

RESEARCH ARTICLE

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Prognostic Value of *FBXO39* and *ETS-1* but not *BMI-1* in Iranian Colorectal Cancer Patients

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Abstract

Background: Colorectal cancer (CRC) is one of the most prevalent cancers worldwide. Despite recent progress in diagnosis and treatment, it remains a major health problem and further studies are needed. We here investigated expression profiles of the *FBXO39*, *ETS-1* and *BMI-1* genes in CRCs to validate any possible diagnostic/prognostic significance. **Material and Methods:** Thirty six patients with locally advanced CRC admitted to Hazrate-Rasoul Hospital-Tehran were enrolled. Initially the expression pattern of *FBXO39*, *ETS-1* and *BMI-1* genes were determined using RT-PCR in CRC tumor and adjacent normal tissues then real-time RT-PCR was employed to quantify *BMI-1* gene expression. **Results:** *FBXO39* expression was restricted to tumor tissues. Interestingly, expression of this gene was detected in all stage-0 tumor samples. There was a significant relation between *FBXO39* gene expression and lymph node involvement. The *ETS-1* gene was expressed in 66% of all tumor tissues with p-value=0.03 for increase as compared to the adjacent normal samples. In addition, there was a significant relation between *ETS-1* gene expression and tumor size and lymph node involvement. RT-PCR demonstrated *BMI-1* gene expression in both tumor and normal tissues and quantification by real-time RT-PCR showed no association between *BMI-1* levels and CRC clinicopathological features. **Conclusion:** Expression of *FBXO39* and *ETS-1* with lymph node involvement may be considered as an alarm for the occurrence of CRC metastasis, and therefore have prognostic value while *BMI-1* appears without importance.

Keywords: Prognostic marker- Colorectal cancer- *FBXO39*- *ETS-1*- *BMI-1*

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Introduction

Colorectal cancer (CRC) is one of the prevalent cancers worldwide. Studies have shown that cancer prevalence in Iran is higher than the global rate (Esna-Ashari et al., 2008; Moradi et al., 2009). Despite recent progression in diagnosis and treatment of cancer, it is still one of the health problems in the world, and therefore requires further studies. In this regards, study of the expressions of the genes involved in CRC is rather important.

Currently The Cancer Genome Atlas (TCGA) research has categorized the types of cancer based on some genetic markers (Verhaak et al., 2012; Marisa et al., 2013). For example studies have shown about 90% of CRC is involved in alteration of genes comprised with Wnt signaling pathway (Najdi et al., 2011). However, more studies are needed to introduce more markers particularly for CRC.

One of the specific tumor markers is Cancer/testis antigens are considered as a group of specific tumor markers. CRC tumors are known as the tumors in which the expression of the genes of the Cancer/testis antigens

family is limited (Caballero and Chen, 2009). Song and colleagues successfully introduced *FBXO39* gene (located on 17p31.1) as a Cancer/testis antigen (Song et al., 2011) which its expression could be detected in some CRC samples.

ETS-1 is located on chromosome 11q24.3 and belongs to the *ETS* family which are a group of the transcription factors. Studies have shown the over-expression of *ETS-1* is associated with progression of various cancers (Dittmer, 2003; Verschoor et al., 2010). *ETS-1* expression is association with tumor invasion and metastasis (Pei et al., 2005). This transcription factor is involved in the control of tumor metastases by targeting crucial genes which role in cancer invasion and metastasis such as matrix metalloproteinases and integrin (Rothhammer et al., 2004; Lefter et al., 2009). Also studies showed the over-expression of *ETS-1* is associated with poor prognosis in patients with breast (Span et al., 2002) and ovarian cancer (Takai et al., 2002).

BMI-1 belongs to the Polycomb repressor complex 1 and is located on chromosome 10p11 (Bhattacharya et al., 2015). This gene as a gene regulatory factor plays

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as an oncogene in cell proliferation and cell movement (Jacobs et al., 1999; Glinsky et al., 2005). The importance of BMI-1 gene as a regulator factor in cell proliferation has been shown in early stages of CRC (Yu et al., 2012). BMI-1 is mostly expressed in primary stage of neuroblastoma compare to the advance stage of the disease (Nowak et al., 2006). Also this gene is associated with advanced stage of prostate (van Leenders et al., 2007) and breast cancer (Guo et al., 2011).

For years our research team has been investigating a panel of genes to find suitable diagnostic/prognostic markers for colorectal and breast cancer (Motalebzadeh et al., 2017). The three above genes were selected and their expression were detected at the mRNA level. We wished to see whether or not they could be used as potential diagnostic/prognostic biomarkers.

Materials and Methods

Patients and tumors characteristics

Thirty six patients with locally advanced CRC between 2011-2013 years admitted to Hazrat-e-rasoul hospital-Tehran were enrolled in this study. The hereditary CRC cases such as FAP, Lynch etc, were excluded. Only Iranian CRC patients were enrolled in this investigation. Fresh tissues (tumor and normal tissue adjacent to the tumor) were collected by the clinicians in separate sterile tubes. Tissue samples were frozen and stored at -70°C. The pathological information of all patients was obtained from pathology department of Hazrat-e-rasoul Hospital. Staging of CRC was performed according to the International Union against Cancer (UICC) which is based on (AJCC-TNM) classification (Edge and Compton, 2010). The patients were followed up and except one patient, others are all still alive.

The patients and tumor characteristics, gathered from the pathology reports. The minimum and maximum age of patients were 29 and 79 years old respectively with the average age 52.5 years. The patients were classified in four groups according to the tumor size: less than 5cm, between 5 and 8cm, between 8 and 10cm and larger than 10cm. Patients were categorized in three groups based on lymph node involvement: N0 (no lymph node involved), N1(1-4 lymph nodes involved) and N2 (more than 4 lymph nodes involved). Ten out of thirty six patients had long distance metastasis (Table 1).

RNA extraction and cDNA synthesis

After confirming histological diagnosis of all samples, RNA was isolated from 100 mg tissue with using Tripure Isolation Reagent (Roche Applied Sciences, Indianapolis, USA). For cDNA synthesis, RNAs were synchronized and according to the concentration of RNAs first-strand cDNA was synthesized as the manufacturer's protocol (Thermo scientific kit).

RT-PCR and Real-time RT-PCR

In this study, the expression pattern of FBXO39 and ETS-1 genes were investigated in CRC tumor and adjacent normal tissues (as control) using RT-PCR. Accuracy of all cDNA samples were checked using GAPDH gene as an internal control. The total volume of the PCR reaction

was 25 µL for each reaction containing PCR buffer, MgCl₂ (1.6 mM), dNTPs (120 µM), Primers (0.4 µM), Enzyme (1 U/25 µl) and template cDNA.

To evaluate BMI-1 gene expression level by real-time RT-PCR using a Rotor gene 6,000 (Corbett Research Pty. Ltd., Australia) system with Fast-Start DNA Master SYBR-Green I kit (YTA, Iran) using GAPDH as an internal control gene. The total volume of the PCR reaction for all samples was 10 µL contained 5 µl SYBR Green PCR master mix, 0.3 µl of each primer (10 µmol/l) and 1 µl template (400 ng/µl). The PCR cycles for each gene are summarized in Table 2.

Primers were designed according to exon-exon junction pattern and specifically for the mentioned three gene mRNAs. Primers sequences are shown in table 3. Samples for no-template were also included in each test to detect any contamination.

Statistical analysis

The data were analyzed using the Statistical Package for Social Sciences (SPSS version 19) software. RT-PCR data were analyzed using the chi-square test. Non-parametric test were used for relative quantification of the gene expression. For comparison of means between two groups Mann WithneyU test was used and for comparison of means among three or more groups Krus-kal-Wallis test was used. A p-value of <0.05 were considered statistically significant.

Results

FBXO39 expression

In this study the relation between FBXO39 gene expression and the clinical risk factors in patients with CRC was examined. Testis tissue was used as a positive control sample. The expression pattern of FBXO39 in six CRC samples on gel electrophoresis was shown in Figure 1.

Our results indicated the expression pattern of FBXO39 gene was restricted to tumor cells and the gene expression was not observed in the adjacent normal tissues (Table 4). This gene was expressed in 64% of all tumoral tissues. Although the expression of this gene was detected in all stage-0 tumor samples but there is no significant relation between the gene expression pattern and stages of CRC. As shown in table 5 a significant relation was observed between FBXO39 gene expression and patients lymph node status (P<0.05).

ETS-1 expression

ETS-1 gene is mostly expressed in CRC tissues (66% of all tumor tissues) with p-value=0.03 compare to the adjacent normal tissues (Table 4). The expression pattern of ETS-1 in five CRC samples was shown in Figure 1.

The expression of ETS-1 in all CRC samples showed there was a significant relationship between the gene expression and tumor size and lymph node involvement with p-value=0.039 and p-value=0.009 respectively. In all metastatic cases ETS-1 was expressed while in 62% of non metastatic patients the expression of this gene was detected (not significant, P>0.05). Also there was

Table 1. The Clinicopathological Information of Patients

characteristics	Groups	Number	Percent
Total patients		36	100
Age	>52	20	56%
	<52	16	44%
Gender	Male	16	43%
	Female	20	57%
Tumor size(cm)	<5	18	50%
	5-8	13	36.10%
	8-10	3	8.30%
	>10	2	5.60%
Lymph node	N0	14	38.90%
	N1	15	41.70%
	N2	7	19.40%
Stage	0	3	8.30%
	I	3	8.30%
	II	7	19.40%
	III	13	36.10%
	IV	10	27.80%
Grade	I	9	25%
	II	18	50%
	Un-known	9	25%
Metastasis	M0 (no metastasis)	26	27%
	M1 (metastasis)	10	73%

not a significant relation between ETS-1 expression and patient's gender and age.

BMI-1 gene expression

Our studies on BMI-1 gene expression level using real time RT-PCR showed there was no significant (p -value=0.708) difference between the gene expression in normal tissues and CRC tumor tissues (Table 4). Also we found no association between the gene expression level and clinicopathological features. Comparison of the expression level of BMI-1 mRNA in tumor and normal tissues was indicated in Figure 3.

All above results are summarized and presented in Table 4 and 5.

FBXO39, ETS-1 and BMI-1 expression and survival

Table 2. The PCR cycles for *FBXO39*, *ETS-1* and *BMI-1*. RT-PCR was used for amplification of *FBXO39* and *ETS-1*. Real time RT-PCR was used for amplification of *BMI-1* mRNA expression level.

Gene	Number of Cycle	Denaturation	Annealing	Extention
<i>FBXO39</i>	35	Time: 40s Temperature: 94°C	Time: 40s Temperature: 59°C	Time: 30s Temperature: 72°C
<i>ETS-1</i>	35	Time: 30s Temperature: 94°C	Time: 30s Temperature: 61°C	Time: 45s Temperature: 72°C
<i>BMI-1</i>	45	Time: 20s Temperature: 95°C	Time: 15s Temperature: 63°C	Time: 20s Temperature: 72°C

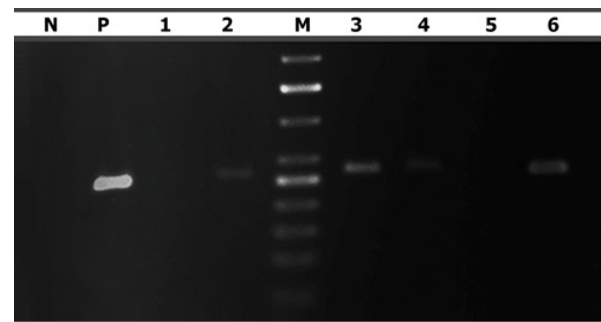


Figure 1. The Expression Pattern of *FBXO39* Gene on 1.5% Agarose Gel. The PCR reaction product length for *FBXO39* is 265 bp. In this figure the result of RT-PCR for six colorectal tumor samples are shown. Testis tissue was used as a positive control sample. N=Negative control, P, Positive control; M, Molecular marker (50 bp).

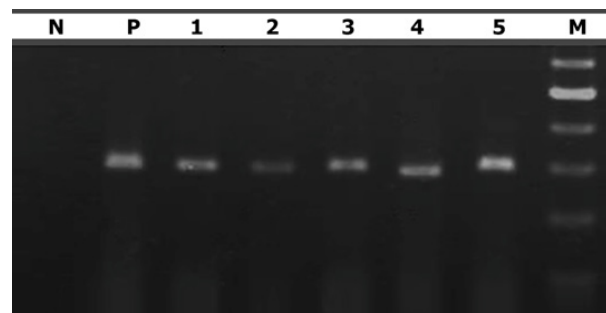


Figure 2. The Expression Pattern of *ETS-1* Gene on 1.5% Agarose Gel. The PCR reaction product length for *ETS-1* gene is 300 bp. In this figure the result of RT-PCR for five CRC samples are shown. Testis tissue was used as a positive control sample. N, Negative control; P, Positive control; M, Molecular marker (100 bp).

Among all the patients enrolled in this study only one case passed away. Both ETS-1 and FBXO39 had expression in tumor tissue of this patient. In addition BMI-1 expression in tumor tissue of this patient was slightly higher than mean expression but of course statistically cannot be interpreted.

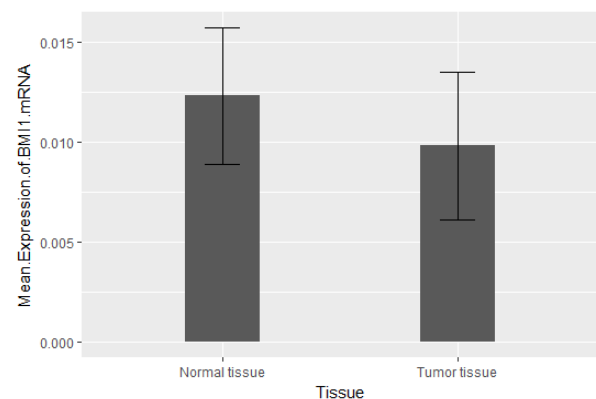


Figure 3. Mean Expression Level of *BMI-1* Gene in Colorectal Tumor and Normal Tissues. Relative quantification of BMI-1 using GAPDH as internal control gene indicated there was no significant difference in BMI-1 gene expression between tumor and normal tissues (p -value=0.708).

Table 3. Sequences of the Primers

Gene		Sequence	Length	Product length
<i>FBXO39</i>	F	5'-GCTGCTTGAGAACTTGTGTGA-3'	21	265 bp
	R	5'-GGGTCTCATTGAACAGTCTGG-3'	21	
<i>ETS-1</i>	F	5'-CCTATCCAGAATCCCGCTAT-3'	20	300 bp
	R	5'-CCCCGAGTTTACCACGAC-3'	18	
<i>BMI-1</i>	F	5'-ATACCTGGAGAAGGAATGGTC-3'	21	210 bp
	R	5'-GAGGAGACTGCACTGGAGTA-3'	20	
<i>GAPDH</i>	F	5'-GCAGGGGGGAGCCAAAAGGGT-3'	21	219 bp
	R	5'-TGGGTGGCAGTGATGGCATGG-3'	21	

Table 4. Comparison of *FBXO39*, *ETS-1* and *BMI-1* Expression between CRC Tumor and the Adjacent Normal Tissues

Gene	p-value	Significancy
<i>FBXO39</i>	<0.01	Significant at 0.01 level
<i>ETS-1</i>	0.03	Significant at 0.05 level
<i>BMI-1</i>	0.708	Not Significant

Discussion

One of the most common causes of the mortality in the world is cancer. In spite of progressions/advancement in CRC treatments, the clinical outcome is far away from expectation. Some of the main reasons are late diagnosis, lack of suitable markers for early detection, prognosis and prediction to treatment.

Cancer biomarkers are discussed as molecular and genetic alterations such as DNA methylation, mutation, copy number and gene expression (Verhaak et al., 2012; Marisa et al., 2013). In this study we aimed to analyze *FBXO39*, *ETS-1*, and *BMI-1* genes expression to find the possible value of them in diagnosis or prognosis in CRC patients.

Cancer/testis antigens (CTAs) are a group of antigens in many kinds of cancerous tissues and normal testis tissue also this group of antigens exist in spleen tissue (Old and Chen, 1998). Song and colleagues introduced *FBXO39* gene as a new Cancer/testis antigene. They found *FBXO39* gene severely expressed in 22 out of 57 samples of colon cancer tissues (Song et al., 2011). In a study by Tarnowski et al., (2016) they found this gene was overexpressed in colon cancer compared to the normal tissues, while they found no association between the gene expression and tumor progression. In the present study we investigated the expression of *FBXO39* gene in CRC and adjacent normal tissues by RT-PCR. *FBXO39* gene expression was observed in 64% of all tumoral tissues. The expression of this gene was seen in all CRC stage-0 samples.

Thus, due to the lack of *FBXO39* gene expression in normal tissue and the expression of the gene in early stage cancerous tissues, the expression of this gene can be considered as a possible diagnostic biomarker for CRC although further study required to completely confirm this data. Furthermore, this study was also conducted to investigate the association between the expression of

Table 5. The Relation between the Gene Expression and Clinicopathological Characteristics in Patients with CRC

Pathological characteristics	<i>FBXO39</i> P-value	<i>ETS-1</i> P-value	<i>BMI-1</i> P-value
Patients' gender	0.608	0.197	0.741
Patients' age	0.958	0.1	0.072
Tumor size	0.764	0.039*	0.949
Lymph node	0.040*	0.009*	0.907
Stage	0.209	0.197	0.928
Grade	0.341	0.409	0.523
Metastasis	0.608	0.197	0.741

this gene and clinicopathological features. We found a significant relation between *FBXO39* gene expression and the lymph node involvement.

ETS-1 is known as a transcription factor gene that plays a role in tumor invasion and angiogenesis. Nakayama et al., (2001) demonstrated that *ETS-1* gene was not expressed in any of 15 healthy colonic mucosa tissues, while in our study the expression of *ETS-1* detected in 45% of all normal adjacent tissues and 66% of all CRC tumors. Peng and colleagues indicated that *ETS-1* was expressed in 57.59% of CRC tissues (significantly higher than its expression in normal tissue).

They found that there was a significant association between gene expression and cancer stage (Peng et al., 2014). Also in another study by Tokuhara et al., (2002) *ETS-1* gene expression was associated with metastasis to lymph nodes in CRC. We found that there was a significant relationship between the gene expression and tumor size and lymph node involvement with p-value=0.039 and p-value=0.009 respectively. In all metastatic cases *ETS-1* was expressed. There was not a significant relation between *ETS-1* expression and patients' gender and age

The other candidate gene, *BMI-1*, initially was examined by RT-PCR but since we found that this gene was expressed in all tumor and normal tissues, we employed real time RT-PCR to quantify the expression level of *BMI-1* mRNA in all samples.

The results of a study by Kim et al., (2004) on *BMI-1* gene expression in CRC tissue indicated increased mRNA levels by 2 to 3 fold compared to normal tissue. Also Lin et al., (2008) showed significantly increased expression of *BMI-1* gene in CRC tumoral tissues and indicated a significant relation of the gene expression with distant

metastasis.

In contrast with the Lin study on BMI-1 gene, we found no difference between the gene expression in normal tissues and CRC tumor tissues. Furthermore, we found no association between BMI-1 gene expression level and clinicopathological features

The summary of association between pathological features and genes expression is as follow:

A) Age: The risk of CRC increases with aging (Amersi et al., 2005). The analysis of our data showed that there was no association between the expression of any of these genes and patient's age .

B) Tumor size: Studies have shown that an increase in tumor size relates to an increased risk of recurrence/metastasis in the most cancers. In our study there was a significant association between ETS-1 gene expression and tumor size , while no association was found with metastasis.

C) Lymph node involvement: One of the important factors in determining prognosis in CRC is lymph node involvement. In the present study data analysis showed that FBXO39 and ETS-1 genes expression were correlated with lymph nodes involvement.

D) Stage: Cancer staging describes the size of tumor and where it has spread. Our results showed there was no significant relation between any of these genes expression and CRC staging.

E) Histological grade: Tumor grade is also an important prognostic variable. In our study there was no association between any of these genes and histological grade.

In conclusion, bases on our study on these three candidate genes in CRC cases we indicated significant association between FBXO39 and ETS-1 genes expressions and lymph node involvement. Since the involvement of lymph nodes can induce metastasis in patients, it is possible that the expression of FBXO39 and ETS-1 genes are an alarm for the occurrence of metastasis and thus may be a potential prognostic biomarkers. Furthermore, FBXO39 gene expression could be a candidate for early detection of CRC. However, due to the small numbers of the samples studied, further investigation on more CRC samples are needed to be able to draw a robust conclusion.

Compliance with Ethical Standards

All authors declare that they have no conflict of interest.

Informed consent was obtained from all individual participants included in the study.

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