

RESEARCH ARTICLE

Homeodomain Protein Transforming Growth Factor Beta-Induced Factor 2 Like, X-Linked Function in Colon Adenocarcinoma Cells

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Abstract

Background: *TGIF2LX* (transforming growth factor beta-induced factor 2 like, X-linked) is a homeodomain (HD) protein that has been implicated in the negative regulation of cell signaling pathways. The aim of this study was to investigate the possible functions of *TGIF2LX* in colon adenocarcinoma cells. **Methods:** The human SW48 cell line was transfected with cDNA for the wild-type *TGIF2LX* gene and gene/protein over-expression was confirmed by microscopic analysis, real time RT-PCR and Western blotting techniques. *In vitro* cell proliferation was evaluated by MTT and BrdU assays. After developing a colon tumor model in nude mice, immunohistochemical (IHC) staining of tumor tissue was carried out for Ki-67 (proliferation) and CD34 (angiogenesis) markers. To predict potential protein partners of *TGIF2LX*, in-silico analysis was also conducted. **Results:** Obtained results showed over-expression of *TGIF2LX* as a potential transcription factor could inhibit either proliferation or angiogenesis ($P < 0.05$) in colon tumors. In-silico results predicted interaction of *TGIF2LX* with other proteins considered important for cellular development. **Conclusions:** Our findings provided evidence of molecular mechanisms by which *TGIF2LX* could act as a tumor suppressor in colon adenocarcinoma cells. Thus, this gene may potentially be a promising option for colon cancer gene-based therapeutic strategies.

Keywords: CD34- Homeodomain protein *TGIF2LX*- HOX gene- Ki-67- Transforming growth factor-beta (TGF- β)

Asian Pac J Cancer Prev, **18** (8), 2101-2108

Introduction

Colorectal cancer (CRC) is the third most common malignant neoplasm worldwide and the second leading cause of cancer-related deaths (Welch and Robertson, 2016; Akbari et al, 2016; Mirzaei et al, 2016). Despite significant developments in therapeutic strategies for CRC in recent years, it still causes extensive mortality (Siegel et al, 2017; Mirzaei et al, 2016; Akbari et al, 2016; Akbari et al, 2014). Pathogenesis of CRC is a complex and multistep process involving dysregulation of diverse genes expression and cell signaling pathways (Akbari et al, 2016; Akbari et al, 2014). Homeobox (HOX) genes encode a series of evolutionarily conserved homeodomain (HD) proteins/transcription factors as developmental regulators (Mobini et al, 2016). These proteins by transcriptional repression/activation affect cell biological processes including growth and differentiation (Li et al, 2006; Chen et al, 2013). Previous studies have showed that HOX genes family could react with the onco/suppressor genes

involved in colon cancer progression (Chen et al, 2013; Ettahar et al, 2013).

5'-Thymine/Guanine -3'-interacting or transforming growth factor beta (TGF- β)-induced factor (TGIF) belongs to a family of evolutionarily conserved HD proteins (Ettahar et al, 2013). Recently, it has been documented that this protein family plays significant roles in the developmental processes, such as proliferation, differentiation and cell fate. It has been shown that TGIF represses gene transcription by acting as a co-repressor of critical co-regulator proteins or signalling transducers for TGF- β signalling pathway (Smads proteins) (Chen et al, 2013, Fadakar et al, 2016). However, the molecular functions of these proteins have been predominantly investigated in cultured cells but not routinely in vivo (Hamid et al, 2009; Seo et al, 2006). Recently, a human TGIF paralog protein (named as TGIF2) has been identified that acts as a transcriptional repressor. It has been understood that TGIF2 suppresses transcription by its interaction with histone deacetylases (HDACs) (Ettahar

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et al, 2013; Hamid et al, 2009). On the other hand, TGIF2 appears to function similar to TGIF by its ability to repress gene expression. A number of results implicate a potential role of human *TGIF2* or *TGIF2LX* in the regulation of cell growth (Glenisson et al, 2007, Raoofian et al, 2013).

Our previous study also suggested that *TGIF2LX* could be involved in negative regulation of cell cycle (Glenisson et al, 2007, Raoofian et al, 2013). Nevertheless, only a few functional studies of human *TGIF2LX* have been reported and underlying mechanisms remain to be addressed. For instance, whether and how *TGIF2LX* is involved in the control of cell proliferation is not known. The present study mainly was aimed to investigate the possible function of *TGIF2LX* and evaluate its role in colon carcinogenesis; cell proliferation and angiogenesis.

Materials and Methods

Cell culture

Human colon adenocarcinoma cell line, SW48, was obtained from National Cell Bank of Iran (NCBI) affiliated to Pasteur Institute (Tehran, Iran). The cells were grown in RPMI-1640 medium (Gibco, Germany) supplemented with 5% (v/v) heat inactivated fetal bovine serum (FBS; Gibco, Germany), 2 mM glutamax (Gibco, Germany), 100 units/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphoterycin (Gibco, Germany) in culture flask 25 cm² (SPL, Life Science, South Korea). The cells were kept at 37 °C in a humidified 95% atmosphere, 5% CO₂ atmosphere incubator designated as culture at a steady-state condition (Faghihloo et al, 2016, Karimi et al, 2015). Cell viability was assessed using trypan blue exclusion test and routinely found in all flasks to contain more than 95% viable cells (Figure 1A).

Overexpression of *TGIF2LX* in SW48 cells

Already, the entire encoding cDNA sequence of *TGIF2LX* had been cloned into the pEGFP-N1 vector by Raoofian et al (Raoofian et al, 2016). We transfected subcultured SW48 cells with either plasmids pEGFP with or without (mock constructs) *TGIF2LX* cDNA sequence using X-tremeGENE siRNA Transfection Reagent (Roche, Applied Science, Germany) according to the manufacturer's instructions. After 48 hour post-transfection, the cells were examined using fluorescence stereoscopic microscopy (Olympus, Tokyo, Japan) to validate green fluorescent protein (GFP) emission as internal control (Figure 1B, C and D).

Establishment of stable cell line

For establishment of SW-48 cell line expressing *TGIF2LX*, 72 hour post-transfection, the highly GFP-expressing cells were cultured in the presence of 750 µg/mL G418 (Sigma Aldrich, Belgium) for 21 days.

Analysis of *TGIF2LX* in transcriptional levels by RT-real time PCR

Total RNA extraction was carried out using HighPure RNA Extraction Kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Total RNA was stored at -70° C until use. Then, the cDNA

was synthesized using 0.5 µg of total RNA as described previously (Akbari et al, 2015; Sheikhzade et al, 2016) and stored at -20°C. Real time RT-PCR was performed to analyze the expression level of *TGIF2LX* gene in the transfected cells as described previously (Raoofian et al, 2013). The PCR products were electrophoresed using gel agarose %2, as previously described (Mohebi et al, 2017).

Analysis of *TGIF2LX* in translational levels by Western blotting

Western blot analysis was conducted as previously described (Raoofian et al, 2013). Briefly, cell lysates were subjected to 12% SDS-PAGE electrophoresis and transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). The membranes were blocked for 1 h with 3% bovine serum albumin (BSA) at 37°C and then incubated with a 1:500 dilution of a polyclonal rabbit anti-*TGIF2LX* antiserum raised against the C-terminus (sc-459, Santa Cruz Biotechnology, Santa Cruz, CA, USA) (2 h, at room temperature). The membranes were then incubated with 1:5,000 dilution of a goat HRP-conjugated anti rabbit IgG secondary antibody (1.5 h) followed by development with 4-chloro-1-naphthol (Immun-Blot, Bio-Rad Laboratories, Hercules, CA, USA).

Colony formation assay

The soft agar colony formation assay was performed using SW-48 cells transfected with *TGIF2LX* by standard techniques (Rafehi et al, 2011). Briefly, SW-48 cells expressing *TGIF2LX* were trypsinized, and 2 × 10⁴ cells were plated in 0.3% top agarose and cultured for 7 day. Colony formation for these cells showed similarly to that observed in control group to develop tumor.

Knockdown of the *TGIF2LX* gene by siRNA

In a 24-well plate, exponentially growing SW-48 cells (8×10⁴cells/well) were seeded in RPMI1640 medium containing 10% (v/v) FBS. After growing the cells overnight, transfection was carried out with siRNA using 2.5 µl X-treme- GENE siRNA Transfection Reagent (Roche) and 0.5 µg of the respective siRNA, according to the manufacturer instructions.

After total RNA extraction and cDNA synthesis with oligo(dT) primers, RT-qPCR was performed using SYBR Green Mastermix (Takara, Japan). PCR reactions were analyzed upon an ABI real-time PCR thermocycle using the following conditions: 95°C for 1 minute, 95°C for 5 minutes, followed by 35 cycles at 95°C for 15 seconds and 61°C for 1 minute (Yazdi et al, 2011, Mohebi et al, 2016). Results were normalized against housekeeping GAPDH gene expression.

Methyl Thiazolyl Tetrazolium (MTT) Assay

To evaluate growth inhibitory effect of *TGIF2LX*, all the three (untransfected SW48, pEGFP-N1-SW48 and pEGFP- *TGIF2LX*-SW48) cells were seeded in 96-well flat-bottom microtitration plates (SPL, Life Science, South Korea) at a density of 3×10³ cells/well (200 µL media/well). After 48 h, the medium was replaced by 100 µL MTT (Sigma-Aldrich, Belgium). Following 3 h incubation at 37 °C, 150 µL DMSO (Sigma Aldrich, Belgium) was

added to each well. Later than gentle mixing for 20 min, the absorbance of converted dye was read at a wavelength of 570 nm using a spectrophotometric microplate reader (BioTek Elx 808).

Bromo-2'-Deoxyuridine (BrdU) Cell Proliferation Assay

The colorimetric immunoassay (Cell Proliferation ELISA System, BrdU assay, Roche, Germany), also was used for the quantification of cell proliferation according to manufacturer's instruction. A primary density of 3×10^3 cells/well (200 μ L media/well) was used for BrdU assay. Cells from the same population that were not BrdU-labeled were used as the background controls. Finally, the absorbance was measured at a wavelength of 570 nm using a spectrophotometric microplate reader (BioTek Elx 808).

Animal model of colon tumor

6-week-old female athymic C56BL/6 nude mice were obtained from Omid Institute for Advanced Biomodels (Tehran, Iran). The protocol of animal experiments was approved by the Committee on the Ethics of Animal Experiments of Tehran University of Medical Sciences (Ethical Code Number; ERC/S/277). Animals were kept under optimized hygienic conditions in an individually ventilated cage system (cancer institute of Imam Khomeini Hospital). The mice were fed with autoclaved commercial diet and water ad libitum. Three groups (n= 8 per group) of mice were selected for inoculation of three cell groups (untransfected SW48, pEGFP-N1-SW48 and pEGFP-*TGIF2LX*-SW48). In order to establish the tumors model, totally 5×10^6 cells, after washing, were resuspended and inoculated subcutaneously at a 200 μ L volume of serum-free medium into the flank of the animals. Tumor growth was measured twice a week until three weeks. The volume of tumors was calculated by standard formula ($\text{Length} \times \text{width}^2 \times 0.52$) and growth curve was drawn (Tomayko, 1989). Xenograft tumors were allowed 21 days to grow, then all mice were sacrificed by CO₂ inhalation according to the recommendations of the ethics committee on animal's experimentation of medical and all efforts were made to minimize suffering. Obtained tumors after isolating from animals were fixed in 10% buffered formalin, embedded in paraffin and subjected to histopathological staining.

Histopathological features

For revision of the histopathological features and confirmation of developed colon tumors, 5 sections (5 μ m) were provided from the formalin fixed paraffin embedded blocks and 2 sections were floated onto charged glass slides (Super-Frost Plus, Fisher Scientific, UK). Both two slides were dried overnight at 60°C and primed to hematoxylin and eosin (H and E) staining.

Immunohistochemistry (IHC) staining

For the evaluation of *TGIF2LX* ectopic over-expression effects on proliferation and angiogenesis, immunohistochemistry (IHC) staining for Ki-67 (proliferation) and CD34 (angiogenesis) markers were carried out. Three sections (5 μ m) floated onto charged

glass slides, were stained immunohistochemically using three steps-indirect streptavidin method. Protocol was performed for Monoclonal Mouse Anti-Human Ki-67 Antigen (MIB-1), clone M 7240 (Dako, Denmark) and Monoclonal Mouse Anti-Human CD34, clone QBEnd-10 (Dako, Denmark). Negative controls were obtained by omitting the primary antibody for the two markers under identical condition. Sections from a lymph node with follicular lymphoid hyperplasia known to be immunoreactive for Ki-67 and CD34 were used as a positive control (as recommended by the manufacturer).

Analysis of immunoreactivity and microvessel density counting

The semi-quantitative analysis of immunoreactivity of proliferating colon adenocarcinoma cells were examined by using a histological score (H-score). The scores were independently obtained by two pathologists without access to and awareness of test results. In the present study, inter-observer differences were less than 3%, and the mean of the two values was obtained. The percentage of immunopositive cases and the H-score values for each sample (mean \pm SEM) were calculated.

We evaluated also the variation in the microvessel density (MVD; microvessel/mm²) by CD34 immunostaining before and after *TGIF2LX* transfection in a matched series of animals. MVD was estimated as a mean of microvessel numbers in three histological fields according to a previous study (Maclean et al, 2005).

In-silico analysis

In order to predict protein-protein interaction network of *TGIF2LX*, STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) (<http://string.embl.de>) was used.

Statistical Analysis

Statistical analysis was performed using the SPSS 21 software. Statistical significance of differences between data was evaluated by Kruskal-Wallis test and one-way analysis of variance (ANOVA) followed by Tukey's post tests for multiple comparisons of differences between groups. Data were expressed as mean \pm SEM. P values less than 0.05 were considered to indicate statistically significant differences between data sets.

Results

TGIF2LX expression in transfected colon adenocarcinoma cells

The expression of *TGIF2LX*-GFP in SW-48 cells was established using microscopic validation of GFP emission. Quantitative transfection efficiency of the cells was approximately 80% (Figure 1 C). The expression of *TGIF2LX* at the gene and protein levels was confirmed by real time PCR (Figure 2) and Western blotting assay, respectively. Microscopic examination revealed that the *TGIF2LX*-GFP was mainly localized in to nucleus (Figure 1D), suggesting this protein as a probable transcription factor.

Table 1. The Oligonucleotide Primers Used in This Study

Gene	Primer sequence	Product size
TGIF2LX	Forward: 5'-CAACAGTAACGATAAGCCTCTTG-3' Reverse: 5'-AAGGCAAGAACTCTGCCTGTA-3'	130 bp
GAPDH	Forward: 5'-CACCAGGGCTGCTTTTAAC-3' Reverse: 5'-ATCTCGCCTCCTGGAAGAT-3'	190 bp
TGIF2LX siRNA	5'-CAUGCAGCCGUAUGCAGUA-3'	
Control siRNA	5'-UAGCGACUAAACACAUCA-3'	-

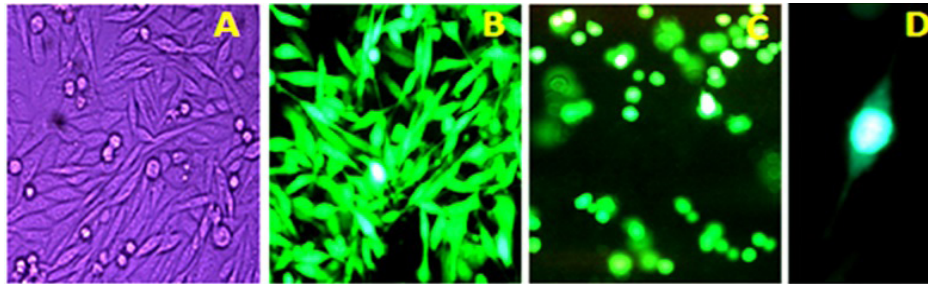


Figure 1. GFP-TGIF2LX Gene Expression Detected with Fluorescent Microscopy to Validate the Transfection Efficiency. Continuous Culture of Un-Manipulated Colon Adenocarcinoma SW48 Cells (A). SW48 Cells Transfected With GFP or Mock Control (B) and TGIF2LX-GFP (C), Three Weeks Post-Transfection Under Fluorescent Microscope (40×). The Single Cell Transfected with TGIF2LX-GFP (D) that Shows TGIF2LX Gene Expresses in Nucleus (400×).

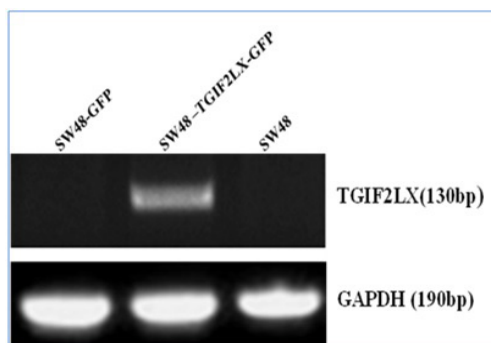


Figure 2. Analysis of TGIF2LX Expression. The quality and integrity of RNAs from three tumor groups (SW48, SW48-TGIF2LX-GFP and SW48-GFP) were confirmed by real time RT-PCR of the GAPDH (190bp) housekeeping control gene (A). PCR products were resolved on a 2 % agarose gel stained with gel red (B).

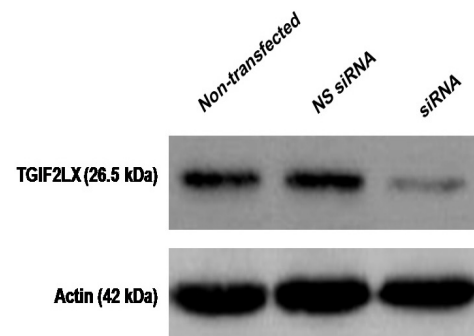


Figure 3. Detection of TGIF2LX Knockdown by siRNA Using Western Blotting Technique. The transfected cells were treated with two siRNA duplexes to TGIF2LX, or with a non-silencing control siRNA (NS siRNA) and protein prepared at 72 hours post-transfection. Protein lysates were analyzed for TGIF2LX expression by western blotting technique.

Inhibition of TGIF2LX on the clonogenic capacity of colon SW48 cells

The results of colony formation assay revealed that the colony forming capacity in *TGIF2LX*-SW48 cells was noticeably lower than the vehicle-treated cells. Additionally, colony forming efficiency of normal SW48 cells was significantly higher than that of *TGIF2LX*-SW48 cells (data not shown).

Knockdown of the TGIF2LX gene by siRNA

Results obtained from RT-qPCR analysis following siRNA knockdown, 72 hours after transfection, showed a significant decreased in *TGIF2LX* mRNA expression level. Successful knockdown of *TGIF2LX* was also observed using the siRNA as seen by the decreased levels of the protein (Figure 3).

MTT Assay and BrdU Assay

In vitro *TGIF2LX* ability to growth inhibition was evaluated by both MTT and BrdU assays. Results revealed a significant change in transfected versus untransfected cells ($P < 0.05$).

TGIF2LX over-expression reduces colon tumor size

21 days after subcutaneous inoculation of cells, comparison of the percentage of tumor size between final and initial tumor volumes indicated an average 100% tumor growth in the transfected cells and an average 175% tumor growth in the control groups. Results showed significant differences in visible size of transfected compared to control tumors ($P = 0.038$) (Figure 4).

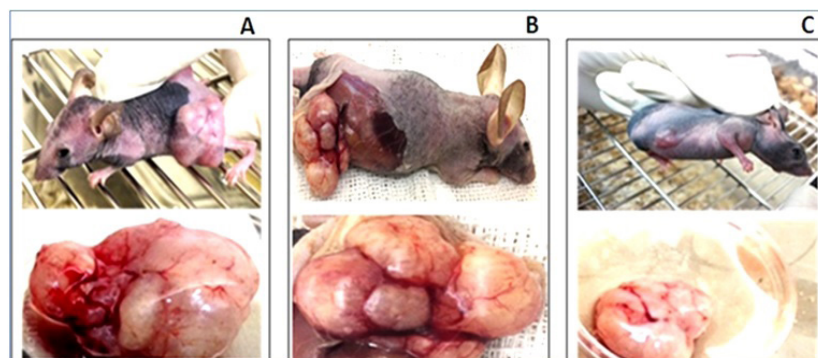


Figure 4. Representative Image of Tumor Size Reduction due to Transfection of TGIF2LX. Untransfected SW48-driving tumors (A), transfected SW48 with empty vector (N cells as control)- driving tumors (B) and transfected with TGIF2LX (C). Homeoprotein TGIF2LX overexpression showed a reduction in mean tumor volume (C) in comparison with untransfected or vehicle-transfected groups as controls (A and B, respectively). Tumor growth is associated with brisk mitotic activity and TGIF2LX transfection could cause a visible reduction in tumor growth.

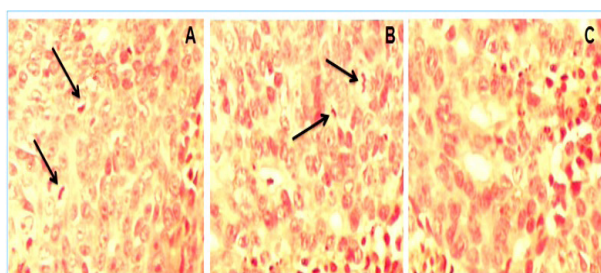


Figure 5. A Representative Result of Pathological Examinations of Colon Tumors. The H&E staining demonstrates marked cellularity with profound hyperchromatism and pleomorphism (arrows). H&E staining of untransfected SW48-driving tumors (A), transfected SW48 with empty vector (N cells as control)-driving tumors (B) and transfected with TGIF2LX (C), confirming colon adenocarcinoma.

Diagnosis of colon adenocarcinoma

The hematoxylin and eosin staining demonstrated marked cellularity with profound hyperchromatism and pleomorphism (arrows in Figure 5). Pleomorphic malignant epithelial cells with the clear nucleolus, a high mitotic index and atypical areas confirmed colon adenocarcinoma. The pattern of adenocarcinoma was identical with the human origin in which the cancerous

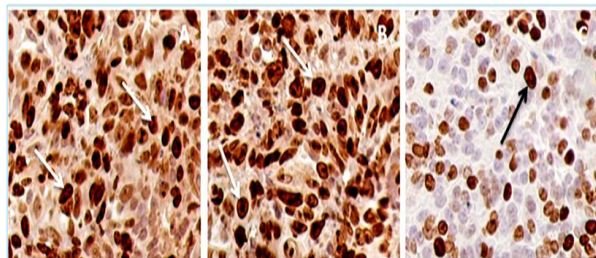


Figure 6. Colon Tumors Stained Immunohistochemically with anti-Ki-67. Positive immunoreactive showing nuclear expression of Ki-67 with large number of brown stained nuclei (arrows) reflecting active cellular proliferation in the untransfected SW48- driving tumors (A), transfected SW48 with empty vector (N cells as control)- driving tumors (B) and transfected with TGIF2LX (C). Immunohistochemical assay showed significant difference between tests and controls in terms of cellular proliferation ($P<0.05$).

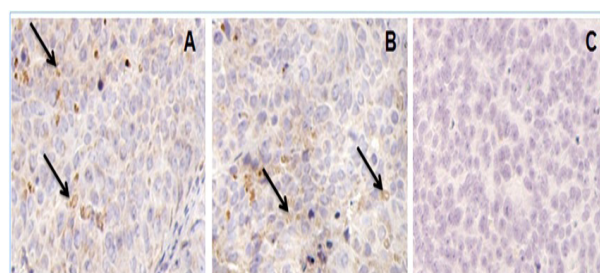


Figure 7. Colon Tumors Stained Immunohistochemically with anti-CD34. Positive immunoreactive showing brown stained cytoplasmic membrane of endothelial cells (arrows), reflecting active angiogenesis in the untransfected SW48- driving tumors (A), transfected SW48 with empty vector (as control)- driving tumors (B) and transfected with TGIF2LX (C). Immunohistochemical assay showed significant difference between tests and controls in terms of positive angiogenesis ($P<0.05$) and MVD counting ($P<0.05$).

cells were poorly differentiated (Figure 6).

TGIF2LX over-expression decreases colon adenocarcinoma cell proliferation

We further hypothesized that the ectopic expression of TGIF2LX may influence cell proliferation in colon tumors. H-scoring analysis of immunoreactivity of transfected and untransfected-driving tumors using anti-Ki67 by two independent pathologists showed significant changes between studied samples (Figure 5, $P<0.05$).

TGIF2LX overexpression decreases angiogenesis in colon adenocarcinoma

To assess whether ectopic expression of TGIF2LX had an influence on angiogenesis in colon tumor or not, the immunohistochemical staining was conducted using CD34 antibodies (Figure. 7). H-scoring analysis of immunoreactivity in transfected and untransfected-driving tumors using anti-CD34 by two independent pathologists showed significant changes between studied samples ($P<0.05$).

In-silico Analysis

Finally, to predict the TGIF2LX-protein interaction

networks, we used the STRING online tool to identify a core network of interacting proteins by submitting the *TGIF2LX*. The in-silico findings showed seven proteins (TGF β I, PABPC5, PCDH11Y, SATL, UBTD2, RHOXF2 and PCDH11X proteins) might be associated with *TGIF2LX*.

Discussion

Understanding the mechanisms through which various tumor repressors and activators regulate tumor development and progression is critical for designing the favorable therapeutic strategy (Mobini et al., 2014; Akbari et al., 2016). Numerous studies showed that dysregulation of homeobox genes is involved in abnormal development and tumorigenesis (Ettahar et al., 2013; Hmid et al., 2013). It is well documented that the majority of homeodomain proteins act as transcription factors and modulate cell growth (Sheikhzade et al., 2016; Raoofian et al., 2013). Moucadel et al., (2002) showed that human homeobox CDX1 gene expression regulates p21, p53 and BCL2 gene expressions in intestinal cells (Moucadel et al., 2002). Also, it has been shown that the function of HOXA10 leads to cell proliferation inhibition in differentiating myelomonocytic cells (Bromleigh et al., 2000).

Most recently, ten colon adenocarcinoma cell line were studied and the lack of expression of *TGIF2LX* only in SW48 cells was reported by our colleagues. We postulated some scenarios including genetic and epigenetic factors to be implicated in the absent expression of *TGIF2LX* in some cancerous cells (Raoofian et al., 2013). In addition, other events such as gene-gene interactions, gene-environment interactions or genetic background of the cell line might be critical for suppression of *TGIF2LX* gene. Given the potential role of *TGIF2LX* in colon tumorigenesis, we initially sought to determine its cellular localization. Our investigations of exogenous *TGIF2LX* distribution by GFP fluorescence revealed that the GFP-*TGIF2LX* protein clearly accumulates in the cell nucleus. Thus, the *TGIF2LX* protein could be actively transported to and can accumulate within the nuclei. These findings imply that the protein (i) consists of a nuclear localization signal (NLS), and may be normally held in the cytoplasm by either protein-protein interaction or posttranslational modifications, suggesting this protein may function as a transcription factor.

In the present study, we reported that overexpression of *TGIF2LX* in CRC could reduce proliferation and angiogenesis. We demonstrated the *in vitro* growth inhibitory effect of *TGIF2LX* on SW48 cell line ($P < 0.05$) as well as the *in vivo* inhibitory effects on proliferation and angiogenesis in heterotopic colon tumors ($P < 0.05$). However, it is unlikely that a dysregulation of a single factor such as *TGIF2LX* can entirely dictate the colon tumorigenesis. A plausible assumption would be that *TGIF2LX*, like the majority of homeobox genes, could potentially function as a tumor suppressor through involving other signaling pathways in cancerous cell.

Former studies have shown that *TGIF2LX* is highly expressed in advanced stages of prostate cancer but not in patients with benign prostatic hyperplasia (Raoofian et

al., 2013). Satoh and colleagues (2008) showed that TGIF and TGIF2 homeodomain proteins suppress the retinoic acid-mediated activation of RXR in target genes involved in cell development (Satoh et al., 2008). Moreover, researchers showed that human TGIF2 inhibited tumor size and proliferation probably through interfering with the cell signaling pathways (Seo et al., 2006; Satoh et al., 2008). A most recent study suggested that overexpression of TGIFLX could lead to down- and up-regulation of BCL2 and BAX, respectively (Raoofian et al., 2013). Also a number of studies have reported that homeobox genes such as BP1, HOXA10 and DLX4 regulate the expression of BCL2, p53 and BAX genes in human cancers (Chu et al., Kim et al., 2007).

According to our findings as well as those have published so far, two main questions needed to be addressed namely: (1) which parts of this protein are implicated in the cellular development? and (2) how can we functionally define the molecular characterization of the *TGIF2LX* protein?

The first question was somewhat addressed by distinct studies on the nature of HD protein properties, suggesting that *TGIF2LX* like other homeodomain transcription factors could regulate its downstream target genes. In order to address the second question, we performed in-silico study to predict *TGIF2LX* protein interaction network as well as microRNAs profiles (data not shown). The in-silico findings using STRING tool showed seven proteins (TGF β I, PABPC5, PCDH11Y, SATL, UBTD2, RHOXF2 and PCDH11X proteins) might be associated with *TGIF2LX* signaling pathway. Here, we would like to discuss about the proteins whose functions could potentially affect the cell signaling pathways in CRC.

PCDH11X belongs to the protocadherin family proteins and is thought to play diverse roles in development and other biological processes. This protein differentially responds to retinoic acid and thought to act in cell-cell recognition (Kim et al., 2007; Kim et al., 2010). Also, several studies showed that PCDHX interacts with β -catenin and induces the Wnt signaling pathway (Priddle et al., 2013). On the other hand, it is well documented that β -catenin is a dual function protein and regulate the coordination of normal development and/or tumorigenesis. Noticeably, regulation of beta-catenin is critical for adenomatous polyposis coli (APC) tumor suppressive effect, a key protein that its loss of function has been frequently reported in CRC (Fodde et al, 2001). Therefore, it can be assumed that the complex of *TGIF2LX*-PCDHX could lead to suppress the tumorigenic activity of β -catenin in CRC.

Our in-silico data also showed that *TGIF2LX* could interact with human RHOXF2 protein. The RHOXF2 gene is expressed in a variety of human cancer tissues including colon and lung cancers. Thus, based on the literature, it has been suggested that this protein may act as homeobox oncoprotein (Maclean et al., 2005; Shibata-Minoshima et al., 2010). So far, two different scenarios have been postulated for RHOXF2 expression, genetic and epigenetic factors (Shibata-Minoshima et al., 2012). Maclean et al., (2012) suggested that RHOXF2 could be activated via Ras pathway, the protein that

its activation plays an important role in carcinogenesis (Maclean et al., 2005). In this regard, our computational analysis suggested that oncogenic activity of RHOXF2 gene could be switched off through its interaction with TGIF2LX.

We concluded that the dysregulation of TGIF2LX might play a role in the pathogenesis of colon cancer. Our results indicated that TGIF2LX is predominantly localized to nuclei. Therefore, TGIF2LX encodes a transcriptional regulatory protein. Taken together, findings supported the molecular mechanisms by which TGIF2LX acts as a tumor suppressor in CRC. Thus, this gene may potentially be a promising option for colon cancer gene-based therapeutic strategies. However, to examine these hypotheses and identify potential target genes, several experimental studies such as cDNA-AFLP, chromatin immunoprecipitation (ChIP) assay and yeast-two hybrid approaches are recommended to be carried out.

Abbreviations

TGIF2LX: TG-interacting factor or transforming growth factor beta-induced factor 2-linked X; TGF- β : Transforming growth factor- β ; CRC: Colorectal cancer; HD: homeodomain ; SW-48: A colon adenocarcinoma cell line; MTT: Methyl thiazolyl tetrazolium; BrdU: Bromo-2'-deoxyuridine; H and E: Hematoxylin and Eosin; IHC: Immunohistochemistry.

Funding

This research received a grant (number 18087) from the School of Advanced Medical Technologies, Tehran University of Medical Sciences (TUMS). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict of interest statement

The authors declare no conflicts of interest in preparing this article.

Acknowledgements

We wish to thank the Tehran University of Medical Sciences (TUMS) for supporting this study. Also, we thank Fatemeh Rafiei for key advices.

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