

## Investigation of mRNA Expression Levels of *TGIFLX* and *OCT1* Homeobox Genes in Colorectal Cancer

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**Abstract-** The OCT1 and TGIFLX transcription factors are members of homeodomains whose expressions have been implicated in normal and abnormal development. However, the expression of *TGIFLX* and *OCT1* in colorectal cancer is unknown. This study aimed to detect the expression of *OCT1* and *TGIFLX* in clinical samples of colorectal cancer. Twenty-six pairs of colorectal cancer tissue and adjacent non-tumoral tissue were obtained at the time of surgery from patients with colorectal cancer. The expression of *TGIFLX* and *OCT1* was detected by real time reverse transcriptase polymerase chain reaction (RT-PCR). *OCT1* was down-regulated in colorectal carcinoma samples in both males (58.33%) and females (57.14%). By contrast, *TGIFLX* was mainly (41.63%) expressed in colorectal tumors of males' samples but not in para-neoplastic normal tissues. *OCT1* expression was not significantly associated with the gender and site of primary tumor ( $P>0.05$ ), but the expression of *TGIFLX* was associated with male patients ( $P<0.05$ ). In conclusion, dysregulation of *OCT1* and *TGIFLX* genes might be novel prognostic biomarkers for patients with colorectal cancer.

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**Keywords:** Colorectal cancer; *TGIFLX*; *OCT1*; Real time RT-PCR

### Introduction

Colorectal cancer is (CRC) a widespread public health problem and many new cases are diagnosed every year (1). In spite of the fact that development of new strategies in diagnosis and therapeutic methods, colorectal cancer in patients show unsatisfactory and poor prognosis (2). So far, the identification and characterization of novel prognostic and predictive biomarkers for CRC have been focused on many studies, and several candidate biomarkers have been reported (3-7). Therefore, recognition of genes whose expressions are implicated in abnormal growth and the development of colorectal cancer are important.

Homeobox genes consist of a large gene family involved in development and differentiation (8-9) which can be subdivided into the clustered and non-clustered homeobox genes (10). The characteristic of homeobox genes is a conserved 180 base pair long motif which encodes a DNA-binding homeodomain with helix-turn-

helix super-secondary structure (11). According to the NMR and X-ray crystallography studies, classic homeodomain is a 60 amino acid structure in which three conserved  $\alpha$  helices surround a hydrophobic center (12). HOX genes are implicated in normal and tumorigenesis.

The OCT1 is a member of POU-domain containing family (13). POU-homeodomain genes are expressed in early developmental processes as transcriptional regulators. *OCT1* belongs to the POU class II and identifies its target sequences by the octamer-binding site (14). Different genes such as *pit1*, histone genes, some interleukins, and immunoglobulins are influenced by OCT1 transcription factor (15). The probable role of *OCT1* in the procedures of malignancy has been indicated, and the inhibition of oncogenesis in mouse embryonic stem cells and mice with the deficiency of p53 observed in the absence of *OCT1* (16). *OCT1* may play an important role in the abnormal expression of cyclinD1 and down-regulation of cyclinD1 has been

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detected in the *OCT1* knockdown CRC cells. Therefore, colorectal cancer cell spreading may be influenced by *OCT1* transcription factor (15,17). Modulating complex functions of POU protein *OCT1* has been proposed in the expression of *CDX2* gene (18).

*TGIFLX* (TG-interacting factor (TGIF)-like X) belongs to the transforming growth-interacting factors group (TGIFs), a member of three amino acid loop extension (TALE) superclass (19-20). In contrast with the classic homeobox genes, TALE superclass encodes a homeodomain with three extra amino acids between helix 1 and 2 (19). DNA binding site is mainly identified by highly conserved helix 3 of TALE homeodomain (19). These proteins function as transcription factors in various important embryonic developmental processes (21-22). The human *TGIFLX* gene includes 2 exons and located in Xq21.3 locus. *TGIFLX* resulted from the retrotransposition of homeobox *TGIF2* gene to the X-chromosome and its specific expression in the adult testis has been indicated (20,23).

## Materials and Methods

### Patients and tumor samples

Matched cancer and normal specimens were obtained from 26 cases of stage I and II of CRC patients. All specimens of CRC were collected and confirmed by histopathological examination at Iran Tumor Bank, Cancer Institute of Tehran University, Imam Khomeini Hospital. Samples were carried in liquid nitrogen and stored at -70 °C.

### Total RNA extraction and cDNA synthesis

Total RNA was extracted using Tripure Isolation Reagent (Roche, Mannheim, Germany) according to the manufacturer’s guidelines and stored at -70 °C for further tests. Total RNA was treated with RNase-free DNase I Enzyme (Fermentas). Quantitative control of

RNA was performed by Spectrophotometry. The cDNA was synthesized using 1 µg of confirmed total RNA with PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara Bio Inc, Shiga, Japan). The cDNA samples with acceptable quality level via GAPDH RT-PCR were stored at -70°C for following investigations.

### Real-Time RT-PCR

To investigate the expression of *OCT1* and *TGIFLX* genes in cancer tissues and adjacent normal tissues real time RT-PCR was performed using specific primers, *OCT1* F 5'- TTCCTCTCGCCGTGTTGTG-3' and *OCT1* R 5'- GTTCCGTCTCCATCCTTTCTTTC-3' and *TGIFLX* primers was ordered from Qiagen company. Every real time RT-PCR reaction was contained 1µl of each primer (5pmoles/µl), 1µl of cDNA and 10 µl of SYBR Premix Ex Taq II (Takara Bio Inc, Shiga, Japan). Primer blast and single peak melting curves confirmed the specificity of primers. In this study, *GAPDH* housekeeping gene used as an internal control and reference gene. The *GAPDH* primers including *GAPF* 5'- CACCAGGGCTGCTTTTAAAC-3' and *GAPR* 5'- ATCTCGCTCCTGGAAGAT-3 designed using Primer3 software.

### Statistical analysis

REST-RG-Version 3 was used for gene expression alterations analysis. Associations between gene expression, gender, and tumor location were analyzed with Statistical Package for Social Science -Version 15 (SPSS) by Chi-Square test.

## Results

The demographic characteristics of the colorectal tumors including age, gender, tumor location, grade of malignancy and histological type are shown in table 1.

**Table 1. Demographic characteristics of the colorectal cancer samples**

		Number	Percentage
<b>Gender</b>	<b>Male</b>	12	46.15%
	<b>Female</b>	14	53.85%
<b>Age</b>	<b>50&lt;</b>	2	7.69%
	<b>50≥</b>	24	92.31%
<b>Tumor location</b>	<b>Colon</b>	17	65.38%
	<b>Rectum</b>	9	34.62%
<b>Grade of Malignancy</b>	<b>I</b>	6	23.08%
	<b>II</b>	20	76.92%
<b>Histological Type</b>	<b>Adenocarcinoma</b>	22	81.62%
	<b>Mucinous (colloid)</b>	4	15.38%

**mRNA expressions of OCT1 and TGIFLX genes in CRC using Real Time RT-PCR**

In order to evaluate the expression of OCT1 and TGIFLX genes at the mRNA levels, the quantity and purity of extracted RNA samples were verified by Spectrophotometer. The mean concentration and optical density 280/260 nm were 667 ng/μl and 1.88, respectively. The results of qualitative control of RNA were examined via GAPDH RT-PCR (data not shown).

Also, single band of real time RT-PCR product of each primer and the expected length of amplified segments according to the primers on an agarose gel

electrophoresis verified the specificity of reactions (data not shown).

**OCT1 gene expression in colorectal cancer**

The mRNA expression of OCT1 was evaluated in both cancerous and non-tumoral adjacent tissues of 24 patients. Real time RT-PCR revealed the OCT1 down-regulation (Gene Fold: -1.545, P: 0.0165) in colorectal carcinoma samples in both males (58.33%) and females (57.14%) (Table 2). But two patients expressed OCT1 only in nontumoral samples. It may be due to mix-up with tumoral adjacent tissues.

**Table 2. OCT1 mRNA expression status in CRC**

	Males	Females	Total patients
<b>Down-regulation</b>	58.33%	57.14%	57.69%
<b>Upregulation</b>	16.66%	14.28%	15.38%
<b>Total Significant Dysregulation</b>	75%	71.42%	73.07%

CRC: Colorectal cancer

Furthermore, the associations between OCT1 gene expression at the mRNA level, gender and tumor location were analyzed with SPSS-Version 15. We found that OCT1 expression was not significantly associated with the gender as well as with site of primary tumor (P>0.05).

**TGIFLX gene expression in colorectal cancer**

In order to analyze the expression of TGIFLX in colorectal cancer and non-tumoral samples in males and

females with different tumor locations, we conducted real time RT-PCR as explained in the materials and methods. Interestingly, in spite of the mainly (41.63%) expressed in colorectal tumors of males, we could not detect TGIFLX mRNA expression in the non-tumoral tissues. Also, as shown in table 3 the expression of TGIFLX was not associated with tumor location while its expression was significantly associated with male patients (P<0.05) (Table 4).

**Table 3. Association between TGIFLX gene expression and tumor location**

	Section		Total	
	Colonic	Rectal		
TGIFLX Expression Status	<b>Positive Count</b>	3	2	5
	<b>%within</b>	60.0%	40.0%	100.0%
	<b>TGIFLX Expression Status %within section</b>	27.3%	25.0%	26.3%
	<b>Negative Count</b>	8	6	14
	<b>%within</b>	57.1%	42.9%	100.0%
	<b>TGIFLX Expression Status %within section</b>	72.7%	75.0%	73.7%
<b>Total Count</b>		11	8	19
	<b>%within</b>	57.9%	42.1%	100.0%
<b>TGIFLX Expression Status %within section</b>		100.0%	100.0%	100.0%

Chi-Square Test					
	Value	df	Asymp.Sig. (2-sided)	Exact.Sig. (2-sided)	Exact.Sig. (1-sided)
Pearson Chi-Square	.012 <sup>b</sup>	1	.912		
Continuity correction*	.000	1	1.000		
Likelihood Ratio	.012	1	.911	1.000	.664
Fisher Exact Test					
Linear-by-Linear Association	.012	1	.914		
N of Valid cases	19				

**Table 4. Significant association between *TGIFLX* gene expression and gender Crosstab**

		Sex		Total
		Female	Male	
TGIFLX Expression Status	Positive Count	0	5	5
	%within	.0%	100.0%	100.0%
	TGIFLX Expression Status	.0%	45.5%	23.8%
	%within sex			
	negative Count	10	6	16
	%within	62.5%	37.5%	100.0%
Total Count	TGIFLX Expression Status	100.0%	54.5%	76.2%
	%within sex			
Total Count	%within	10	11	21
	TGIFLX Expression Status	47.6%	52.4%	100.0%
Total Count	%within sex	100.0%	100.0%	100.0%
	TGIFLX Expression Status			

Chi-Square Test					
	Value	df	Asymp.Sig. (2-sided)	Exact.Sig. (2-sided)	Exact.Sig. (1-sided)
Pearson Chi-Square	5.966 <sup>b</sup>	1	.015		
Continuity correction*	3.723	1	.054		
Likelihood Ratio	7.895	1	.005	.035	.023
Fisher Exact Test					
Linear-by-Linear Association	5.682	1	.017		
N of Valid cases	21				

## Discussion

CRC is common in both men and women. Recently the prevalence of CRC has increased and is one of the most common reasons for death from cancer in western and developing regions (1). Some investigations verified the homeobox genes functions in gastrointestinal evolvment and cancer (24). These genes are involved in the developmental and differential process as transcription factors by activation or repression of the target genes expression (25-26). Although various reports indicating about homeobox genes pathological roles in cancer, unfortunately, are not complete and comprehensive. In addition, it has been shown that they

act as tumor suppressor genes at least in some cases whereas, in other cases play oncogenic roles in human tumors.

### *OCT1* gene expression in colorectal cancer

In the present study, *OCT1* mRNA was evaluated in both cancerous and non-tumoral adjacent tissues of 24 patients. Our findings revealed a significant down-regulation of *OCT1* expression with *P*: 0.0165 was generally observed in cancerous samples. But two patients expressed *OCT1* only in non-tumoral samples. This may be due to mix-up of tumoral with non-tumoral tissues. The results of different studies suggest two opposite protumorigenic and tumor suppressive

functions for *OCT1* gene in cancers.

#### ***OCT1* as a protumorigenic gene**

Some examinations propose protumorigenic role of *OCT1* gene. The expression of *OCT1* target genes has been shown to upregulate in lung cancer (27). Also, there is an interaction between *OCT1* and some metabolic enzymes in a glycolytic complex (28). Metabolism often switches from oxidative reactions to the glycolysis pathway for adjustment to hypoxia in primary and metastatic tumor cells (29). From the other point of view, *OCT1*-negative cells produce their energy through the oxidative pathway. The absence of *OCT1* alters the expression of some metabolic regulatory genes and prevent tumorigenesis by interference with glycolytic metabolic shifts. So a control point may be active in the metabolism of stimulated cells by oncogenic signals (16). Also, *OCT1* upregulation and its *CDX2* promoter-binding ability have been recognized in an investigation on intestinal gastric cancer and metaplasia. But *OCT1* role in the induction of *CDX2* expression and IM initiation remained unclear (30). According to several studies Cyclin D1 overexpression has been observed in CRC (31-32). *OCT1* may influence colorectal cancer cell propagation by a probable role in the abnormal cyclin D1 expression which controls cell cycle (15,17).

#### ***OCT1* as a tumor suppressor gene**

It should be noted that some studies suppose *OCT1* tumor suppressive roles. Findings show that *OCT1* and BRCA-1 tumor suppressor gene association stops growth and activates *GADD45* gene transcription (33-34). So the probable tumor suppressive function of *OCT1* in response to DNA damage can be concluded (35). On the other hand, irregular responses to radiation and dysregulation of several cellular stress-associated genes have been observed in *OCT1*-negative fibroblasts. They have suggested that *OCT1* transcription factor acts as a stress sensor and regulates target genes expression which results in a proper response (36).

According to the studies and considering two aforesaid opposite hypotheses our results can represent the lack of *OCT1* functional independence as a new hypothesis. This hypothesis is, in fact, originated from the nature of the homeobox genes. Some studies have shown these proteins in tumor suppressor mode, but sometimes they have played oncogenic role in other investigations. The *OCT1* performance is not clear exactly, and meta-analysis suggests that this gene has a dual dependent function in different tissues. From the

evidence, it seems that *OCT1* as an indicator which involved in stem cell differentiation has a very important and vital role so that the absence of this gene in the mouse fetus can cause death from lack of cell differentiation (37). Our studies are in agreement with this hypothesis, because even though we observed the diversity of expression at the level of *OCT1* mRNA in cancerous tissues, but considerably and significantly *OCT1* expression had been reduced in comparison with normal tissues. Also, this conclusion is in full agreement with the results of Shahriar *et al.*, (Unpublished data). Our results have been obtained from the comparison of *OCT1* expression in cancerous and normal adjacent colorectal samples, and more studies are needed about the *OCT1* function and its expression pattern in more and different tissues.

#### **mRNA expression of *TGIFLX* in CRC**

Also in this study, *TGIFLX* gene was not expressed in both males and females' adjacent non-tumoral tissues. While aberrant *TGIFLX* expression was observed in 41.63% of colorectal tumors of males' samples.

The function of *TGIFLX* has not been clearly known, but some studies have revealed its testis-specific expression in adult males (20). The probable role of *TGIFLX* gene in differentiation and spermatogenesis (38) and also the association of *TGIFLX* expression with prostate cancer (39) progression have been suggested. According to Aarabi's study, the pattern of *TGIFLX* gene expression can be influenced by gender. On the other hand, investigations revealed that *TGIFLX* normally expressed in adults' testis (20). Its abnormal expression in 41.63% of our cancerous male samples reconfirms probable gender-influenced *TGIFLX* expression pattern. The *TGIFLX* gene had expressed only in the male cancerous samples, and its mRNA wasn't detected in the female colorectal tumors and paraneoplastic normal tissues in our investigation. According to our results and the investigations of Blanco-Arias (2002), Ousati Ashtiani (2009) and Aarabi (2008) the role of aberrant *TGIFLX* expression in cancer development is proposed. X chromosome defects can result in aberrant *TGIFLX* expression in males if gender influences *TGIFLX* expression pattern. So the frequency of aberrant expression in studied male samples compared to female tissues is justifiable. As well as the results suggest that *TGIFLX* may play a role as a tumor suppressor gene and its aberrant expression can be caused by abnormal activity of gene regulatory regions.

So finding the exact role of *TGIFLX* gene in the



pathway of cancer and even use of it as a probable biomarker can be the aims of future studies.

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