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Interleukin-1β, interleukin-6 and interleukin-10 polymorphisms in Tunisian patients with colorectal cancer and liver metastasis

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Abstract: The balance between pro- and anti-inflammatory cytokine expression is essential for an efficient immune response and for the regulation of cancer development and progression. This study analyzed the expression and genetic variation in *IL-1β*, *IL-6* and *IL-10* genes and the possible associations with colorectal cancer (CRC) and colorectal liver metastases (CRLM). We examined *IL-1β*, *IL-6* and *IL-10* mRNA expression and three gene variants: *IL-1β* (rs1143627), *IL-10* (rs1800872) and *IL-6* (rs1800795), in 198 CRC, 65 CRLM patients and 230 controls. Carriers of the C/T genotype of *IL-1β* (rs1143627) have an increased risk of developing CRC and CRLM. T/T genotype carriers have a higher risk of CRLM incidence. For *IL-10* (rs1800872), patients harboring the C/A genotype have a lower risk of CRC and CRLM occurrence. For *IL-6* (rs1800795), the C/C genotype heightens the risk of CRLM development. Overall survival analysis showed that carriers of the C/T genotype of *IL-1β* (rs1143627) have a worse overall survival in CRC patients. It can be concluded that interleukin genetic variants can be used as biomarkers to detect and predict clinical outcomes and prognostic factors for CRC and CRLM.

Keywords: colorectal cancer; liver metastasis; gene polymorphism; interleukins

INTRODUCTION

Colorectal cancer (CRC) is currently a major health problem. With nearly 1.9 million new cases diagnosed each year, it is the 3rd most frequent malignant tumor in the world in terms of incidence, and the 2nd in terms of mortality, with an estimated 916,000 cancer-related deaths [1]. CRC prevalence in Tunisia has increased in recent years from 12.4/100,000 in 2009 compared to 6.4/100,000 in 1994, with a projected incidence rate of 39.3/100,000 in 2024 [2,3]. Due to its anatomical location, the liver is the most common metastatic site in patients with CRC, and ultimately about 70% of patients develop liver metastasis [4]. Given the severity of such disease, its progression rate and morbidity require therapeutic emergency. Therefore, identifying prognostic biomarkers for CRC and CRLM has been the subject of many diverse investigations aiming at establishing reliable and effective screening tests [5].

Cytokines are small molecules secreted by immune, stromal and/or tumor cells that coordinate and modulate immune responses [6,7]. Their expression levels may represent interesting biomarkers for the early detection of CRC and CRLM, the monitoring of pathogenesis and/or predicting the response to

treatment [8,9]. The identification of such biomarkers will allow for the development of tests that can help clinicians identify patients at high risk of developing CRC and CRLM at an early stage, thus establishing better, more personalized treatments.

Interleukins are a group of cytokines that play an important role in cancer cell growth, differentiation, survival and migration [10]. It is widely accepted that immune dysfunction, aberrant expression of interleukins or an imbalance between pro- and anti-inflammatory interleukin actions strongly correlates with CRC development and progression [6,7,11]. Interleukin-1β (IL-1β), interleukin-6 (IL-6) and interleukin-10 (IL-10) are involved in promoting tumor progression, invasion and metastasis [7]. The pro inflammatory IL-1β, which is mainly produced by activated macrophages, induces the expression of another pro inflammatory cytokine, IL-6, by stimulating monocytes, fibroblasts, endothelial cells, macrophages, T cells and B lymphocytes. The anti-inflammatory IL-10 produced by T helper 2 cells (Th2), B cells, tumor cells and macrophages is a potent inhibitor of pro inflammatory cytokines [6,11-13]. Therefore, the balance between pro- and anti-inflammatory interleukins is critical for the efficacy of the immune response and protection against underlying colorectal tissue damage. Several investigators have reported an increase in the expression of interleukins in CRC and CRLM tissues [13-19].

Genetic divergence within our genome can alter neoplasm onset and development risk, treatment, and outcome. These polymorphisms reshape interleukin gene sequences and consequently influence their role where they can have a paradoxical impact either by repressing carcinogenesis when implicated in immune response activation or by promoting tumor growth when associated with chronic inflammation [5].

Numerous studies have reported many single nucleotide polymorphisms (SNPs) mainly within the interleukin gene promoter regions. We chose to evaluate the most representative SNP impacting transcription and protein expression for each gene [20-22]. Several studies have investigated these SNPs, *IL-1β* (-31C>T), *IL-6* (-174G>C) and *IL-10* (-592C>A), to understand their significance as influencers of CRC or CRLM risk; however, the results obtained are inconclusive and/or controversial [20,23,24]. Although serum levels of interleukins have been widely studied, their mRNA expression has rarely been investigated and most genetic studies of CRC targeted alterations acquired from primary tumors. Nonetheless, the genetic profiles of primary and metastatic tumors were examined [25]. In the current study we investigated the expression of *IL-1β*, *IL-6* and *IL-10* in tumor tissues and three polymorphisms: *IL-1β* -31C>T, *IL-6* -174G>C and *IL-10* -592C>A, to evaluate them as diagnostic and/or prognostic biomarkers for CRC and CRLM.

MATERIALS AND METHODS

Patients and tumor samples

The study protocol was approved by the National Ethics Board (IRB: ISA/2016/03.1). The data collected included sex, age, tumor location, histological type, differentiation and tumor node metastasis (TNM stage) (stages I, II, III, and IV). TNM staging was based on the American Joint Committee on Cancer (AJCC) Staging Manual, 8th edition [26]. This retrospective study was performed on 263 Tunisian patients (198 with primitive CRC and 65 who developed liver metastases). The control group consisted of 230 healthy blood donors who had neither gastrointestinal disease nor a history of tumor disease.

RNA extraction and reverse transcriptase polymerase chain reaction

RNA extraction and RT-PCR were performed as described [27]. The PCR program was as follows: 94°C for 30 s; (annealing temperature of each primer for 30 s; and 72°C for 30 s. The procedure was repeated for 30 cycles. The primer sequences were as follows: for *IL-1β* (R: TTGCTGTAGTGGTGGTCGGAGAT, F: GCTGATGGCCCTAAACAGATGA), *IL-6* (R: GCCTCTTTGCTGCTTTCACA, F: CAGCCACTCACCTCTTCAGAAC), *IL-10* (R: TAGAGTCGCCACCCTGATGTC, F: TGATGCCCCAAGCTGAGAACC) and the housekeeping gene *GAPDH* (R: GACTGTGGTCATGAGTCCT, F: GGGTGTGAACCATGAGAAGT).

DNA extraction

Genomic DNA was extracted from frozen and/or paraffin-embedded tissue blocks using the PureLink Genomic DNA mini kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For the 230 healthy blood donors, genomic DNA was extracted from peripheral blood using the Wizard Genomic Purification Kit (Promega, Madison, WI, USA). DNA sample concentrations were assessed using the Qubit dsDNA HS (high sensitivity) Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) on a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Genotyping of *IL-6* (-174G>C) polymorphism by PCR-SSP and *IL-1β* (-31C>T) and *IL-10* (-592C>A) by PCR-RFLP

The mixture for polymerase chain reaction (PCR) contained 100 ng DNA, 10×PCR buffer (Promega, Madison, WI, USA), 10 μM dNTPs (PureLink, Invitrogen, CA, USA), 50 μM $MgCl_2$ (Promega, Madison, WI, USA), 1 μM of each primer (Invitrogen, Carlsbad, CA, USA) and 1 U Taq DNA polymerase (Biomatik, Wilmington, DE, USA) and H_2O in the final reaction volume of 25 μL. The PCR program was as follows: 94°C for 5 min, 30 cycles of 94°C for 30 s, the annealing temperature of each primer was 72°C for 1 min, with a final extension step at 72°C for 10 min. Then, PCR products were checked on a 3% agarose gel stained with ethidium bromide under UV light. For PCR-RFLP, 10 μL of each PCR product was digested with 1 μL of the appropriate restriction enzyme (New England Biolabs, New England, USA), 5 μL CutSmart Buffer (New England Biolabs, New England, USA), and H_2O in a final digestion reaction volume of 25 μL. The incubation time for the digestion reaction was 20 min at 37°C, followed by inactivation at 80°C for 20 min, according to the manufacturer's instructions. *IL-1β*, *IL-6*, and *IL-10* promoter regions were amplified with specific primers; for rs1800795 (*IL-6* (-174G>C)) polymorphism, a region covering 234 bp was amplified using the following primers F: GAGCTTCTCTTTCGTTCC, R1: CCTAGTTGTGTCTTGCC and R2: CCCTAGTTGTGTCTTGCG. The human growth hormone (HGH) was used as positive control F: GCCTTCCCAACCATTCCCTTA, R: TCACGGATTTCTGTTGTGTTTC). For rs1143627 (*IL-1β* (-31C>T)) polymorphism, a region covering 234 bp was amplified using the following primers R: AGCACCTAGTTGTAAGGAAG; F: AGAAGCTTCCACCAATACTC. The change of C to T creates an AluI digestion site, yielding products of 137 and 97 bp. The major homozygous genotype C/C yields only a 234 bp product. The heterozygous genotype C/T shows three bands 234, 137 and 97 bp, and the minor T/T genotype is represented by 137 and 97 bp bands. For rs1800872 (*IL-10* (-592C>A)) polymorphism, a region covering 412 bp was amplified using the following primers R: CCTAGGTCACAGTGACGTGGAGCACCTAGTTGTAAGGAAG, F: GGTGAGCACTACCTGACTAGC. The change of C to A creates an RsaI digestion site, yielding products of 236 and 176 bp. The major homozygous genotype C/C yields only a 412 bp product. The heterozygous genotype C/A has three bands, 412, 236 and 176 bp, and the minor A/A genotype is identified by the presence of 236 and 176 bp bands. PCR-SSP and PCR-RFLP products were analyzed using the On-Chip Electrophoresis system (Agilent Technologies, Santa Clara, CA, USA). Chips were loaded with samples and the appropriate well was filled with the gel-dye mix as recommended by the manufacturer.

Statistical analysis

Statistical analysis was conducted using SPSS software V20 (SPSS, Inc, Chicago, IL, USA). Associations between interleukin gene status, mRNA expression and different clinicopathological variables were assessed using the chi-square ($\chi 2$) test. The odds ratio (OR) test was used to evaluate the risk. When the P-value was less than 0.05, the test was considered statistically significant.

RESULTS

Patients and tumor features

Clinic pathological criteria are presented in Table 1. The gender ratio for CRC patients was 1.2, with 108 males and 90 females with a mean age of 61.6±11.4 years ranging from 29 to 92 years. As for CRLM patients, a predominance of males was noted with a sex

Table 1: Clinic pathological criteria of CRC and CRLM patients.

Clinic pathological criteria	CRC Patients (n=198)	(%)	CRLM Patients (n=65)	(%)
Gender				
Male	108	54.5	42	64.6
Female	90	45.5	23	35.4
Age				
≥60 years	118	59.6	22	33.8
<60 years	80	40.4	43	66.2
Tumor location				
Right colon	47	76.2	27	41.5
Left colon	151	23.8	38	58.5
Histological type				
NMC	119	60.1	48	73.9
MA	51	25.8	10	15.4
MC	28	14.1	7	10.8
Differentiation				
Well	160	80.8	24	36.9
Moderate	30	15.1	35	53.8
Poor	8	4.1	6	9.3
Tumor invasion depth				
pT1+pT2	30	15.1		
pT3+pT4	168	84.9		
Lymph node invasion				
N0	104	52.5		
N1	59	29.8		
N2	33	16.7		
N3	2	1		
TNM stage				
I	20	10.1		
II	80	40.4		
III	89	45		
IV	9	4,5		

CRC–colorectal cancer; CRLM –colorectal cancer liver metastasis;n–number of subjects; NMC–non-mucinous carcinoma; MA–mucinous adenocarcinoma; MC–mucinous component;pT –primary tumor invasion depth.

ratio of 1.82 (42 males and 23 females), and mean age of 55.6±10.2 years with extremes of 26 and 80 years. For healthy controls, a predominance of females was noted with a sex ratio of 0.79 (102 males and 128 females), with a mean age of 46.75±12.74 years with extremes of 17 and 87 years. The healthy controls were matched by sex and age to the group of CRC patients as well as to the group of CRLM patients.

As regards tumor location, 47 tumors originated in the right colon and 151 in the left colon, including sigmoid and rectal cancers in CRC cases, and 27 had a primary right colon cancer and 38 a primary left colon cancer in CRLM samples. Our study included 119 (60.1%) non-mucinous carcinomas (NMC), 51 (25.8%) mucinous adenocarcinomas (MA) and 28 (14.1%) adenocarcinomas with a mucinous component (MC) (<50%). As for the CRLM, 48 (73.9%) were NMC, 10 (15.4%) MA and 7 (10.8%) MC. Tumor grading was performed according to WHO criteria [28]. Accordingly, CRC and CRLM cases were divided into 160 (80.8%) and 24 (36.9%) well-differentiated, 30 (15.1%) and 35 (53.8%) moderately differentiated, and 8 (4.1%) and 6 (9.3%) poorly differentiated cases, respectively.

Concerning the pathological classification of the tumors, cases were assessed according to the international TNM staging system [26]. When a tumor invades the sub muscular and muscular, the invasion's depth leads to a disease upstaged as pT1+pT2 (in 30 or 15.1% of subjects), and when the tumor invades the sub-serosa and/or other organs, the depth of the invasion leads to a disease upstaged to pT3+pT4 (in 168 or 84.9% of subjects). According to TNM staging, 20 (10.1%) of primary CRC cases were stage I, 80 (40.4%) were stage II, 89 (45%) were stage III, and 9 (4.5%) were stage IV; among them, 94 subjects had lymph node invasion [59 N1 (29.8%) + 33 N2 (16.7%) + 2 N3 (1%)] and 104 (52.5%) did not (N0).

Distribution of *IL-1β* (-31C>T), *IL-10* (-592C>A) and *IL-6* (-174G>C) polymorphism frequencies in CRC, CRLM patients and healthy controls

Fig.1A, B and C show the different genotyping profiles obtained for *IL-1β* (-31C>T), *IL-10* (-592C>A) and *IL-6* (-174G>C) variants. *IL-1β* (-31C>T) genotyping was performed on 198 CRC patients; it showed that 64 (32.3%) of individuals were homozygous for the C/C major genotype and 134 (67.7%) were heterozygous C/T. None of CRC patients carried the T/T minor homozygous genotype. As for CRLM, 6 (9.2%) patients had the major C/C genotype, 39 (60%) had a heterozygous C/T genotype and 20 (30.8%) had a homozygous minor genotype T/T. For healthy controls, 106 (46 %) were C/C, 118 (51.3 %) C/T and 6 (2.7 %) T/T. Comparison of the *IL-1β* (-31C>T) genotype frequencies between CRC cases and healthy controls showed that carriers of the C/T genotype were at a 1.881-fold higher risk (P=0.002, 95% CI=1.265-2.797) of developing CRC. The same outcome was noted for CRLM (P=0.000, OR= 5.839, 95% CI=2.377-14.342).

Table 2: Distribution of polymorphism frequencies in CRC, CRLM patients and healthy controls.

SNPs		Controls n (%)	CRC Patients n (%)	P	OR[1] [CI 95%]	CRLM Patients n (%)	P	OR[2] [CI 95%]
rs1143627 *IL-1β* (-31 C>T)	C/C	106 (46.1)	64 (32.3)	-	-	6 (9.2)	-	-
	C/T	118 (51.3)	134 (67.7)	**0.002**	1.881 [1.265-2.797]	39 (60)	**0.000**	5.839 [2.377-14.342]
	T/T	6 (2.6)	-	-	-	20 (30.8)	**0.000**	58.889 [17.242-201.137]
	C/T+T/T	124 (53.9)	134 (67.7)	**0.004**	1.790 [1.206-2.656]	59 (90.8)	**0.000**	8.406 [3.490-20.245]
	C	330 (71.7)	262 (66.2)	-	-	51 (39.2)	-	-
	T	130 (28.3)	134 (33.8)	0.078	1.298 [0.971-1.736]	79 (60.8)	**0.000**	3.932 [2.619-5.904]
rs1800872 *IL-10* (-592C>A)	C/C	93 (40.4)	141 (71.2)	-	-	50 (77)	-	-
	C/A	123 (53.5)	45 (22.7)	**0.000**	0.241 [0.157-0.371]	15 (23)	**0.000**	0.227 [0.120-0.429]
	A/A	14 (6.1)	12 (6.1)	0.166	0.565 [0.250-1.276]	-	-	-
	C/A+A/A	137 (59.6)	57 (28.8)	**0.000**	0.274 [0.183-0.411]	13 (20)	**0.000**	0.204 [0.108-0.384]
	C	309 (67.2)	327 (82.6)	-	-	115 (88.5)	-	-
	A	151 (32.8)	69 (17.4)	**0.000**	0.431 [0.312-0.597]	15 (11.5)	**0.000**	0.266 [0.150-0.473]
rs1800795 *IL6* (-174 G>C)	G/G	164 (71.3)	135 (68.2)	-	-	38 (58.5)	-	-
	G/C	64 (27.8)	63 (31.8)	0.399	1.196 [0.789-1.812]	16 (24.6)	0.819	1.079 [0.562-2.070]
	C/C	2 (0.9)	-	-	-	11 (16.9)	**0.000**	23.737 [5.051-111.546]
	G/C+C/C	66 (28.7)	63 (31.8)	0.483	1,160 [0.767-1.754]	27 (41.5)	0.049	1.766 [0.998-3.122]
	G	392 (85.2)	333 (84.1)	-	-	92 (70.7)	-	-
	C	68 (14.8)	63 (15.9)	0.648	1.090 [0.751-1.582]	38 (29.3)	**0.000**	2.380 [1.508-3.759]

CRC–colorectal cancer; CRLM –colorectal cancer liver metastasis; n–number of subjects; P–Pearson's chi square test (P<0.05). Major alleles are indicated in bold for each SNP. OR[1]–odds ratio CRC patients versus controls; OR[2]–odds ratio CRLM patients versus controls; CI–confidence interval.

Moreover, patients harboring the homozygous minor T/T genotype were at a very high risk of developing CRLM (P=0.000, OR=58.889, CI=17.242-201.137) (Table 2).

As regards *IL-10* (-592C>A) genotyping, 141 (71.2%) cases carried the major C/C genotype, 45 (67.7 %) the heterozygous C/A genotype and 12 (6.1%) the homozygous A/A minor genotype for CRLM; 50 (77%) patients had the C/C major genotype and 15 (23%) the heterozygous C/A genotype versus 93 (40.4 %) C/C, 123 (53.4 %) C/A and 14 (6.2 %) A/A of healthy controls. Analysis of genotype frequencies between CRC cases and healthy controls highlighted that the C/A genotype was negatively associated with both CRC and CRLM occurrence (P=0.000, OR= 0.241, 95% CI=0.157-0.371 and P=0.000, OR= 0.227, 95% CI=0.120-0.429), respectively (Table 2).

Genotyping of *IL-6* (-174G>C) revealed that 135 CRC cases (68.2%) were homozygous for the major G/G genotype, 63 (31.8 %) were heterozygous G/C

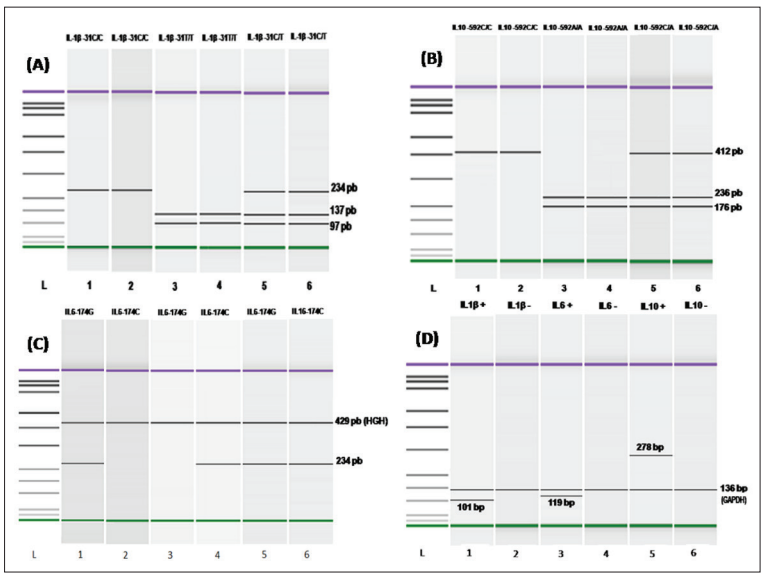

Fig. 1.A– Amplification of rs1143627 by PCR-RFLP: wells 1 and 2 correspond to carriers of the C/C genotype; wells 3 and 4 correspond to carriers of the T/T genotype, wells 5 and 6 correspond to carriers of the C/T genotype. **B**– Amplification of rs1800872 by PCR-RFLP: wells 1 and 2 correspond to carriers of the C/C genotype; wells 3 and 4 correspond to carriers of the A/A genotype, wells 5 and 6 correspond to carriers of the C/A genotype. **C** – Amplification of rs1800795by PCR-SSP: wells 1 and 2 correspond to carriers of the G/G genotype, wells 3 and 4 correspond to carriers of the C/C genotype, wells 5 and 6 correspond to carriers of the G/C genotype. The 429 bp band represents the internal control gene human growth hormone (HGH). **D** – Amplification of interleukin gene by RT-PCR: wells 1, 3 and 5 correspond to positive mRNA expression of *IL-1β, IL-6* and *IL-10*, respectively, wells 2,4 and 6 correspond to negative mRNA expression of *IL-1β, IL-6* and *IL-10*, respectively. The 136 bp band represents the housekeeping gene GAPDH.L: DNA ladder in the range of 25 to 1000 bp.

and none carried the C/C minor homozygous genotype. Meanwhile, analysis of CRLM cases revealed that 38 (58.5%) of patients had the major G/G genotype, 16 (24.6%) had the heterozygous G/C genotype and 11 (16.9%) had the homozygous minor C/C genotype. As for healthy controls, 164 (71.3 %) were G/G, 64 (27.8 %) G/C and 2 (0.9 %) C/C. No statistically significant association was found between genotype frequencies of CRC cases and healthy controls. However, carriers of the C/C minor genotype had an increased risk of CRLM occurrence (P=0.000, OR=23.737 95% CI=5.051-111.546) (Table 2).

Association of *IL-1β*, *IL-10* and *IL-6* mRNA expression with clinic pathological criteria

The profile of interleukin transcript expression is shown in Fig.1D, and the results are summarized in Table 3 for CRC patients and in Table 4 for CRLM patients. For CRC cases, 132 (66.7%) expressed *IL-1β* transcripts (+), while the remaining 66 (33.3%) did not (-). As regards the 65 CRLM cases, mRNA (+) was detected in 51 cases (78.5%) and absent (mRNA (-)) in 14 (21.5%). *IL-1β* mRNA expression was correlated with tumor invasion depth (pT3+pT4; P=0.012) for CRC patients and with age for those under 60 years (P=0.038) for CRLM patients.

IL-6 mRNA expression analysis showed that 129 (65.2%) of CRC cases and 50 (76.9%) of CRLM cases expressed this cytokine; the remaining 69 (34.8%) and 15 (23.1%) did not. This expression was significantly associated with the advanced tumor stages III+IV (0.033) for CRC patients. No significant association was found for CRLM patients.

IL-10 mRNA expression analysis for CRC revealed that 116 (58.6%) cases were mRNA (+) versus 41 (63.1%) that were mRNA (-). As regards the 65 CRLM cases, 41 (63.1%) were mRNA (+) and 24 (36.9%) were mRNA (-). Association analysis did not reveal any significant correlation between *IL-10* mRNA expression and the clinic pathological criteria for CRC and CRLM.

Comparison of mRNA expression for *IL-1β*, *IL-10* and *IL-6* between cases stratified according to their genotypes for each variant did not reveal any significant association (Table 5).

Overall survival statistical analysis

Among 198 CRC patients, 23 were lost at follow-up, and hence were excluded from the survival curve analysis. The average overall survival rate (OSR) of the 175 remaining CRC patients was 26.3±8.6 months (from 1 to 167 months), whereas all 65 CRLM patients were followed-up with an OSR average of 17.3±9 months (from 1 to 72 months). The OSR was better for CRC cases with the *IL-1β* (**-31C**>T) C/C major genotype (P=0.033) than individuals carrying the heterozygous

Table 3. Association between *IL-1β, IL6* and *IL-10* mRNA expression and clinic-pathological criteria in CRC patients.

Clinic pathological criteria	*IL-1β*			*IL6*			*IL-10*		
	mRNA (+) (n=132)	mRNA (-) (n=66)	P	mRNA (+) (n=129)	mRNA (-) (n=69)	P	mRNA (+) (n=116)	mRNA (-) (n=82)	P
Gender			0.069			0.430			0.429
Male (n=108)	66	42		73	35		66	42	
Female (n=90)	66	24		56	34		50	40	
Age			0.413			0.120			0.531
≥60 years (n=118)	76	42		82	36		67	51	
<60 years (n=80)	56	24		47	33		49	31	
Tumor location			0.125			0.570			0.240
Right colon (n=47)	27	20		29	18		31	16	
Left colon (n=151)	105	46		100	51		85	66	
Histological type			0.918			0.291			0.934
NMC (n=119)	79	40		81	38		70	49	
MA + MC (n=79)	53	26		48	31		46	33	
Differentiation			0.799			0.638			0.407
Well (n=160)	106	54		103	57		96	64	
Moderate + Poor (n=38)	26	12		26	12		20	18	
Tumor invasion depth			**0.012**			0.064			0.864
pT1+pT2 (n=30)	14	16		24	6		18	12	
pT3+pT4 (n=168)	118	50		105	63		98	70	
Lymph node invasion			0.421			0.085			0.757
Absence (n=104)	72	32		62	42		62	42	
Presence (n=59)	34	60		67	10		54	40	
TNM stage			0.315			**0.033**			0.905
I +II (n=100)	70	30		58	42		59	41	
III+IV (n=98)	62	36		71	27		57	41	

n–number of subjects; P– Pearson's chi square test (P<0.05); NMC–non-mucinouscarcinoma; MA–mucinousadenocarcinoma; MC–mucinous component; (+)–positive mRNA expression; (-)–negativemRNA expression

Table 4: Association between IL-1β, IL6 and IL-10 mRNA expression and clinic pathological criteria in CRLM patients.

Clinic pathological criteria	*IL-1β*			*IL6*			*IL-10*		
	mRNA (+) (n=51)	mRNA (-) (n=14)	P	mRNA (+) (n=50)	mRNA (-) (n=15)	P	mRNA (+) (n=41)	mRNA (-) (n=24)	P
Gender			0.062			0.421			0.418
Male (n=42)	30	12		31	11		28	14	
Female (n=23)	21	2		19	4		13	10	
Age			**0.038**			0.232			0.090
≥60 years (n=22)	14	8		15	7		17	5	
<60 years (n=43)	37	6		35	8		24	19	
Tumor location			0.618			0.890			0.304
Right colon (n=27)	22	5		21	6		19	8	
Left colon (n=38)	29	9		29	9		22	16	
Histological type			0.650			0.471			0.314
NMC (n=48)	37	11		38	10		32	16	
MA + MC (n=17)	14	3		12	5		9	8	
Differentiation			0.252			0.778			0.941
Well (n=24)	17	7		18	6		15	9	
Moderate + Poor (n=41)	34	7		32	9		26	15	

N– Number of subjects; P– Pearson's chi square test (P<0.05); NMC–non-mucinous carcinoma; MA–mucinous adenocarcinoma; MC–mucinous component; (+)–positive mRNA expression; (-)–negative mRNA expression.

Table 5: Association of *IL-1β*, *IL6* and *IL-10* mRNA expression in CRC and CRLM patients according to the genotypes of analyzed variants.

SNPs	Genotypes	CRC patients (n=198)	*IL-1β* mRNA (+) (n=132)	*IL-1β* mRNA (-) (n=66)	P	CRLM patients (n=65)	*IL-1β* mRNA (+) (n=51)	*IL-1β* mRNA (-) (n=14)	P
***IL- 1β*rs1143627**	C/C	64	42	22	0.830	6	4	2	0.461
	C/T	134	90	44		59	47	12	
			***IL-6* mRNA (+) (n=129)**	***IL-6* mRNA (-) (n=69)**			***IL-6* mRNA (+) (n=50)**	***IL-6* mRNA (-) (n=15)**	
***IL-6* rs1800795**	G/G	135	85	50	0.344	38	27	11	0.183
	G/C	63	44	19		27	23	4	
			***IL-10* mRNA (+) (n=116)**	***IL-10* mRNA (-) (n=82)**			***IL-10* mRNA (+) (n=41)**	***IL-10* mRNA (-) (n=24)**	
***IL-10* rs1800872**	C/C	141	81	60	0.609	50	31	19	0.743
	C/A+A/A	57	35	22		15	10	5	

N–number of subjects; P– Pearson's chi square test (P<0.05); (+)–positive mRNA expression; (-)–negative mRNA expression.

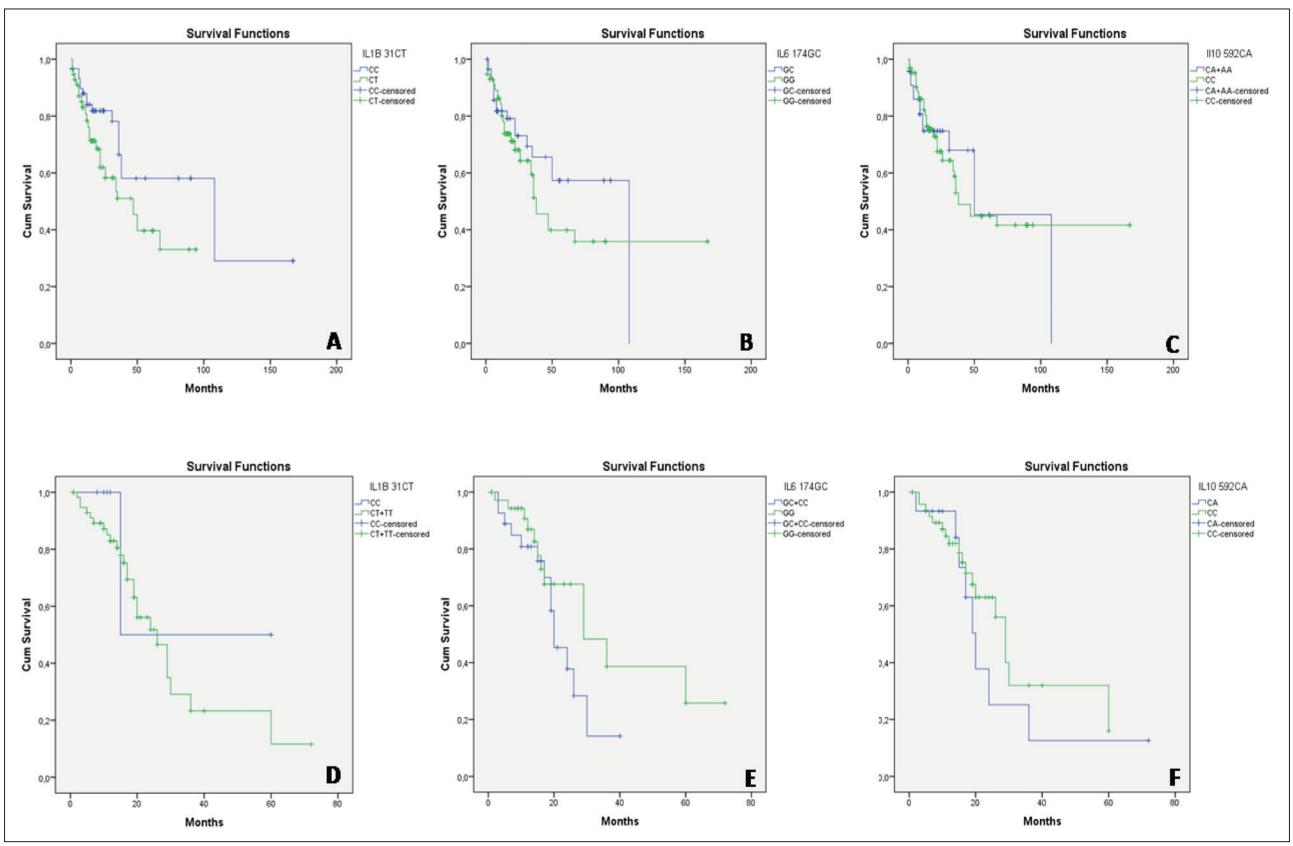

Fig. 2. A, **B**, **C** – Overall survival based on Kaplan-Meier curves for CRC patients according to each polymorphism genotype. **D**, **E**, **F**– Overall survival based on Kaplan-Meier curves for CRLM patients according to each polymorphism genotype.

C/T genotype (Fig.2A). For *IL-6* (**-174G**>C) and *IL-10* (**-592C**>A) polymorphisms, the Kaplan Meir test did not show any significant association for CRC patients (Fig.2B, C). Similarly, no significant association was found for CRLM cases according to the different variants (Fig.2D, E and F).

DISCUSSION

Several studies have highlighted the immune system's pivotal role in the development and progression of CRC. It is generally accepted that deregulation of the balance between anti-inflammatory and pro inflammatory cytokines promotes the development and progression of CRC and CRLM [7]. In this context, the pro- and anti-inflammatory interleukins IL-1β, IL-6 and IL-10 are the most involved in the development of these neoplasms and are activated by various signaling pathways during CRC progression, supporting their crucial role in pathogenesis [7,12,13]. In this regard, several studies have reported the association of different interleukin gene polymorphisms, particularly those involved in modulating cytokine expression, such as *IL-1β* (-31C>T), *IL-6* (-174G>C) and *IL-10* (-592C>A) variants with CRC risk [24,29–32]. However, to date there have been only a few studies focusing on CRLM. The current study targeted these polymorphisms (*IL-1β* -31C>T, *IL-6* -174G>C and *IL-10* -592C>A) and *IL-1β*, *IL-6* and *IL-10* expression in tumor tissue to evaluate them as diagnostic and/or prognostic biomarkers for CRC and CRLM.

Comparison of the *IL-1β* (-31C>T) genotype frequencies between CRC cases and healthy controls showed that carriers of the C/T genotype were at much higher risk of developing CRC. Data in the literature are few and contradictory and a recent multi-population cohort meta-analysis found a significant association with the dominant model (C/C + C/T vs T/T) and in the heterozygous model (C/T vs T/T) with a reduced risk of developing CRC [32], while research conducted in northeastern Scotland showed that C/C genotype carriers are at higher risk of developing CRC than other genotype carriers [33]. Other Indian and Chinese reports did not identify any significant associations at all [34-36]. Moreover, IL-1β -31C/T variants were linked to various neoplasms, and the T/T genotype was reported to be associated with an increased risk of hepatocellular [5], gastric [23] and lung cancers [21]. As for the C/T genotype, cancer occurrence risks were correlated with prostate cancer [5]. Similarly, the C/C genotype was reported to be involved in breast cancer [37,38]. In Tunisia, the distribution of these genotypes and allelic frequencies did not highlight any relationship with acute heart failure [39].

For *IL-10* (-592 C>A), comparison of genotype frequencies between CRC cases and healthy controls showed that carriers of the C/A genotype were negatively associated with CRC occurrence. This was consistent with the meta-analysis conducted in a Chinese population where it was stated that this variant was associated with a significantly reduced CRC risk [31]; similar findings were reported in an ethnic Kashmiri population where the carriers of the minor allele A were associated with a reduced CRC risk [40]. Conversely, two meta-analyses found no correlation [24,41]. In Tunisia, the A/A genotype and A allele were associated with a higher risk of head and neck and nasopharyngeal cancers but not laryngeal cancer [42]. As for the C/C genotype, it seems to be protective against the development and progression of hepatocellular carcinoma [43].

For *IL-6* -174G>C, we found no statistically significant correlation between genotype frequencies of CRC cases and healthy controls, with similar results reported in multi-population studies [29,30]. However, *IL-6* -174G>C polymorphism was found to be associated with either an increased risk of CRC occurrence in different cohorts [44-46] or a decreased risk in an ethnic Kashmiri population [47]. This polymorphism was associated with a high risk of breast, stomach and bladder cancers [5]. In Tunisia, the -174G>C promoter variant was not associated with either coronary heart [48] or Behçet's diseases [49].

In CRLM patients, comparison of the *IL-1β* (-31C>T) genotype frequencies showed that carriers of the C/T genotype had a significantly higher risk of developing CRLM. In addition, patients carrying the homozygous minor T/T genotype had a very high risk of developing CRLM. Regarding the *IL-10* -592 C>A genotype, comparison of frequencies between CRLM cases and healthy controls showed that carriers of the C/A genotype were negatively associated with CRLM occurrence. For *IL-6* -174G>C, the carriers of the C/C minor genotype were strongly associated with an increased risk of CRLM occurrence.

To the best of our knowledge, this study is the first to analyze the association between CRLM and these interleukins gene variants. These variants can be used to identify patients' susceptibility to develop CRLM; however, results should be verified with a larger number of patients and studies from other different ethnicities.

Although the serum level of interleukins has been well studied, their expression in cancer tissues has rarely been elucidated. Therefore, we investigated the tissue expression of *IL-1β*, *IL-6* and *IL-10* in CRC and CRLM to determine potential prognostic significance. The RT-PCR results showed that *IL-1β*, *IL-6* and *IL-10* mRNA levels were expressed in most tissues samples for CRC and CRLM. Their mRNA expression was higher in CRLM than in CRC patients. This result is consistent with several works in which interleukin levels were elevated in advanced stages and metastatic tumors [50-55]. While many studies showed that mRNA expression was increased in CRC and CRLM tissues, other studies showed that it was co-expressed in both tumor tissue and in normal colonic mucosa [50-52,56]. The correlation between the clinic pathological criteria of CRC and CRLM cases and the RT-PCR results showed that mRNA expression of *IL-1β* correlated with tumor invasion depth pT3+pT4. For CRLM, we found a significant correlation between its mRNA expression and patients who were under 60 years. In the literature, several reports showed that the IL-1β serum level was associated with lymph-vascular invasion, young patients [12] and advanced TNM staging [53]. As for *IL-6* mRNA expression, a significant association with advanced stages in CRC patients was noted. *IL-6* showed a statistically significant association with advanced tumor stage, tumor invasion depth pT3+pT4, tumor size, liver metastasis, lymph node metastasis and tumor histological differentiation [20,50,51,57,58]. As for *IL-10* mRNA expression, no significant association was found with clinic pathological criteria for either CRC or for CRLM patients in the present study, with similar results obtained by Abtahi et al. [59]; however, a recent study revealed that *IL-10* expression was associated with tumor metastasis and TNM stage [54].

Considering the rarity of analyses evaluating interleukin mRNA expression in liver metastatic tissue, we did not find any data in the literature. Overall survival analysis showed that carriers of the C/T genotype for the *IL-1β* rs1143627 polymorphism had a worse overall survival than the major genotype C/C in CRC patients. This is the first study showing that this polymorphism can be used to predict survival outcome for CRC patients, but further investigation should be carried out on a larger sample size.

CONCLUSION

This study shows that interleukin gene variants might be useful as biomarkers for detecting patients at high or low risk of developing CRC and CRLM. Moreover, for the first time, these SNPs were evaluated in CRLM tissues where it was observed that *IL-1β* (-31C>T) and *IL-6* (-174G>C) increased CRLM occurrence, and *IL-10* (-592C>A) lowered the risks. This is the first report of the association of the common SNP rs1143627 in the *IL-1β* gene that can serve to predict the overall survival in CRC patients. More studies should be carried out with large sample sizes to further investigate these associations.

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Data availability: Data underlying the reported findings have been provided as part of the submitted article and are available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Hazgui%20et%20al_7826_Data%20Report.pdf

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