

Gene's Expression Status of ZBTB2 and TRAF6 in Breast Cancer Patients

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Abstract - Breast cancer is the most common type of cancer and the most common cause of cancer-related mortality among women worldwide. However, the burden is not evenly distributed and according to the best available data, there are large variations in the incidence, mortality, and survival between different countries and regions and within specific regions. Current predictions and statistics suggest that both worldwide incidence of breast cancer and related mortality are on the rise. So that many studies have been conducted on various genes in breast cancer that can help to treat this cancer. Also in this paper we investigated status expression of ZBTB2 and TRAF6 gene in 35 breast cancer tissues by Real time-PCR method. The results obtained showed that there was no significant difference of expression level of these genes between tumor samples and normal samples ($P > 0.05$).

Keywords - ZBTB2, TRAF6, Real time-PCR method, breast cancer.

I. INTRODUCTION

Breast cancer (BC) is the most common cancer among women worldwide (Liu, Kimball, Liu, Holowatyj, & Yang, 2015). The incidence is rising in most countries and is projected to rise further over the next 20 years despite current efforts to prevent the disease (Eccles et al., 2013; Rahib et al., 2014). Globally, breast cancer is estimated 1.38 million new cases per year. There are 458,000 deaths per year from breast cancer worldwide making it the most common cause of female cancer death in both the developed and developing world (Ferlay et al., 2010). In the UK, the age-standardized incidence of breast cancer in women has increased by 6% over the last decade, between 1999 to 2001 and 2008 to 2010 (Maddams et al., 2009). Also approximately 182,000 women with breast cancer are diagnosed in the United States annually, accounting for roughly 26% of the incidence of cancers among women, and 40,000 women die of breast cancer every year and the second cause of death Cancer in American women after lung cancer (Jemal et al., 2008). BC is a complex and heterogeneous disease with a variety of histopathological

and molecular sub forms with diverse clinical outcomes and relationships with established risk factors (Althuis et al., 2004; Weigelt & Reis-Filho, 2009). One important sub classification of clinical breast tumors is based on the presence or absence of estrogen (ER) and progesterone (PR) receptors (Rakha et al., 2007). Breast cancer patients with tumors that are estrogen receptor (ER)-positive and/or progesterone receptor (PR)-positive have lower risks of mortality after their diagnosis compared to women with ER-and/or PR-negative disease (Anderson, Chu, Chatterjee, Brawley, & Brinton, 2001; Fisher, Redmond, Fisher, & Caplan, 1988). Studies show that genetic, epigenetic and environmental factors affect the incidence and development of cancers (Tao et al., 2015). Recently, many studies have examined on various genes in breast cancer. In this study, ZBTB2 and TRAF6 genes expression were investigated in breast cancer. The ZBTB2 gene is a member of the POK transcription factor family and is a strong repressor of the

important pathway of ARF-HDM2-p53-p21 in cell cycle regulation, this gene suppresses the transcription of ARF, P51, and P21 genes, but activates the HDM2 gene. ZBTB2 directly interacts with Sp1 via its POZ domain and zinc fingers, which is important in the repression of transcription activation by Sp1, also ZBTB2 directly interacts with p53 via its zinc fingers, inhibiting p53 binding and repressing transcription activation by p53. In particular, the ZBTB2 gene is a potent transcriptional suppressor that inhibits p53 and sp1, leading to the arrest of the p21 cell cycle (Gylfe et al., 2013; Jeon et al., 2009; Lohrum, Ludwig, Kubbutat, Hanlon, & Vousden, 2003). Recent studies have shown that TRAF6 is involved in some cancers. TRAF6 is a component of intracellular signal transduction proteins and is part of the TNFR family members (Chung, Park, Ye, & Wu, 2002) and this gene plays an important role in inherent immune responses (Lee & Lee, 2002). Differences in the expression of TRAF protein have been reported in human cancers and TRAF2 and TRAF6 have the highest levels of expression in human cancer cells (Rajandram, Bennett, Morais, Johnson,

& Gobe, 2012; Zapata et al., 2000). The expression patterns of TRAF protein vary widely so that highest levels of TRAF1 and TRAF5 expression exist in the tonsils, spleen, lungs, testicles, and thymus (Ishida et al., 1996; Rothe, Wong, Henzel, & Goeddel, 1994). This gene also plays important role in the growth, inflammation, metastasis, apoptosis and tumor progression (Kobayashi, Walsh, & Choi, 2004).

II. MATERIAL AND METHODS

A. Human specimens

Human breast cancer specimens (n = 35) and adjacent non-tumor tissues were obtained from patients at Imam Khomeini Hospital, Tehran, Iran. With informed consent from each patient. Patient demographic and clinic pathologic characteristics are shown in Table 1.

Table 1. Clinic pathologic characteristics of thirty-five breast cancer patients.

Variable	Value (n = 35)
Gender	
Female	35 (100%)
Smoking history	
Never-smoker	28 (80%)
Smoker	7 (20%)
Age	
30-40	5 (14.2%)
40-50	10 (28.6%)
50-60	12 (34.3%)
60 < n	8 (22.9%)
Stage	
I/II	19 (54.3%)
III	16 (45.7%)

B. RNA extraction, cDNA preparation and Real-time quantitative PCR

Total RNA was isolated from each tumor tissue and adjacent non-tumor tissue by using RiboEx (GeneAll, Korea) according to the manufacturer's specifications. The concentration of total RNA in the final eluate was determined by spectrophotometry and the absorbance 260/280 ratio was controlled between 1.8 and 2.0. The synthesis of cDNA (240 ng of total RNA per 20 µL reaction

mixture) was performed using the Prime Script RT reagent kit (Perfect Real Time) RR037A (Takara, Japan) according to the manufacturer's specifications. The obtained cDNAs were stored in -80°C until use. Real-time PCR was performed using an StepOnePlus™ Real-Time PCR Systems (ABI Applied Bio-systems, Thermo Fisher Scientific, USA) in a 15-µl reaction containing 7.5-µl of RealQ Plus 2x Master Mix Green High ROX™ (Ampliqon, Denmark), 1-µl of cDNA, 5.5-µl of H₂O and 1-µl of mixed

forward and reverse primers (3 Pmol/μl concentration). Real-time PCR amplifications were done as follows: for two selected genes, PCR amplification was set to an initial 95°C for 15 min and then for ZBTB2 and TRAF6 genes, a total of 40 cycles, 95°C for 15 seconds and 60°C for 1 min (step and hold). All samples were analyzed in duplicate. GAPDH was

used as an internal control. Gene expression was calculated using the comparative threshold cycle ($2^{-\Delta\Delta CT}$) method. The primers used for real-time PCR are listed in Table 2.

Table 2. Primer sequences of two selected genes and GAPDH.

Target genes		Sequences (5'→3')	Product length
TRAF6	Forward	TTGCCATGAAAAGATGCASAG	85bp
	Reverse	AGCCTGGGCCAACATTCTC	
ZBTB2	Forward	CCCGTAGTAACCTAAGGCAACA	114bp
	Reverse	CATTAAGGGGATGCACTTCACC	
GAPDH	Forward	CATCAAGAAGGTGGTGAAGCA	120bp
	Reverse	GCGTCAAAGGTGGAGGAGTG	

C. Statistical Analysis

Statistical analysis was performed using the GraphPad Prism v7.03 (GraphPad Software Inc., USA) and T-test. For all tests, a *P* value <0.05 was considered statistically significant.

III. RESULTS

A. Expression changes of ZBTB2 gene in patients with breast cancer by Real time-PCR method

In this research, the status of ZBTB2 gene expression was evaluated in 35 breast cancer specimens and 35 adjacent normal samples. According to Fig. 1A, it is clear that the expression of this gene in 18 (51.4%) tumor samples has decreased in comparison with the normal sample and also the expression of ZBTB2 in 17 (48.6%) tumor samples has increased in comparison with the normal sample. Therefore, it can be concluded that there is no significant difference between of expression level of this gene in tumor samples and normal samples (*P*=0.8). (Fig. 1A and 2A show the results of the ZBTB2 gene).

B. Expression changes of TRAF6 gene in patients with breast cancer by Real time-PCR method

The study on TRAF6 gene expression in 35 breast cancer tissues and 35 adjacent normal tissues showed that the expression level of this gene has increased in 19 (54.3%) tumor samples comparison with normal samples and the expression level has decreased in 16 (45.7%) tumor samples comparison with normal samples. Finally, according to the results it can be said that there is no significant difference between the expression level of this gene in tumor samples

compared to normal samples (*P*=0.9). (Fig. 1B and 2B show the results of the TRAF6 gene).

IV. DISCUSSION

Over the last few years, a great number of studies have reported aberrant patterns of gene expressions in various cancers including breast cancer. Therefore, understanding of the different mechanisms involved in the onset and progression of breast cancer can provide the basis for better treatment. In this study, we have analyzed the expression level of ZBTB2 and TRAF6 genes in thirty-five breast cancer patients by real-time quantitative PCR.

Earlier researches on these genes determine different results in various cancers. Aberrant gene expressions of TRAF6 have been reported in several human cancers. Meng et al. demonstrated that the expression rate of TRAF6 mRNA was significantly increased in osteosarcoma tissues rather than normal bone tissues (Meng et al. 2012). TRAF6 was also overexpressed in pancreatic cancer tissues (Rong et al. 2014). In another study, Sun et al. showed that the TRAF6 is upregulated in colon cancer which is associated with higher tumor grade (Sun et al. 2014). This gene was significantly upregulated in muscles of gastric cancer compared to normal controls (Sun et al. 2012). Investigation of 339 NSCLC patients showed that the expression of TRAF6 was increased in lung cancer (Zhang et al. 2014a). Considering the mentioned studies and the roles of this gene in normal breast tissues, we expect the overexpression of TRAF6 gene occurs in these patients, but in this case, overexpression of TRAF6 gene was observed only in 19 breast cancer patients (54.3%).

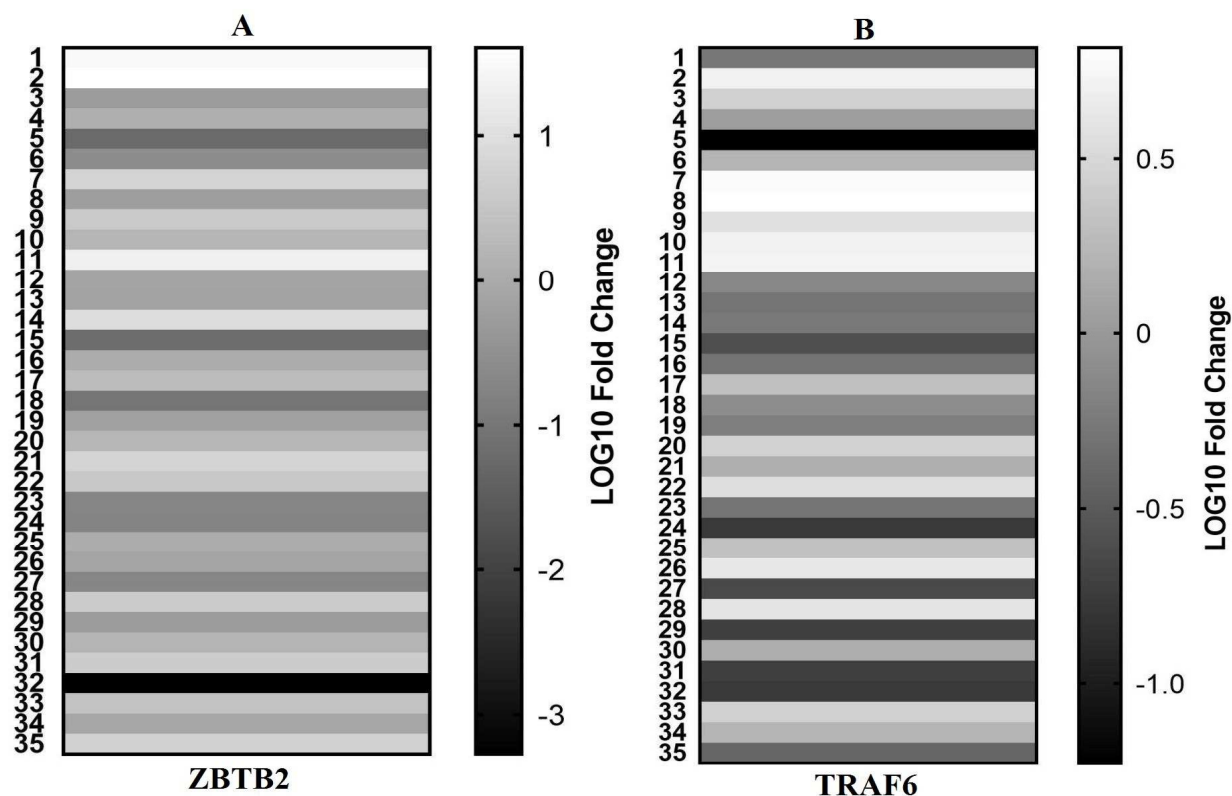


Fig.1 The diagram (A) related to ZBTB2 gene expression and the diagram (B) related to TRAF6 gene expression

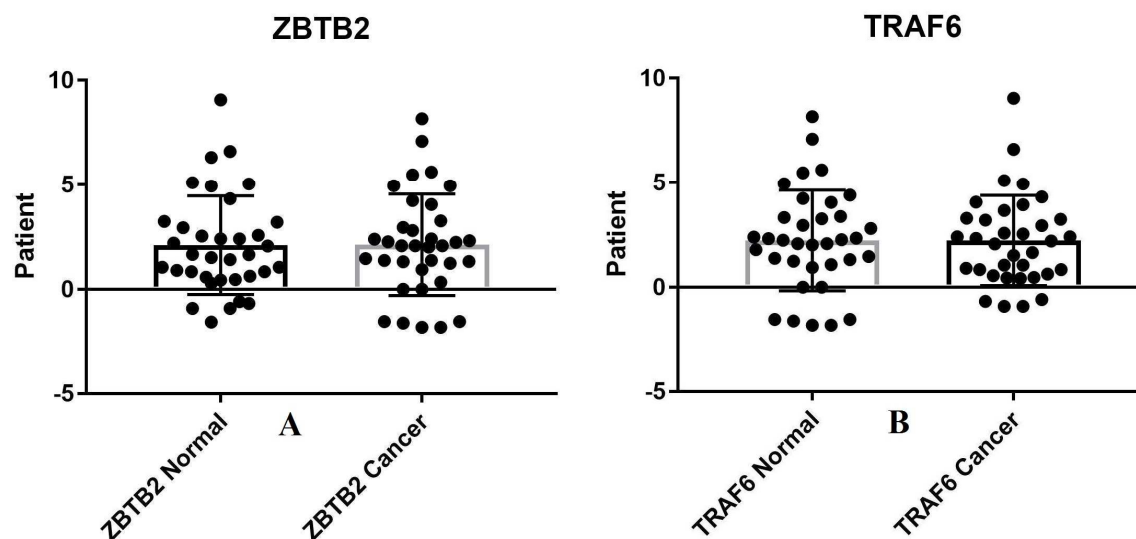


Fig.2. The graph (A) related to comparison of ZBTB2 gene expression between tumor samples and adjacent normal samples and graph (B) related to comparison of TRAF6 gene expression between tumor samples and adjacent normal samples

The role of ZBTB2 gene in gastric cancer has already been studied and the results showed that the ZBTB2 gene expression was significantly reduced by miR-149 (Wang et al. 2012). In this study, we didn't see any significant difference in expression level between tumor and normal breast tissues for this gene, but the ZBTB2 expression was reduced in 18 out of 35 (51.4%) breast cancer tissues compared to adjacent normal tissues.

However, the roles of the ZBTB2 and TRAF6 genes in the evolution of breast cancer are complicated and require more research and a larger population. This study was designed as a pilot study, and further investigations are required to confirm our findings.

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