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A Study on STAT3 Gene Expression in Malignant Tissue of Breast Cancer

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Abstract: The *Activating transcription factor 3* increases the cell survival, growth and immunity tolerance in body. Moreover, STAT3 is active as an oncogene and its high expression is reported around 70 percent of solid and bloody tumors. It is likely to increase expression in breast cancers, and this increase in the expression of STAT3 gene may be helpful in early diagnosis of breast cancer. First, HRRT1 gene was selected as a control gene, and then the condition of STAT3 gene expression was studied in 30 samples of malignant tissue and 10 samples of normal tissues using the Real Time PCR technique. Increased expression of STAT3 gene was seen in 26 out of 30 samples using the Real Time PCR technique. Among all STAT family members, STAT3 is often associated with tumor genesis and is considered as an oncogene. In general, STAT3 turned to the active form in a wide range of tumors in the cell, and especially, the structural activity of STAT3 which is reported in different types of tumors including multiple myeloma, lymphoma and leukemia, prostate cancer, ovary cancer, melanoma, kidney cancer, and colorectal cancer. In this study, STAT3 gene expression has a considerable expression in the malignant tissue of women with breast cancer compared with the expression of the same gene in normal breast tissue, and it can be considered as a prognosis and biomarker.
Keywords: breast cancer, STAT3 gene, Real Time PCR, gene expression study, malignant tissue.

Introduction

Breast cancer is the second reason of death resulting from cancers among women after death from the lung cancer, and it is the most prevalent cancer in women after non-melanoma skin cancer. Based on the statistics of the World Health Organization (WHO), more than 1.2 million cases of breast cancer are diagnosed and more than 500000 people die of this illness each year (Alexander, 2002). The state of being affected by this illness is increasing in the developing countries, contrary to the United States (2). Iran is also experiencing the same trend. This type of cancer is the cause of 21.4 percent of all malignant cases and is the most prevalent cancer in Iranian women (3). Besides high prevalence of breast cancer in Iran, paying attention to the fact that Iranian women are affected by this cancer at least one decade sooner that women in developed countries doubles its significance(4). There are various risk factors in incidence of cancer and it is suggested that approximately 5-10 percent of breast cancers resulted from inherited and exclusive mutations (5). Three groups of regulatory natural genes including growth promoting proto-oncogenes, cancer suppressor genes (growth inhibitor) and the genes regulating the programmed death or apoptosis are the main goals of genetic damages (6 , 7). The investigations showed that the increased risk of breast cancer among the people has both the genetic susceptibility and history of being in contact with environmental factors (4). Therefore, investigating the environmental factors leading to breast cancer, and hereditary susceptive factors provide a new opportunity for understanding the risk of breast cancer. ***Activating transcription factor STAT3*** is one of

these factors. Signal convertors and transcription activators are a family of transcription factors that were first determined by biochemical factors involved in regulating IFN (Interferon) gene expression. The family members of these convertors were identified by separating EST (Expressed Sequence Tag) for associated genes through identifying their activity in connection with induced DNA. This family is composed of seven members that are localized to three chromosomal regions with respect to genetic (15). In many human cancers such as breast cancer, the STAT rout is always active. The cell culture medium studies proved that STATs may change the programmed cell death routs or prevent the cell death. Studies showed that the substitution of two cysteines in the terminal loop of terminal C region produced a molecule from STAT3 which has the ability of automatic dimerization, and can enter to the nucleus after dimerization and attaches DNA (8) and finally activates the transcription. STAT3 in fibroblasts inhibits the Apoptosis process and causes tumor. Haan and Haan. (6) showed that more than 40 various polypeptide ligands cause phosphorylation of STAT through cytokine receptors besides associated JAK (Janus kinase) (9). STAT3 molecule is inactive in the monomer form. When the extracellular ligand are attached to growth factors receptors, interleukin 6 and factors necrosis tumor cause signal transfer into cell and finally cause JACK2 automatic phosphorylation (10 , 11). When the SH2 domain of JAK2 is phosphorylated, it transfers this ability to the subordinate molecules, and causes the phosphorylation of SH2 domain of STAT3 protein. Phosphorylation of STAT3 causes the dimerization of STAT3, and activates it. STAT3 can directly enter to the nucleus, and connect to special regions of DNA, and cause an increase in the transcription of gene (16 , 17).
STAT3 increases the cell cycle by activating C-myc which is a regulator gene that codes for A transcription factor (12 , 13). Subsequently, STAT3 proteins become inactive by tyrosine dephosphorylation and return to cytoplasm. A group of proteins such as SOCS (Suppressor of cytokine signaling) has SH2 domain and has the property of tyrosine kinase. This protein causes the phosphorylation of SATA3 through interaction with STAT3 and makes it inactive (17 , 18). Heat shock proteins (Shp1, Shp2) also have phosphatase property and cause STAT3 dephosphorylation. Activation of STAT3 often causes distinction and growth regulation through ligands but permanent activation of STAT is often associated with interaction with growth regulation (7, 14).

Methods

To collect the samples of the study, 30 malignant tumor tissues and 10 normal breast tissues were selected and they were collected in sterile microtubes and were transferred to a research lab immediately and were kept in a freezer of -70 degree centigrade. Then, we referred to the archive of surgery section in hospitals including Al Zahra, Sadughi, Seyyedoshohada, Isfahan, Iran, and the women with breast cancer were studied who underwent a mastectomy surgery from March 2014 to October 2014. Then, some information about personal details, condition of biomarkers of tumor type, treatment type, clinic-pathologic properties of tumor including tumor size, number of involved lymphoma nodes, primary metastasis as well as the patient's condition in successive followings with respect to relapse of illness and patient death were extracted and recorded. In order to extract DNA, extraction kit of Gene All Hybrid-R™ , Total RNA Purification Kit, made in Korea was used based on the protocol available in the kit. To determine the amount and quality of extracted RNA, the nanodrop machine was used. RNA concentration level and absorption ratio of 260 to 280 were investigated which were in the normal range of 1.7-2 in most studied samples. All Gene Synthesis HyperScript TM First Strand was used for synthetizing the cDNA. In order to ensure the formation of cDNA, its absorption was checked through spectrophotometer, and the absorption level was about 2000 nanograms in microliter in all samples. Primer designing was conducted by a tool named Primer Blast of NCBI base and structural analysis was conducted by Oligo 7 software. Both primers were designed so that they have the sequence among exon.

Table A1.Primer Pair 1: STAT3

Forward	**5'-CAAGGAGGAGGCATTCGGAAAGT-3'**
Reverse	**5'- GTATTGCTGCAGGTCGTTGGTGT-3'**

Table A2.

Primer 2: HPRT1 (housekeeping gene)

Forward	5'- CCTGGCGTCGTGATTAGTGATGAT-3'
Reverse	5'- GCAAGACGTTCAGTCCTGTCCAT-3'

Conducting the Technique of Real Time PCR

In order to conduct the technique, Real Time PCR Corbett system made in Australia was used. Sample analysis was in the form of Doplicat and leads to more accurate results. Vials related to SYBER Green master mix were kept away from light, and the content of the tubes were mixed by shaking the vials. Other materials were prepared for being used by the help of Vortex and fast spin. The reaction mixture was prepared for conducting Real Time PCR according to Table 3, and was placed in Real time PCR apparatus quickly for further analysis.

Table A3.Reaction Mixture for Conducting Real Time PCR

Mixtures	Values
RealQPCR2x Maste Mix (SYBR Green)	12.5 µL
Primer Forward(10µM)	0.5µL
Primer Reverse(10µM)	0.5 µL
Nuclease-free Water	Up to 25
cDNA	Equal to 100 nanograms

Table A4. Optimal Program for Real Time PCR

Stage	Temperature	Time	Round
Primary Denaturation	C°95	5 min	**1**
Denaturation	C°94	30 sec	**40**
Annealing	C°58	30 sec	
Extension	C°72	50 sec	
	Plate read		

Statistical Analysis and the Way of Investigating the Expression of a Given Gene

Real Time PCR is a powerful method for studying the level of gene expression which is conducted based on an available protocol. Especial primers for each gene and the given apparatus were used for this study. HPRT1 was used as internal control of selection, and designed primers were used for quantifying the gene expression. Standard curves were drawn using successive dilutions, and then the reaction and gene expression output were determined. For each gene, the standards curve using three dilutions including 1/1000, 1/100 and 1/10 were applied. The gene expression level was measured and the obtained level was divided by HPRT1 gene to obtain the gene expression for each sample in Means±SD. Results were investigated in tumor sample group using Kolmogorov-Smirnov test, and were analyzed using Student-t test.

Melting Curve Diagram

Melting curve of STAT3 gene primer couple and the curve of HPRT1 primer couple are provided in Figure 2 and 3 in different concentrations of cDNA. The presence of just one peak in the concentration of 1000 nanogram indicates the appropriateness of primers for operating Real Time PCR by SYBER Green. Therefore it was shown that the best concentration of cDNA for multiplying both STAT3 and HPRT1 genes is at 1000ng/µl.

Figure A1. The Melting Curve of STAT3 Gene in Normal Sample

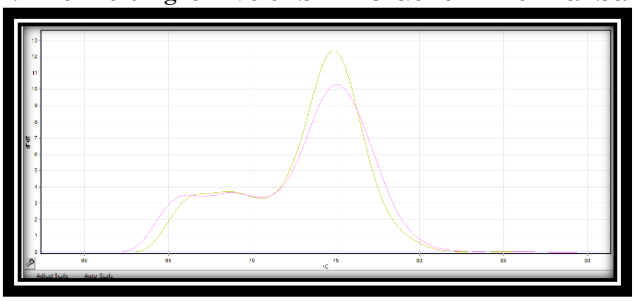
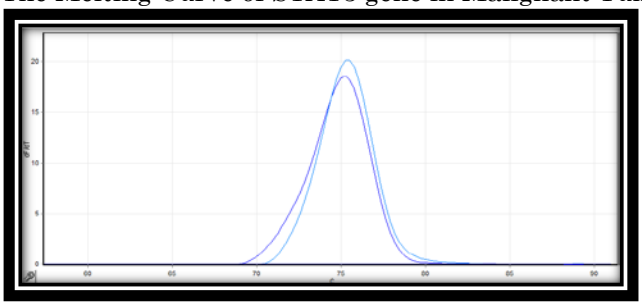


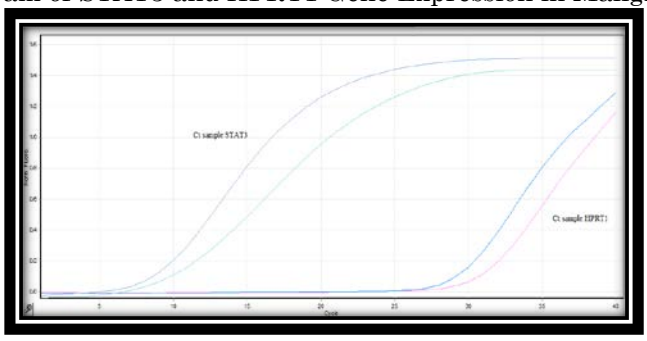
Figure A2. The Melting Curve of STAT3 gene in Malignant Tumor Sample



Multiplication Diagram

Multiplication diagram in Figure 3 shows that in some tumor samples, the differences in cDNA multiplication curves of STAT3 and HPRT1 genes are low, and C_t level associated with STAT3 in these samples are close to C_t level associated with HPRT1. These variations show that the number of copies of STAT3 gene is increased in these samples, and since HPRT1 has fixed transcripts number in tumor and normal samples, STAT3 gene expression is increased in tumor samples.

Figure A3.The Diagram of STAT3 and HPRT1 Gene Expression in Malignant Tissue Samples



Results

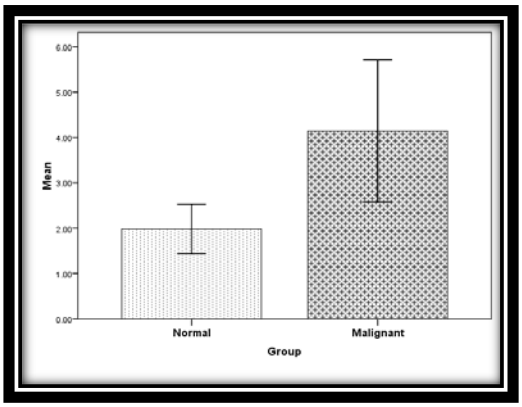
Thirty fresh tissues of breast malignant tumor were studied to diagnose increased expression of STAT3 gene using Real Time PCR. After drawing the multiplication curve of each gene, C_t level of each gene in each tumor sample was obtained. The expression level of STAT3 gene was calculated in each sample using C_t of each gene in normal sample by using $2^{-\Delta\Delta Ct}$ method (Equation 1-4).

$$2^{-\Delta\Delta Ct} = 2^{-[\text{ Ct (STAT3)-Ct (HPRT1), Tumor}]-[\text{Ct (STAT3)-Ct (HPRT1), Normal}]}$$

Comparing STAT3 Gene Expression Level in Normal and Malignant Samples

The average level of STAT3 gene expression, calculated by $2^{-\Delta\Delta Ct}$, showed a normal expression level in normal samples. Among 10 normal tissue samples, just two cases had expression level close to increased expression limit, while in malignant tissues, 26 cases of total 30 samples showed increased expression of STAT3. Therefore, with more than 95 percent confidence, this gene has increased expression in malignant tissues, and the expression level of this gene in malignant tumor tissue is 2.16 times more than normal tissues (Figure 4).

Figure A4. The Diagram of STAT3 Gene Expression Level in Malignant Tissue of Breast Compared with Normal Tissue



Discussion and Interpretation

Berclaz et al. (2001) stated that STAT3 activation has a role in tumor genesis and active protein of STAT plays a role in human cancers. They also speculated that there might be a very strong relationship between nuclear STAT3 and EGFRQ expression in breast cancer. These results clearly show the strong relationship between STAT3 activation and breast tumor and confirm that STAT3 activation may has an important role in tumor genesis in breast tissues because of signaling routs and tyrosine kinase. Pensa (2009) compared STAT1 and STAT3 and concluded that these two genes have opposite roles in tumor. While STAT3 causes cell survival increase and growth and immunity of body and it is considered as an oncogene, ATAT1 causes increase in inflammation, inherent and acquired immunity and stimulation in most anti-developmental cases and answers causing Apoptosis in tumor cells. Moreover, they stated that STAT3 is active as an oncogene and it is reported in about 70 percent of solid and bloody tumors. Tania et al. (2014) showed that tyrosine kinase activity of STAT subunits may have increased compared with normal tissue. They also stated that the best sample is the activation of STAT1, 3 by epidermal growth factors, and continued that JAK family is not involved in this activation. It is also shown that Interleukin 7 or epidermal growth factor causes phosphorylation of STAT protein, and permanent activation of STAT3 may play a role in the formation of breast tumor cells, and may affect the regulation of involved gene expression in cell cycle and Apoptosis. In this study, the role of STAT3 was studied by blocking this gene using antisense therapy. It is to be noted that when we talk about controlling the gene expression, we mean controlling it at transcription level. STAT3 exerts its operation at transcriptional level. Therefore, it seems logical to study the expression of this gene simultaneously with radiotherapy and chemotherapy in patients with breasts cancer in early stages of tumor formation, and in the intermediate stages of metastasis in a relatively bigger society to obtain a more accurate estimation of medicine dosage, its effect in various stages, and the level of gene expression control and their association with curing the patient.

References

Alexander, W. (2002). Suppressors of cytokine signalling (SOCS) in the immune system. *Journal of Nature Immunology, 2,* 410-416.

Berclaz, G., Altermatt, H., Rohrbach, V., & Sirgusa, A. (2001). EGFR dependent expression of STAT3 (but not STAT1) in breast cancer. *Journal of Oncology, 19*, 1155-1160.

Chang, K., Wu, M., & Jones, D. (2006). Activation of STAT3 in thymic epithelial tumors correlates with tumor type and clinical behavior. *Journal of Pathology, 2*, 224–233.

Casanova, J., Holland, S., & Notarangelo, L. (2012). Inborn errors of human JAKs and STATs. *Journal of Immunity, 36,* 515–528.

Ho, H., & Ivashkiv, L. (2006). Role of STAT3 in type I interferon responses: Negative regulation of STAT1-dependent inflammatory gene activation. *Journal of Biology Chemistry, 281*, 14111–8.

Haan, C., & Haan, I. S. (2010). Perspectives for the use of structural information and chemical genetics to develop inhibitors of Janus kinases. *Journal Cell Medical*, *14*, 504–527.

Kortylewski, M., Jove, R., & Yu, H. (2005). Targeting STAT3 affects melanoma on multiple fronts. *Journal of Cancer Metastasis Review, 2*, 315–327.

Kumar, V., Cotran, R., & Robbins, SL. (2003). *The female genital system and breast in Robbins basic pathology* (7th ed), Philadelphia, Saunders.

Levy, D., & Inghirami, G. (2006). STAT3: A Multifaceted Oncogene. *Journal of Proceeding of The National Academy of Sciences of The United States of America, 103,* 10151-10152.

Li, WX. (2008). Canonical and non-canonical JAK-STAT signaling. *Journal of Trends Cell Biology, 18*, 545–551.

Levy, D., & Darnell, J. (2002). Signaling: Stats: transcriptional control and biological impact. *Journal of Nature Biology, 3,* 651–662.

Meterissian, SH. (1997) Apoptosis: its role in the progression of and chemotherapy for carcinoma. *Journal of American College of Surgeons, 184 (6),* 658-66.

Marotta, L., Almendro, V., Marusyk, A., Shipitsin, M., & Schemme, J. (2014). The JAK2/STAT3 Signaling Pathway Is Required For Growth Of CD44+CD24– Stem Cell–like breast cancer cells in human tumors. *Journal of Clinical Investigation, 121,* 2723-2735.

Pensa, S., Regis, G., Boselli, D., Novelli, F., & Poli, V. (2009). STAT1 and STAT3 in Tumor genesis: Two Sides of the Same Coin?. *Journal of Land Bioscience,* 101-118.

Polyak, K. (2011). Heterogeneity in Breast Cancer. *Journal of Clinical Investigation, 121,* 3786-3788.

Tanya, G., Ann, W., James, T., Satoshi, K., & Tammy, B. (2014). Persistent Activation of Stat3 Signaling Induces Surviving Gene Expression and Confers Resistance to Apoptosis in Human Breast Cancer Cells. *Journal of Human Cancer Biology, 12*, 11-19.