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Up-regulation of Bax-interacting factor-1 in Iranian Colorectal Cancer Patients

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ABSTRACT

Background: Colorectal cancer (CRC) is one of the most common cancers worldwide and can be caused by a variety of genetic and acquired/environmental factors. Bax-interacting factor-1 (*Bif-1*) is an apoptosis inducer gene that interacts with the Bcl2 protein family and triggers apoptosis via direct contact or by changing into the Bax protein conformation using the phosphorylation mechanism. *Bif-1* also interacts with Beclin-1, a protein that plays a central role in autophagy through mediation of UVRAG (ultraviolet irradiation resistant-associated gene), a positive regulator of phosphatidylinositol 3-kinase complex 3 (PI3KC3), thereby inducing autophagy in mammalian cells. Considering the dual role of *Bif-1* in many tumors of different origins, in this study we assessed *Bif-1* gene expression to investigate its potential role as a possible prognostic biomarker in Iranian colorectal cancer patients.

Methods: *Bif-1* gene expression in tumors and normal adjacent tissues in 50 colorectal cancer patients were quantified using Real-time RT-PCR. Also, the association between *Bif-1* gene expression levels with the histopathological characteristics of patients was assessed.

Results: The results indicated an overall upregulation of the *Bif-1* gene in colorectal tumors compared with normal adjacent tissues ($p < 0.0001$). Also, Bif-1expression was significantly elevated in stages II and III compared with stage I, and down-regulated in stage IV patients with distant metastasis. A positive association was also observed between lymph node involvement and tumor size ≥ 5 centimeters with *Bif-1* overexpression ($P < 0.001$).

Conclusion: In conclusion, up-regulation of the *Bif-1* gene could be considered as a possible prognostic candidate in colorectal cancers associated with nodal metastasis and greater tumor size. Further validation of these results are recommended in studies with larger sample sizes.

Keywords: Colorectal cancer, Bax-interacting factor-1 (*Bif-1*), gene expression, Biomarker

INTRODUCTION:

Colorectal cancer (CRC) is one of the most important causes of cancer death worldwide. Changes in expression of apoptosis-related proteins play a key role in both CRC progression and response to chemotherapy[1]. Determination of alterations in gene expression during colorectal cancer progression may lead to cancer clinical management[2].
Bax-interacting factor-1 (*Bif-1*, also known as endophilin B1 and SH3GLB1), a member of the membrane curvature driving endophilin family of proteins, is associated with the proapoptotic Bcl-2 family protein Bax[3]. *Bif-1* promotes Bax conformational changes to induce apoptosis[4]. Inhibition of *Bif-1* expression in vitro abrogates cytochrome *c* release and caspase-3 activation induced by various intrinsic apoptosis signals, and *Bif-1* knockout mouse shows delayed mitochondrial apoptosis[5]. These findings support an important role for *Bif-1* in apoptotic activation, since the loss of this molecule is involved in tumorigenesis. *Bif-1* also regulates the induction of autophagy[4]. Autophagy, an evolutionarily conserved catabolic process, is involved in the regulation of a variety of pathological and physiological processes such as cell death, immunity, energy homeostasis, cell differentiation and carcinogenesis[6]. *Bif-1* also plays an important role in regulation of lipid catabolism to prevent obesity development and insulin resistance[7]. *Bif-1* is a multifunctional protein. The unique neuroprotective role for *Bif-1* is described by Wang et al. in 2015 and *Bif-1* has also been introduced as a potential therapeutic target for the treatment of neurological diseases, especially degenerative disorders characterized by alterations in mitochondrial dynamics[8]. The human *Bif-1* gene is located on chromosome 1p22[9].
Altered *Bif-1* expression was found in cancer cells compared to adjacent normal tissues in various human malignancies, including gastric cancer[10], prostate cancer[6,11], invasive bladder cancer[12], pancreatic cancer[13], and CRC[2,14]. However, the clinical implications of *Bif-1* expression are controversial[2,6,14].
In this study *Bif-1* gene expression in tumors and normal adjacent tissues in 50 patients with colorectal cancer was quantified using RT- PCR. Also, the association of *Bif-1* gene expression levels with the histopathological characteristics of patients was assessed.

METHODS:

Patients and Specimens

Tissues from 50 colorectal tumors and their adjacent normal tissues were removed surgically from patients admitted to Rasoul-Akram Hospital from 2011-2015. The clinical characteristics of patients are summarized in **Table 1**. Written informed consent was obtained

Table 1:Clinical characteristics of patients with colorectal cancer

Clinicopathological features	Number (%)
Age:	
Less than 55 years	24 (48%)
More than 55 years	26 (52%)
Gender:	
Male	29 (58%)
Female	21 (42%)
Tumor size (cm):	
<5	26 (52%)
≥5	24 (48%)
Lymph node status:	
Positive	21 (42%)
Negative	29 (58%)
Tumor Stage:	
Stage I	4 (8%)
Stage II	16 (32%)
Stage III	24 (48%)
Stage IV	6 (12)

from patients who participated in this study prior to sample collection. Fresh tissue samples were frozen in liquid nitrogen and stored at -70°C. Two pathologists confirmed the cancerous tumors and normal tissues. Staging of the CRC was performed according to the Union for International Cancer Control (UICC) which is based on (AJCC-TNM) classification. All procedures were approved by the local ethical standards of National Institute of Genetic Engineering and Biotechnology (NIGEB) based on the Helsinki declaration.

Primer designing

The mRNA sequences of *Bif-1* and *GAPDH* , as the reference gene, were obtained from the Gene Bank. After identifying the exon/intron junctions, suitable reverse and forward primers were manually designed using gene runner software. The selected sequences were evaluated for hairpin and duplex formation stability. BLASTN searches were conducted to approve gene specificity of the primer sequences (**Table 2**).

RNA extraction and cDNA synthesis

TriPure Isolation Reagent (Roche Applied Sciences, Germany) was used for total RNA extraction of colorectal tissue samples. Electrophoresis, using agarose gel and ethidium bromide staining, were used to determine the quality of the RNA samples. The concentration of RNA was measured by Nano Drop spectrophotometer. 1 µg of RNA from each sample was used to synthesize cDNA using First Strand cDNA Synthesis Kit, Fermentas, USA.

Standard curve construction

Amplification efficiency for each primer pair was determined by the amplification of a linear standard curve (from 0.24 to 1,000 ng) of total cDNA assessed with the use of an ultraviolet spectrophotometer. Standard curves showed good linearity and amplification efficiency (100%) for each primer set of experimental (*Bif-1*) and reference (*GAPDH*) genes.

Real-time RT-PCR

All PCRs were performed using a Light Cycler TM system (Rotor gene, Corbett, Germany). For each sample, 500 ng/ µl of total cDNA was used. cDNA was mixed with 0.3 µM of each forward and reverse primer with 10 µl of Sybr green 1 master mix (Roche, Germany) to a final reaction volume of 20 µl. The thermal cycling conditions comprised of an initial denaturation step at 95˚C for 10 min and 45 cycles at 95˚C for 10 s and 61˚C for 30 s and 72˚C for 20 s. Experiments were performed with duplicates for each data point. As a negative control, each sample was previously run with *GAPDH* primers without reverse transcription in order to detect genomic DNA contamination; moreover, negative test controls were assayed in each reaction and for each primer set to detect DNA contamination of reagents. Using the $2^{-\Delta\Delta CT}$ method[15], the data were presented as the change in gene expression normalized to an endogenous reference gene (*GAPDH*) and relative to

Table 2:Sequences of the primers

Gene		Sequence	Amolicon size
Bif-1	F	5’- AGCCCAGATGACTTACTATGC -3’	125 bp
	R	5’- CTGATGGTACAGGTGTCACAG -3’	
GAPDH	F	5›-GCAGGGGGGAGCCAAAAGGGT-3’	219 bp
	R	5’-TGGGTGGCAGTGATGGCATGG-3’	

bp: base pair

the controls. We considered two-fold or greater RNA expression as up-regulation, between 0.5- and 2-fold as normal and 0.5-fold or less as down-regulation.

Statistical Analyses

Statistical computations were performed using SPSS version 16.0 (SPSS, Chicago, IL). Comparison of the data between different patient and control groups was carried out using the Kruskal Wallis test. The Mann Whitney test was performed for comparisons between two groups. For all analyses, differences were accepted as statistically significant at $p < 0.05$. Numerical data are presented as mean ± standard deviation (SD).

RESULTS:

Bif-1 gene expression analysis in normal and tumor tissues

Analysis of Real Time- RT PCR results confirmed by REST 2009 revealed a significant increase in the level of *Bif-1* gene expression in tumor tissues ($p < 0.0001$). The mean of *Bif-1* gene expression in tumor colorectal tissues showed a 7.32 ± 7 fold change increase compared with normal adjacent tissues. The range of gene expression was between 0.45 to 29 fold. As shown in **Figure 1**, approximately 54% of colorectal cancer tissues showed up-regulation of *Bif-1* gene and 21.62%

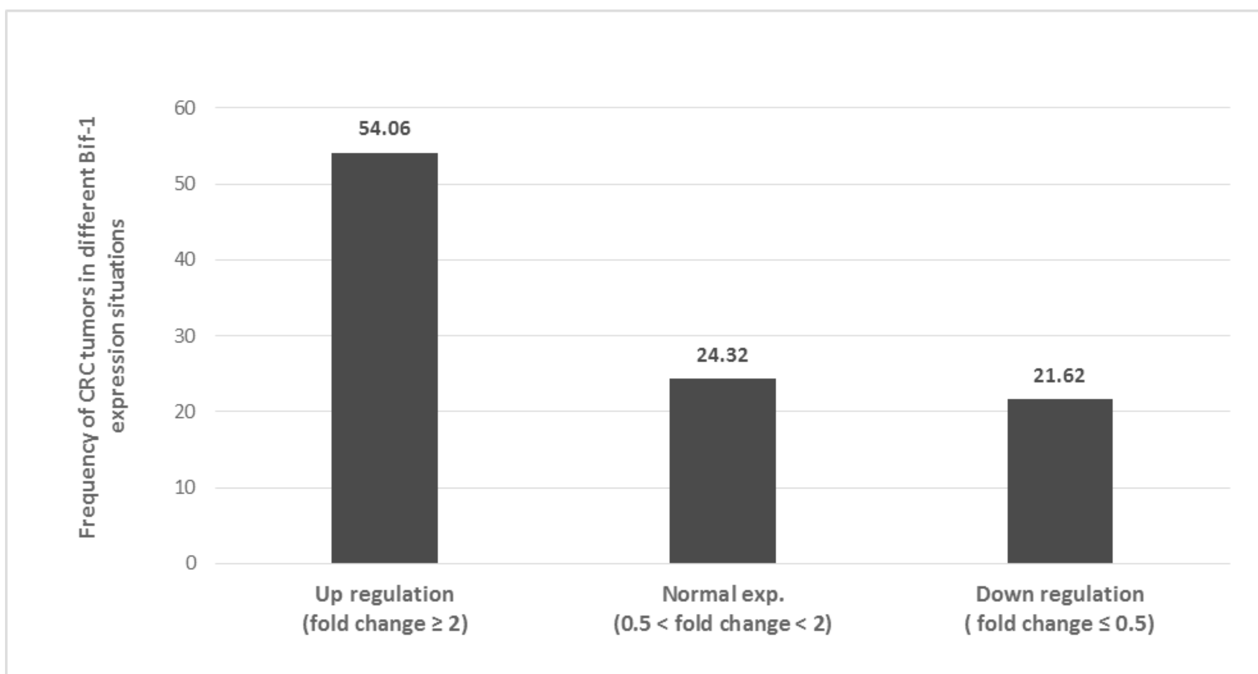

Figure 1. Frequency of colorectal cancer tumors in different *Bif-1* expression situations assessed by Real-time RT-PCR analysis. *Bif-1* expression was classified as up- regulation (fold change ≥ 2), Normal expression (0.5 < fold change < 2) and down regulation (fold change ≤ 0.5). Results are expressed as fold number increase versus control assumed as 1. *Bif-1* were previously normalized to *GAPDH* RNA values.

and 24.32% showed down-regulation and normal expression of the gene, respectively.

Bif-1 **expression and lymph node involvement**

As shown in **Figure 2**, the data indicated that *Bif-1* gene expression was significantly higher in patients with lymph node involvement (P < 0.001). The mean of *Bif-1* mRNA expression was calculated to be 14.65± 6.96 and 2.47 ± 2.91 in lymph node positive and negative groups respectively.

Bif-1 **expression and tumor size**

Figure 2 shows that the mRNA expression of *Bif-1* was dramatically increased in patients with colorectal tumors of more than five centimeters compared to smaller ones (P < 0.0001). The mean of *Bif-1* expression level was 13.71 ±7.28 and 2.33 ± 2.96 in tumor size ≥ 5 and less than 5 centimeters, respectively.

Bif-1 **expression and CRC pathological stages**

When the pathological stages of the disease were considered, the data indicated up-regulation of *Bif-1* in stages II and III compared with stage I, and sudden down-regulation of this gene in stage IV with distant metastasis and invasion. The mean of mRNA expression was 2.29 ±1.68, 9.53 ±8.45, 8.4 ±7.52 and 0.47± 0.01, in stages I to IV, respectevely.

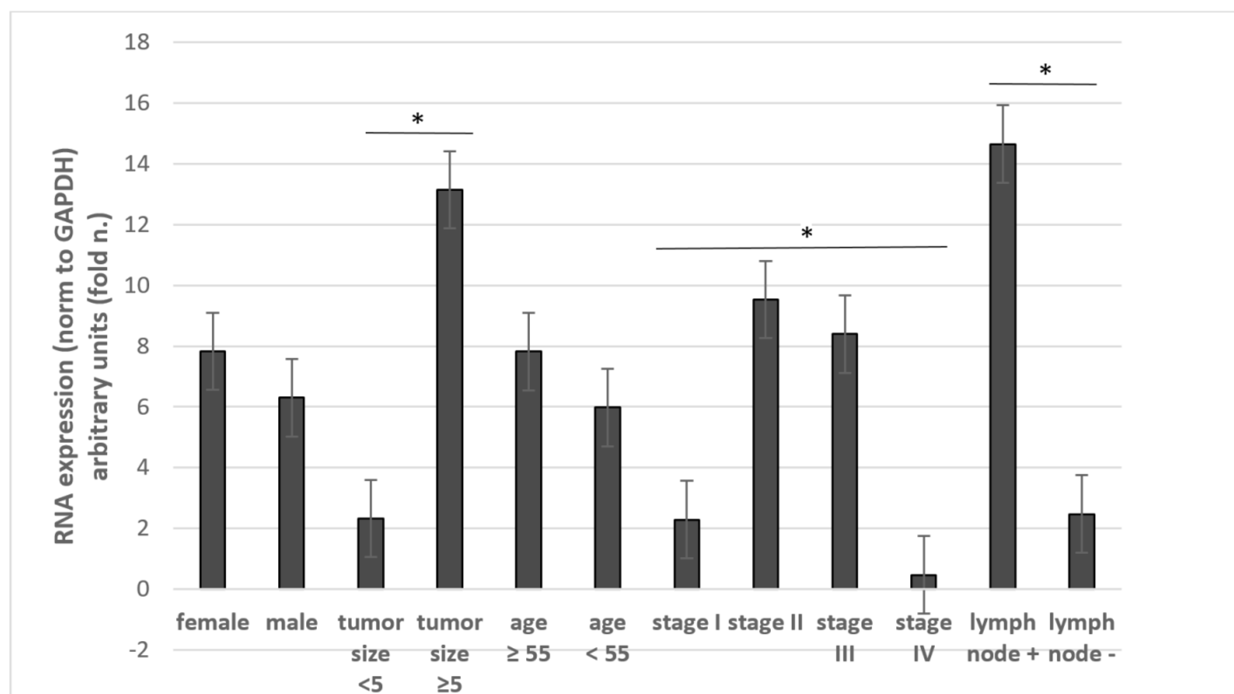

Figure 2. Real -time RT-PCR analysis of *Bif-1* expression in colorectal cancer patients classified based on clinicohistological characterization.
Results are expressed as fold number increase versus control assumed as 1. *Bif-1* value were previously normalized to *GAPDH* RNA values.
* : P < 0.001

***Bif-1* expression and patients, demographic characteristics**

As shown in **Figure 2**, there were no statistically significant differences in *Bif-1* mRNA expression between patient groups based on sex and age (P > 0.05). Patients were sorted into two groups , ≥ 55 and < 55 years old based on their age at diagnosis. *Bif-1* expression was 5.98 ± 6.33 and 7.82 ± 8.1 at < 55 and ≥55 years old, respectively. Patients were categorized based on gender to male and female groups, with *Bif-1* mean expression being 6.3 ± 6.4 in males and 7.8 ± 8.4 in females.

DISCUSSION:

Loss of *Bif-1* tumor suppressor activity has been reported in a variety of tumor types and plays an important role in carcinogenesis[9,16]. However, the clinical value of *Bif-1* expression in various types of solid cancer remains controversial. *Bif-1* expression was down-regulated through in situ clinical progression to metastatic carcinoma in an experimental metastatic model study of breast cancer[17]. Furthermore, no correlations have been reported between *Bif-1* protein expression, clinical outcomes and histological characteristics in various tumors, such as invasive bladder cancer[12], pancreatic cancer[13] and gastric cancer[18]. However, the clinical prognostic significance of *Bif-1* expression in CRC needs to be clarified due to controversial results. Several pathological features, such as tumor grade, invasion status, nodal involvement, and curative resection status have been considered as prognostic clinicopathological parameters in patients with CRC. In the present study, results indicated that high levels of *Bif-1* mRNA expression were associated with poor clinical outcomes in CRC, and higher levels of *Bif-1* expression were observed more frequently in nodal metastasis and greater tumor size and more progressed stages (stages II and III compared with stage I, however, sudden down-regulation was observed in stage IV patients with distant metastasis.) Chromosome 1p22, where *Bif-1* is localized, has been postulated as more common in metastatic CRC compared with primary CRC tumors[9]. This report was somewhat in line with results observed in the present study that showed down-regulation of *Bif-1* in distant metastasis and stage IV. This sudden down-regulation may be due to deletions in the *Bif-1* gene, or cancerous cells becoming out of control in stage IV of the disease.

In an immunohistochemistry tissue microarray study carried out in resected specimens from CRC patients by Ko et al. in 2013, low *Bif-1* protein expression was observed in 52.2% and high expression in 47.8% of patients. No significant differences were observed in clinicopathological parameters between patients with high and low *Bif-1* expression[2]. Another study confirmed the tumor suppressor role of *Bif-1* in CRC tissue samples by IHC based expression assay[6]. Also, low expression of *Bif-1* was reported in human pancreatic ductal carcinoma using immunohistochemistry and tissue microarray techniques[13].

The loss of *Bif-1* protein expression in cancer cells could be functionally interpreted in several ways. *Bif-1* could act as a tumor suppressor gene due to Bax regulation via accelerating Bax conformational changes either through a phosphorylation-dependent mechanism or by enhancing the kinetics of apoptosis induction in response to intrinsic apoptotic signals. This is achieved by direct binding to Bax, resulting in an increased permeability of the outer mitochondrial membrane[10].

In contrast, in patients with hepatocellular carcinoma (HCC), high-intensity *Bif-1* expression was correlated with a shorter survival time compared to patients with low-intensity expression[18]. These conflicting results may be caused by the complexity of *Bif-1* biological

functions.

Another theory for the discrepancy in *Bif-1* expression involves autophagy. Nowadays, autophagy has been extensively studied in a variety of tumors, e.g., breast, pulmonary, prostate, brain and colorectal[14,19]. Up until now, autophagy in carcinogenesis has been described as a double-edged sword due to its dual function. On the one hand, autophagy protects cells against neoplastic transformation by maintaining intracellular homeostasis, but, on the other hand, this may result in cancer cells being more likely to survive than normal cells under adverse circumstances, such as starvation and hypoxia, as well as during anticancer therapy[19,20]. To date, the results of many studies on autophagy in CRC have been inconclusive and conflicting. Autophagy, also known as type II programmed cell death, is usually activated in response to adverse circumstances during which cytoplasmic materials are enclosed in double membrane-bound vesicles targeted by the lysosome for degradation[21]. Beclin 1 that is an essential autophagy regulator, is monoallelically deleted in many human breast, ovarian and prostate cancers. In addition, mutant mice with heterozygous disruption of beclin 1 are prone to develop spontaneous tumors[22]. *Bif-1* interacts with beclin 1 through UVRAG, which is a positive regulator of PI3KC3, resulting in autophagy induction in mammalian cells. Also, *Bif-1* is required for trafficking of Atg9, an autophagy essential transmembrane protein, as well as Golgi membrane fission during autophagy induction[23]. Thus, the loss of *Bif-1* significantly inhibits PI3KC3 activation and the formation of autophagosomes in cancer cells.

Our findings demonstrate an increased expression of *Bif-1* in stages II and III of CRC, where autophagy may be required to provide essential nutrients to cells in the inner part of solid tumors that lack direct access to adjacent tumor vasculature[24]. There have been discrepant reported results in studies analyzing the association between *Bif-1* expression and TNM staging in CRC patients[2,6,14].These discrepant results in CRC may be attributable at least in part to differences in methodology and patient demographics.

In this study we investigated *Bif-1* expression as a possible molecular prognostic indicator and found that high levels of *Bif-1* expression were an independent negative prognostic marker in CRC patients in relation to nodal metastasis and tumor size. Distant metastasis was associated with down-regulation of *Bif-1* expression in our study. We assumed that up-regulation of *Bif-1* would be correlated with induction of autophagy. Emerging evidence indicates that autophagy has a context-dependent role in cancer. The prosurvival role of autophagy under stressful conditions, such as hypoxia or cancer treatment, can promote tumor development [21]. Our findings may have clear clinical implications for CRC, although the relatively small sample sizes in each stage did not allow for definite conclusions regarding the prognostic value of *Bif-1* expression. Further studies with larger numbers of patients are required to determine the role of *Bif-1* expression in CRC.

CONCLUSION:

In conclusion, up-regulation of the *Bif-1* gene may have potential to be considered as a possible prognostic candidate in colorectal cancer associated with nodal metastasis and larger tumor size. Further validation of these results are recommended in larger sample sizes.

CONFLICT OF INTEREST:

No potential conflicts of interests were disclosed by the authors.

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