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Association between Inflammatory Cytokines, and Growth Factors in Early Detection and Progression of Colorectal Cancer

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Abstract: ***Background: Prevalence of colorectal cancers (CRC) is continuously elevating during the recent last decades. Objective: The main aim of this survey was to study the association between inflammatory cytokines, interleukins and growth factors in early detection and progression of colorectal cancer. Methodology: A total of 300 subjects (150diagnosed patients with CRC as cases + 150 healthy persons as controls) were included in this study. Growth factors and cytokines levels had been determined in all participated subjects' serum by using Luminex Technology Multiplex Assay (ELISA). A logistic regression analysis had been used for determining the association between CRC and plasma interleukins' levels after adjusting for some potential confounding factors. Results: 60% of the studied population were Saudis. Male : female ratio was 8 : 7. Subjects' age ranged from 45 to 86 years (mean ±SD: 64.4 ± 9.6). The mean body mass index (BMI) was 24.1 kg/m^2 (±2.9). Significant differences were observed in the mean plasma levels between the CRC patients and controls for 13 plasma cytokines, namely IL-4, IL-8, IL-9, IL-17A, Eotaxin, G-CSF, IFN-γ, TNF-α, IP-10, MIP-1α, MIP-1β, IFNG, and RANTES (p < 0.05). Conclusion: The significant elevation of serum inflammatory cytokines and growth factors in CRC patients suggesting the strong association and crucial role in early detection of CRC in humans.***

Keywords: Inflammatory Cytokines; Growth Factors; Early Detection; Progression of Colorectal Cancer

1. Introduction

Various health and research organizations have conducted studies about the prevalence, diagnostics and distribution of colorectal cancer (CRC) in the Kingdom of Saudi Arabia (KSA). One such study was conducted by the World Health Organization in 2014, which found that general cancer mortality in males is slightly higher (4, 900 deaths) than females (4, 300 deaths). Within this sample however, CRC accounts for 12.5% of cancer deaths in males while, 11.1% of cancer deaths in females is associated with CRC.1, 2 Compared to other popular types of cancers, the mortality associated with CRC can be said to be very high, threatening and a worrying situation. The situation is even worse when it comes to cancer incidence, as CRC in males is the highest with incident cases of 1, 168 per year. Even though breast cancer has the highest incidence in females, CRC comes next with 879 cases a year. Among other things, Zubaidi et al. lamented that in the KSA population, not much has been done to understand the risk factors associated with CSC and how the disease manifests. This situation has been attributed to the high incidence and mortality with CRC.3

There are number of risk factors that the World Health Organization associates with increasing rate of CRC in KSA. Some of these risks are tobacco smoking, physical inactivity, obesity, and diet. Between males and females Saudis, the level of manifestation of these risks factors differ significantly. For example, whereas 37.9% of males are at risk due to smoking, there is less than 1% of females at risk because of smoking. Physical inactivity is however the highest risk factor in both males and females with 52.1% and 67.7% respectively.4Bresnick, Weber and Zimmer noted that these earlier risk factors can generally be controlled if Saudis adopted the right behaviors. Meanwhile, there are other risk factors that can hardly be controlled. Some of these include age, personal history of inflammatory bowel disease, family history, and personal history of the disease. The fact that personal history of inflammatory bowel disease exposes one to CRC makes it very important for further studies into the role of growth factors, inflammatory cytokines and other protein related conditions such as interleukins to be taken very seriously.5, 6

The incidence of colorectal cancer has quadrupled in the last two decades. According to the World Health Organization, colorectal tumors are among the most common tumors, accounting for 10% of the incidence of new cancer cases annually, and the second highest incidence among males and third among females globally.7 This type of cancer is associated with aging, as about 90% of people diagnosed with over the age of 50 years. In 2018, 3 million people were diagnosed with colorectal cancer, and there were more than 696, 000 deaths from the disease worldwide. It is also the fourth leading cause of death from cancers globally, and mortality increases with age, and occurs among men more than women.8

2. Methodology

Study Population

The population of the current study included patients (cases) of the oncology centre of King Abdullah Medical City in Makkah and Jeddah in addition to health matched persons as controls. By adopting a random sampling technique, a total of 300 subjects were recruited to participate. A self reporting questionnaire had been used for collecting demographic data beside blood samples

Blood sample collection

One to three milliliter of blood was collected in sterile tubes after overnight fast. The blood samples were then centrifuged (3500 rpm for15 min) and the separated serum

was transferred to appropriatelylabeled 1.5-mL Eppendorf tubes and stored at 80^{o} C until use in experiments.

Instruments and software's

Luminex Analysis

- Analyzer Luminex® 200™.
- Luminex 200 Calibration kit and Performance Verification kit.
- Luminex Sheath Fluid.
- Human cytokine multiplex magnetic kits containing assay diluent, beads with capture antibodies, biotinylated detection antibodies, streptavidin-phycoerythrin conjugate, cytokine standard mix, wash buffer, 96-well flat-bottom plate.
- Refrigerated centrifuge with fixed angle rotor for microtubes, up to 16, 000 × *g* at 4 °C.
- Sonicating water bath.
- Orbital 96-well plate shaker.
- Handheld magnetic 96-well separator.
- Calibrated adjustable 10 µL to 25 mL precision pipets with tips (200 µL multichannel pipet and electronic repetitive pipet is advantageous, e. g., Gilson Repetman).

Software

- xPonent® software (description of procedures in protocol based on version 3.1) installed on acquisition computer connected to Luminex® 200™.
- R interactive statistical programming environment.
- Tidyverse set of packages (tidyverse) for data manipulation and graphing.
- Cowplot package for publication ready graphical outputs from R.
- Packages specific for reading multiplex data exported from xPonent—drLumi and standard curve fitting—drLumi and nCal.
- RStudio (RStudio) —not necessary, but useful, IDE (Integrated development environment) for R, provides syntax highlighting for R scripts, autocompletion and interactive R console as well as support for viewing and saving graphics and imported datasets.

Preparation of Luminex System

At first we turned on the Luminex instrument, XY platform and sheath fluid delivery system. Allow the lasers warm up for 30 min. then adjustment needle height according to the plate type used. Wash the instrument with 70% ethanol or isopropanol to remove bubbles, followed by two water washes using commands in the Maintenance menu of the xPonent software. then we Calibrated the Luminex system according to the manufacturer's instructions using Luminex® 100/200 Calibration Kit and Luminex® 100/200 Performance Verification kit. Calibration is valid for maximum 1 week provided that the stable temperature (±2 °C) is guaranteed.

Statistical analysis

All statistical analyzes were performed using the Social Sciences Statistical Package (SPSS) version 25 (Advanced Analytics, Inc. Tokyo, Japan). Unless otherwise noted, persistent data is summarized as the mean standard deviation (SD). A value of p <0.05 was taken to represent the statistical significance. In statistical analysis, plasma cytokine levels underwent a logistical as well as normative shift, those below the detection limit were given a value of half of the limit for the detection.

Before building the prediction model, we compared the average plasma levels of each cytokine between cases and controls using multiple linear regression analysis with gender, age and hospital adjustments. Subsequently, a multiple logistic regression analysis was performed to examine the association between plasma cytokines and the presence of the CRC after adjusting for gender, age and Khartoum-Petal. Odds ratios (OR) and 95% confidence intervals (95% CI) were estimated for the presence of CRC for each plasma cytokines. Correlation analysis was performed for all pairs of cytokines to assess the linear relationship between them. To construct the prediction model, candidate plasma cytokines were chosen according to the following procedures. First, we filtered as a plasma cytokine primary filter that demonstrated the smallest value of p in the above-mentioned multiple logistic regression analysis, and we excluded all the cytokines whose binding coefficients with the specified cytokine exceeded 0.7. Next, we determined the other candidate cytokine with the smallest value of the following p, and we removed some of the remaining cytokines in the case where their binding parameters with the second specific cytokine were higher than 0.7. These procedures were repeated until the history of cytokines with sweets were completed. By applying the reverse cancellation method to the logistical registration model with all potential candidates for plasma cytokines and fixed variables for gender, age, and body mass, we built a prediction model for the presence of colon and rectal cancer.

3. Results

As presented in table (2) 300 participants were enrolled in this study (150 patients diagnosed with CRC as cases + 150 healthy matched individuals as controls). A written informed consent was obtained from all subjects. It shows that 60% of the studied population were Saudis. Male: female ratio was 8: 7. Subjects' age ranged from 45 to 86 years (mean ±SD: 64.4 ± 9.6). The mean body mass index (BMI) was 24.1 kg/m^2 (±2.9). In regard to the tumors' locations among the cases, the primary lesion was located in the cecum and ascending colon in 32 patients (21.3%), the transverse colon in 23 (15.3%), descending and sigmoid colon in 30 (20%) and finally, in the rectum in 65 (43.3%).

Table 1: Adjusted calibration standard preparation for more precise quantification of interleukins in blood serum

	Recommended in kit		Adjusted			
	pg/mL	Dilution	pg/mL	Dilution	Assay Diluents (µL)	Previous STD (µL)
STD 1	30, 700.00		30,700.00			
STD 2	7675.00	4-fold	10, 233.33	3-fold	400	200
STD 3	1918.75	4-fold	3411.11	3-fold	400	200
STD 4	479.69	4-fold	1137.04	3-fold	400	200
STD 5	119.92	4-fold	379.01	3-fold	400	200
STD 6	29.98	4-fold	126.34	3-fold	400	200
STD 7	7.50	4-fold	63.17	2-fold	250	250
STD 8			31.58	2-fold	250	250

STD 9			15.79	2-fold	250	250
STD 10			7.90	2-fold	250	250
STD 11			3.95	2-fold	250	250

Table 1: Demographic characteristics of the study population (n=300)

Variables characteristics	
Nationality	
Saudi/ Non-Saudi 180/120	
Age (years)	
Mean (±SD)	64.4 (± 9.6)
Median	62.5
Range	45–86
Gender	
Male/female	160/140
BMI (kg/m^2)	
Mean (±SD)	24.1 (± 2.9)
Median	23.5 (5)
Location of colorectal tumor [n (%)]	
Cecum & Ascending colon32 (21.3%)	
Transverse colon23 (15.3%)	
Descending& Sigmoid colon 30 (20%)	
Rectum 65 (43.3%)	

Table 2: Multi-analysis of plasma cytokines in the CRC patients and control

Cytokine	Serum (pg/mL)				p-value
	Control		CRC		
	Mean ± SD	Max—Min	Mean ± SD	Max—Min	
IL-1ra	57.8± 58.37	477.25–3.88	56.28±28.9	165.95–10.78	0.767
IL-1β	1.8± 1.92	14.40–0.04	1.66±0.084	3.94–0.51	0.571
IL-4	1.49± 0.67	3.31–0.23	2.09±0.67	3.67–0.88	<0.001
IL-5	8.86± 6.26	33.42–0.68	9.67±5.66	29.07–1.76	0.201
IL-6	4.98± 8.03	39.30–0.11	4.69±7.85	46.64–0.31	0.622
IL-7	9.78± 9.91	80.81–0.20	10.69 ± 6.97	28.29–0.37	0.398
IL-8	7.3± 3.03	20.05–2.38	9.78±5.15	32.86–0.61	0.007
IL-9	20.6± 11.97	63.08–2.44	32.37±17.03	113.69–10.00	<0.001
IL-10	7.69 ± 12.82	81.98–0.08	6.57±5.24	25.46–0.20	0.555
IL-12	8.40 ± 10.91	83.62–0.12	9±6.87	36.60–0.39	0.213
IL-13	21.08± 22.09	144.17–0.54	21.01±19.65	96.01–1.54	0.779
IL-17A	36.43± 32.34	137.78–0.01	56.89±45.41	197.97–0.73	0.009
Eotaxin	35.9± 16.99	145.14–8.90	43.61±17.19	139.12–21.34	0.001
FGF-2	33.18± 50.44	469.35–1.47	30.06±10.31	56.93–9.97	0.302
G-CSF	28.94± 15.51	75.48–2.25	39.38±14.48	80.45–9.24	<0.001
GM-CSF	16.17± 26.90	150.57–0.07	10.66±10.74	55.00–0.27	0.857
IFN-γ	40.07± 30.59	230.87–3.36	46.01±23.25	115.14–14.32	0.040
TNF-α	40.23± 27.98	232.33–8.35	44.74±15.19	97.54–20.04	0.021
IP-10	377.59± 256.98	171.98–116.17	501.43±316.08	178.90–143.82	0.002
MIP-1α	2.66± 1.65	13.48–0.71	2.99±1.08	9.09–1.41	0.007
MIP-1β	27.14± 10.56	60.13–9.19	35.14±19.5	138.81–12.35	0.002
IFNG	128.85± 157.64	736.91–0.69	242.81±245.57	1196.18–9.50	<0.001
RANTES	2335.75± 1132.53	7825.46–84.60	2850.85±1215.08	5794.57–181.78	0.031
VEGF	7.18± 7.74	31.57–0.11	6.68±8.58	44.10–0.19	0.624

Significant differences were observed in the mean plasma levels between the CRC patients and controls for 13 plasma cytokines, namely IL-4, IL-8, IL-9, IL-17A, Eotaxin, G-CSF, IFN-γ, TNF-α, IP-10, MIP-1α, MIP-1β, IFNG, and RANTES ($p < 0.05$). An additional comparison was conducted among the controls, stage 0 –II cases, and stage III–IV cases, and all 13 plasma cytokines, except IFN-γ and RANTES, showed an increasing trend according to tumour progression (Table 4). The associations of the above 13 plasma cytokines with the presence of CRC were all statistically significant Table (3).

Construction of a prediction model for the presence of CRC

As seen in table 3, IL-6and IL-4 showed the smallest p-values (< 0.00001) and were selected as the initial candidates for the IL-6and IL-4 models, respectively. Seven plasma cytokines were nominated as additional candidates for each model: Eotaxin, G-CSF, TNF-α, IP-10, MIP-1α, MIP-1β, and IFNG for the IL-6model; and IL-8, Eotaxin, TNF-α, IP-10, MIP-1α, MIP-1β, and IFNG for the IL-4 model. Finally, the backward elimination method revealed two multivariable prediction models (logistic models) for the presence of CRC as: IL 9 model: z = 1.45 IL 9 + 0.87 Eotaxin +0.65 G –CSF+ 0.098 TNF – 0.024 gender-.024age IL 4 model: z = 1.34 IL4+ 0.86 IL 8 +0.759 Eotaxin +0.009 IP 10-0.98 gender-0.08 age

Table 4: Results of ROC analysis of two multivariable prediction models and each cytokine

	Sensitivity	Specificity	Area Under the ROC Curve (AUC)
Model IL-9	0.652	0.839	0.819
IL-9	0.848	0.552	0.755
Eotaxin			
G-CSF			
TNF-α			
Eotaxin	0.758	0.667	0.714
G-CSF	0.788	0.563	0.695
TNF-α	0.455	0.839	0.657
Model IL-4	0.742	0.767	0.832
IL-4	0.576	0.791	0.736
IL-8	0.591	0.736	0.68
Eotaxin	0.758	0.667	0.714
IP-10	0.652	0.678	0.681
TNF-α	0.455	0.839	0.657

As presented in table (4) ROC analysis performed to closely evaluate the prediction models for the presence of CRC. In the IL-6model, the AUC, sensitivity, and specificity of the optimal cut-off points were 0.819, 0.652, and 0.839, respectively, while the corresponding values in the IL-4 model were 0.832, 0.742, and 0.767, respectively. Both models thus demonstrate "good" capability for discriminating between CRC patients and controls. A comparative prediction model without cytokines (model 0) was constructed by using gender, age, and hospital as variables. Its AUC, sensitivity, and specificity of the optimal cut-off points were 0.559, 0.485, and 0.655, respectively, therefore, the model demonstrated "poor" capability. All of the evaluation parameters for the IL-6and IL-4 models were better than those for model 0. Also, single-cytokinemodels presented lower AUCs (0.657–0.755) than the multi-cytokine IL-6and IL-4 models.

4. Discussion

The study results supported our working hypothesis that the levels of some plasma cytokines vary depending on the presence of CRC. Even after controlling for gender, age, and hospital, the plasma levels of CRC patients and controls differed significantly in terms of the following 13 cytokines: IL-4, IL-8, IL-9, IL-17A, Eotaxin, G-CSF, IFN-γ, TNF-α, IP-10, MIP-1α, MIP-1β, IFNG, and RANTES ($p < 0.05$). The combinatorial assessment of some of these plasma cytokines showed promise for detecting the presence of CRC. In fact, the ROC analysis showed that the IL-6and IL-4 models had "good" capability for discriminating between CRC patients and controls. These two models showed similar AUC values, although some differences were observed; the performance of the IL-6model was excellent with respect to specificity, while the IL-4 model balanced both sensitivity and specificity.

In our research, the logistic regression analysis revealed that the cytokines IL-6and IL-4 had potential association with the presence of CRC. IL-6is a cytokine produced by $CD4^{+}$Th2 cells as well as by some B lymphomas; it has been shown to induce an increase in the proliferation of CRC cells and to promote tumorigenesis in CRC cells.[9, 10]

Kantola et al. conducted a screening cohort study using cytokines in serum, including IL-9, and concluded that cytokine biomarkers might be a promising tool for the detection of CRC.1[1, 12] Broadly, IL-4 can be categorized as a type of anti-inflammatory cytokine. IL-4 and IFN-γ are the most frequently described cytokines in the inflammatory process. Szylberg et al. analyzed 144 colorectal polyps and showed a significantly increased level of IL-4 in adenomas, serrated adenomas, and hyperplastic polyps compared with the control group[13, 14]Sharp et al. reported that levels of the anti-inflammatory cytokine IL-4 were significantly elevated in advanced CRC, whereas IFN-γ levels were not statistically different.1[5, 16]Recent studies have found that IL-4 levels in colorectal polyp-derived serum were significantly higher than those in serum from healthy volunteers.[17]

Other cytokines included in the IL-6and IL-4 models warrant mention. There is evidence that the cytokine Eotaxin is strongly associated with primary and metastatic tumors of colorectal origin.[18]Both IL-4 and IL-13 synergistically enhance TNF-α–induced Eotaxin production.[19]. Natori et al. suggested that G-CSF may have the potential to promote tumor growth, at least in part, by stimulating angiogenesis.[20] The level of G-CSF has been examined in CRC patients and found to be significantly higher than in healthy subjects. Despite the small sample size, another study found significantly higher levels of G-CSF in CRC patients before surgery compared with controls at baseline.[21, 22]

TNF-α is a potent pro-inflammatory cytokine thought to be involved in the pathogenesis of inflammatory bowel disease and has been reported to promote inflammation and colitis-associated cancer.2[3, 24]In the blood of the patients with CRC, a significant elevation has been reported in the levels of TNF-αDimberg et al. analyzed 50 CRC patients and found a significantly higher IL-8 (CXCL 8) level in cancer tissue compared with paired normal tissue, and showed that CRC patients exhibited significantly higher plasma levels than healthy controls.2[5]

Crucitti et al. conducted screening of 30 CRC patients; although the sample size was small, significantly higher levels of IL-1β, IL-7, IL-8, G-CSF, IFN-γ, and TNF-α were detected in CRC patients compared with controls at baseline.2[6, 27]. The CXCL10 (IP-10) /CXCR3 axis of inflammatory mediators is one of the most important chemokine axes and has been proven to be a lymphocyte-associated metastasis mediator in several tumors, although in one, report serum levels of IP-10 were of measureable concentration but did not differ between the control and CRC groups.2[8]

5. Conclusion and Recommendation

Interleukins generated considerable interest in their use for detecting the presence of CRC early in its development. The hope is that the convenience of screening blood tests would allow for early diagnosis and treatment and lead to significant reductions in cancer-related morbidity and mortality. Of the various pathways to tumor formation and progression, the inflammatory pathway has stimulated particular attention. Several excellent reviews have described the cellular and molecular roles of inflammation in the development of cancer. A central feature of activated immune cells is the production and release of growth factors and cytokines that modulate the inflammatory milieu in tumor tissues. Currently, more than 300 different cytokines have been identified. CRC has been linked to systemic and local changes in the cytokine profile, and recent work indicates that multiple pro-tumorigenic and also antitumorigenic cytokines are differently expressed in distinct CRC tissues. Chemokines, small peptides that are structurally and functionally similar to growth factors, are also among the key players that promote cancer cell metastasis in some types of cancers. Chemokine ligand-receptor interactions have been reported to be involved in CRC progression. Thus, the differential expression of the blood cytokines could have the potential in the early detection of CRC. To explore this, we car-ried out a multicenter, hospital-based case-control study to examine the associations between plasma cytokine levels and the presence of CRC. We hypothesized that the levels of some

plasma cytokines would fluctuate depending on the presence of CRC and combinatorial cytokines would have greater discriminant ability.

The levels of several plasma cytokines varied significantly between CRC patients and control subjects, suggesting the possibility of differentially expressed plasma cytokines as potential biomarkers for detecting the presence of CRC.

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