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ORIGINAL ARTICLE

The Relationship of CXCR1, IκBα and HIF-1α Expression Levels with Clinicopathological Parameters in Colorectal Cancer

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SUMMARY

Background: **The role of C-X-C motif chemokine receptor 1 (CXCR1), inhibitor of kappa B (IκBα), and hypoxia-inducible factor 1 alpha (HIF-1α) have been reported to promote tumorigenesis and progression in colorectal cancer (CRC). This study is to evaluate the expression of CXCR1, IκBα, and hypoxia-inducible factor 1 alpha (HIF-1α), in combination, in CRC tissues. It also aims to analyze the relationship of these three factors with clinicopathological characteristics.**
Methods: **CRC and tumor-adjacent tissues were surgically collected from CRC patients. All patients were diagnosed by pathological examination, and none of the patients had received preoperative chemotherapy or radiotherapy. RNA extraction and cDNA synthesis were performed, and the transcription of CXCR1, IκBα, HIF-1α, and β-actin was quantified by RT-qPCR.**
Results: **The significant increase of CXCR1, HIF-1α, and decrease of IκBα mRNA expression level were observed in tumor samples of CRC patients ($p < 0.05$). In addition, CXCR1 expression level was correlated with lymph node metastasis ($p = 0.013$). Also, results demonstrated a relationship between HIF-1α expression and TNM stage and lymph node metastasis ($p = 0.047$ and $p = 0.005$, respectively). CXCR1 and HIF-1α simultaneous expression demonstrated the significant relationship with lymph node metastasis in CRC.**
Conclusions: **Our findings indicated that CXCR1, HIF-1α, and IκBα can be used as potential prognostic factors indicating tumors in the advanced stage in patients with CRC.**
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INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers occurring in men and women worldwide [1]. The identification of genetic biomarkers is crucial to assist the process of choosing appropriate treatment strategy for various patients [2] and ascertains whether some tumor metastasis-associated genes can be utilized as prognostic markers for metastasis of CRC or not.
A complex interaction of genetic and environmental factors determines the risk of cancer invasion [3]. Inflammation is one of the factors that has significant effects on the development and progression of many hu-

man cancers [4]. A variety of studies show that the chronic inflammation of the large intestine, such as ulcerative colitis and Crohn's disease, has a correlation with the subsequent development of CRC [5,6]. Chronic inflammation provides a susceptible microenvironment for tumor development. This microenvironment is susceptible owing to the presence of different growth factors, cytokines, and harmful by products of cellular respiration like free radicals [7].

Some evidence has been obtained regarding the relationship between inflammation and angiogenesis [8]. Also, oxygen deficiency (hypoxia) is an essential signal for the induction of angiogenesis. Angiogenesis process is a major factor in the progression of cancer [9]. Hypoxia-inducible factor 1 alpha (HIF-1α) and vascular epithelial growth factor (VEGF) are significant angiogenic factors regulated by hypoxia [10]. HIF-1 is a heterodimeric transcription factor combining HIF-1α and HIF-1β subunits. HIF-1 can directly operate the expression of several proangiogenic factors. Binding HIF-1α to the VEGF promoter region is the actual activator of expression of VEGF gene. These are necessary factors for the spread of tumor and metastasis [11,12].

Interleukin-8 (IL-8), is one of the chemotactic and inflammatory cytokines that is produced by macrophages and other cell types such as endothelial and epithelial cells in this microenvironment [13]. Moreover, the high expression of it and its receptor have been determined in cancerous cells. This chemokine promotes neutrophil chemotaxis and degranulation. IL-8 induces activation of two cell surface G protein-coupled receptors, C-X-C motif chemokine receptors, CXCR1 and CXCR2, and downstream signal pathways. It also can increase the expression of many genes such as nuclear factor kappa B (NF-κB), HIF-1, and activator protein 1 (AP-1) that are associated with regulation of cell survival, apoptosis, and angiogenesis [14,15].

Another target of CXCR1 and CXCR2 is NF-κB. This protein sets up a family of transcription factors that contribute to several biological processes, such as inflammation and immune response. NF-κB signaling pathways comprise the classical (canonical) and alternative pathway. In the classical pathway, Rel A/p50 transcription factor is bound and inhibited by IκB proteins. On the other hand, alternative pathway activates the RelB/p52 transcription factor using a mechanism that depends on the inducible processing of p100 (precursor of p52) instead of degradation of IκBα [16]. The NF-κB classical pathway is triggered by Toll-like receptors (TLRs) and pro-inflammatory cytokines such as IL-1 and TNFα. The activated IKKβs phosphorylate the IκBs inhibitory proteins and this action results in ubiquitination and subsequent degradation by the proteasome. The nf-κb transcription factor, free of IκB, translocates to the nucleus, where it operates the transcription of target genes, including angiogenesis, cytokines, and anti-apoptotic genes [15,17].

According to the mediatory function of CXCR1 in response to IL-8 (an important inflammatory chemokine) and the relationship of IκBα and HIF-1α proteins with inflammation response and angiogenesis downstream of this receptor, it seems that the combination of CXCR1 and IκBα besides HIF-1α expression in tumor tissue could be useful biomarkers of prognosis monitoring of CRC. Therefore, the expression pattern of CXCR1, IκBα, and HIF-1α with clinicopathological factors of CRC was investigated.

MATERIALS AND METHODS

Patients and tissue samples

CRC and tumor-adjacent tissues were surgically collected from 47 patients with CRC who were admitted to Shahid Sadoughi Hospital (Yazd, Iran) between March 2016 and April 2017. All patients were diagnosed by pathological examination, and none of them had received preoperative chemotherapy or radiotherapy. The case group included the tumor tissues, while the control group included adjacent non-cancerous colorectal tissues gathered from > 2 cm away from the tumor. The samples were rapidly transferred to liquid nitrogen after surgery.

The study was approved by the ethics committee of Shahid Sadoughi University (ID: IR.SSU.MEDICINE.REC.1394.536) and written informed consent was obtained from all patients. Also, we gathered the following information from the patients' medical records: age, gender, tumor size, TNM staging, lymph node status, distant metastasis, and perineural invasion.

RT-qPCR

The experimental procedures of RNA extraction were performed according to the manufacturer's direction. Briefly, 100 mg tissue was sufficiently homogenized with 1 mL Trizol LS (Invitrogen) reagent for each sample and final precipitated RNA was dissolved in 30 µL DEPC-treated water. To remove DNA contamination, RNA solutions were treated with DNase I. The concentration and quality of RNA were analyzed by a Nano-Drop Spectrophotometer (A260/A280 = 1.8 - 2; A260/A230 > 1.7) and samples were preserved at -80°C until further use.

cDNA synthesis was performed by HyperScript TM first strand synthesis kit (Geneall Biotechnology Co., Korea). CXCR1, IκBα, HIF-1α, and β actin expression levels were evaluated by real-time PCR using SYBR Green master mix (Ampliqon A/S, Denmark).

The primers for CXCR1 were
5′-GAGACAAGCAGCCCTTAGCC-3′ and
5′-GGACAAGCACGGAACAGAAG-3′.
The primers for IκBα were
5′-TCAGAGTTCACGGAGTTCACA-3′ and
5′-ATGTTCTTTCAGCCCCTTTG-3′.
The primers for HIF-1α were
5′-TTTGGCAGCAACGACACAGA-3′ and
5′-TGGGTGAGGGGAGCATTACA-3′.
β actin gene was used as an endogenous control for

sample normalization.
The primers for β actin were
5′-TTCCTATGTGGGCGACGAGG-3′ and
5′-CAGTTGGTGACGATGCCGTG-3′.
Triplicate qPCR measurements were carried out on the cDNA of samples. Data were collected and quantitatively evaluated by Rotor-Gene Q Series Software (Rotor-Gene), and the RT-qPCR data analyses were performed by ΔΔCT method.

Statistical analyses
The statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Normal distribution was assessed and differences in mRNA expression levels were evaluated by parametric test (paired *t*-test). The association of clinicopathological features with candidate gene expressions were analyzed using the χ^2 test. $p < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Patient characteristics
The analyses include all 47 patients with CRC (Table 1). In the samples, 27 patients were males and 20 were females. The age range was between 48 - 73 years old. The mean age of the patients was 58.3 ± 7.3 years old. Twenty-one samples had the tumor diameter of ≤ 5 cm, and 26 had the tumor diameter more than 5 cm. The tumor size range was between 2 cm and 7 cm. The postoperative stages of patients were I - II and III - IV in 24 and 23 patients, respectively. Thirty-four patients had lymph node metastasis. The distant metastasis and non-distant metastasis were found in 11 and 36 patients, respectively. The number of patients with perineural invasion was 20, and 27 patients had no perineural invasion. The statistical analysis indicated that the expression levels of the CXCR1 and HIF-1α were associated with lymph node metastasis and HIF-1α was correlated with TNM stage in CRC ($p < 0.05$).

CXCR1, IκBα, and HIF-1α expression levels in CRC tissues vs. adjacent normal colorectal tissue
In order to evaluate the expression of CXCR1, IκBα, and HIF-1α in the corresponding specimens, we analyzed real-time PCR data generated from 47 pair samples, CRC tissue samples and their adjacent normal ones. The results represented that expression levels of CXCR1 and HIF-1α increased in CRC tissues in comparison with adjacent normal colorectal tissue (1.53 ± 0.10 vs. 1.16 ± 0.07, and 2.22 ± 0.14 vs. 1.18 ± 0.09, $p < 0.05$, respectively). However, the relative expression level of IκBα mRNA decreased (0.80 ± 0.12 vs. 1.15 ± 0.07, $p < 0.05$) (Figure 1).

Clinicopathological significance of CXCR1, IκBα, and HIF-1α expression
The associations between expression levels of CXCR1and IκBα as well as HIF-1α and clinicopathological parameters were shown in Table 1. The expression of CXCR1 and HIF-1α was positively observed in 72% and 85% of the patients, respectively; the low expression rate of IκBα was 60%. Results revealed CXCR1 expression level was correlated with lymph node metastasis ($p = 0.013$). Also, results indicated association between HIF-1α expression and TNM stage and lymph node metastasis ($p = 0.047$, and $p = 0.005$, respectively). There were no statistically significant differences in expression of CXCR1, IκBα, and HIF-1α with regard to patient age, gender, tumor size, distant metastasis, and perineural invasion.

Association of single or combined CXCR1, IκBα, and HIF-1α expression with lymph node metastasis
Results showed that the increased incidence of lymph node metastasis in CRC patients correlated with over expression of HIF-1α and CXCR1 ($p = 0.005$ and $p = 0.013$; Table 1). In addition, there were three correlation scenarios of lymph node metastasis of CRC with combined expression, namely CXCR1/IκBα, CXCR1/ HIF-1α, and IκBα/HIF-1α. Considering the above-mentioned scenarios, CXCR1/HIF-1α expression was more prevalent compared to others ($p = 0.033$, Table 2).

DISCUSSION

In the present retrospective study, results showed CXCR1 expression level in CRC tissues was significantly higher than in comparison with normal margin tissues ($p < 0.05$), and the expression of CXCR1 significantly correlated with lymph node metastasis ($p < 0.05$). C-X-C motif chemokine ligand 8 (CXCL8) signaling is regulated by CXCL8 (IL-8), CXCR1, and CXCR2 expression in normal tissue. CXCL8 signaling is induced by steroid hormones, inflammatory signals (e.g., TNF-α and IL-1), death receptors, and reactive oxygen species [15,18,14]. Also, CXCL8 signaling may be induced by multiple environmental stresses, such as hyperglycemia, hypoxia, acidosis, radiation, and cytotoxic chemotherapies [18]. Also, the gain of function mutations in oncogenic genes and loss of function of tumor suppressor genes can lead to disorders of particular signaling pathways that regulate CXCL8, CXCR1, and CXCR2 expression. Several studies have shown hypoxia conditions can stimulate the CXCL8 signaling pathway in hypoxic cells [19] and IL-8 induces an increase of HIF-1, AP-1, and NF-κB transcription factors that can augment expression of genes associated with the regulation of cellular metabolism and apoptosis. Also, the CXCL8 promoter comprises binding sites for HIF-1 and AP-1, and NF-κB transcription factors can underpin transcription of the IL-8 gene [20-22].

Table 1. Correlation of CXCR1, IKBα, and HIF-1α expression with clinicopathologic features in colorectal cancer.

Clinicopathologic Parameters	Case No.	CXCR1 expression		p-value	IKBα expression		p-value	HIF-1α expression		p-value
		Low	High		Low	High		Low	High	
Age										
≤ 60	28	9	19	0.404	16	12	0.680	3	25	0.329
> 60	19	4	15		12	7		4	15	
Gender										
Male	27	6	21	0.333	16	11	0.959	3	24	0.397
Female	20	7	13		12	8		4	16	
Tumor size										
≤ 5 cm	21	5	16	0.596	14	7	0.373	4	17	0.472
> 5 cm	26	8	18		14	12		3	23	
TNM stage										
I - II	24	9	15	0.123	15	9	0.676	6	18	0.047 *
III - IV	23	4	19		13	10		1	22	
Lymph Node Metastasis										
Yes	34	6	28	0.013*	20	14	0.865	2	32	0.005 *
No	13	7	6		8	5		5	8	
Distant metastasis										
Yes	11	2	9	0.422	9	2	0.086	0	11	0.113
No	36	11	25		19	17		7	29	
Perineural invasion										
Yes	20	3	17	0.095	11	9	0.582	1	19	0.101
No	27	10	17		17	10		6	21	
Total cases	47	13	34		28	19		7	40	

Table 2. Correlation of combined expression of CXCR1, IKBα, and HIF-1α with lymph node status.

	Lymph node metastasis		p-value
	Positive	Negative	
CXCR1/IKBα			
(1) Both CXCR1/IKBα high expression	12	1	0.079
(2) One of CXCR1/IKBα high expression	18	9	
(3) Both CXCR1/IKBα low expression	4	3	0.638
CXCR1/HIF-1α			
(1) Both CXCR1/HIF-1α high expression	28	5	0.033*
(2) One of CXCR1/HIF-1α high expression	4	4	
(3) Both CXCR1/HIF-1α low expression	2	4	0.533
IKBα/HIF-1α			
(1) Both IKBα/HIF-1α high expression	14	3	0.439
(2) One of IKBα/HIF-1α high expression	18	7	
(3) Both IKBα/HIF-1α low expression	2	3	0.166

In this study, results revealed expression of HIF-1α is significantly more frequent in CRC tissues than in normal tissues ($p < 0.001$), and a significant association was considered between high expression levels of HIF-

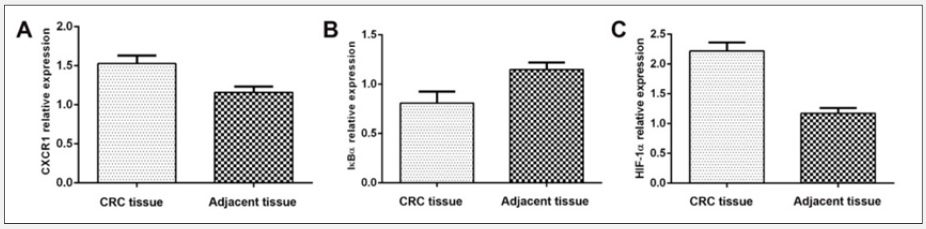

Figure 1. Expression of CXCR1, IκBα, and HIF-1α mRNA in colorectal cancer tissues compared with adjacent normal colorectal tissue by real-time PCR (Mean ± S.E., $p < 0.05$).

1α and tumor TNM stage ($p < 0.05$) and lymph node metastasis ($p < 0.01$). The HIF-1α expression level has been investigated in various human cancers and its expression can be correlated with angiogenesis [19,23]. Hence, by considering HIF-1α as a marker involved in angiogenesis and metastasis of tumors, accompaniment of the expression level of CXCR1 and IκBα was examined. HIF-1 is a heterodimeric transcription factor that responds to a hypoxic cellular microenvironment. In normoxic conditions, HIF-1α subunits are hydroxylated by prolyl hydroxylases and after ubiquitination, proteasomal degradation mediated by the Von-Hippel Lindau-dependent pathway [24,25]. Under hypoxic conditions, HIF prolyl-hydroxylase is inhibited, and HIF-1α translocates to the nucleus and dimerizes with HIF-1β. HIF-1 heterodimeric transcription factor binds to promoters of HIF-responsive element (HREs) genes which are essential for proliferation, angiogenesis, metastasis, and apoptosis [26,27]. Forsythe et al. identified a functional HRE in the 5′ flanking region of the VEGF human promoter [28]. Several studies have shown that VEGF is an important angiogenic factor and has a key role in cancer progression [29].

Several studies showed that NF-κB is a direct modulator of HIF-1α expression in the presence of normal and hypoxic oxygen pressure [17,30]. In the canonical pathway, many pro-inflammatory stimuli (e.g., TNF-α and IL-1), lipopolysaccharide (LPS), and different drugs can activate the NF-κB signaling pathway. Constitutively activated NF-κB is reported in various cancer cells [31-34]. NF-κB transcription factor (RelA/p50) is inhibited by IκB in the cytoplasm and triggers signal transduction of the NF-κB pathway activating an IκB kinase (IKK). Activated IKK phosphorylates the IκB inhibitory protein and ultimately results in degradation of IκB, and free NF-κB dimers enter to the nucleus [17]. NF-κB activates transcription of target genes, including angiogenesis, anti-apoptosis, and metastasis genes. Angiogenic factors such as CXCL8 are considered to be regulated by NF-κB [31]. Several studies represented the critical role of NF-κB in the regulation of HIF-1α expression level [17,30]. Also, VEGF is partially regulated by NF-κB. The results showed that expression of IκBα is significantly less frequent in CRC tissues than in normal tissues ($p < 0.001$).

The mRNA levels of CXCR1, HIFα, and IκBα were evaluated by RT-qPCR and analyzed solely and in combination with the clinicopathologic characteristics of CRC samples. Statistical analyses showed CXCR1/HIF-1α high expression correlates significantly with lymph node metastasis ($p < 0.05$).

CONCLUSION

CXCR1 and HIF-1α were highly expressed and IκBα expression decreased in CRC samples. CXCR1 and HIF-1α expression, all together, revealed the significant association with lymph node metastasis in CRC. Therefore, CXCR1 in conjunction with HIF-1α and IκBα could be used as a potential prognostic factor for indicating tumors. Considering the obtained results and limitations in collecting fresh tissue samples, further studies are needed to verify our findings.

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Declaration of Interest:
The authors have no conflicts of interest to disclose.

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