Iranian J Publ Health, Vol. 42, No. 11, Nov 2013, pp.1242-1252

**Original Article**

# Induction of Apoptosis and Growth Suppression by Homeobox Gene TGIFLX in Prostate Cancer Cell Line Lncap

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**(Received 19 Jun 2013; accepted 12 Sep 2013)**

**Abstract**

**Background:** TGIFLX, a Homoproteins cluster member located on the X chromosome, has a critical role in male reproduction and prostate development. Previous studies have shown the erratic expression of TGIFLX gene in a large proportion of prostate tumors. However TGIFLX function in prostate development remains unknown. The purpose of this study was to evaluate the consequences of TGIFLX expression on prostate cancer cell lines (LNCaP).

**Method:** Inducible Tet-On gene expression system was used with a regulatory capability by doxycycline induction. In this system, stable LNCaP cells with TGIFLX tet-on plasmid were able to induce TGIFLX expression by doxycycline treatment. TGIFLX gene expression was confirmed by RT-PCR.

**Results:** Induction of gene expression caused cell proliferation decrement and apoptosis increment in LNCaP TGIFLX cells compared with control cells ($P<0.01$). Also, by using PEGFPN1 plasmid protein in this study localization was shown in nucleus. The gene was cloned in the plasmid and transfected to LNcap cells with plasmid PEGFPN1 TGIFLX and the plasmid was PEGFPN1. The TGIFLX expression was confirmed by RT-PCR and fluorescent microscopy.

**Conclusion:** TGIFLX expression demonstrated a tumor suppressor characterization in a prostatic cancer cell line with low grade of tumorigenicity (LNCaP). More cell lines with different level of tumorogenicity need to be investigated for further clarification of the TGIFLX gene function.

**Keywords:** TGIFLX gene, Prostate cancer, LNCaP, Stable cell line, Tet-On vector

## Introduction

Prostate cancer is one of the most common cancers and the second cause of death after lung cancer in industrial countries with a higher frequency after the 7$^{th}$ decade of life. It is estimated that about 40% of men over 50 year's old, show a cancerous transformation which should be detected with histological observation (1). Prostate cancer is a heterogeneous and multi-factorial disease in which several factors such as life style, environment, hormone and genetics could be involved (2). Although many genes have been identified in having a role in prostate cancer progression, the precise molecular mechanism(s) responsible for initiation and progression of the disease are still not well understood.

In homeobox genes, especially its HOX subgroup, there are coding transcription factors which are responsible for coordination and regulation of cellular physiology such as growth, apoptosis, etc. in gestational life and many evidences indicate a that there is a relationship between dysregulation

of these genes and initiation of neoplasia in breast, colorectal, kidney and also leukemia (3).

Several studies have shown role of HOX genes such as HOXC4, HOXC5, HOXC6, and HOXC8 in prostate cancer (4). They are coding regulatory proteins which control the primitive evolutionary reactions in various tissues and contain about 60 amino acids called homo-domain. They have a helix-turn-helix structure which binds to the DNA, rolling as a transcription factor. Mutation in such genes, leads to sever alterations in the morphology of organism (5). They are categorized into six groups. One of them is coding for TALE proteins. TGIF belongs to TALE proteins as a suppressor of TGF-β transcriptional inducer. TGIF2 has a substantial homology with TGIF, located in the long arm of chromosome 20, expressing in most of human body tissues with higher expression level in heart, kidney, testis and ovarian cancer (6, 7). TGIFLX is produced from TGIR2 retro-transposition and is located in chromosome X. It is more likely a regulatory transcription factor which might perform a function in association with other homo-domain proteins. The biological role of TGIFLX gene is not completely known except its exclusive expression in adult testis (8). A study has shown strong relationship between loss of TGIFLX expression and infertility in men (9). Moreover, another study has demonstrated that its abnormal expression has an important role in malignancy and progression of prostate cancer (10).

This study investigated the effect of in vitro gene expression on prostate cancer cell line with TGIFLX transfection into the prostate cancer cell line (LNCaP) in order to prepare a stable cell line. The study and analysis of TGIFLX effect on this cell line can help to elucidate the basis of molecular biology of such genes on cancer.

## Materials and Methods

### *Cell line and Primers*

The LNCaP cell line was prepared by the Pasteur Institute of Iran (Institute Pasteur d'Iran). It was cultured in culture media containing RPMI 1640 and 10% Fetal Bovine Serum (FBS), 100ug/ml Penicillin, 100ug/ml streptomycin in 37 C and 5% $CO_2$. Primers were designed based on the genomic database of given in NCBI (http://-www.ncbi.nlm.nih.gov/) and Gene Runner (http://www.generunner.net/) designing software. PGEFp-N1 and inducible tet-ON plasmid was used (Takara, Japan) for stable gene expressing eukaryotic cell.

### *RT-PCR*

After 80-90% confluence RNA was extracted by Tripure Isolating Reagent (Roche, USA) according to manual protocol. The amount of 1ug of RNA was used for preparation of cDNA by using cDNA synthesis kit (Fermentase Thermo scientific, USA) based on the instruction protocol. The TGIFLX expression of LNcap was analyzed by using PCR reaction with primers (Table 1). The PCR reaction is demonstrated in Table 2. PCR products were run through a 2% agarose gel and DNA band intensity was observed by Gel Doc (Gel logic 212 PRO, USA).

**Table 1:** Primers used for this study

| Name | Description | Sequence |
|---|---|---|
| TGIFLX forward | For Amplification (EcorI Restriction site) | **5'-GGGAATTCATGGAGGCCGCTGCGGAC-3'** |
| TGIFLX reverse | For Amplification (EcorV Restriction site) | **5'-GGGATATCTCATGGATTAGGCTCTTG-3'** |
| TGIFLX forward: TGX F | For expression analysis | **5'-CAACAGTAACGATAAGCCTCTTG -3'** |
| TGIFLX reverse: TGX R | For expression analysis | **5'-AAGGCAAGAACTCTGCCTGTA -3'** |
| GAPDH forward | House keeping | **5'-CACCAGGGCTGCTTTTAAC-3'** |
| GAPDH reverse | House keeping | **5'-ATCTCGCCTCCTGGAAGAT-3'** |

### *Cloning*

To make a recombinant TGIFLX expression vector, the gene was amplified by PCR from a TA vector which had been prepared before and then sub-cloned to the inducing vector pTet-On (P-Tight). Cloning primers were used and EcoRI restriction site in 5' of forward primer and EcoRV restriction site in 5' of reverse primer (Table 1) were designed for multiple cloning sites in order to amplify TGIFLX from TA vector (Table 2) which was sub-cloned before (11). The sequence accuracy was confirmed by DNA sequencing. Amplified product was digested by the mentioned restriction enzymes and run through 0.7% gel electrophoresis, separated by QIAQuick Gel Extraction kit (Qiagen, USA) based on the manual instruction. The separated fragment was ligated into the p-Tet-On vector by using T4 DNA Ligase (invitrogen, USA),

Ligation reaction was transformed into the Top10 f (Invitrogen, USA) by heat shock method and the accuracy of clones was confirmed by double digestion. The sequence of inserted product was confirmed also by DNA sequencing. Number of 25x $10^4$ LNcap cell lines were seeded in each 6 well plates and waited for 70% confluency.

**Table 2:** PCR programs

| For TGIFLX Amplification | **1cycle** | | **20 cycle** | | | **16cycle** | | **1cycle** |
|---|---|---|---|---|---|---|---|---|
| | 96ºC | 96ºC | 66 ºC | 72 ºC | 96ºC | 54 ºC | 72 ºC | **72 ºC** |
| | 5' | 30" | 30" | 2' | 30" | 30" | 2' | **7'** |

| For TGIFLX Expression Analysis | 1cycle | | 35 cycle | | **1cycle** |
|---|---|---|---|---|---|
| | 96ºC | 96ºC | 55 ºC | 72 ºC | **72 ºC** |
| | 5' | 30" | 30" | 30" | **7'** |

In a period of 24 hours before lipofection, culture media was changed with antibiotic free RPMI 1640. Afterwards, 5 ug of purified regulatory plasmid (p-rtTA) was mixed with 250 ul of FBS free RPMI without antibiotic and was incubated at room temperature before usage. On the other hand, 5 ul of lipofctamine 2000 (Invitrogen, USA) was mixed with 250 ul of serum free RPMI without antibiotic at room temperature for 5 minutes. In the next step, both of solutions (DNA and lipofectamine) were mixed and incubated for 20 minutes at room temperature. Here, cell culture media was replaced with the mixture and incubated for 3 minutes in $CO_2$ incubator. Then 2 ml of antibiotic free RPMI containing 10% FBS was added to the cells and transferred to the incubator. After 4-6 hours, the media was replaced with antibiotic free RPMI containing 10% FBS.

Number of $2x10^5$ LNCaP cells was inserted in each well of 6 well plates and after 70% confluency, the media was replaced with G418 culture media. Then the killing curve of antibiotic was analyzed. Antibiotic containing cell culture media was refreshed every 48 hours. The minimal concentration which caused to complete cell death within one week was used for the next step which was 400 ug/ml. Forty eight hours after lipofection with regulatory plasmid (p-rtTA), the culture media was replaced with RPMI containing 10% FBS and 400ug/ml G418 and was refreshed every 48 hours for 3 weeks until a clone of G418 resistant cell was found in the plate. The selected stable cell

line was transfected with responding plasmid p-Tight, cloned with TGIFLX and selected with 250 ug/ml (optimized by killing curve same as above) of hygromycin for 3 weeks.

### TGIFLX induction expression

A number of $2x10^5$ double transfected cell lines were seeded in 6 well plates. After 24 hours three different dilutions of doxycyclin (500 ng/ml, 800 ng/ml and 1ug/ml) were added to different wells. Then after 48 hours, RNA of the cells were extracted and the expression of TGIFLX gene with our without presence of doxycyclin was analyzed by cDNA synthesis and RT-PCR reaction. Equal amount of double transfected cells were distributed in 6 well plates and different dilutions of doxycyclin (200 ng/ml, 400 ng/ml, 800 ng/ml, 1ug/ml) were added to different wells. The total of RNA was extracted after 48 hours for TGIFLX gene expression analysis with or without presence of doxycyclin.

### MTT assay

A number of $5\text{-}10x10^4$ stable TGIFLX inducible cells, LNCaP with p-Tet on empty vector and LNCaP cells were seeded in each wells of 96 well plates and incubated for 24 hours. Then doxycyclin with concentration of 1ug/ml was added to each well and 50ul of 0.5mg/ml MTT solution was added and incubated at 37 °C for 1 hour. After discarding the solution by inversion, the amount of 100 ul of DMSO was added to the purple precipitation of wells and dissolved by shaking. Finally the absorbance rate in 550nm to 630nm as background was analyzed by ELISA reader (Anthos 2020, Austria).

### BRDU assay

A number of $5\ x10^5$ cells were seeded in each of the 96 well plates as triplicate for each sample (TGIFLX cells, p-Tight empty vector cells and LNCaP cells) and treated with doxycyclin for 48 hours. BRDU (Roche, Germany) was added to culture media and incubated for a definite time at 37 °C. Then it was discarded by the inverting culture plate and fixDenta (Roche, Germany) was added as the fixing solution. After 30 min incubation at room temperature, the fixing solution was discarded and Anti-BRDU-POD (Roche, Germany) was added. It was incubated for 30 to 90 min at room temperature and the antibody was discarded and wells were washed for three times by washing buffer. The TMB substrate (Roche, Germany) was added and incubated for 5-20 min at room temperature. Lastly, 450 nm of absorbance was measured.

### Apoptosis and caspase assay

Cultured cells were treated by doxycyclin for 48 h and after synchronization for 12 hours (culturing in FBS free media), tripsinized and span down at 600g in 4°C for 5 min before the supernatant were discarded. The cells were washed with 1 ml PBS and span down again. The amount of 100 ul of cell lysis buffer was added to $2x10^6$ cells after discarding the supernatant and placed on ice for 15 to 20 min. Then the lyzed cells were centrifuged for 10 to 15 min at 20000 g in 4 °C and supernatant was transferred to new tubes and incubated at -70 °C.

The caspase 3 enzyme activity test was conducted by calorimetric method. The amount of protein was calculated by using A280 wave length of Nano-drop (NaNoDrop 1000, Thermo scientific, USA). Different concentrations of protein were checked to optimize the protein concentration. The concentration of 30 mg/ml was defined as the optimized concentration. The appropriate amount of protein and reaction buffer was added to each well to a final volume of 90 ul. The reaction was initiated by adding 10 ul of caspase 3 substrate. The plate was moved gently without making bubbles. It was covered with foil aluminum and incubated at 37°C for 18 hours. The sample absorbance was measured at 405 nm.

### TGIFLX localization

TGIFLX amiplified coding sequence was amplified with primers containing BglII and EcorI restriction sites in 5' direction from TA cloning vector. It inserted into the GFP expressing vector pEGFP (Clontech, USA) in order to investigate the localization of TGFILX protein and be transfected into LNCaP cells the same as it was men-

tioned before. Transfected cells (pEGFP-TGIFLX called LNGFLX and pGEFP-N1 as an empty vector LNGFN) were selected by G418 for 21 days. Green fluorescence signaling was investigated by using fluorescent microscope (0lympus IX71, Japan) using different magnifications.

### *Statistical analysis*

The average and standard deviation (SD) of all variables from triplicate samples were calculated by using Excel 2010 (Microsoft, USA) and *P* value calculated by *t*-test.

## Results

### *TGIFLX expression and LNCap*

TGIFLX gene expression was investigated in LNCaP cell lines. Data of RT-PCR demonstrated no expression of this gene in LNCaP cell line. GAPDH was used as a house keeping gene (Fig. 1A).

### *TGFLX amplification by PCR*

TGIFLX gene was amplified by primers with EcoRI and EcoRV restriction sites as mentioned in the material and methods. The size of PCR product was 728 base pair and was recovered in 2% agarose gel which is shown in figure1B. Digested plasmid (p-Tight responding plasmid) and amplified PCR fragment which purified by using PCR gel extraction were confirmed by using agarose. Plasmid and fragment were ligated in 1:3 molar ratios. Ligation mixture was transformed into competent cells and 10 antibiotic resistant colonies were selected and cultured. Afterwards, plasmids were extracted and PCR products were recovered by using 1% agarose gel which is shown in Fig. 1C. Plasmids with higher molecular weight were selected and digested in a single and double manner. Finally plasmids with product size of 728 bp after double digestion with restriction enzymes were chosen as recombinant plasmids. The validity of TGIFLX sequence was confirmed by DNA sequence analysis.

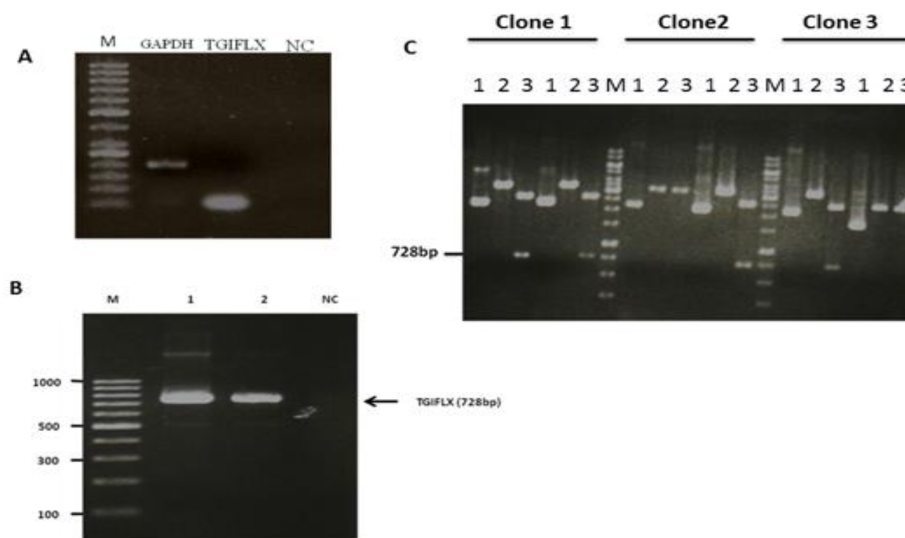


**Fig. 1: (A)** Agarosis gel electrophoresis of RT-PCR products for TGIFLX expression in Lncap cell line stained with ethidium bomide showed no band for TGIFLX expression. GAPDH as house keeping and NC as template negative control were shown in this picture defined by 50 bp DNA ladder (M). **(B)** TGIFLX product was amplified by using specific primers as mentioned in materials and methods. Wells number 1 and 2 were used for PCR product using 80 and 20 nanogram/ul respectively. DNA marker as 100bp ladder (M). NC as negative control for PCR. **(C)** Electrophoresis for single and double digestion of pTet-On recombinant plasmid with EcoRI as mentioned in materials and methods. The lines containing undigested plasmid (1), single digested plasmid (2) and double digested plasmid containing the 728 bp cloned fragment (3) and 1Kb DNA marker (M)

### Recombinant plasmid containing TGIFLX

Double transfected stable cell line (named LNX-1) was treated with different dosages of 200ng/ml, 400 ng/ml and 1ug/ml. PCR products of treated and untreated cells and also LNCaP transfected with empty vector (named LNN-1) has been illustrated in Fig. 2. While untreated double transfected stable LNCaP cell line and empty vector transfected cells have shown no band for TGIFLX, the same stable cell lines treated with doxycyclin have shown the TGIFLX amplification band in a dosage dependent pattern. The gene expressing bands were normalized by using housekeeping gene GAPDH which has been detailed above.

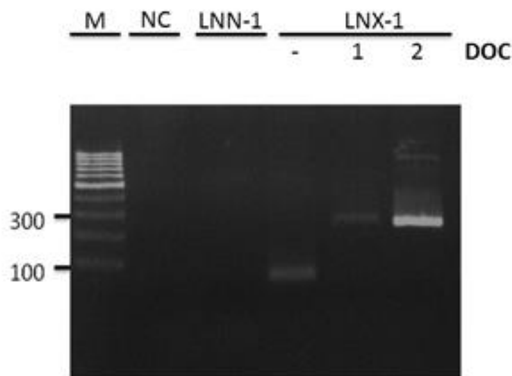


**Fig 2:** Agarose gel electrophoresis of TGIFLX expression in stable cell lines. There is no expression in LNN-1 empty vector Lncap cell line. Inducible cell lines showing expression of TGIFLX after treatment with 400ng/ml (1) and 1 ug/ul (2) Doxycyclin in a dose dependent manner compared with untreated cells (-). NC as negative control and 100 DNA ladder as marker (M) was included in data

### MTT cell viability

MTT assay was investigated in 550nm absorbance. As it is shown in Fig. 3, it was 0.37, 0.41 and 0.34 for LNCaP, LNN-1 and untreated LNX-1, while this range was reduced to 0.25 after LNX-1 TGIFLX induction by doxycyclin ($P$<0.005). Data has shown lower metabolic activity in LNX-1 treated with doxycyclin compared with untreated cells LNN-1.

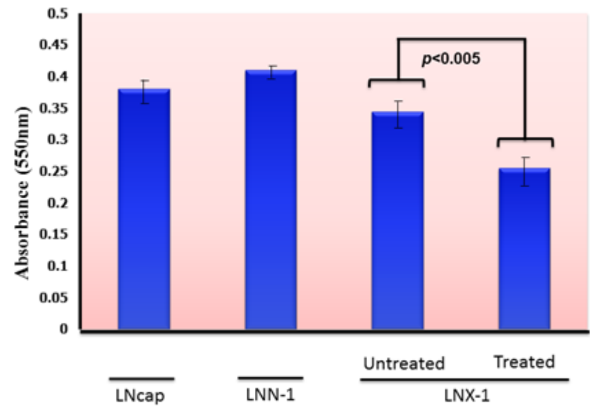


**Fig. 3:** MTT assay has shown a dramatic effect of TGIFLX expression on cell viability in TGIFLX expressing (LNX-1) cells compared with wild type (Lncap) and empty vector (LNN-1) stable cell lines ($P$<0.05)

### BRDU and cell proliferation

BRDU assay by recording 450 nm absorbance has shown these amounts to be 0.25, 0.24, 0.24 for LNCaP- LNN-1 and untreated LNX-1, respectively, while the amount of absorbance has shown a dramatic decrease for treated LNX-1 low to 0.15 ($P$<0.05). Figure 4 demonstrates a considerable reduction in cell proliferation by TGIFLX gene induction.

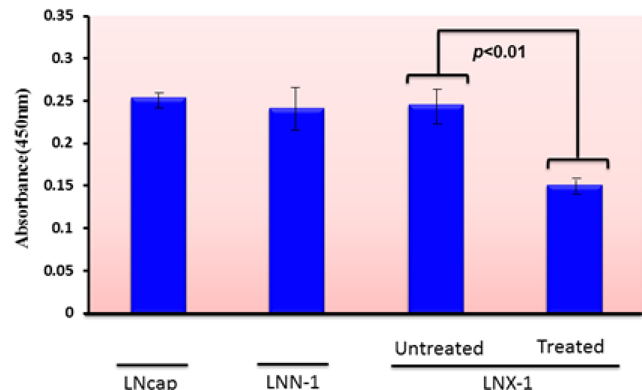


**Fig. 4:** TGIFLX expression leads to suppression of growth by BrdU assay. As shown in figure the absorbance is clearly deceased in treated LNX-1 cells compared to untreated LNX-1, LNN-1 and Lncap cells ($P$<0.05)

### *Apoptosis by caspase*

Caspase 3 has a critical role in the process of nuclear apoptsis such as chromatin aggregation, DNA fragmentation and cell bubbling based on caspase 3 activities according to hydrolysis of Ac-DEVD-pNA by caspse3 and releasing of p-Nitroaniline (PNA) which could be calculated by 450 nm absorbance recording. The absorbance was recorded as 0.11 and 0.13 for LNCaP and untreated LNX-1 respectively, while this amount increased to 0.3 in LNX-1 cells after 48 hours treatment with 1ug/ml of doxycyclin (Fig. 5). The results demonstrate a dramatical enhancement of caspase activity in TGFLX induced cells ($P$<0.005).

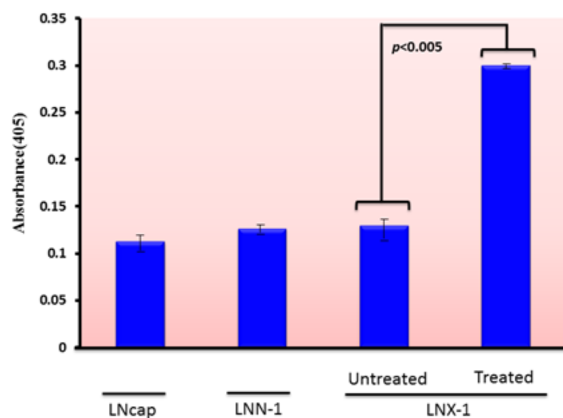


**Fig. 5:** Apoptosis induction by TGIFLX is shown in this figure. Treated LNX-1 compared with untreated LNX-1 and LNN-1, Lncap cells demonstrated a significant increase in caspase activity ($P$<0.05)

### *Protein localization*

Transfection of LNCaP cells with TGIFLX recombinant pEGFPN1 plasmid has revealed the GFP signaling from the nucleus which may suggest the TGIFLX localization is in the nucleus, while GFP expression of empty pEGFPN1 transfected was defined in the cytoplasm (Fig. 6).

## Discussion

Primary studies have shown that TGIFLX gene expresses only in adult testis which could be included in its role in spermatogenesis (8, 12). In this regard a study has shown that in non-obstructive azospermic testis samples, TGIFLX has not expressed compared with a normal person, which clarifies the relationship between the spermatogenesis and TGIFLX expression (9). In another study it has been shown that in prostate sample of patients with BPH (Benign Prostate Hyperplasia), TGIFLX gene is not expressing while in advanced stages of prostate cancer it has high level of expression.

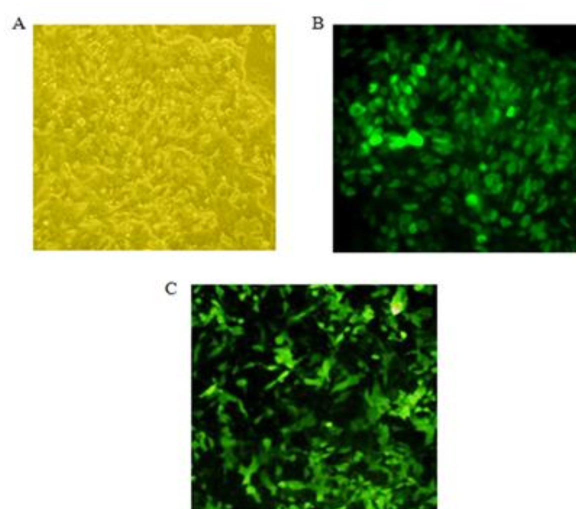


**Fig. 6:** Lncap cells Transfected with PGEFP-TGIFLX and PGEFP as empty plasmid (10x magnification). **(A)** PGEFP-TGIFLX transfected cells on light microscopy. **(B)** PEGFP-TGIFLX transfected cells on fluorescent microscope showing GFP signal (TGIFLX localization) in nucleus. **(C)** PEGFP transfected cells on fluorescent microscope showing GFP signal of empty vector (localization) in cytoplasm

It is suggested that TGIFLX has a role in the tumoregenesis path of prostate cancer (10). Based on the previous data, this study was conducted in order to find out the possible correlation of TGIFLX gene in the process of initiation and progression of prostate cancer. So a decision was made to study the effect of TGIFLX expression on apoptosis and cell death in a prostate cancer cell line.

Homeobox genes belong to a huge family of growth and differentiation regulatory genes with

various functions in embryogenic period (13). Several studies with regulation of these genes' function have shown that the expression of Homeobox proteins can have an inhibitory or activatory effect on cell cycle (14). However, a precise molecular mechanism which could explain discrepant activity of these genes is still unclear. For instance Msx, Msx1 and Msx2 have the capability to suppress the gene expression by interaction with main components of transcription (15) and studies have shown that Msx1 genes cause promotion of cell proliferation (16). Elevation of cell proliferation could decrease cell differentiation through cyclin D1 over expression. In fact, Msx1 prevents the skip from cell cycle through up regulation of cyclin D1 expression which eventually leads to cell proliferation (17).

On the other hand, expression of HOXA10 and P21, which induce differentiation in the primitive period of life, could be coordinated, inhibiting cell proliferation through binding HOXA10 on P21 promoter region and transcribing activatory function which leads to cell proliferation inhibition (18). In addition, another study has shown the role of HOX11 in growth and development of spleen by cell survival enhancement (19). It demonstrates the opposite role of Homeobox genes in cells which can cause cell proliferation and in some cases inhibition in a contrary direction.

TGIFLX belongs to Homeobox genes and could have an inhibitory role in proliferation of LNCaP which is a prostate cancer cell line with low grade of invasion. As it was mentioned before, TGIFLX belongs to TALE proteins and TGIF family. TGIF has several transcription inhibitory domains and suppressing transcription induced by TGF-β through different mechanisms. Transcription inhibition could happen through histon deacetylase dependent or independent mechanisms (20). TGIF interacts with HDACs through its carboxil terminal and has a PLDLS (Pro-Leu-Asp-Leu-Ser) motive at the amino acid end, which is necessary for the inhibitory role of CtBP (carboxyl terminus-binding protein). It leads to transcription down regulation in HDACs independent mechanism (21).

Among the several domains of TGIF, one of the suppressor domains (RD-2b) has a moderate homology with the same region in TGIF2 (22). Hence it seems that TGIF2 acts very similarly to TGIF. Therefore, it is likely that TGIF2 could be able to act as a transcription suppressor (21). Also, TGIF and TGIF2 have the capability of binding to Smads active complexes and suppress TGF-β related transcription. However, TGIF2 can only suppress transcription through HDAC independent recruitment due to lack of PLDLS motifs (23).

On the other hand, TGIF mutation that causes holoprosencephaly disease is due to loss of transcription suppressing ability of this gene. However, it is not clear how it can influence through TGF-β dependent or independent pathways. The findings show that TGIF and TGIF2 could compensate TGIF deficiency in the TGF-β dependent or independent pathways. Therefore, differences in the severity of the holoprosencephaly phenotype could suggest that some factors inside the cell can compensate TGIF deficiency (23).Given the above we can suggest that TGIF2 is a particular form of TGIF and is a transcription suppressor (23).

Moreover, TGIF and TGIF2 can down regulate transcription by direct binding to DNA (21). Also it seems that the c-terminal region of TGIF2 plays more determinative role in transcription repression compared to other regions (24). RD-2b region of the TGIF2c-terminal is also relatively protected in TGIFLX. So we can conclude that TGIFLX could participate in the transcription repression similar to TGIF2 (8).

The other data has shown the apoptotic of TGIFLX in LNCaP cells. Homeobox large family of genes is a potential regulator which has a role in the cell cycle, differentiation and cell morphology. One of these genes is HOXD10. Several studies have shown that the expression of this gene is down regulated in gastric cancer cells, while restoring the expression will cause cell survival inhibition, apoptosis induction, invasion decrement and thus, acting as a tumor suppressor (24).

Studies have shown that activation of Smad proteins through TGF-β pathway causes the expres-

sion of proteins which directly activates apoptosis in the cell. Activated genes in this pathway include the TGF-β-inducible early response gene-1 (TIEG1), GADD45b, and Bcl2 (25, 26). Each one of these genes reactivates the other genes and causes apoptosis.

In prostate cancer the role of TGF-β as an apoptosis inducer has been studied. For example, losing TGFβRI, RII activity or decreasing their expression is shown in prostate cancer. Also recent studies have shown high expression of TGF-βRII in LNCaP which was not sensitive to TGF-β due to cells response to TGF-β which leads to growth arrest and apoptosis induction (27, 28). Cells which responding to TGF-β show a decreased expression of bcl2 and induced caspase1 activity (29).

In a prostate cancer cell, increasing the level of bcl2 causes progression of tumour to androgen independency and poor prognosis (30, 31). Apoptosis occurring through caspase activity and activated caspase, could degrade proteins involved in structure, cell cycle regulation, DNA repair and cell function which in turn leads to releasing of cytochrome c from mitochondria (32, 33). On the other hand, caspase repressors such as bcl2 and bcl-xL could prevent apoptosis by inhibiting cytochrome c release (34, 35).

TGIF family are known as transcription suppressor through TGF-β. Like the other homeodomain, TGFILX could also act as both repressor and enhancer of transcription factor possibly through repression of genes involved in cell growth such as c-myc or genes which have a role in immortality, e.g. bcl2 which initiates the process of apoptosis in cells.

In this study, it was demonstrated that TGIFLX expression leads to apoptosis in LNCaP. Our findings show that in transfection of cells with vectors containing GFP and TGIFLX (TGIFLX-GFP), green signalling was observed in the nucleus under fluorescent microscopy. However, cells transfected with empty vectors containing GFP demonstrated expression in cytoplasm. These data suggest that TGIFLX protein is transported to the nucleus after gene expression which might represent a transcription factor.

As it was mentioned before, homeodomain proteins have a critical role in proliferation, development and cell destiny as a transcription factor (36). One of the homeodomain protein subgroups are TALE (three-amino-acid loop extension) (36). TGIF genes belong to TALE family proteins, including TGIF1, TGIF2, TGIFLX, TGIFLY (37). TGIF and TGIF2 have well-conserved homeodomain which suggests that the interactions with other proteins are similar in this region. Also the third helix in these two proteins are very similar and can suggest that they are binding to similar DNA sequences and in fact acting as a repressor transcription factor (24). Indeed, this area is involved in DNA recognition (8).

There are also evidences that TGIF2 can be localized in the nucleus which can indicate its action as a transcription factor (38). In addition, nucleus localization signaling which was predicted in TGIF2 sequence analysis by PSPORTII program existed in TGIF and TGIFLX and was replaced with similar amino acids. This region in TGIF2 includes RKRR which is located in N-terminal and RRRR and KKRK for TGIF and TGIFLX respectively. In fact all of these evidences indicate that TGIFLX is same as TGIF and TGIF2 in acting as a transcription factor and localized in the nucleus as demonstrated by this study.

## Conclusion

This study demonstrated a decrease in cell proliferation which could be the cause of transcription repression of genes involved in cell cycle pathway. Still, more investigations seem necessary to clarify this claim. In addition, apoptosis was enhanced in LNCaP inducing TGIFLX showing that this gene, just like the other proteins of homeobox superfamily, is involved in apoptosis pathway and could have a tumor suppression role in LNCaP cells which have a low level of tumorogenesis.

Studies have shown that nucleus localization is one of the important factors that all of transcription factors should have (39). Therefore, even

though In Silico studies have recommended that TGIFLX is coding a transcription factor, nuclear localization studies using GFP expressing vector has shown that this evidence is in need of more studies for confirmation. In conclusion, all data demonstrated that TGIFLX, same as the other homebox super-family, has a critical role in gene regulation evidences in LNCaP cancer cell line accompanied by a nuclear localization which may indicate a transcriptional activity role.

## Ethical considerations

Ethical issues (Including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

## Acknowledgement

The authors would like to thank Seyed Muhammed Hussein Mousavinasab for his sincere cooperation in editing this text. This study was supported by Cancer Research Center of Cancer Institute of Iran (Tehran University of medical Sciences). The authors declare that there is no conflict of interest.

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