


Fluoropyrimidine therapy induced alterations in interleukins expression in colorectal cancer patients

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Abstract

This study monitored the changes in the expression of inflammatory IL-6 and IL-1 β during the treatment period of Fluoropyrimidine (FP) based therapy. RNA was extracted from the peripheral blood of 102 CRC patients before treatment with FP therapy, and from 48 and 32 patients after 3 and 6 months of treatment, respectively. The genetic transcription of IL-6 and IL-1 β was determined by real time PCR. Patients were stratified according to their levels of IL-6 and IL-1 β genes expression for subgroup and survival analyses. Baseline CRC patients showed overexpression of IL-6 and IL-1 β compared to healthy control. FP therapy significantly induced IL-6 and IL-1 β expression. Subgroup analysis showed that patients with right colon tumors had significant elevation in both IL-6 and IL-1 β with FP therapy. FP therapy significantly induced IL-1 β expression in patients ≤ 45 years, smokers, with high baseline level of CA19.9, right colon tumors, low grade pathology, T3 tumors and positive lymph nodes. Survival analysis showed that baseline levels of interleukins expression had insignificant effect on overall survival and event free survival. FP therapy has an impact on the level of interleukins expression declared in certain clinicopathological subgroups of CRC patients, but without a prognostic significance on patients' survival.

Keywords

colorectal cancer, fluoropyrimidine therapy, interleukins expression

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Introduction

Colorectal cancer (CRC) is the most frequently diagnosed type of cancer in developing populations. Fluoropyrimidine (FP) based therapy described as single agent of capecitabine or 5-fluorouracil (5-FU), and in combination with oxaliplatin, is still the gold standard in CRC treatment.¹

The transition of normal epithelium into malignancy is not driven only by the intrinsic genetic mutations, but also by the interaction of tumor cells with the components of tumor microenvironment.² Interleukins are small molecule cytokines expressed by tumor-associated macrophages and

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neutrophils. Interleukins possess dichotomous functions depending on the phase of the disease, and their receptors were found on both intestinal epithelia and tumor cells.³

Cytokines secreted by the activated tumor stroma have role in initiating and sustaining the chronic inflammation of gut.⁴ Cytokines modulate tumor growth and enhance the invasiveness of tumor cells through the activation of nuclear factor-kappa B (NF- κ B) by tumor necrosis factor alpha (TNF α) and interleukin-1 β (IL-1 β), and also through the activation of STAT3 by interleukin-6 (IL-6).⁵⁻⁷

Nagasaki et al.⁸ demonstrated that stromal fibroblasts isolated from colon cancer produced significant amounts of IL-6 which enhanced VEGF production by fibroblasts, thereby inducing angiogenesis. IL-6 mediated the upregulation of integrin β 6, which was involved in the invasion and epithelial-mesenchymal transition of CRC cells.⁹ The progression of CRC disease was correlated with the significant overexpression of IL-6 in serum¹⁰ and in CRC tissues.¹¹ IL-6 was positively correlated with CRC tumor TNM stage, depth of invasion and lymph node metastasis.¹¹ An association was shown between serum values of carcinoembryonic antigen (CEA) and IL-6.¹² Also an association was shown between high IL-6 expression and risk of relapse¹³ and poor overall survival (OS) and disease-free survival (DFS).¹⁴

The action of IL-1 β in CRC is mediated through the NF- κ B pathway.¹⁵ IL-1 β increased the expression of miR-181a, leading to the inhibition of tumor suppressor (PTEN) expression and the promotion of colon cancer cells proliferation.¹⁵ Also it is documented that IL-1 β increased the expression of MiR301A in intestinal epithelial cells in colitis-associated cancer patients, thus inhibiting BTG anti-proliferation factor 1 (BTG1) expression.¹⁶ High IL-1 β expression was correlated with high expression of human leukocyte antigen class II molecules and monocyte activation in CRC intestinal mucosa.¹⁷ IL-1 β , produced by neutrophils in the intestinal mucosa, can induce mononuclear phagocytes to produce IL-6, thereby promoting tumor initiation and progression.¹⁸

Accordingly, this study tracked the changes of IL-6 and IL-1 β expression in the peripheral blood of CRC patients at baseline and after 3 and 6 months of treatment with FP therapy, to be correlated with

their clinicopathological characters and their survival.

Patients and methods

Ethics approval and consent to participate

This study was conducted according to Good Clinical Practice guidelines. The study was approved by the Institutional Human Research Ethics Committee of NCI, Egypt, and conducted in accordance with the Declaration of Helsinki and an informed consent was taken from each patient.

Patients

This is a prospective study in which 102 CRC patients were enrolled at baseline and after 3 and 6 months during the course of FP based therapy. Patients included in this study were admitted to the National Cancer Institute, Cairo University during the period from February 2014 to December 2014. A written informed consent was taken from the participated patients, and the full clinicopathological information was recorded from the patients' files. Thirty-two healthy individuals, matched in sex and age with our patients, were recruited as control. The mean \pm Standard deviation of age of healthy controls was 39 ± 13.8 and the male:female ratio=1:1.6, $P=0.16$. Peripheral blood samples were collected in EDTA tubes under complete aseptic conditions. Mononuclear cells were isolated from the whole blood samples using hemolysin buffer (8.46 g, 84 ammonium chloride, 1 g potassium bicarbonate and 1 g ethylene diamine disodium salt dissolved in 11 water, and the pH was adjusted at 7-7.2), as described in our previous work in Fouad et al.¹⁹

Patients included in this study had the following criteria: newly diagnosed with CRC, at age ≥ 18 years, with performance status 1, 2, and 3 by Eastern Cooperative Oncology Group (ECOG) scale, not received chemotherapy before, had curative fluoropyrimidine based treatment for colorectal cancer, and no psychological or geographical barriers for regular follow up of the patients. Event free survival (EFS) and the hazard of recurrence and progression were the primary end points. The exploratory measure in the first publication was the impact of epigenetic (global DNA methylation:

5-methyl cytosine and DNA methyl transferases) in the response of colorectal cancer patients to fluoropyrimidine therapy.¹⁹ However, in this one, and on the same cohort of patients, the change in interleukins (IL-6 and IL-1 β) expression was evaluated in response to fluoropyrimidine therapy.

RNA extraction and c-DNA synthesis

Total RNA was extracted from the lymphocytic cell pellet with total RNA purification kit (Direct-Zol RNA Kit, Zymo Research, Germany) in which isopropanol (300 μ l) was added to the cell lysate to extract the nuclear RNA. After several steps of centrifugations and washings of spin column placed in a 2 ml collection tube, pure RNA was separated and eluted with 40 μ l of elution buffer into RNase-free microcentrifuge tube. cDNA synthesis was performed according to the manufacturer's instructions using Revert Aid First Strand cDNA synthesis kit (ThermoFisher, UK) in which RNA sample (10 μ l) was added to 10 μ l reaction 2 \times Reverse Transcription Master mix: 2 μ l of RT buffer, 0.8 μ l dNTP mix, 2 μ l random primers, 1 μ l reverse transcriptase, 1 μ l RNase inhibitor and 3.2 μ l nuclease-free water were mixed together. The tubes were loaded to the thermal cycler with the following conditions: 25 $^{\circ}$ C (10 min), 37 $^{\circ}$ C (120 min), and 85 $^{\circ}$ C (60 min).

Quantitative evaluation of interleukins expression with real time PCR

Quantitative real time PCR was conducted according to manufacturer's instructions by Applied Biosystems syber green PCR master mix (USA) in which 2 μ l of cDNA was added to 18 μ l of the reaction master mixture: 10 μ l Syber green mix, 0.5 μ l Passive Reference Dye, 0.4 μ l forward primer, 0.4 μ l reverse primer and 6.7 μ l nuclease free H₂O. Initial denaturation at 95 $^{\circ}$ C for 10 min, followed by 40 cycles of denaturing at 95 $^{\circ}$ C for 15 s, and annealing at 62 $^{\circ}$ C for 1 min was performed for all analyses in triplicate on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Reverse and forward sequences of primers genes encoding for mRNA transcript of IL-6 and IL-1 β genes were designed by NCBI-NIH tool and the sequences were summarized as follows: IL-6 (Sequence ID: NM_000600.5, Region: 546–565) forward primer: GAGACTTGCCTGGTGAAAAT,

reverse primer: CAGGGGTGGTTATTGCATCT. IL-1 β (Sequence ID: XM_017003988.2, Region: 467–486) forward primer: GGACAAGCTGAGGAAGATGGC, reverse primer: TTTTTTGCTGTGAGTCCCGG. β -actin (Sequence ID: NM_001100.3, Region: 286–304) forward primer: CCAGAGCAAGAGAGGTATCC, reverse primer: CTGTGGTGGTGAAGCTGTAG. CT values were normalized to housekeeping gene (β -actin) ($2^{-\Delta Ct}$) in order to calculate the relative expression of each gene. Also the fold change of genes expression ($2^{-\Delta\Delta Ct}$) was calculated after normalization to the genes expression in baseline patients and in healthy controls.²⁰ In the analysis of baseline IL-6 and IL-1 β genes expression (Figures 1a and 2a), and in the subgroup analysis of the two genes (Tables 2 and 3), the normalization was relative to the healthy control levels of the genes. However, in the effect of fluoropyrimidine therapy on interleukins expression in Figures 1b and 2b, the CT values of genes expression was normalized to the level of the genes in baseline patients before fluoropyrimidine therapy.

Survival analysis

After 3 years of follow up, OS and EFS of CRC patients were calculated by Kaplan-Meier method of analysis for the subgroups of patients with the over- and under-expression levels of interleukins. Patients were stratified around their median expression baseline level of interleukin, where patients with relative IL-6 expression level ≤ 1.72 and IL-1 β ≤ 1.77 were considered with under-expression, while patients with IL-6 expression level > 1.72 and IL-1 β ≤ 1.77 were considered with over-expression. OS was calculated from the date of diagnosis till the date of last visit or the date of death, while EFS was calculated from the date of resection or neoadjuvant therapy to the date of recurrence, progression, or death, which occurred first. EFS for patients who neither progressed, relapsed, nor died, was censored at last assessment prior to loss to follow-up. COX proportional-hazards model was used to determine the independent significant risk of individual factors.

Statistical analyses

IBM SPSS statistical package version 24 was used in data manipulation. Statistical power analysis

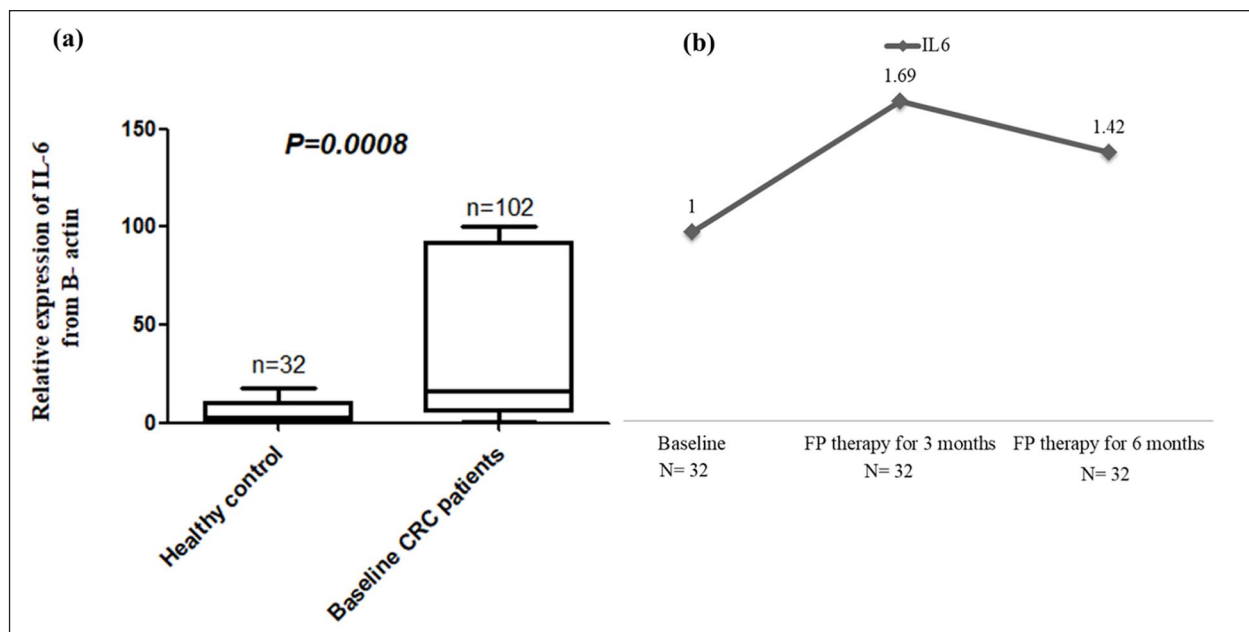


Figure 1. (a) IL-6 expression in healthy control and baseline CRC patients. (b) The change in IL-6 expression after 3 and 6 months of FP therapy normalized to their baseline levels in CRC patients.

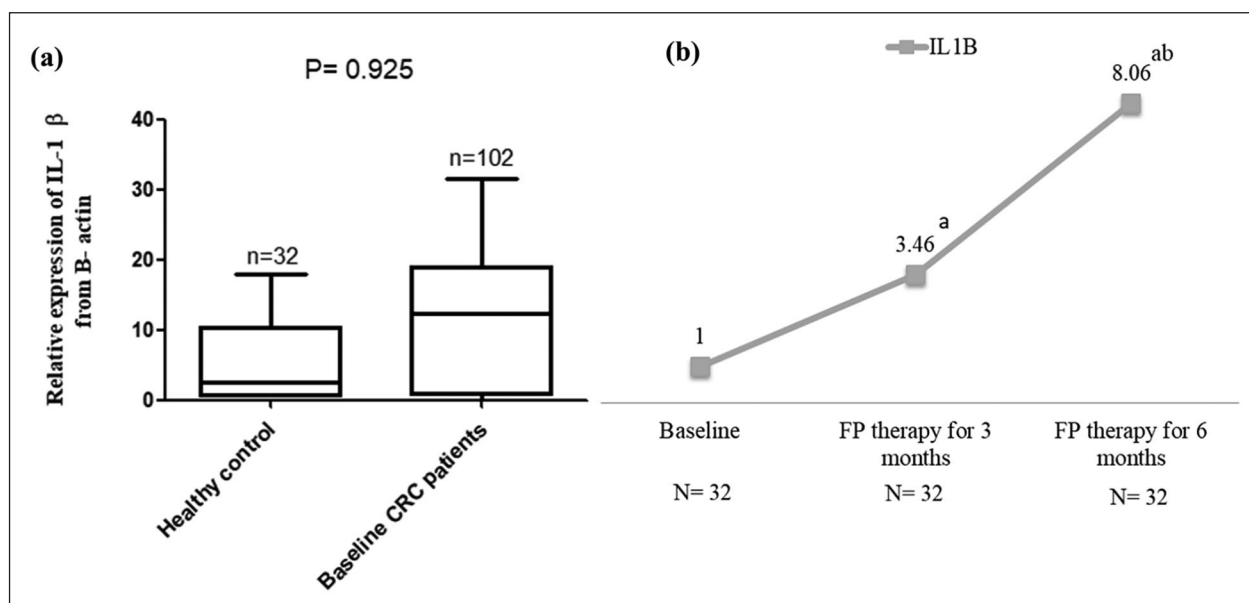


Figure 2. (a) IL-1 β expression in healthy control and baseline CRC patients. (b) The change in IL-1 β expression after 3 and 6 months of FP therapy normalized to their baseline levels in CRC patients.

^aSignificant difference when CRC patients after 3 and 6 months of FP therapy were compared with their baseline level, P value ≤ 0.05 .

^bSignificant difference when CRC patients after 6 months of FP therapy were compared with their level after 3 months of FP therapy, P value ≤ 0.05 .

was used for the determination of the effective sample size of CRC patients in order to detect the true effect of fluoropyrimidine therapy on interleukins expression over the treatment period of 6 months. Numeric data explored for normality using Kolmogorov-Smirnov test and Shapiro-Wilk

test. Categorical data were expressed as count of patients, while numerical data were summarized as 25 percentile, median, and 75 percentile. Patients were stratified into subgroups according to their clinicopathological. The comparison between subgroups of CRC patients was tested by Chi-Square

test for the categorical data. Kruskal Wallis was used to test the significance for more than two subgroups of numerical data, and then Mann-Whitney was conducted for pairwise testing. The effect of FP therapy on genes expression over time (3 and 6 months) in certain subgroup of CRC patients was tested by Friedman, followed by pairwise comparison with Wilcoxon matched test. All P-values were two-sided and P -values ≤ 0.05 were considered significant.

Results

Patients

The full clinicopathological characters of our 102 CRC patients were demonstrated in Table 1. Nearly half of the patients (46%) were ≤ 45 years with slight male predominance (53.92%). Most of the

Table 1. Patients' clinicopathological characteristics and treatment protocol.

Variables	N (%)
Total number of patients	102 (100)
Age	
≤ 45 years	47 (46.07)
> 45 years	55 (53.92)
Ratio (young:old)	1:1.2
Median age	46
Range	19–72
Mean age	45 ± 13.7
Gender	
Male	55 (53.92)
Female	47 (46.07)
Ratio (male:female)	1:0.85
Comorbidities ⁺⁺	
Hypertension	8 (7.84)
Diabetes mellitus	7 (7.84)
Hepatitis C infection	7 (7.84)
Smoking	
No	71 (69.60)
Yes	31 (30.39)
Complains ⁺⁺	
Bleeding/rectum	56 (54.90)
Abdominal pain and swelling	45 (44.11)
Constipation	17 (16.67)
Diarrhea and vomiting	12 (11.76)
Urine retention and stool incontinence	2 (1.96)
Anemia	1 (0.98)
Performance status	
I	85 (83.33)
II	15 (14.70)

(Continued)

Table 1. (Continued)

Variables	N (%)
III	2 (1.96)
CEA level	
Normal	46 (58.22)
High	33 (41.77)
CA19.9 level	
Normal	57 (79.16)
High	15 (20.83)
Surgery	
No surgery	28 (27.40)
Surgery	74 (72.55)
Tumor location	
Right colon	30 (29.41)
Left colon	25 (24.50)
Rectum	40 (39.21)
Pathology	
Adenocarcinoma	71 (69.60)
Mucinous and signet ring	31 (30.39)
Grade	
II	82 (80.39)
III	20 (19.60)
Tumor size	
T2	15 (14.70)
T3	65 (63.72)
T4	19 (18.62)
Lymph nodes	
Negative	41 (40.19)
Positive	39 (38.23)
Metastasis	
No	71 (69.90)
Yes	31 (30.39)
Sites of metastases ⁺⁺	
Liver	20 (19.60)
Peritomeum	17 (16.67)
Lung	8 (7.84)
Others	16 (15.68)
Stage	
II	32 (31.37)
III	39 (38.23)
IV	31 (30.39)
FP therapy ⁺⁺	
No FP therapy	5 (4.90)
Neoadjuvant FP therapy	35 (34.31)
Adjuvant FP therapy	81 (79.41)
Protocol of FP therapy ⁺⁺	
Single agent	28 (27.45)
Combination	81 (79.41)
After treatment toxicities ⁺⁺	
GIT	28 (27.20)
Non-GIT	29 (28.30)

Data presented as counted number of patients and percentage N (%) to the total number of patients (102).

CEA: carcinoembryonic antigen; CA19.9: carbohydrate antigen 19.9; FP therapy: fluoropyrimidine therapy.

⁺⁺More than one event per patient was recorded.

Table 2. IL-6 relative expression in subgroups of Egyptian CRC patients at baseline and after treatment with FP therapy for 3 and 6 months.

		IL-6 fold change relative to healthy control ($2^{-\Delta\Delta CT}$) in CRC patients						P-value
		At baseline		After 3 months of FP therapy		After 6 months of FP therapy		
		N	Median (IQR)	N	Median (IQR)	N	Median (IQR)	
Age	≤45	47	1.72 (0.13–2.73)	23	2.88 (0.60–4.63)	15	12.25 (0.84–18.78)	0.584
	>45	55	1.46 (0.57–3.29)	25	2.06 (0.10–3.56)	17	12.52 (1.30–19.54)	0.526
			P-value		0.682		0.992	
Sex	Female	47	1.25 (0.25–3.62)	20	1.49 (0.32–3.22)	13	12.27 (0.80–18.93)	0.779
	Male	55	1.72 (0.60–2.73)	28	3.32 (1.96–6.53)	19	12.39 (1.57–18.91)	0.148
			P-value		0.930		0.762	
Smoking	Non smoker	71	1.75 (0.53–2.87)	34	2.13 (0.68–4.22)	20	12.37 (1.53–18.64)	0.810
	Smoker	31	0.58 (0.17–3.61)	14	1.88 (0.35–3.95)	12	9.98 (0.73–26.44)	0.197
			P-value		0.770		0.923	
Baseline CEA	Normal	33	1.75 (1.46–4.29)	5	1.83 (0.10–4.63)	8	15.57 (0.63–26.44)	0.526
	High	46	1.79 (0.53–2.87)	13	2.20 (2.06–4.66)	4	12.45 (1.07–18.64)	0.641
			P-value		0.550		0.704	
Baseline CA19.9	Normal	15	1.32 (0.53–2.50)	7	2.06 (0.10–3.56)	3	12.30 (0.69–18.78)	0.202
	High	57	4.94 (2.40–7.89)	12	3.18 (0.10–8.40)	11	12.46 (0.65–18.64)	0.717
			P-value		0.234		0.924	
Site of tumor	Right colon	30	1.74 (0.30–3.96)	15	2.06 (0.10–3.26)	16	12.18 ^{ab} (0.80–18.45)	0.048
	Left colon	25	1.91 (1.17–1.95)	15	2.93 (1.39–16.93)	6	10.08 (0.95–18.64)	0.472
	Rectum	40	1.01 (0.60–2.62)	18	3.18 (2.20–4.63)	10	13.44 (1.07–23.92)	0.135
			P-value		0.898		0.868	
Pathology	Adenocarcinoma	66	1.91 (0.60–3.63)	32	2.50 (1.85–4.44)	22	12.45 (2.55–18.91)	0.513
	Mucinous	34	0.62 (0.17–1.85)	16	0.86 (0.20–3.56)	10	9.90 (0.63–23.92)	0.459
			P-value		0.201		0.550	
Grade	Low grade	82	1.85 (0.42–3.66)	34	2.06 (0.80–4.44)	24	12.58 (2.17–19.12)	0.444
	High grade	20	0.85 (0.39–2.70)	14	2.20 (0.20–3.56)	8	6.00 (0.45–12.45)	0.529
			P-value		0.682		0.176	
T	T2	15	2.70 (0.60–3.61)	12	4.66 (2.06–11.08)	9	13.72 (11.97–25.86)	0.276
	T3	65	1.76 (0.35–3.18)	30	1.96 (0.15–3.59)	18	12.15 (0.95–18.37)	0.627
	T4	19	0.85 (0.30–1.75)	6	0.80 (0.60–2.20)	5	8.33 (0.31–25.86)	0.819
			P-value		0.785		0.382	
N	Negative	41	1.74 (0.60–3.63)	26	2.06 (0.20–4.44)	22	12.57 (2.17–18.93)	0.348
	Positive	39	0.50 (0.13–2.49)	22	2.95 (0.92–4.00)	10	7.57 (0.54–18.91)	0.459
			P-value		0.343		0.482	
M	Negative	71	0.96 (0.30–2.73)	38	2.06 (0.20–3.56)	24	12.18 (0.65–25.46)	0.259
	Positive	31	2.49 (0.85–3.61)	10	3.26 (2.06–4.66)	8	13.06 (1.57–18.37)	0.565
			P-value		0.390		0.987	
Stage	II	32	0.96 (0.30–3.63)	20	1.85 (0.10–2.95)	13	12.25 (0.80–25.86)	0.062
	III	39	1.21 (0.24–2.34)	18	3.59 (0.92–4.63)	11	12.15 (0.95–19.12)	0.607
	IV	31	2.49 (0.85–3.61)	10	3.26 (2.06–4.66)	8	12.51 (1.53–18.37)	0.565
			P-value		0.484		0.946	

Data presented as median (interquartile range:IQR) of IL-6 gene expression in 102 CRC patients at baseline, 48 and 32 patients after treatment with FP therapy for 3 and 6 months, respectively.

IL: interleukin; CEA: carcinoembryonic antigen; CA19.9: carbohydrate antigen 19.9; NA: not applicable; T: tumor burden; N: lymph node; M: metastasis.

^aSignificant difference when gene level after 3 and 6 months of FP therapy was compared with its baseline level, P-value ≤0.05. Significant P-values marked **bold** and *italic*.

^bSignificant difference when gene level after 6 months of FP therapy was compared with its level after 3 months of FP therapy, P-value ≤0.05. Significant P-values marked **bold** and *italic*.

cases had good performance status (ECOG I). High initial CEA and CA19.9 levels were recorded in 41.7% and 20.8% of patients, respectively. It was

found that about 24.50% of the patients had their primary tumor located in the left side while the right side location was encountered in 29.41% and

Table 3. IL-1 β relative expression in subgroups of Egyptian CRC patients at baseline and after treatment with FP therapy for 3 and 6 months.

		IL-1 β fold change relative to healthy control ($2^{-\Delta\Delta CT}$) in CRC patients						P-value
		At baseline		After 3 months of FP therapy		After 6 months of FP therapy		
		N	Median (IQR)	N	Median (IQR)	N	Median (IQR)	
Age	≤45	47	0.42 (0.25–1.42)	23	1.87 (0.13–2.63)	15	19.77 ^{ab} (5.84–95.45)	0.010
	>45	55	3.00 (0.62–3.93)	25	1.22 (0.41–3.18)	17	13.19 (5.90–85.14)	0.135
	P-value		0.150		0.582		0.581	
Sex	Female	47	0.99 (0.35–3.50)	20	2.00 (0.77–3.40)	13	19.00 (9.00–59.39)	0.053
	Male	55	2.57 (0.56–17.86)	28	1.22 (0.28–2.57)	19	14.03 (4.20–88.76)	0.368
	P-value		0.299		0.272		0.272	
Smoking	Non-smoker	71	1.85 (0.36–3.93)	34	2.48 (0.60–2.94)	20	14.03 (5.94–83.29)	0.058
	Smoker	31	0.62 (0.42–3.41)	14	0.45 (0.28–1.22)	12	23.54 ^{ab} (5.00–100.00)	0.034
	P-value		0.394		0.070		0.661	
Baseline CEA	Normal	46	1.77 (0.36–3.67)	13	2.54 (0.92–3.95)	4	12.61 (5.03–95.39)	0.169
	High	33	3.10 (1.42–31.56)	5	1.52 (0.41–2.70)	8	17.76 (8.86–83.29)	0.135
	P-value		0.162		0.148		0.684	
Baseline CA19.9	Normal	57	0.81 (0.35–3.84)	12	1.21 (0.35–2.82)	11	20.53 (6.23–95.39)	0.264
	High	15	2.55 (0.25–6.58)	7	1.36 (0.16–2.57)	5	26.50 ^{ab} (14.03–70.00)	0.029
	P-value		0.361		0.589		0.812	
Site of tumor	Right colon	30	2.60 (0.42–3.93)	15	1.22 (0.28–3.01)	16	7.99 ^{ab} (2.00–41.00)	0.038
	Left colon	25	4.68 (2.28–13.21)	15	7.34 (4.72–19.56)	6	91.19 (19.00–99.00)	0.174
	Rectum	40	0.56 (0.19–1.99)	18	2.49 (0.46–2.63)	10	26.54 (10.39–89.70)	0.236
	P-value		0.825		0.441		0.436	
Pathology	Adenocarcinoma	66	1.85 (0.40–3.93)	32	1.69 (0.43–2.63)	22	17.76 (8.86–49.34)	0.052
	Mucinous	34	0.73 (0.36–2.89)	16	1.69 (0.28–3.01)	10	13.57 (4.20–87.00)	0.067
	P-value		0.242		0.289		0.607	
Grade	Low grade	82	2.28 (0.40–3.93)	34	2.00 (0.41–2.70)	24	15.96 ^{ab} (5.74–93.00)	0.011
	High grade	20	0.56 (0.36–3.41)	14	0.46 (0.15–9.92)	8	13.39 (6.23–42.81)	0.234
	P-value		0.545		0.190		0.054	
T	T2	15	3.06 (1.45–13.79)	12	2.49 (0.41–3.40)	9	14.29 (9.30–100.00)	0.156
	T3	65	1.21 (0.36–3.93)	30	1.22 (0.43–2.70)	18	13.90 ^{ab} (5.13–79.00)	0.022
	T4	19	0.42 (0.14–2.55)	6	1.45 (0.46–2.28)	5	39.50 (7.00–100.00)	0.549
	P-value		0.067		0.569		0.485	
N	Negative	41	0.99 (0.40–3.84)	26	1.87 (0.77–2.57)	22	17.63 (7.73–93.00)	0.065
	Positive	39	2.01 (0.39–3.67)	22	0.46 (0.15–3.01)	10	14.03 ^{ab} (4.20–88.76)	0.045
	P-value		0.322		0.311		0.889	
M	Negative	71	0.86 (0.36–3.62)	38	1.61 (0.35–2.60)	24	19.00 (5.74–39.00)	0.070
	Positive	31	2.89 (0.62–3.93)	10	1.69 (0.41–3.01)	8	12.64 (7.00–82.71)	0.102
	P-value		0.390		0.289		0.168	
Stage	II	32	0.99 (0.40–3.84)	20	1.85 (0.43–2.57)	13	19.00 (7.73–95.51)	0.155
	III	39	0.50 (0.25–3.93)	18	1.51 (0.30–3.68)	11	14.03 (4.20–70.00)	0.069
	IV	31	2.60 (0.42–3.93)	10	1.69 (0.35–2.85)	8	13.02 (7.00–79.00)	0.102
	P-value		0.474		0.348		0.309	

Data presented as median (interquartile range: IQR) of IL-1 β gene expression in 102 CRC patients at baseline, 48 and 32 patients after treatment with FP therapy for 3 and 6 months, respectively.

IL: interleukin; CEA: carcinoembryonic antigen; CA19.9: carbohydrate antigen 19.9; NA: not applicable; T: tumor burden; N: lymph node; M: metastasis.

^aSignificant difference when gene level after 3 and 6 months of FP therapy was compared with its baseline level, P -value ≤ 0.05 .

^bSignificant difference when gene level after 6 months of FP therapy was compared with its level after 3 months of FP therapy, P -value ≤ 0.05 .

rectal cancer represented 39.2% of all CRC patients. Adenocarcinoma (69.60%), grade II (80.39%), and T3 tumors (63.72%) were the most

common pathological subtypes. Thirty-one patients presented with metastatic disease where the liver was the most frequent site followed by the

peritoneum then the lung. Out of 102 patients, 97 patients received FP based therapy either as single agent of capecitabine (22 % of patients) or combined with oxaliplatin (78 % of patients).

IL-6 expression at baseline and after FP-therapy in CRC patients

Figure 1a showed the mRNA expression of IL-6 in baseline CRC patients was significantly higher than in healthy control (*medians* = 15.96 vs 2.47 folds, $P=0.0008$). The expression of IL-6 was insignificantly increased after 3 and 6 months of FP-therapy, Figure 1b. The increase of IL-6 level with FP-therapy was significant only in patients with right colon tumors (*medians* = 12.18 folds after 6 months of FP-therapy vs 1.74 folds at baseline, 2.06 folds after 3 months, $P=0.048$), as presented in Table 2.

IL-1 β expression at baseline and after FP-therapy in CRC patients

Figure 2a showed the mRNA expression of IL-1 β was insignificantly higher in baseline CRC patients than in healthy control (*medians* = 12.35 vs 5.8 folds, $P=0.92$). Significant induction in IL-1 β expression by 3.46 and 8.06 folds was revealed after treatment with FP-therapy for 3 and 6 months, respectively (Figure 2b). The increase of IL-1 β was significantly apparent after 6 months of therapy in patients ≤ 45 years, smokers, with high baseline level of CA19.9. Also FP therapy induced IL-1 β elevation in patients with right colon tumors, low grade, and T3 tumors and with positive lymph nodes (Table 3).

Survival analysis

The mean OS of all patients was 18.43 months, while the mean EFS of all patients was 28.33 months. There was no significant association between OS rate and baseline IL-6 expression level [25.17 months in patients with under-expression of IL-6 (IL-6 level ≤ 1.72) compared to 24.64 months in patients with over-expression of IL-6 (IL-6 level > 1.72), $P=0.673$, Figure 3a], and also for IL-1 β [26.55 months in patients with under-expression of IL-1 β (IL-1 β level ≤ 1.77) compared to 24.57 months in patients with over-expression of IL-1 β (IL-1 β level > 1.77), $P=0.164$, Figure 3b]. Similarly, there was no significant

association between EFS rate and baseline IL-6 expression level [19.97 months in patients with under-expression of IL-6 (IL-6 level ≤ 1.72) compared to 18.33 months in patients with over-expression of IL-6 (IL-6 level > 1.72), $P=0.465$, Figure 3c], and IL-1 β [22.23 months in patients with under-expression of IL-1 β (IL-1 β level ≤ 1.77) compared to 16.33 months in in patients with over-expression of IL-1 β (IL-1 β level > 1.77), $P=0.094$, Figure 3d].

Multivariate COX regression analysis revealed that poor tumor grade is independent prognostic factors for poor OS of CRC patients (HR=1.17, 95% CI=1.04–1.70, $P=0.014$), Table 4.

Discussion

Interleukins are the key regulators of inflammation-cancer transformation.²¹ In spite of their role in the induction of CRC growth and progression, they have also a well-established role in the maintenance of normal gut homeostasis.²² Because of their double sword nature, CRC treatment becomes difficult.²³

Before FP therapy, our CRC patients showed upregulation of both IL-6 and IL-1 β levels compared to healthy control. Tumor biopsies of patients with metastatic CRC have increased transcripts of IL-1 β which promote the recruitment of myeloid-derived suppressor cells to tumors and support disease progression.²⁴ IL-6 is a growth factor for human colon cancer cells. It protects cells from Fas-induced apoptosis through upregulation of bcl-x.²⁵ Tumor derived IL-6 was shown to enhance the self-seeding of solid tumors, a process whereby IL-6 promote tumor growth and enhance angiogenesis of circulating tumor cells.²⁶

Several experimental and clinical studies have linked the pleiotropic nature of IL-6 cytokine to the pathogenesis of sporadic and inflammation-associated CRC.²⁷ IL-6 directly promotes tumor cell proliferation and inhibits apoptosis. It activates transmembrane glycoprotein 130 subunit on the surface of tumor cells, switching on the subsequent intracellular signal of Janus kinases (JAKs), and signal transducer and activator of transcription 3 (STAT3).²⁸ In addition IL-1 β has an indirect effect in the progression of CRC. It promotes the growth of CRC tumors through the induction of expression of several proinflammatory mediators (TNF α , IL-6, IL8, IL-17, COX-2, and PGE2) in CRC tumor cells.^{29,30}

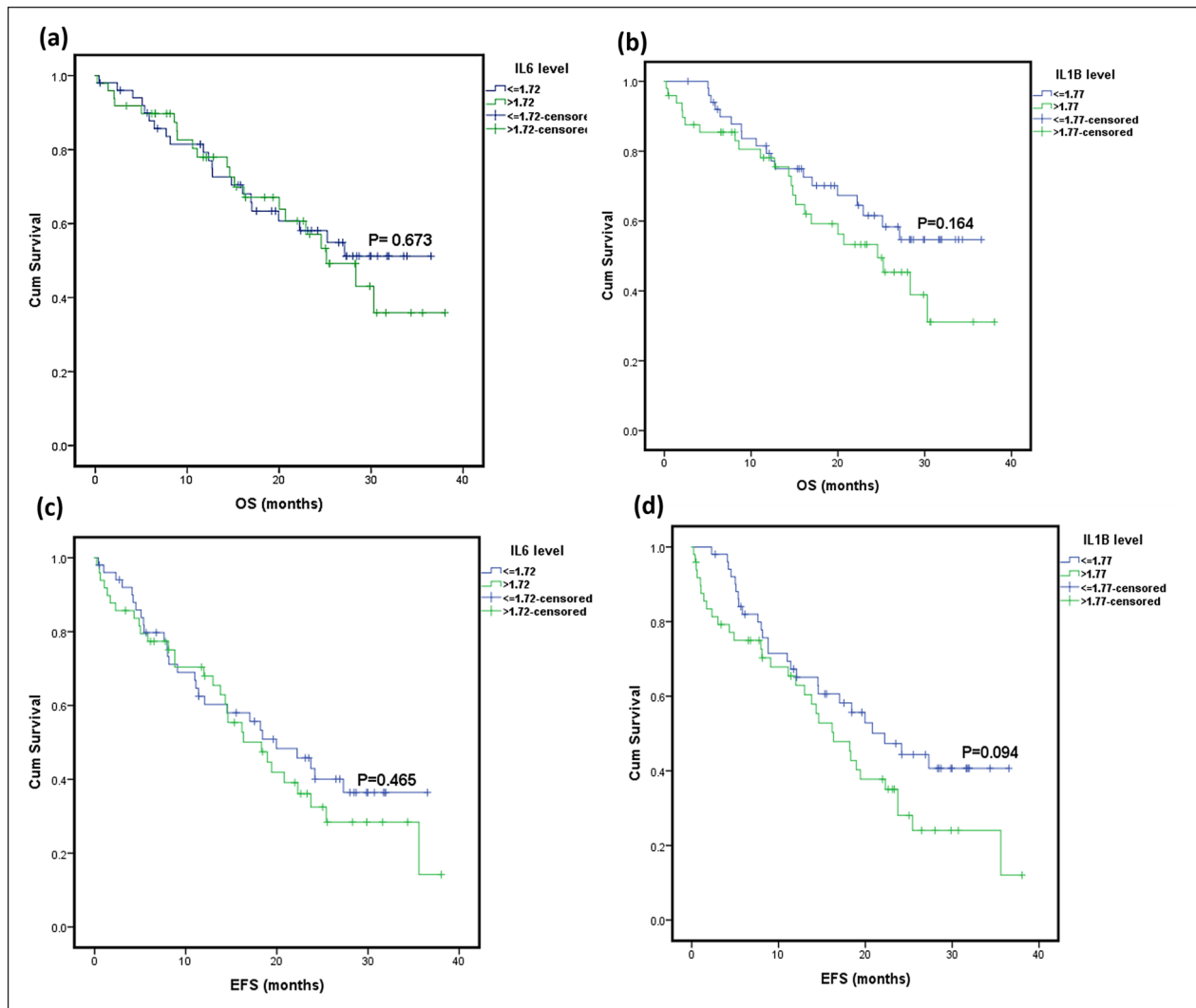


Figure 3. Kaplan-Meier analysis of OS for IL-6 (a) and IL-1 β (b), EFS for IL-6 (c), and IL-1 β (d). Baseline CRC patients were stratified into two groups of over-expression and under-expression around their median expression level of IL-6 and IL-1 β normalised to β -actin.

The results of this study showed the effect of FP based therapy in interleukins expression. Significant elevation in IL-1 β level with FP based therapy was exhibited particularly in patients' ≤ 45 years, smokers, with high baseline level of CA19.9, right colon tumors, low grade pathology, T3 tumors, and positive lymph nodes. Morgillo et al.³¹ correlated IL-1 β overexpression with the resistance to chemotherapy. It was found that IL-1 β activates Zinc Finger E-Box Binding Homeobox 1 (Zeb1), causing an increase in the self-renewal of colon stem cells and subsequent activation of the epithelial mesenchymal transition.³¹ Zaki et al.³² reported the activation of IL-1 β in response of cell stress or infection, in which leucine-rich repeat

inflammasome caused caspase-mediated cleavage and activation of IL-1 β . Also, IL-1 β has an essential role in the chronic proliferation required to repair the damage of the epithelial monolayer caused by constant inflammation.³³ It induces acute phase cytotoxic response through enhancing the expression of adhesion molecules, stimulating the proliferation of fibroblasts and activating multiple pro-coagulant factors.³³

Chemotherapy induces reactive oxygen species and proinflammatory cytokines such as IL-1 β , IL-6, and TNF- α leading to an increase in the mucosal damage.³⁴ It was claimed that 5-FU increased the myeloperoxidase activity in tissues and the proinflammatory cytokine secretion in the

Table 4. Multivariate COX regression for the hazard ratio (HR) of OS and EFS rates for subgroups of baseline CRC patients.

Subgroup of patients	HR of OS	95.0% CI for exp(B)		P-value
		Lower	Upper	
Age (>45 years vs ≤45 years)	0.64	0.25	1.68	0.370
Sex (male vs female)	1.02	0.33	3.18	0.967
Smokers vs non-smokers	1.43	0.44	4.67	0.553
PS III vs PS I&II	0.08	0.00	2.56	0.151
Initial high vs low CEA	1.61	0.41	6.30	0.492
Initial high vs low CA19.9	1.41	0.38	5.23	0.605
Rectum and left vs right tumor location	1.92	0.63	5.81	0.249
Mucinous and signet ring vs adenocarcinoma pathology	0.32	0.07	1.46	0.142
Grade III vs II	1.17	1.04	1.70	0.014
T3 & T4 vs T2	1.52	0.44	5.26	0.511
N (positive vs negative)	1.32	0.16	10.93	0.795
M (yes vs no)	2.47	0.31	19.64	0.393
Stages III & IV vs II	0.39	0.04	4.34	0.446
Over-expression vs under-expression of IL-6	1.30	0.32	5.36	0.717
Over-expression vs under-expression of IL-1β	2.38	0.86	6.54	0.094

Subgroup of patients	HR of EFS	95.0% CI for exp(B)		P-value
		Lower	Upper	
Age (>45 years vs ≤45 years)	1.00	0.41	2.43	0.994
Sex (male vs female)	1.14	0.46	2.81	0.781
Smokers vs non-smokers	1.60	0.61	4.17	0.335
PS III vs PS I & II	0.10	0.01	1.93	0.127
Initial high vs low CEA	0.88	0.29	2.64	0.817
Initial high vs low CA19.9	1.03	0.32	3.33	0.956
Rectum and left vs right tumor location	1.79	0.69	4.65	0.235
Mucinous and signet ring vs adenocarcinoma pathology	0.90	0.27	3.04	0.864
Grade III vs II	0.56	0.17	1.82	0.334
T3 & T4 vs T2	0.76	0.30	1.93	0.561
N (positive vs negative)	1.54	0.28	8.38	0.620
M (yes vs no)	1.32	0.25	7.06	0.743
Stages III & IV vs II	0.68	0.11	4.06	0.670
Over-expression vs under-expression of IL-6	0.98	0.32	2.95	0.967
Over-expression vs under-expression of IL-1β	1.87	0.16	3.82	0.065

sera of CRC patients leading to systemic inflammation.³⁵ The activation of systemic inflammatory response to tumors has been shown to be a risk factor for inferior survival and poor treatment outcome in CRC.³⁶

In this study, it was found an association between young age (≤45 years) and the induction of IL-1β expression by the treatment with FP therapy. Recently, it is reported that young CRC patients have distinct molecular and clinical features than elderly ones which control their response to 5-FU therapy.^{37–39} In Cheong et al.³⁷ study, CRC patients ≤50 years showed higher incidences of mucin production, high microsatellite instability, and N2 stage. Also, in the retrospective review of Willauer

et al.,³⁸ early-onset CRC patients were more likely to have synchronous metastatic disease, primary tumors in the distal colon or rectum, and fewer BRAF V600 mutations in comparison with patients 50 years old or older. Mauri et al.³⁹ described the characters of early-onset CRC by a more advanced stage at diagnosis, poorer cell differentiation, higher prevalence of signet ring cell histology, and left colon-sided location of the primary tumor.

Furthermore, it was noticed an increase of both IL-6 and IL-1β with FP therapy in patients with right sided tumors. The difference in immune landscape, genetic make-up, and response to therapy between right sided and left sided was well documented in Zhang et al.⁴⁰ and Baran et al.⁴¹ CRC

immune microenvironment in right side tumor was characterized by increased infiltration of immune cells with enhanced cytotoxic function, based on higher cytotoxic activity scores and interferon- γ signatures.⁴⁰ Molecularly, distal (right) tumors have a higher frequency of chromosomal instability, p53 mutations, and defects in mismatch repair-related genes as well as MLH1 silencing, but a lower frequency of microsatellite instability and CpG island methylator phenotype compared with proximal (left) tumors.⁴²

In spite of the increase of interleukins expression with FP therapy was associated with certain subgroups of CRC patients, however it was not associated with a significant prognostic value. Suggesting for future investigation for the use of naturally or synthetic immunomodulatory agents in combination with FP therapy in order to improve its effectiveness in CRC patients. Undeniably, the main drawback of the current study is the limitation of the method of analysis to genetic expression without post-translational confirmation of interleukins protein levels. In addition, the sample collected was heterogeneous (from patients with different tumor locations and at different stages of the disease); however subgroup analysis overwhelmed that limitation. Also, it is recommended to replicate that work with broader array of tumor microenvironment associated genes in order to determine their paracrine role in the treatment response of CRC patients to therapy. Furthermore, this study lack of information about point mutations in interleukins expression and its association with treatment outcome. So, future investigation, with gene sequencing technology, for inflammatory and immunoregulatory panels of genes will improve the already followed steps in CRC personalized therapy, and will enhance the treatment precision through careful selection of CRC patients who are eligible for fluoropyrimidine therapy after pharmacogenetic investigation.

Conclusion

Before FP therapy, CRC patients showed upregulation of both IL-6 and IL-1 β levels compared to healthy control. FP therapy induced the expression of IL-1 β particularly in patients' \leq 45 years, smokers, with high baseline level of CA19.9, right colon tumors, low grade pathology, T3 tumors, and positive lymph nodes. The expression of both IL-6 and

IL-1 β were increased with FP therapy in patients with right sided tumors. Both types of interleukins were not associated with significant effect on patients' survival, and poor (grade III) was the only independent predictor for the patients hazard of death during the OS time.

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Ethical approval

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Informed consent

Written informed consent was obtained from all subjects before the study.

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References

1. Sadeghi Gandomani H, Aghajani M, et al. (2017) Colorectal cancer in the world: incidence, mortality and risk factors. *Biomedical Research and Therapy* 4: 1–20.
2. Arvelo F, Sojo F and Cotte C (2015) Biology of colorectal cancer. *Ecancermedicalscience* 9: 520.
3. Zhong X, Chen B and Yang Z (2018) The role of tumor-associated macrophages in colorectal carcinoma progression. *Cellular Physiology and Biochemistry* 45: 356–365.

4. Klampfer L (2011) Cytokines, inflammation and colon cancer. *Current Cancer Drug Targets* 11: 451–464.
5. Mager LF, Wasmer MH, Rau TT, et al. (2016) Cytokine-induced modulation of colorectal cancer. *Frontiers in Oncology* 6: 96.
6. Waldner MJ, Foersch S and Neurath MF (2012) Interleukin-6—a key regulator of colorectal cancer development. *International Journal of Biological Sciences* 8: 1248–1253.
7. Fisher DT, Appenheimer MM and Evans SS (2014) The two faces of IL-6 in the tumor microenvironment. *Seminars in Immunology* 26: 38–47.
8. Nagasaki T, Hara M, Nakanishi H, et al. (2014) Interleukin-6 released by colon cancer-associated fibroblasts is critical for tumour angiogenesis: Anti-interleukin-6 receptor antibody suppressed angiogenesis and inhibited tumour–stroma interaction. *British Journal of Cancer* 110: 469–478.
9. Sun Q, Shang Y, Sun F, et al. (2020) Interleukin-6 promotes epithelial-mesenchymal transition and cell invasion through integrin $\beta 6$ upregulation in colorectal cancer. *Oxidative Medicine and Cellular Longevity* 2020: 13.
10. Chung YC and Chang YF (2003) Serum interleukin-6 levels reflect the disease status of colorectal cancer. *Journal of Surgical Oncology* 83(4): 222–226.
11. Zeng J, Tang ZH, Liu S, et al. (2017) Clinicopathological significance of overexpression of interleukin-6 in colorectal cancer. *World Journal of Gastroenterology* 23(10): 1780–1786.
12. Belluco C, Nitti D, Frantz M, et al. (2000) Interleukin-6 blood level is associated with circulating carcinoembryonic antigen and prognosis in patients with colorectal cancer. *Annals of Surgical Oncology* 7: 133–138.
13. Olsen J, Kirkeby LT, Olsen J, et al. (2015) High interleukin-6 mRNA expression is a predictor of relapse in colon cancer. *Anticancer Research* 35(4): 2235–2240.
14. Xu J, Ye Y, Zhang H, et al. (2016) Diagnostic and prognostic value of serum interleukin-6 in colorectal cancer. *Medicine (Baltimore)* 95(2): e2502.
15. Hai Ping P, Feng Bo T, Li L, et al. (2016) IL-1 β /NF- κ B signaling promotes colorectal cancer cell growth through miR-181a/PTEN axis. *Archives of Biochemistry and Biophysics* 604: 20–26.
16. He C, Yu T, Shi Y, et al. (2017) MicroRNA 301A promotes intestinal inflammation and colitis-associated cancer development by inhibiting BTG1. *Gastroenterology* 152: 1434–1448.
17. Sconocchia G, Eppenberger-Castori S, Zlobec I, et al. (2014) HLA class II antigen expression in colorectal carcinoma tumors as a favorable prognostic marker. *Neoplasia* 16: 31–42.
18. Wang Y, Wang K, Han GC, et al. (2014) Neutrophil infiltration favors colitis-associated tumorigenesis by activating the interleukin-1 (IL-1)/IL-6 axis. *Mucosal Immunology* 7: 1106–1115.
19. Fouad MA, Salem SE, Hussein MM, et al. (2018) Impact of global DNA methylation in treatment outcome of colorectal cancer patients. *Frontiers in Pharmacology* 9: 1173.
20. Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) method. *Methods* 25(4): 402–408.
21. Mantovani A, Dinarello CA, Molgora M, et al. (2019) Interleukin-1 and related cytokines in the regulation of inflammation and immunity. *Immunity* 50(4): 778–795.
22. Mager LF, Wasmer MH, Rau TT, et al. (2016) Cytokine-induced modulation of colorectal cancer. *Frontiers in Oncology* 6: 96.
23. Rizzo A, Pallone F, Monteleone G, et al. (2011) Intestinal inflammation and colorectal cancer: A double-edged sword? *World Journal of Gastroenterology* 17(26): 3092–3100.
24. Sieminska I and Baran J (2020) Myeloid-derived suppressor cells in colorectal cancer. *Frontiers in Immunology* 11: 1526.
25. Yuan H, Liddle FJ, Mahajan S, et al. (2004) IL-6-induced survival of colorectal carcinoma cells is inhibited by butyrate through down-regulation of the IL-6 receptor. *Carcinogenesis* 25(11): 2247–2255.
26. Ara T and Declerck YA (2010) Interleukin-6 in bone metastasis and cancer progression. *European Journal of Cancer* 46(7): 1223–1231.
27. Terzić J, Grivennikov S, Karin E, et al. (2010) Inflammation and colon cancer. *Gastroenterology* 138(6): 2101–2114.
28. Johnson DE, O’Keefe RA and Grandis JR (2018) Targeting the IL-6/JAK/STAT3 signalling axis in cancer. *Nature Reviews Clinical Oncology* 15(4): 234–248.
29. Voronov E and Apte RN (2015) IL-1 in colon inflammation, colon carcinogenesis and invasiveness of colon cancer. *Cancer Microenvironment* 8(3): 187–200.
30. Molina-Holgado E, Ortiz S, Molina-Holgado F, et al. (2000) Induction of COX-2 and PGE(2) biosynthesis by IL-1 β is mediated by PKC and mitogen-activated protein kinases in murine astrocytes. *British Journal of Pharmacology* 131(1): 152–159.
31. Morgillo F, Dallio M, Della Corte CM, et al. (2018) Carcinogenesis as a result of multiple inflammatory and oxidative hits: A comprehensive review from tumor microenvironment to gut microbiota. *Neoplasia* 20: 721–733.
32. Zaki MH, Lamkanfi M and Kanneganti TD (2011) Inflammasomes and Intestinal Tumorigenesis. *Drug Discovery Today. Diseases Mechanisms* 8: e71–e78.

33. Li Y, Wang L, Pappan L, et al. (2012) IL-1 β promotes stemness and invasiveness of colon cancer cells through Zeb1 activation. *Molecular Cancer* 11: 87.
34. Chang C-T, Ho T-Y, Lin H, et al. (2012) 5-Fluorouracil induced intestinal mucositis via nuclear factor-kb activation by transcriptomic analysis and in vivo bioluminescence imaging. *PLoS One* 7: e31808.
35. Melo ML, Brito GA, Soares RC, et al. (2008) Role of cytokines (TNF-a, IL-1b and KC) in the pathogenesis of CPT-11-induced intestinal mucositis in mice: effect of pentoxifylline and thalidomide. *Cancer Chemotherapy and Pharmacology* 61: 775–784.
36. Tuomisto AE, Mäkinen MJ and Väyrynen JP (2019) Systemic inflammation in colorectal cancer: Underlying factors, effects, and prognostic significance. *World Journal of Gastroenterology* 25(31): 4383–4404.
37. Cheong C, Oh SY, Kim YB, et al. (2019) Differences in biological behaviors between young and elderly patients with colorectal cancer. *PLoS One*. 14(6): e0218604.
38. Willauer AN, Liu Y, Pereira AAL, et al. (2019) Clinical and molecular characterization of early-onset colorectal cancer. *Cancer* 25(12): 2002–2010.
39. Mauri G, Sartore-Bianchi A, Russo AG, et al. (2019) Early-onset colorectal cancer in young individuals. *Molecular Oncology* 13(2): 109–131.
40. Zhang L, Zhao Y, Dai Y, et al. (2018) Immune landscape of colorectal cancer tumor microenvironment from different primary tumor location. *Frontiers in Immunology* 9: 1578.
41. Baran B, Mert Ozupek N, Yerli Tetik N, et al. (2018) Difference between left-sided and right-sided colorectal cancer: a focused review of literature. *Gastroenterology Research* 11: 264–273.
42. Cisyk AL, Penner-Goeke S, Lichtensztejn Z, et al. (2015) Characterizing the prevalence of chromosome instability in interval colorectal cancer. *Neoplasia* 17: 306–316.