Transforming Growth Factor Beta-Induced Factor 2-Linked X (TGIF2LX) Regulates Two Morphogenesis Genes, Nir1 and Nir2 in Human Colorectal

Gholam Reza Mobini[1], Mohammad Hossein Ghahremani[2], Saeid Amanpour[3], Ahmad Reza Dehpour[4,5], Abolfazl Akbari[1,6], Seyed Mojtaba Hoseiniharouni[1], Samad Muhammadnejad[3], Maryam Sheikhzade[7], Hoda Abedkhojasteh[8], Masoumeh Mohebi[8], Manzar Bolhassani[9], and Mansour Heidari[5,9]

[1]Department of Molecular Medicine, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran

[2]Department of Pharmacology and Toxicology, School of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

[3] Cancer Models Research Center, Cancer Institute of Iran, Tehran University of Medical Sciences, Tehran, Iran

[4] Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

[5]Experimental Medicine Research Center, Tehran University of Medical Sciences, Tehran, Iran

[6] Colorectal Research Center, Rasoul-e-Akram Hospital, Iran University of Medical Sciences, Tehran, Iran

[7]Department of Biochemistry and Genetics, School of Medicine, Arak University of Medical Sciences, Arak, Iran

[8]Farabi Eye Research Center, Farabi Eye Hospital, Tehran University of Medical Sciences, Tehran, Iran

[9] Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

Received: 15 Sep. 2014; Accepted: 02 Feb. 2015

Abstract- A member of homeodomain protein namely TGIF2LX has been implicated as a tumor suppressor gene in human malignancy as well as in spermatogenesis. However, to our knowledge, dynamic functional evidence of the TGIF2LX has not yet been provided. The aim of the present study was to investigate the human TGIF2LX target gene(s) using a cDNA-AFLP as a differential display method. A pEGFP-TGIF2LX construct containing the wild-type TGIF2LX cDNA was stably transfected into SW48 cells. UV microscopic analysis and Real-time RT-PCR were used to confirm TGIF2LX expression. The mRNA expressions of TGIF2LX in transfected SW48 cells, the cells containing empty vector (pEGFP-N), and untransfected cells were compared. Also, a Real-time PCR technique was applied to validate cDNA-AFLP results. The results revealed a significant down-regulation and up-regulationby TGIF2LX of Nir1 and Nir2 genes, respectively. The genes are engaged in the cell morphogenesis process. Our findings may provide new insight into the complex molecular pathways underlying colorectal cancer development.

Acta Med Iran, 2016;54(5):302-307.

Keywords: cDNA-AFLP; *TGIF2LX*; Transcription factor; Tumor suppressor; Target genes; Colorectal cancer

Introduction

Homeodomain transcription factors play fundamental roles in cellular proliferation, differentiation as well as cell fate (1). The homeobox genes are a diverse group of genes characterized by a conserved 180 base-pair motif encoding a homeodomain with three structurally conserved helices (2). These genes have a particular DNA sequence, the homeobox, which encodes a very variable protein domain named homeodomain. Many homeodomain proteins are transcription factors with important roles in embryonic development and cell differentiation, several of which are involved in human disease and congenital abnormalities (3,4).

One subfamily of homeobox genes encodes proteins with atypical homeodomain referred to as three amino acid loop extension (TALE) which are described by the presence of three extra amino acids between helix 1 and helix 2(3,5). The third helix has amajor role in DNA-binding site recognition (5). TALE homeodomain proteins are crucial transcription factors for embryonic progress and early development (6,7). Four TALE classes have been identified in animals: *PBC*, *MEIS*,

Corresponding Author: M. Heidari
Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
Tel: +98 21 88953005, Fax: +98 21 88953005, E-mail address: mheidari@sina.tums.ac.ir

TGIF and *IRO* (Iroquois) (8).

TGIF (referred to as transforming growth factor-β-induced factor 5'-TG-3' interacting factor) genes encode *TALE*-class homeodomain proteins including *TGIF*1, *TGIF2, TGIF2LX* (transforming growth factor-β-induced factor 2-like, X-linked) and*TGIF2LY* (transforming growth factor-β-induced factor 2-like, Y-linked), that act as multifunctional repressors of TGF-βsignaling. These transcription factors function in normal and abnormal developments (3,9-11).

TGIF2 is frequently expressed in human tissues, with especially high expression in the heart, kidney, and testis (1).

The human *TGIF2LX* gene is located on X-chromosome (Xq21.3) and is suggested to have originated from the retrotransposition of autosomal *TGIF2*.The*TGIF2LX* has 2 exons with a 96 base-pair intron. Although *TGIF2* and *TGIF2LX*show extensive variations, they share conservationally within the homeodomain, and the C-terminus region (12).

An abnormal expression of *TGIF2LX* gene in some prostate tumors, but not in normal and benign prostate hyperplasia, has been shown (13).Moreover, a possible associationof*TGIF2LX/Y* mRNA expression with human azoospermia has been reported in the Iranian patients affected by infertility (14). Another study showed that *TGIF2LX* gene is differentially expressed in glioblastoma and astrocytoma cell lines suggesting a potential function of this gene in brain tumorigenesis (15).

The present study aimed at identifying potential human*TGIF2LX* target gene(s) using cDNA-AFLP as a differential display method.

Materials and Methods

Cell culture

The colon adenocarcinoma cell line, SW48, was grown in RPMI-1640 medium (Gibco, Germany) containing 25 mM D-glucose, 4 mM L-glutamine and 1 mM sodium pyruvate and supplemented with 5% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco, Germany) 2 mM glutamate (Gibco, Germany), 100 units/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin (Gibco, Germany) in culture flask 25 cm2 (SPL Life Sciences, South Korea). The cells were incubated at 37°C in a humidified 95% atmosphere, 5% CO2 atmosphere incubator designated as theculture at a steady-state condition (16,17).

Cell transfection

In our previous study, we designed and cloned the entire coding region of *TGIF2LX* into the pEGFP-N1vector (15). We transfected subcultured SW48 cells using X-treme GENE siRNA Transfection Reagent (Roche, Applied Science, and Germany) either with pEGFP carrying *TGIF2LX* cDNA sequence or empty pEGFP plasmid according to the manufacturer's instructions. After 24 to 48 hours, transfection was examined using an Olympus fluorescent inverted microscope. The following day, the cells were cultured in the presence of 800 µg/mL G418 (Life Technologies) for 21 days to select highly expressing GFP cells. As a maintenance dose, the cells were cultured in the presence of 200 µg/mL of G418 (Life Technologies).

RNA extraction and cDNA synthesis

Total RNA was extracted by the TriZol reagent (Invitrogen, USA) following the manufacturer's instructions. The quality and quantity of total RNA were verified by gel electrophoresis and optical density reading with a NanoDrop (ThermoScientific2000, USA).Total RNA was treated with *DNaseI* (Invitrogen, USA). Then, 5 µg of total RNA was used to synthesize cDNA fragments using random sequence hexamer primers and OligodT with a cDNA synthesis system kit (Vivantis, USA), according to the manufacturer's protocol.

cDNA-AFLP

A modified cDNA-AFLP procedure was performed as described previously (18-20). Double-stranded cDNA (dscDNA) was synthesized at 16 °C for 2 h with *DNA polymeraseI* (Thermo Scientific, USA). The resulting double-stranded cDNA was then digested with 5 U *EcoRI*, (Fermentas, Canada) at 37 °C for 2 hand PCR templates were generated. Subsequently, the adaptors were ligated to*T4-DNA ligase* (Vivantis, USA) overnight at 16°C, and one-tenth of this reaction volume was used for pre-amplification with *PrEcoRI* primers (Table1) in a 50 µl reaction volume.

The PCR condition was as following steps: initial denaturation at 95°C for 5 min followed by 30 amplification cycles at 94°C for 40 s, 60°C for 40 s, and 72°C for 1.5 min, and a final extension at 72°C for 7 min. The sensitive amplification was also carried out by sensitive primers. These primers contained adaptor sequences plus one variable nucleotide at the 3′ end. The products resulting from sensitive amplifications were separated on 10% non-denaturing polyacrylamide gel

electrophoresis (PAGE) and stained with silver nitrate according to the protocol (21). Finally, the gels were scanned and checked for the presence of differentially expressed transcription-derived fragments (TDFs).

Isolation, cloning, and sequence analysis of transcript-derived fragments

Based on their intensity of up-regulation and down-regulation, the bands that were obviously changed were selected and cut from the gels with a surgical blade. The gel was dissolved in 100 µl sterile water with vigorous vortexing for 1 min, and incubated in boiling water for 30 min. A 5 µl portion of each dissolved band was re-amplified using appropriate, sensitive adaptors. After checking PCR products on 1.5% agarose gel, they were cloned into a *pTZ57R/T* Vector (Thermo scientific, USA). There combinant plasmid containing the TDF of interest was subjected to sequencing using universal primers (*M*13 (-20) forward and reverse primer) (Fermentase,USA); this was done to identify the isolated TDFs. Sequence data were analyzed in a non-redundant nucleic and protein databases, namely BLAST (Basic Local Alignment Search Tool) (http://www.ncbi.nim.nih.gov/BLAST).

Real-time RT-PCR analysis

Real-time RT-PCR was conducted to confirm the differences in expression patterns of TDF-derived genes, For expression analysis, specific primers were designed using the NCBI primer–BLAST program (http://www.ncbi.nih.gov/primer-BLAST). A quantitative Real-time PCR was performed in a total volume of 20 µl that contained 2 µl cDNA, 10 µl of *SYBR Green I Taq* Mix (Takara, Dalian, China), 1 µl (0.2 mM) of each primer, and 6 µl ddH2O. Experiments were performed in triplicate using a CFX96™ Real-Time System (C1000TM Thermal Cycler) (Bio-Rad, Hercules, CA, USA). The PCR condition followed this steps: initial denaturationat 95°C for 3 min followed by45 amplification cycles containing denaturation at 95°C for 10 s, annealing at 60°C for 32 s and extension at 72° for 1.5 min, and a final extension at 72°C for 7 min. The amplification products were heated to 95°C to determine their melting curves and confirm their specificity. Glyceraldehyde 3-phosphate dehydrogenase *(GAPDH)* gene expression was used as a housekeeping gene to normalize data. The cycle thresholds (CTs) of the identified genes were compared with that of the house keeping gene *(GAPDH),*the relative expression level of each target gene was analyzed by using the 2 - ΔΔCT method (22,23).

Results

The entire coding region of *TGIF2LX* was cloned into the *pEGFP-N1* vector. The *TGIF2LX* expression was detected by RT-PCR. Plasmids containing the correct sequence were transfected into SW48 cell line, and the *TGIF2LX-GFP* fusion protein is expressed. The fluorescent microscopic study revealed that the TGIF2LX-GFPwasmainly localized to nuclei (Figure 1).

Figure 1. *TGIF2LX* gene expression detected by fluorescent microscopy. SW48 cells 21 days after post-transfection with a light microscope (A). SW48 cells transfected with empty plasmid *pEGFP* (B), and with plasmid *pEGFP- TGIF2LX* (C) under a fluorescent microscope (×20).

cDNA-AFLP analyses were carried out on SW48 with either pEGFP plasmid with *TGIF2LX* cDNA sequence or pEGFP plasmid without *TGIF2LX* cDNA sequence; this was done to investigate target gene(s) of the TGIF2LXThe cDNA AFLP products from different primer combinations were resolved on 10% non-denaturing PAGE (Figure 2) different bands containing TDFs were extracted from PAGEs, amplified and subcloned into T-vector for sequencing so that it was possible to detect TGIF2LX target genes. The sequence obtained for each TDF was compared with the Gen Bank non-redundant public sequence database BLAST program (24).

In this study, different mRNA expression levels of*Nir1* and *Nir2*genes, and two other unknown genes were detected in SW48 cells containing plasmid pEGFP-TGIF2LX compared to cells containing the empty pEGFP plasmid.

Validation of cDNA AFLP results using real time PCR

After identification by cDNA-AFLP, gene expression was further investigated in order to identify two TDFs using qRT-PCR by specific primers (Table 1). These TDFs were selected based on the expression intensity and differential patterns of expression in the cDNA-AFLP experiment. According to the qRT-PCR results, *Nir1* and *Nir2* were found to be down-regulated and up-regulated, respectively(Figure 3).

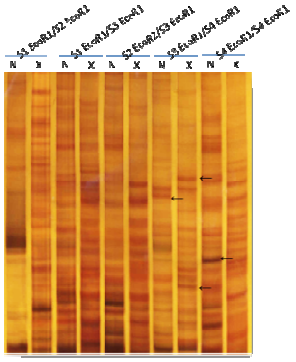

Figure 2. The pattern of TDFs extracted from cDNA-AFLP PAGE. Sensitive amplification of cDNA-AFLP on a PAGE from different primer combinations: S1 *Eco*RI /S2 *Eco*RI, S1 *Eco*RI /S3 *Eco*RI, and S2 *Eco*RI /S3 *Eco*RI, and S3 *Eco*RI /S4 *Eco*RI, and S4 *Eco*RI /S4 *Eco*RI. The arrows indicate extracted TDFs. N: SW48 cells transfected with empty plasmid *pEGFP,* X: SW48 cells transfected with *pEGFP- TGIF2LX.*

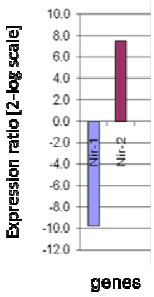

Figure 3. Relative expression pattern of *Nir1* and *Nir2* genes by real time RT-PCR. *Nir2* gene showed increased expression level, and *Nir1* gene showed decreased expression level after normalization with internal control (*GAPDH*)(*P*<0.001).

Table 1. Sequences of adaptors and primer used in this study

Primer name	Sequences	PCR product size (bp)	Primer name
Housekeeping	**GAPDH-F**	5’-CACCAGGGCTGCTTTTAAC-3’	190 bp
	GAPDH-R	5’-ATCTCGCCTCCTGGAAGAT-3’	
cDNA AFLP Adaptorsand Primers	**AdL Ecor 1**	5’-ACCGACGTCGACTATCCATGAAG -3’	--
	AdS EcoR 1	5’- AATTCTTCATGG -3’	--
	Pr EcoR 1	5’- ACCGACGTCGACTATCCATGAAGAATTC -3’	--
	S1 EcoR 1	5’- ACCGACGTCGACTATCCATGAAGAATTCC -3’	--
	S2 EcoR 1	5’- ACCGACGTCGACTATCCATGAAGAATTCG -3’	--
	S3 EcoR 1	5’- ACCGACGTCGACTATCCATGAAGAATTCA -3’	--
	S4 EcoR1	5’- ACCGACGTCGACTATCCATGAAGAATTCT -3’	--
Target Gene	**Nir 1 – F**	5’- TATCGGATCGCCAAGTTGCT-3’	169 bp
	Nir 1 – R	5’-TTGCAGAATCACCCCAAAGC-3’	
	Nir 2 – F	5’- GTAGAACTGAACATCGTGGCCG-3’	151 bp
	Nir 2 – R	5’- CCACATAGCCGTCTGACAGGA-3’	

Discussion

Colorectal cancer is one of the common malignancies worldwide, with one million new cases each year. Determination of new target genes as molecular markers is useful for the cancer prognosis and helps to select therapeutic strategies (25,26).

The present study was conducted to identify

downstream genes regulated by *TGIF2LX*. It is possible that *TGIF2LX,* like the majority of homeobox genes, activates or represses downstream target gene(s) via direct or indirect manners. It was found in the present study that *Nir1* (also known as *PITPNM*3) and *Nir2* act as two*TGIF2LX* target genes. It was also revealed that *Nir1*was down-regulated while *Nir2* wasup-regulated.

The *Nir* family members have been favorably conserved during evolution. *PITPNM*3 contains 20 exons spanning approximately 101 kb of genomic sequence and encodes a protein belonging to phosphatidyl-inositol transfer protein (*PITP*) family. *Nir* proteins show tissue-specific expression pattern. The *Nir1*gene is expressed in the human retina, brain, spleen, ovary and breast cancer cells (27-29). Whereas *Nir2* is expressed in various tissues and cell types, including neuronal, epithelial, and hematopoietic cells (30). Evidence suggests that *Nir2* is an important regulator of cell morphogenesis and its ectopic expression remarkably affects cell morphology (31). It has been revealed that *Nir2*is essential for cytokinesis; blocking of Nir2 function by PITPNM1 antibody injection results in the production of multinucleated cell formation. Overall, any changes in *Nir2* structure lead to an abnormalcytokinesis (32).

It has been demonstrated that *Nir1* is a functional receptor for CCL18 (a Tumor-associated macrophages-derived cytokine). Expression and functional role of *PITPNM3* in breast cancer cells support the correlation of *CCL*18-expressing TAM count with prognosis due to increased CCL18 production that may have a stronger metastasis-promoting effect on breast cancer cells. CCL18 promotes breast cancer invasion, metastasis, and reduces patient survival; whereas *PITPNM*3 suppression would reverse these effects (33). In contrary, *PITPNM*3 is scarcely detected in gastric cancer cells (34).

It has been documented that *Nir1* and *Nir2* have different expression patterns in retinal cells. Their characteristic and expression pattern may indicate several roles in cellular processes. While *Nir1* is predominantly expressed in premature cells, *Nir2* is homogeneously distributed in undifferentiated neuroblasts and ganglion cells at birth but later becomes distinctly distributed in newly differentiated neuronal cells (35). The results obtained in the present study are in agreement with the results of the above-mentioned study except that in the present study *Nir2* as a *TGIF2LX* target was up-regulated in colorectal adenocarcinoma grade 4 which resulted in the increased in tissue differentiation and decrease in cancerogenesis. On the other hand, lack of *TGIF2LX* expression leads to carcinogenesis. Therefore, it seems that *TGIF2LX* acts as tumor suppressor gene, but *Nir1* is a proto-oncogene.

Overall, the present study identified two target genes of *TGIF2LX,* which would be involved in the cell morphogenesis processes. These findings may provide new insight into the complex molecular pathways underlying colorectal cancer development.

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