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Role of Hematopoietic Growth Factors as Immune Modulators (GM-CSF & IL-3) in Newly Diagnosed Colorectal Cancer Patients and their Correlation with P53 Expression and Global DNA Methylation

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Abstract

Colorectal Cancer (CRC) is a global medical problem that has a high rate of mortality. Interestingly, evaluating the hematopoietic growth factors is useful in the early diagnosis of CRC. Henceforth, this study was designed to measure the potential role of Granulocyte-macrophage colony-stimulating factor (GM-CSF) and Interleukin 3 (IL-3) in CRC progress and their relation to P53 expression and global DNA methylation (5mC). Blood samples were collected from a total of 60 Iraqi patients who participated in this study and were diagnosed with CRC, and from 30 healthy volunteers who served as control for comparison purposes. Serum levels of GM-CSF and IL-3 were assessed quantitatively using enzyme linked immunosorbent assay (ELISA). Also, DNA and RNA were extracted from the whole blood samples and DNA methylation and gene expression of P53 were estimated. There were significant differences in the serum levels of GM-CSF and IL-3 between CRC patients and controls. Also, a positive link between serum GM-CSF and IL-3 was detected. Regarding demographic characteristics of the patients (age, gender and CRC status) and GM-CSF, IL-3 were found to have statistically highly significant differences ($P \leq 0.001$). No significant correlation was found between levels of P53 expression, global DNA methylation between hematopoietic growth factors as immune modulators. The differences of GM-CSF and IL-3 at serum levels may be used as useful biomarkers for CRC progression.

Keywords: Colorectal cancer, GM-CSF, IL-3, P53 expression, Global DNA methylation.

دور عوامل النمو المكونة للدم (GM-CSF و IL-3) كمُعدِّلات مناعية في المرضى المشخصين حديثا بسرطان القولون والمستقيم وارتباطهما بتعبير P53 ومثيلة الحمض النووي

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الخلاصة

يعتبر سرطان القولون والمستقيم (CRC) مشكلة طبية عالمية وله معدل وفيات مرتفع. ومن المثير للاهتمام أن تقييم عوامل النمو المكونة للدم مفيدة في التشخيص المبكر لـ CRC . لذلك تم تصميم هذه الدراسة لقياس الدور المحتمل لعامل تحفيز مستعمرات الخلايا المحببة والبلاعم GM-CSF و IL-3 في تقدم مرض سرطان المستقيم وعلاقتها بتعبير P53 ومثيلة الحمض النووي. تم جمع عينات دم من إجمالي 60

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مريضاً عراقياً شاركوا في هذه الدراسة تم تشخيصهم بمرض CRC وايضا 30 شخصا متطوعا سليمًا كمجموعة سيطرة لأغراض المقارنة. تم تقييم مستويات GM–CSF و IL–3 في مصل مجاميع الدراسة كمياً باستخدام تقنية الامتزاز المناعي (ELISA)، أيضًا تم استخلاص الحمض النووي والحمض النووي الريبي من عينات الدم الكاملة من جميع المشاركين في هذه الدراسة فضلا عن تقدير مثيلة الحمض النووي والتعبير الجيني لجين *P53*. كانت هناك فروق ذات دلالات إحصائية في مستويات المصل في كل من GM–CSF و IL–3 بين مرضى CRC ومجموعة السيطرة. فيما يتعلق بالخصائص الديموغرافية للمرضى (العمر والجنس وحالة CRC)، تم ايجاد فروق ذات دلالات احصائية معنوية عالية ($P \leq 0.001$) في مستويات GM–CSF و IL–3. لم تكشف الدراسة عن وجود ارتباط معنوي بين مستويات التعبير الجيني لجين *P53* و مثيلة الحمض النووي وبين عوامل النمو المكونة للدم كمُعدِّلات مناعية. يمكن استخدام الاختلافات في المستويات المصلية لل GM–CSF و IL–3 كمؤشرات حيوية مفيدة لتقدم مرض سرطان القولون والمستقيم.

1. Introduction

Colorectal cancer (CRC) is the third leading cancer causing mortality and the most prevalent cancer type globally [1]. Chronic inflammation is closely related to colon cancer. Colorectal cancer biomarkers are required not only for early diagnosis but also for prognostic stratification and patients monitoring. According to Binefa *et al.* [2], the development of CRC is due to accumulation of genetic and epigenetic changes that transform normal colonic mucosa into aggressive cancer cells. Commonly, one of the riskiest symptoms of CRC is diagnosed at high level stage. Prolonged chronic inflammation has been shown to have a significant importance in the etiology of several cancers, including colorectal cancer, and may even promote proinflammatory cytokines production in the tumor area. Through the migration of tumor cells, this process primarily contributes to the start, develop and advance tumors. Additionally, a growing tumor has the capacity to trigger both local and systemic inflammatory reactions [3].

Additionally, inflammation-related cancer is linked to immune cells infiltration such as tumor infiltrating leukocytes, cytokines and tissue remodeling factors [4]. A number of researchers have used immunohistochemistry to assess the expression and serum concentration of stem cell factor (SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF) in CRC to subjects with benign colon lesions, and healthy volunteer's tissues in order to determine their roles in the pathogenesis of CRC [4]. Hematopoietic cytokines (HCs) have a main role in regulating and proliferation of hematopoietic progenitor cells. In addition, cancer growth can be promoted by HCs through circulating cytokines concentrations elevation in CRC patients [5]. Large amounts of HCs have been shown to be secreted from a number of malignant tumor cell lines. Furthermore, elevation in cytokine concentrations has been associated with clinicopathological features, implying that determining serum cytokine levels may have a diagnostic value [6].

Cytokine release syndrome (CRS) is distinguished by an elevation in serum levels of inflammatory factors such as C-reactive protein, cytokines and chemokines [7]. It is well understood that the onset and progression of cancer disrupts normal epigenetic processes, including normal DNA methylation. Indeed, the Cancer Genome Atlas group was the first to describe CpG island methylator phenotypes (CIMP) in colon cancer [8, 9]. According to controls applied in terms of costs, data storage and analysis of whole genome, epigenetics has gained significant attention as it added a new dimension to genomic and proteomic research [10]. Atherogenesis, thrombosis process and dysfunctional endothelial cells can be controlled by systemic inflammation and DNA methylation [11]. Tumor cells deactivate p53 function

through somatic mutations in its tumor suppressor gene, resulting in the loss or reduction of wild-type p53 activity. Increasing mutant protein leads to tumor progression and high resistance to anticancer drugs [12]. However, GM-CSF and IL-3 role in CRC progression is still not clear. Henceforth, this study was designed to measure the potential role of GM-CSF and IL-3 in CRC progress and their relation to P53 expression and global DNA methylation.

2. Materials and Methods

2.1. Subjects

Sixty patients with colorectal cancer (32 males, 28 females) who attended Oncology Teaching Hospital in Baghdad, Iraq during February 2022 to September 2022, participated in this study. In addition, 30 healthy controls (12 males, 18 females) were involved in this study. This study excluded any patient under therapy. All studied groups ranged between <40-80 years of age.

All participants in this study provided signed, fully informed permission. This study was approved by ethical committee of Department of Biology, College of Science, University of Bagdad, Baghdad, Iraq with authorization reference number CSEC/1021/0098 on October 29, 2021. Participants' baseline blood samples were taken in order to estimate serum GM-CSF and IL-3 levels, gene expression of P53 by RT-PCR and global 5-Methylcytosine (5mC). Participants were staged, grading and status, according to staging system of cancer (tumor, lymph node, metastasis) (TNM), World Health Organization (WHO) and Eastern Cooperative Oncology Group (ECOG) respectively [13].

2.2. Blood Samples

2.2.1. Assay serum levels of GM-CSF and IL-3

Five ml of blood was drawn from the redial vein using disposable syringes. Gel clot activator vacuum tubes were used for clotting. Following that, at 3000 rpm for 10 minutes the samples were centrifuged to aspirate the serum. The serum was then dispensed into Eppendorf tubes using a micropipette which were then refrigerated at -20ºC for later immunological investigation of the serum GM-CSF and IL-3 levels using (Human Fine Test Biotech, ELISA kit, China).

2.2.2. Extraction of DNA

The remaining 2ml of blood was drawn and placed in an EDTA-containing tube for extracting the DNA by using Quick-DNA™ Miniprep Kit; ZYMO RESEARCH; USA). A nanodrop spectrophotometer (Thermo, USA) was used to confirm the presence and purity of extracted DNA. The DNA concentrations (ng/µl) were calculated and the purity of DNA was assessed at 260/280 nm absorbance. No DNA damage was confirmed by staining DNA with ethidium bromide when running on 1% agarose gels at 80 V for 30 min.

2.2.3. Assay of DNA Methylation

The quantification kit of 5-methylcytosine (Epigentek, USA) was used to estimate the global DNA methylation in all blood samples. One hundred ng of genomic DNA was used for each sample. A standard curve was generated according to manufacturer's instructions by using a methylated polynucleotide (contained 50% of 5-methylcytosine) which served as a positive control. The DNA samples were added to their corresponding wells and then measured at 450 nm of absorbance by using ELISA reader.

2.2.4. Assay of P53 Gene Expression

The Direct-zol™ RNA MiniPrep was used to isolate total RNA from blood samples. RNA purification was done in high-quality from samples in TRI Reagent. After preparing samples in TRI reagent, they were then spun, washed and eluted for the the RNA which was suitable for molecular manipulation and analysis (including RT-PCR). The master mix was prepared for all the samples in a final volume of 20 ul of isolated RNA and the relative quantification of gene expression was measured using the $2^{-\Delta\Delta CT}$ method. The TRI Reagent®; ZYMO RESEARCH; USA was supplied as a 2X master mix with integrated Kapa Sybr Fast qPCR Master Mix (2X) Universal, forward primer, reverse primer, nuclease-free water, template DNA sample volume, the master mix containing RT buffer. For run samples, reverse transcriptase activation was performed at 50°C for 30 min. Then, activation was conducted at 95°C for 15 min and denaturation at 95°C for 1 min. Followed by 39 cycles of denaturation at 95°C, annealing at 55°C, and extension at 72°C for 1 min before a final elongation step at 72°C for 10 min.

Table 1: Oligonucleotide primers of P53 used in the qPCR

Gene	Sequence	Tm	GC%	Reference
P53	AGA GTC TAT AGG CCC ACC CC	60	55	[14]
	GCT CGA CGC TAG GAT CTG AC	61	50	
GAPDH	AATGGGCAGCCGTTAGGAAA	57	50	[15]
	GCGCCCAATACGACCAAATC	56	50	

2.2.5. Statistical Analysis

SPSS version 24.0 program was used for all statistical analysis. For all numerical parameters, the mean and the standard error (SE) were also determined. Three parameters or more were compared using the ANOVA test. T. student tests were additionally utilized to compare the two numerical or categorical parameters. The threshold of $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. Evaluation of Serum GM-CSF and IL-3 Levels

Table 2 explains the variation of serum (GM-CSF and IL-3) between patients and controls. Results showed that GM-CSF levels increased in CRC patients and differed significantly from the control group, ($P \leq 0.001$). Also, a significant difference was found between CRC patients and healthy control in IL-3 level ($P \leq 0.001$).

Table 2: Serum levels of GM-CSF and IL-3 levels in colorectal cancer patients and healthy control

Parameters	CRC Patients	Healthy Control	*P-value*
	Mean and S.E.		
GM-CSF (pg/ml)	298.96±17.24	75.35±3.79	0.001
IL-3 (pg/ml)	848.84±22.66	266.74±15.51	0.001

3.2. Relationship between GM-CSF, IL-3 and Gender in CRC Patients

The 60 CRC patients (33 men and 27 women) samples were matched with serum levels of GM-CSF and IL-3 (Table 3). Results showed that GM-CSF levels increased in males in both low and high level of disease progress, while IL-3 levels increased in females in both low and high level of disease progress and there was a significant difference at $P \leq 0.00$.

Table 3: Statistical analysis according to the gender of GM-CS and IL-3 levels values in colorectal cancer patients in relation to clinic-pathological variables of tumor

Gender			
Variable		**GM-CSF (pg/ml) Mean±S.E.**	**IL-3 (pg/ml) Mean±S.E.**
Low L. (I+II)	**Male**	333.79±43.82 ^{a}	766.70±47.50 ^{a}
	Female	248.11±46.95 **b**	953.54±28.44 ^{a}
P-value		**0.001**	**0.001**
High L. (III+IV)	**Male**	293.48±33.12 ^{c}	815.34±42.26 ^{b}
	Female	301.63±23.60 ^{c}	896.65±36.58 ^{b}
P-value		**0.001**	**0.001**
The results are presented as mean ± standard error. ***Different letters mean there is significant differences between genders**			

3.3. Correlation between GM-CSF, IL-3 and Age Groups in CRC Patients

The groups of 60 CRC patients were matched based on age groups (<40, 40-60, and 61-80 years) (Table 4). According to the serum level of GM-CSF, the highest level was recorded in 61-80 years age group in both low- and high-level stages. Also, there was a significant difference between two levels (P≤0.001). In the low-level stage, IL-3 was recorded with the highest level in 61-80 years age group, while the highest mean level of IL-3 was recorded in <40 years age group according to high level patients of CRC, and there were statically significant differences (P≤0.001).

Table 4: Statistical analysis according to the age groups of GM-CS and IL-3 levels values in colorectal cancer patients in relation to clinic-pathological variables of tumor

Age Groups			
Variable		**GM-CSF (pg/ml) Mean±S.E.**	**IL-3 (pg/ml) Mean±S.E.**
Low L. (I+II)	**<40**	237.87±0.00 ^{a}	839.09±0.00 ^{a}
	40-60	281.1±43.85 ^{b}	803.79±67.53 ^{b}
	61-80	336.5±57.86 ^{b}	874.78±30.85 ^{b}
High L. (III+ IV)	**<40**	311.10±46.16 ^{b}	922.09±51.47 ^{c}
	40-60	285.73±23.93 ^{b}	843.12±41.77 ^{c}
	61-80	303.55±39.46 ^{b}	839.26±50.81 ^{c}
P-value		**0.001**	**0.001**
The results are presented as mean ± standard error. *** Different letters mean there is significant differences between age groups**			

3.4. Correlation between GM-CSF, IL-3 and different Parameters in CRC Patients

There was a significant difference in GM-CSF and IL-3 serum levels in CRC patients with low and high stage levels (*P≤ 0.001*). The current study also compared GM-CSF and IL-3 levels in CRC patients with low invasion and high invasion tumors. There was no significant increase in GM-CSF and IL-3 levels in low and high invasion tumors at *P≥0.05*.

Also, CRC were classified as low-level grade (well differentiated, moderately differentiated) and high-level grade (poorly differentiated) based on histopathological differentiation (Table 5). According to cell differentiation grades, the results observed no significant elevation in GM-CSF and IL-3 levels in low and high cell differentiation grade tumors (Table 4).

Table 5: GM-CSF and IL-3 in colorectal cancer patients in relation to clinic-pathological variables of tumor

Variables	GM-CSF (pg/ml)	IL-3 (pg/ml)
TNM Stage		
Low level (I+II)	302.22±33.27	835.53±37.70
High level (III+ IV)	297.45±20.26	855.00±28.44
P-value	**0.001**	**0.778**
Grading		
Grade 1-2	354.25± 37.90	885.88± 29.59
Grade 3-4	345.97± 30.70	948.10± 26.04
P-value	**0.869**	**0.129**
Tissue invasion		
Low invasion T1+T2	279.17±26.92	856.69± 41.55
High invasion T3+T4	296.04±25.53	860.04± 32.39265
P-value	**0.661**	**0.949**
The results are presented as mean ± standard error. *P > 0.05 non-significant differences, P < 0.05 significant differences		

In the current study, using the Pearson correlation to evaluate the strength of the relationship and the percentages are presented as median or individual as individual dots. The results of Figure 1(A) show that GM-CSF and P53 expression in the tumor area correlated negatively and no significant associated was found with control ($P \geq 0.05$). Also, negative correlation was found between GM-CSF and DNA methylation in the tumor area and no significant associated was detected with control (Figure 1(B)). No correlation between IL-3 and P53 expression in the tumor area was detected (Figure 1(C). Figure 1(D) shows that there was positive correlation between IL-3 and DNA methylation in the tumor area at ($P \geq 0.05$). Similar results were indicated between GM-CSF and IL-3 in the tumor area with significant association with the control at ($P \leq 0.001$).

Discussion

Hematopoietic cytokines such as IL-3, GM-CSF and M-CSF, are members of the glycoproteins group. A study by Ardekani *et al*. [16] found significant increases in GM-CSF levels in the sera of patients with CRC, compared to healthy age/sex matched controls. The results of this study matched the results of the current study. Evidence suggests that GM-CSF have a pro-tumorigenic function and is an independent prognostic factor which supports tumor development which has been categorized as immunomodulatory [16]. Recent studies

have concluded that skin carcinoma and CRC can be caused by aberrant expression of GM-CSF and its receptors [17].

Colorectal cancer can be detected through some biochemical markers such as GM-CSF and IL-3. A previous study showed significantly lower serum level of IL-3 in the patients with CRC in comparison to the healthy controls. In contrast, another study indicated that increase in IL-3 level is associated with clinicopathological features with diagnostic value [5]. Also, it has been reported that large amounts of hematopoietic cytokines are secreted from many malignant tumor cells which leads to alter levels of these cytokines in the serum of cancer patients [18]. However, megakaryocyte proliferation stimulated by pro-inflammatory cytokines such as IL-1, IL-3 and IL-6, are released in response to systemic inflammation [19]. It has been suggested that CRC patients younger than 45 have relative risks similar to those CRC patients older than 45 years in response to modifiable factors [20, 21]. These results matched the results of the current study. Local and systemic inflammations are important in the carcinogenesis and development of the cancer diseases and that IL-1, IL-3 and IL-6 are released during systemic inflammation [19].

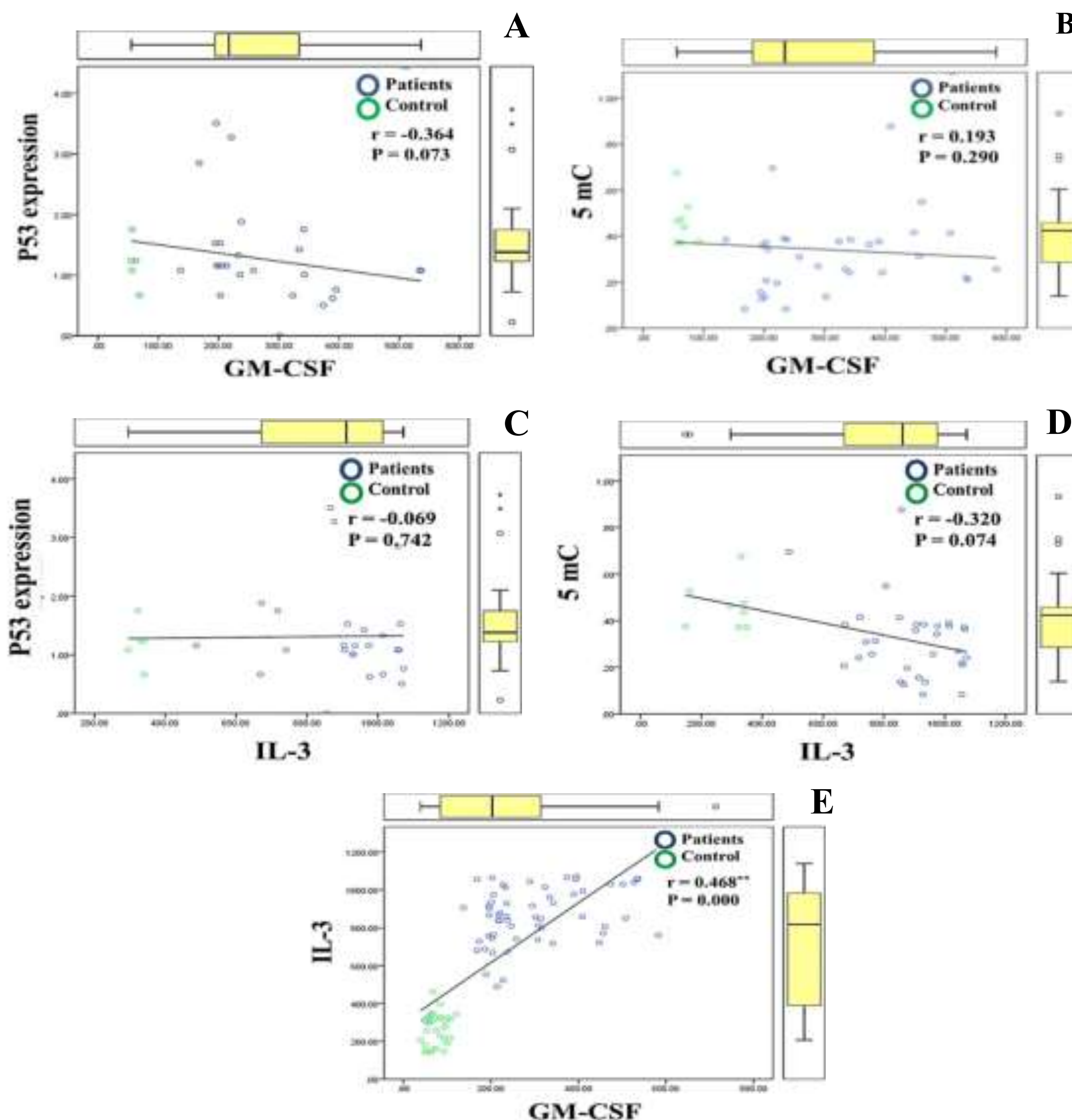

Figure 1: Pearson correlation was used to evaluate the strength relationship between parameters studied and the percentages are presented as median of individual dots. The results of correlation are significant at the level of 0.05.

Early diagnosis of colorectal cancer is very important in reducing mortality and morbidity [12]. This study observed elevation in the GM-CSF levels in grade I and II, high depth of invasion (T3+T4) and stages I and II in sera of CRC patients and that this can be accompanied by CRC progression. Numbers of studies have shown that GM-CSF has stimulatory effects in tumor progression and metastasis, and is also considered as having anti-proliferative or anti-apoptotic effects, depending on the tumor status [22].

Fluctuation in GM-CSF levels, like other soluble immune mediators, has effects on cancer aggressiveness. Low concentrations of GM-CSF prevents appropriate innate immune cells production and activation of adaptive anti-cancer immune responses. Whereas, their high concentrations exhaust immune cells and encourage cancer growth. This effect signals on cancer progression, cancer type and tumor microenvironment [7, 18]. Vasiliades *et al.* [18] concluded that hematopoietic cytokines (HCs) such as SCF, M-CSF, GM-CSF and IL-3, do not affect the tumor location, stage, tumor type, tumor size, perineural invasion, vascular invasion and lymph node involvement. The findings of this study agree with results of the current study. However, it has also been reported that IL-3 and IL-5 and GM-CSF are involved in proliferation and differentiation of myeloid precursors and that the GM-CSF is produced and secreted in seventy percent of human and murine CRC [23]. Both genetic and epigenetic factors can influence cytokines production. Surprisingly, epigenetic events are associated with the onset of inflammatory diseases [24]. The study of pathway of DMP/DMR genes has revealed a significant enrichment in the IL3, and GM-CSF levels [25]. Many studies in both normal hematopoiesis and hematological malignancies (HMs) have showed that epigenetic changes such as DNA methylation, play an important role in stem cells self-renewal, differentiation and malignancy pathogenesis [26]. In the current study we tested whether variation in the 5mC of blood immune cells is correlated with serum hematopoietic growth factors (GM-CSF & IL-3). IL-3, GM-CSF and DNA methylation in the tumor area were negative and no significant association was detected with control. However, a previous study reported that DNA methylation sites were significantly correlated with circulating levels of the inflammatory acute-phase protein (CRP), TNF, IL-6, IL-8 and IL-10 [27, 28]. Both genetic and epigenetic factors can influence cytokine production. Surprisingly, epigenetic events are associated with the onset of inflammatory diseases [24]. Moreover, gene expression may not be related to the disease progression. A recent study detected that the reduction in BRCA1 expression was negatively associated with the disease grades, as in breast cancer patients with the advanced stage III [29, 30]. This study also focused on the relation between IL-3, GM-CSF and P53 expressions and showed no correlation and significantly no association with the control ($P \geq 0.05$). At the same time, Zheng *et al.* [31] concluded that late IL-10 transcription can be regulated by miRNA. Future research is still needed to explain the mechanism of circRNA and IL-10 that are related to immune inflammation as their significance in recent times is still unclear. According to Huang *et al.* [32], GM-CSF reduced accumulation of p53 protein in rat SCI model. Further studies are needed to understand the signal pathways mediating its anti-apoptotic activity related proteins by GM-CSF [22, 32].

Conclusion

Current study indicated statistically significant differences between the analyzed groups of CRC patients' age groups, gender, stage of the disease, and no significant results were found between low- and high-levels of grade and invasion. Serum levels of GM-CSF and IL-3 in sera can be used as prognostic risk factors that may be utilized in early diagnosis of CRC cancer.

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