British Journal of Medicine & Medical Research
3(4): 953-961, 2013

SCIENCEDOMAIN *international*
www.sciencedomain.org

Impact of TGIFLX Expression on the Regulation of *BCL2* and *BAX* in Prostate Cancer Cell Line (LNCaP)

Reza Raoofian[1,2], Majid Mojarrad[2] and Mansour Heidari[1,3*]

[1]Department of Medical Genetics, Tehran University of Medical Sciences, Tehran, Iran.
[2]Department of Medical Genetics, Mashhad University of Medical Sciences, Mashhad, Iran.
[3]Department of Ophthalmology, Eye Research Center, Tehran University of Medical Sciences, Tehran, Iran.

Authors' contributions

This work was carried out in collaboration between all authors. Authors have read and accepted the content of final manuscript.

Research Article

Received 24th December 2012
Accepted 2nd March 2013
Published 13th March 2013

ABSTRACT

Aims: The homeoprotein TGIFLX (transforming growth factor-β-induced factor 2-like, X-linked), which is essential in male reproduction and development and likely oncogenic when aberrantly expressed in prostate. We have previously shown an aberrant expression of TGIFLX in the majority of human prostate tumors. However, mechanism by which TGIFLX acts in prostate cancer is unknown. The aim of this study was to investigate the effects of overexpression of wild-type TGIFLX (wt-TGIFLX) on LNCaP, human prostate adenocarcinoma cells.
Study Design: As a prospective study, we used adenovirus expression system for evaluation of TGIFLX expression effects on mammalian cells.
Place and Duration of Study: Medical Genetics Department, Tehran University of Medical Sciences (TUMS), between December 2009 and July 2012.
Methodology: We cloned entire coding sequence of *TGIFLX* gene into adenovirus and subsequently LNCaP cells were transfected with the recombinant virus harboring *TGIFLX* cDNA or control. The TGIFLX expression was confirmed by microscopic analysis and RT-PCR technique. Following molecular cloning and characterization of *TGIFLX* transcription factor, we then studied the effects of overexpression of TGIFLX in LNCaP cells on mRNA expression of BAX and BCL2 genes.

**Corresponding author: Email: mheidari@sina.tums.ac.ir;*

Results: Our results showed that overexpression of TGIFLX downregulated *BCL2* gene (P<0.05) and upregulated BAX gene (P<0.05) at transcript level. Our results suggested that TGIFLX could be a tumor suppressor gene and might be involved in initiation and/or development.
Conclusion: TGIFLX can play a role as a transcriptional modulator of the genes involved in cell cycle pathway. But still more investigations are necessitated for clarifying this claim.

Keywords: TGIFLX; adenovirus; LNCaP cell line; prostate cancer.

1. INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed non-cutaneous cancer and the second leading cause of malignancy death of men living in the USA and also worldwide [1,2]. Understanding molecular mechanisms involved in this carcinoma leads to design new therapeutic strategies with fewer side effects. Although tremendous progress has been made over the past few years in the understanding of the molecular processes involved in PCa initiation, metastasis and local spread, many other genes need to be defined. It has been well documented that one group of genes implicated in the development of prostate cancer are homeobox genes which encode homeodomain proteins. These transcription factors are highly conserved developmental control genes that are involved in the process of pattern formation in most—if not all metazoan phyla [3,4]. Among these, TALE superfamily (three amino acid loop extension) serves as a multifunctional repressor of TGF beta-induced transcription. These transcription factors act in normal and abnormal developmental processes [5,6]. TGIF (named after transforming growth factor-β-induced factor or 5'-TG-3' interacting factor) genes such as *TGIF1*, *TGIF2*, *TGIFLX* and *TGIFLY* are TALE-class homeodomain protein and play critical roles in normal and abnormal developments. Human *TGIFLX* gene encodes a member of the TALE/TGIF homeobox family of transcription factors. This gene is located on X-chromosome and thought to have originated from the retrotransposition of *TGIF2* at least 80 million years ago [7]. We recently reported an aberrant expression of *TGIFLX* gene in some prostate tumors, but not in normal and benign prostate hyperplasia [8]. It has been suggested that this gene implicated in normal spermatogenesis development as well as in prostate cancer genesis and progression [7-9].

The purpose of this work was to better understand the function(s) of human TGIFLX homeobox gene and its possible function in prostate cancer; we evaluated the effect of TGIFLX mRNA overexpression on apoptosis in LNCaP prostate cancer cells.

2. MATERIALS AND METHODS

2.1 Cell Culture

Adherent human LNCaP cells were purchased from Pasteur Institute of Iran. Cells were cultured at 37ºC in a humidified CO_2-controlled atmosphere in RPMI1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and an appropriate antibiotic (including Penicillin/Streptomycin with 100U/ml and 100µgr/ml at final concentration).

2.2 Ad-GFP-TGIFLX and Ad-GFP Infection

The Ad-GFP and Ad-GFP-TGIFLX produced as previously described [20]. LNCaP cells 1 × 10^6 were plated in 10 cm dishes. Infection of the cells was carried out as described by Gomez-Manzano et al. [14]. Briefly, viral stock was diluted to certain concentrations, added to cell monolayers (0.5 ml/10 cm dishes), and incubated at 37ºC for 30 min with brief agitation every 5 min. This was followed by the addition of culture medium and the return of the infected cells to the 37ºC incubator. At 48 h post-infection, cells were examined using UV-fluorescent microscope. We also included a mock-control using LNCaP cells which was treated with culture medium instead of the recombinant adenovirus.

2.3 RT-PCR

The transcriptional activity of the reporter gene was evaluated by RT-PCR of TGIFLX mRNA and compared to constitutive GAPDH (glyceraldehyde-phosphate dehydrogenase) mRNA as an internal control. Briefly, total RNA extraction was carried out using High Pure RNA Isolation Kit (Roche) according to the manufacturer's instructions. cDNA was synthesized using 1µg of total RNA as described previously [8,9]. Specific primers for application in this study were designed using Primer3 (v. 0.4.0) (http://frodo.wi.mit.edu/primer3/) and presented in Table 1. RT-PCR was conducted as described previously [10].

2.3.1 Methyl Thiazolyl Tetrazolium (MTT) assay

$3x10^3$ LNCaP cells were transfected with either wt-TGIFLX (Ad-TGIFLX-GFP), vector controls (Ad-GFP) or culture medium (mock- control) in 96 well plates as described in above section. After confirmation of Ad-TGIFLX-GFP by UV-microscope study, MTT assay was performed. Briefly, 50 µl of MTT solution (0.5 mg/ml) was added to each well and cells were further incubated at 37ºC for 1 h. Following solubilization of precipitated formazan with 100 µl DMSO, the optical densitometry was measured at a wavelength of 550 nm by ELISA reader (Anthos 2020, Austria). The inhibition rate (IR) of TGIFLX was evaluated using the following equation: $IR(\%)=1-OD_{exp}/OD_{con}\times100$ (where OD_{exp} and OD_{con} are the optical densitometries of treated and untreated cells, respectively).

2.3.2 Western blot analysis

The evaluation of the TGIFLX-GFP expressing cells was finalized at a translational level by western blot analysis. Briefly, total proteins were extracted [11] and western blot analysis was conducted as previously described [12] with some modifications. About 15 µl whole cell lysate of each cells were electrophoresed through 12.5% SDS-PAGE gels and transferred to a nitrocellulose membrane (Amersham Pharmacia, USA). The membranes were blocked for 1 h with 3% Bovine Serum Albumin (BSA) at 37ºC and then incubated with a 1:500 dilution of a polyclonal rabbit anti-TGIFLX antiserum (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (2h, at room temperature). The membranes were then incubated with 1:5,000 dilutions of a goat HRP-conjugated anti-rabbit IgG secondary antibody (1.5 h) prior to development with 4-chloro-1-naphthol (Immun-Blot, Bio-Rad Laboratories, USA).

2.4 Real -Time PCR

Real Time RT-PCR was performed to study the potential effects of *TGIFLX* gene expression on *BAX* and *BCL2* genes in transfected cells with Ad-TGIFLX-GFP. The reaction mixture in

real time RT-PCR contained 1µl of each primer (10pmoles/µl) (Table 1) 1µl of cDNA and 5 µl of 2× SYBR Green PCR Master Mix (Takara). The amplification program included an initial denaturation at 95ºC for 10 min, followed by 30 cycles of a two-stage PCR consisting of 95°C for 10 sec and 60ºC for 30 sec. Specificity of primers was verified by observing a single peak dissociation curve for each run. All reactions were performed in triplicate. The absence of contamination was verified using non-template controls.

Table 1. List of primers used in this study

Primers	Cloning Primers	PCR Product (bp)
TGIFLXF	5'-CGAG ATCTATGGAGGCCGCTGCGGA-3'	**730**
TGIFLXR	5'-GCGATATCATCATGGATTAGGCTCTTGC-3′	
Real Time RT-PCR Primers		
BCL2 F	5'- GGAGGATTGTGGCCTTCTTT -3'	**176**
BCL2 R	5'- GCCGTACAGTTCCACAAAGG -3'	
BAX F	5'- GATGCGTCCACCAAGTCGCT-3'	**170**
BAX R	5'- CGGCCCCAGTTGAAGTTG-3'	
pEGFPF	5'-TTCAGGACCTGTTAAGGCTACA-3'	**130**
TGIF2LXR	5'-ATTTGAAGGACACAAGACCCG-3'	
GAPDHF	5'-CACCAGGGCTGCTTTTAAC-3'	**190**
GAPDHR	5'-ATCTCGCTCCTGGAAGAT-3'	

2.5 Statistics Analysis

Statistical tests were conducted in two steps. First, factorial analysis of variance was used to test for overall statistical interaction. Second, interactions significant at $P<0.05$ were followed by simple contrast tests. All data distributions were checked for consistency with statistical assumptions.

3. RESULTS AND DISCUSSION

To identify genes targeted by wild-type TGIFLX, overexpression of TGIFLX was carried out using a mammalian expression vector containing wt-TGIFLX gene in LNCaP prostate cancer cell line. The LNCaP cells were transfected with either wt- TGIFLX (Ad-TGIFLX-GFP), vector controls (Ad-GFP) or culture medium (mock- control). After 48h post-transfection, cells were examined by UV-fluorescent microscopy; the transfected LNCaP cells with Ad-TGIFLX-GFP, Ad-GFP and negative control are shown in Fig. 1. Subsequently, total RNA was extracted from the cell lines and cDNAs were synthesized. Our results show that a high level of TGIFLX mRNA could be detected in post transfected cells by Ad-TGIFLX-GFP construct. The integrity of cDNA was checked using the house keeping gene GAPDH primers (Fig. 2). Prior to investigating the effects of overexpression of wt-TGIFLX on apoptosis in LNCaP cells, it was important to provide further evidence that TGIFLX is overexpressed in the transfected LNCaP cells with Ad-GFP in comparison with controls. In this regards, western blot analysis (Fig. 3) and quantitative RT-PCR was conducted using an equal amount of total RNA from three different samples and specific primers for amplification of partial TGIFLX coding regions (Table 1). As shown in Fig. 4A the TGIFLX mRNAs were overexpressed LNCaP cells containing Ad-TGIFLX-GFP in comparison with controls (infected LNCaP cells with Ad-GFP and mock control).

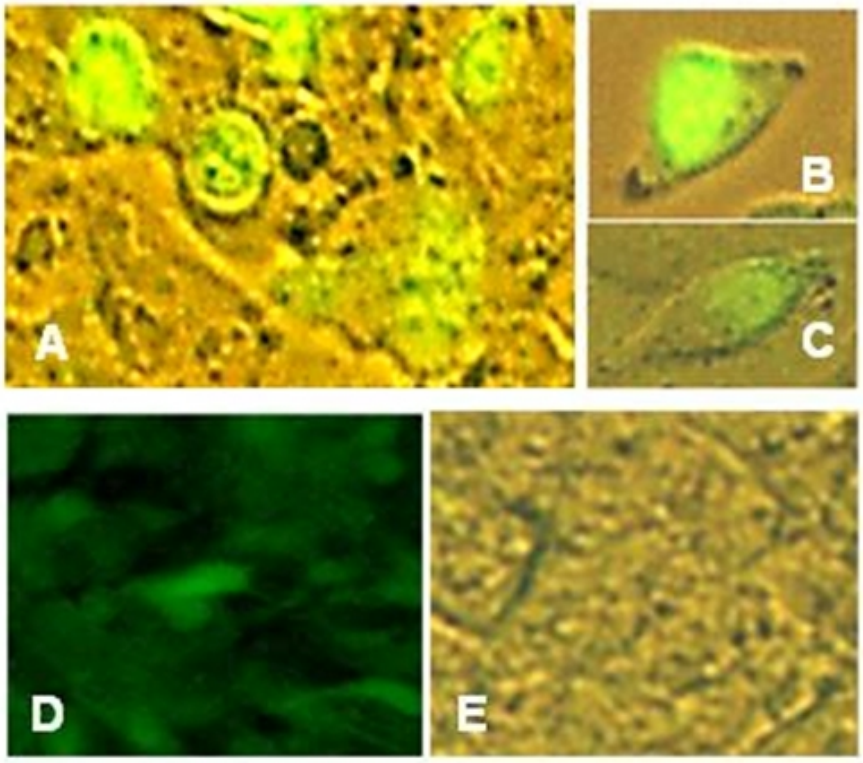

Fig. 1. GFP-TGIFLX gene expression detected with fluorescent microscopy.
LNCaP *cells three weeks post-transfection under fluorescent microscope (×20). LNCaP cells transfected with Ad-TGIFLX-GFP (A); single cell transfected with Ad-TGIFLX-GFP (B,C); Ad-GFP (D) and mock control (E).*

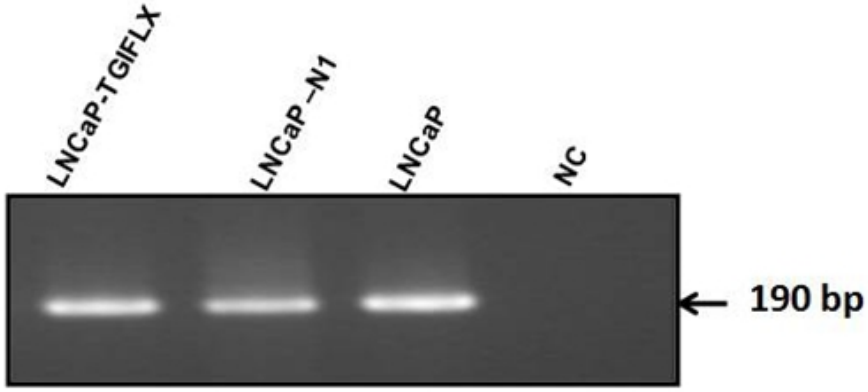

Fig. 2. Gel electrophoresis of RT-PCR products for GAPDH from total RNA of prostate Cancer-Derived Cells.
The quality and integrity of RNAs from ten LNCaP cell line were confirmed by RT-PCR of the GAPDH (190bp) housekeeping control gene. PCR products were resolved on a 2% agarose gel stained with ethidium bromide. Negative control (NC) (ddH_2O).

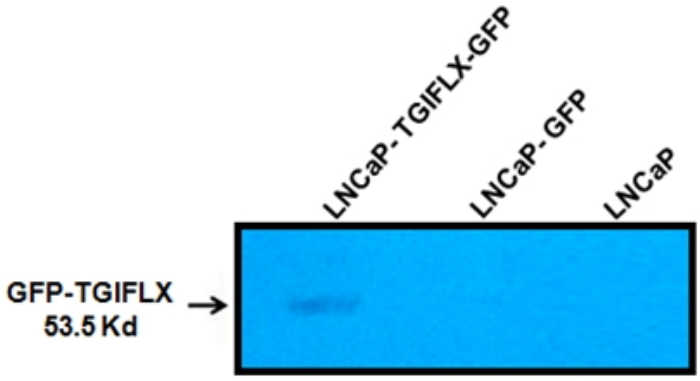

Fig. 3. Overexpression of TGIFLX in LNCaP cells

*Western blotting assay shows protein expression of TGIFLX (53.5 kD) in the transfected LNCaP cells in the transfected LNCaP with Ad-TGIFLX-GFP vector (**A**) but not in Ad-GFP (**B**) and mock control (**C**).*

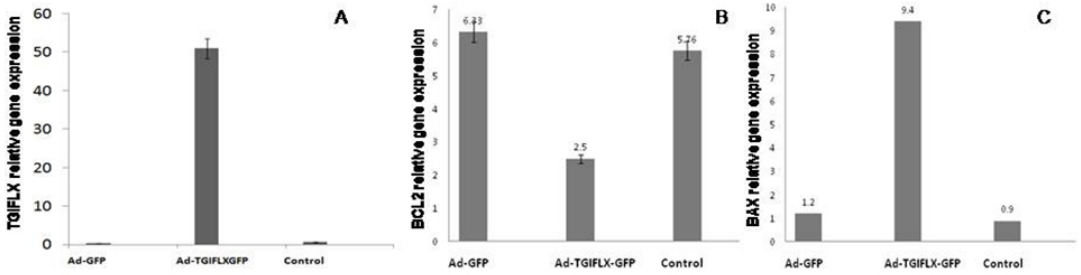

Fig. 4. Gene expression in LNCaP cell lines.

*Real-time PCR result with specific, TGIFLX, BCL2 and BAX primers. Each Real-time PCR was performed in triplicate and experiments were repeated twice with the mean±SD. Expression of TGIFLX mRNA (**A**). Expression of BCL2 mRNA (**B**). Expression of BAX mRNA (**C**).*

3.1 Overexpressed-TGIFLX Upregulates BAX but downregulates BCL2 mRNA Expression

In order to evaluate the impact of TGIFLX overexpression in LNCaP cells, real time RT-PCR was conducted using specific primers corresponding to coding sequence of BCL2 and BAX genes ($P<0.05$). As shown in Fig. 4, overexpression of TGIFLX significantly downregulates BCL2 (Fig. 4B) gene expression in LNCaP containing Ad-TGIFLX vector in comparison to controls. However, the expression of BAX gene at the mRNA level was increased in the LNCaP cells harboring Ad-TGIFLX-GFP vector but not in the controls (Fig. 4C).

TG-interacting factor (TGIF), which is a TALE superfamily, containing TGIF1, TGIF2, TGIFLX and TGIFLY, serves as a multifunctional repressor of TGF-ß signaling [7,13-15]. TGIF-like homeodomain proteins are those that have homology with TGIF. In the present study, we report nuclear localization of TGIFLX in LNCaP cells. The TGIFLX nuclear localization suggested being performed using N-terminal residues (KKRK) in TGIFLX [7]. The TGIFLX nuclear localization confirmed that TGIFLX as a homeodomain transcription factor could act as transcriptional activators or repressors.

The entire coding sequence of TGIFLX was cloned into an adenovirus and the overexpression effects of TGIFLX were studied in LNCaP cells. To date, adenoviruses are the most commonly used vectors for clinical gene, identification of target genes and therapy applications. It has been reported that gene transfer approach using adenovirus accounting for 24% of all clinical trials [18]. Our results using adenoviral gene transfer system in LNCaP cell represented a potential TGIFLX localization in nuclear compartment. The TGIFLX expression indicated that could be involved in the regulation of cell cycle processes.

Results presented in this work also revealed that *BCL2* dramatically downregulated in the transfected LNCaP cells with Ad-TGIFLX-GFP vector but not in controls. To confirm tumor suppressor activity of TGIFLX, MTT assay was investigated in 550nm absorbance. As it is shown in Fig. 5, it was about 0.325, 0.42 and 0.43 for LNCaP –TGIFLX-GFP, LNCaP-Mock and LNCaP-GFP, respectively. Indeed, a significant reduction was occurred in LNCaP harboring Ad-TGIFLX-GFP ($p<0.05$). A large body of evidence has implicated homeodomain proteins as playing critical roles in promoting G1-S cell cycle transitions [16,17] and human cancer [8,19]. TGIFLX is not the first homeobox gene that regulates cell cycle control genes, various studies reported that homeobox genes such as *BP1*, *HOXA10* and *DLX4* regulate the expression of *BCL2* [20], *p53* [21] and *BAX* genes [22] in human cancer cell lines, respectively. In agreement with our results, Moucadel et al [23] showed that human homeobox *CDX1* gene expression regulates *p21*, *p53* and *BCL2* gene expressions and they found that induction of *CDX1* gene was negatively regulated target of *p53* in intestinal cells. *CDX1* regulates *p21* and *BCL2* expressions through physical interaction with *p21* TATA box and consensus CDX1-binding sites, respectively [23]. Also, it has been shown that p21 could be a transcriptional target of HOXA10 in differentiating myelomonocytic cells. This regulation could be coordinated through binding HOXA10 on *p21* promoter region and its function leads to cell proliferation inhibition [24]. In addition, another study has shown the role of HOX11 in growth and development of spleen by cell survival enhancement [25]. It demonstrates the opposite role of Homeobox genes in cells which can cause cell proliferation and in some cases inhibition in a contrary direction.

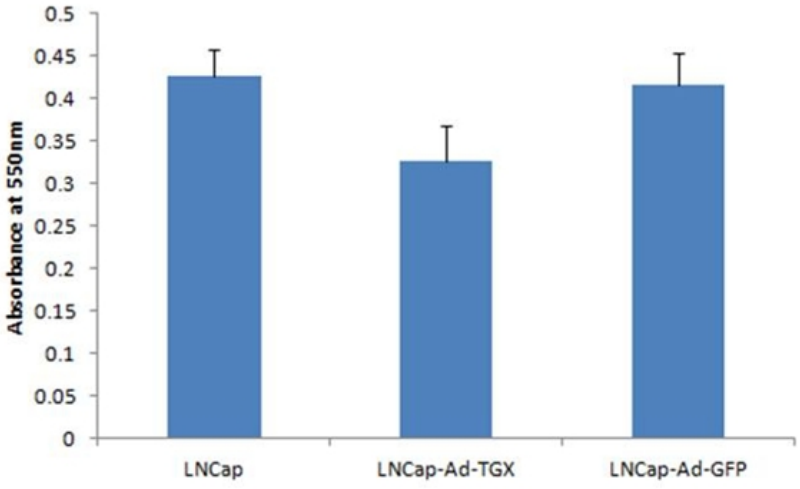

Fig. 5. Effect of TGIFLX expression in LNCaP cells

Viable cells were determined by MTT assay. LNCaP cells (5×10^3cells/well) were seeded and cultured for 24h. Cultured cells were transfected with Ad-TGIFLX-GFP or Ad-GFP. Cell viability was quantified by MTT assay for transfected cells in comparison with control. Data are expressed as means±SD

4. CONCLUSION

In conclusion, though this study we demonstrated ectopic expression of TGIFLX could be implicated in transcription repression of genes involved in cell cycle pathway. Still, more investigations seem necessary to clarify this claim.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin. 2012;62:10-29.
2. DeMarzo AM, Nelson WG, Isaacs WB, Epstein JI. Pathological and molecular aspects of prostate cancer. Lancet. 2003;361(9361):955-64.
3. Akam M. Hox and HOM: homologous gene clusters in insects and vertebrates. Cell. 1989;57(3):347-9.
4. De Robertis EM. Evolutionary biology. The ancestry of segmentation. Nature. 1997;387(6628):25-6.
5. Argiropoulos B, Humphries RK. Hox genes in hematopoiesis and leukemogenesis. Oncogene. 2007;26(47):6766-76.
6. Yu YY, Pan YS, Zhu ZG. Homeobox genes and their functions on development and neoplasm in gastrointestinal tract. Eur J Surg Oncol. 2007;33(2):129-32.
7. Blanco-Arias P, Sargent CA, Affara NA. The human-specific Yp11.2/Xq21.3 homology block encodes a potentially functional testis-specific TGIF-like retroposon. Mamm Genome. 2002;13(8):463-8.
8. Ousati Ashtiani Z, Ayati M, Modarresi MH, Raoofian R, Sabah Goulian B, Greene WK, et al. Association of TGIFLX/Y mRNA expression with prostate cancer. Med Oncol. 2009;26(1):73-7.
9. Aarabi M, Ousati-Ashtiani Z, Nazarian A, Modarressi MH, Heidari M. Association of TGIFLX/Y mRNA expression with azoospermia in infertile men. Mol Reprod Dev. 2008;75(12):1761-6.
10. Saffari M, Dinehkabodi OS, Ghaffari SH, Modarressi MH, Mansouri F, Heidari M. Identification of novel p53 target genes by cDNA AFLP in glioblastoma cells. Cancer Lett. 2009;273(2):316-22.
11. Heidari M, Rice KL, Phillips JK, Kees UR, Greene WK. The nuclear oncoprotein TLX1/HOX11 associates with pericentromeric satellite 2 DNA in leukemic T-cells. Leukemia. 2006;20(2):304-12.
12. Heidari M, Rice KL, Kees UR, Greene WK. Expression and purification of the human homeodomain oncoprotein HOX11. Protein Expr Purif. 2002;25(2):313-8.

13. Holland PWH, Booth HAF, Bruford EA. Classification and nomenclature of all human homeobox genes. BMC Biol. 2007;5:47.
14. Hyman CA, Bartholin L, Newfeld SJ, Wotton D. Drosophila TGIF proteins are transcriptional activators. Mol Cell Biol. 2003;23(24):9262-74.
15. Wang Y, Wang L, Wang Z. Transgenic analyses of TGIF family proteins in Drosophila imply their role in cell growth. J Genet Genomics. 2008;35(8):457-65.
16. Sikder HA, Devlin MK, Dunlap S, Ryu B, Alani RM. Id proteins in cell growth and tumorigenesis. Cancer Cell. 2003;3(6):525-30.
17. Yeh B-W, Wu W-J, Li W-M, Li C-C, Huang C-N, Kang W-Y, et al. Overexpression of TG-interacting factor is associated with worse prognosis in upper urinary tract urothelial carcinoma. Am J Pathol. 2012;181(3):1044-55.
18. Seregin SS, Aldhamen YA, Appledorn DM, Zehnder J, Voss T, Godbehere S, et al. Use of DAF-Displaying Adenovirus Vectors Reduces Induction of Transgene- and Vector-Specific Adaptive Immune Responses in Mice. Hum Gene Ther. 2011;22(9): 1083-94.
19. Cillo C, Cantile M, Faiella A, Boncinelli E. Homeobox genes in normal and malignant cells. J Cell Physiol. 2001;188(2):161-9.
20. Awwad RT, Do K, Stevenson H, Fu SW, Lo-Coco F, Costello M, et al. Overexpression of BP1, a homeobox gene, is associated with resistance to all-trans retinoic acid in acute promyelocytic leukemia cells. Ann Hematol. 2008;87(3):195-203.
21. Chu MC, Selam FB, Taylor HS. HOXA10 regulates p53 expression and matrigel invasion in human breast cancer cells. Cancer Biol Ther. 2004;3(6):568-72.
22. Sun Y, Lu X, Yin L, Zhao F, Feng Y. Inhibition of DLX4 promotes apoptosis in choriocarcinoma cell lines. Placenta. 2006;27(4-5):375-83.
23. Moucadel V, Totaro MS, Dell CD, Soubeyran P, Dagorn JC, Freund JN, et al. The homeobox gene Cdx1 belongs to the p53-p21(WAF)-Bcl-2 network in intestinal epithelial cells. Biochem Biophys Res Commun. 2002;297(3):607-15.
24. Bromleigh VC, Freedman LP. p21 is a transcriptional target of HOXA10 in differentiating myelomonocytic cells. Genes Dev. 2000;14(20):2581-6.
25. Lonyai A, Kodama S, Burger D, Davis M, Faustman DL. The promise of Hox11+ stem cells of the spleen for treating autoimmune diseases. Horm Metab Res. 2008;40(2):137-46.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=205&id=12&aid=1096