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ORIGINAL ARTICLE

Association of *IL-12Bpro* polymorphism with tumor-infiltrating dendritic cells in colorectal cancer

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Summary

Purpose: *Chronic inflammation is a key component in the development and progression of colorectal cancer (CRC). A notable hallmark of the inflammation process is the release of pro-inflammatory cytokines by infiltrating cells of the immune system. Defects in dendritic cells (DCs) recruitment, maturation and cytokine release are a hallmark of the CRC strategy to escape immune surveillance.The purpose of our study was to evaluate the possible role of IL-12B polymorphism in the promoter region of the IL-12B gene (rs17860508) as a genetic factor contributing to the risk for CRC development. Additionally, we aimed to evaluate the influence of this polymorphism on DCs infiltration in tumor microenvironment.*

Methods: *IL-12Bpro polymorphism was genotyped by Amplification Refractory Mutation System- Polymerase Chain Reaction (ARMS-PCR). Immunohistochemistry was performed for DCs infiltration.*

Results: *Statistically significant correlation between the expression of S100 and CD1a DCs and the 11- genotype of the studied polymorphism was found. No statistically significant difference in genotype distribution between cases and controls was observed (p=0.163). Analysis of the overall survival (OS) of genotyped patients revealed a tendency among the carriers of the 22-genotype to have shorter survival of 36 months versus the 11- and 12-cariers- 61 months (log rank, p=0.117).*

Conclusions: *The IL-12Bpro polymorphism does not constitute a risk factor for CRC development. However, genotype-11 might have a complex role in the recruitment and maturation of DCs in tumor microenvironment.*

Key words: *colorectal cancer, dendritic cells, gene polymorphism*

Introduction

CRC is one of the most common malignancies associated with high rates of morbidity and mortality worldwide. Its etiology is complex and involves both genetic and environmental interactions. A well estimated contributor to CRC development and progression is chronic inflammation [1-3]. The cellular component of the inflammatory microenvironment in the tumor is represented by the host immune cells including DCs- the most potent antigen-presenting cells (APCs) which are differentially distributed in the tumor and might alter the tumor-host interactions. The inflammatory process is driven by the release of pro-inflammatory cytokines by tumor-infiltrating DCs among which interleukin (IL)-12 is considered most effective in the defense against carcinogenesis. The antitumor activity of this cytokine arises from its ability to present a linkage between innate and adaptive immunity through stimulation of interferon (IFN)-γ secretion and promoting Th1- and cell-mediated immunity [4]. Defects in DCs recruitment, maturation and cytokine release are a hallmark of the CRC strategy to escape immune surveillance, probably as a result of the DCs' switch from immunogenity to tolerogenicity. Moreover, tumor-associated DCs show loss of

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APC function, inefficient motility and decreased production of IL-12 [5].

Human IL-12 (IL-12p70) is a 70kDa heterodimeric protein composed of two subunits (p40 and p35) each encoded by separate genes (*IL12B* and *IL-12A*, respectively). Several polymorphisms have been described in the *IL-12B* gene including a complex polymorphism in the promoter region located 2.7 kb upstream of the transcription initiation site, resulting from a 4bp microinsertion combined with an AA/GC transition (rs17860508). This polymorphism is commonly referred to as *IL12B* promoter (*IL12Bpro*) polymorphism and more recently it has been reported to influence IL-12p70 secretion [6-10], but not IL-12p40 production [9]. The p40 chain of IL-12 is predominantly expressed by DC and activated macrophages [11], but the assembly with the p35 is subject to a complex regulation at the genetic level with evidence for posttranscriptional mechanisms to regulate the secretion of p70 [12,13].

In light of these findings, we have designed a case-control study for a group from the Bulgarian population to investigate the relationship between *IL-12Bpro* polymorphism and the presence and maturation status of tumor-infiltrating DCs in different tumor compartments.

Methods

Study subjects

A total of 134 Bulgarian patients were studied. Cases with new diagnosis of CRC attending the University Hospital in Stara Zagora were selected. The histopathological examination confirmed the diagnosis. The subjects' group consisted of 75 male (56%) and 59 (44%) female patients. The mean age at diagnosis of the total group of CRC patients was 67±9 years. The mean age at diagnosis of male versus female among the cases was 67±9 years versus 66 ±8 years (p=0.579, *t*-test). The available demographic and clinicopathological data of the patients at the time of diagnosis are presented in Table 1.

Ninety-eight healthy individuals with no previous cancer diagnosis were selected from the same cohort as cases. The group consisted of 48 males and 50 females with mean age of the total group 58±15 years.

DNA extraction and genotyping of IL12B pro polymorphism

Genomic DNA was extracted from fresh frozen tissues of subjects' tumor biopsies using a genomic DNA purification kit (NucleoSpin Tissue, Macherey-Nagel, Duren, Germany) and DNA from the controls was isolated from blood samples (NucleoSpin Blood L, Macherey-Nagel, Duren, Germany) according to manufacturer's protocol.

Extracted DNA was stored at -20°C until further use. The concentration of resulting DNA was measured spectrophotometrically at 260 nm by NanoVue TM Spectrophotometer (Healthcare, Buckinghamshire, UK). The ratio of absorptions at 260 nm vs 280 nm was calculated to assess the purity of DNA samples.

Genotyping for the *IL12Bpro* (rs17860508) polymorphism was performed by ARMS-PCR. The sequences of used primers were as follows: CTCTAA allele, marked as *IL12Bpro-1*: 5'-TGTCTCCGAGAGAGGCTCTAA-3'; GC-allele, marked as *IL12Bpro-2*: 5'- TGTCTCCGAGAGAGGGCTGT-3'; and *IL12Bpro* generic primer 5'-TGGAGGAAGTGGTTCTCGTAC-3'. Amplification was carried out in a total volume of 10 ⌈l containing 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 mM of each primer and 1U/rcn Taq Polymerase. The reagents for PCR

Table 1. Demographic and clinical data for CRC patients' group

Parameters	*n (%)*
Demographic data	
Gender	n=134 (100)
Male	75 (56)
Female	59 (44)
Age (years)	n=128
Median (range)	68 (35-88)
Clinical data	
Location of primary tumor	n=101 (100)
Colon	39 (39)
Sigmoid	12 (12)
Rectum	50 (49)
Local tumor invasion	n=100 (100)
T 1-2	24 (24)
T 3-4	76 (76
Lymph node metastases	n=105 (100)
Yes	36 (24)
No	69 (66)
Distant metastases	n=100 (100)
Yes	24 (24)
No	76 (76)
CRC stage	n=100 (100)
Stage I + II	57 (57)
Stage III + IV	43 (43)
Survivors at the end of follow-up	n=97 (100)
Alive	46 (47)
Deceased	51 (53)
Survival after the operation	n=97
median (months) (range)	14.69 (0.62-126.4)
Histological data	
Histological type	n=73 (100)
Type I	11 (15)
Type II	15 (20)
Type III	35 (48)
Type IV	12 (17)
Grade of differentiation	n=95 (100)
Low	16 (17)
Moderate	76 (80)
High	3 (3)
Inflammatory infiltrate	n=79 (100)
No (-)	8 (10)
Weak (+)	33 (42)
Moderate (++)	23 (29)
Strong (+++)	15 (19)

mixture were supplied by Thermo Fisher Scientific (Waltham, MA, USA). The thermal protocol for amplification included an initial denaturation step of 15 min at 95°C; 30 cycles of 30 s at 95°C; 30 s at 65°C; 30 s at 72°C; and a final extension step of 7 min at 72°C.

In this case of ARMS-PCR the two alleles were detected not only by the presence/abscence of amplification product, but also the amplicons differed in size as allele-1 generates a 196 bp product and allele-2 a 192 bp product (Figure 1).

Amplification was performed on QCycler System (QuantaBiotech Ltd, UK). The PCR products were visualized on a 2.5% agarose gel stained with ethidium bromide (0.5 mg/ml). In each PCR run, heterozygous control template as well as negative template control were used to ensure accuracy.

Immunohistochemistry

For immunohistochemical staining, the paraffin blocks were prepared using tumor tissues from the periphery of the tumor adjacent to the normal tissues.

Specimens were fixed in 10% buffered formalin, embedded in paraffin and then cut to 4 μm thickness sections. Next step was dewaxing and endogenous peroxidase was blocked for 5 min with blocking reagent according to the protocol. Then the slides were washed 3 times with phosphate buffered saline (PBS) and incubated with primary antibody for 1 hr. After being washed 3 times the slides were incubated with marked polymer and then washed again. In the last step they were incubated with DAB substrate-chromogen and washed again. Finally, they were counter stained with Mayer's hematoxylin.

Immunohistochemical reactions were carried out using: Monoclonal Mouse Anti-Human CD1a, CD68, S100 (DAKO, Denmark), CD11c and CD123 (Leica Biosystems) all in dilution 1:100, as well as detection system EnVision™ FLEX+, Mouse, High pH (Link) (DAKO, Denmark). For negative control, the primary antibody was replaced with PBS. Cell counting was performed in the tumor stroma and at the tumor border on 5 fields of vision in the areas with most intensive cell recruitment (hot spots) at a magnification (x 320, 0.74 mm^2 area). In the surrounding normal tissue the same cells were counted at 5 fields of vision, chosen at random, at a magnification (x 320, 0.74 mm^2 area). The number of the positive cells was calculated and presented as cells/mm^2.

Statistics

Statistical analysis was carried out using SPSS software, version 16 (IBM,Chicago,IL). The descriptive statistical tests, including mean, standard deviation and median, were calculated according to standard methods. Kolmogorov–Smirnov's test and Shapiro-Wilks' W-test were used for analyzing the normality of the distribution. Continuous variables were compared between the independent groups by Student's t-test, ANOVA test, Mann-Whitney U test and Kruskal-Wallis test, depending on the variables' distribution. Allelic and genotype frequencies were compared between groups by x^2 test or Fisher exact test (as appropriate). Logistic regression analysis was performed and the odds ratios (OR) with corresponding 95% confidence intervals (CI) were evaluated. Hardy-Weinberg equilibrium was tested among controls and patients using x^2 test.

Cumulative survival curves were drawn by the Kaplan-Meier method and the difference between the curves was analyzed by the Mantel-Cox (log-rank) test. Factors with $p<0.05$ were considered statistically significant.

Results

Correlation of genotypes with dendritic cells density

Immunohistochemistry was performed with antibodies against CD1a, CD11c, CD68, CD123 and S100 protein in samples of CRC patients, also investigated for the *IL-12Bpro* polymorphism. Summarized correlations between the number of infiltrating DCs and genotypes are presented in Table 2.

Our results indicate that the *IL12Bpro* genotypes did not correlate with the number of CD11c and CD123 in the tumor stroma or in the tumor border ($p>0.05$, ANOVA). We found a statistically significant association between the infiltration of

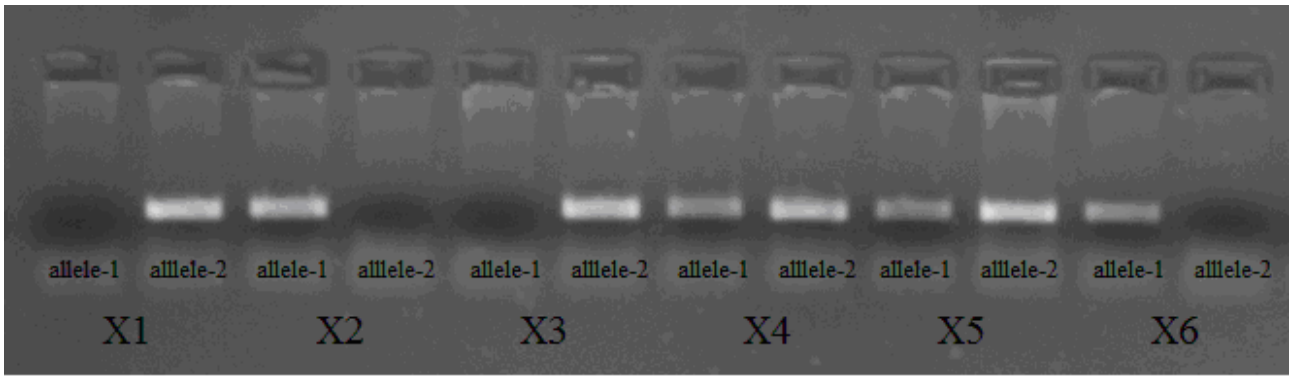

Figure 1. Agarose gel electrophoresis of the *IL-12Bpro* polymorphism illustrating the 11-genotype (X2 and X6), the 12-genotype (X4 and X5) and the 22-genotype (X1 and X3).

S100 (PAM marker for DCs) expressing DC in the tumor stroma and the studied polymorphism. It appeared that the carriers of the 11-genotype had significantly higher density of infiltrating S100 DCs (p=0.011, ANOVA; Figure 2) The expression of CD1a (a marker for immature DCs) analysis revealed significantly lower density of CD1a+ DCs in the invasive margin of the tumor among the carriers of at least one 2-allele (12- + 22-genotypes) (p=0.051, ANOVA) (Figure 3).

Distribution of IL-12Bpro polymorphism

Genotype and allele frequencies of *IL-12Bpro* polymorphism in healthy donors and CRC patients are presented in Table 3. The genotype distribution was in agreement with Hardy-Weinberg equilibrium (x^2=5.082, df=2, p=0.075).

In patients with CRC the 11-genotype was identified in 30.6% of the cases, the heterozygous 12-genotype in 40.3% and the 22-genotype in 29.1%. In the control group the 11-genotype was identified in 26.3%, the 12-genotype in 52.5% and the 22-genotype in 21.2% (Figure 4).

No statistically significant difference in genotype distribution between cases and controls was observed (p=0.163), neither was such difference observed in the allele frequencies distribution (p=0.482). Analysis of the OS of genotyped patients revealed no statistical significance (log rank, p=0.272), though there was a tendency among the carriers of the 22-genotype to have-shorter survival of 36 months versus the 11- and 12-cariers- 61 months (log rank, p=0.117). With respect to the 3-year overall survival rate of the patients we found a tendency in 2-allele (12- and 22-genotype) carriers for shorter survival rate (x^2, p=0.089). When analysis was performed on the combined group of 11-genotype and 22-genotype carriers a statistically significant value emerged (x^2, p=0.024). (Figure 5).

Furthermore, we investigated the *IL-12Bpro* polymorphism with regard to clinical data. The results showed no correlation between the studied polymorphism and clinical and some histopathological characteristics, such as tumor invasion level, presence of lymph node metastases, distant metastases, pTNM stage and grade of differentiation of primary tumors.

Table 2. Correlations between the number of infiltrating DCs and genotypes

	IL-12Bpro polymorphism			
Dendritic cells (mean±SE)	*Genotype-11*	*Genotype-12*	*Genotype-22*	*p value*
CD1a stroma	3.96 (±2.00)	3.32 (±0.56)	2.19 (±0.70)	0.612
CD1a border	3.51 (±1.23)	8.45 (±1.52)	5.45 (±7.27)	0.056
CD11c stroma	53.55 (±9.40)	53.51 (±5.26)	62.24 (±18.87)	0.835
CD11c border	97.60 (±15.02)	140.02 (±16.67)	106.80 (±16.33)	0.148
CD123 stroma	6.39 (±0.93)	8.3 (±1.64)	7.08 (±1.61)	0.65
CD123 border	12.90 (±1.88)	13.16 (±2.52)	10.75 (±1.99)	0.724
s100 stroma	17.98 (±3.00)	10.23 (±1.57)	9.10 (±1.68)	0.011
s100 border	26.10 (±3.93)	19.23 (±4.00)	15.16 (±1.93)	0.144

SE: standard error

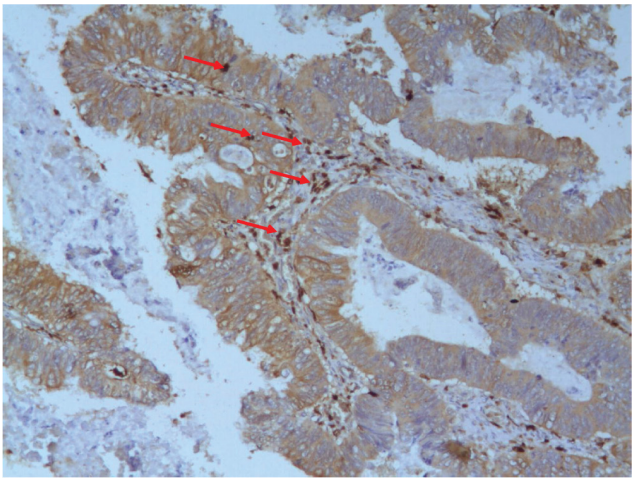

Figure 2. Immunohistochemical expression of S100 positive dendritic cells (arrows) (x200).

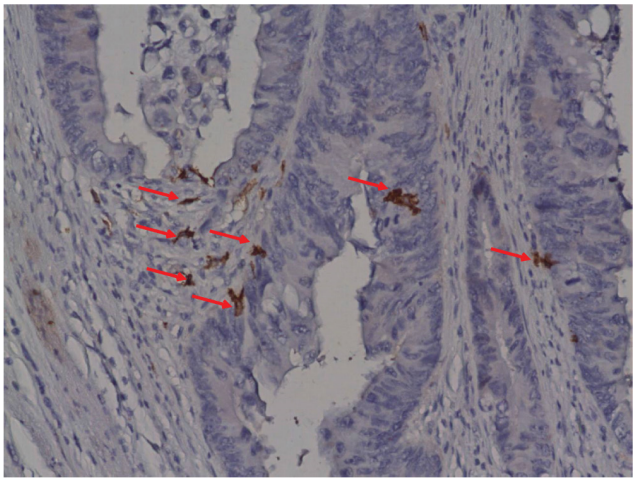

Figure 3. Immmunohistochemical expression of CD1a positive dendritic cells (arrows) (x200).

Table 3. Genotype and allelic frequencies of *IL-12Bpro* polymorphism among CRC patients and controls

IL-12Bpro polymorphism	*CRC patients*		*Controls*		*OR (95% CI)*
	n	*Frequency*	*n*	*Frequency*	
	n=134		*n=98*		
Genotype frequency					
11	41	0.31	26	0.27	1.0 (referent)
12	54	0.40	52	0.53	0.659 (0.337-1.284) p=0.211
22	39	0.29	21	0.20	1.178 (0.537-2.588) p=0.715
Allelic frequency					
Allele-1	136	0.50	104	0.53	1.0 (referent)
Allele-2	132	0.50	94	0.47	1.074 (0.731-1.578) p=0.709

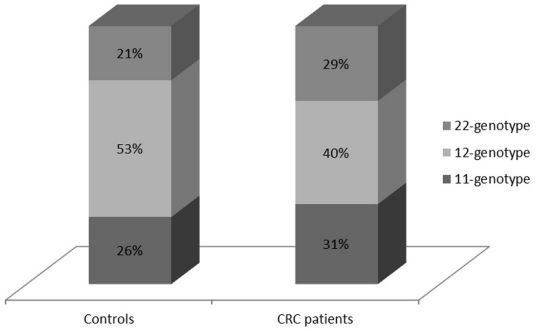

Figure 4. Differences between genotypes distribution among CRC cases and controls.

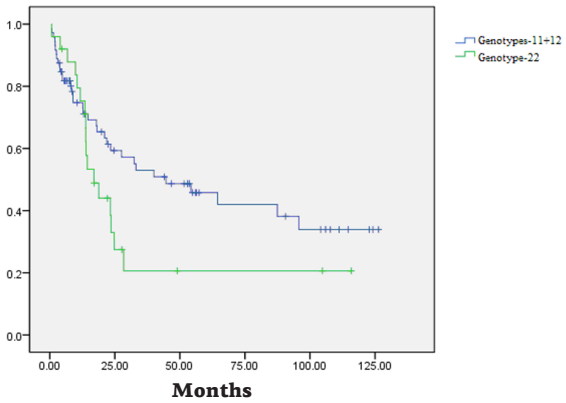

Figure 5. Kaplan-Meier 3-year cumulative survival (p=0.024).

Discussion

The molecular pathogenesis of CRC is associated with a wide array of factors among which is the inflammation. It involves a well-coordinated cooperation of the components of the innate and adaptive immune system [10]. The cellular component of the innate immunity (e.g., macrophages, mast cells, dendritic cells and natural killer cells) initiates the inflammatory response by releasing cytokines. Thereby genetic variations in inflammation-related genes, especially cytokines, might well contribute to the susceptibility to CRC [14]. Additionally, we have reported that impairment of DCs function in the tumor microenvironment is associated with tumor stage and metastatic ability of CRC [15].

Given its pluripotent role in the immune function, *IL-12B* gene is an obvious candidate gene for investigation in CRC. In accordance with this, we have conducted a case-control study to investigate the possible role of a genetic variation in the promoter region of the *IL-12B* gene in CRC susceptibility. We have also hypothesized that this polymorphism might correlate with the tumor-infiltrating DCs' density.

Our results demonstrated that the studied polymorphism does not constitute a genetic risk factor for the development and progression of CRC. No differences in genotype and allelic frequencies of the *IL-12B* polymorphism between patients with CRC and controls were found, either when patients were analyzed as a whole group or when they were studied according to clinical and histopathological characteristics. Our findings are similar to the results reported by Miteva et al. in 2009 [16]. The authors have studied the *IL-12Bpro* polymorphism and a single nucleotide variation in 3'-untranslated region of the same gene and they have concluded that both studied polymorphisms can be excluded as risk factors for CRC. These data are in agreenment with the results reported by Navaglia et al., regarding polymorphisms in the *IL-12B* gene and intestinal metaplasia [17].

However, these findings do not exclude a key role for IL-12 in the development and progression of CRC. In the present study, we demonstrated a positive association between the increased num-

ber of S100 DCs in the tumor stroma and the carriers of the low-producing *IL-12B* genotype-11. Genotype-11 was also positively correlated with lower infiltration of immature DCs ($CD1a^+$) in the invasive margin of the tumor. Some recent evidence has suggested a possible autocrine regulation of DCs function by IL-12 [18]. Dadabayev et al. have reported that the correlation between the degree of tumor infiltration by DCs and the disease prognosis depends on the level of maturation of these cells [19]. Previous studies have also demonstrated lower density of mature DCs in both human colon cancers and rat colon models [20].

In conclusion, on the basis of our data we may suggest that the *IL-12Bpro* polymorphism does not constitute a risk factor for CRC development. However, our study indicates that this genetic variation might not disturb the recruitment of DCs in tumor microenvironment, but genotype-11 may play a role in DCs maturation.

Conflict of interests

The authors declare no confict of interests.

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