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COMBINED HAPLOTYPE ANALYSIS OF THE INTERLEUKIN-19 AND INTERLEUKIN-20 GENES: RELATIONSHIP TO PLAQUE-TYPE PSORIASIS

Master's thesis in Developmental Biology

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Abbreviations

ARMS-PCR	amplification refractory mutation system polymerase chain reaction
CRF 2	cytokine receptor family type 2
GM-CSF	granulocyte-macrophage colony-stimulating factor
HIV	human immunodeficiency virus
IL-10, -19, -20	interleukin-10, interleukin-19 and interleukin-20, respectively
IL-20Rα,β	respectively, α- or β-subunit of interleukin-20 receptor complex
LD	linkage disequilibrium
LPS	bacterial lipopolysaccharide
PASI	psoriasis area and severity index
rs#	dbSNP assigned reference SNP ID ('#' representing multiple numbers)
T_m	melting temperature
SNP	single nucleotide polymorphism
STAT	signal transducer and activator of transcription
UTR	untranslated region

Introduction

Psoriasis is an universal chronic inflammatory skin disease, which is mainly characterized by T cell-mediated hyperproliferation of keratinocytes. Multiple genes are involved in psoriasis interacting not only with each other but also with the environment to cause disease expression. Several triggering factors have been found to initiate psoriasis, and although psoriasis is heterogeneous with respect to genetic and environmental components, it is shown that final common pathogenic pathway involves various cytokines and chemokines released by activated, skin-homing pathogenic T cell population.

There is increasing evidence to suggest that the newly discovered cytokines of IL-10 family − interleukin-19 (IL-19) and interleukin-20 (IL-20) − have a role in the function of epidermis and in psoriasis. The *IL-19* and *IL-20* genes are mapped to a gene cluster of IL-10 family cytokines in q31-q32 region on human chromosome 1. Linkage to several common autoimmune diseases have been detected in this region and recent data indicate that this region could be related to psoriasis susceptibility.

Previously, SNPs of human *IL-20* gene had been analyzed in respect to their involvement in susceptibility to psoriasis, and significant association between patients with psoriasis and the G allele of SNP rs2981572 and GAA haplotype was established. The aim of the present study was to clarify the role of *IL-19* gene, which is preceding *IL-20* gene in the 1q32 region, in predicting risk for psoriasis. Combining previously obtained data of *IL-20* gene study and new data attained in this study for *IL-19* gene, extended association, linkage disequilibrium and haplotype analyses of *IL-19* and *IL-20* genes was performed.

Review of literature

Psoriasis

Psoriasis (OMIM 177900) is an universal inflammatory disease, initially described at the beginning of medicine in the *Corpus Hippocratum*. Hippocrates used the term *psora*, which in translation means 'to itch' (Nickoloff *et al*. 2004).

Psoriasis is mainly characterized by T cell-mediated hyperproliferation of keratinocytes (Lebwohl 2003). Multiple genes are involved, interacting not only with each other but also with the environment to cause disease expression (Barker 2001). Examination of individuals from the Danish Twin Registry, has shown concordance for psoriasis in 63% of monozygotic twins compared with 15% for dizygotic twins (Brandrup *et al*. 1982). However, it is evident that the concordance rates do not reach 100%, indicating that the environment plays a significant role in disease expression (Barker 2001).

Several triggering factors have been found to initiate or exacerbate psoriasis, including bacterial pharyngitis, stress, HIV infection, various medications (*e.g.*, lithium and β-blockers) and alcohol abuse (Lebwohl 2003, Gudjonsson *et al*. 2004, Nickoloff *et al*. 2004). Although psoriasis is heterogeneous with respect to genetic and pathogenic components, several studies have confirmed that final common pathogenic pathway involves specific antigen (*e.g.*, streptococcal M proteins and those keratins that are up-regulated in psoriatic lesions) recognition by T cells and activation of T cells leads to release of cytokines resulting in stimulation of keratinocytes proliferation.

In the normal skin the ratio of proliferating to non-proliferating keratinocytes is around 60% while in case of psoriatic skin it is almost 100%. In psoriatic lesions the mean cell cycle time is reduced from 311 h to 36 h (Gudjonsson *et al*. 2004). The symptoms vary from isolated scaling erythematous plaques on elbows, knees, scalp, and intergluteal cleft, to cases where patients can have up to 100% of their cutaneous surface affected. Severity of psoriasis case is mainly quantified by the score of Psoriasis Area and Severity Index (PASI) which is calculated based on the degree of erythema, desquamation and infiltration of lesions combined with the surface extension (Fredriksson *et al*. 1978).

Primarily taken as skin disorder, it is now known that psoriasis is a chronic systemic disease which in addition to skin aberrations may eventually cause nail changes (10-30%,

Nickoloff *et al*. 2004) and affect joints − from 5% to 42% of psoriasis patients have psoriatic arthritis, a destructive and occasionally disabling joint disease (Gladman *et al*. 2000).

Estimated prevalence of psoriasis varies from 0.5% to 4.6%. While rates vary between countries and races (more frequent at higher latitudes and affects Caucasians more than other races), the prevalence is almost equal in men and women (Lebwohl 2003). Most common form of psoriasis is plaque-type psoriasis which occurs in more than 80% of psoriasis cases, outnumbering less frequent cases of guttate (10%), erythrodemic and pustular (both occurring in less than 3% of the cases) psoriasis (Lebwohl 2003). Two peaks of age on onset of have been described: 20-30 years and a smaller peak at 50-60 years, suggesting that two forms of the disease exist. Described by Henseler *et al*. (1985), psoriasis patients can be divided into two subgroups − patients with type I psoriasis, if they have early onset of disease and familial psoriasis (at least one first- or second-degree relative with psoriasis), and patients with type II psoriasis, if they have late onset of the disease and sporadic form of the disease.

The precise set of genes making individuals predisposed to psoriasis has not been identified, but several candidate genes have been suggested. Zhou *et al*. (2003) identified, with the help of 63,100-element oligonucleotide array, 1,338 genes with potential roles in psoriasis pathogenesis/maintenance, and revealed many perturbed biological processes, including immune and inflammatory responses. Using linkage disequilibrium studies, psoriasis susceptibility loci have been mapped to several regions on different chromosomes (Gudjonsson *et al*. 2004). One of the loci, 1q31-32, has been shown to be associated with several autoimmune diseases and has also been shown to be associated with psoriasis based on transmission/disequilibrium test (Hensen *et al*. 2003).

The clinical impact of gene discovery, and with it identification of disease-specific biological pathways, is potentially immense (Barker 2001). Resolving the system of cytokines and chemokines playing a central role in the disease process, is an ongoing challenge which is expected to provide novel therapeutic targets for treatment of psoriasis (Gudjonsson *et al*. 2004).

Cytokines

In the epidermis, keratinocytes, melanocytes, and dendritic Langerhans cells act in conjunction with dermal elements such as mast cells, tissue macrophages, granulocytes, dermal dendritic cells, and fibroblasts to deliver skin immunity. Such communication and interaction between these cells is done through the diverse group of molecules of cytokines, which are soluble glycoproteins produced by different cell types in all organs. They are

classified as interleukins, colony-stimulating factors, interferons, and tumor necrosis factors. Subtle changes in cytokine expression can cause modulation of immune response or inflammation. Cytokines can induce or inhibit the production of other cytokines, and one of the central roles of cytokines is to regulate cell differentiation and growth (Weiss *et al*. 2004). There is increasing evidence to suggest that IL-10 family cytokines IL-19 and IL-20 have a role in the function of epidermis and in psoriasis (Romer *et al*. 2003, Blumberg *et al*. 2001).

Blumberg *et al*. (2001) mapped IL-10 family genes to a 195 kb region on 1q31-q32, and deduced the gene order as *IL-10*, *IL-19*, *IL-20* and *IL-24*. Such data indicate that four members of the IL-10 family comprise a cytokine cluster (Blumberg *et al*. 2001). Functional relevance of the *IL-10* gene polymorphisms has been demonstrated by their involvement in determining susceptibility and severity to a number of immune-inflammatory diseases. Protective effect of *IL-10* gene microsatellite polymorphism against psoriasis has been shown by Hensen *et al*. (2003). Kingo *et al*. (2004) showed that SNPs of the *IL-20* gene could have a role in determining the susceptibility to plaque-type psoriasis, but no polymorphisms of *IL-19* gene had been investigated in complex diseases.

The predicted helical structure of interleukins IL-10, IL-19 and IL-20 is conserved but certain receptor-binding residues are variable and define the interaction with specific heterodimers of different type 2 cytokine receptors, leading to diverse biological effects. Signalling through the heterodimeric cytokine receptor family type 2 (CRF 2) complexes (Langer *et al*. 2004) involves the activation of Jak kinases and the phosphorylation of signal transducer and activator of transcription (STAT) factors, which induce γ-activated sequence-dependent or otherwise STAT-dependent transcription (Fickenscher *et al*. 2002).

Romer *et al*. (2003) found IL-19 and IL-20 being expressed in psoriatic lesions, while no expression of IL-19 and IL-20 was detected in uninvolved psoriatic skin. Similarly, they found expression of receptor subunits IL-20Rα and IL-20Rβ in psoriatic skin with lower expression in uninvolved skin. These findings demonstrate IL-19 and IL-20 having pathogenic role in psoriasis (Romer *et al*. 2003).

Interleukin-19

Interleukin-19 (IL-19) was first characterised by Gallagher *et al*. (2000) in result of a study where they screened sequence databases for interleukin-10 homologs. IL-19 was identified as a secreted protein and a cytokine based on its production by immune cells, its ability to be secreted from cells, and its capacity to induce Jak-STAT signal transduction pathway through a specific receptor complex (Gallagher *et al*. 2000, Dumoutier *et al*. 2001). The transcription

of the *IL-19* gene has been detected in granulocyte-macrophage colony-stimulating factor (GM-CSF) and lipopolysaccharide (LPS) activated monocytes and resting monocytes and, at lower level, in B cells (Wolk *et al*. 2002).

IL-19, novel homolog of IL-10 is mapped to same region of 1q31-32 with IL-10, being positioned next to *IL-10* towards telomere. *IL-10* and *IL-19* are transcribed in opposite directions − while *IL-10* is being transcribed towards centromere, *IL-19* is transcribed towards telomere. Initially, *IL-19* seemed to comprise of five exons similarly to *IL-10*, but Liao *et al*. (2002) showed *IL-19* having two additional exons 5' from the first coding exon found earlier and confirmed suggestion of Gallagher *et al*. (2000) that alternative splicing or promoter usage occurs in the 5' region of the *IL-19*. Regardless of many similarities in the structure of IL-10 and IL-19, it was also reported that considering differences in the region which is interacting with IL-10 receptor, IL-19 would not likely use same receptor complex with IL-10 (Gallagher *et al*. 2000).

Indeed, IL-19 was shown to bind to receptor complex IL-20Rα/IL-20Rβ, which belongs to CRF 2 family, as simultaneous presence of both receptor subunits in a cell is required for IL-19 activity (Dumoutier *et al*. 2001) and such binding results in rapid activation of the transcription factor STAT3 (Gallagher *et al*. 2004). The expression of the IL-19 receptor subunits, IL-20Rα and IL-20Rβ, has been found to be up-regulated in psoriatic skin, whereas normal skin has low levels of receptor expression. Since the same receptor complex is shared by both IL-19 and IL-20, and also IL-24, it is suggested that IL-19 might have overlapping biological activities with IL-20 and may be involved in psoriatic lesions.

Unlike IL-10, protein of IL-19 was found to be monomer both in the solution and in the crystal. Such finding suggests that IL-19 and other cytokines structurally distinct from IL-10, should be regarded as separate subgroup of IL-10 family cytokines (Chang 2003). Hence, Gallagher *et al*. (2004) suggested dividing IL-10 family cytokines into two subgroups based on receptor-chain sharing between the cytokines − IL-10 subgroup comprising of IL-10, IL-22 and IL-26, and subgroup of IL-19 comprising of IL-19, IL-20 and IL-24. The three members of IL-19 subfamily are all able to bind to and signal through the receptor originally defined as IL-20 receptor (Gallagher *et al*. 2004).

Interleukin-20

Interleukin-20 (IL-20) was first identified and characterized by Blumberg *et al*. (2001). Like in case of discovery of IL-19, IL-20 also was found in database search using special algorithm aimed at finding new IL-10 homologs. Analysis of the entire coding sequence of IL-20

confirmed IL-20 classifying as a helical cytokine and also confirmed IL-20 being a member of IL-10 family of cytokines. IL-20 was found to be expressed at very low levels in skin, trachea, and in other tissues (Pestka *et al*. 2004).

Alike with IL-19, IL-20 receptor complex IL-20Rα/IL-20Rβ was found to be required for IL-20-mediated STAT3 activation (Blumberg *et al*. 2001), but in addition to IL-20 binding to IL-20Rα/IL-20Rβ receptor complex which it shares with IL-19 and IL-24, IL-20 also binds to IL-22R/IL-20Rβ complex which is shared with IL-24 but not with IL-19 (Dumoutier *et al*. 2001). The IL-20 heterodimeric receptor is structurally similar to other class 2 cytokine receptors and is expressed in skin where the activity of the IL-20 ligand has also been demonstrated. Both IL-20Rα and IL-20Rβ mRNAs are markedly up-regulated in human psoriatic skin compared to normal skin, providing evidence for them having role in psoriasis (Blumberg *et al*. 2001).

Histological analysis of transgenic mice overexpressing both human and mouse IL-20, showed thickened epidermis and hyperkeratosis among other skin abnormalities which characterize psoriatic skin (Blumberg *et al*. 2001).

Goal of the study

In a previous study the LD pattern within the SNPs of *IL-20* gene, and association analysis was conducted in respect to the their involvement in susceptibility to psoriasis. The goal of this study was to extend the association study by including the SNPs of the *IL-19* gene, which is preceding *IL-20* in the IL-10 family cluster of genes − to describe the linkage disequilibrium pattern within *IL-19* and *IL-20* gene SNPs and determine the effect of extended haplotypes in susceptibility to psoriasis.

Materials and methods

Ethical consideration

The Ethics Review Committee on Human Research of University of Tartu approved the study protocols and the informed consent forms. All participants signed the written informed consent.

Study participants

All subjects who participated in this study − patients with psoriasis and healthy control subjects − were Caucasians living in Estonia.

Unrelated patients with plaque-type psoriasis from the Department of Dermatology, University of Tartu, were divided into subgroups according to the age of disease onset and family history of psoriasis. Patients with disease onset below the age of 40 years were assigned as early onset psoriasis (n=180). The group included 94 male and 86 female subjects and mean age of the group was 20 years. Patients with onset of the disease at the age of 40 years and later were assigned as late onset disease (n=74). The group of late onset psoriasis included 41 male and 33 female subjects and mean age of the group was 53 years. The age of disease onset was defined as age when patients first became aware of psoriatic skin lesions. Dividing patients into subgroups based on familial or sporadic form of the disease, patients were considered to have familial psoriasis if they had at least one first- or second-degree relative with psoriasis (n=101), and to have sporadic psoriasis if they had no known relatives with psoriasis (n=153). Based on classification initiated by Henseler *et al*. 1985, patients were also divided into subgroups of type I and type II psoriasis − patients were assigned to subgroup of type I psoriasis (n=87) if they had early onset of the disease and familial psoriasis, and to subgroup of type II psoriasis (n=61) if they had late onset late onset of the disease and sporadic form of psoriasis.

Control group (n=148) comprised of healthy volunteers, free from positive family history of psoriasis, and included 57 male and 91 female subjects. Individuals with a history of other dermatoses were not included in the control group.

DNA isolation

Whole blood (approximately 14 ml) from psoriasis patients and controls was collected in disodium EDTA containers. Genomic DNA was extracted from the whole blood. Standard

protocol was used by which firstly the cell membranes were lysed, then the nuclei were lysed and thereafter DNA was precipitated by ethanol. Formed precipitate was washed with 70% ethanol and then dissolved in Tris/EDTA. Average yield of the DNA isolation varies from 80 ng/µl to 260 ng/µl, which is admissible for PCR amplification.

Database search to select SNPs

To map and analyze the disease loci we used bi-allelic SNP markers because of their high variability and abundance – number of SNPs with occurrence frequency of minor allele above 10% in human population is expected to be 5.3 million (Kruglyak *et al*. 2001). We used reference sequence NT_021877 to investigate SNPs of *IL-19* gene. Using the list of all possible SNPs, positioned either within the gene or in adjacent 5' or 3' region, to conduct subsequent investigation of the SNPs. We used publicly available dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/) to determine the validation statuses of the SNPs, and within the validated SNPs we determined their exact chromosomal positions and previously established allelic frequencies. To compose final set of SNPs we selected SNPs with minor allele frequency at least above 10%, preferably above 20%. The next criteria in selecting SNPs was chromosomal position from adjacent SNPs with preferred distance 1,000 bp and above. Although our primary intention was to analyze SNPs spread evenly across the gene and adjacent regions, the SNPs matching our criteria, were confined to a shorter 3' region of the *IL-19* gene. Hence, the set of selected SNPs for analyzing was following − rs2243158, rs2243168, rs2243191, rs2073186, rs2243174, rs2243188, and rs2243193.

Primer design

To design primers for selected SNPs we used publicly available online software − http://cedar.genetics.soton.ac.uk/public_html/primer1.html, which is was developed and is provided by Ye *et al*. (2001). This program requires user to enter the nucleotide sequence with the SNP of interest within it. Maximum length of the sequence is 1,000 bp, and the longer the entered sequence is, the better chance it is to find the best set of primers for the SNP. The form of the program also requires to specify the position of the SNP within the entered sequence, and two allelic forms of the SNP. Although the program allows user to customize number of other parameters, like minimum/maximum/optimum primer and amplicon size, primer T_m and GC content, *et cetera*, we would recommend leaving those parameters to their defaults as they are already optimal, except for the number of outputs, which will give better selection of primer sets when changed from 10 to 30 (or any other convenient number higher

than 10). While choosing from the list of primer sets generated by the software, we tried avoiding sets, if possible, in which the difference in estimated melting temperatures of the primers exceeded 6°C or in which the estimated melting temperature was 72°C or higher, to prevent complications in optimizing PCR reaction for the set of primers. Additionally, we used BLAST search to analyze the potential primer set against human genomic sequence, and excluded sets, in which one or more primers were likely to anneal to several unspecific locations across the human genome, since unspecific annealing is expected to competitively inhibit the production of desired/specific amplicon.

Tetra-primer ARMS-PCR

For detecting the allelic variations of *IL-19* gene SNPs in the samples, we used a tetra-primer Amplification Refractory Mutation System-Polymerase Chain Reaction (tetra-primer ARMS-PCR) which is novel rapid, high throughput and low cost method for analyzing bi-allelic SNPs (Ye *et al*. 2001). Same method was used for genotyping the *IL-20* gene polymorphisms (Kingo *et al*. 2004). With such method, a set of four oligonucleotide primers is used to amplify the two different alleles of a SNP in a single reaction. Either of the allele-specific amplicons are generated using one allele-specific inner primer and one non-allele-specific outer primer of opposite direction. Allele-specificity of inner primers is determined by last nucleotide in 3' terminus of the primer − if the 3' terminal nucleotide matches the nucleotide in the same position on genomic DNA sequence, proper annealing of the primer onto template DNA can occur allowing extension of the primer and thereby generating the allele-specific amplicon, whereas in case of a 3' mismatch the proper annealing and extension of the primer are obstructed. In order to enhance allele specificity, a second mismatch at position -2 from the 3' terminus is also incorporated in both inner primers. Outer primers are used to generate non-allele-specific control product, which itself is also adequate template for allele-specific amplicons.

Figure 1 Selected SNPs are illustratively shown on the *IL-19* and *IL-20* genes (represented as striped boxes of horizontal bar, representing noncoding region, whereas dark stripes on the genes represent exons). SNPs are represented by their ID numbers in the public SNP database. Allelic variations are shown for each SNP next to the SNP ID, and distances with adjacent SNPs are shown below.

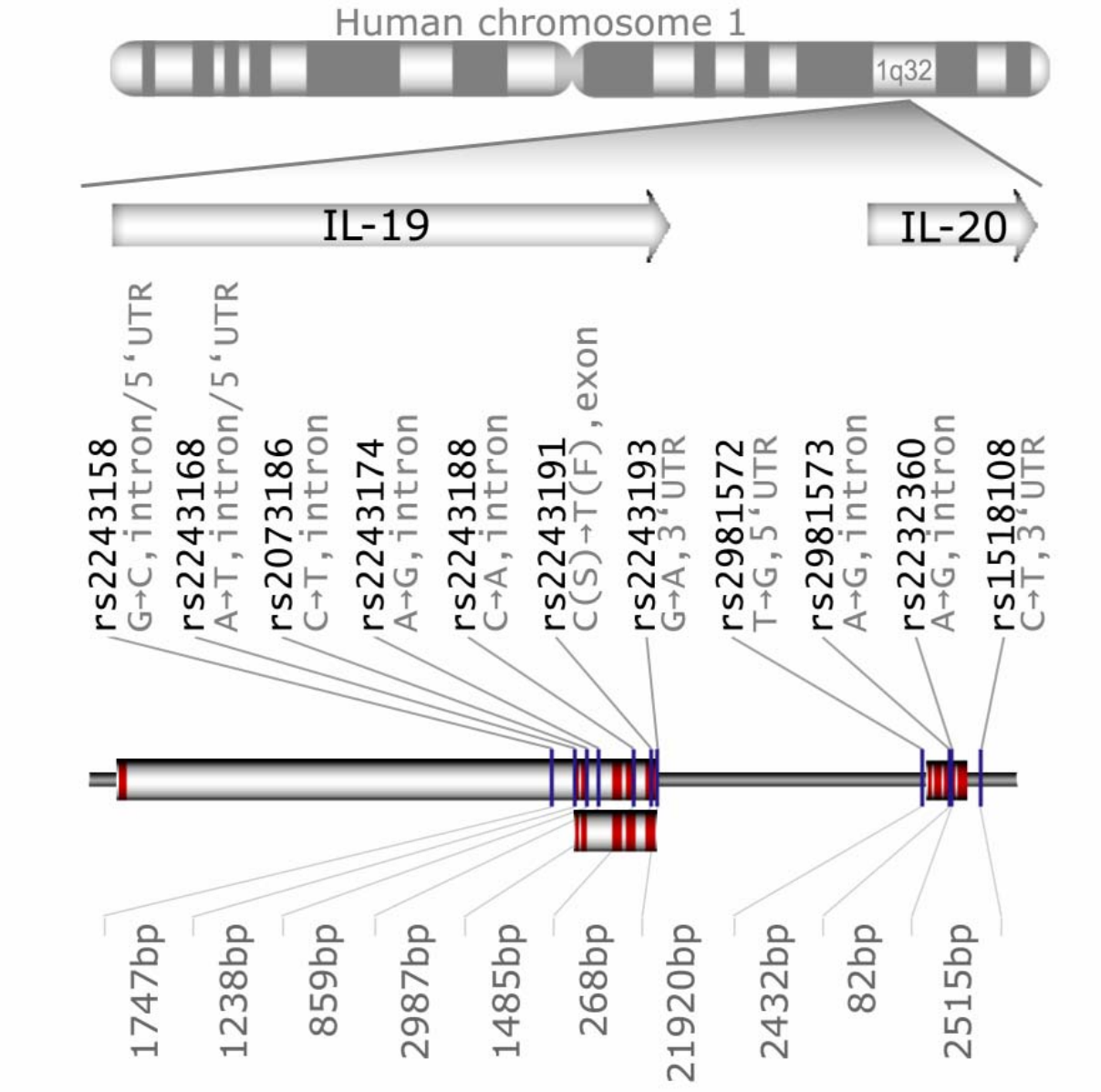
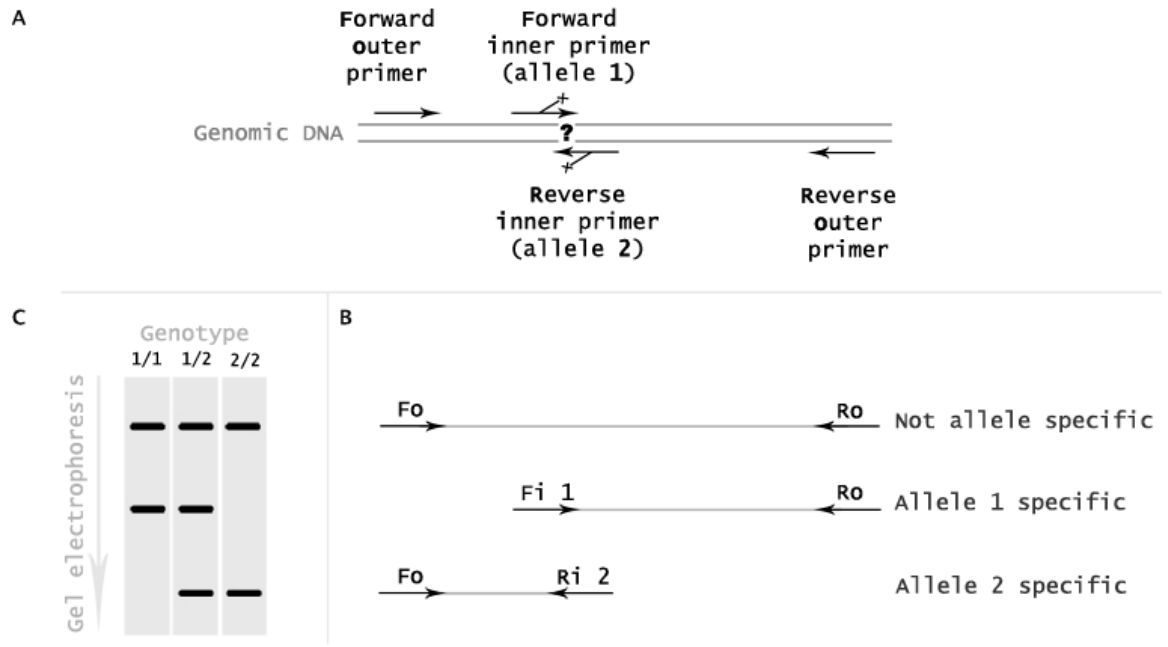


Figure 2 Schematic presentation of the tetra-primer ARMS-PCR method. A) This method employs set of four primers to detect two different alleles of a Single Nucleotide Polymorphism (SNP; shown as '?'). Two inner primers are designed to match two different alleles of the SNP. In case the 3' nucleotide of one of the inner primers matches the allelic variant of the SNP, annealing of the respective inner primer to template DNA will be sufficient to amplify the PCR product of the respective inner primer and opposite outer primer (B). In case the 3' nucleotide of the inner primer is different from what is presented at the respective position of genomic DNA, the annealing will be insufficient and the allele specific amplicon can not be synthesized. Outer primers are needed to amplify the allele specific products by being counter parts to opposite inner primers but additionally the outer primers also amplify the specific region surrounding the SNP, thus giving it an advantage over non-specific PCR products. C) As the three different amplicons are designed to differ in length, they can be discriminated by gel electrophoresis.



PCR reaction conditions

Each PCR reaction was carried out in a total volume of 20 µl, containing approximately 100 ng of template DNA, 20 pmol of each inner primer, 2 pmol of each outer primer, 0.2 mM dNTP, 1×PCR reaction buffer (75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 0.01 mg/ml Tartrazine, 1% Sucrose; Naxo, Tartu, Estonia) and 0.5 U Smart-Taq DNA polymerase (Naxo, Tartu, Estonia). Concentrations of $MgCl_2$ and annealing temperatures, optimized separately for each set of primers, and sequences of the primers which were used to genotype the respective polymorphisms of *IL-19* gene, are shown in Table 1.

As advised by Ye *et al*. (2001) to increase the specificity, the thermal cycling program included touchdown cycles − initial denaturation at 95°C for 2 min followed by 10 cycles of denaturation at 95°C for 1 min, annealing at temperature 10°C higher than the annealing temperature of main cycles for 1 min (decreasing by 1°C per cycle) and extension at 72°C for 1 min. Following 25 main cycles were performed using constant annealing temperature (Table 1), and final extension was performed at 72°C for 10 min.

Direct sequencing

We used direct sequencing of incidental DNA samples to confirm the results obtained from tetra-primer ARMS-PCR, and to simultaneously validate the SNP under investigation. Product of the outer primers of appropriate SNP set was used as template. Direct sequencing was performed using ABI Genetic Analyzer 310 (Applied Biosystems, forster City, CA, USA). The results of the tetra-primer ARMS-PCR were completely consistent with the results of direct sequencing.

Visualizing the PCR products

The three possible amplicons synthesized in the PCR reaction differ in sizes and can therefore be discriminated on agarose gel-electrophoresis. We used 2% agarose (agarose from Naxo, Tartu, Estonia) gel with TBE buffer (Tris-Borate-EDTA; Naxo, Tartu, Estonia). PCR products, separated on agarose gel-electrophoresis and stained with ethidium bromide (Naxo, Tartu, Estonia), were visualized and photographed under UV illumination.

Table 1 Primers and conditions for tetra-primer ARMS-PCR used for *IL-19* genotyping.

Genetic polymorphism	Primers (5' - 3')	T_m	Annealing temperature	Mg^{2+}	Amplicon size
rs2243158 G/C	Forward inner primer (C allele): GGT GGA TCC ACC CAG CAA ACC TTC AC	72˚C	68˚C	2,5 mM	487 bp - control
	Reverse inner primer (G allele): TTT TAT TCA GGT GGA TAA GAG GAA ATG GTC	66˚C			290 bp - G allele
	Forward outer primer: GCC ACA GCT CTC AGG AAA GTG ACC TAA G	69˚C			253 bp - C allele
	Reverse outer primer: CCA GCA TCT GGA ACA TCA TAG CCA TAC A	69˚C			
rs2243191 C/T	Forward inner primer (C allele): CTG GAT TAA TAA GAA TCA TGA AGT AAT TTC	59 ˚C	60˚C	2,5 mM	436 bp - control
	Reverse inner primer (T allele): ATA CAG GTT CCT TGT CAT CAA GCT GCG A	70˚C			279 bp - T allele
	Forward outer primer: CCT GCG AGG AAA ATA ATA TTG AGT CTG T	64˚C			215 bp - C allele
	Reverse outer primer: CAC AGT AGA CTT TTT GGC TAT TTT CAA GC	64˚C			
rs2243168 A/T	Forward inner primer (T allele): GGA AGT TGC CAA GCT GCC CTC TAT CT	69˚C	60˚C	3,5 mM	328 bp - control
	Reverse inner primer (A allele): CAA TAA GGA GCT AGG GGA AGA AGC CGA T	69˚C			215 bp - T allele
	Forward outer primer: AGA AGG GTA AGA GAA TGA GAA GCG GTG G	69˚C			167 bp - A allele
	Reverse outer primer: TGG TTT TTG ATG TTT GCC CCT GAA ATA A	69˚C			
rs2073186 T/C	Forward inner primer (C allele): AGG TGC TCA GAG GGG ACA GGA TTG CC	73˚C	65˚C	2,5 mM	295 bp - control
	Reverse inner primer (T allele): ACC TTC CAA AAT TAC CCC CAA GCC CCA	73˚C			209 bp - C allele
	Forward outer primer: TGT TAG GGC ACG CTA GTG TCC CAG GGA TA	73˚C			139 bp - T allele
	Reverse outer primer: TCC TTT GGG TCA CAA ACC TGG TCA CCT C	73˚C			
rs2243174 A/G	Forward inner primer (A allele): TGG TGC TGT TCT TAC AAT GGA CAC CA	68˚C	60˚C	2,5 mM	407 bp - control
	Reverse inner primer (G allele): GAT CTT GTC ACT GGC TTT CCT GCC TAA C	68˚C			272 bp - G allele
	Forward outer primer: TTG GAA TCC CTG GGA GGA ATT AAA GAA G	68˚C			189 bp - A allele
	Reverse outer primer: AGA ATA CTT CCC AGG ACT GGA GGA GCT C	68˚C			
rs2243188 C/A	Forward inner primer (C allele): TGG GGA AGA TGG AAG ATG AGA GGT AGA CCC	72˚C	65˚C	2,5 mM	366 bp - control
	Reverse inner primer (A allele): AGG AGG CTG AAG GCC TGG CAC CCT TCT	76˚C			238 bp - A allele
	Forward outer primer: AAT GTC ACT TCT CAT GTG GGG AGG CAG GA	74˚C			185 bp - C allele
	Reverse outer primer: AAT GAT GGA GAT GGG GAG CCC AGG AGA T	74˚C			
rs2243193 G/A	Forward inner primer (A allele): AAG GGC TGC CTT CCC ATC TAA TTT ATT TTA	67˚C	60˚C	2,5 mM	414 bp - control
	Reverse inner primer (G allele): TCA CAT CAC AGA CAT GGA CTA TAT GAC GTC	67˚C			281 bp - G allele
	Forward outer primer: GGA ACC TGT ATA GTG ATC CAG GGA TGA A	67˚C			193 bp - A allele
	Reverse outer primer: CTG TCA GAA ACA CCC TGT CCT CAG TCT T	67˚C			

Statistical analysis

For statistical analysis of the genotype-phenotype association a package of population genetics software GENEPOP version 3.3 (Raymond 1995) was used. This is a freeware which can be downloaded (http://wbiomed.curtin.edu.au/genepop/genepop_3_3.zip) and installed on a personal computer and is also available in web-based version (http://wbiomed.curtin.edu.au/genepop/genepop_op1.html). With the help of this program, a variety of different population genetic parameters for co-dominant markers can be computed, including exact tests for Hardy-Weinberg equilibrium, population differentiation etc.

Haplotype-based association analysis was performed using the THESIAS program (available at http://www.genecanvas.org). This program is based on the maximum likelihood model (Tregouet *et al*. 2002) and being linked to the Stochastic-EM algorithm (Tregouet *et al*. 2004) it also allows the simultaneous estimation of haplotype frequencies and of their associated effects on the phenotype of interest. Same program was used to compute the pairwise LD matrix. The extent of disequilibrium was expressed in terms of standardized *D*' characteristic. An absolute value of *D*' of 1 indicates complete LD and 0 corresponds to no LD.

The significance level of the tests for declaring a probability value as significant was set to 0.05.

Results

Association, linkage disequilibrium and haplotype analyses of the IL-19 gene

For an association study of seven SNPs of *IL-19* gene − rs2243158, rs2243168, rs2243191, rs2073186, rs2243174, rs2243188 and rs2243193 − we used same study population that was used for association study of *IL-20* gene.

Genotype distributions of the seven analyzed *IL-19* gene polymorphisms had no deviation from Hardy-Weinberg equilibrium. Allele frequencies for both alleles of all studied SNPs of *IL-19* gene are shown in Table 2.

Comparing psoriatic patients with healthy controls, we established that the patients with psoriasis had a lower frequency of the SNP rs2243188 minor allele A (19.5% in patients *versus* 26% in controls, $p<0.05$), suggesting the minor allele having protective effect against susceptibility to psoriasis. We also observed lower representation of the minor allele A at this position in patients with late onset psoriasis (16.0% in late onset psoriasis patients *versus* 26.0% in controls, $p<0.02$) and in sporadic psoriasis (19.3% in sporadic psoriasis patients *versus* 26% in controls, $p<0.05$). There were also fewer persons with early onset and with familial type of psoriasis possessing the minor allele A of the SNP rs2243188, but the differences were not statistically significant. We established SNP rs2243158 being associated with type II phenotype of psoriasis as the prevalence of minor allele C at position rs2243158 was significantly higher in controls compared to patients with type II phenotype (10.1% in controls *versus* 4.1% in patients, $p<0.05$). The prevalence of the minor allele T at position rs2243168 was significantly higher in controls than in he subgroup of patients with late onset of psoriasis (8.8% in controls *versus* 3.3% in patients, $p<0.05$) and in the subgroup of patients with type II phenotype (8.8% in controls *versus* 2.5% in patients, $p<0.02$). Therefore, SNPs rs2243158 and rs2243168 represent potential subtype specific markers. All other SNPs of *IL-19* gene − rs2073186, rs2243174, rs2243191, and rs2243193 − resulted in negative findings for both allele distributions when comparison between patients and controls was performed.

To investigate whether the individual protective effects of the IL-19 polymorphisms, observed in single-marker association analysis, depends on the haplotypic background by which they are carried, linkage disequilibrium (LD) and haplotype analyses of the *IL-19* gene were executed. The pairwise LD matrix revealed that nearly complete LD ($|D'|$ between 0.88

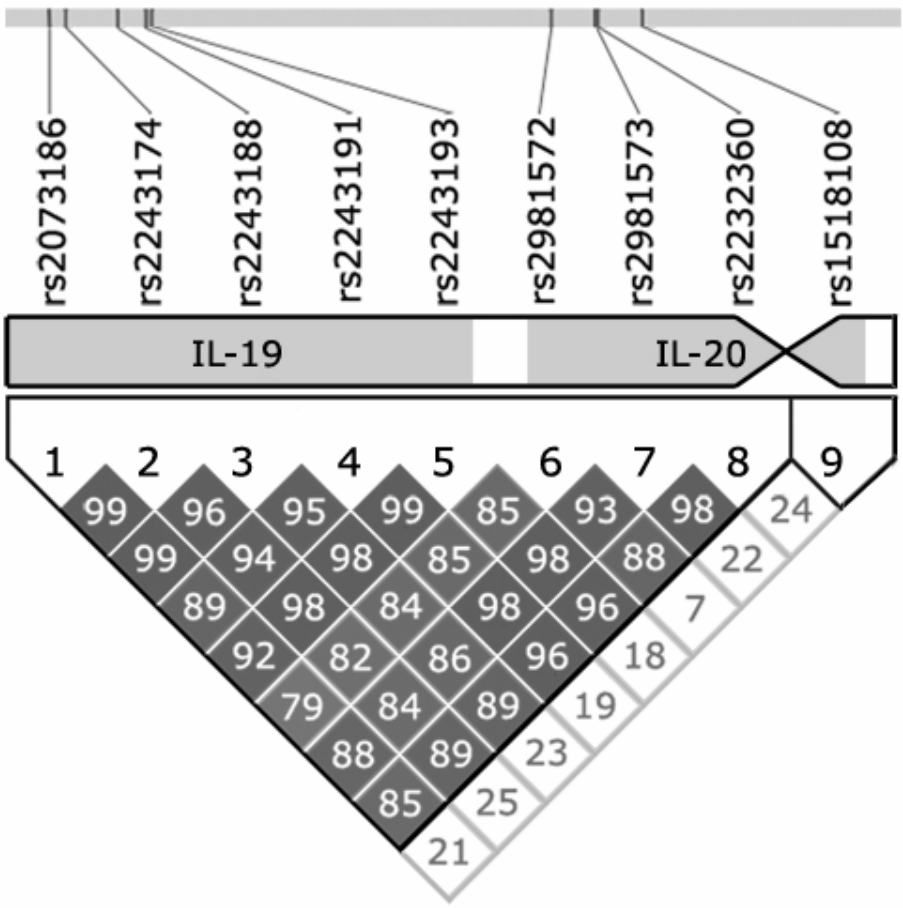
and 0.99) existed between the SNPs of the *IL-19* gene (Figure 3). SNPs rs2243158 and rs2243168 were excluded from the further haplotype analysis as the frequencies of minor alleles of these SNPs were lower that 10%. Using five remaining SNPs with minor allele frequencies above 10% − rs2073186, rs2243174, rs2243188, rs2243191, and rs2243193 − we established presence of five common haplotypes with frequencies ≥1% − HT1 CACCG, HT2 TGATA, HT3 CACTA, HT4 TAATA, and HT5 TACCG. These five most frequent haplotypes accounted for 97.7% of all haplotypes in the pooled samples. When frequencies of the haplotypes were compared between healthy controls and patients with psoriasis, we found the control group having a higher frequency of the haplotype HT2 TGATA, but the difference was not statistically significant (p=0.09, OR 0.737, 95% CI 0.516-1.053). However, statistical significance was found when control group was compared with subgroup of patients having late onset of psoriasis, with haplotype HT2 TGATA being more frequent in controls (p=0.05, OR 0.58, 95% CI 0.335-1.000). The frequencies of haplotypes in control group and patients with late onset of psoriasis, and p-values are shown in Table 3.

Table 2 Results of association analysis of *IL-19* gene SNPs in plaque-type psoriasis. Allele frequencies of the seven studied SNPs of *IL-19* gene were compared between the psoriatic patients (n=154) and the control group (n=148), p-values for allelic association were calculated using Fischer's exact test. The significance level for all statistical tests was 0.05.

SNP ID	Polymorphism		Allele frequency in controls (%)		Allele frequency in patients (%)		p-value
	1[a]	2[b]	1[a]	2[b]	1[a]	2[b]	
rs2243158	G	C	89.9	10.1	91.9	8.1	0.36
rs2243168	A	T	91.2	8.8	93.3	6.7	0.33
rs2073186	C	T	73.0	27.0	78.3	21.7	0.08
rs2243174	A	G	76.7	23.3	81.1	18.9	0.14
rs2243188	C	A	74.0	26.0	80.5	19.5	0.029*
rs2243191	C	T	74.0	26.0	78.0	22.0	0.23
rs2243193	G	A	73.0	27.0	77.0	23.0	0.21

* − $p<0.05$ allelic differences compared to controls
1[a] – most frequent allele
2[b] – least frequent allele

Figure 3 The extent of LD values within the fragment of human chromosome 1 region q32, encompassing genes *IL-19* and *IL-20*. Vertical stripes on the upper band represent SNPs (distances between the stripes are in proportion with the actual distance between the SNPs) with SNP IDs shown below the band. SNPs rs2243158 and rs2243168 were excluded from further haplotype analysis and therefore also from this figure because of minor allele frequency below 10%. The broader band is added to illustrate to which gene the SNPs are positioned (gene areas on the broad band do not reflect the actual length of the genes). The narrowness occurring on the broad band in the region of *IL-20* gene, represents a very low LD value between the two SNPs, suggesting occurrence of recombination hot-spot in that area. |*D*'| values on the squares have been multiplied by 100 for better visibility of the image.



Associations of combined IL-19 and IL-20 haplotypes with psoriasis

Previously, Kingo *et al*. (2004) analyzed four SNPs of *IL-20* gene − rs2981572, rs2981573, rs2232360, and rs1518108 − and demonstrated that nearly complete LD (|*D*'| between 0.879 and 0.985) was present within the first three of the SNPs (rs2981572, rs2981573, and rs2232360). The fact that *IL-19* gene maps next to the *IL-20* gene on human chromosome 1 region q32, gives reason to anticipate high LD between these genes. Hence, we performed LD analysis of IL-19 and IL-20 SNPs to examine the linkage disequilibrium between the SNPs of these genes. The pairwise LD matrix of the IL-19 and IL-20 SNPs showed all studied SNPs,

except SNP rs1518108 of *IL-20* gene, were in significant LD with each other (|*D*'| between 0.78 and 0.99) (Figure 3).

Subsequently, eight-marker haplotype analysis with five SNPs across the *IL-19* gene − rs2243191, rs2073186, rs2243174, rs2243188, and rs2243193 − and three SNPs across the *IL-20* gene − rs2981572, rs2981573, and rs2232360 − was performed. We observed five common haplotypes with frequencies ≥1% − HT1 CACCGTAA, HT2 TGATAGGG, HT3 CACCGGAA, HT4 CACTAGGG, and HT5 TAATAGGG. These haplotypes account for 91.86% of all possible marker combinations in pooled samples. Results of extended haplotype analysis of the *IL-19* and *IL-20* genes in control group and patients with psoriasis, are shown in Table 4. We found that patients with psoriasis had a significantly higher frequency of the haplotype HT3 CACCGGAA ($p<0.01$, OR 2.548, 95% CI 1.379-4.706) when compared with the control group. Same, HT3 CACCGGAA haplotype was also associated with an increased risk of early onset of psoriasis ($p<0.02$, OR 2.225, 95% CI 1.175-4.213) and late onset of psoriasis ($p<0.05$, OR 2.467, 95% CI 1.1258-5.405), and also with familial psoriasis ($p<0.02$, OR 2.424, 95% CI 1.199-4.903) and sporadic psoriasis ($p<0.01$, OR 2.877, 95% CI 1.478-5.601). This association mainly reflects a significant individual effect of G allele at position rs2981572 of *IL-20* gene ($p<0.01$, OR 2.548, 95% CI 1.379-4.706). No significant association was observed comparing patients with controls in relation to other four common haplotypes − HT1 CACCGTAA, HT2 TGATAGGG, HT4 CACTAGGG, and HT5 TAATAGGG.

The protective effect of the *IL-19* gene haplotype TGATA on late onset of psoriasis did not withstand after the extended haplotype analysis of *IL-19* and *IL-20* genes. The frequency of combined haplotype TGATAGGG did not differ significantly ($p=0.34$, OR 0.722, 95% CI 0.367-1.422) among patients with late onset of psoriasis and control group. Detailed results of the haplotype analysis of *IL-19* gene and combined haplotype analysis of *IL-19* and *IL-20* genes among patients with late onset of psoriasis and control group are given in Table 3.

Table 3 Results of IL-19 haplotype analysis and extended haplotype analysis of the *IL-19* and *IL-20* genes in patients with late onset psoriasis. The haplotype frequencies (%) and haplotypic OR-s with their 95% CI-s and p-values by comparison to the reference haplotype in late onset psoriasis patients compared with controls are indicated. Haplotype frequencies and haplotype-phenotype associations were estimated using maximum likelihood method.

IL-19 haplotypes	**Controls (n=148)**	**Late onset psoriasis patients (n=74)**	**Haplotypic OR (95% CI)**	**p-value**
CACCG	70.7	77.3	[a]	
TGATA	21.8	13.4	0.581 (0.335-1.000)	0.05
TAATA	2.7	0.6	0.182 (0.022-1.501)	0.11
CACTA	1.0	3.2	2.690 (0.549-13.17)	0.22
TACCG	1.4	1.3	1.580 (0.277-8.999)	0.61
IL-19 and IL-20 combined haplotypes				
CACCGTAA	63.5	61.1	[a]	
TGATAGGG	16.7	11.8	0.722 (0.367-1.422)	0.34
CACCGGAA	5.9	14.2	2.467 (1.126-5.405)	0.02
TAATAGGG	2.7	0.7	0.260 (0.031-2.146)	0.21
TACCGTAA	2.4	0.7	0.318 (0.035-2.869)	0.31

[a] – The haplotype combining the most frequent alleles at each site is chosen as the reference haplotype.

Table 4 Results of extended haplotype analysis of the *IL-19* and *IL-20* genes in patients with plaque-type psoriasis. The haplotype frequencies (%) and haplotypic ORs with their 95% CIs and p-values by comparison to the reference haplotype in psoriasis patients compared with controls are indicated. Haplotype frequencies and haplotype-phenotype associations were estimated using maximum likehood method.

Haplotype	rs2073186	rs2243174	rs2243188	rs2243191	rs2243193	rs2981572	rs2981573	rs2232360	Controls (n=148)	Psoriasis patients (n=254)	Haplotypic OR (95% CI)	p-value
HT1	C	A	C	C	G	T	A	A	64.6	60.3	[a]	
HT2	T	G	A	T	A	G	G	G	17.4	15.5	0.947 (0.639-1.403)	0.78
HT3	C	A	C	C	G	G	A	A	5.6	13.5	2.548 (1.379-4.706)	<0.01
HT4	C	A	C	T	A	G	G	G	1.4	2.0	1.597 (0.420-6.073)	0.49
HT5	T	A	A	T	A	G	G	G	2.4	0.8	0.342 (0.100-1.172	0.09

[a] − The haplotype combining the most frequent alleles at each site is chosen as the reference haplotype.

Discussion

IL-10 itself possesses a multitude of effector functions, and its immunoregulatory properties suggest a possible role in the initiation or resolution of various skin diseases (Weiss *et al*. 2004). Novel cytokines , IL-19 and IL-20 along with IL-24, have been mapped to same region with IL-10 − 1q31-q32, comprising cluster of IL-10 family cytokines (Blumberg *et al*. 2001). The IL-10 gene cluster has shown to have role in number of chronic pathological processes (Shin *et al*. 2000, Johanneson *et al*. 2002). This cluster has previously been associated with psoriasis through a microsatellite marker of IL-10 (Hensen *et al*. 2003). While the central role of IL-10 in immunity has been well established (Weiss *et al*. 2004), but no polymorphisms of *IL-19* and *IL-20* genes had been investigated in complex diseases. It has been elucidated by Blumberg *et al*. (2001) that IL-20 and its receptor complex play a part in epidermal function by regulating keratinocytes proliferation and differentiation, and by overexpressing IL-20 in transgenic mice, the mice had skin abnormalities similar to psoriatic skin. Receptor complex IL-20Rα/IL-20Rβ is shared by IL-19 and IL-20, suggesting existence of overlapping biological activities of IL-19 and IL-20. Expression of both of the IL-20Rα/IL-20Rβ receptor complex subunits has been shown to be up-regulated in psoriatic skin (Blumberg *et al*. 2001, Romer *et al*. 2003). Such data suggest that IL-19 and IL-20 may have a pathogenic role in psoriasis.

Results of previous study by Kingo *et al*. (2004) proved an association of the *IL-20* gene GAA haplotype in patients with plaque-type psoriasis. Taking this into consideration, we hypothesized that association and haplotype analyses of the *IL-19* gene and extended haplotype analysis across a region encompassing the *IL-19* and *IL-20* genes might indicate additional markers of psoriasis susceptibility.

Association analysis of the *IL-19* gene demonstrated that minor alleles of *IL-19* gene SNPs − rs2243158, rs2243168, and rs2243188 − revealed protective effect to psoriasis, especially to late onset psoriasis and type II psoriasis. Protective cytokine loci have been shown to have a complex genetic basis in several diseases (Smith *et al*. 2004). Data of the present study in accordance with association analysis in previous study by Kingo *et al*. (2004) reveal that different loci in the q32 region of human chromosome 1 have different effects in susceptibility to psoriasis.

We used an indirect approach of an association study, meaning testing polymorphisms under the assumption that if a risk polymorphism exists, it either will be genotyped directly or by way of strong LD with one of the genotyped polymorphisms (Collins *et al*. 1997). Several

studies confirm that single SNPs do not represent the primary basis of the disease and rather SNP combination should be considered. Pairwise LD matrix of the *IL-19* gene SNPs revealed that nearly complete linkage disequilibrium was present within the polymorphisms of *IL-19* gene. Linkage disequilibrium (LD) matrix reflects non-random association of alleles at selected markers. Subsequent pairwise LD matrix of SNPs of *IL-19* and *IL-20* genes showed that *IL-19* and *IL-20* genes form one block of LD, except for IL-20 SNP rs1518108 which has been found to form another block of LD with SNPs of *IL-24* gene (Koks *et al*. 2005). Block of LD is a discrete chromosome region of high LD and low haplotype diversity, separated by possible hotspots of recombination and a breakdown of LD.

The novelty of this study lies in the extension of the risk haplotype already described in *IL-20* gene (Kingo *et al*. 2004) with new SNPs within the proximal IL-19 gene. Estimating extended haplotype-phenotype association of *IL-19* and *IL-20* genes, we established haplotype HT3 CACCGGAA being associated with an increased risk of psoriasis, reflecting the possible role of this haplotype in determining susceptibility to plaque-type psoriasis. Haplotype analysis of the *IL-19* gene indicated significant protective effect of the TGATA haplotype in case of late onset psoriasis. However, combined haplotype analysis of *IL-19* and *IL-20* genes demonstrated that the protective effect of *IL-19* gene is secondary to the susceptibility effect of *IL-20* gene. Herein, we did not analyze functional significance of the IL-19 and IL-20 haplotypes. Persons with different haplotypes may have different expression levels of IL-19 and IL-20 as it has been shown for IL-10 (Crawley *et al*. 1999). Hence, it is reasonable to expect that haplotypes described in this study, do determine the differences in IL-19 and IL-20 expression levels. Further studies are needed to support the functional role of the *IL-19* and *IL-20* genes in the pathogenesis of psoriasis.

While we have verified significant genetic influence of the studied region on the development of psoriasis, our goal for future studies is to analyze SNPs of other potential loci of psoriasis susceptibility, and to combine SNP and haplotype data with gene expression studies with the aim of moving from genotyping to functional studies.

Conclusion

We established nearly complete linkage disequilibrium of *IL-19* and *IL-20* genes and described five major haplotypes. We observed that extended *IL-19* and *IL-20* haplotype HT3 CACCGGAA is related to increased risk (OR 2.548) for psoriasis in sample of unrelated patients and controls. We were not able to confirm the protective effect of *IL-19* gene, which we found analyzing *IL-19* haplotypes, in context of extended haplotype analysis of *IL-19* and *IL-20* genes in plaque-type psoriasis. Family-based studies are needed to confirm the impact of *IL-19* and *IL-20* genes in susceptibility to psoriasis.

Summary in Estonian

Interleukiin-19 ja intereukiin-20 haplotüüpide efektid naastulise psoriaasi avaldumisel

Psoriaas on krooniline põletikuline dermatoos, millele tüüpiliste kliiniliste tunnuste (põletikulise infiltraadi, epidermise hüperproliferatsiooni ja keratinotsüütide diferentseerumise häire) kujunemisel on juhtiv roll T-rakkudel ning neist vabanevatel tsütokiinidel ja kemokiinidel. Interleukiin-19 (IL-19) ja interleukiin-20 (IL-20) on hiljuti avastatud IL-10 perekonda kuuluvad tsütokiinid ning mitmetes uuringutes on tõestatud nende olulisus epidermise funktsioonis ja psoriaasi kujunemisel. Nimetatud tsütokiine kodeerivad geenid paiknevad 1 kromosoomi pikas õlas q31-q32 lookuses. Antud regioonis paikneva *IL-10* geeniga on seotud mitmete autoimmuunsete haiguste avaldumine, sealhulgas ka psoriaasi perekondliku vormi avaldumine. Lisaks on varasemas uuringus näidatud, et *IL-20* geeni SNP-de rs2981572, rs2981573 ja rs2232360 alleelsetest variantidest kombineerunud GAA haplotüüp on riskihaplotüübiks psoriaasi avaldumisel ($p<0.01$, OR 2.341, 95% CI 1.346-4.074).

Käesoleva töö eesmärgiks oli uurida *IL-19* geeni ühenukleotiidsete polümorfismide (SNP-de) seoseid psoriaasiga. Kuna geen, mis kodeerib IL-19 produkti, paikneb *IL-20* geeni läheduses, viisime täiendavalt läbi *IL-19* ja *IL-20* geenide kombineeritud aheldus- ja haplotüübianalüüsi.

254 naastulise psoriaasiga haigel ja 148 tervel kontrollil analüüsisime seitset *IL-19* geeni SNP-d nelja praimeriga ARMS-PCR meetodil, mis võimaldab ühes PCR-i reaktsioonis korraga amplifitseerida kumbagi SNP alleeli.

Antud töö tulemused näitavad, et *IL-19* ja *IL-20* geeni SNP-d moodustavad ühe aheldunud bloki ja esinevad viie sagedasema haplotüübina. *IL-19* ja *IL-20* geenide SNP-de kombineeritud haplotüüp CACCGGAA osutus riskihaplotüübiks psoriaasi avaldumisel (OR 2,548). *IL-19* geeni kaitsev efekt psoriaasile ei kinnitunud *IL-19* ja *IL-20* geenide laiendatud haplotüübianalüüsis.

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FULL PAPER

Combined haplotype analysis of the interleukin-19 and -20 genes: relationship to plaque-type psoriasis

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There is increasing evidence to suggest that the newly discovered cytokines interleukin (IL)-19 and -20 have a role in the function of epidermis and in psoriasis. The genes encoding these cytokines locate into the genomic IL-10 region on human chromosome 1. The aim of the present study was to analyze whether single-nucleotide polymorphisms (SNPs) in these genes have an impact on the susceptibility for psoriasis. From pairwise linkage disequilibrium (LD) matrix of the IL-19 and -20 gene polymorphisms, what reflects the nonrandom association of alleles at these markers, it was apparent that IL-19 and -20 genes form one block of LD. We found that the HT3 CACCGGAA haplotype of the IL-19 and -20 genes was associated with an increased risk of psoriasis, reflecting its role in determining susceptibility to plaque-type psoriasis. Although association analysis of the IL-19 gene indicated that minor alleles of the IL-19 gene SNPs (rs2243188, rs2243169 and rs2243158) revealed protective effect to psoriasis and haplotype analysis of the IL-19 gene proved significant protective effect of the TGATA haplotype in case of late-onset disease, combined haplotype analysis of the IL-19 and -20 genes demonstrated that protective effect of the IL-19 gene is secondary to the susceptibility effect of the IL-20 gene.
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Introduction

The molecular basis of the pathogenesis of psoriasis, the chronic inflammatory skin disease, remains unclear, but principal clinical features of psoriasis (inflammatory infiltrate and epidermal hyperproliferation with abnormal keratinocyte differentiation) appear to be driven mainly by various cytokines and chemokines released by the activated, skin-homing pathogenic T-cell population.[1–4] There is increasing evidence to suggest that newly discovered cytokines interleukin-19 (IL-19) and interleukin-20 (IL-20) have the role in the function of epidermis and in psoriasis.[5,6]

IL-19 and -20 are members of the IL-10 family that were initially identified during a sequence database search aimed to find potential IL-10 homologs.[6,7] IL-20 has been found to be preferentially expressed in monocytes and its main targets are keratinocytes, where IL-20 binds type I IL-20R (IL-20Rα and -20Rβ) and type II IL-20R (IL-20Rβ and -22R) complexes.[6,8,9] Binding of the IL-20 in human HaCaT keratinocytic cell line results in STAT 3 phosphorylation and activation of a promoter including STAT-binding sites.[6] Microarray and RT-PCR analyses in HaCaT cells have demonstrated that the expression of several genes involved in inflammation are increased in response to IL-20 and therefore this cytokine may modulate the inflammatory response in the skin.[6] Furthermore, Blumberg *et al*[6] have shown that overexpression of IL-20 under different promoters in transgenic mice caused neonatal lethality with skin abnormalities, similar to those found in psoriatic skin. IL-19 has been detected in immune cells, such as LPS- or GM-CSF-activated and resting monocytes, and at lower level in resting and stimulated B cells.[7,8] This cytokine binds to the type I IL-20R complex and modulates gene expression in responsive cell types through activation of the STAT 1 and STAT 3 signal transduction pathway.[9–11] Sharing the same receptor complex with IL-20 suggests that IL-19 may have partially overlapping biological activities with IL-20. Moreover, *in vitro* data have proved that IL-19 acts as proinflammatory cytokine or modulator of the inflammatory response.[12,13] Romer *et al*[5] have confirmed the pathogenic role of described cytokines in psoriasis demonstrating the higher expression of IL-19 and -20 and their receptors IL-20Rα and -20Rβ in involved psoriatic skin in contrast to uninvolved psoriatic skin.

Together with the genes encoding IL-10 and MDA-7, IL-19 and -20 genes are found within a 200 kb region of chromosome 1 in q31–32 locus.[6,7,14,15] Linkage to several common autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis, have been detected in this locus.[16–18] In addition, protective effect of microsatellite marker IL-10.G9 allele 3 for familial psoriasis has been observed using the transmission/

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disequilibrium test (TDT) in persons with a positive family history of psoriasis.[19] These results indicate that locus q31–32 on chromosome 1 could be related to psoriasis susceptibility.

In our previous study, we analyzed the frequency of single-nucleotide polymorphisms (SNPs) of the human IL-20 gene in an association case–control study involving 254 patients with plaque-type psoriasis and 148 unrelated healthy volunteers. A significant association between patients with psoriasis and the G allele at position −1053 and GAA haplotype was established.[20] In the present study, we attempted to clarify the role of IL-19 gene in predicting risk for psoriasis using study population identical to the one used for IL-20 gene investigation. We analyzed seven SNPs of the IL-19 gene in patients with psoriasis and in healthy controls. Association and haplotype analysis of the IL-19 gene and combined haplotype analysis of the IL-19 and -20 genes were performed.

Results

In an initial study a database search to find the SNPs of the IL-19 gene (dbSNP, reference sequence NT_021877) was performed. We chose the rs2243158, rs2243168, rs2243191, rs2073186, rs2243174, rs2243188 and rs2243193 SNPs of the IL-19 gene. Frequency of minor allele, distance between SNPs and validation status of particular SNPs were taken as selection criteria (Figure 1).

Genotype distributions of the seven analyzed IL-19 gene polymorphisms had no deviation from Hardy–Weinberg equilibrium. Allele frequencies of IL-19 SNPs in controls and cases are reported in Table 1. Comparing psoriatic patients with controls, patients with psoriasis had a lower frequency of the SNP rs2243188 minor A allele (19.5 *vs* 26.0%, $P<0.05$), suggesting a protective effect of this minor A allele to psoriasis. Likewise, we observed lower-representation of the A allele at this position in patients with late-onset psoriasis (16.0 *vs* 26.0%, $P<0.02$) and in sporadic disease (19.3 *vs* 26.0%, $P<0.05$). Although there appeared to be fewer persons possessing the SNP rs2243188 A allele in the early onset and familial psoriasis group, the differences were not statistically significant. SNP rs2243158 was associated with type II phenotype as the prevalence of minor allele at position rs2243158 was significantly higher in controls than in patients with type II phenotype (10.1 *vs* 4.1%, $P<0.05$) and SNP rs2243168 was associated with late-onset psoriasis and type II phenotype as the prevalence of minor allele at position rs2243168 was significantly higher in controls than in patients with late-onset disease (8.8 *vs* 3.3%, $P<0.05$) and in patients with type II phenotype (8.8 *vs* 2.5%, $P<0.02$). Owing to these reasons, rs2243158 and rs2243168 represent potential subtype specific markers. All other IL-19 gene SNPs resulted in negative findings for both allele distributions when comparison between psoriatic patients and controls was performed.

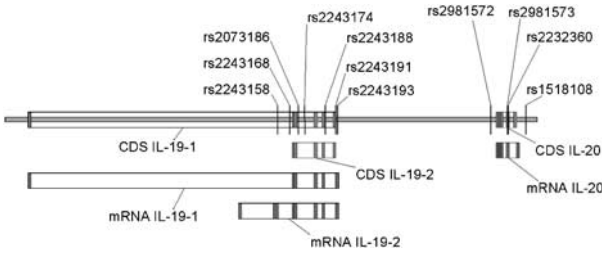

Figure 1 Selected SNPs of IL-19 and -20 genes. 76 075 bp fragment of human chromosome 1, locus 1q32. Selected SNPs are represented on the illustration by their cluster ID numbers in public Single Nucleotide Polymorphism database. Coding regions (CDS) and mRNAs of respective genes are also shown in the illustration.

To test whether the individual protective effect of the IL-19 polymorphisms, observed in single-marker association analysis, depends on the haplotypic background by which they are carried, LD and haplotype analyses of the IL-19 gene were executed. The pairwise LD matrix showed that the nearly complete linkage disequilibrium (D' between 0.88 and 0.99) was present within the polymorphisms of the IL-19 gene. We excluded SNP rs2243158 and SNP rs2243168 from the further haplotype analysis, because the minor allele frequencies of these polymorphisms were lower than 0.10. The presence of five haplotypes (HT1 CACCG, HT2 TGATA, HT3 CACTA, HT4 TAATA, and HT5 TACCG) with a frequency $\geqslant 1\%$ was established. These most frequent haplotypes accounted for 97.7% of all haplotypes in the pooled samples. Psoriasis patients had a decreased frequency of the HT2 TGATA haplotype compared to controls, but the differences were not statistically significant ($P=0.09$, OR 0.737, 95% CI 0.516–1.053). However, our data showed that HT2 TGATA haplotype was significantly more frequent in controls compared to late-onset psoriasis group ($P=0.05$; OR 0.58, 95% CI 0.335–1.00).

In our previous study, we have demonstrated that the nearly complete LD (D' between 0.879 and 0.985) occurred within the polymorphisms at positions −1053 (rs2981572), 1380 (rs2981573) and 1462 (rs2232360) of the IL-20 gene. As IL-19 gene maps close to the IL-20 gene on human chromosome 1q32, the measure of LD for all pairs of IL-19 SNPs and IL-20 SNPs studied was implemented

Table 1 Results of association analysis of interleukin-19 gene SNPs in plaque-type psoriasis

SNP ID	*Polymorphism*		*Allele frequency in controls (%)*		*Allele frequency in cases (%)*		*P-value*
	1[a]	*2*[b]	*1*[a]	*2*[b]	*1*[a]	*2*[b]	
rs2243158	G	C	89.9	10.1	91.9	8.1	NS
rs2243168	A	T	91.2	8.8	93.3	6.7	NS
rs2073186	C	T	73.0	27.0	78.3	21.7	0.08
rs2243174	A	G	76.7	23.3	81.1	18.9	NS
rs2243188	C	A	74.0	26.0	80.5	19.5	0.029*
rs2243191	C	T	74.0	26.0	78.0	22.0	NS
rs2243193	G	A	73.0	27.0	77.0	23.0	NS

Allele frequencies of the seven studied polymorphisms of the IL-19 gene were compared between the psoriatic patients ($n=254$) and the controls ($n=148$), *P*-values for allelic association were calculated using Fisher's exact test. The significance level for all statistical tests was 0.05.

*$P<0.05$ allelic differences compared to the controls.

[a]1—most frequent allele.

[b]2—least frequent allele.

in the present study. The pairwise LD matrix of the IL-19 and -20 polymorphisms showed that IL-19 and -20 SNPs were in significant LD with each other (D' between 0.78 and 0.99).

Thereafter, eight-marker haplotype analysis with five SNPs across the IL-19 gene (rs2243191, rs2073186, rs2243174, rs2243188 and rs2243193) and three SNPs across the IL-20 gene (rs2981572, rs2981573 and rs2232360) was performed. We observed five major haplotypes (HT1 CACCGTAA, HT2 TGATAGGG, HT3 CACCGGAA, HT4 CACTAGGG, HT5 TAATAGGG) with a frequency $\geqslant 1\%$ that account for 91.86% of all possible marker combinations in the pooled samples. The frequencies for these haplotypes among patients and controls and haplotype effects are presented in Table 2. We found that patients with plaque psoriasis had a higher frequency of the HT3 CACCGGAA haplotype ($P<0.01$, OR 2.547, 95% CI 1.379–4.706), compared to control group. Likewise, the HT3 CACCGGAA haplotype was associated with an increased risk of early-onset psoriasis ($P<0.02$, OR 2.225, 95% CI 1.175–4.213) and late onset of disease ($P<0.05$, OR 2.467, 95% CI 1.1258–5.405), familial psoriasis ($P<0.02$, OR 2.424, 95% CI 1.199–4.903) and sporadic disease ($P<0.01$, OR 2.877, 95% CI 1.478–5.601). This association mainly reflects a significant individual IL-20 SNP effect at position −1053 ($P<0.01$, OR 2.548, 95% CI 1.379–4.706). Comparing psoriatic patients with controls no significant association was observed concerning the other haplotypes. Protective effect of the IL-19 gene did not withstand the analysis after stratification for the known IL-20 susceptibility factor.

In addition, the protective effect of the IL-19 TGATA haplotype to late-onset psoriasis did not withstand after combined haplotype analysis of the IL-19 and -20 genes. The frequency of haplotype TGATAGGG among patients and controls did not differ significantly ($P=0.34$, OR 0.722; 95% CI 0.367–1.422). Detailed results of the haplotype analysis of the IL-19 gene and combined haplotype analysis of the IL-19 and -20 gene among patients with late-onset disease and controls are given in Table 3.

Discussion

Chromosome 1 contains several genes regulating the immune responses and IL-10 gene cluster of this chromosome is a key regulator in a number of chronic pathological processes.[21–24] The genes encoding IL-19

Table 2 Results of extended haplotype analysis of the IL-19 and -20 genes in patients with plaque-type psoriasis

Haplotype	*rs2073186*	*rs2243174*	*rs2243188*	*rs2243191*	*rs2243193*	*rs2981572*	*rs2981573*	*rs2232360*	*Controls (n=148)*	*Psoriasis patients (n=254)*	*Haplotypic OR (95% CI)*	*P-value*
HT1	C	A	C	C	G	T	A	A	64.6	60.3	*	
HT2	T	G	A	T	A	G	G	G	17.4	15.5	0.947 (0.639–1.403)	0.78
HT3	C	A	C	C	G	G	A	A	5.6	13.5	2.548 (1.379–4.706)	<0.01
HT4	C	A	C	T	A	G	G	G	1.4	2.0	1.597 (0.420–6.073)	0.49
HT5	T	A	A	T	A	G	G	G	2.4	0.8	0.342 (0.100–1.172)	0.09

The haplotype frequencies (%) and haplotypic ORs with their 95% CIs and *P*-values by comparison to the reference haplotype in psoriasis patients compared with controls are indicated. Haplotype frequencies and haplotype–phenotype associations were estimated using maximum likelihood method.
*The haplotype combining the most frequent alleles at each site is chosen as the reference haplotype (CACCGTAA).

Table 3 Results of IL-19 haplotype analysis and extended haplotype analysis of the IL-19 and -20 genes in patients with late-onset psoriasis

	Controls (n=148)	*Late-onset psoriasis patients (n=74)*	*Haplotypic OR (95% CI)*	*P-value*
IL-19 haplotypes				
CACCG	70.7	77.3	*	
TGATA	21.8	13.4	0.581 (0.335–1.000)	0.05
TAATA	2.7	0.6	0.182 (0.022–1.501)	0.11
CACTA	1.0	3.2	2.690 (0.549–13.17)	0.22
TACCG	1.4	1.3	1.580 (0.277–8.999)	0.61
IL-19 and -20 combined haplotypes				
CACCGTAA	63.5	61.1	*	
TGATAGGG	16.7	11.8	0.722 (0.367–1.422)	0.34
CACCGGAA	5.9	14.2	2.467 (1.126–5.405)	0.02
TAATAGGG	2.7	0.7	0.260 (0.031–2.146)	0.21
TACCGTAA	2.4	0.7	0.318 (0.035–2.869)	0.31

The haplotype frequencies (%) and haplotypic ORs with their 95% CIs and *P*-values by comparison to the reference haplotype in late-onset psoriasis patients compared with controls are indicated. Haplotype frequencies and haplotype–phenotype associations were estimated using maximum likelihood method.
*The haplotype combining the most frequent alleles at each site is chosen as the reference haplotype.

and -20 also locate into the genomic IL-10 region of the human chromosome 1. Blumberg *et al*[6] have demonstrated that IL-20 and its receptor complex play a part in epidermal function by regulating keratinocyte proliferation and differentiation. IL-19 is proinflammatory cytokine, which also may have role in the development of psoriasis. *In vitro* data have suggested that IL-19 induces IL-6 and TNF-α production and apoptosis in monocytes.[12] The involvement of the cytokines like IL-6 and TNFα has been clearly demonstrated in the pathogenesis of psoriasis. In addition, focal suprapapillary epidermal expression of IL-19 and -20 has been detected.[5] These data suggest that IL-19 and -20 may play a pathogenetic role in psoriasis. Furthermore, the results of our previous study proved an association of the IL-20 gene GAA haplotype in patients with plaque-type psoriasis.[20] Based on this knowledge, we hypothesized that association and haplotype analyses of the IL-19 gene and extended haplotype analysis across a region encompassing the IL-19 and -20 genes might indicate supplementary markers of disease susceptibility.

Association analysis of the IL-19 gene in the present study demonstrated that minor alleles of the IL-19 gene SNPs (rs2243188, rs2243169 and rs2243158) revealed protective effect to psoriasis, especially to late-onset disease and type II phenotype. Protective cytokine loci have been shown to have a complex genetic basis in several diseases.[25,26] In psoriasis, certain HLA alleles with a protective effect have been identified.[27] Moreover, IL-10.G polymorphism from the IL-10 promoter region has been identified to be protecting against psoriasis.[19] Hensen *et al*[28] have detected the existence of both susceptible and protective loci in the chromosome 19p13 in patients with plaque-type psoriasis. Similarly, data of the present study in accordance with association analysis in our previous study reveal that in the chromosome 1q32 region different loci have different effects in susceptibility to disease.

Several studies confirm that single SNPs do not represent the primary basis of the disease and rather SNP combination should be considered. From the pairwise linkage disequilibrium (LD) matrix of the IL-19 gene polymorphisms, what reflects the nonrandom association of alleles at these markers; it was apparent that the nearly complete linkage disequilibrium was present within the polymorphisms of the IL-19 gene. Moreover, the pairwise LD matrix of the IL-19 and -20 polymorphisms showed that IL-19 and -20 genes form one block of LD. Block of LD is a discrete chromosome region of high LD and low haplotype diversity, separated by possible hotspots of recombination and a breakdown of LD.

The novelty of the present study lies in the extension of the risk haplotype already described in IL-20 gene with new polymorphisms within the proximal IL-19 gene. Estimating combined haplotype–phenotype association of IL-19 and -20 genes, we found that the HT3 CACCGGAA haplotype was associated with an increased risk of psoriasis, reflecting the possible role of this haplotype in determining susceptibility to plaque-type psoriasis. Although haplotype analysis of the IL-19 gene proved significant protective effect of the TGATA haplotype in case of late-onset disease, combined haplotype analysis of the IL-19 and -20 genes demonstrated that protective effect of the IL-19 gene is secondary to the susceptibility effect of the IL-20 gene.

In the present study, we did not analyze functional significance of the IL-19 and -20 haplotypes. Persons with different haplotypes could have different IL-19 and -20 expression levels. Similar genetically determined differences have been shown nicely in case of IL-10.[21] It is reasonable to expect that described haplotypes really determine the differences in IL-19 and -20 expression levels. Supportive to this functional hypothesis is that we described two haplotypes with opposite effects—one protective and another one for susceptibility. We suppose that IL-19 TGATA haplotype might induce lower level of IL-19 expression than combined IL-19 and -20 haplotype CACCGGAA. However, further studies are needed to support the functional role of the IL-19 and -20 genes in the pathogenesis of psoriasis.

In conclusion, we established linkage disequilibrium of IL-19 and -20 genes and described five major haplotypes. Moreover, we found that HT3 CACCGGAA is related to increased risk (OR 2.548) for psoriasis in sample of unrelated patients and controls. We were not able to prove the protective effect of the IL-19 gene in context of extended haplotype analysis of the IL-19 and -20 genes in plaque-type psoriasis. Family-based studies should be performed additionally to confirm the impact of IL-19 and -20 haplotypes in susceptibility to psoriasis.

Materials and methods

Unrelated patients ($n = 254$) with chronic plaque psoriasis from the Department of Dermatology, University of Tartu, were divided into the subgroups according to the age of disease onset and family history of psoriasis. Patients with disease onset below the age of 40 years were assigned as early-onset psoriasis ($n = 180$), while patients with onset of disease at the age of 40 years and later years were referred to as the late-onset disease ($n = 74$). The mean age at early-onset disease group was 20 years and the group included 94 male and 86 female subjects. The mean age at late-onset disease group was 53 years and the group included 41 male and 33 female patients. Patients were considered to have familial psoriasis if they had at least one first- or second-degree relative with psoriasis ($n = 101$), or to have sporadic disease, if they had no affected relatives ($n = 153$). Patients were regarded to have type I psoriasis, if they had early onset of disease and history with affected parents ($n = 87$), or type II psoriasis, if they had late onset and sporadic form of disease ($n = 61$). Caucasian healthy volunteers, living in Estonia, and free from the positive family history of psoriasis, served as a control group ($n = 148$). The control group included 57 male and 91 female subjects. Individuals with a history of other dermatoses were not included in the control group.

To detect the nucleotides at the specific positions we applied the tetraprimer ARMS-PCR method.[29] This method uses four different primers, two (the so-called inner primers) are allele specific and two (outer primers) are control primers. Primers were designed by using the program at http://cedar.genetics.soton.ac.uk/public_html/primer1.html. Primers we used for SNP detection are shown in Table 4. Each PCR reaction was carried out in total volume of 20 μl, containing 100 ng of template DNA, 20 pmol of each inner primer, 2 pmol of each outer primer, 0.2 mM dNTP and $MgCl_2$ according to Table 4.

Table 4 Primers and conditions for tetra-ARMS-PCR used for IL-19 genotyping

Genetic polymorphism	*Primers (5′–3′)*	T_m	*Annealing temperature*	Mg^{2+} *(mM)*	*Amplicon size*
rs2243158 G/C	Forward inner primer (C allele): GGT GGA TCC ACC CAG CAA ACC TTC AC	72°C	68°C	2.5	487 bp—control
	Reverse inner primer (G allele): TTT TAT TCA GGT GGA TAA GAG GAA ATG GTC	66°C			290 bp—G allele
	Forward outer primer: GCC ACA GCT CTC AGG AAA GTG ACC TAA G	69°C			253 bp—C allele
	Reverse outer primer: CCA GCA TCT GGA ACA TCA TAG CCA TAC A	69°C			
rs2243168 A/T	Forward inner primer (T allele): GGA AGT TGC CAA GCT GCC CTC TAT CT	69°C	60°C	3.5	328 bp—control
	Reverse inner primer (A allele): CAA TAA GGA GCT AGG GGA AGA AGC CGA T	69°C			215 bp—T allele
	Forward outer primer: AGA AGG GTA AGA GAA TGA GAA GCG GTG G	69°C			167 bp—A allele
	Reverse outer primer: TGG TTT TTG ATG TTT GCC CCT GAA ATA A	69°C			
rs2073186 C/T	Forward inner primer (C allele): AGG TGC TCA GAG GGG ACA GGA TTG CC	73°C	65°C	2.5	295 bp—control
	Reverse inner primer (T allele): ACC TTC CAA AAT TAC CCC CAA GCC CCA	73°C			209 bp—C allele
	Forward outer primer: TGT TAG GGC ACG CTA GTG TCC CAG GGA TA	73°C			139 bp—T allele
	Reverse outer primer: TCC TTT GGG TCA CAA ACC TGG TCA CCT C	73°C			
rs2243174 A/G	Forward inner primer (A allele): TGG TGC TGT TCT TAC AAT GGA CAC CA	68°C	60°C	2.5	407 bp—control
	Reverse inner primer (G allele): GAT CTT GTC ACT GGC TTT CCT GCC TAA C	68°C			272 bp—G allele
	Forward outer primer: TTG GAA TCC CTG GGA GGA ATT AAA GAA G	68°C			189 bp—A allele
	Reverse outer primer: AGA ATA CTT CCC AGG ACT GGA GGA GCT C	68°C			
rs2243188 C/A	Forward inner primer (C allele): TGG GGA AGA TGG AAG ATG AGA GGT AGA CCC	72°C	65°C	2.5	366 bp—control
	Reverse inner primer (A allele): AGG AGG CTG AAG GCC TGG CAC CCT TCT	76°C			238 bp—A allele
	Forward outer primer: AAT GTC ACT TCT CAT GTG GGG AGG CAG GA	74°C			185 bp—C allele
	Reverse outer primer: AAT GAT GGA GAT GGG GAG CCC AGG AGA T	74°C			
rs2243191 C/T	Forward inner primer (C allele): CTG GAT TAA TAA GAA TCA TGA AGT AAT TTC	59°C	60°C	2.5	436 bp—control
	Reverse inner primer (T allele): ATA CAG GTT CCT TGT CAT CAA GCT GCG A	70°C			279 bp—T allele
	Forward outer primer: CCT GCG AGG AAA ATA ATA TTG AGT CTG T	64°C			215 bp—C allele
	Reverse outer primer: CAC AGT AGA CTT TTT GGC TAT TTT CAA GC	64°C			
rs2243193 G/A	Forward inner primer (A allele): AAG GGC TGC CTT CCC ATC TAA TTT ATT TTA	67°C	60°C	2.5	414 bp—control
	Reverse inner primer (G allele): TCA CAT CAC AGA CAT GGA CTA TAT GAC GTC	67°C			281 bp—G allele
	Forward outer primer: GGA ACC TGT ATA GTG ATC CAG GGA TGA A	67°C			193 bp—A allele
	Reverse outer primer: CTG TCA GAA ACA CCC TGT CCT CAG TCT T	67°C			

The reaction buffer and *Taq* polymerase (AmpliTaq DNA Polymerase, Applied Biosystems, Foster City, CA, USA) were added according to the manufacturer's guidelines. To increase PCR reaction specificity, we applied touchdown cycles: initial denaturation at 95°C for 2 min followed by 10 cycles of 1 min denaturation at 95°C, annealing at 10°C higher than annealing temperature (in Table 4) for 1 min (decreasing by 1°C per cycle) and extension at 72°C for 1 min. The following 25 cycles were performed at appropriate annealing temperature, followed by final extension at 72°C for 10 min. PCR products were analyzed by gel electrophoresis using 2% agarose gel. To control the tetraprimer ARMS-PCR method and to validate the polymorphisms, direct sequencing of incidental DNA samples were performed, using ABI Genetic Analyzer 310. Results of the tetraprimer ARMS-PCR were completely consistent with the results of the direct sequencing.

Statistical analysis of the genotype–phenotype associations was performed using GENEPOP Version 3.3 software. For haplotype analysis, THESIAS software was used. Pairwise LD was estimated by a log-linear model and the extent of disequilibrium was expressed in terms of standardized *D′* characteristic. Haplotype analysis was performed using maximum likelihood method for estimating simultaneously haplotype frequencies and haplotype–phenotype association as described by Tregouet *et al*.[30]

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