RESEARCH ARTICLE

Expression Analysis of Two Cancer-testis Genes, FBXO39 and TDRD4, in Breast Cancer Tissues and Cell Lines

Mahnaz Seifi-Alan[1&], Roshanak Shamsi[1&], Soudeh Ghafouri-Fard[1*], Reza Mirfakhraie[1], Davood Zare-Abdollahi[1], Abolfazl Movafagh[1], Mohammad Hossein Modarressi[2], Golnesa Kazemi[3], Lobat Geranpayeh[4], Mitra Najafi-Ashtiani[5]

Abstract

Breast cancer accounts for one third of new cancer cases among women. The need for biomarkers for early detection is the stimulus to researchers to evaluate altered expression of genes in tumours. Cancer-testis (CT) genes are a group with limited expression in normal tissues except testis but up-regulation in a wide variety of cancers. We here evaluated expression of two CT genes named FBXO39 and TDRD4 in 32 invasive ductal carcinoma samples, 10 fibroadenomas and 6 normal breast tissue samples, in addition to two breast cancer cell lines, MCF-7 and MDA-MB-231, by the means of quantitative real time RT-PCR. FBXO39 showed significant up-regulation in invasive ductal carcinoma samples in comparison with normal samples. It also was expressed in both cell lines and after RHOXF1 gene knock down it was down-regulated in MCF-7 but up-regulated in the MDA-MB-231 cell line. TDRD4 was not expressed in the MCF-7 cell line and any of the tissue samples except testis. However, it was expressed in MDA-MB-231 and was up-regulated after RHOXF1 gene knock down. Our results show that FBXO39 but not TDRD4 can be used for cancer detection and if proved to be immunogenic, might be a putative candidate for breast cancer immunotherapy.

Keywords: Breast cancer - cell lines - cancer-testis genes - FBXO39 - TDRD4

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Introduction

Statistics estimate that breast cancer will be the most commonly diagnosed type of cancer among women in 2013 accounting for about one third of all new cancer cases. It also continues to be the second most common cause of cancer death in women (Siegel et al., 2013). Although in general incidence of breast cancer is decreasing in the United States during the last decade, the incidence of the biologically aggressive estrogen receptor (ER) negative, progesterone receptor (PR) negative breast cancer in women younger than 40 has been increasing in African Americans in the US, as well as some other populations (Kakarala et al., 2010). The age of onset of breast cancer in Iran is at least one decade younger than their counterparts in developed countries (Harirchi et al., 2004). The commonest histological types of breast carcinoma are invasive ductal carcinomas not otherwise specified (IDC-NOS) or of no special type (IDC-NST), invasive lobular carcinoma, pure tubular carcinoma, invasive cribriform carcinoma, medullary carcinoma and mucinous carcinoma, with the first one accounting for 65-80% of cases (Weigelt et al., 2010). In spite of increasing survival rate for breast cancer patients in many countries, it seems that the survival in Iranian breast cancer is poor and early detection of cancer is an important step in improvement of survival (Vahdaninia and Montazeri, 2004).

Cancer-testis (CT) antigens are a group of tumor antigens attracting oncologists and cancer immunologists in recent years for their special characteristics: being expressed in gametogenic tissues, absence from almost all other normal tissues and aberrant expression in different tumor types. These features besides the presence of blood testis barrier make them suitable biomarkers and targets for therapeutic cancer vaccines (Ghafouri-Fard and Modarressi, 2009). Early detection of breast cancer is an important challenge in any cancer control program; so the role of CT antigens in this regard has been highlighted. Few researches have been conducted on the evaluation of CT antigens expression in breast cancer mostly focusing on MAGE and NY-ESO-1 CT antigens (Bandić et al., 2006; Mischo et al., 2006; Theurillat et al., 2007; Chen et al., 2011; Curigliano et al., 2011). Some of these results

*[1]Department of Medical Genetics, Shahid Beheshti University of Medical Sciences, [2]Department of Medical Genetics, [4]Department of Surgery, Sina Hospital, Tehran University of Medical Sciences, [3]Genetics Research Center, University of Social Welfare and Rehabilitation Sciences, Tehran, [5]Shahid Madani Hospital, Karaj, Iran [&]Equal contributors *For correspondence: ghafourifard@razi.tims.ac.ir*

show that breast cancer is among the tumors with the most frequent expression of CT antigens, providing for many patients potential candidates for vaccine trials (Mischo et al., 2006). In addition, considering the limited treatment options for ER/PR/HER2 triple-negative breast cancer, preferential expression of CT antigens in these tumor types, necessitate the exploration of their potential use in immunotherapeutic approaches (Chen et al., 2011). A CT antigen named TSGA10 was found to be expressed in about two third of breast tumor samples examined and mostly in invasive ductal carcinoma (Mobaseri et al., 2007; Dianatpour et al., 2011). A subset of patients also showed anti TSGA10 antibodies. So it was suggested for detection and immunotherapy of breast cancer (Dianatpour et al., 2011).

Breast tumors are considered to be diverse in their natural history and in their responsiveness to treatments basically as a result of variation in transcriptional programs (Perou et al., 2000). ER negative and PR negative breast cancer cell lines and primary breast carcinomas have been shown to express CT antigens of X chromosome (CT-X antigens) at a significantly higher frequency. Immunohistochemical staining has confirmed the correlation of CT-X antigen expression and ER negativity in breast tumors showing a trend for their coexpression with basal cell markers. Breast cancers with basal-like features are generally of higher grade with a tendency to spread to brain. Constituting a subset of triple negative breast cancers, they carry a very poor prognosis. Because the therapeutic options for ER negative breast cancers are limited; CT-X based cancer vaccines may be useful in this regard (Grigoriadis et al., 2009). In addition, a high CT antigen expression rate has been reported in BRCA-associated breast cancer despite the lack of expression of these antigens in benign breast tissue of carriers. So, CT antigens have been proposed as potential vaccine targets for breast cancer prevention in people carrying these mutations (Adams et al., 2011).

To identify novel CT antigens in breast cancer, we examined mRNA expression of two previously found CT antigens Tudor domain-containing protein 4 (TDRD4) and F-box protein 39 (FBXO39) in invasive ductal carcinoma of breast, fibroadenoma and normal breast tissues in addition to two breast cancer cell lines MCF-7 and MDA-MB-231 which were transfected with Rhox homeobox family, member 1 (RHOXF1) shRNA constructs. TDRD4 (RNF17 ring finger protein 17) codes for a RING-finger protein which has interaction with Mad proteins to move them to the cytoplasm. As Mad proteins inhibit the transcriptional activity of the c-Myc oncoprotein, TDRD4 promotes c-Myc function (Yin et al., 2001). TDRD4 has been shown to be essential in differentiation of male germ cells (Pan et al., 2005) and is expressed in the testis but not in other normal tissues and in some liver cancer samples (Yoon et al., 2011). FBXO39 (BCP-20) is a member of F-box protein family, an expanding family of eukaryotic proteins distinguished by an about 40 amino acid motif, which are one of the four subunits of ubiquitin protein ligases (Cenciarelli et al., 1999). FBXO39 has been shown to be expressed highly only in normal testis among normal tissues and in 39% of colon tumor samples examined by quantitative real-time and conventional RT-PCR analysis. FBXO39 has also been expressed in different cancer cell lines including one of the 6 examined breast cell lines (BT00474, HTB131, MCF7, MCF7/MDR, MDA-MB-231, and SK-BR-05), colon, renal, thyroid, ovarian, and lung cancers. As ELISA analysis has shown that the levels of FBXO39 antibody in patients with colon cancer is significantly higher than healthy donors, it has been suggested as a new CT antigen useful for immunotherapy and cancer detection (Song et al., 2011).

Materials and Methods

Tissue samples

Thirty two invasive ductal carcinoma of breast and 10 fibroadenoma samples were obtained from patients in Shahid Madani hospital under the protocols of Medical Ethics Committee. All patients had written informed consent. Normal testis tissue obtained from a prostate cancer patient following orchiectomy was used as positive control for analysis of testis specific genes expression. Six normal breast tissues also were used for gene expression analysis. Tissues were frozen in liquid nitrogen for further analysis.

Cell culture

The human breast cancer cell lines MDA-MB-231and MCF-7 were obtained from Pasteur Institute of Iran and cultured according to the manufacturer's instruction. In brief, cells were cultured in DMEM medium (Invitrogen, USA) supplemented with 10% fetal bovine serum, 100U/ml penicillin, and 100μg/ml streptomycin. The cells were plated in an incubator containing 5%CO_2/95% humidity at 37°C.

shRNA constructs and transfection

Four unique 29mer RHOXF1 shRNA constructs in retroviral GFP vectors were used for cell lines transfection as described previously (Ghafouri-Fard et al., 2012).

RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted from tissue samples and cells using easy-BLUE™ Total RNA Extraction Kit (iNtRON BIOTECHNOLOGY) as instructed by manufacturer. RNA extracts were analyzed by Biowave II spectrophotometer (Biochrom Cambridge, England) to determine purity and concentration and electrophoresd to check integrity. Then, 1 μg of RNA was applied to cDNA synthesis by Fermentas RevertAidTM H Minus First Strand cDNA Synthesis Kit. Synthesized cDNA was analyzed spectrophotometrically to verify concentration and stored in aliquots at -20°C until use. Quantitative real time RT-PCR reaction was performed on a rotor gen 6000 corbette detection system using AccuPower® 2× Greenstar qPCR Master Mix (BIONEER, USA) following thermal cycling conditions: an initial activation step for 5 min at 95ºC followed by 40 cycles including a denaturation step for 10s at 95ºC and a combined annealing/extension step for 30s at 56ºC. No template control (NTC) consisting of H_2O for target and reference genes were included in each run. Beta 2 microglobulin (B2M) gene was used as normalizer. Primer

Table 1. Primer Sequences

Primers name	Primers sequences	Product sizes
B2M-F	5'-AGATGAGTATGCCTGCCGTG-3'	105 bp
B2M-R	5'-GCGGCATCTTCAAACCTCCA-3'	
FBXO39-F	5'-TAGATCTCCTGCCCACCTTCC-3'	167 bp
FBXO39-R	5'-ACTCTTCAGGATCCGCTCCA-3'	
TDRD4-F	5'-ATCGTGGCAAGGTGATGGAG-3'	186 bp
TDRD4-R	5'-TCTGGTTGCCAGACATTCCC-3'	

sequences are listed in Table 1. Melting curve analysis was done to confirm specificity of PCR products. Real time RT-PCR products were also electrophoresed on 2% agarose gel to verify product sizes and specificity.

Statistical analysis

Fold changes in gene expression were calculated by LinRegPCR(2) (Software for analysis of quantitative real-time PCR data) and Relative Expression Software Tool-RG©-version 3 (Calculation Software for the Relative Expression in real-time PCR using Pair Wise Fixed Reallocation Randomization Test©). The amounts of mentioned mRNAs in the tissue, standardized to the B2M mRNA, were calculated as follows: -ΔCT=-(CT Gene of interest -CT B2M). The level of probability was set at $p<0.05$ as statistically significant.

Results

Expression of TDRD4 and FBXO39 in MCF-7 and MDA-MB-231 cell lines

Normal testis cDNA was used as a positive control for TDRD4 and FBXO39 expression. Real time PCR showed that both cell lines expressed FBXO39 mRNA, but TDRD4 was just expressed in MDA-MB-231. The sizes of products were similar in testis and cancer cell lines (Figure 1).

The relative TDRD4 and FBXO39 expression ratio in MCF-7 and MDA-MB-231 cell lines

TDRD4 and FBXO39 expressions were evaluated in MCF-7 and MDA-MB-231 cell lines before transfection by quantitative real time PCR. Real time RT-PCR results showed that FBXO39 expression when normalized with B2M expression was not significantly different in two cell lines. TDRD4 was not expressed in MCF-7 but was expressed in MDA-MB-231.

The relative TDRD4 and FBXO39 expression ratio in MCF-7 and MDA-MB-231 cell lines after RHOXF1 knock down

FBXO39 was down-regulated in MCF-7 after transfection by a mean factor of 0.057 (p value=0.000) but up-regulated in MDA-MB-231 by a mean factor of 4.062 (p value=0.000). TDRD4 was up-regulated in MDA-MB-231 after transfection by a mean factor of 14 (p value=0.000).

TDRD4 and FBXO39 expression in breast tissue samples

None of normal, fibroadenoma and ductal invasive breast samples showed TDRD4 expression. Twelve out of 32 (37.5%) breast tumor samples showed FBXO39

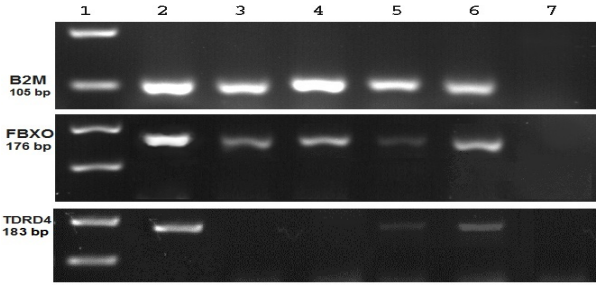

Figure 1. RT-PCR Products for MCF-7 and MDA-MB-231 Cell Lines Electorophoresed on 2% Agarose Gel. Lane 1: DNA size marker, lane 2: testis , lane 3: MCF-7 before transfection, lane 4: MCF7 after transfection, lane 5: MDA-MB-231 before transfection, lane 6: MDA-MB-231 after transfection, lane 7: negative control

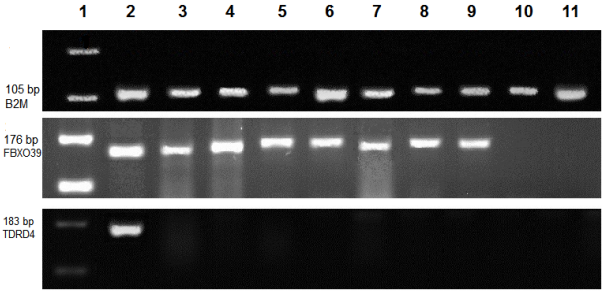

Figure 2. Genes Expression Results in Tissue Samples. lane 1: DNA size marker, lane 2: testis, lanes: 3-7: tumor samples, lanes 8-9: fibroadenoma samples, lanes 10-11: normal breast samples

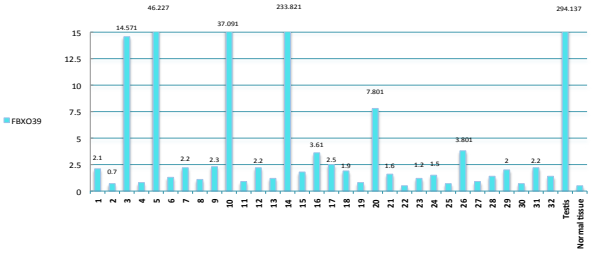

Figure 3. Quantitative Real Time RT-PCR Analysis of mRNA Expression in Breast Tumor Samples. Over expression in cancer tissues was defined as 3-fold higher expression than the expression in normal tissue, excluding the testis. 34 cDNA samples composed of 32 breast cancer specimens, one normal tissue, and one testis tissue sample were analyzed by real time RT-PCR

expression (Figure 2). Quantitative real-time RT-PCR expression analysis showed no significant difference between cancer samples and fibroadenoma samples.

FBXO39 expression ratio in cancer samples and normal tissues

Quantitative real time RT-PCR analysis of mRNA expression in breast tumor samples showed that 7 samples had significant FBXO39 over expression which was defined as 3-fold higher expression than the expression in normal tissue, excluding the testis (Figure 3).

Discussion

Breast cancer is among the first cancers which showed evidences of presence of cancer stem cells

(CSC) (Tabarestani and Ghafouri-Fard, 2012). It has been speculated that the presence of CT gene-expressing cells in tumor cell population is the consequence of clonal proliferation of an aberrant CSC (Ghafouri-Fard and Modarressi, 2012). Identification of CT antigens expressed in breast cancer can pave the way for immunotherapy and cancer prevention especially in high-risk individuals.

Although breast cancer can be classified into biologically and clinically meaningful subgroups according to histological grade and histological type, expression analysis of genes in breast cancer can be a valuable tool for classification of breast cancer and even evaluation of survival and prognosis. In fact, some oncologists expect the time to pack up the old-fashioned morphological classification and go straight to the microarray chip (Cummings et al., 2011).

FBXO proteins are important parts of the SCF (Skp1-CUL-1-F-box protein) type E3 ubiquitin ligase complex entailed in detection and recruitment of substrates for ubiquitination. These proteins have role in the regulation of many cellular processes such as the cell cycle, immune responses, signaling and development through mediating proteasomal degradation of target proteins including cyclins, cyclin-dependent kinase inhibitors, IjB-a, and b-catenin and particularly p53 (Song et al., 2011). As the SCF complex is a negative regulator of p53, FBXO proteins can be involved in the process of tumorigenesis. In another study, it has been shown that depletion of Kelch domain-containing F-box protein induces cell apoptosis and detains cells in the G1 phase (Sun et al., 2009). In addition, deregulation of SCF proteolytic system may cause uninhibited proliferation, genomic instability and cancer as shown in various human tumors. Although FBXO39 has been shown to be expressed in a subset of colon cancer samples, this is the first report of showing its expression in breast cancer. Significant over expression of FBXO39 in breast cancer samples in comparison with normal breast tissue, makes it an appropriate target for detection of cancer. In addition, proteins involved in ubiquitylation system are targets for research in the field of anti cancer drugs (Nakayama and Nakayama, 2006). As FBXO39 is a testis specific gene, it can be used for immunotherapy of cancer. So future researches should focus on evaluation of its immunogenicity in cancer patients.

RHOXF1 is a transcription factor expressed in reproductive tissues and different cancer cell lines. It has been suggested to have an antiapoptotic effect and moderate RHOXF1 knock down has significantly down regulated expression of Bcl-2, an oncogene with a critical function in regulation of apoptosis (Ghafouri-Fard et al., 2012). Here, we observed that RHOXF1 knock down resulted in up regulation of TDRD4 and FBXO39 in MDA-MB-231 but down regulation of FBXO39 in MCF-7. It seems that signaling pathways regulating expression of these 2 genes in mentioned cell lines are different. MCF-7 is an ER positive cell line, while MDA-MB-231 is ER negative (Weigel and deConinck, 1993). MDA-MB-231 has a more invasive phenotype in comparison with MCF-7 according to expression profile of genes having role in cell migration, invasion, metastasis and inhibition of apoptosis (Zheng et al., 2006). As TDRD4 promotes c-Myc function, expression of TDRD4 in MDA-MB-231 but not in MCF-7, is in line with the previous findings. In conclusion, this research provides data for future studies to evaluate function of these two CT antigens in breast cancer samples and cell lines and evaluate FBXO39 suitability for immunotherapy.

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