

Original Article

Running Title: Human Homeobox TGIFLX Regulates Colorectal Cancer

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Human Homeobox TGIFLX Regulates CDX1, CDX2, and OCT1 genes Expression in Colorectal Cancer Cell Lines

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Abstract

Background: Homeodomain transcriptional regulatory proteins, which are encoded by Homeobox (HOX) genes, play critical roles in both normal development and carcinogenesis. Previous studies have shown that the expression of HOX genes is deregulated in numerous tumors and this expression is specific to each cancer based on the arising embryonic origin tissue and the site of tumor.

Method: In this in vitro study, the expression levels of HOXA10, CDX1, CDX2, TGIFLX, TGIFLY, and OCT1 genes were compared across 10 different human colorectal cancer cell lines with different differentiation stages. Subsequently, the effect of TGIFLX siRNA-mediated knockdown on the expression levels of CDX1, CDX2, and OCT1 genes was analyzed in SW948 cell line.

Results: The obtained results revealed that these homeobox genes were differentially expressed in different colorectal cancer cell lines. Furthermore, the siRNA-mediated knockdown of TGIFLX led to higher levels of CDX1, CDX2, and OCT1 expression.

Conclusion: Our data suggested that TGIFLX plays an important role in the upstream regulation of CDX1, CDX2, and OCT1 genes.

Keywords: Colorectal neoplasms, Homeobox genes, RNA, Small interfering, Mutation

Introduction

Based on the recent reports of cancer statistics in the USA, colorectal cancer (CRC) is estimated as the third leading malignancy regarding the incidence of new cases and cancer-related deaths.¹ Recent advances regarding the understanding of the

molecular mechanisms involved in tumorigenesis has resulted in new improvements in early detection and treatments of early-stage cancers, by which the CRC mortality and morbidity rates have significantly reduced.² The vast majority of CRCs arise from CRA, which later progress

to adenocarcinomas through a process called adenoma-carcinoma sequence using distinct genetic pathways.³ Malignant transformation of adenoma to CRC thus makes available a very useful paradigm for studying the molecular genetics of cancer.⁴ Moreover, CRC provides an important system to investigate genetic events associated with multiple steps of tumorigenesis. In fact, it is one of the most widely studied epithelial tumor types at both cytogenetic and molecular levels.⁵⁻⁷ In spite of the growing body of evidence about different aspects of colorectal cancer, our knowledge of the perturbations of gene expression, which occurs in colorectal tumor development and/or progression, remains a major challenge in CRC research.

In general, at least two classes of genes, including tumor suppressor genes and oncogenes, are involved in CRC tumorigenesis due to loss of function and activation, respectively.⁸ Several studies have shown that homeoproteins mostly play important roles as transcription factors (TF) in organogenesis and in the development of human cancers.^{9,10} These homeoproteins contain a highly conserved DNA-binding domain, the homeodomain, and are key components of master regulatory pathways.¹¹ The homeobox genes are classified into clustered and non-clustered groups. The first group of homeobox genes, namely the HOX (homeobox) genes, contain a 180-bp DNA sequence (homeobox) encoding a highly conserved 60 amino-acid homeodomains. The HOX genes regulate their target gene(s) via binding of their HD to a specific DNA target sequences.^{12,13} One group of non-clustered homeobox genes encode proteins, called TALE group, has a 3-amino-acid insertion between helices 1 and 2 of the homeodomain, resulting in a 63-amino acid homeodomain.^{14,15}

TGIFLX (TGFB-induced factor homeobox 2 like X-linked) gene, which encodes a

member of the TALE/TGIF homeodomain family of transcription factors, is located on X-chromosome and thought to have originated from the retrotransposition of TGIF2.¹⁶ CDX1 (caudal-related homeobox protein 1) and CDX2 (caudal-related homeobox protein 2) homeobox genes encode DNA-binding proteins that regulate intestine-specific gene expression and play important roles in intestinal cell proliferation and differentiation and in CRC development.¹⁷⁻¹⁹ In addition, human OCT1 (Organic cation transporter 1), a member of the POU homeodomain family, is ubiquitously expressed in adult tissues. The overexpression of OCT1 has been detected in several cancers, such as gastric carcinomas.²⁰⁻²² Moreover, it has been revealed that inappropriate expressions of clustered and non-clustered homeobox genes is associated with malignancies, including brain, prostate, kidney, blood, colon, and bladder cancers, compared to normal tissues from which they were derived.^{10,23,24} Aberrant expression of the Hox genes have been reported in many types of human cancers; these genes could act as either oncogenes or tumor suppressors.²⁵ These genes have many important roles in the regulation of apoptosis, differentiation, signaling pathways, cell motility, and angiogenesis.²⁶

Materials and Methods

Cell culture

In the present in vitro study, the human colon cancer cell lines, Caco-2, SW48, SW1116, SW948, SW742, HT-29, LS180, LS174T, HCT116, and HT29/219, were obtained from National Cell Bank of Iran (NCBI) affiliated to Pasteur Institute (Tehran, Iran). Table 1 represents different pathologic grades for original tumors of all the mentioned cell lines. These cell lines were cultured in RPMI 1640 medium (Biosera, Sussex, UK) supplemented with

10% heat-inactivated Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (Life Technologies, Darmstadt, Germany) at 37°C in a humidified atmosphere with 5% CO₂.

RNA extraction and cDNA synthesis

We carried out total RNA extraction using RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In order to eliminate any genomic contaminations, total RNA was treated with RNase-free DNase I on RNeasy Mini columns. Afterwards, RNA was dissolved in 30 µl of DEPC-treated water and stored at -70°C for downstream experiments. cDNA was synthesized using 1 µg of total RNA, as described previously.²⁷ The cDNA qualities of the samples were confirmed via GAPDH1 RT-PCR (Reverse transcription polymerase chain reaction). The samples were stored at -20°C for subsequent Q-PCR.

Semi-quantitative RT-PCR

Specific primer sets for expression evaluation in this study were designed utilizing Primer3,²⁸ which are presented in Table 2. Semi-quantitative RT-PCR was conducted as described previously.²⁹

Quantitative expression analysis of mRNA levels

To evaluate the expression levels of CDX1, CDX2, OCT1, and TGIFLX genes in normal tissue and colon cancer-derived cells, we used the real-time RT-PCR. The reaction mixture in real-time RT-PCR contained 1 µl of each primer (10pmoles/µl), 1 µl of cDNA, and 5 µl of 2× SYBR quantitative real-time RT-PCR in a Rotor-gene 6000 instrument (Corbett life science, Sydney, Australia) employing SYBR Premix ExTaqII (TAKARA, Japan). Relative gene expression changes between different samples were determined applying the $\Delta\Delta C_t$ method in which the GAPDH gene was used as internal reference control.

Mutation screening

To address the existence of mutations in the TGIFLX coding sequences of TGIFLX in SW948 cells, the whole TGIFLX coding sequence in SW948 cells was screened via direct Sanger sequencing. In order to conduct TGIFLX mutation screening, the genomic DNA was extracted from SW948 cells. The TGIFLX coding sequences were then amplified with PCR and direct Sanger sequencing was performed on amplified products. Sequencing data were searched using the BLAST tool against the non-redundant nucleic and protein databases (<http://www.ncbi.nlm.nih.gov/BLAST>).

siRNA transfection

The siRNAs against the coding sequence of TGIFLX gene (NM_138960) were designed with the Ambion siRNA search engine (http://www.ambion.com/techlib/misc/siRNA_finder.html). After evaluation of gene suppression potential of siRNAs, TGIFLX-si2 (5'-CAAGUUUCCCUUGCGCUUCUU-3') was selected for RNA interference. The scramble sequence was designed according to the TGIFLX-si2 sequence (5'-GAAACGUGCAGCGAAAGUAGA-3'). Typically, 24 hours prior to each transfection experiment, 1.5×10^5 SW948 cells were seeded in a 6-well plate, in complete RPMI-1640 without antibiotics. We performed the transfection experiments utilizing Lipofectamine® 2000 Transfection Reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Following the transfection, the cells were cultivated in standard conditions and collected at time point of 36 h. Total RNA was then isolated and cDNA synthesis were performed as described in the above section. Furthermore, as the control, the scramble was employed with the same transfection conditions.

Statistical analysis

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

Results

Total RNA was isolated from CRC cell lines and cDNA was synthesized. The integrity of cDNA in samples was initially examined using GAPDH primer in an RT-PCR experiment (Figure 1a). The quantitative real-time PCR with the GAPDH, as the reference gene, was performed in the Corbett (Corbett Life Science, Australia) instrument. Therefore, the mRNA expression levels of TGIFLX, CDX1, CDX2, and OCT1 in CRC cell lines were compared to those of Caco2 cells, a well-differentiated cell line, via semi-quantitative (Figure 1b) and real-time RT-PCR (Figure 2).

mRNA expression of TGIFLX gene in CRC cell lines

The Q-PCR showed a great discrepancy of TGIFLX mRNA expression among CRC cell lines in comparison to Caco2 cells (Figure 2a). Generally, except for SW948 and SW48 cells, all the other CRC cell lines showed a significant reduction (downregulation) of TGIFLX expression and for SW948, the expression level was much higher. Although a variable TGIFLX mRNA expression pattern has been shown in colorectal cell lines, we were unable to find any relationships between TGIFLX and cellular differentiation status of cell lines; for example, the TGIFLX expression in the SW948 and SW1116 cell lines, as human colon carcinoma cells (grade III), was higher than that in others with different cellular differentiation conditions (Figure 3A). To address the existence of any mutations in the

TGIFLX coding sequences of SW948 cell line, the whole TGIFLX coding sequence in SW948 cells was screened using direct Sanger sequencing; we detected no mutations in the coding sequences (data not shown).

mRNA expression of CDX1 gene in CRC cell lines

A variable expression of the CDX1 mRNA was shown in CRC cell lines. The mean level of CDX1 mRNA expression was again significantly higher in SW948, SW1116, LS180, and LS174T cell lines than that in Caco2 cells. In contrast, we found the lowest level of CDX1 transcript in SW48 and in SW742 cell lines compared to Caco-2 (Figure 2).

mRNA expression of CDX2 and OCT1 in CRC cell lines

The expression analysis of CDX2 and OCT1 indicated lower expression of both genes in all the cell lines compared to the Caco2 cell line. For CDX2, the least expression levels were detected in HT29, HT29/219, and the highest levels belonged to SW1116 compared to Caco2 (Figure 2c). The OCT1 expression patterns in CRC cell lines SW48 and SW948, which is characterized by the same cellular differentiation condition, had the highest level of OCT1 mRNA expression compared to Caco-2; meanwhile, the lowest expression level was observed in SW1116 cells (Figure 2d).

Expression of TGIFLX target genes following siRNA-mediated TGIFLX silencing

A concordant overexpressed TGIFLX, OCT1, CDX1, and CDX2 genes was detected in five CRC cell lines, namely SW948, SW1116, LS180, LS174T, and HT29/219 cells whereas discordant CDX1 and CDX2 expression patterns were detected in the other lines. In order to find any relationships between TGIFLX and other homeobox genes, we performed a knockdown experiment assay of TGIFLX

gene. The SW948 cell line was transfected with TGIFLX siRNA and scrambled siRNA. To assess the efficiency of silencing, expression of TGIFLX was monitored with Q-PCR after transfection. Approximately 94% silencing of TGIFLX transcript was achieved in SW948 cell line compared with scrambled siRNA and untransfected SW948 cell line (Figure 3a). Silencing TGIFLX resulted in a 90%, 87%, and 36% decrease in CDX2, CDX1, and OCT1 expressions in SW948 cell line, respectively (Figure 3b-d). All the changes listed were specific to TGIFLX silenced cells and the cells transfected with scrambled siRNA or untransfected cells did not show similar trends.

Discussion

In the present study, we tried to elucidate whether differential expressions of four homeobox genes CDX1, CDX2, OCT1, and TGIFLX genes are involved in the development and progression of CRC. We demonstrated that TGIFLX, CDX1, CDX2, and OCT1 are differentially expressed in colon carcinoma cell lines. In particular, we found significant down-regulation of TGIFLX in all CRC cell lines. In contrast, a higher level of TGIFLX expression was detected in SW948 cell line, which is poorly differentiated (grade III). We assumed that the TGIFLX expression decreases with tumor progression and that it may act as a tumor suppressor in the majority of cell lines. We also observed a concordant and/or discordant overexpression and down-regulation of homeobox genes in CRC cell lines; concordant overexpression of TGIFLX and CDX1 genes was particularly detected in SW948 cells. Moreover, we revealed discordant CDX1 and CDX2 expression patterns in 50% of CRC cell lines.

Several studies have suggested that deregulation of homeobox genes could be

involved in abnormal development and tumorigenesis.^{25,30} We previously described that an abnormal expression of human TGIFLX gene was associated with prostate cancer male infertility.^{17,28} Our work is not the first one showing that altered expression of human homeobox genes indicates implications in the physiopathology of colorectal cancer. To date, the inappropriate mRNA expression of CDX1 and CDX2, and OCT1 has been previously reported in human colon tumors.^{19,31,32} Mallo et al. (1998) analyzed the expression of CDX1 and CDX2 genes in colon cancer-derived cells via RT-PCR. They suggested that the decrease in both CDX1 and CDX2 expressions is associated with tumor development and invasiveness in colorectal tumors by independent pathways.³³ Our results are not in agreement with certain claimed evidence on consistent down-regulation of these genes in CRC cell lines. We found the overexpression of CDX1 gene in a significant proportion of cell lines (30%), including SW948, LS174T, SW1116, and LS180 cells. However, in agreement with previous publications, our results confirmed that CDX2 mRNA expressions are either absent or downregulated in all the studied cell lines. There are available data supporting the fact that down-regulation and/or up-regulation of homeobox genes are involved in the process of cancer genesis. The inactivation of CDX1 in CRC development suggests a tumor suppressor function for this gene;^{19,20} in certain cases, it acts as an oncogene.^{18,34} Wong et al. (2004) studied the loss of CDX1 mRNA expressions in 37 CRC cell lines and found that the inactivation was attributable to promoter methylation and neither mutation nor loss of heterozygosity (LOH).³⁵ Similarly, the gain and loss-of-function study of CDX1 gene on mouse models in intestinal development and

cancers suggested that its overexpression could increase malignancy in early stages of tumorigenesis.³⁶ Another study implied the overexpression of CDX1 gene in more than one-third of CRC specimens.¹⁸ It has been well documented that the majority of homeobox genes that encode homeodomain proteins act as transcription factors which bind to their DNA target sequences containing AT-rich sequences.³⁷ Studies have shown that cellular target genes of these transcription factors are best understood in the context of interacting partner proteins which form transcriptional complexes assembled on gene promoters and/or enhancers. Soubeyran et al. (2001) determined that CDX1 regulates the Ras, Rho, and PI3-kinase pathways, leading to malignant transformation of intestinal epithelial cells in mice.³⁸ Furthermore, it has been demonstrated that mutation and methylation are involved in the gene regulation of homeobox genes; for example, promoter methylation plays critical roles in the CDX genes inactivation.³⁹

In addition, in our study, we observed the low levels of CDX1 and CDX2 transcripts in HCT116 and HT29 cell lines, yet none of CDX1 and CDX2 mRNAs were detected in HT29/219 cells. We also found altered regulation of the OCT1 gene among CRC cell lines. Expression of OCT1, a member of the POU homeodomain family, is important for differentiation and tumorigenesis. The overexpression of OCT1 has been detected in several cancers, such as gastric carcinomas,²³ endocrine-related pituitary, and breast tumors. It has been revealed that OCT1 induces the activation of oncogene cyclin D1 expression. It also activates H2B and snRNA genes which are essential for cell cycle regulation.⁴⁰ Zhou et al. (2008) studied the OCT1 expression in 71 colorectal tumors with matched normal tissues. They found that OCT1 was overexpressed in the majority of tumor

samples. Additionally, they suggested that OCT1 is involved in cell proliferation and tumor progression through transactivation of hPTTG1 transcription, which is a key component of the spindle checkpoint pathway.²⁰ As mentioned earlier, the highest level of TGIFLX mRNA belonged to SW8 cell line. To test whether TGIFLX variants are involved in the overexpression, the entire coding sequences of TGIFLX were screened, yet no mutations was observed. It seems as though other genetic and epigenetic factors could be involved in the up-regulation of TGIFLX gene in SW948 cells. Interestingly, we observed that TGIFLX overexpressing cell line, SW948 cell line, displayed a higher level of CDX1, CDX2, and OCT1 compared to Caco2 cells. We hypothesized that TGIFLX might be involved in the regulation of CDX1, CDX2, and OCT1. To address this, we evaluated the expression CDX1, CDX2, and OCT1 genes after silencing siRNA-mediated TGIFLX. Our results revealed that TGIFLX siRNA-mediated knockdown led to considerable down-regulation of CDX1, CDX2, and OCT1 genes. According to these results, TGIFLX seems to play a key role in CRC development via regulation of CDX1, CDX2, and OCT1 genes, which are almost associated with cellular differentiation and tumorigenesis. However, direct or indirect effects of TGIFLX on CDX1, CDX2, and OCT1 genes need to be further clarified. Different techniques, such as chromatin immunoprecipitation assay (ChIP Assay) and protein-protein interaction, employing specific antibodies, could be useful strategies for identification of their downstream target genes and protein partners, respectively.

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Conflict of Interest

None declared.

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Table 1. List of colon cancer derived cells and differentiated classification

Cell line CRC	Differentiation status (Duke's Class)
Caco2	+++*
SW1116	+++
HT29	++**
SW948	++
LS174T	++/+++
LS180	++/+++
SW48	+***
HCT116	++/+
SW742	+/>++
HT29/219	NC****

* differentiated; ** Moderately differentiated; *** Poorly differentiated; **** NC: Not characterized CRC: Colorectal cancer

Table2. List of real-time PCR primer sets used in this study

Primer	Forward Primer	Reverse Primer
GAPDH	5' CACCAGGGCTGCTTTTAAAC 3'	5' ATCTCGCCTCCTGGAAGAT 3'
CDX1	5' TCGGACCAAGGACAAGTACC 3'	5' CTGTTGCTGCTGCTGTTTCT 3'
CDX2	5' -CAGTCGCTACATCACCATCC-3'	5' -TTTCCTCTCCTTTGCTCTGC-3'
TGX	5' -CAACAGTAACGATAAGCCTCTTG-3'	5' -AAGGCAAGAACTCTGCCTGTA-3'
OCT-1	5' -TGGCACCCCTCACAGTTTG-3'	5' -TTGCTGGTGAGCAGAGAGAG-3'

PCR: Polymerase chain reaction

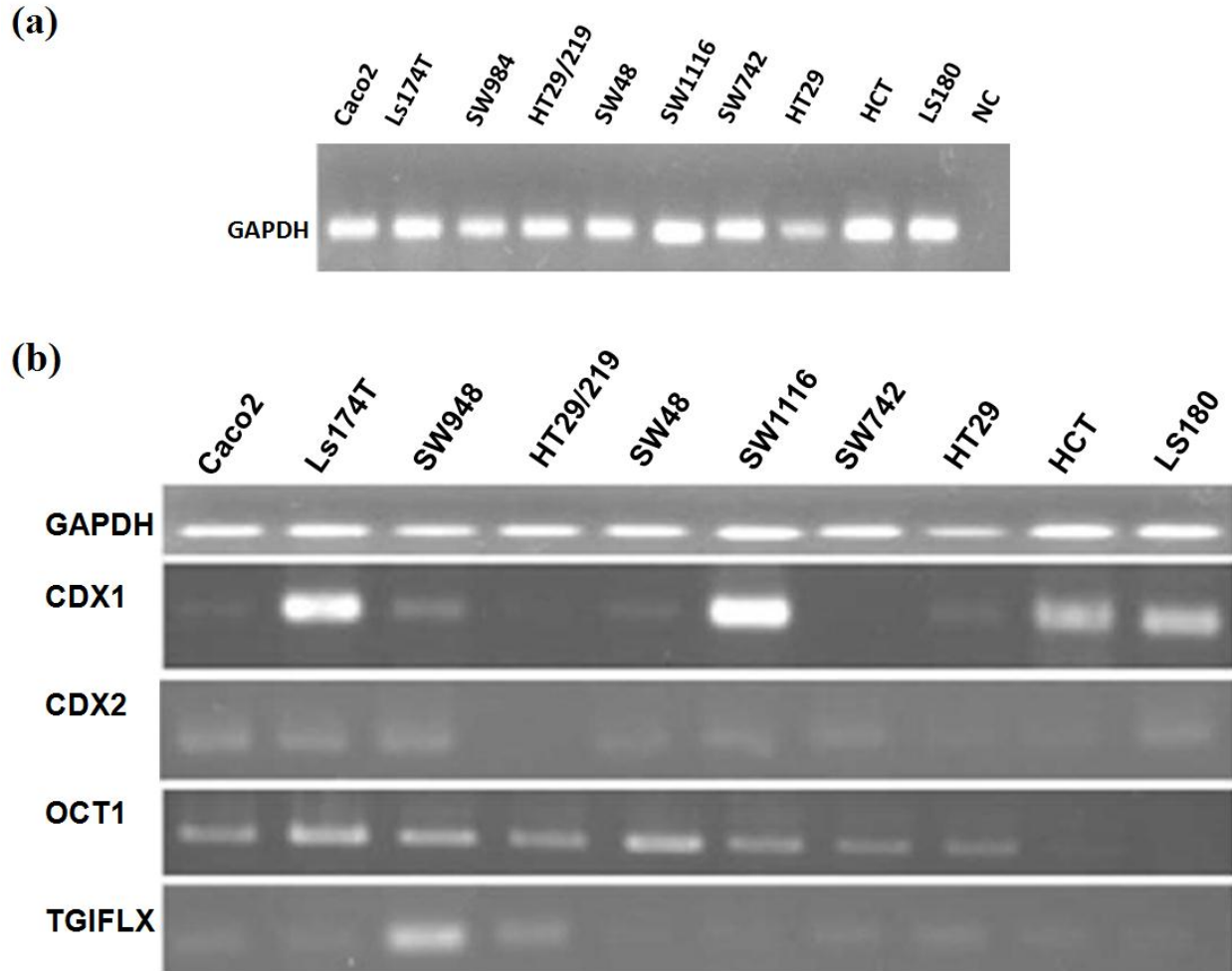


Figure 1. Evaluation of mRNA expression of TGIFLX target genes in colorectal cancer cell lines with semi-quantitative RT- PCR technique on a 2% agarose gel, stained with ethidium bromide. a) the cDNA quality control before real-time PCR was performed, and the quality and integrity of prepared cDNAs from 10 CRC cell lines (Caco2, Ls174T, SW948, HT29/219, SW48 SW1116, SW742, HT29, HCT1116 and LS 180) were confirmed via RT-PCR of the *GAPDH* (191bp) gene. (NC is negative control which ddH₂O was added to the PCR reaction instead of cDNA. b) Semi-Quantitative RT-PCR of *CDX1* (211bp), *CDX2* (109bp), *OCT1* (257bp), and *TGIFLX* (160bp) transcripts in CRC cell lines.

RT- PCR: Reverse transcription polymerase chain reaction; PCR: Polymerase chain reaction

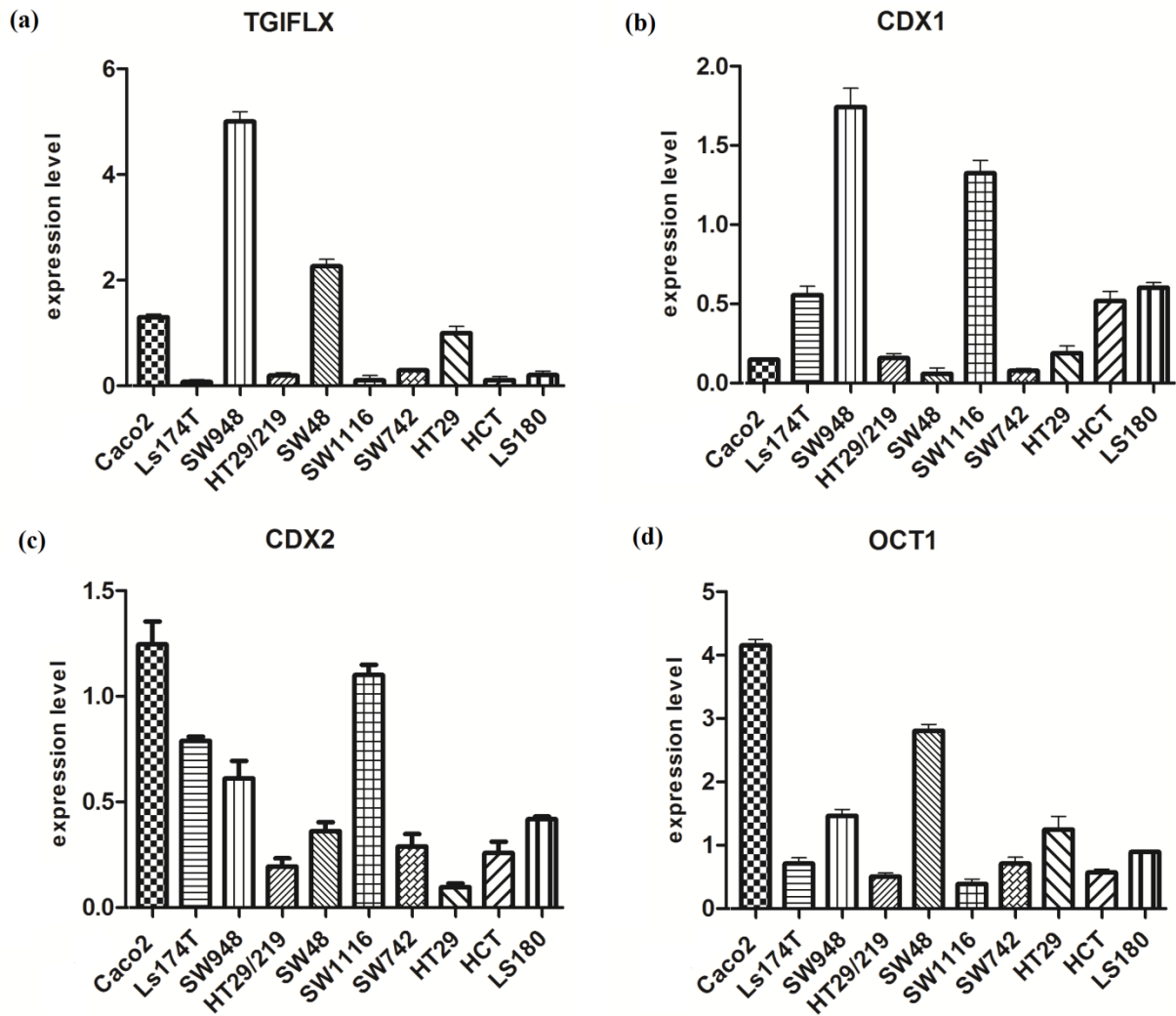


Figure 2. Evaluation of mRNA expression levels of *TGIFLX* target genes in colorectal cancer cell lines with real-time PCR method. a) expression of *TGIFLX* mRNA was highest in SW948 cell line compared to Caco2 cell line. b) expression of *CDX1* gene. c) expression of *CDX2*, d) expression *OCT1* genes. The expression of all the studied genes were performed in triplicate and the experiments were repeated twice with the mean \pm SD. PCR: Polymerase chain reaction

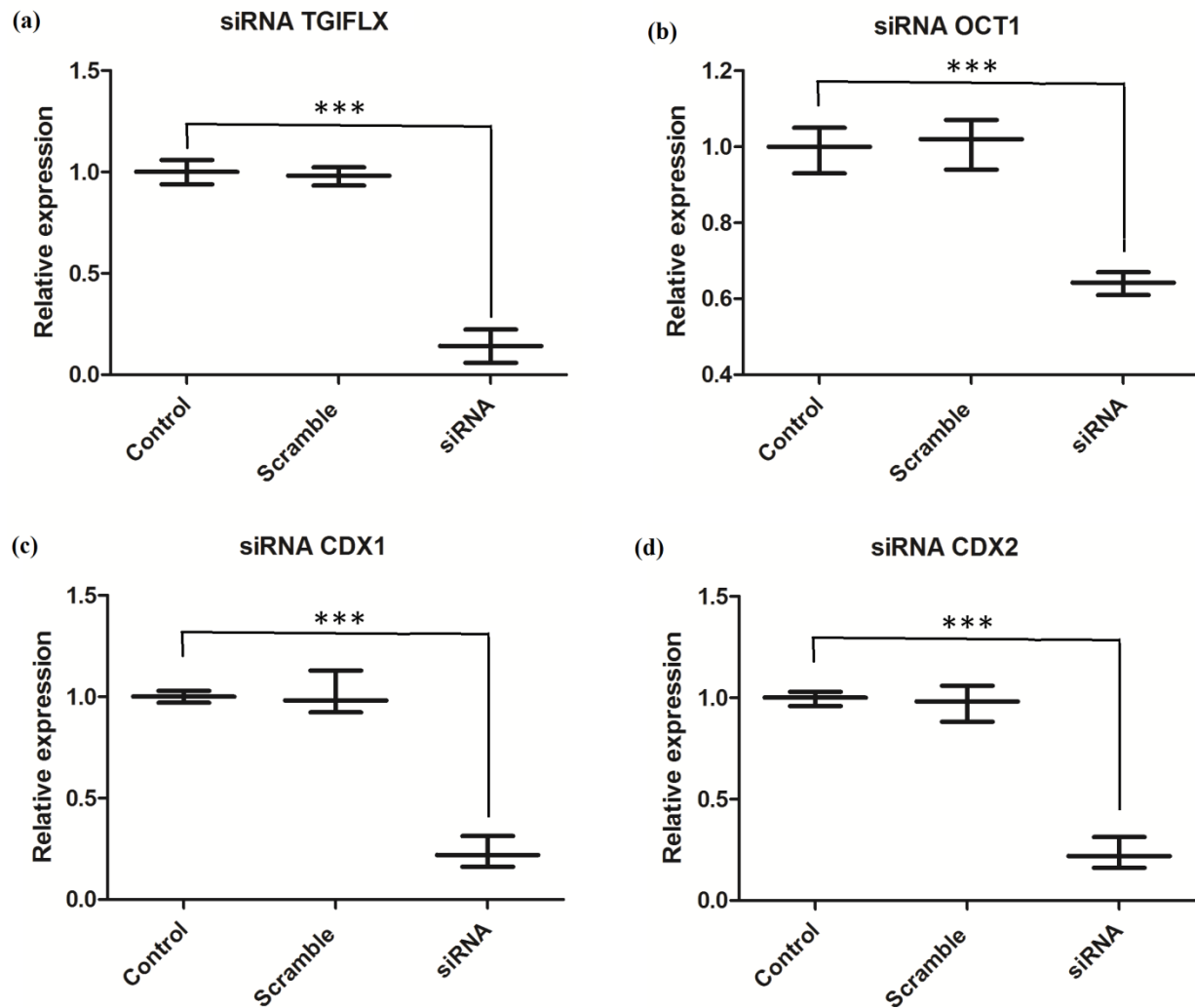


Figure 3. The effects of siRNA-mediated TGIFLX silenced in SW948 cells. The SW948 cell line was transfected with TGIFLX siRNA and scrambled siRNA. The results showed the TGIFLX (a), *OCT1*(b), *CDX1* (c), and *CDX2* (d), expression levels. Each real-time PCR was conducted in triplicate and the experiments were repeated twice with the mean \pm SD. siRNA: Small interfering RNA; PCR: Polymerase chain reaction