Association of Haplotypes of Interleukin-10 Gene with the Risk of Cancer

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Polymorphisms of promotor region of IL-8, IL-10, and IL-12 genes were analyzed in cancer patients and subjects without history of cancer. The distribution of alleles of the analyzed polymorphisms in the control group coincided with that in other Caucasian populations. The incidences of three *IL-10* gene polymorphisms (G-1082A, C-819T, and C-592A) significantly differed in controls and patients. Of 8 theoretically probable *IL-10* gene haplotypes determined by these polymorphisms, 3 variants were revealed. Haplotype ACC was more incident in cancer patients, while ATA haplotype was rarer. The results are in line with the findings of other studies indicating the involvement of the immune system genes in the pathogenesis of cancer.

Key Words: *haplotypes; interleukin-10; genetic predisposition; cancer*

The role of genome mutations in the pathogenesis of cancer in humans has been long studied only as exemplified by rare hereditary syndromes, such as hereditary forms of breast cancer, colorectal cancer (familial adenomatous polyposis, Lynch syndrome) responsible for no more than 5% cases of these diseases according to different estimations [7,11]. Point mutations responsible for these diseases are highly penetrant and the probability of disease development in a carrier of this genetic variant reaches 80-90%. For example, study of mutations in *BRCA1* and *BRCA2* genes [11] demonstrated a 7-fold increase in the probability of breast cancer in comparison with controls. On the other hand, the incidence of even the most prevalent mutations in *BRCA1* and *BRCA2* gene in the population is very low (<0.1%).

Recent studies showed that genetic factors largely determine the development of sporadic forms of cancer [13]. The genetic risk of sporadic forms of cancer is associated with combination of frequent gene polymorphisms, each being low penetrant if present alone [9]. A certain contribution to predisposition to cancer development was proven not once for detoxification gene polymorphisms (cytochrome P450 and glutathione transferases gene families) [13].

Other most probable candidates are gene polymorphisms of proteins involved in the immune response, *i.e.* genes encoding IL, TNF, IFN, *etc.* For example, IL-10 is characterized by immunosuppressive action and suppresses the proliferation and activity of T-cells. Tumors producing IL-10 are characterized by more aggressive growth [1]. IL-8 is essential for proliferation and functional activity of immunocompetent cells and promotes angiogenesis. Hyperexpression of IL-8 is associated with aggressive tumor growth and development of relapses [15]. IL-12 induces production of other cytokines and promotes differentiation of immature T lymphocytes into type 1 T helper cells. Experiments on transgenic mice showed that IL-12 deficit is linked

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with better survival of transplanted tumor cells and higher percentage of metastases [10].

In order to evaluate the role of genetic factors in the pathogenesis of cancer, we analyzed 9 point nucleotide polymorphisms, located in the promotor regions of IL-10 (*IL-10*), IL-8 (*IL-8*), and IL-12 (*IL-12*) genes. The choice of promotor region polymorphisms as the object of the study is explained by higher probability of their potential effects on changes in the levels of expression of the respective genes and hence, on the probability of cancer.

MATERIALS AND METHODS

The age- and sex-matched groups of oncological patients (n=120) and controls (n=600) were formed from a sampling of 9600 residents of Novosibirsk, participants of the International Program "Cardiovascular Risk Factors in Eastern Europe". The presence of cancer was confirmed by the data from the Medical Register for Cancer in the city of Novosibirsk. Additional analysis of *IL-10* gene polymorphisms was carried out in 251 patients from the main sampling, who had a history of asthma (according to interviews). Each patient gave informed consent to examinations, collection and analysis of biological materials.

Genome DNA isolated from 5 ml blood by the standard method (phenol-chloroform extraction) was analyzed. Polymorphism alleles were determined by the minisequencing reaction with subsequent mass-spectrometry of the reaction products on a time-lapse mass-spectrometer (MALDI-TOF minisequencing). Gene sites carrying the analyzed polymorphisms were amplified by a preliminary PCR. Amplification was carried out in reaction mixture containing 66 mM Tris-HCl (pH 9), 16.6 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 100 μM each dNTP, 1 U Taq polymerase (Promega), and 2 pmol each primer; reaction volume 10 μl. Amplification reaction (35 cycles) was carried out in a DNA Engine Tetrad 2 amplifier (MJ Research) at the following regimen: 15 sec at 94°C, 15 sec at 58°C, and 16 sec at 72°C. For subsequent minisequencing reaction the terminal dNTP phosphate groups were dephosphorylated in a postamplification mixture. The samples were incubated with 1 U Antarctic shrimp phosphatase (New England BioLabs) at 37°C for 30 min, the enzyme was inactivated by 10-min heating at 85°C. The minisequencing reaction was carried out in 20 μl reaction mixture: 66 mM Tris-HCl (pH 9), 16.6 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.2 mM each needed ddNTP and/or dNTP, 3 pmol each oligonucleotide probe, and 2 U TermiPol DNA Polymerase (Solis Biodyne) with amplified DNA fragments serving as the template. Minisequencing products were produced under the universal conditions: 5 sec at 94°C, 20 sec at 58°C, and 5 sec at 72°C (40 cycles). The products of minisequencing were purified using SpectroCLEAN Kit (Sequenom). The adsorbent from this kit (8 mg) was dissolved in 16 μl ultraclean water (Merck) and the resultant suspension (24 μl) was added into the tube with the minisequencing reaction products. The contents of the tube was thoroughly mixed and incubated at ambient temperature for 15 min. The adsorbent was then precipitated by centrifugation (5 min, 1000 rpm). The supernatant was used for mass-spectrometric analysis.

An aliquot of the sample (0.2-1.0 μl) was applied onto a matrix prepared from saturated 3-hydroxypicolinic acid (Fluka) in 50% acetonitrile (Merck) with 10 g/liter dibasic ammonium citrate (Fluka) and dried on an AnchorChip plate (600 μ; Bruker Daltonics), and the aliquot was dried on air. The spectra were obtained using MALDI TOF mass-spectrometer Reflex-IV (Bruker Daltonics) fitted with a nitrogen laser (λ=337 nm) at pulse frequency of up to 20 Hz. All measurements were carried out in a linear mode, positive ions were detected at ascending voltage of 20 kV by an accumulating electrode 18.65 kV, focusing lens 9.2 kV, and at analyzer delay time 400 nsec. Fifty laser pulses with radiation power set up at the minimum threshold value, sufficient for the sample desorption-ionization were used for obtaining each mass spectrum. The mass spectra were recorded, processed, and analyzed using Bruker Daltonics: flexControl 2.4 (Build 38) and flexAnalysis 2.4 (Build 11) software. The nucleotide context in the position was evaluated by the presence of peaks of the reaction products corresponding to ions of a certain expected molecular weight in mass spectra.

Haplotypes were evaluated using HAPLOVIEW software (Broad Institute of MIT and Harvard). The significance of differences in the distribution of polymorphism alleles and/or haplotypes in analyzed samplings was evaluated using χ^2 test.

RESULTS

The following polymorphisms were analyzed: 3 polymorphisms of *IL-10* gene promotor region: G-1082A, C-819T, C-592A; 2 polymorphisms of *IL-8* gene promotor region: A-251T, C-781T; and 4 polymorphisms of *IL-12* gene promotor region: C-1340G, A-2055G, C-2437T, and A-6110G (Table 1). The incidence of alleles in the control group corresponded to their incidence in other European populations [3,5,14], significant differences between the groups were detected only for *IL-10* gene C-

TABLE 1. Incidence of Allele Polymorphisms in Analyzed Groups

Polymorphism	Polymorphism identifier in NCBI dbSNP data base	Incidence of rare allele in control group (*n*=1200), %	Incidence of rare allele in patients (*n*=240), %	Significance of differences
IL-10 G-1082A	Rs1800896	45.9	47.5	—
IL-10 C-819T	Rs1800871	24.7	17.1	χ^2=6.804, *p*=0.01
IL-10 C-592A	Rs1800872	24.7	17.1	χ^2=6.804, *p*=0.01
IL-8 A-251T	Rs4073	45.5	45.8	—
IL-8 C-781T	Rs2227306	42.4	44.0	—
IL-12 C-1340G	Rs3212217	19.0	16.5	—
IL-12 A-2055G	Rs1433048	18.8	19.8	—
IL-12 C-2437T	Rs730691	42.7	43.0	—
IL-12 A-6110G	Rs2546890	32.7	32.9	—

Note. *n:* number of chromosomes in the group.

819T and C-592A polymorphisms. These polymorphisms were linked: polymorphism C-819T allele T was detected only in the presence of polymorphism C-592A allele A. Presumably, only one of them is functionally significant, while changed incidence of the other polymorphism is due to the link.

The presence of a link between these polymorphisms was confirmed by subsequent analysis of haplotypes of *IL-10* gene promotor region. Three promotor region polymorphisms analyzed in this study can theoretically form eight haplotypes. However, analysis detected only 3 variants: GCC (major alleles of all polymorphisms), ACC (G-1082A minor allele and C-819T and C-592A major alleles), and ATA (minor alleles of all three polymorphisms).

The incidence of *IL-10* gene haplotypes in the control group were similar to the incidence in other European populations and differed significantly from haplotype incidence in Asian populations, for which the most incident haplotype is ATA [5,6,12]. Comparison of the distributions of all detected haplotypes in the control group and in cancer patients detected statistically significant differences (χ^2=7.84, *p*=0.0025). Comparison of the incidence of individual haplotypes showed significant difference for ATA haplotype (*p*=0.01) due to difference in the incidence of C-819T and C-592A polymorphism alleles and for ACC haplotype (*p*=0.06). Despite the borderline value of significance for the differences in the incidence of ACC haplotype, great difference between the significance values for the distribution of all haplotypes and the most significant of them (ATA) indicates that ACC haplotype is presumably also significant, which is confirmed by the findings of other authors. For example, ACC haplotype is significantly more incident in the Mongoloids with hepatocellular carcinoma [12].

Analysis of haplotype combinations in the groups showed the most significant differences for ATA/

TABLE 2. Incidence of Haplotypes and Their Combinations in Analyzed Groups

Haplotype/ combination of haplotypes	Incidence in control group (*n*=600), %	Incidence in cancer patients (*n*=120), %	Incidence in patients with asthma (*n*=251), %
GCC	45.9	47.5	45.8
ACC	29.4	35.4	27.7
ATA	24.7	17.1	26.5
GCC/GCC	22.1	20.0	21.9
GCC/ACC	25.8	37.5	26.7
GCC/ATA	21.7	17.5	21.1
ACC/ACC	8.9	10.8	6.8
ACC/ATA	15.1	11.7	15.1
ATA/ATA	6.4	2.5	8.4

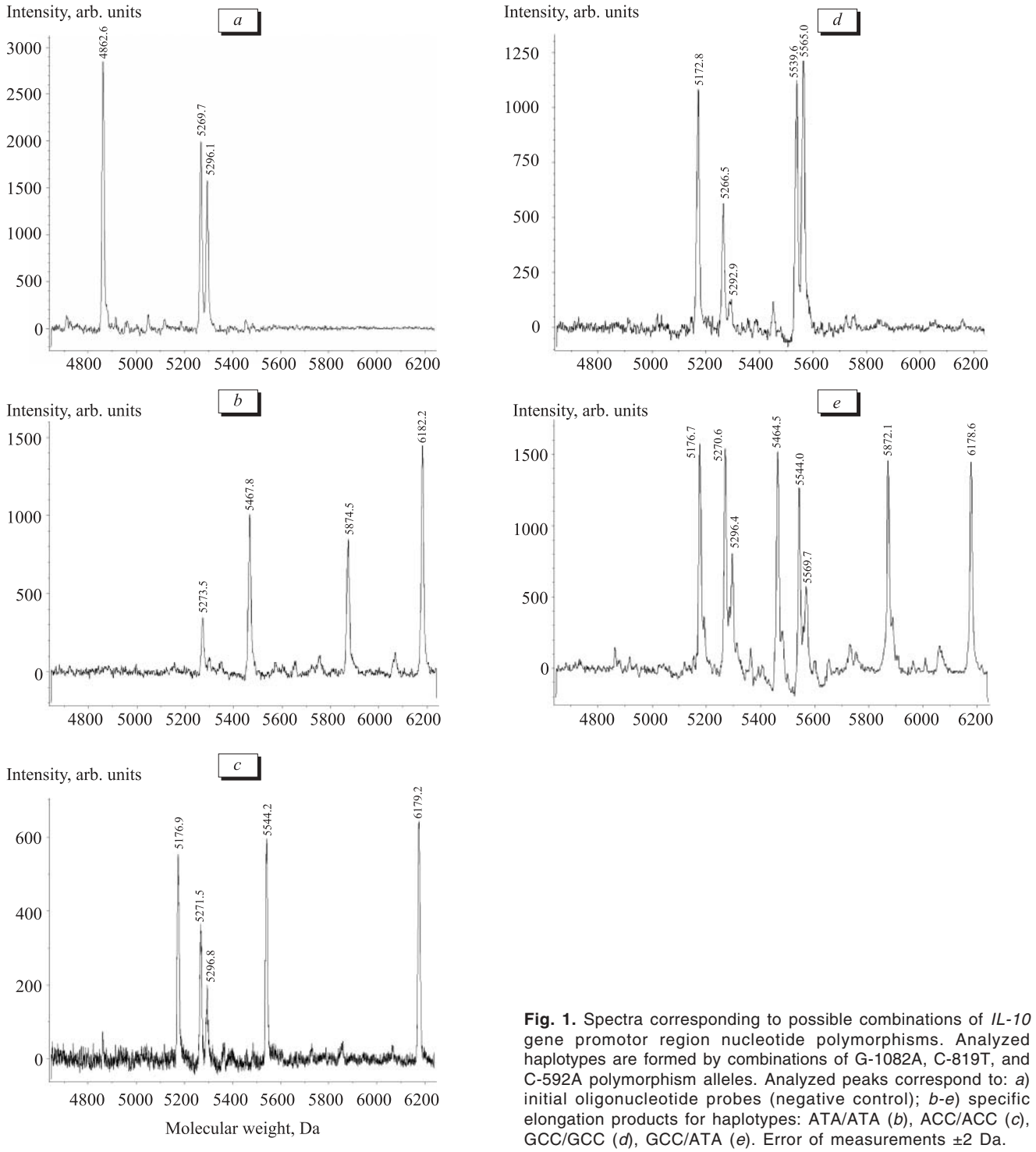

Fig. 1. Spectra corresponding to possible combinations of *IL-10* gene promotor region nucleotide polymorphisms. Analyzed haplotypes are formed by combinations of G-1082A, C-819T, and C-592A polymorphism alleles. Analyzed peaks correspond to: *a*) initial oligonucleotide probes (negative control); *b*-*e*) specific elongation products for haplotypes: ATA/ATA (*b*), ACC/ACC (*c*), GCC/GCC (*d*), GCC/ATA (*e*). Error of measurements ±2 Da.

ATA haplotype combination (Table 2). We previously analyzed *IL-10* gene haplotypes in a group of patients with a history of asthma (n=251) from the main sampling. We expected to detect differences in this group opposite to differences in cancer patients. The distributions of individual alleles and individual haplotypes in patients with asthma were similar to the distributions in the control group. However, analysis of haplotype combinations showed diverse changes from cancer patients: the incidence of ATA/ATA haplotype combination was maximum in the group of asthmatics and minimum in cancer patients. On the other hand, ACC/ACC haplotype combination was the rarest in the group of patients with asthma and the most incident in cancer patients (Table 2). Hence, *IL-10* gene ATA

haplotype is presumably associated with low, while ACC with high expression of the gene.

These data agree with the findings of other studies in Caucasian populations. Analysis of *IL-10* gene promotor region polymorphisms in 500 women with breast cancer and 500 healthy women in Germany showed that ATA haplotype homozygotes were almost 2-fold more incident in the control group (7.3 *vs.* 4.3%) [6]. Comparison of *IL-10* gene C-592A polymorphism allele incidence in 264 patients with colorectal cancer and 408 healthy residents of North-Eastern Scotland showed that the risk of disease was 2-fold lower for carriers of allele A (present only in ATA haplotype) [8]. Analysis of G-1082A polymorphism showed that AA homozygotes (the greater part are ACC haplotype carriers) were more incident among renal cancer patients [4]; the prognosis for this population in case of melanoma diagnosed at late stages, relapses, and metastases was worse [2].

We also analyzed the haplotype combinations for various genes. No differences in the distribution of genotypes of other analyzed genes (*IL-8* and *IL-12*) in combination with certain *IL-10* gene genotypes were detected in the majority of cases. However, the fact that all *IL-10* gene ATA haplotype homozygotes in the group of cancer patients were also carriers of two alleles A of *IL-8* gene polymorphism T-251A, while in controls this combination was rare, is worthy of note. Small size of the sample does not allow us to make conclusions about the significance of differences, but this fact is interesting in the light of other findings. Analysis of the probability of colorectal cancer relapses after therapy in 90 patients in the USA showed that 88% AA homozygotes (*IL-8* gene polymorphism T-251A) developed relapses, *vs.* only 38% cases with relapses among TT homozygotes [3]. It was shown *in vitro* that allele A of this polymorphism was associated with high expression of *IL-8* gene [15]. Presumably, due to its potent angiogenic effect, high level of IL-8 promotes rapid tumor growth.

Hence, it seems that of the polymorphisms analyzed in this study, *IL-10* gene promotor region polymorphisms are largely essential for genetic predisposition to oncological diseases. Presumably, *IL-8* gene polymorphism T-251A is characterized by a modulating effect.

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