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Clinicopathological Implications of Interleukin-6 Expression in Colorectal Cancer Patients

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A B S T R A C T

Interleukin-6 (IL-6) is a cytokine that plays a significant role in the immune response and inflammation. IL-6 is regarded as an important tumor-promoting factor in various human malignancies including colorectal cancer (CRC). In CRC, IL-6 expression has been implicated in cancer progression, including proliferation, migration, and angiogenesis. Numerous studies described an association of increased IL-6 expression with poor survival and unfavorable clinical outcome in CRC patients. Hence, present study aimed to examine IL-6 mRNA expression in CRC patients and further to correlate the results with clinicopathological parameters and survival. A total of 50 histologically confirmed CRC patients and 10 healthy controls were enrolled in the study. The relative expression of IL-6 mRNA was studied by reverse transcription quantitative real-time PCR and the fold change IL-6 expression was determined using 2-$\Delta\Delta$CT method. The data was analyzed using SPSS software and P value $\leqslant$ 0.05 was considered as significant. Out of 50 CRC patients, upregulation of IL-6 mRNA expression was detected in 92% (46/50) of patients, while only 8% (4/50) of patients showed IL-6 downregulation as compared to controls. In relation to clinicopathological parameters, the fold change IL-6 mRNA expression was found to be significantly higher in patients with positive lymph node status (P=0.047), advanced stage (P=0.047), moderately/poorly differentiated tumors (P=0.017) and presence of necrosis (P=0.045) as compared to their respective counterparts. Moreover, a trend of higher IL-6 mRNA fold change expression was found in female patients as compared to male patients (P=0.097); and in patients with tumors located on the right side as compared to left side located tumors (P=0.051). Further, IL-6 mRNA expression was not significantly correlated with relapse-free survival (RFS) and overall survival (OS). It can be concluded that correlation of higher IL-6 mRNA expression with high-risk clinicopathological factors suggests its role as a useful biomarker to predict poor prognosis in CRC patients.

Keywords: Interleukin-6, mRNA expression, Real-time PCR, Colorectal Cancer, Clinicopathological Parameters, Survival

1. Introduction

The prevalence of colorectal cancer (CRC) has been dramatically increasing in recent years with an estimated 1.93 million new cases diagnosed worldwide, accounting for 10% incidence, and it remained the second deadliest cancer accounting for 9.4% mortality rate according to GLOBOCAN 2020 [1]. Various genetic, epigenetic as well as environmental and life-style related factors such as lack of exercise, poor diet, obesity, diabetes mellitus, smoking, alcohol consumption have been shown to be responsible for the onset and progression of CRC [2]. Chronic inflammation has also been regarded as an important risk factor for the development of CRC [3] and growing evidence shows its association with dysregulation of numerous signaling pathways, among which IL-6 signaling pathway play significant role to promote CRC cell proliferation and survival [4]. Experimental and clinical studies demonstrated a clear association of sporadic CRC and inflammation-associated CRC with IL-6 signaling, although the specific mechanism through which IL-6 plays a role during CRC onset and progression has not been completely clarified [5].

Interleukin-6 (IL-6) is a pleiotropic cytokine having pro- or anti-inflammatory roles, produced by various cell types, such as stromal, hematopoietic, epithelial, and muscle cells. Its anti- and pro-inflammatory properties make it able to alter the responses to various diseases, including immune diseases; chronic inflammatory diseases; and different cancers [5]. Numerous studies have prescribed a probable responsibility of IL-6 in colon cancer initiation and progression. Increased serum IL-6 levels have been reported in various malignancies and higher IL-6 levels correlates with metastasis and unfavorable prognosis. It has been demonstrated that serum levels of IL-6 were significantly higher in CRC patients than healthy controls [6]. The plasma concentration of IL-6 was significantly higher in the CRC cases compared to the controls [7] and it was associated with colon cancer risk [8]. Further, scientific evidence described the upregulation of serum IL-6 in CRC, and its correlation with unfavorable prognosis in metastatic colon cancer patients and treatment-refractory carcinomas. In-vitro studies also showed that IL-6 promotes the growth of epithelial colon cancer cells. These observations suggest the role of IL-6 serum levels to be a diagnostic and prognostic biomarker in CRC patients that correlates with relapse-free survival and recurrence [5].

However, there is comparatively little work performed on the analysis of IL-6 mRNA expression by real time PCR and its association with clinicopathological features and survival in CRC patients. Several studies indicated the role of IL-6 expression as poor prognostic indicator. Higher tissue IL-6 expression was observed in CRC patients as compared to healthy colon mucosa and increased IL-6 expression positively correlated with the age, tumor TNM stage and grade [9]. IL-6 was found to be a risk indicator for CRC [10]. Further, high IL-6 expression showed association with risk of relapse [11]. These findings suggest that a high tumor tissue IL-6 expression might be a useful marker to predict poor prognosis in patients with CRC. Additionally, IL-6 might be suggested to be used as a potential therapeutic target in CRC [12]. Hence, present study aimed to evaluate the role of IL-6 mRNA expression in CRC patients and its association with established clinicopathological parameters and prognosis.

2. Material and Methods

2.1 Patients

A total 50 untreated histologically confirmed colorectal cancer patients at Gujarat Cancer and Research Institute between 2017 and 2021, and 10 age-matched healthy controls were enrolled in this study. A detailed clinicopathologic history of patients was obtained from case files maintained at the institute. The study was approved by institutional review and ethics committee.

2.2 Sample Collection

Written consent was taken from all the patients prior to sample collection. Primary tumor tissue specimens of all 50 CRC patients were collected on ice directly from the operation theatre. Tumor part from collected specimen was selected by a pathologist and divided into 2 portions. One portion was submitted for routine histopathological evaluation. Another tumor portion was collected in a vial containing RNA later reagent and then snap frozen in liquid nitrogen and preserved at -80°C till further analysis. Further, peripheral blood samples of healthy controls were collected.

2.3 Real time PCR

Total RNA extraction from tumor tissue samples as well as blood samples of healthy controls was performed using RNA isoplus extraction method. The extracted RNA was quantified fluorometrically using Qubit Spectrofluorometer 3.0 (Invitrogen). Reverse transcription quantitative real-time PCR (RT-qPCR) was performed to quantify the IL-6 mRNA expression. RNA was reverse transcribed to cDNA using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, cat # 4368814) as per manufacturer's protocol. The quantitative real-time PCR of cDNA samples was performed using QuantiNova SYBR Green PCR Kit (Qiagen cat #208052) and 1 μg of total RNA was added per reaction. Primers for IL-6 were as follows: Forward-5' AAA CAA CCT GAA CCT TCC AAA GA 3' and Reverse- 5' GCA AGT CTC CTC ATT GAA TCC A 3'. GAPDH was used as the internal control for normalization. The following conditions were applied: Initial activation step at 95ºC for 2 min, 2-step cycling (40 cycles) at 95ºC denaturation for 5 sec, combined annealing/ extension at 60ºC for 10 sec. The Ct values were obtained for IL-6 and GAPDH for both patients and healthy controls. The levels of IL-6 were analysed quantitatively relative to GAPDH by $2^{-\Delta\Delta Ct}$ method using the following equation.

$\Delta Ct = (Ct_{IL\text{-}6} - Ct_{GAPDH})$

$\Delta\Delta Ct = (\Delta Ct_{patient} - \Delta Ct_{Control})$, Average ΔCt of controls was taken to calculate $\Delta\Delta Ct$ values.

2.4 Statistical Analysis

Data was evaluated statistically using SPSS (Statistical Package for the Social Sciences) software. Association of relative expression (Mean ± SE of fold change) of IL-6 with the clinicopathological parameters of colorectal cancer patients was calculated using Independent-Samples T-test. Relapse free survival and overall survival was calculated using Kaplan-Meier method and Log rank test. P value ≤0.05 was considered as significant.

3. Results

3.1 Incidence of IL-6 mRNA Expression in CRC Patients

Among the studied 50 CRC patients, majority of the patients (92%; 46/50) showed upregulation of IL-6 mRNA expression, while only 8% (4/50) of patients showed downregulation of IL-6 mRNA expression as compared to controls.

3.2 Correlation of IL-6 mRNA Expression with Clinicopathological Parameters

In relation to clinical parameters, relative IL-6 mRNA expression showed a trend towards significance with gender and tumor location. A higher trend of IL-6 mRNA fold change expression was found in female patients as compared to in male patients (P=0.097). Moreover, a trend of higher IL-6 mRNA expression was observed in patients having tumors located on the right side as compared to those having tumors located on the left side (P=0.051) (Table 1).

With regard to pathological parameters, IL-6 mRNA expression showed significant association with nodal status, TNM stage, histological grade and necrosis. A significantly higher IL-6 expression was noted in patients with lymph node metastasis as compared to those without lymph node metastasis (P=0.047). The relative IL-6 expression was significantly higher in advanced (III) stage patients as compared to early (I+II) stage patients (P=0.047). Additionally, a significant higher fold change IL-6 mRNA expression was observed in patients having moderately/poorly differentiated tumors as compared to those with well differentiated tumors (P=0.017). Moreover, in patients having presence of necrosis, the relative IL-6 mRNA expression was significantly higher as compared to its expression in patients having absence of necrosis (P=0.045) (Table 2).

Table 1 - Correlation of IL-6 expression with clinical parameters in CRC patients (N=50)

Characteristics	**N**	**IL-6 mRNA Expression**	
		Mean ± SE	**P- value**
Age (years)			
<56	24	74.72 ± 22.84	0.555
≥56	26	118.29 ± 67.21	
Gender			
Female	16	185.60 ± 427.47	0.097
Male	34	55.86 ± 99.16	
Habit*			
No	31	132.42 ± 57.39	0.223
Yes	19	40.20 ± 15.72	
Family history			
No	46	102.91 ± 39.49	0.612
Yes	04	33.77 ± 14.43	
Diet			
Vegetarian	37	102.80 ± 47.81	0.805
Vegetarian + Non-vegetarian	13	81.95 ± 35.73	
Tumor site			
Colon	36	117.21± 49.27	0.388
Rectum	14	46.39 ± 27.61	
Tumor location#			
Right side	18	191.85 ± 94.95	**0.051**
Left side	32	44.24 ± 15.50	

*Tobacco chewing, smoking, alcohol, snuff (anyone or in combination)

Right side: cecum, ascending colon and transverse colon

Left side: descending colon, sigmoid colon and rectum

Table 2 - Correlation of IL-6 expression with pathological parameters in CRC patients (N=50)

Characteristics	**N**	**IL-6 mRNA Expression**	
		Mean± SE	**P- value**
Tumor size			
Small (T1+ T2)	05	34.95 ± 17.47	0.573
Large (T3+T4)	45	104.31 ± 40.33	
Nodal status			

Characteristics	**N**	**IL-6 mRNA Expression**	
		Mean± SE	**P- value**
Negative	22	23.56 ± 6.27	**0.047**
Positive	28	155.38 ± 63.16	
TNM stage			
Early (I+II)	22	23.56 ± 6.27	**0.047**
Advanced (III+IV)	28	155.38 ± 63.16	
Tumor differentiation			
Well	06	7.29 ± 1.48	**0.017**
Moderate + poor	44	109.66 ± 41.08	
Histologic type			
Adenocarcinoma	41	103.92 ± 44.06	0.706
Mucinous/Signet ring cell	09	67.59 ± 27.64	
Mucin			
Absent	40	105.38 ± 45.13	0.665
Present	10	65.39 ± 25.34	
Lymphatic permeation			
Absent	25	109.96 ± 69.47	0.733
Present	25	84.79 ± 23.97	
Vascular permeation			
Absent	34	121.28 ± 52.75	0.344
Present	16	46.59 ± 16.52	
Lymphocytic stromal response			
Absent	22	125.60 ± 78.53	0.498
Present	28	75.21 ± 22.06	
Perineural invasion			
Absent	42	97.92 ± 42.35	0.973
Present	08	94.54 ± 53.43	
Pre-op circulating CEA (ng/ml)			
≤ 5.0	23	39.56 ± 15.32	0.145
> 5.0	27	146.63 ± 65.23	

3.3 Survival Analysis

Out of total 50 patients enrolled, 42 patients could be followed for the period of 24 months or until death within that period and were included for Overall survival (OS) analysis. Out of these 42 patients, 35 patients were alive and 7 patients were dead. For relapse–free survival (RFS) analysis, out of total 42 patients, 2 patients with persistence disease were excluded. Hence, 40 patients were included for RFS analysis. Out of these 40 patients, 12 patients had local/distant metastasis, while the rest 28 patients had no recurrence. For survival analysis, median IL-6 relative quantification was used as cut-off to divide the patients into low and high IL-6 mRNA expression groups. Kaplan-Meier univariate survival analysis for RFS showed no significant difference in the incidence of disease relapse between patients with low IL-6 mRNA expression 21% (04/19) and those with high expression 38% (08/21) (Log rank=1.295, df=1, P=0.255) [Fig. 1]. For OS also, no significant difference in the incidence of death was observed between patients with low IL-6 mRNA expression 11% (02/19) and those with high expression 22% (05/23) (Log rank=1.108, df=1, P=0.292) [Fig. 1].

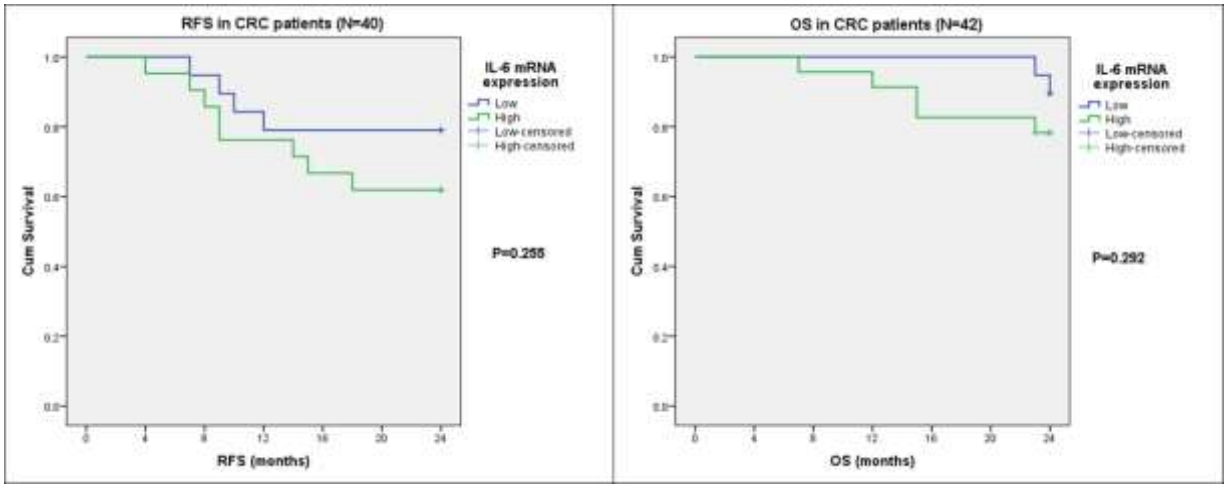

Fig. 1: Kaplan-Meier univariate survival analysis for RFS and OS in relation to IL-6 mRNA expression in CRC patients

4. Discussion

Colorectal cancer is one of the most common malignancies and the fourth leading cause of cancer related death worldwide. Different factors have been shown to be liable for the accumulation of mutations in CRC including inheritance and environmental factors (e.g composition of diet, obesity, diabetes mellitus, smoking, alcohol consumption). Also chronic inflammation is regarded as an important risk factor for the development of cancer [2]. Tumor promoting inflammation is one of the hallmarks of cancer and data propose a direct effect of inflammation on tumor growth. Many previous studies have shown that neoplasms arise at sites of chronic inflammation. Further, it was observed that upregulation of inflammatory cytokines secreted by inflammatory cells, other mesenchymal cells and tumor cells could facilitate tumor initiation and enhance tumor cell proliferation and invasion. Among the numerous inflammatory cytokines, interleukin-6 has continually attracted extensive attention [12].

A multifunctional cytokine IL-6 takes on pro- or anti-inflammatory roles in various biological mechanisms, such as immune responses, cell survival, apoptosis, and proliferation. Today, IL-6 is regarded as an important tumor promoting factor in various types of human cancer including glioma, lymphoma, melanoma as well as breast, ovarian, pancreatic, prostate, renal and colorectal cancer. Clinical and experimental data strongly propose a contribution of IL-6 signaling to the development of both sporadic and inflammation-associated CRC development [5].

To evaluate the clinical value of IL-6 expression in CRC, present study analyzed IL-6 mRNA expression by RT-qPCR technique in tumor tissues of 50 CRC patients. IL-6 mRNA expression was found to be upregulated in 92% of CRC patients, while it was downregulated in only 8% of patients as compared to controls. Accordingly, one study evaluated IL-6 mRNA by real time PCR and IL-6 mRNA expression was significantly upregulated in tumor tissues of CRC patients compared to normal adjacent tissues ($p< 0.001$) [11]. Moreover, several studies have investigated the expression of IL-6 in CRC and found that IL-6 expression was elevated in CRC compared with normal mucosa [12]. In addition, one recent study by observed 78% IL-6 immunopositivity in CRC tumor tissues as compared to normal tissues [9].

Majority of the studies in CRC examined serum circulating levels of IL-6 and observed elevated IL-6 levels in patients. Higher serum IL-6 levels were found in colon cancer patients than healthy controls [13]. Waldner et al also found elevated levels of IL-6 in the serum of patients with CRC and in tumor tissue [2]. Median IL-6 level was significantly higher in patients with CRC than in normal controls [14]. A previous study by Kinoshita et al in CRC examined serum and tissue levels of IL-6 in 70 CRC patients and demonstrated that the concentration of serum IL-6 in the patients was significantly higher than that in normal controls. Also, the concentration of IL-6 in tumor tissue was significantly higher than that in normal mucosa, and was correlated strongly with the serum IL-6 concentration [15]. Moreover, it has been recorded that IL-6 levels in serum samples was associated with an increased risk of colorectal tumors [16,17]. Thus, consistence with above studies, present results also showed higher IL-6 expression in majority of CRC patients as compared to age-matched healthy controls.

Further, present study correlated relative IL-6 mRNA expression with clinicopathological parameters in CRC patients. Higher IL-6 expression in patients with elder age group was observed as compared to younger age group. However, the association was not significant (P=0.555). Similarly, advanced age was positively associated with a higher score of IL-6 in CRC patients [9]. Further, on the subject of gender, current study found a trend of higher IL-6 expression in female patients as compared to male patients (P=0.097). Contradictorily, the expression of IL-6 was higher in males than in females [9]. However, Hsu et al found no correlation of IL-6 with gender [18]. These inconsistent results might be due to the use of different study populations or methodologies, or different etiologies of CRC patients.

Current study also showed a trend of higher IL-6 mRNA expression in patients having tumors located on the right side as compared to those having tumors located on the left side (P=0.051). Further, there was a significant association of IL-6 mRNA expression with nodal status, tumor stage, histological grade and necrosis. The fold change IL-6 mRNA expression was found to be significantly higher in patients with positive lymph node status (P=0.047),

advanced stage (P=0.047), moderately/poorly differentiated tumors (P=0.017) and presence of necrosis (P=0.045) as compared to their respective counterparts. In accordance with present study, recent report showed that patients with advanced stage and poorly differentiated tumors had a significantly high tumor IL-6 expression [9]. Zeng et al also described that the levels of IL-6 expression were positively associated with TNM stage, whereas inversely associated with histological differentiation. They also observed association of IL-6 expression with invasion depth and lymphnode metastasis in CRC, which is in accordance to present study [12]. However, in a cohort of 189 CRC patients, no significant association was found between IL-6 mRNA expression and tumor differentiation grade or cancer stage [11]. Moreover, consistent with present results, previous study demonstrated that patients with stage III and IV disease had a significant higher IL-6 serum concentration than those with stage I and II disease [19]. Other studies also described that higher IL-6 levels were associated with increasing tumor stages, tumor size, tumor local invasion, metastasis, and decreased survival [13,20]. Previously, the serum IL-6 concentration was correlated with tumor size and liver metastases, and it may reflect the proliferative activity of the tumor in patients with colorectal carcinoma [15]. A study by Chung-Chang et al revealed that IL-6 played a role in colon cancer patients with high serum levels (>12 pg/mL) correlating with larger tumor size, elevated serum CRP levels, and liver metastasis [14]. In CRC, serum IL-6 levels were also associated with high circulating CEA levels [19], which is in accordance to present study showing non-significant but higher IL-6 expression in patients having high circulating CEA levels (P=0.145).

These results imply that IL-6 might have a role in CRC invasion and metastasis and it may be involved in CRC progression. IL-6 is a critical tumor promoter during early CRC tumorigenesis [12]. A study by Miller et al [21] denotes that IL-6 may be implicated in CRC progression. Moreover, it was reported that IL-6 has important roles in cancer progression, including proliferation, migration, and angiogenesis in several cancers, and it deteriorates cancer prognosis [22,23,24]. These observations suggest that disease progression may be initiated through the ability of IL-6 to induce migration and proliferation, which has been shown in several colonic cancer cell line studies [25,26]. In a view of above studies, present results described association of higher IL-6 mRNA expression with high-risk prognostic parameters in CRC patients, which suggests the role of IL-6 in disease aggressiveness and progression.

Present study also examined association of IL-6 mRNA expression with survival. However, IL-6 expression showed no significant association with RFS and OS in CRC patients. Discordant to this, previous observations in CRC stated association of high serum IL-6 levels with poor prognosis. CRC patients with higher serum IL-6 level had a worse OS (P = 0.0027) and DFS (P < 0.001) [27]. Another record demonstrated association of elevated IL-6 levels with tumor recurrences [28]. Further, high IL-6 expression was significantly associated with risk of relapse in colonic cancer, even when adjusted for clinicopathological characteristics [11]. This might imply a role for treatment with antibodies against IL-6 in CRC. Commercially available monoclonal antibodies against IL-6 are being tested in various advanced solid tumors such as ovarian and prostate cancer. Moreover, IL-6 has been proposed as a possible target in colonic cancer therapy [11]. Further, growing evidence shows a critical role for IL-6 signaling in the development of both sporadic and inflammation-associated CRC. Hence, targeting this pathway for therapeutics could be promising options for CRC patients [12]. Hence, further studies including large cohort are required to establish the role of IL-6 as a potential therapeutic as well as prognostic marker in CRC.

5. Conclusion

Correlation of higher IL-6 mRNA expression with high-risk clinicopathological parameters suggests its association with disease aggressiveness. Hence, increased IL-6 expression might have a role in CRC progression and may be used as a biomarker to predict poor prognosis in CRC patients. However, IL-6 mRNA expression had no significant association with survival in studied CRC patients.

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