Interleukin-6 Expression in Colorectal Tumors in Iraqi Patients

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ABSTRACT

Background: *Colorectal cancer ranks as the 7th most common cancer among Iraqis. This task intended to check out the tissue level of Interleukin-6 (IL-6) in colorectal tumors using IL-6 specific antibodies and Immunohistochemistry (IHC).* **Methods:** *IHC staining was performed using Interleukin-6 (IL6) specific antibodies. Tissue samples included 50 cases of colorectal cancer, 8 cases of colon adenomas in addition to 20 samples of normal colon tissues as a control.* **Results:** *Total 39 out of 50 colorectal cancer cases (78%) demonstrated positive expression of IL-6 specific antibody. While only 2 out of 8 adenomas (25%) exhibited positive outcome when stained with this marker, comparing to 20 normal colon tissue samples which revealed a negative expression of IL-6. Results disclosed significant association between IL-6 expression and clinicopathological traits including age, sex as well as tumor stage and grade.* **Conclusion:** *Our findings revealed that IL-6 might be a valuable indicator for predicting the prognosis for patients with colorectal cancer and might act as a future therapeutic approach in colorectal cancer.*

Keywords: Interleukin-6, Tumor markers, Colon cancer, Immunohistochemistry

INTRODUCTION

Colorectal cancer is the third most common malignancy worldwide, and the 7th in Iraq. It is a major cause of cancer-related deaths; a lot of research has been focused on the uses of new biomarkers to improve the diagnostic process [1].

Interleukin-6 (IL-6) is a versatile cytokine that is implicated in tumor growth, invasion, and metastasis in human malignancies [2,3]. A significant role of IL-6 in the tumor initiation and progression of a variety of cancers had been suggested. For instance, Nguyen, et al., denoted that IL-6 is an essential modifier in the establishment of prostate tumorigenesis, tumor growth, metastasis, and resistance to chemotherapy [4]. Zhang, et al., reported that IL-6 is crucial for keeping and development of pancreatic intraepithelial neoplasia [5]. Taniguchi, et al., reviewed that serum IL-6 levels showed a relationship with poor prognosis, tumor burden, survival and advanced stages of the disease in a variety of cancers including lung, esophagus, mammary gland, ovary, and kidney cancer. Besides, numerous latest studies have prescribed a probable responsibility of IL-6 in colon cancer initiation and progression [6]. It turned out that serum levels of IL-6 were raised in colorectal cancer (CRC) patients [7]. Furthermore, it appears that IL-6 promotes the growth of colorectal cancer epithelial cells *in vitro*. However, there is comparatively little work on the application and analysis of IL-6 by immunohistochemistry and its' correspondence with clinicopathological features of CRC [8].

The aim of this study is to assess the immunohistochemical expression of this marker in colorectal tumors in Iraqi patients and its association with clinicopathological parameters.

MATERIALS AND METHODS

Total 50 specimens of colorectal cancer from Iraqi patients and other 8 tissue samples of colonic adenomas in the form of paraffin-embedded tissue blocks were collected from histopathology unit of Al-Yarmouk teaching hospital,

Baghdad, in addition to 20 colonic biopsies with no significant pathology used as control cases. The clinical records about patient's age, sex and site of the tumor as well as pathological stage and grade were collected from the existing histological reports. Each block was sliced at a thickness of 5 μm. Sections were then placed on salinized coated slides and were heated at 58°C for 24 hours.

Sections were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol (100%, 90%, 80%, and 70%). Antigen was retrieved by antigen retrieval solution (ready to use) inside the pressure cooker and heated in the scientific microwave for 10 min. Sections were encircled with a wax pencil around the tissues. All slides were incubated in peroxidase-blocking solution for 10 minutes, followed by washing with distilled water (DW). Slides were incubated with a primary antibody specific for IL-6 [mouse monoclonal antibodies, Abcam 9324, diluted (1:250) using antibody diluent] for 1 hour at room temperature (25°C). After that, sections were washed with buffer and incubated with biotinylated link antibody (ready to use) for 15 min at room temperature (25°C), followed by 3 items of washing. Next, sections were incubated with streptavidin for 15 min and were then washed 3 times in wash buffer. Sections were incubated with diaminobenzidine (DAB) peroxidase for 10 min and then washed 3 times with wash buffer. The slides were then counterstained in hematoxylin, dehydrated in increased series of alcohol (70%, 80%, 90%, and 100%) and finally coverslipped. Slides were scanned and scored by consultant pathologist. Positive controls of known positive tissues (human spleen formalin fixed paraffin embedded tissue section) and negative controls with primary antibody replaced with TBS were run with the patient slides.

IL-6 Scoring System

Cells with visible brown particles in the cytoplasm were taken as positive. The immunohistochemical results were determined according to Zeng, et al., depending on the intensity of IL-6 staining (weak=1, intense=2), and the percentage of positive tumor cells (0%=0, 1%-50%=1, 51%-75%=2, >75%=3). The eventual score of each sample was determined by multiplying intensity by percentage score [8], and the tumors were ultimately categorized negative expression: score=0; low expression: score ≤ 3; or high expression: score >3.

Statistical Analysis

Statistical analysis and reporting of obtaining data were carried out by using SPSS version 20. The statistical significances of difference (comparison) between the study groups, discrete (qualitative) variables of 2 groups were assessed by using the Chi-square test. Statistical tests were approved by assuming a null hypothesis of no difference between variables, a probability was considered statistically significant at p ≤ 0.05.

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RESULTS

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- **Age:** Range of patient's age was 20-75 years, with a mean of (54.93 ± 14.24) years of patients with colonic carcinoma. The mean age of patients with colonic adenomas was (50.00 ± 17.93) years

- **Sex:** Of the total 50 cases of carcinoma, 36 (72.2%) were males, while 14 (27.8%) were females

- **Tumor site:** Malignant tumors from right colon encompassed (40%), while tumors from left colon comprised 60% of cases (Figure 1)
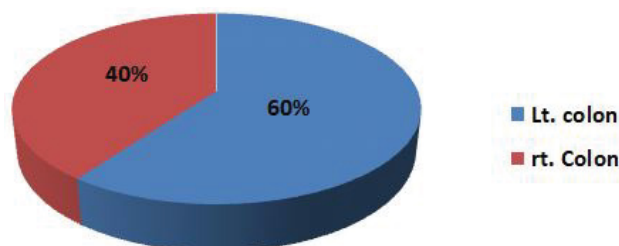

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Figure 1 Distribution of cases according to the site of the tumor

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- **Tumor stage:** According to Dukes classification, 16% of malignant cases were staged A, 20% were stage B1, 24% were stage B2, 10% were stage C1 and 30% of cases were stage C2 (Figure 2)
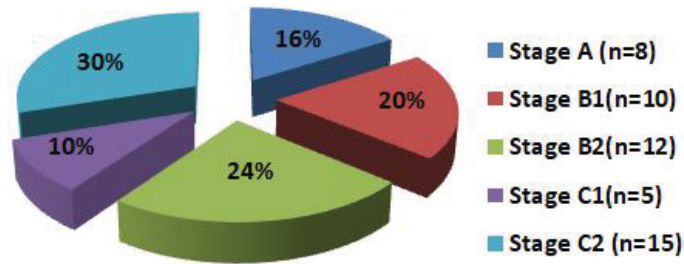
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Figure 2 Distribution of cases according to the stage of tumor

- **Tumor grade:** According to WHO grading, 28% of malignant cases were well differentiated, 24% were moderately differentiated and 48% were poorly differentiated (Figure 3)
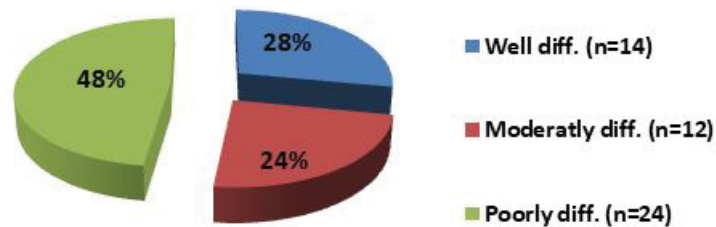

Figure 3 Distribution of cases according to the grade of the tumor

Immunohistochemical Expression of IL-6 in Groups of Study

Results showed that 78% (39/50) of malignant cases showed positive expression of IL-6, whereas it was detected in only 25% (2/8) of adenomas cases. IL-6 was totally negative in normal colon tissues (Figure 4).
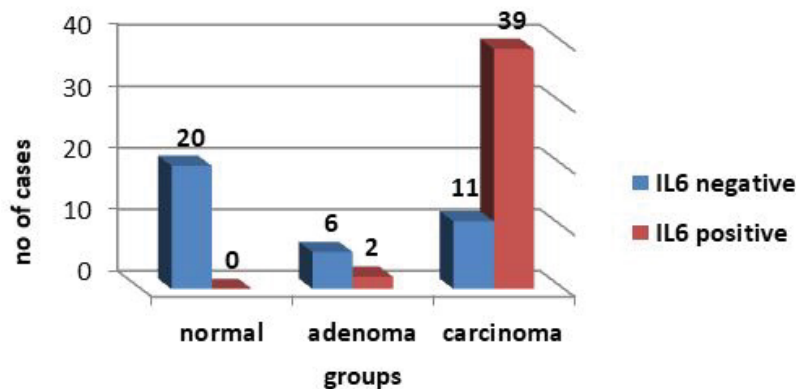

Figure 4 Immunohistochemical expression of IL-6 in groups of study

Concerning the score of IL-6, results showed that the positive malignant cases were distributed as 14% (7 cases) within score 1, 26% (13 cases) within score 2 and 38% (19 cases) showed score 3 (Figures 5 and 6).
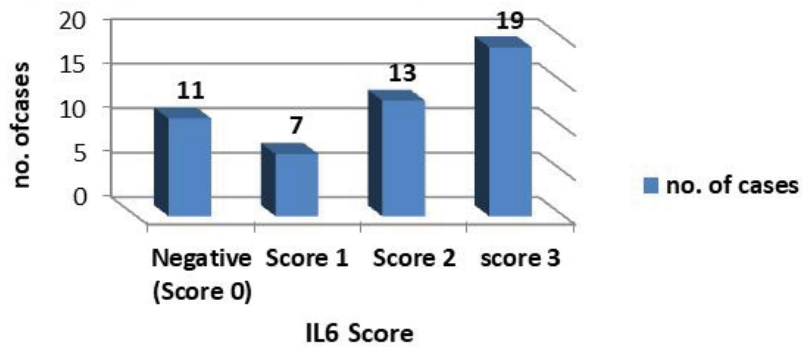
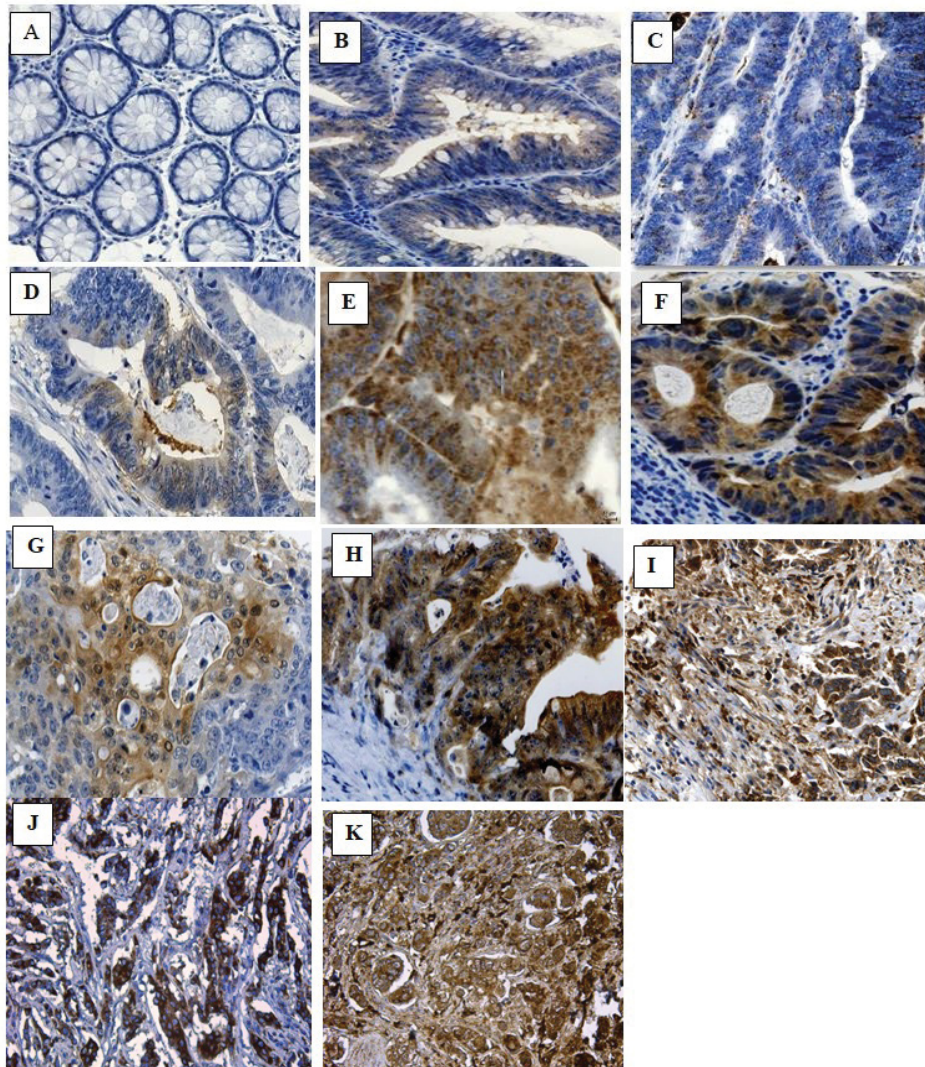**Figure 5 Distribution of malignant cases according to score**

Figure 6 Immunohistochemical expression of IL-6 in sections of colon tissues. A: normal colon tissue showing negative IL-6 expression. B and C: Adenoma (villous and tubular respectively) with positive IL-6 expression, score1 (weak<50%) D: Moderately differentiated adenocarcinoma (Dukes staging B), score1 (weak<50%). E: Moderately differentiated adenocarcinoma (Dukes staging B) score 3 (intense>76%). F: Moderately differentiated adenocarcinoma (Dukes staging C) score 3 (intense>76%). G: Poorly differentiated adenocarcinoma (Dukes staging C) score 2 (moderate 51%-75%). H, I, J and K: Poorly differentiated adenocarcinoma (Dukes staging C) Score 3 (intense>76%)

Association between the Expression of IL-6 and Clinicopathological Features

- **Age:** Results showed a significant correlation in the expression of IL-6 among age groups. i.e. the frequency of highest score (score 3) of IL-6 was seen at age group (60-69 years) rather than other groups (Table 1)

Table 1 Association between age and expression of IL-6 in study groups

Study Groups	Age Groups	Negative (Score 0)	Score 1	Score 2	Score 3
Normal (n=20)	<39 years	9	-	-	-
	40-49years	7	-	-	-
	50-59 years	4	-	-	-
Adenoma (n=8)	<39 years	2	-	-	-
	40-49 years	2	-	-	-
	50-59 years	1	-	-	-
	60-69 years	-	1	-	-
	>70 years	1	1	-	-
Carcinoma (n=50)	<39 years	-	1	2	1
	40-49 years	2	-	3	2
	50-59 years	2	2	4	3
	60-69 years	7	2	3	10
	>70 years	-	2	1	3
χ^2 test=0.0110843; p< 0.05 (significant); p<0.01 (highly significant)					

- **Sex:** Results showed a significant correlation in the expression of IL-6 between males and females, it was higher in males than in females and the vast majority of score 3 was found among males (Table 2)

Table 2 Association between sex and expression of IL-6 in cancer group

IL6 Score	Males (n=36)	Females (n=14)
Negative (Score 0) (n=11)	9	2
Score 1 (n=7)	6	1
Score 2 (n=13)	7	6
Score 3 (n=19)	14	5
p-value (χ^2-test) 0.02811 (significant)		

- **Tumor site:** No significant association was found between the expression of IL-6 and tumor site as shown in Table 3

Table 3 Association between site of tumor and expression of IL6 in cancer group

Study group	Tumor site	Negative (Score 0)	Score 1	Score 2	Score 3
(n=50)Carcinoma	Lt. colon	6	4	8	12
	Rt. Colon	5	3	5	7
χ^2 test (P)=0.4819756; p>0.05 (not significant)					

- **Tumor grade:** Results showed a highly significant correlation between the expression of IL-6 and tumor grade. The highest score of IL-6 was found with poorly differentiated tumors (Table 4)

Table 4 Association between tumor grade and expression of IL6 in cancer group

Grade	Negative (Score 0)	Score 1	Score 2	Score 3
Normal (n=20)	20	0	0	0
Adenoma (n=8)	6	2	0	0
Carcinoma (n=50) Well diff. (n=14)	5	4	5	0
Moderately diff. (n=12)	3	2	3	4
Poorly diff. (n=24)	3	1	5	15
p-value (χ^2-test): 0.00000109 (very high significant)				

- **Tumor stage:** Results demonstrated highly significant differences in the expression of IL-6 among study groups in regards to different stages. IL-6 was highly expressed in stage C cases in comparison to earlier stages

DISCUSSION

Colorectal cancer, the 7th most common malignancy in Iraq, affects male above 60 years and most are presented with left-sided tumors at an advanced stage and poor differentiation. Similar results were reported in the previous study from Iraq [9]. Many studies have been focused on the biological functions of pro-inflammation, anti-inflammation, and angiogenesis, regulated by several biological molecules, of them Interleukin-6 which is liberated in reply to infection, burns, trauma, and neoplasia and can be generated by T-lymphocytes, B lymphocytes, macrophages, endothelial cells, keratinocytes, and mesangial cells in normal tissues, as well as by hematologic neoplasms and human cancers [8,10,11].

It is common knowledge that chronic inflammation is one of the risk factors for many cancers including CRC [4], in which the inflammatory cells infiltrate to the tumor region and secrete inflammatory cytokines, which contribute to cancer development. However, even in sporadic CRC with no preceding chronic inflammation, inflammatory cells acting the same, and one of the important secretions is IL-6 which imposes proliferation and anti-apoptotic effects in tumor cells [4,12]. It has been recorded that IL-6 expression in serum samples from patients was associated with an increased risk of colorectal tumors [13,14]. Elevated levels of IL-6 had been found in the serum of patients with CRC and in tumor tissue [15]. A study by Chien-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 and liver metastasis [16]. Although data on IL-6 serum levels in sporadic CRC are well proven, there is a continuing debate about the source of IL-6 expression in non-inflammation-associated cancer [15]. Moreover, it had been proven that the growth of colon cancer epithelial cells in a cell culture system *in vitro* is promoted by IL-6 [16]. An additional mechanism could be an amplification of the IL-6 gene, as reported in patients with glioblastoma, although, this mechanism has not been shown for CRC so far [17]. Another explanation of increased IL-6 serum levels could be the infiltration of tumors with IL-6 secreting inflammatory cells as seen in colitis-associated cancer (CAC) [18].

Up till now, and according to the existing data, there have been no pertinent studies in our country analyzing the levels of IL-6 expression in CRC tissue samples using IHC combined with biostatistics and clinicopathological associations.

This effort scanned the expression of IL-6 in colorectal tumor cells and found that IL-6 expression was positive in 78% of CRC tissue samples compared to normal tissues, which is harmonious with previous studies [19,20]. Our results have the same findings as Kinoshita, et al., who reported that 60% of colorectal carcinoma samples had positive IL-6 expression [21]. Whereas other result stated that 46.3% of specimens were positive for IL-6 antibodies [22]. The discrepancy among results may be due to sample size, tumor grade and stage at the time of presentation of the patients, methods, and techniques of evaluation, and divergence in the usage of monoclonal and polyclonal antibodies in immunohistochemistry.

Our outcomes demonstrated significant correspondence in the expression of IL-6 among age groups. That is, advanced age was positively associated with a higher score of IL-6. These findings agree with Tzu-Chi, et al., who reported a significant correlation between age and postoperative raise of IL-6 in CRC patients [23].

On the subject of gender, our upshot found that the expression of IL-6 was higher in males than in females, and this contradicts with what was recorded by the previous study which found no correlation [23]. This is an inconsistency between outcomes which might have resulted from the use of different study populations or methodologies, or from different CRC patients' etiologies. In the current study, the tissue expression of IL-6 did not correlate with the tumor site.

As well, we found that the levels of IL-6 were positively related to histological differentiation and tumor stage. More patients with advanced stage of the disease had a significantly high tumor IL-6 expression. These findings are consistent with previous observations stated that high serum IL-6 levels were associated with poor prognosis [24]. While another record demonstrated an elevated IL-6 level associated with tumor recurrences but not with stages of the disease [16]. A study by Miller, et al., denotes that IL-6 may be implicated in CRC progression. In a point of view, IL-6 is highly elevated in the CRC cases with parameters of poor prognosis, and it is copious at the tumor microenvironment, where it plays a role in cancer metastasis via downregulation of E-cadherin [25]. Lately, 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 [26-30]. One of the most important steps of progressions is angiogenesis; as the aggressive growth of solid tumors depends on angiogenesis. And it was recorded recently that

IL-6 acts as an angiogenic cytokine [31]. Several IL-6-related angiogenic pathways have been reported including IL-6/HIF signaling in ovarian cancer and Stat3/VEGF signaling in breast cancer [20,30]. Yet, the mechanism by which IL-6 stimulates angiogenesis is not entirely understood. Nevertheless, the present study is the first to report that the tissue expression of IL-6 is directly correlated with high grade, advanced stage CRC, well-known parameters of poor prognosis in colorectal carcinoma.

CONCLUSION

We disclosed that the IHC is a specific method to investigate the tissue expression of IL-6 in colorectal carcinoma, and IL-6 expression was correlated with disease progression which puts forward that IL-6 may serve as a useful prognostic marker in the clinical management of patients.

DECLARATIONS

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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