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Original article

In vivo identification of novel TGIF2LX target genes in colorectal adenocarcinoma using the cDNA-AFLP method

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ABSTRACT

Background and study aims: Homeobox-containing genes are composed of a group of regulatory genes encoding transcription factors involved in the control of developmental processes. The homeodomain proteins could activate or repress the expression of downstream target genes. This study was conducted to *in vivo* identify the potential target gene(s) of TGIF2LX in colorectal adenocarcinoma.
Methods: A human colorectal adenocarcinoma cell line, SW48, was transfected with the recombinant pEGFPN1-TGIF2LX. The cells were injected subcutaneously into the flank of the three groups of 6-week-old female athymic C56BL/6 nude mice (n = 6 per group). The transcript profiles in the developed tumours were investigated using the cDNA amplified fragment length polymorphism (cDNA-AFLP) technique.
Results: The real-time RT-PCR and DNA sequencing data for the identified genes indicated that the N-terminal domain-interacting receptor 1 (Nir1) gene was suppressed whereas Nir2 and fragile histidine triad (FHIT) genes were upregulated followed by the overexpression of TGIF2LX gene.
Conclusion: Downregulation of Nir1 and upregulation of Nir2 and FHIT genes due to the overexpression of TGIF2LX suggests that the gene plays an important role as a suppressor in colorectal adenocarcinoma.

Introduction

Homeobox genes contain a family of regulatory genes encoding transcription factors with a role in the regulation of developmental processes. This group of genes comprises a conserved sequence of 183 nucleotides, the homeobox, which encodes homeodomain, a 60 amino acid helix-turn-helix type of DNA-binding motif [1,2]. Homeodomain proteins are greatly conserved in evolution and are regularly found in animals, plants, and fungi. These proteins have been reported to regulate various embryonic developmental processes such as axis formation, limb development, and organogenesis [3]. A number of gene alterations in homeobox genes have been established to be involved in diseases such as neuroblastoma, leukaemia, and cancer [4–7]. Homeodomain proteins are categorized into diverse classes on the basis of their sequences and conserved domains [5]. The three-amino acid loop extension (TALE) superclass consists of members contains three extra residues between helix 1 and helix 2 of the homeodomain [8]. Some TALE

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homeodomain proteins function as cofactors for the HOX protein clusters are involved in patterning along the anterior-posterior body axis [9,10]. The TALE superclass is classified into four classes in animals: PBC, MEIS, TGIF, and IRO (Iroquois) [4]. TG-interacting factors (TGIFs) comprise a family of TALE-homeodomain proteins that contains several members. Four TGIFs in human are TGIF, TGIF2, TGIF2LX, and TGIF2LY (TGIF2 like on X or Y chromosomes). Previous studies on cultured cells demonstrated that TGIF2 acts as a transcriptional repressor and antagonizes TGF-beta-activated gene expression [11,12]. Moreover, the *tgif2* gene was found to beamplified and over-expressed in various ovarian cancer cell lines [13]. Whether and how it is implicated in the regulation of cell proliferation and its molecular mechanism is not fully known. This study was conducted to identify the possible target gene(s) of TGIF2LX in colorectal adenocarcinoma using cDNA-AFLP, a gene expression analysis assay that is not required prior to gene sequencing.

Materials and methods

Cell culture

Human colorectal adenocarcinoma cell line, SW48, was acquired from the National Cell Bank of Iran (NCBI) affiliated to the Pasteur Institute (Tehran, Iran). The cell line was cultured in RPMI-1640 medium (Gibco, Germany) supplemented with 5% fetal bovine serum (FBS) (Gibco, Germany), 0.03% L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (Gibco, Germany) at 37 °C in a 5% CO2 atmosphere, as previously described [14,15].

Cloning of TGIF2LX gene in an expression vector

After amplification of TGIF2LX coding sequence, the PCR product was purified and cloned into pEGFP-N1, a eukaryotic expression vector. The recombinant pEGFPN1-TGIF2LX was transfected into SW48 cells using X-tremeGENE siRNA Transfection Reagent (Roche, Germany). An empty vector pEGFP-N1 was employed as a mock control. Following 48 h, the transient transfection efficiency was evaluated by a UV-fluorescent microscope (Olympus, Japan). The transfected cells were next cultured in the presence of 400 μg/ml G418 (Neomycin antibiotic) (Life Technology, California, USA) for 21 days to select the cells with overexpressing GFP (a marker protein).

Animal study

An animal study was completed as previously explained [16]. Briefly, 6-week-old female athymic C56BL/6 nude mice (n = 6 per group) were obtained from the Omid Institute for Advanced Biomodels (Tehran, Iran). All animal experiments were conducted according to to the Ethical Committee of Tehran University of Medical Sciences (ethics code: ERC/S/277). After cell culture, 5×10^6 cells were transfected with the pEGFP-N1 (empty vector) and pEGFP-TGIF2LX. The transfected cells and untransfected cells were injected subcutaneously at a 200 μl volume of the serum-free medium into the flank of the three groups of animals. After three weeks and confirmation of tumour growth, the mice were anesthetized and the tumours were completely isolated.

RNA extraction, Real-time RT-PCR and western blotting

Total RNA was extracted by TriZol isolation reagent (Invitrogen, USA) according to the manufacturer's instructions. Total RNA was DNase-treated with DNase I)Invitrogen, USA) and 5 μg of total RNA was used to synthesize cDNA fragments using random sequence hexamer primers and Oligo(dT) with a cDNA synthesis system kit (Vivantis, USA), following the manufacturer's protocol. The quality of cDNA was checked using the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) RT- PCR.

Real-time RT-PCR was performed to verify the expression level of TGIF2LX gene in colorectal adenocarcinoma cells. The reaction mixture contained 1 μl of each primer (10 pmol/μl), 1 μl of cDNA and 5 μl of 2 × SYBR Green PCR Master Mix (SYBR Premix Ex Taq II, Takara). The primers are listed in Table 1. The amplification plan 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 s and 60 °C for 30 s. Specificity of primers was verified by observing a single peak in the dissociation curve for each run. All the reactions were done in triplicate.

In order to evaluate the expression level of TGIF2LX protein, Western blot analysis was conducted. Briefly, cell lysates for western blotting were prepared from SW48 cells transfected with the pEGFP-N1 (empty vector), pEGFP-TGIF2LX transfected cells, and untransfected cells. Afterward, SW48 cell lines were subjected to 10% 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 incubated with a 1:500 dilution of a polyclonal rabbit anti-TGIFLX antiserum (Santa Cruz Biotechnology, Santa Cruz, USA) for 2 h at room temperature. The membranes were incubated with a goat HRP conjugated anti-rabbit IgG secondary antibody (1:5000) for 1.5 h prior to the development with 4-chloro-1-naphthol (4CN) (Immun-Blot, Bio-Rad Laboratories, USA).

Total RNA extraction and cDNA synthesis

Total RNA of tumours transfected with pEGFPN1-TGIF2LX and empty vector was extracted by the TriZol isolation reagent (Invitrogen, USA) according to the manufacturer's instruction. cDNA was synthesized using 1 μg of total RNA, as described previously [17,18].

cDNA amplified fragment length polymorphism (cDNA-AFLP)

The summary of the cDNA AFLP strategy is shown in Fig. 1. Double strand cDNA (dscDNA) synthesis was carried out using DNA polymerase I enzyme (Thermoscientific, USA) at 16 °C for 2 h. The synthesized dscDNA was purified and quantified by spectrophotometry and gel agarose electrophoresis and subjected to restriction enzyme digestion. About 5 μg of dscDNA was digested with 5 U EcoRI restriction enzyme (Fermentas, Burlington, Canada)

Table 1
Oligonucleotides used in this study.

Oligonucleotides	Name	Sequences
cDNA AFLP Adaptors and Primers	AdL EcoR 1	5′-ACCGACGTCGACTATCCATGAAG-3′
	AdS EcoR 1	5′-AATTCTTCATGG-3′
	Pre-EcoR 1	5′-ACCGACGTCGACTATCCATGAAGAATTC-3′
	S1 EcoR 1	5′-ACCGACGTCGACTATCCATGAAGAATTCC-3′
	S2 EcoR 1	5′-ACCGACGTCGACTATCCATGAAGAATTCG-3′
	S3 EcoR 1	5′-ACCGACGTCGACTATCCATGAAGAATTCA-3′
	S4 EcoR1	5′-ACCGACGTCGACTATCCATGAAGAATTCT-3′
Target Genes	Nir1-F	5′-TATCGGATCGCCAAGTTGCT-3′
	Nir1-R	5′-TTGCAGAATCACCCCAAAGC-3′
	Nir2-F	5′-GTAGAACTGAACATCGTGGCCG-3′
	Nir2-R	5′-CCACATAGCCGTCTGACAGGA-3′
	FHIT-F	5′-ATCATGGAGGCCATGGAACAC-3′
	FHIT-R	5′-CATTCTTCAGCATGTGCGTGG-3′
Housekeeping Gene	GAPDH-F	5′-CACCAGGGCTGCTTTTAAC-3′
	GAPDH-R	5′-ATCTCGCCTCCTGGAAGAT-3′