Improved colorectal cancer screening by adding noninvasive serum-based biomarkers

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Received: 28 October 2022
Revised: 4 December 2022
Accepted: 12 December 2022
Published: 9 June 2023

The Egyptian Journal of Surgery 2023, 42:1–9

Background
Colorectal cancer (CRC) is one of the most common causes of cancer-related morbidity and mortality worldwide. The most widely used CRC diagnostic tests either noninvasive or invasive are limited by insufficient performance or patient compliance. Profiling inflammatory mediators with carcinogenic roles could aid in CRC diagnosis. We aimed to use multiplex bead assay in evaluating the utility of serum eotaxin-1, macrophage-inflammatory protein-1β (MIP-1β), granulocyte colony-stimulating factor (G-CSF), vascular endothelial growth factor A (VEGF-A), and Fas ligand (FasL) as potential biomarkers in CRC.
Patients and methods
The study was conducted on 87 patients. Based on colonoscopy findings, patients were divided into 35 CRC and 52 nonmalignant patients (nine with colon polyp, 24 with colitis, and 19 with normal mucosa). Multiplex assay was used for measuring the studied biomarkers.
Results
The median values of eotaxin-1, MIP-1β, G-CSF, and VEGF-A were significantly higher in patients with CRC compared with the nonmalignant group. The area under the receiver operating characteristics curve for eotaxin-1, MIP-1β, G-CSF, and VEGF-A was 0.863. The area under the ROC for occult blood in stool was only 0.597. Moreover, significantly higher levels of G-CSF and VEGF-A were found in patients with CRC than the precancerous colon polyp group.
Conclusions
Serum profiling of eotaxin-1, MIP-1β, G-CSF, and VEGF-A could be used as potential biomarkers in early CRC diagnosis with better discriminatory power than stool occult blood and may increase occult blood performance, thus reducing false-positives rates and unneeded colonoscopy. Multiplexing bead technology represents a promising approach for CRC screening and diagnosis.

Keywords:
colorectal cancer, multiplex bead assay, serum biomarkers

Egyptian J Surgery 42:1–9

1110-1121

Introduction

Colorectal cancer (CRC) is one of the most common causes of cancer-related morbidity and mortality worldwide, ranking as the third most frequently diagnosed cancer and the second in mortality [1]. CRC is mostly asymptomatic in early stages, whereas common symptoms and signs associated with advanced stages of CRC include change in bowel habits, such as diarrhea or constipation, rectal bleeding, abdominal pain, unexplained iron-deficiency anemia, chronic fatigue, and unexplained weight loss. Sometimes patients with CRC are presented with symptoms of obstruction, including abdominal distention and/or nausea and vomiting [2]. CRC outcomes could be improved by early diagnosis. The five-year survival rate depends on tumor stage at the time of diagnosis, which is about 90% for stage I and 10% for stage IV [3].

Tumor microenvironment consists mainly of cancer cells and recruited host stromal cells interacting with each other secreting many inflammatory mediators that promote cancer growth and progression. Profiling these inflammatory mediators, which have a carcinogenic role, could aid in CRC diagnosis [4].

Eotaxin-1 is a potent chemo-attractant cytokine for eosinophils, one of chemokines with two adjacent cysteine residues, known as CC chemokine ligands-11 [5]. Eotaxin-1 can induce angiogenesis and metastasis through proangiogenic mediators and macrophage recruitment and protumor M2

 DOI: 10.4103/ejs.ejs_262_22

polarization [6]. Tissue eosinophilia in CRC is a good prognostic marker; it decreases with the adenoma-carcinoma progression [7].

Macrophage-inflammatory protein-1β (MIP-1β) is a chemo-attractant cytokine for macrophages, known as CC motif chemokine ligand-4. Recruited macrophages into hypoxic tumor microenvironment are repolarized from M1 phenotype into M2 phenotype, known as tumor-associated macrophages, which are essential in CRC microenvironment [6,8].

Granulocyte colony-stimulating factor (G-CSF) is a hematopoietic cytokine, controlling the production and differentiation of neutrophils, which play a role in CRC development with other immune cells. These cells promote CRC progression through release of metalloproteinase into the extracellular matrix.

Vascular endothelial growth factor A (VEGF-A) is a heparin-binding glycoprotein with a potent angiogenic activity. It induces endothelial cell proliferation, migration, differentiation, and secretion of matrix metalloproteinases. In addition, anti-VEGF drugs have been used alone or in combination with chemotherapy in treating CRC and have improved CRC patient survival [9].

Fas ligand (FasL) is a transmembrane protein, known as cluster of differentiation 95 ligand. It binds to Fas receptor and initiates a cascade of caspases, leading to DNA degradation and apoptosis initiation, especially Fas positive antitumor lymphocytes, thus promoting tumorigenesis [10]. Moreover, FasL can facilitate colorectal hepatic metastasis through apoptosis of normal hepatocytes allowing tumor invasion and hepatic colonization [11].

The most widely used diagnostic tests include noninvasive tests as radiological imaging and stool-based tests limited by their insufficient performance, in addition to invasive endoscopic techniques, which are limited by patient noncompliance [12].

We aimed to use multiplexing technology, which allows measurement of multiple analytes in a single sample simultaneously with lower sample consumption in a rapid, efficient, and cost–effective way. We aimed to study the benefits of using multiplex assay for a serum panel of five inflammatory biomarkers in the diagnosis of CRC and improving patients compliance by decreasing unnecessary endoscopy, in addition studying its role in improving the decreased performance of occult blood in diagnosis of CRC.

Patients and methods

This case–control study was conducted after approval of ethical committee of MRI. Written consent was taken from 87 Egyptian patients complaining of gastrointestinal symptoms. Patients with known malignancy other than colorectal and who had a history of CRC therapy were excluded from the study. Patients were subjected to routine investigations and occult blood in stool. Based on colonoscopy findings and histopathological assessment, patients were divided into 35 CRC and 52 nonmalignant cases. Nonmalignant patients were further subdivided into nine with colon polyp, 24 with inflamed mucosa (colitis), and 19 with normal mucosa. Metastatic status were obtained from the MRI of Department of Pathology.

Blood samples were withdrawn from each patient and dispensed into a serum separator tube. The blood was allowed to clot and then centrifuged for 15 min at 1000*g*. The obtained serum was immediately separated and stored at −20°C till time of assay of C-reactive protein by means of auto-analyzer OLYMPUS AU400 and serum eotaxin-1, MIP-1β, G-CSF, VEGF-A, and FasL on the Luminex 200 platform (Olympus au400. Japan). The multiplex assay principle is based on using magnetic beads to measure multiple analytes simultaneously, where specifically colored microspheres are coated with specific antibodies, and the results were read by flow cytometry, which distinguishes bead fluorescence.

Eosinophils were counted in histopathological cancerous sections stained with hematoxylin and eosin, and they were classified into absent eosinophilia (<5), low (5–10), moderate (10–50), and high (>50) cells observed in the examined field.

Statistical analysis was done using the Statistical Package for the Social Sciences Program, version 20.0 (Statistical Package for Social Sciences, Chicago, Illinois, USA). The qualitative variables were summarized using frequency and percentage. χ^2 test was used to determine whether the observed frequencies differed significantly from expected frequencies among nominal variables. The quantitative variables were explored for normality distribution and revealed abnormal distribution, so nonparametric tests were done. Quantitative variables were summarized using median and range. Mann–Whitney *U* test was used for comparing between two groups. Kruskal–Wallis test was used for comparing more than two groups. Spearman's

correlation was done to correlate the quantitative variables to each other. Level of significance of 5% was used on all of the tests of association or tests of comparison. Receiver operating characteristics curve was created by plotting diagnostic sensitivity on *y* axis versus 1-specificity on *x* axis at different cutoff values and was used to calculate area under the curve (AUC), as well as the cutoff values for a variable and its diagnostic accuracy.

Results

There was a statistically significant difference in the median age between patients with CRC (60 years) and the nonmalignant group (43 years) ($P<0.001$) (Table 1).

All resected CRC cases were histopathologically classified as adenocarcinoma, with 85.7% of cases moderately differentiated type, 8.6% having well-differentiated type, and 5.7% with poorly differentiated type.

The panel of the studied serum biomarkers showed significantly higher median values of eotaxin-1 in patients with CRC than their corresponding median values in nonmalignant group and both colitis and control subgroups ($P<0.001$, $P=0.001$, and $P=0.001$, respectively). The median value of MIP-1β was also significantly higher in patients with CRC than the corresponding nonmalignant, colitis, and control groups ($P<0.001$, $P=0.003$, and $P<0.001$, respectively). In addition, patients with CRC had significantly higher median serum levels of G-CSF than the nonmalignant group and all nonmalignant subgroups, namely, colitis, polyp, and control groups ($P<0.001$, $P=0.003$, $P=0.001$, and $P<0.001$, respectively). VEGF-A also showed significantly higher median values in patients with CRC than that seen in nonmalignant group and nonmalignant subgroups (colitis, polyp, and control groups) ($P<0.001$ and 0.001, respectively). However, median values of serum FasL did not show significant difference neither between patients with CRC and nonmalignant groups ($P=0.104$) nor between patients with CRC and nonmalignant subgroups ($P=0.221$). Serum levels of C-reactive protein (CRP) in patients with CRC were significantly higher than those seen in nonmalignant group and in both colitis and control subgroups ($P<0.001$, 0.001, and 0.001, respectively) (Table 2).

Regarding diagnostic performance of studied serum panel to detect patients with CRC from nonmalignant group, (Table 3) the ROC curve generated cutoff value for serum eotaxin-1 of 113.31 pg/ml, serum MIP-1β of 96.074 pg/ml, serum G-CSF of 23.353 pg/ml, and serum VEGF of 195.94 pg/ml. AUC for combined serum panel was 0.863 ($P<0.001$), and on combining studied panel with occult blood, it improved AUC for occult blood from 0.597 to 0.875 ($P<0.001$) (Fig. 1).

Correlation studies showed significant positive association between eotaxin-1 and MIP-1β ($P=0.006$), eotaxin-1 and VEGF-A ($P=0.001$), MIP-1β and G-CSF ($P<0.001$), G-CSF and VEGF-A ($P=0.043$), and VEGF-A and CRP ($P=0.033$) among patients with CRC (Table 4).

No significant association was found between tumor degree of differentiation and tissue eosinophil count among patients with CRC ($P=0.196$) and no significant association between tumor degree of differentiation and serum eotaxin-1 level among patients with CRC ($P=0.880$). (Table 5). No significant association was found between levels of eotaxin-1, MIP-1β, G-CSF, VEGF-A, and FasL and metastasis in patients with CRC (Table 5).

Table 1 Comparison between colorectal cancer and nonmalignant groups concerning age and sex

Parameter	Cancer ($N=35$) (mean±SD) [*n* (%)]	Nonmalignant ($N=52$) (mean±SD) [*n* (%)]	*P* value	Statistics (degree of freedom)
Age (years)				
Less than or equal 50	8 (22.9)	33 (63.5)		
More than 50	27 (77.1)	19 (36.5)		
Minimum–maximum	25.0–82.0	18.0–79.0	$P<0.001$**	$\chi^2=13.842$*
Median (IQR)	60.0 (52.5–69.5)	43.0 (33.0–54.0)		$t=4.196$*
Sex				
Male	13 (37.1)	29 (55.8)	$P=0.088$	$\chi^2=2.907$
Female	22 (62.9)	23 (44.2)		

χ^2, χ^2 test; *t*, Student's *t* test statistics. *P values less than 0.05 were considered statistically significant. **P values of less than 0.001 were considered highly significant.

Table 2 Comparison between patients with colorectal cancer and nonmalignant subgroups concerning studied serum panel and C-reactive protein

Biomarker	Cancer (*N*=35)	Nonmalignant			Statistic	*P*
		Colitis (*N*=24)	Polyp (*N*=9)	Control (*N*=19)		
Eotaxin-1 (pg/ml)						
Minimum–maximum	74.02–630.2	10.10–311.3	10.10–390.6	10.1–284.7		
Median (IQR)	171 (119.2–220.3)	99.90 (53.2–129.6)	128.1 (110.2–174.7)	87.27 (37.0–122.1)	*H*=16.227	0.001*
*P*0		0.001*	0.314	0.001*		
MIP-1β (pg/ml)						
Minimum–maximum	32.99–469.7	15.5–1231.4	31.56–356.7	15.5–140.9		
Median (IQR)	172.4 (112.9–281.8)	75.46 (23.5–190.6)	96.07 (81.0–105.2)	31.56 (17.2–49.4)	*H*=35.745	<0.001**
*P*0		0.003*	0.051	<0.001**		
G-CSF (pg/ml)						
Minimum–maximum	6.32–387.5	0.95–139.3	0.95–74.99	0.95–29.0		
Median (IQR)	40.88 (31.1–66.2)	16.69 (7.8–40.9)	13.59 (2.1–19.7)	8.67 (1.0–17.1)	*H*=31.83	<0.001
*P*0		0.003*	0.001*	<0.001**		
VEGF-A (pg/ml)						
Minimum–maximum	27.86–854.9	11.31–826.8	5.76–230.7	16.5–247.4		
Median (IQR)	230.6 (111.1–309.5)	71.73 (47.3–130.3)	50.77 (20.2–86.7)	51.3 (32.0–91.6)	*H*=31.035	<0.001**
*P*0		<0.001**	<0.001**	<0.001**		
FasL (pg/ml)						
Minimum–maximum	20.42–161.3	19.69–130.9	31.33–119.4	16.77–152.3		
Median (IQR)	72.83 (61.2–83.7)	58.66 (39.3–73.1)	73.42 (39.3–87.2)	68.35 (50.0–83.6)	*H*=4.404	0.221
CRP (mg/l)						
Minimum–maximum	2.30–339.10	4.80–294.70	10.30–25.30	4.0–160.80		
Median (IQR)	36.60 (17.15–98.90)	10.35 (6.50–24.80)	16.70 (14.70–21.0)	8.70 (6.70–15.85)	*H*=20.843*	<0.001*
*P*0		<0.001*	0.133	<0.001*		

Pairwise comparison between each two groups was done using post-hoc test (Dunn's for multiple comparisons test). *H*, Kruskal–Wallis test statistics. *P* value for comparing between the subgroups. *P*0 value for comparing between cancer and each other subgroup. **P* values less than 0.05 were considered statistically significant. ***P* values of less than 0.001 were considered highly significant.

Table 3 Agreement (sensitivity, specificity) for eotaxin-1, macrophage-inflammatory protein-1β, granulocyte colony-stimulating factor, vascular endothelial growth factor A, and stool occult blood to detect colorectal cancer cases from nonmalignant patients

	AUC	*P*	95% CI		Cut off#	Sensitivity	Specificity	PPV	NPV
			LL	UL					
Eotaxin-1 (%)	0.737	<0.001*	0.634	0.840	>113.31 pg/ml	77.14	65.38	60.0	81.0
MIP-1β (%)	0.822	<0.001*	0.735	0.908	>96.074 pg/ml	91.43	71.15	68.1	92.5
G-CSF (%)	0.826	<0.001*	0.738	0.914	>23.353 pg/ml	82.86	76.92	69.2	83.3
VEGF-A (%)	0.840	<0.001*	0.753	0.928	>195.94 pg/ml	65.71	92.31	85.2	80.0
Stool occult blood (%)	0.597	0.127	0.477	0.717	–	82.86	36.54	46.77	76.0

	AUC	*P*	95% CI	
			LL	UL
Combination of markers	0.863	<0.001*	0.787	0.940
Occult blood	0.597	0.127	0.477	0.717
Combination of four markers and occult blood	0.875	<0.001*	0.804	0.946

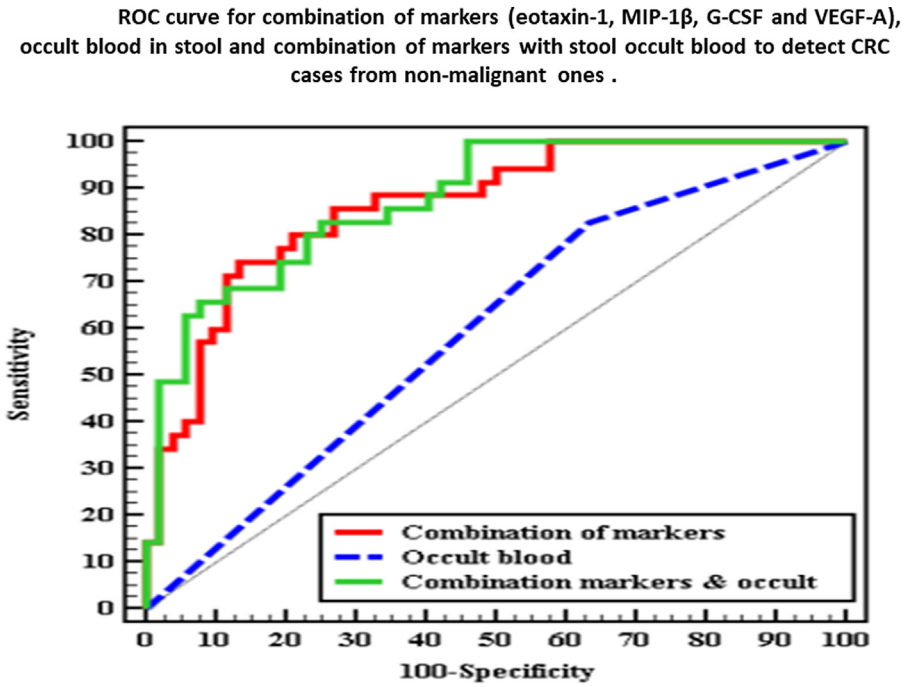
AUC, area under a curve; CI, confidence intervals; NPV, negative predictive value; PPV, positive predictive value; LL and UL, Luminex, Austin, TX. *Statistically significant at *P* value less than equal to 0.05. #Cutoff was choose according to Youden index.

Discussion

CRC is one of the most common and deadly cancers worldwide. It has the second highest mortality rate, accounting for 9.2% of all cancer deaths [1], which is most probably due to high rate of metastasis, especially to liver [13]. CRC has many risk factors, which include advanced age, family history, male sex, colon polyps, long-standing inflammatory bowel diseases (IBD), and lifestyle factors. The risk of CRC development in males is 1.5-fold higher than that in females [2]. Early diagnosis of CRC can reduce mortality and recurrence rates through primary prevention by early removal of small localized lesions [14].

Owing to the heterogeneous nature of CRC, a panel of minimally invasive biomarkers is needed rather than using a single biomarker as a promising approach for

Figure 1



ROC curve for combination of markers (eotaxin-1, MIP-1β, G-CSF, and VEGF-A), occult blood in stool, and combination of markers with stool occult blood to detect CRC cases from nonmalignant ones. AUC for combined serum panel was 0.863 (P<0.001), and on combining studied panel with occult blood, it improved AUC for occult blood from 0.597 to 0.875 (P<0.001). AUC, area under curve; CRC, colorectal cancer; G-CSF, granulocyte colony-stimulating factor; MIP-1β, macrophage-inflammatory protein-1β; ROC, receiver operating characteristic curve; VEGF-A, vascular endothelial growth factor A.

Table 4 Correlation between eotaxin-1, macrophage-inflammatory protein-1β, granulocyte colony-stimulating factor, vascular endothelial growth factor A, Fas ligand, and C-reactive protein among patients with colorectal cancer (*N*=35)

	Eotaxin-1	MIP-1β	G-CSF	FasL	VEGF-A	CRP
Eotaxin						
r_s	1.000	0.454	0.290	0.130	0.532	–0.014
P		0.006*	0.091	0.458	0.001*	0.935
MIP-1β						
r_s		1.000	0.562	0.049	0.038	–0.070
P			<0.001*	0.780	0.829	0.689
G-CSF						
r_s			1.000	–0.084	0.344	0.266
P				0.631	0.043*	0.122
FasL						
r_s				1.000	0.045	–0.097
P					0.798	0.578
VEGF-A						
r_s					1.000	0.362
P						0.033*
CRP						
r_s						1.000
P						

CRP, C-reactive protein; FasL, Fas ligand; G-CSF granulocyte colony-stimulating factor; MIP-1β, macrophage-inflammatory protein-1β; VEGF-A, vascular endothelial growth factor A; r_s, Spearman coefficient. *Statistically significant at *P* value less than equal to 0.05.

CRC screening in high-risk patients, CRC diagnosis, and personalized medicine implementation. In addition, the panel is needed to overcome the disadvantages of occult blood in CRC diagnosis and decrease the need for other invasive techniques.

The tumor microenvironment consists of cancer cells and recruited host stromal cells that interact with each other and secrete inflammatory mediators that promote cancer growth and progression. These mediators can promote cell mutation and prevent their apoptosis;

Table 5 Relation between malignancy /eosinophils differentiation and possible marker

Relation between degree of differentiation with eosinophil count and serum eotaxin-1 among patients with colorectal cancer.					
	Degree of differentiation				
	Poor (*N*=3) [*n* (%)]	Moderate (*N*=28) [*n* (%)]	Well (*N*=4) [*n* (%)]	Test of significance	*P*
Eosinophil count					
<5	0	16 (57.15)	2 (50.0)		
5–10	2 (66.67)	2 (7.15)	2 (50.5)	χ^2=7.750	^{MC}P=0.196
10–50	1 (33.33)	7 (25.0)	0		
>50	0	3 (10.7)	0		
Eotaxin-1					
Minimum–maximum	171.0–188.2	74.02–630.2	94.43–256.3	*H*=0.255	0.880
Relation between metastasis and studied serum panel in patients with CRC					
	Metastasis				
	M0 (*N*=26)		M1 (*N*=9)	*U*	*P*
VEGF-A					
Minimum–maximum	30.43–854.93		97.24–383.94	22.0	1.000
Median	218.14		180.51		
G-CSF					
Minimum–maximum	17.34–387.51		6.32–75.61	11.0	0.177
Median	40.88		23.15		
Eotaxin-1					
Minimum–maximum	74.02–247.57		84.55–188.23	21.50	0.949
Median	121.30		129.57		
FasL					
Minimum–maximum	33.55–97.52		20.42–83.0	18.50	0.661
Median	72.83		67.02		
MIP-1β					
Minimum–maximum	98.89–423.03		32.99–192.15	12.0	0.226
Median	150.93		117.78		

CRC, colorectal cancer; FasL, Fas ligand; G-CSF granulocyte colony-stimulating factor; MIP-1β, macrophage-inflammatory protein-1β; VEGF-A, vascular endothelial growth factor A.

thus, profiling of inflammatory mediators could have a potential in CRC diagnosis [4].

Eotaxin-1 is a chemokine that acts as a potent chemoattractant for eosinophils, which infiltrate different tumors [15]. MIP-1β is a chemokine that recruits protumorigenic macrophages and facilitates tumor development and metastasis [16]. G-CSF is a hematopoietic cytokine that regulates neutrophil development and function and shows overexpression in various tumors [17]. VEGF-A is a potent angiogenic cytokine. VEGF-A and its receptor are expressed in ~50% of CRCs. It has a potential role in metastasis, which accounts for most of CRC-related mortality cases [18]. FasL is a transmembrane protein that leads to apoptotic cell death induction and tumor maintenance [19]. Blood-based tests with higher performance rates can avoid unnecessary colonoscopy interventions.

Old age is prevalent in patients with CRC; this goes with the present study. There was a statistically highly significant difference between CRC and nonmalignant groups concerning age (P<0.001). Although CRC is more common in males; only 37.1% of patients with CRC included in the present study were males. This could be attributed to small sample size included in the current study.

A previous study reported that serum eotaxin-1 levels were elevated in precancerous bowel conditions such as patients with IBD when compared with the control group (P<0.01) [20]. The same findings were found in another study, showing higher serum eotaxin-1 levels in patients with IBD in comparison with the control group (P<0.0001) [21]. Both studies go with the present study results. In contrast to our results, plasma levels of eotaxin-1 in Swedish patients with CRC were lower than those in the control group (P<0.0001) [22].

Concerning eotaxin-1 and adenoma-carcinoma progression, a study from Korea reported decrease in eotaxin-1 expression at glandular cells of neoplastic lesions during progression from low-grade to high-grade dysplasia and finally adenocarcinoma (P<0.001), in addition to low infiltrating eosinophils causing CRC escape from antitumoral eosinophils. However, higher

expression of eotaxin-1 was found in tumor stromal cells increasing eotaxin-1 level; thus, the source of high serum eotaxin-1 level in patients with CRC could be the recruited stromal cells [23]. Moreover, several studies reported decrease in numbers of tissue-infiltrating eosinophils with the adenoma-carcinoma progression with lower numbers in high-grade dysplasia and adenocarcinoma in comparison with those in low-grade dysplasia [7,23].

Regarding MIP-1β, a previous study showed that CCR5 (MIP-1β receptor) was found to be overexpressed in colonic tissues of patients with IBD, which is a precancerous risk, in comparison with normal tissues in a corresponding control group ($P<0.05$) [24]. This was also shown in a study among Chilean patients, demonstrating higher MIP-1β in tumor tissue in comparison with normal tissue ($P<0.05$). In addition, a positive correlation was found between MIP-1β and the protumor macrophages marker CD163 ($P=0.0443$). This could explain the role of MIP-1β in recruitment of macrophages, which stimulate tumorigenesis. Moreover, the same study found a positive correlation between plasmatic MIP-1β measured by multiplex assay and VEGF-A, which has a main role in angiogenesis, metastasis, and poor prognosis in patients with CRC [25]. On the contrary, our study showed a negligible nonsignificant correlation between serum MIP-1β and VEGF-A ($r_s=0.038$).

Serum G-CSF findings in the present study showed results that are consistent with a study from Poland, showing patients with CRC with a significantly higher median value than the corresponding control group ($P<0.05$). On the contrary, it was reported that serum G-CSF differed significantly between patients with adenoma and control group but no significant difference was found between patients with CRC and patients with adenoma [26]. Moreover, another study from United States that used different sample types reported that 90% of colon tumors had high G-CSF receptor expression. The study revealed that neoplastic colon tissues showed up to 30% increase in G-CSF level in cell culture supernatants by bead-based multiplex assay and twofold increase in G-CSF receptor expression in comparison with matched normal tissues. G-CSF with its receptor promotes CRC progression and can be used as target therapy [27].

The findings of VEGF-A in many previous studies go with our results, as it was reported that there were significantly higher levels of VEGF in patients with CRC than controls [28,29], with *P* values less than 0.0001, 0.001, 0.05, and 0.05, respectively. In addition, it was found that VEGF-A levels decreased significantly after curative surgery ($P<0.001$) and it had a significant predictive role in CRC prognosis ($P=0.001$) [28]. High VEGF-A level was found to be associated with overall survival and could predict poor treatment outcome [30].

In contrast to the present study, another study failed to confirm a significant difference in serum VEGF-A level between patients with CRC and control group ($P>0.05$) [31].

The current study failed to find an association between VEGF-A and CRC metastasis. This goes with a previous study from Turkey, reporting that there was no significant correlation between serum VEGF-A level and the overall survival in patients with CRC ($P=0.064$) [32]. However, another study from Italy reported that serum VEGF-A levels showed significant increase in advanced CRC stages ($P=0.04$) [33].

The association between FasL and CRC was not proven in the present study. This finding with the positive findings of the studied markers in addition to significant difference of CRP between patients with CRC and controls could explain the role of inflammation in cancer development and the role of cytokines and chemokines in facilitating CRC pathogenesis and progression.

In contrast to our results, serum levels of soluble Fas ligand were significantly elevated in all patients with CRC ($P<0.05$) [34]. A previous study showed FasL role in hepatic metastasis of CRC, with high tissue expression of FasL in late CRC stage and in liver metastasis ($P=0.024$) [11].

The concerning studies included measurement of more than one biomarker, similar to our study. A study from Italy assessed serum eotaxin-1, G-CSF, MIP-1β, and VEGF-A in patients with CRC and control patients using multiplexed bead-based assay. Only G-CSF levels were significantly higher in patients with CRC than control patients ($P=0.003$) [35].

In addition, a study from United States reported no significant association between multiple cytokines including MIP-1β and VEGF-A and colorectal adenomas. Comparing serum levels of MIP-1β ($P=0.65$) and VEGF-A ($P=0.97$) using multiplexing technology showed no statistically significant

difference between patients with adenomas and patients with normal colonoscopy findings [36]. A study from Malaysia compared serum levels of eotaxin-1, G-CSF, VEGF-A, MIP-1β, and FasL between controls, patients with colon polyps, and patients with CRC using bead-based multiplex assay. The study reported that patients with CRC showed significantly higher serum levels of eotaxin-1 ($P<0.01$), G-CSF ($P<0.01$), VEGF-A ($P<0.05$), and MIP-1β ($P<0.01$) as compared with the control group and significantly higher levels of eotaxin-1 ($P<0.01$) and G-CSF ($P<0.05$), as compared with the polyp group. However, serum MIP-1β ($P<0.0001$) and FasL ($P<0.001$) levels were significantly higher in the polyp group as compared with the control group [37].

Our study reported that patients with CRC showed significantly higher serum levels of G-CSF ($P=0.001$) and VEGF-A ($P<0.001$) as compared with patients with precancerous colon polyp. However, no statistically significant difference was found between patients with colon polyps and patients with CRC regarding serum levels of eotaxin-1 ($P=0.314$) and MIP-1β ($P=0.051$). A larger sample size is needed to assess the significance of the measured biomarkers in differentiating premalignant polyp from CRC.

In Poland, a study reported high serum levels of MIP-1β and G-CSF in patients with CRC using multiplexing technology [38]. Moreover, eotaxin-1, G-CSF, and MIP-1β varied significantly between Japanese patients with CRC and control patients using multiplex bead assay ($P=0.001$, 0.0002, and 0.003, respectively) [39].

When studying ROC of the combined panel, it was shown that MIP-1β had the highest sensitivity (91.43%) and NPV (92.5%), whereas VEGF-A had the highest specificity (92.31%) and PPV (85.2%). In addition, AUC was 0.863 ($P<0.001$). Thus, this panel has a good discriminatory power to discriminate between CRC and nonmalignant cases. Moreover, on combining these studied panel with occult blood, the area under the curve improved from 0.597 to 0.875 ($P<0.001$). Thus, this panel could be used with occult blood to increase its power to discriminate between CRC and nonmalignant cases. As increase in sensitivity tends to decrease in specificity, simultaneously panels of serum protein biomarkers can be used to decrease the likelihood of false results [40].

A significant association was found between eotaxin-1 and MIP-1β ($P=0.006$), eotaxin-1 and VEGF-A ($P=0.001$), MIP-1β and G-CSF ($P<0.001$), and G-CSF and VEGF-A ($P=0.043$). Therefore, assessing eotaxin-1 and G-CSF simultaneously with MIP-1β and VEGF-A can strengthen the discriminatory power to discriminate between CRC and nonmalignant patients.

The current study showed that the discriminatory power of the panel of markers (eotaxin-1, MIP-1β, G-CSF, and VEGF-A) to discriminate between CRC and nonmalignant cases is better than that of routinely used occult blood in stool and could increase the diagnostic performance of occult blood if used in combination with it to reduce false-positives rates and unneeded colonoscopy and biopsies. Moreover, the panel of markers add to the promising approach on using multiplexing bead assay for CRC screening in high-risk patients, CRC diagnosis, and personalized medicine implementation.

Acknowledgements

The present article is particularly important for old age population in generally and patients with colorectal cancer specifically, as these populations are targeted for routine endoscopic screening for early detection of colorectal cancer, which is an inconvenient technique for most people; thus, the technique presented in this study may reduce the need for endoscopic maneuvers and the annoying experience accompanying it. In addition, it may increase the benefits of routinely used occult blood screening test through both cost and time saving.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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