagues (Horne et al., 2018) had demonstrated that down-regulation of E-cadherin is one of the hallmarks of breast cancer tissues. They had shown that E-cadherin in breast cancerous cells is not stable, and there is a down-regulation of this gene at the invasive stage of cancer (Fulga et al., 2015). Yet another study had documented that the tumor cells are able to accumulate all E-cadherin in their cytoplasm, but it is not expressed at the cell membranes (Kowalski et al., 2003). In fact, E-cadherin is one of the most important factors in breast cancer progression (Berx et al., 2001). Also, E-cadherin level could be correlated with a histological type of breast cancer. However, Qureshi and others (Qureshi et al., 2006) has shown that it is not useful as a prognostic biomarker, while Kim and Sahin (Kim & Sahin, 2005) revealed that down regulation of E-cadherin is associated with metastasis.

ZEB-1 is another EMT-related gene which acts as a tumor suppressor and its expression is related to inhibition of cancerous conditions (Zhang et al., 2018). One of the oncogenes, named Ribonucleic acid export 1 (RAE1), is over-expressed in breast cancer due to changes in the level of ZEB-1 (Oh et al., 2019). Yu and others (Yu et al., 2018) documented that the hTERT promoter is stimulated by ZEB-1, leading to triggering of a sequence of breast cancer-related signaling pathways. Another mechanism contributing to breast cancer may involve stimulation of the androgen receptor by ZEB-1 (Graham et al., 2010). Since PTBP3 tends to regulate ZEB-1 in breast cancer, it can be used as a suitable target in cancer therapy (Hou et al., 2018). However, ZEB-1 is able to increase expression of VEGF in breast cancer tissues, and stimulate growth of breast cancer tumor (Liu et al., 2016). Soini and others also found that ZEB-1 is up-regulated in breast cancer (Soini et al., 2011). It is also known that ZEB-1 may be targeted by neurogenin-3, which results in breast cancer progression (Zhou et al., 2017). Moreover, ZEB-1 may be one of the causes of E-cadherin expression repression (Singh et al., 2011; Sánchez-Tilló et al., 2010).

Down-regulation of Snail can reduce breast cancer cell motility. In fact, in breast cancer, Snail increases the RhoA GTPase expression and is associated with initiation of breast cancer (Zhang et al., 2013). Activation of nuclear ERK2 can be achieved by Snail, which is also related to breast cancer (Smith et al., 2014). Ganesan, et al. found that the damage of Slug and Snail involved in the activation of phospholipase D (PLD) promoter; therefore, breast cancer progression is stimulated (Ganesan et al., 2016). Lundgren and others (Lundgren et al., 2009) indicated that hypoxia can partially induce Snail expression in the breast cancer tissues. The over-expression of Snail tends to repress p53 at the posttranslational level in breast cancer tissues (Ferrarelli et al., 2016). Also Burton and others (Burton et al., 2015) had demonstrated that the complex of Snail-Cathepsin L can induce breast cancer progression.

Interestingly, it seems that another mechanism of E-cadherin expression repression is related to the Snail expression, which has a potential to reduce E-cadherin expression (Cano et al., 2000).

Our data indicated that there was no significant change in expression of DNMT1 and DNMT3A in the breast cancer tissues. However, we revealed that there was a significant up regulation in DNMT3B expression.

Sun and others (Sun et al., 2012) had demonstrated that there is a strong relationship between the heterogeneous genotypes of rs16999593 in DNMT1, rs2424908 in DNMT3B and breast cancer. Probably, in breast cancer tissues, there are some oncogenes, especially MUC1-C, which up-regulate the DNMT1 and DNMT3B expression, and are associated with breast cancer progression (Rajabi et al., 2016). Also, DNMT1 upregulation may lead to methylation of CpG islands in ERX, and increase cancer progression; thus, it may be possible to diagnose breast cancer by detecting expression of DNMT1 (Zhang et al., 2016). Shin and co-authors confirmed that there is an extensive effect of DNMT1 in breast cancer (Shin et al., 2016). Nevertheless, Tang and others (Tang et al., 2014) had shown that the mechanism which contributes to breast cancer may be the effect of miR-185 on E2F6, DNMT1 and BRCA1. Moreover, DNMT3B and DNMT3B can reduce the level of E-cadherin expression (Chen et al., 2016).

CONCLUSION

In this study, we found Snail and ZEB-1 to be up-regulated, and there is a decrease in E-cadherin expression in the breast cancer tissue. Taken together, our findings indicate that Snail and ZEB-1 can act as inhibitors of E-cadherin; therefore, they can induce tumor cell metastasis in breast cancer. Furthermore, DNMT3B has some effects on this type of cancer. It may be possible that DNMT3A and DNMT3B can repress E-cadherin expression by methylation of the E-cadherin promoter; therefore, it seems that more assessments for finding the relationship between expression of E-cadherin and DNMT3A and DNMT3B are needed.

Conflict of Interest

The authors declare no conflicts of interests.

Authors’ Contributions

E.F., S.H.T. contributed in the study design and performed cell culture and molecular experiments. H.G. performed statistical analyses. All authors read and approved the final version of the manuscript.

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