

Influence of interleukin-8 and interleukin-10 on sporadic colon cancer development and progression

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Cytokines produced in the tumour microenvironment have an important role in cancer pathogenesis. Altered cytokine expression may result in increased susceptibility to and/or poor prognosis in certain cancers. Therefore, the aim of this study was to investigate the influence of interleukin (IL)-8 and IL-10 on sporadic colon cancer development and progression. In our study, a statistically significant increase in *IL-8* messenger RNA (mRNA) expression and decrease in *IL-10* mRNA expression in tumour tissue compared with normal mucous tissue was observed ($P = 0.003$; $P = 1.3 \times 10^{-9}$). No association was found between *IL-8* –251 A/T genotypes and *IL-8* mRNA expression in tumour and corresponding normal mucous tissue, as well as susceptibility to sporadic colon cancer. Positive immunohistochemical *IL-8* staining was more frequent in moderately and poorly differentiated tumours compared with well-differentiated tumours ($P = 0.024$). Finally, *IL-8* significantly stimulated invasion of HT-29 cells *in vitro* ($P = 0.000172$). Significant association of *IL-10* –1082 A/G, –819 T/C and –592 A/C genotypes and *IL-10* mRNA expression in tumour tissue was observed ($P = 0.022$; $P = 0.013$; $P = 0.02$). Significant association of –819 T/C and –592 A/C genotypes and *IL-10* mRNA expression in corresponding normal mucous tissue was observed ($P = 0.01$; $P = 0.04$) as well. *IL-10* single-nucleotide polymorphism (SNP) promoter genotypes associated with low *IL-10* mRNA expression (–819 TT; –592 AA) were also associated with increased risk of sporadic colon cancer compared with high-expression genotypes [odds ratio, 5.53; 95% confidence interval (CI), 1.53–20.1; odds ratio, 4.07; 95% CI, 1.28–12.96]. Positive *IL-10* immunohistochemical reaction was more frequent in well-differentiated and moderately differentiated tumours compared with poorly differentiated tumours ($P = 0.036$). In Dukes' C tumours, positive *IL-10* immunohistochemical reaction was less frequent compared with Dukes' A and B tumours ($P = 0.023$). Taken together, our results point to possible tumour promoting role of *IL-8* and potential protective role of *IL-10* in sporadic colon cancer.

Introduction

Colorectal cancer is among the most common cancers and one of the leading causes of death in the Western world (1). During the last two decades, many of the 'key-player' genes in colorectal tumorigenesis have been identified (2). As cancer is a complex genetic disease, it is probable that besides few highly penetrant genes a number of low-penetrant genes contribute to cancer susceptibility in a larger population of patients (3).

Tumour microenvironment is composed of many cell types that participate in tumour progression through interactions of numerous

signalling molecules that they produce. A group of molecules that has received much attention due to its role in tumorigenesis are cytokines. They act as a part of a highly complex and coordinated networks in which they modulate their own synthesis as well as that of other cytokines and their receptors (4).

Interleukin (IL)-8 is produced by a wide variety of normal as well as tumour cells and its principal role is in the initiation and amplification of acute inflammatory reactions. IL-8 has also been implicated in chronic inflammatory processes and diseases with a chronic inflammatory component such as cancer (5).

IL-10 is an immunoregulatory cytokine and its main biological function is limitation and termination of inflammatory responses. IL-10 also regulates differentiation and proliferation of several immune cells (6). Antiangiogenic properties of IL-10 have also been described (7). Thus, its dual role as immunosuppressive and antiangiogenic cytokine may have both promoting and inhibiting effect on tumour development and progression (8).

As the role of IL-8 and IL-10 in tumorigenesis is still not very well defined, the aim of this study was to investigate the influence of IL-8 and IL-10 on sporadic colon cancer development and progression.

Local production of cytokines within the tumour microenvironment is an important modulator of tumorigenesis (9). Increased IL-8 expression was found in various tumours and in some studies IL-8 serum and/or tissue levels correlated with tumour progression and metastasis (10–12). IL-10 overexpression as well as deficiency were found under different pathophysiological conditions depending upon the tumours analysed (13). Consequently, we have decided to examine *IL-8* and *IL-10* messenger RNA (mRNA) expression in sporadic colon tumours and corresponding normal mucous tissue. IL-8 and IL-10 protein expression and their pathological significance in sporadic colon tumours were analysed as well.

Cytokines and their receptors are often encoded by highly polymorphic genes. Single-nucleotide polymorphisms (SNPs) in cytokine genes potentially affect their production by either creating or eliminating key binding motifs within promoter or other regulatory sequences. Polymorphism in genes regulating the immune response and cell growth may result in increased susceptibility to and/or poorer prognosis in certain cancer patients (14).

Based on these findings, we hypothesized that regulatory SNP present in the *IL-8* and *IL-10* genes might influence colon cancer susceptibility. The human *IL-8* gene maps to 4q13–q21 and consists of four exons and three introns (15). In our study, the best-described SNP in the *IL-8* promoter, –251 A/T was, selected because of its possible influence on IL-8 expression (16).

The human *IL-10* gene is located on chromosome 1 (1q31–32) (17). The *IL-10* promoter is highly polymorphic and best-described SNPs in this region so far are –1082 A/G, –819 T/C and –592 A/C (18–20). Biological relevance of these SNPs is not clear and limited studies have suggested their influence on IL-10 production (21).

Several studies in recent years have investigated possible associations between *IL-8* and *IL-10* SNPs and cancer susceptibility (8,22,23). Nevertheless, there is still considerable controversy in the literature regarding the influence of regulatory SNPs on susceptibility to various diseases. In addition, results of *in vitro* expression analysis are not always in accordance to the *in vivo* mRNA expression in tissues due to different experimental settings, inducers of mRNA expression and cell types analysed (24). Therefore, we have decided to examine the influence of *IL-8* and *IL-10* promoter SNPs on mRNA expression in sporadic colon cancer tissue as well as association of these polymorphisms with susceptibility to sporadic colon cancer.

Cancer cell migration and invasion into adjacent tissues and intravasation into blood/lymphatic vessels are important steps for metastasis of adenocarcinomas (25). Stimulatory effect of IL-8 on tumour

Abbreviations: CI, confidence interval; IL, interleukin; mRNA, messenger RNA; PCR, polymerase chain reaction; RT, reverse transcription; SNP, single-nucleotide polymorphism.

cell migration and invasion has been demonstrated for human melanoma and prostate cell lines *in vitro* (26,27). While the significance of IL-10 mRNA and protein expression in various tumours remains controversial, several studies suggest IL-10 antimetastatic and antitumour activity *in vivo* (28,29). Since cell migration and invasion are essential for tumour progression, we have decided to examine the influence of IL-8 and IL-10 on these processes *in vitro*.

Materials and methods

Patients and controls

In our study, the population control group consisting of 160 unrelated healthy volunteers and a group of 160 patients with sporadic colon cancer were analysed. Specimens (tumour and adjacent normal colon tissue) from patients with sporadic colon cancer and blood samples of control subjects used in our study were obtained from the Croatian Tumour Bank, Ruder Bošković Institute, Zagreb, Croatia (30). During the collection of samples from cases and controls for each subject, demographic characteristics, age, sex and family history of cancer were recorded. The female versus male distribution among cases was 46.9 versus 53.1% (75 females versus 85 males) and among the controls 46.3 versus 53.7% (74 females versus 86 males). The mean age at sampling of cases was 64.5 years (42–80) and of controls 63.1 (40–78). Case group consisted of patients with sporadic colon cancer, with negative family history of hereditary cancer. All specimens were obtained during routine surgery of patients with colon adenocarcinoma. None of the patients underwent preoperative irradiation or chemotherapy. Diagnoses were established by standard diagnostic procedures and confirmed histopathologically. Fresh samples of resected colon carcinoma were snap frozen in liquid nitrogen and stored at -80°C until further use for RNA and DNA extraction. Before use in the study, each specimen was verified by the histopathologist (Š.K.). All specimens were examined by routine haematoxylin and eosin staining. Patients' DNAs were extracted from histologically normal colon mucosa adjacent to adenocarcinoma. DNA extraction was performed using proteinase K digestion and phenol–chloroform extraction. Population control group DNAs were extracted from blood samples of control subjects. Control subjects used in our study were randomly selected among unrelated volunteers without a history of tumours or other serious illnesses. Exclusion criteria were malignant, autoimmune, inflammatory or infectious disease and immunodeficiency or immunosuppressive therapy. Ethics Committee approval was obtained and written consent was provided by all subjects.

RNA extraction and reverse transcription

Total RNA was extracted from 60 pairs of resected colon carcinoma and corresponding normal tissue using Trizol reagent (Invitrogen, Carlsbad, CA) and 10 μg of RNA were used for reverse transcription (RT) (High-Capacity cDNA Archive Kit, Applied Biosystems, Foster City, CA) according to the manufacturer's protocols. Number of tumour samples analysed was dependent upon sufficient RNA quality for real-time RT–polymerase chain reaction (PCR) analysis.

Real-time RT–PCR analysis of IL-8 and IL-10 mRNA expression

Real-time RT–PCR analysis for IL-8, IL-10 and RPLP0 (house keeping gene) was performed using an ABI PRISM 7000 SDS (Applied Biosystems) and predeveloped TaqMan assay reagents, Hs_00174128 (IL-8), Hs00174086_m1 (IL-10) and Hs99999902_m1 (RPLP0) (Applied Biosystems). PCR was carried out according to the manufacturer's protocol. Briefly, 5 μl of complementary DNA was used as a template in a reaction volume of 50 μl containing 25 μl of TaqMan Universal PCR Master Mix, 2.5 μl of 20 \times Gene Expression Assay and 17.5 μl of nuclease-free water. All experiments were done in triplicates. No template controls were included in each experiment. To compensate for inter-PCR variation, normalization of the target gene (IL-8 or IL-10) with an endogenous control (RPLP0) was performed. Results are shown as ΔCt values, which is the difference in cycle number required to raise the amount of PCR product above threshold. ΔCt is therefore inversely proportional to the amounts of mRNA (31).

Real-time PCR–SNP analysis of IL-8 –251 A/T and IL-10 –1082 A/G promoter polymorphisms

Real-time PCR–SNP analysis of IL-8 –251 A/T (rs4073) and IL-10 –1082 A/G (rs1800896) was performed using an ABI PRISM 7000 SDS (Applied Biosystems) and predeveloped TaqMan SNP genotyping assays C_11748116_10 (–251 A/T) and C1747360_10 (–1082 A/G) (Applied Biosystems). PCR was carried out according to the manufacturer's protocol. For quality control, 15% of randomly selected samples of both cases and control were analysed a second time, without finding any discrepancies. Control samples covering three possible SNP genotypes and no template control were run in parallel with tested samples in each experiment.

PCR and restriction fragment length polymorphism analysis of IL-10 –819 T/C and IL-10 –592 A/C promoter polymorphisms

For –819 T/C (rs1800871) and –592 A/C (rs1800872) PCR–restriction fragment length polymorphism analysis was carried out as described previously (32). Briefly, PCR was performed using oligonucleotide primers 5'-AACTTCTTCACCCCATCTTT-3' and 5'-ATCCTCAAAGTTCCCAAG-CAG-3'. Genomic DNA (100 ng) was used as a template in a reaction volume of 25 μl containing 5 pmol of each primer, 50 μM of each deoxynucleoside triphosphate and 1 U Taq Gold DNA polymerase (Applied Biosystems). PCRs were carried out in an Applied Biosystems GeneAmp PCR System 2400 for 30 cycles with annealing temperature at 55°C (optimized in pilot studies before processing experimental samples).

For restriction fragment length polymorphism analysis, 5 μl of the PCR product was digested overnight with 2 U *MaeIII* at 55°C (New England Biolabs, Ipswich, MA) or *RsaI* (Roche, Mannheim, Germany) at 37°C in a volume of 25 μl for –819 T/C and –592 A/C SNP analyses, respectively. After addition of loading buffer, the samples were analysed by submerged gel electrophoresis on a precast Spreadex EL 800 (–819 T/C) and EL 1200 gels (–592 A/C) (Elchrom Scientific, Cham, Switzerland). Electrophoresis was performed in 0.75 \times TAE (Tris–acetate–EDTA) buffer for 85 min (–819 T/C) or 55 min (–592 A/C) at 10 V/cm, at 55°C . The gels were SyberGold stained (Molecular Probes, Leiden, The Netherlands). Gels were reviewed by at least two readers blinded to case–control status. For quality control, 15% of randomly selected samples of both cases and control were analysed a second time, without finding any discrepancies. Control samples covering three possible SNP genotypes and no template control were run in parallel with tested samples in each experiment.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue. Expression of IL-8 and IL-10 in 160 samples of sporadic colon cancer was analysed using mouse monoclonal antibodies for IL-8 (MAB208) (R&D Systems, Minneapolis, MN, USA) and IL-10 (E-10) (Santa Cruz Biotechnologies, Santa Cruz, CA), respectively. Paraffin-embedded tissue sections of normal human appendix stained with IL-8 antibody or normal human lymph node stained with IL-10 antibody served as positive control. Negative controls were performed by omission of the primary antibody. After deparaffinization in xylene, slides were rehydrated in ethanol and washed in phosphate-buffered saline (3 \times 3 min). The endogenous peroxidase activity was quenched by 15 min incubation in methanol with 3% hydrogen peroxide (Sigma Chemical Co., Munich, Germany). Non-specific binding was blocked by applying DAKO® Protein Block Serum-Free (DAKO, Glostrup, Denmark) in a humidity chamber for 10 min at room temperature. Slides were blotted, and the primary mouse monoclonal antibody at concentration of 25 $\mu\text{g}/\text{ml}$ (IL-8) or 4 $\mu\text{g}/\text{ml}$ (IL-10) was applied overnight at 4°C . Slides were then washed three times in phosphate-buffered saline. DAKO EnVision™ + System, HRP (DAB) (DAKO) was used for visualization of positive reaction according to the manufacturer's instructions. The slides were counterstained with haematoxylin for 30 s, dehydrated and mounted in Canada balsam. Each slide was evaluated in the entire tumour area. Results were expressed as negative or positive staining.

Cell culture

All experiments were performed on human colon adenocarcinoma cell line HT-29 purchased from American Type Culture Collection (ATCC, Rockville, MA, USA). Cells were seeded in tissue culture flasks (Sarstedt, Numbrecht, Germany) in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM glutamine, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin in a humidified atmosphere with 5% CO_2 at 37°C .

Cell invasion–migration assay

In vitro cell migration and invasion were determined using 24-well BioCoat Matrigel Invasion Chambers (Becton Dickinson, Franklin Lakes, NJ) according to the manufacturer's protocol. Briefly, cells were re-suspended in serum-free medium with 0.1% bovine serum albumin at a concentration of 6.7×10^5 cells/ml and 500 μl of cell suspension was seeded in the upper compartments. Experiments were done in parallel for the untreated control, IL-8 and IL-10 treatment. After 24 h, in IL-8 experiments human recombinant IL-8 (Chemicon International, Temecula, CA) was added to the wells of the BD Falcon TC Companion Plate at final concentration of 1 $\mu\text{g}/\text{ml}$. In IL-10 experiments, after 24 h recombinant human IL-10 (Chemicon International) was added in the upper compartment at final concentration of 100 ng/ml. After treatment, cells were incubated in a humidified atmosphere at 37°C in 5% CO_2 for 48 h. After incubation, non-invading cells on the upper side of membrane were wiped off and cells that traversed the membrane and spread on the lower surface of the membrane were fixed with formaldehyde and stained with 0.1% crystal violet. Invading cells were counted under a light microscope. Experiments were done in triplicate. Results are shown as the percent of migration or invasion of cells

treated with IL-8 or IL-10 relative to the migration or invasion of untreated cells.

Statistical analysis

The Hardy–Weinberg equation was used to determine whether the proportion of each genotype obtained was in agreement with expected values calculated from allele frequencies. Allele and genotype frequencies were compared between patient and control groups and by chi-square test. A chi-square P -value <0.05 was considered statistically significant. Associations between genotypes and sporadic colon cancer were calculated as odds ratios with 95% confidence intervals (CIs) by logistic regression. In all cases, homozygosity for the most common allele in Caucasians was used as the reference category.

Correlations of mRNA expression in normal and tumour tissue were analysed with the Student's t -test. Correlations of mRNA expression and genotypes were analysed with the analysis of variance. A P -value <0.05 was considered statistically significant. Box–Whisker plots were generated in the basic module of the program Statistica. All evaluations were performed using the computer program SAS Stat v 6.12.

Results

Case–control study

The frequencies of *IL-8* –251 A/T, *IL-10* –1082 A/G, –819 T/C and –592 A/C promoter SNPs were analysed in a group of colon cancer patients in comparison with a group of control individuals.

Using TaqMan SNP genotyping assays (*IL-8* –251 A/T and *IL-10* –1082 A/G) and PCR–restriction fragment length polymorphism analysis (*IL-10* –819 T/C and –592 A/C) 160 patients and 160 unrelated control subjects were genotyped. All SNPs' genotype frequencies in both sporadic colon cancer patients and control subjects were in agreement with the Hardy–Weinberg equilibrium. None of the *IL-8* –251 A/T genotypes showed significant association with sporadic colon cancer susceptibility. *IL-10* –1082 A/G genotypes were not associated with a risk of sporadic colon cancer as well. However, a statistically significant association between *IL-10* –819 T/C and –592 A/C genotypes and risk of sporadic colon cancer was observed (*IL-10* –819 TT versus CC: odds ratio, 5.53; 95% CI, 1.53–20.1; $P = 0.003$ and *IL-10* –592 AA versus CC: odds ratio, 4.07; 95% CI, 1.28–12.96; $P = 0.009$). Results of this case–control study are summarized in Table I.

IL-8 and *IL-10* mRNA expression in sporadic colon cancer

In our study, we have analysed *IL-8* and *IL-10* mRNA expression in 60 pairs of colon tumours and corresponding normal mucous tissue by

real-time RT–PCR. Number of tumour samples analysed was dependent upon sufficient RNA quality for real-time RT–PCR analysis.

In tumour tissue, a statistically significant increase in *IL-8* gene expression ($P = 0.003$) (Figure 1A) and a statistically significant decrease in *IL-10* gene expression ($P = 1.3 \times 10^{-9}$) (Figure 1B) compared with the corresponding normal tissue was observed. No correlation was found between *IL-8* or *IL-10* mRNA expression and histological grade or Dukes' stage of tumours in both tumours and corresponding normal mucous tissue.

Correlation of *IL-8* and *IL-10* mRNA expression with *IL-8* and *IL-10* SNP genotypes

In our study, we have examined the influence of *IL-8* –251 A/T, *IL-10* –1082 A/G, –819 T/C and –592 A/C promoter SNPs on *IL-8* and *IL-10* mRNA expression in tumour and corresponding normal mucous tissue.

No association of *IL-8* –251 A/T genotypes and *IL-8* mRNA expression in colon tumour as well as corresponding normal mucous tissue was observed ($P = 0.821$ and $P = 0.25$, respectively) (Figures 2A and 3A).

A statistically significant correlation between *IL-10* mRNA expression in colon tumour tissue and all three *IL-10* SNP polymorphisms was observed. *IL-10* –1082 AA genotype was associated with lower *IL-10* mRNA expression, whereas –1082 GG genotype was associated with higher *IL-10* mRNA expression ($P = 0.022$) (Figure 2B). *IL-10* –819 TT genotype was associated with lower *IL-10* mRNA expression, whereas –819 CC genotype was associated with higher *IL-10* mRNA expression ($P = 0.013$) (Figure 2C). *IL-10* –592 AA genotype was associated with lower *IL-10* mRNA expression, whereas –592 CC genotype was associated with higher *IL-10* mRNA expression ($P = 0.02$) (Figure 2D).

Expression of *IL-10* mRNA in corresponding normal mucous tissue in relation to *IL-10* SNP polymorphisms was examined as well. There was no association of *IL-10* –1082 A/G genotypes and *IL-10* mRNA expression ($P = 0.87$, Figure 3B). A statistically significant association of *IL-10* –819 T/C and –592 A/C genotypes with *IL-10* mRNA expression was present in normal mucous tissue ($P = 0.01$ and $P = 0.04$, respectively) (Figure 3C and D, respectively).

Immunohistochemical analysis of *IL-8* and *IL-10* protein expression

Expression of *IL-8* and *IL-10* in sporadic colon cancer was analysed by immunohistochemistry on formalin-fixed, paraffin-embedded

Table I. Genotype and allele frequencies of *IL-8* and *IL-10* promoter polymorphisms in patients with sporadic colon cancer and controls

SNP genotype		Controls, n (%)	Colon cancer, n (%)	Odds ratio (95% CI)
<i>IL-8</i> –251	T/T	53 (33.1)	46 (28.8)	1
	A/T	73 (45.6)	75 (46.9)	1.18 (0.71–1.97)
	A/A	34 (21.3)	39 (24.3)	1.32 (0.72–2.42)
	T	179 (55.9)	167 (52.2)	1
<i>IL-10</i> –1082	A	141 (44.1)	153 (47.8)	1.16 (0.85–1.59)
	A/A	43 (26.9)	54 (33.7)	1
	A/G	92 (57.5)	86 (53.8)	0.74 (0.45–1.22)
	G/G	25 (15.6)	20 (12.5)	0.64 (0.31–1.30)
<i>IL-10</i> –819	A	178 (55.6)	194 (60.6)	1
	G	142 (44.4)	126 (39.4)	0.81 (0.59–1.11)
	C/C	106 (66.3)	83 (51.9)	1
	C/T	51 (31.9)	64 (40.0)	1.60 (1.00–2.57)
<i>IL-10</i> –592	T/T	3 (1.9)	13 (8.1)	5.53 (1.53–20.1)
	C	263 (82.2)	230 (71.9)	1
	T	57 (17.8)	90 (28.1)	1.81 (1.24–2.63)
	C/C	104 (65.0)	83 (51.9)	1
<i>IL-10</i> –592	A/C	52 (32.5)	64 (40.0)	1.54 (0.97–2.46)
	A/A	4 (2.5)	13 (8.1)	4.07 (1.28–12.96)
	C	260 (81.2)	230 (71.9)	1
	A	60 (18.8)	90 (28.1)	1.70 (1.17–2.46)

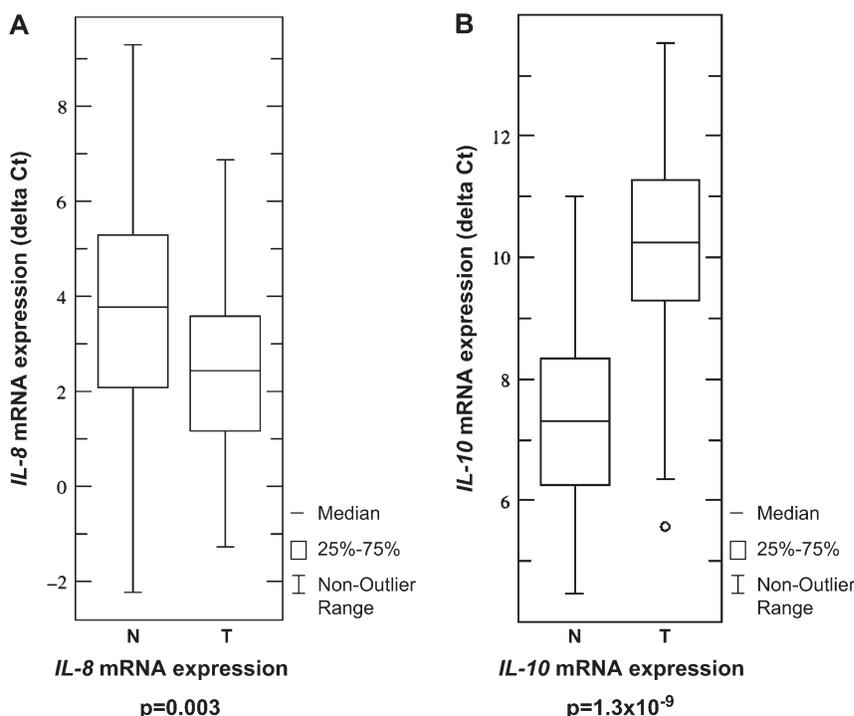


Fig. 1. *IL-8* and *IL-10* mRNA expression in sporadic colon cancer and corresponding normal mucous tissue. *P*-values were calculated using *t*-test. N, normal colon tissue; T, tumour tissue. (A) *IL-8* mRNA expression. (B) *IL-10* mRNA expression.

tissue sections. Results of immunohistochemical analysis were correlated with histological grade and Dukes' stage of tumours.

Paraffin-embedded tissue sections of human appendix stained with IL-8 antibody served as positive control of IL-8 immunohistochemical reaction. The usual pattern of positive staining for IL-8 in sporadic colon cancer was cytoplasmatic. Paraffin-embedded tissue sections of human lymph node stained with IL-10 antibody served as positive control of IL-10 immunohistochemical reaction. The usual pattern of positive staining for IL-10 in sporadic colon cancer was cytoplasmatic. In the positive control tissue (lymph node), IL-10-positive cells were present predominantly in germinal centres (Figure 4).

Of 160 sporadic colon cancers studied, 74/160 (46.3%) stained positively for IL-8. A statistically significant correlation of IL-8 protein expression and tumour differentiation was observed ($P = 0.024$). Positive immunohistochemical reaction for IL-8 protein was more frequently observed in moderately and poorly differentiated tumours compared with well-differentiated tumours. Seventeen of 54 (31.5%) well-differentiated (grade 1), 40 of 72 (55.6%) moderately differentiated (grade 2) and 17 of 34 (50.0%) poorly differentiated (grade 3) tumour samples stained positively for IL-8 (Figure 5A). No statistically significant correlation of IL-8 protein expression with Dukes' stage of tumours was observed ($P = 0.842$). Positive IL-8 staining was observed in 45.5% (10/22) Dukes' A, 48.1% (39/81) Dukes' B and 47.4% (27/57) Dukes' C tumour samples.

Of 160 sporadic colon cancers studied, 68/160 (42.5%) stained positively for IL-10. A statistically significant correlation of IL-10 protein expression and tumour differentiation was observed ($P = 0.036$). Positive immunohistochemical reaction for IL-10 protein was more frequently observed in well-differentiated and moderately differentiated tumours compared with poorly differentiated tumours. Twenty-three of 54 (42.6%) well-differentiated (grade 1) and 36 of 72 (50.0%) moderately differentiated (grade 2) tumour samples stained positively for IL-10. In poorly differentiated tumour samples (grade 3), positive staining for IL-10 was observed in 23.5% (8/34) of tumour samples (Figure 5B). In tumours classified as Dukes' C (metastatic tumours), positive immunohistochemical reaction for IL-10 protein was less frequently observed compared with Dukes' A

and B tumours and this difference was found to be statistically significant ($P = 0.023$) (Figure 4C). Positive staining was observed in 50.0% (10/20) Dukes' A, 50.0% (40/80) Dukes' B and 31.7% (19/60) Dukes' C tumour samples.

Influence of IL-8 and IL-10 on HT-29 migration and invasion in vitro
In our study, we have examined the influence of IL-8 and IL-10 on human adenocarcinoma cell line HT-29 migration and invasion *in vitro*.

Migration and invasion properties of HT-29 cells were assessed by using BD BioCoat Matrigel Invasion and Control Chambers. Migration of HT-29 cells *in vitro* was not significantly altered by IL-8 ($P = 0.4071$) or IL-10 treatment ($P = 0.795$). IL-8 treatment significantly stimulated invasion of HT-29 cells (invasion index = 1.57, $P = 0.000172$). IL-10 treatment had a weak inhibitory effect on invasiveness of HT-29 cells (invasion index = 0.85); however, this effect was not statistically significant ($P = 0.143$) (Figure 6).

Discussion

In recent years, efforts have been made to identify genes involved in the genetic predisposition or progression of particular types of cancer. Cytokines present in tumour microenvironment have gained much attention due to their influence on cell activation, growth, differentiation or cell migration and they are increasingly recognized as potential cancer modifying genes (4).

Since the role of cytokines in tumorigenesis is still far from being well defined, we have decided to analyse the influence IL-8 and IL-10 on sporadic colon cancer development and progression.

IL-8 is a pro-inflammatory cytokine and elevated IL-8 levels have been reported in many different tumours (33–35). Sugiyama *et al.* (36) analysed patterns of gene expression in colon cancer cells and corresponding normal mucous cells by DNA microarray analysis and found an increase in IL-8 expression in cancer cells compared with the adjacent normal mucosa.

In our study, a significantly higher *IL-8* mRNA expression in tumour tissue compared with corresponding normal mucous tissue was detected as well ($P = 0.003$). Positive IL-8 immunohistochemical

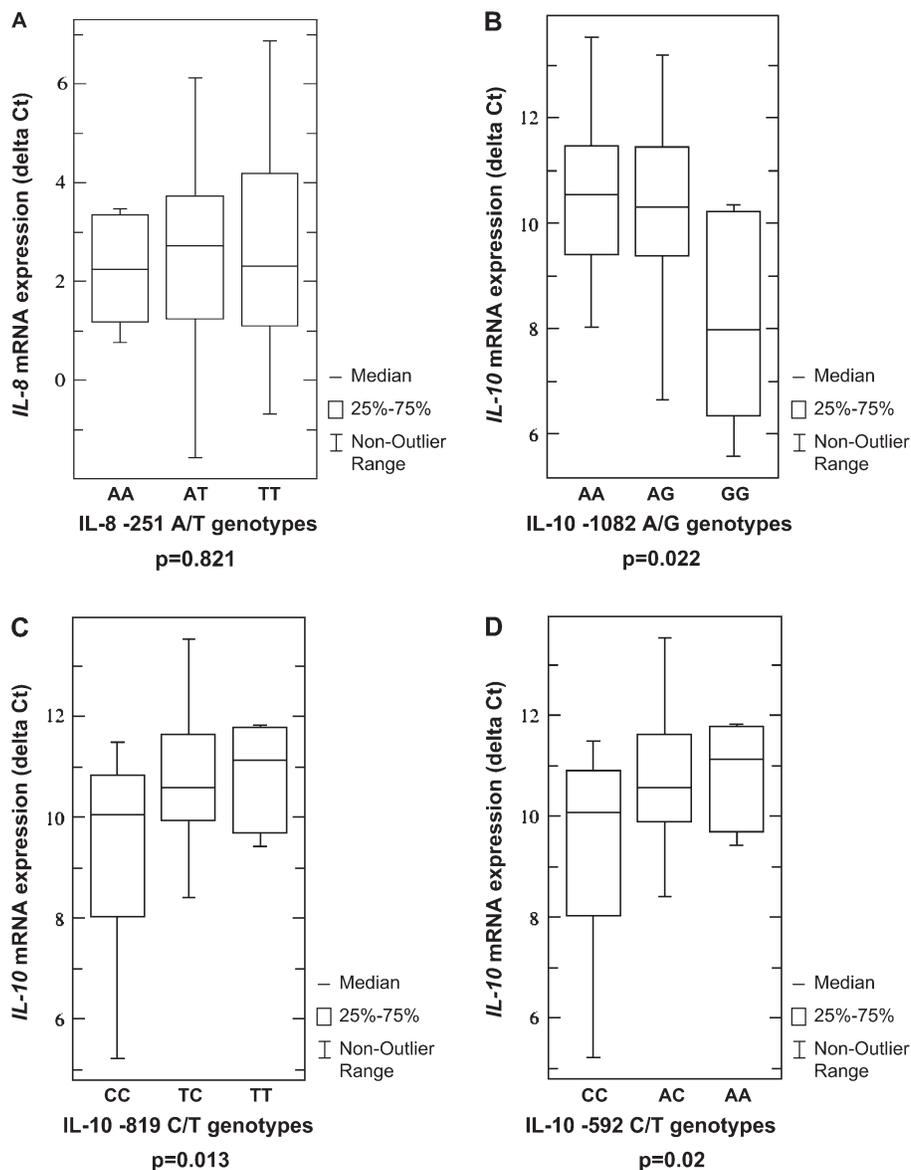


Fig. 2. *IL-8* and *IL-10* promoter SNPs' genotypes and mRNA expression in sporadic colon cancer. *P*-values were calculated using analysis of variance. (A) *IL-8* -251 A/T genotypes and *IL-8* mRNA expression. (B) *IL-10* -1082 A/G genotypes and *IL-10* mRNA expression. (C) *IL-10* -819 C/T genotypes and *IL-10* mRNA expression. (D) *IL-10* -592 C/T genotypes and *IL-10* mRNA expression.

staining was more frequently observed in moderately and poorly differentiated tumours compared with well-differentiated tumours ($P = 0.024$); however, no correlation with the Dukes' stage of tumours was observed.

IL-10 is a pleiotropic cytokine, and although it was first described as immunosuppressive and anti-inflammatory, recent findings suggest its stimulatory properties depending upon the tumour and disease state analysed (13).

Contradictory findings are present in the literature concerning *IL-10* systemic or tissue levels and survival of cancer patients as well (37,38). For instance, Mocellin *et al.* (39) found that *IL-10* overexpression within the tumour microenvironment was implicated in cancer immune rejection. In the study of Uwatoko *et al.* (40), *IL-10* gene expression in renal cell carcinoma was inversely correlated with incidence of distant metastasis. Contrary to these findings, for instance, Nemunaitis *et al.* (41) showed that elevated *IL-10* levels correlated with poor survival in melanoma patients.

As shown previously by Autschbach *et al.* (42), *IL-10* is constitutively expressed at both mRNA and protein level in normal human

colon tissue with intestinal cells being a major source of this cytokine. In our study, we have examined *IL-10* mRNA and protein expression in sporadic colon cancer. There was a statistically significant decrease in *IL-10* mRNA expression in tumour tissue compared with corresponding normal colon tissue ($P = 1.3 \times 10^{-9}$). Positive immunohistochemical reaction for *IL-10* protein was more frequently observed in well-differentiated and moderately differentiated tumours compared with poorly differentiated tumours and this difference was found to be statistically significant as well ($P = 0.036$). In tumours classified as Dukes' C (metastatic tumours), positive immunohistochemical reaction for *IL-10* protein was less frequently observed compared with Dukes' A and B tumours and this difference was also statistically significant ($P = 0.023$). Taken together, these findings suggest that *IL-10* might have a protective role in sporadic colon cancer development and progression.

While numerous factors influence the inflammatory response in cancer, the role of an individual's genetic background has recently received increasing attention (43). In investigating disease-gene associations, there is a strong argument for focusing on polymorphisms of

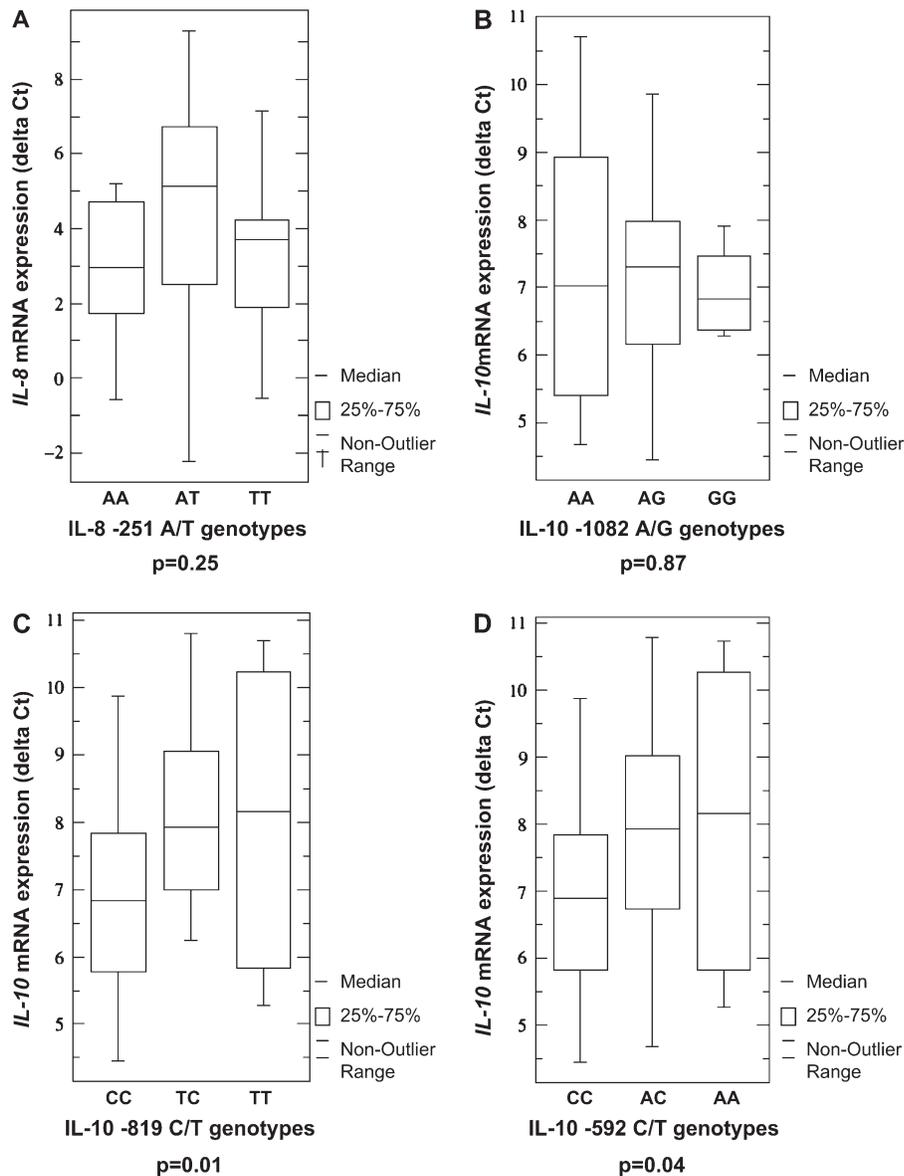


Fig. 3. *IL-8* and *IL-10* promoter SNPs' genotypes and mRNA expression in corresponding normal mucous tissue. *P*-values were calculated using analysis of variance. (A) *IL-8* -251 A/T genotypes and *IL-8* mRNA expression. (B) *IL-10* -1082 A/G genotypes and *IL-10* mRNA expression. (C) *IL-10* -819 C/T genotypes and *IL-10* mRNA expression. (D) *IL-10* -592 C/T genotypes and *IL-10* mRNA expression.

functional significance, for instance SNP polymorphisms in promoter region as they might alter transcriptional regulation (44). The role of natural variation in the systemic level of cytokines is still controversial. The relevance of cytokine levels within the tumour microenvironment has never been comprehensively studied in human beings and some authors suggest that their influence should be studied at the tumour site (39). Therefore, in order to achieve full informativity, genetic studies should be complemented with gene expression analysis and proteomics (45).

Until present date, several functional polymorphisms have been characterized in the *IL-8* and *IL-10* genes. Among these polymorphisms, the presence of *IL-8* -251 A/T in the transcription start site has been associated with various diseases; however, results of different studies are often contradictory. In the study of Gunter *et al.* (46), homozygous carriers of the *IL-8* -251 A allele were at 2.7-fold increased risk of colon adenoma. Landi *et al.* (22), however, found association between *IL-8* -251 A allele and decreased risk of colon cancer. On the contrary, Theodoropoulos *et al.* (23) did not find any association of *IL-8* genotypes and colorectal cancer risk. In our study,

IL-8 -251 A/T did not influence sporadic colon cancer susceptibility as well.

The influence of *IL-8* -251 A/T SNP on *IL-8* production was assessed by functional studies using lipopolysaccharide-stimulated *IL-8* production in whole blood (16). However, in most studies analysing the association of SNPs with various diseases, specific SNPs are presumed to alter mRNA expression of analysed genes and these studies are rarely (if at all) accompanied with the mRNA expression analysis in the tissue of interest. In our study, both *IL-8* mRNA expression in colon tumour and corresponding normal mucous tissue and its correlation to *IL-8* -251 A/T genotypes of patients were analysed; however, no correlation was observed.

Furthermore, we have analysed three *IL-10* SNPs, -1082 A/G, -819 T/C and -592 A/C, in the promoter region of this gene. Previous studies have indicated an association of GCC and ATA haplotypes with high and low *IL-10* production in peripheral blood cell cultures, respectively (21); however, it is important to note that *in vitro* expression of cytokines is stimulus dependent, and different experimental systems may yield different results for different SNPs.

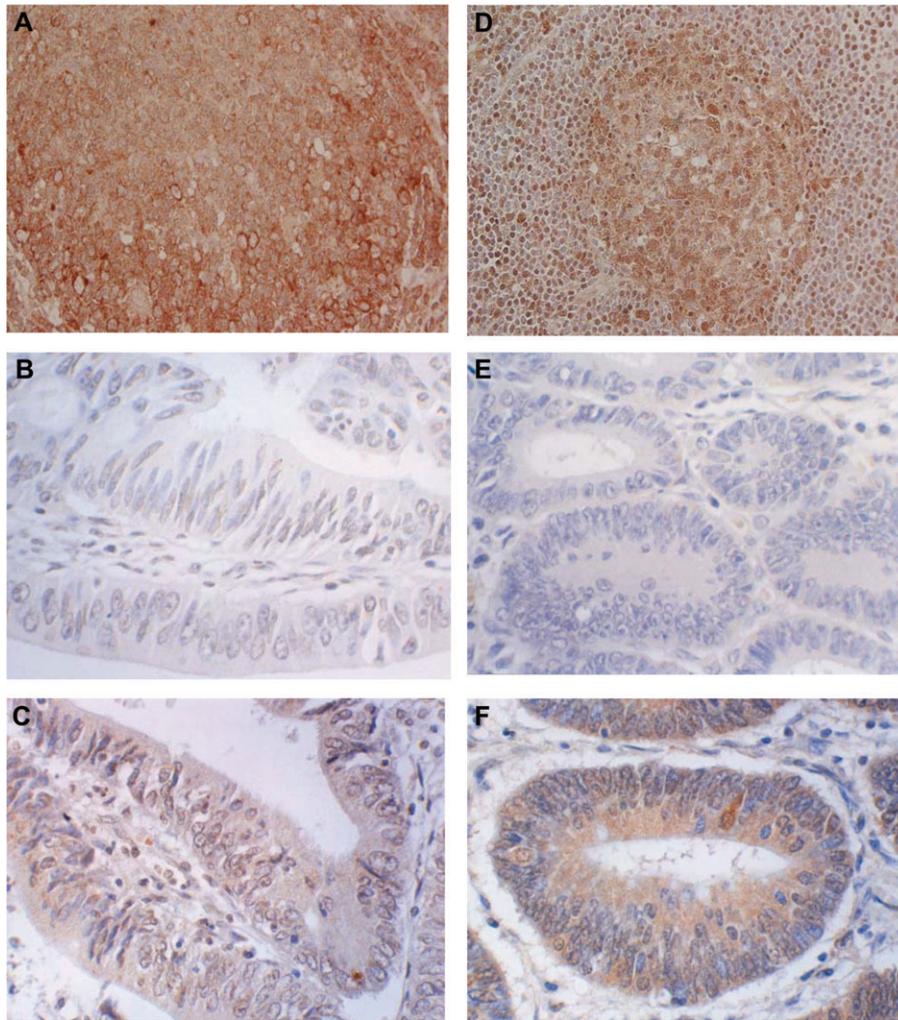


Fig. 4. IL-8 and IL-10 protein expression in sporadic colon cancer. Immunohistochemical staining patterns of IL-8 (A–C) and IL-10 (D–F). (A) Appendix, positive control of IL-8 immunohistochemical reaction. (B) Tumour negative for IL-8 expression. (C) Tumour positive for IL-8 expression with most cancer cells expressing IL-8 in the cytoplasm. (D) Lymph node, positive control of immunohistochemical reaction with IL-10-positive cells in the germinal centre. (E) Tumour negative for IL-10 expression. (F) Well-differentiated adenocarcinoma with most cancer cells expressing IL-10 in the cytoplasm (original magnification $\times 400$).

Association between *IL-10* genotype and IL-10 expression may differ between tissues and disease states as well (19). Genotypes associated with low IL-10 expression were described as risk factors for cancer development and/or progression in some types of cancer (breast and prostate), whereas in other cancers (cervical and hepatocellular) genotypes associated with high IL-10 expression were found to be a risk factor. However, none of these studies included both SNP genotyping of patients and *IL-10* mRNA expression analysis in the tumour tissue (45).

In our study, IL-10 –1082 AA genotype was associated with lower *IL-10* mRNA expression, whereas –1082 GG genotype was associated with higher *IL-10* mRNA expression in tumour tissue ($P = 0.022$). However, these genotypes were not associated with *IL-10* mRNA expression in corresponding normal mucous tissue. In a group of colon cancer patients, an increased frequency of the homozygous –1082 AA genotype (genotype associated with low *IL-10* mRNA expression in tumour tissue) compared with control group was observed; however, this difference was not statistically significant and IL-10 –1082 G/A SNP did not influence sporadic colon cancer susceptibility.

In our study, low *IL-10* mRNA expression in tumour and corresponding normal mucous tissue was associated with –819 TT and –592 AA genotypes, whereas high mRNA expression was associated with –819 CC and –592 CC genotypes. The ‘low-producer geno-

types’ –819 TT and –592 AA were present more frequently in colon cancer patients and this difference in genotype distribution was statistically significant. Therefore, ‘high-producer genotypes’ of IL-10 might be considered as protective factor in sporadic colon cancer susceptibility.

The influence of IL-8 on tumour cell migration and invasion is of great importance in tumour progression and metastasis formation. Li *et al.* (47) observed significant differences in the invasive potential of colon carcinoma cells expressing different levels of IL-8. Cell line KM12C (low IL-8 expression) was less invasive than KM12L4 cell line (high IL-8 expression). In our study, IL-8 stimulated HT-29 cell invasion *in vitro* ($P = 0.000172$).

Adris *et al.* (48) reported that murine colon carcinoma cell line CT26 transduced with a recombinant retrovirus expressing IL-10 displayed decreased invasive and migration ability *in vitro*. Stearns *et al.* (49) demonstrated IL-10-mediated down-regulation of matrix metalloproteinase-2 and membrane type 1 matrix metalloproteinase in primary prostate tumour cells, a molecular complex strongly associated with tumour invasion and metastases. In our study, colon tumours classified as Dukes’ C (metastatic tumours) were found to be less frequently positive for IL-10 protein expression compared with Dukes’ A and B tumours. This result might point to a possible anti-metastatic role of IL-10 in colorectal cancer progression; however,

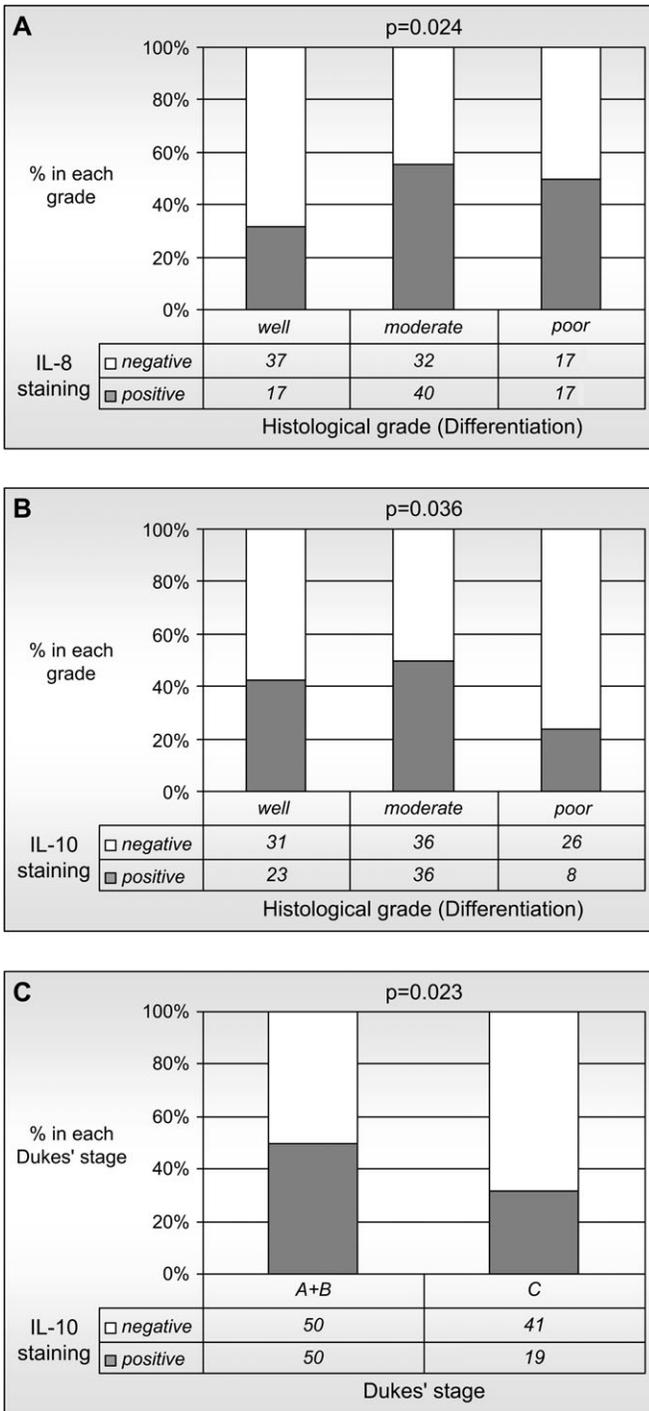


Fig. 5. IL-8 and IL-10 protein expression and clinicopathological features (histological grade and Dukes' stage) of sporadic colon tumours. *P*-values were calculated using chi-square test. (A) IL-8 protein expression and histological grade of tumours. (B) IL-10 protein expression and histological grade of tumours. (C) IL-10 protein expression and Dukes' stage of tumours.

IL-10 showed only weak inhibitory effect on HT-29 cell migration and invasion *in vitro*.

In conclusion, results obtained from our study of IL-8 -251 A/T polymorphism and susceptibility to sporadic colon cancer provide no evidence that particular IL-8 -251 genotypes are associated with disease susceptibility. However, to our best knowledge, this is the first study to analyse the influence of this polymorphism on IL-8 mRNA expression in colon cancer tissue. Elevated IL-8 mRNA levels in

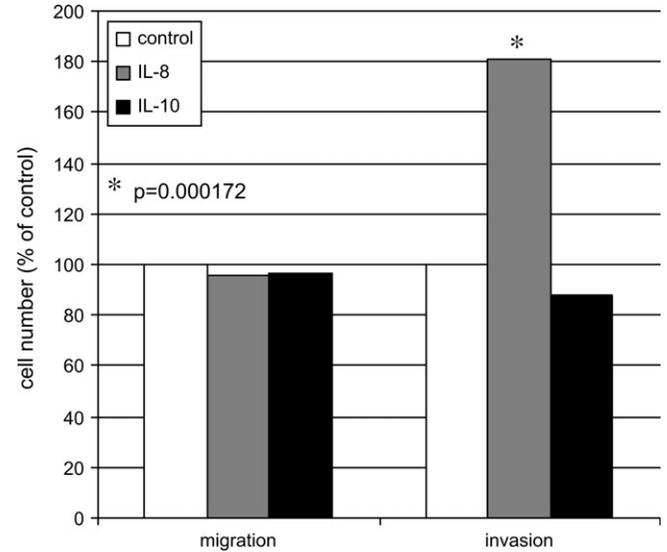


Fig. 6. Influence of IL-8 and IL-10 on HT-29 migration and invasion *in vitro*. *P*-value was calculated using chi-square test.

tumour tissue compared with corresponding normal mucous tissue, more frequent positive staining for IL-8 protein observed in moderately and poorly differentiated tumours compared with well-differentiated tumours together with the stimulatory effect of IL-8 on human colon adenocarcinoma cell line HT-29 invasion *in vitro* underline the importance of IL-8 in colon cancer development and progression.

This is also the first study to analyse the influence of IL-10 promoter polymorphisms on IL-10 mRNA expression in sporadic colon cancer and based on our findings, we can conclude that IL-10 SNP promoter genotypes associated with low IL-10 mRNA production (-819 TT and -592 AA) are found more frequently in a group of sporadic colon cancer patients. Low IL-10 mRNA expression in tumours and less frequent positive immunohistochemical staining for IL-10 protein in a group of poorly differentiated tumours and tumours classified as Dukes' C all point to a possible protective role of IL-10 in sporadic colon cancer. Finally, we can conclude that results of our study contribute to a rising pool of knowledge about the role of cytokines in cancer and open the door to further research of IL-8 and IL-10 role in the colon cancer tumorigenesis.

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