ORIGINAL REPORT

Differential Expression of Human Homeodomain TGIFLX in Brain Tumor Cell Lines

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Abstract- Glioblastoma is the most common and the most lethal primary brain cancer. This malignancy is highly locally invasive, rarely metastatic and resistant to current therapies. Little is known about the distinct molecular biology of glioblastoma multiforme (GBM) in terms of initiation and progression. So far, several molecular mechanisms have been suggested to implicate in GBM development. Homeodomain (HD) transcription factors play central roles in the expression of genomic information in all known eukaryotes. The TGIFX homeobox gene was originally discovered in human adult testes. Our previous study showed implications of *TGIFLX* in prostate cancer and azoospermia, although the molecular mechanism by which TGIFLX acts is unknown. Moreover, studies reported that HD proteins are involved in normal and abnormal brain developments. We examined the expression pattern of TGIFLX in different human brain tumor cell lines including U87MG, A172, Daoy and 1321N1. Interestingly, real time RT-PCR and western blot analysis revealed a high level of *TGIFLX* expression in A172 cells but not in the other cell lines. We subsequently cloned the entire coding sequence of *TGIFLX* gene into the pEGFP-N1 vector, eukaryotic expression vector encoding eGFP, and transfected into the U-87 MG cell line. The TGIFLX-GFP expression was confirmed by real time RT-PCR and UV-microscopic analysis. Upon transfection into U87 cells, fusion protein TGIFLX-GFP was found to locate mainly in the nucleus. This is the first report to determine the nuclear localization of TGIFLX and evaluation of its expression level between different brain tumor cell lines. Our data also suggest that *TGIFLX* gene dysregulation could be involved in the pathogenesis of some human brain tumors.

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Keywords: Glioblastoma; Homeobox gene; Nuclear localization; TGIFLX

Introduction

Glioblastoma multiforme (GBM) is a fast-growing type of tumor that is the most common and most lethal primary brain malignancy in adults (1-4). This rare metastatic tumor is highly locally invasive (5) spreading into the brain tissue and placing cells outside the margin of therapeutic resection (6). A study reported that more than 22,000 Americans were estimated to diagnose with brain tumors in 2010 (7). Despite several genetic and cellular pathways have been suggested to implicate in GBM (4,8-13), still little is known about the distinct biology of glioblastoma development. Various molecular genetic approaches such as DNA microarray have proven to be very useful in identification of genes in brain tumor formation (13-15). It has been accepted that understanding of the molecular mechanism involved in tumorigenesis, in turn, would lead to the discovery of new therapeutic strategies based on particular tumor biology (6,14,16,17).

Homeobox genes encode homeodomain (HD), a 180-bp DNA sequence encoding a highly conserved 60 amino-acid domain, transcription factors and play vital roles in the expression of genomic information in all

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metazoans (18-23). These genes are classified into clustered and non-clustered groups (24). These transcriptional regulatory proteins modulate their target genes) through binding of their HD to a specific DNA target sequences (25).

TGIFLX gene originally discovered in human adult testes by comparative DNA analysis and encodes a member of the TALE/TGIF homeodomain family of transcription factors (26-28). TALE (three-amino-acid-loop-extension) superfamily is an ancient member of divergent homeodomain containing genes encodes proteins that have a 3-amino-acid insertion between helices 1 and 2 of the homeodomain, (24, 29-30).

The human TGIFLX is located on X-chromosome (Xq21.3) and suggested to be originated from the retrotransposition of TGIF2 about 80 million years ago [16]. The TGIFLX consists of two exons, the first exon contains the 5' untranslated region (5'UTR) while coding region being located within exon 2. The TGIFLX coding region is 726bp, which expresses an approximately 27kD protein of 241 aa (31). More recent study reported an aberrant expression of TGIFLX gene in some prostate tumors, but not in normal and benign prostate hyperplasia (32). Also, in the recent study by Aarabi *et al.,* (2008), it was reported a possible association TGIFLX/Y mRNA expression with human azoospermia (33).

Moreover, it has been well documented that homeobox genes are critical determinants of mouse and human brain development (34-35). These genes regulate the patterning of the forebrain along the dorsal/ventral and rostral/caudal axis and are also essential for the differentiation of specific neuronal subtypes. Peukert *et al*., explained that Lhx2 and Lhx9 determine neuronal differentiation in the forebrain by regulating Wnt signaling (36). Inhibitory interneurons that arise from the ganglionic eminences and migrate tangentially to the neocortex and hippocampus are dramatically affected by mutations in several homeobox genes. In human, mutations of homeobox genes expressed in the forebrain have been shown to result in mental retardation, epilepsy or movement disorders (37). The number of homeobox genes currently linked to human nervous system disease is surprisingly low, perhaps reflecting the essential functions of these genes throughout embryogenesis or the degree of functional redundancy during central nervous system development.

In this study, we describe the first report of the different expression pattern of *TGIFLX* in brain tumor cell lines as well as its subcellular localization.

Materials and Methods

Cell culture

Human tumor cell lines, U-87MG, A172, Daoy and 1321N1 were obtained from National Cell bank of Iran (NCBI) affiliated to Pasteur Institute (Tehran, Iran). The pathologic differentiation grades of the original tumors of the cell lines are shown in Table 1. These cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum with an appropriate penicillin and streptomycin (100 U/ml and 100ug/ml, respectively) in humidified CO_2 incubator.

Total RNA extraction and cDNA synthesis

Total RNA extraction was carried out using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. In order to eliminate any genomic contamination, total RNA was treated with on-column RNase-free DNase I. Then, RNA was dissolved in 30 µl of nuclease free elution buffer and stored at -70° C for further tests. cDNA was synthesized using 1 µg of total RNA as described previously (38). Samples with similar cDNA quality via GAPDH RT- PCR (Figure 1) were stored at -70°C for following investigation.

Table 1. List of cell lines and their characteristics (National Cell Bank of Iran, Pasteur Institute of Iran).

Designation	Gender	Morphology	Tumor	Reference
U-87MG	F	Epithelial-like	Malignant glioma, Grade IV	Ponten *et al.* (58)
A172	M	glial	Malignant glioma, Grade IV	Giard *et al.* (57)
1321N1	F	Glial-like	Malignant glioma	Ponten *et al.* (58)
Daoy	M	Polygonal adherent	desmoplastic cerebellar medulloblastoma	Jacobsen *et al.* (59)

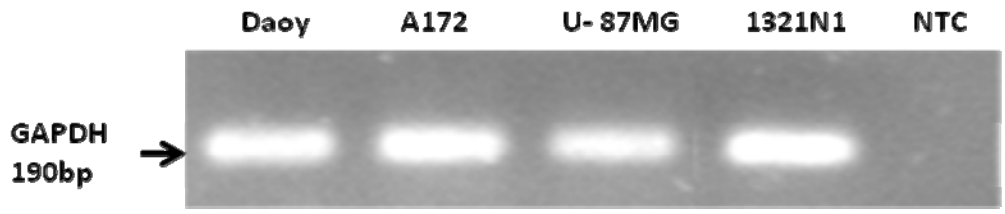

Figure 1. Gel electrophoresis of RT-PCR products for GAPDH gene from total RNA of Brain Cancer-Derived Cell lines. The quality and integrity of RNAs from four cell lines (Daoy, A172, U-87MG and 1321N1) were confirmed by RT-PCR of the GAPDH (190bp) housekeeping control gene. PCR products were resolved on a 1.8 % agarose gel stained with ethidium bromide. Non-Template Control (NTC) (ddH_2O).

Table 2. List of oligonucleotides that have been used in this study.

Primer	Sequence (5'----> 3')	PCR product size
GAPDH-F	CACCAGGGCTGCTTTTAAC	190
GAPDH-F	ATCTCGCTCCTGGAAGAT	
TGX-F	CAACAGTAACGATAAGCCTCTTG	164
TGX-R	AAGGCAAGAACTCTGCCTGTA	
TGX-GFP-F	CAGCAGTACAAAGGGCTGCCGA	133
TGX-GFP-R	AACAGCTCCTCGCCCTTGCTCA	
TGX-Bgl TGX-	CATGAGATCTATGGAGGCCGCTGCGGACGG	743
EcoRI	CATGGAATTCGTGGATTAGGCTCTTGCTTCT	

Analysis of TGIFLX mRNA levels by real-time RT-PCR

To verify the differential expression of TGIFLX gene in glioblastoma-derived cells real time RT-PCR was performed. The reaction mixture in real time RT-PCR contained 1µ of each primer (10pmoles/µl), 1µl of cDNA and 5 µl of 2× SYBR Green PCR Master Mix (SYBR *Premix Ex Taq* II, Takara). The primers are listed in Table 2. The amplification program included an initial denaturation at 95°C for 10 min, followed by 45 cycles of a two-stage PCR consisting of 95°C for 10 sec and 60°C for 30 sec. Specificity of primers was verified by observing a single peak in dissociation curve for each run. All the reactions were performed in triplicate. The absence of contamination was verified using non-template controls (NTCs).

Construction of pTGIFLX-EGFP-N1 vector and Establishment of SW48 cell line stably expressing TGIFLX

The TGIFLX coding sequence was amplified by specific forward and reverse primer that introducing 730 bp insert size (as shown in Table 2). The underlined sequences indicate the introduced *Bgl*II and *EcoR*I restriction endonuclease sites to facilitate cloning. TGIFLX coding sequence was digested with *Bgl*II and *EcoR*I and cloned into pEGFP-N1, eukaryotic expression vector. The obtained vector was sequenced, and after confirmation it was transfected into U-87 MG cells using lipofectamine 2000 (Invitrogen, Carlsbad, CA). An "empty" vector pEGFP-N1 was utilized as a negative control. After 24 to 48 hr, the transient transfection efficiency was determined using an Olympus fluorescence inverted microscope. The next day, cells were then cultured in the presence of 800 µg/mL G418 (Life Technology) for 21 days for selection of highly expressing GFP cells. Total RNA was isolated and quantitative RT-PCR performed to detect the mRNA level of TGIFLX in stably expressing cells as described above. Each sample was measured in triplicate manner.

Western blotting

Western blot analysis was conducted as previously described (39-40). Briefly, cell lysates for Western blotting were prepared from U-87 MG cells transfected either with the pEGFP-N1 (empty vector) or pEGFP-TGIFLX or in untransfected cells and U-87 MG cell line and Daoy, A172, and 1321N1 were subjected to 12% SDS–PAGE electrophoresis and transferred to nitrocellulose membrane (Amersham Biosciences, 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-TGIFLX 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) prior to the development with 4-chloro-1-naphthol (4CN) (Immun-Blot, Bio-Rad Laboratories, USA).

Statistical 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.

Results

TGIFLX expression in brain tumor cell lines

In order to explore TGIFLX expression levels in brain tumor, a panel of brain tumor cell lines was analyzed for TGIFLX expression by Western blotting and TGIFLX transcript level was determined by quantitative RT-PCR and western blotting. We found variable expression of *TGIFLX* in the tumor cell lines evaluated with highest expression in A172 cells but not in U87, Daoy and 1321N1 cells (Figure 2).

Subcellular distribution of GFP-TGIFLX fusion proteins

We sought to determine its cellular localization in U-87 MG. we cloned the entire coding region of *TGIFLX* into the pEGFP-N1 vector. The TGIFLX expression was detected by realtime RT-PCR and western blot analysis (Figure 3). Plasmids containing correct sequence were transfected into U-87 MG cells, which then express the TGIFLX-GFP fusion protein. Fluorescent microscopic study revealed that the TGIFLX-GFP mainly is localized to nuclei (Figure 4).

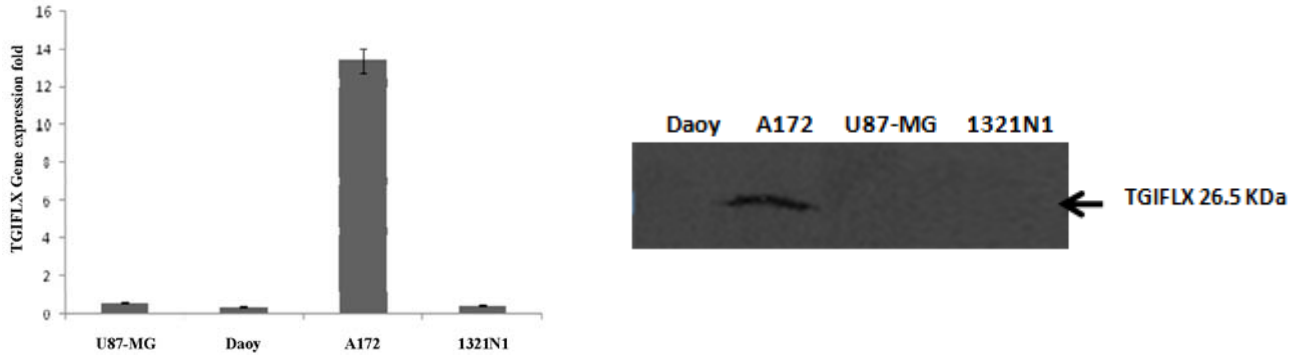

Figure 2. Expression of TGIFLX gene in Brain tumor cell lines. A. Real-time PCR result with specific TGIFLX primer. Each Real-time PCR was performed in triplicate and experiments were repeated twice with the mean±SD. B. Western blotting assay shows significantly over expression of TGIFLX (26.5KDa) in A172 cells in comparison with other brain tumor cell lines.

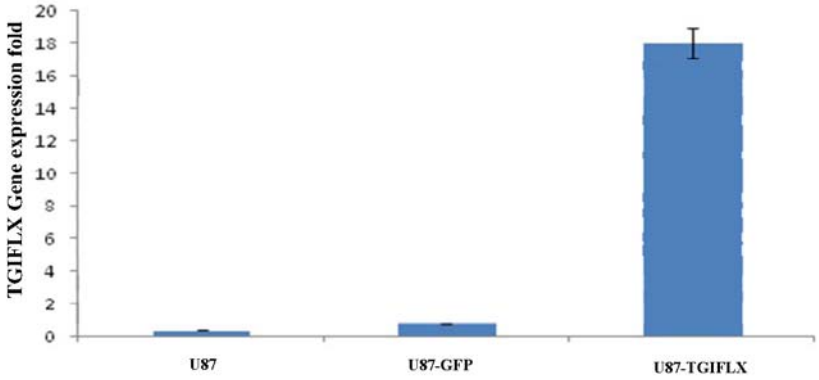

Figure 3. Expression of TGIFLX gene in stably expressing U-87 MG cell lines. Real-time PCR result with specific TGIFLX primer for fusion protein and the wild type. Each Real-time PCR was performed in triplicate and experiments were repeated twice with the mean±SD. Expression of TGIFLX mRNA.

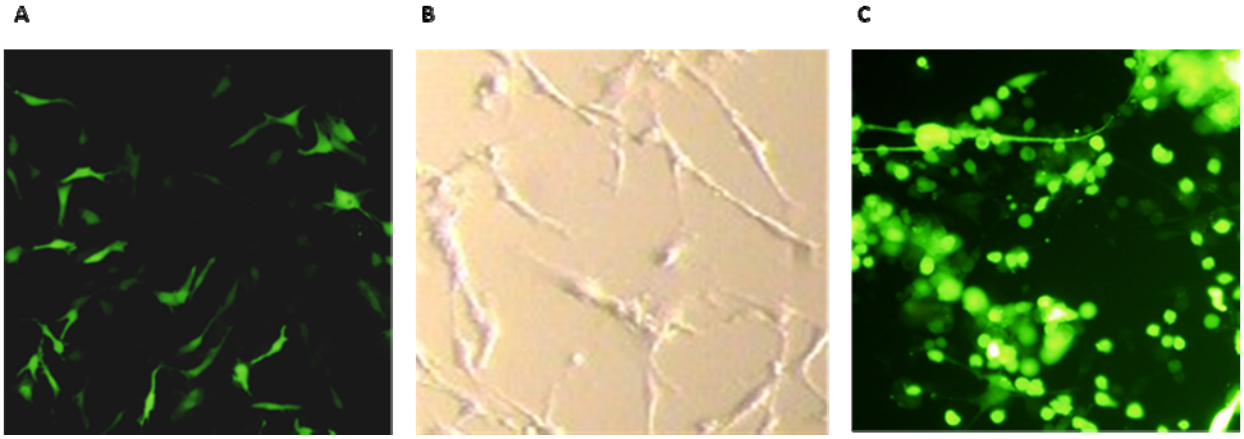

Figure 4. TGIFLX gene expression detected with fluorescent microscopy. U-87 MG cells 21 days after post-transfection. U-87 MG cells transfected with pEGFP-N1 (A), light microscope (B) and pEGFP- TGIFLX (C) under a fluorescent microscope (×20).

Discussion

Homeobox genes are a large family of regulatory genes implicated in the control of normal development, malignant transformation and progression of different types of tumors (18, 41-46). In spite of intensive efforts to delineate the expression profiles of HOX genes in other cell types, nothing is known regarding the TGIFLX expression profile in human astrocytes and astrocytomas. In this study, we analyzed the TGIFLX nuclear localization and its gene expression pattern in brain tumor cell lines. Our data showed that TGIFLX protein is almost exclusively localized to the nuclei. The U-87 MG has been reported to derive from a Caucasian female with grade IV malignant glioma. Although it likely appears that the TGIFLX protein has DNA-binding capacity, this ability still remains to be demonstrated.

Also, we analyzed the expression profile of the TGIFLX in U-87 MG, A172, Daoy and 1321N1, well-established brain tumor cell lines, using real time RT-PCR and western blot analyses. Our findings revealed differential expression of TGIFLX gene. The data showed that TGIFLX gene is differentially expressed in glioblastoma and astrocytoma cell lines suggesting a potential function for this gene in brain tumorigenesis. In spite of the fact that all the GBM cell lines were originally isolated from astrocytic tumors that were designated as GBMs, TGIFLX gene expression at a high level was detected in A172. However, the expression of TGIFLX in other brain tumor cell lines, Daoy, U-87 MG and 1321N cells was not detected. Several studies demonstrated that glioblastoma cells differentially express human homeobox genes (47-49). Abdel-Fattah et al (2006) studied the differential expression of HOX genes in neoplastic and non-neoplastic human astrocytes using RT-PCR, quantitative real-time PCR, immunocytochemistry and western blot analyses. They have shown that nine HOX genes (A6, A7, A9, A13, B13, D4, D9, D10, and D13) were differentially expressed in normal human astrocytic cell lines and non-neoplastic temporal lobe specimens (49). The data showed that multiple HOX genes were overexpressed in glioblastoma cell lines, astrocytomas (II/III), and glioblastoma multiform. Their findings suggested a role for these genes in brain tumorigenesis. They indicated an association of glioblastoma with deregulated HOX network in brain tumors. In agreement with previous reports, the TGIFLX like other homeobox genes might be implicated in brain malignant transformation at least in some cases (A172 cells). Moreover, we found the lack of expression of TGIFLX in other brain tumor cells including Daoy, U-87 MG and 1321N1 cell lines. The variable expression of TGIFLX in these cell lines might be depended on cellular context, genetic heterogeneity and epigenetic features (50). Vladimirova *et al.,* (2009) reported an aberrant methylation and reduced expression of LHX9 (human Lim-homebox 9) in malignant childhood gliomas (51). In this regard, apart from TGIFLX dysregulation in gliomas, several studies have also demonstrated an aberrant expression of clustered and non-clustered homeobox genes in a variety of human cancers including HOXA9, A10, and A13 in MLL and AML, CDX1and CDX2 in colorectal cancer, HOXA9 in lung cancer, HOXB7 in pancreatic cancer (52-56).

These important findings raise questions related to possible implication of TGIFX expression in brain tumorigenesis. 1) what then are the factors that cause the TGIFLX overexpression in A172 cells and lack of its

expression in U-87 MG cell lines? As we pointed out these cell lines have been classified with the same pathological grade (grade IV), but with different genders, A172 cell lines and U-87 MG had been characterized as female and male, respectively (57,58). One possibility is that dysregulation of TGIFLX in brain tumor cell line could be due to gene-gene interactions, gene-environment interactions or genetic background of each cell line. 2) what is the impact of variable TGIFLX gene expression in cancer? It is unlikely that a dysregulation of a single factor such as TGIFLX can entirely dictate the brain tumorigenesis. A plausible assumption would be that *TGIFLX* like the majority of homeobox genes function in terms of activation or repression downstream target gene(s) via direct or indirect modes of the action. Transcriptional regulation is mediated by selective recognition of DNA sequences (homeodomain) in nuclei. Given the potential role of TGIFLX in brain tumorigenesis, we sought to determine its cellular localization. Our investigations of exogenous TGIFLX distribution by GFP fluorescence revealed that the GFP-TGIFLX protein clearly accumulates in the cell nuclei. Thus, the TGIFLX protein could be actively transported to and can accumulate within the nuclei, implying that the protein (i) consists of a nuclear localization signal (NLS), and (ii) may be normally held in the cytoplasm (partially) of cells by either protein-protein interaction or posttranslational modifications.

We conclude that the dysregulation of TGIFLX might play critical roles in the pathogenesis of brain cancer. Our result indicated that TGIFLX predominantly localized to nuclei. Therefore, TGIFLX encodes a transcriptional regulatory protein. In order to define the pathogenic relevance of this HD-containing protein several molecular studies are required. For example, identification of TGIFLX potential target genes using cDNA-AFLP, chromatin immunoprecipitation assay, ectopic expression and gene silencing approaches could help to delineate its function. Also, for better understanding of its biological significance, discovery of TGIFLX protein partners using co-immunoprecipitation assay would be useful.

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