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Gene Polymorphisms of 22 Cytokines in Macedonian Children with Hyperimmunoglobulinemia E

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Abstract

INTRODUCTION: For some time it is known that cytokines and their receptors are encoded by highly polymorphic genes. These polymorphisms can be responsible for differences in the production of cytokines between individuals. Large number of the polymorphisms within the regulatory regions of the cytokine genes is in correlation with the production and there are variations among populations.

AIM: The aim of this study was to analyze association between polymorphisms in the IFN-gamma, IL-1alpha, IL-1beta, IL-1R, IL-1RA, IL-2, IL-4, IL-4R alpha, IL-6, IL-10, IL-12B, TGF-beta1 and TNF-alpha and hyperimmunoglobulinemia E.

MATERIAL AND METHODS: The study included 28 unrelated patients with high IgE levels in serum and the control group consisted of 301 unrelated healthy individuals. Cytokine genotyping was performed with PCR-SSP method. We analyzed the allele frequencies, genotypes, haplotypes and diplotypes of the cytokine genes. The differences were analyzed using $\chi 2$ test, odds ratio and Confidence Interval.

RESULTS: Susceptible association with hyperimmunoglobulinemia E was found for four different cytokine alleles (*IL-4 -33/T, TGF-beta1 cdn25/C, IL-1 alpha -889/T* and*TNF-alpha -238/A*), ten different genotypes (*IL4 -1098/G:G, IL4 -33/T:T, IL-1 alpha -889/C :T, IFN gamma utr5644/A:T, TGF-beta1 cdn25/C:G, IL-6 -174/G:G, IL-1 beta -511/C:T, IL-10 -1082/A:G, TNF alpha -238/A:G* and*IL-1 beta +3962/C:T*) and five different combinations of haplotypes (*IL-4/GTT, IL-4/TCT, IL-6/TCC, TNF-alpha/GA* and *TGF-beta1/CC*). Protective association with hyperimmunoglobulinemia E was found in four cytokine alleles (*IL-4 -33/C, TGF-beta1 cdn25/G, IL-1 alpha -889/C* and*TNF-alpha -238/G*), three genotypes (*IL-10 -1082/A:A, IL-1 alpha -889/C:C* and *IL4 -33/C:C*) and for only one haplotype (*IL-4/GCC*).

CONCLUSION: Several susceptible and protective associations between cytokine gene polymorphisms and hyperimmunoglobulinemia E were found. However, it is still speculative weather these polymorphisms contribute to susceptibility/protection from hyperimmunoglobulinemia E or they might be in significant linkage disequilibrium with some unknown gene responsible for the disease. It is also possible that different ethnical groups show different association with cytokine polymorphisms.

Introduction

Hyperimmunoglobulinemia E Syndrome (HIES) was described by Davis and Wedgwood in 1966 [1, 2]. Through the years different groups further characterized the syndrome by reporting immunological and clinical features of Hyperimmunoglobulinemia E and established two forms of the Syndrome: a dominant and a recessive form [3–13].

Minegishi et al. in 2007 found that eight out of fifteen unrelated non-familial HIES patients had heterozygous STAT3 mutations, but their parents and siblings did not have the mutant STAT3 alleles, suggesting that these were *de novo* mutations. Five different mutations were established**,** all of which were located in the STAT3 DNA-binding domain. In the patients' peripheral blood cells they found impaired responses to cytokines, including IL-6 and IL-10. They also discovered that the DNA-binding ability of STAT3 in these cells was very moderate. All of them demonstrated dominant-negative effects when they were co-expressed with wild-type STAT3. These

results emphasize the multiple roles played by STAT3 in humans, and indicate the involvement of multiple cytokine pathways in the pathogenesis of HIES [14]. Also in 2007, Holland et al. reported clinical data from HIES patients and their families. They measured the cytokine levels exuded by stimulated leukocytes. They also measured the gene expression in resting and stimulated cells. In the HIES patients the levels of proinflammatory gene transcripts in neutrophils and mononuclear cells from the peripheral blood were increased and they suggested that there is a defect in the IL-6 signaling pathway. The defect was in the signaling through the downstream mediators**,** one of which is STAT3. They sequenced the STAT3 gene from the HIES patients and found missense mutations and single-codon in-frame deletions within STAT3. They suggested that eighteen discrete mutations, five of which were hot spots**,** are involved in affecting DNA binding and SRC homology 2 (SH2) domains. They found that STAT3 mutations are present in sporadic and dominant forms of the hyper-IgE syndrome [15]. From this, the conclusion was made that a dominant form is caused by mutations in STAT3.

In 2009, Zhang et al. reported 11 patients with the AR form of HIES and discovered homozygosity or compound heterozygosity for deletions or mutations in the DOCK8 gene. The protein function was lost and they could not detect the DOCK8 protein itself in primary T-cell cultures or in transformed lymphocyte lines [16, 17]. In continued investigations by Alsum et al., 25 patients with the AR form of HIES were described and three novel DOCK8 mutations and two large deletions in thirteen patients were identified [18].

It is known that cytokines and their receptors are coded by highly polymorphic genes. These polymorphisms are responsible for individual differences which appear in the production of the cytokines and maybe this represents one of the mechanisms which are responsible for the defective Th1/Th2 imbalance. Because of this, in the last years there is an increase of the studies which analyzed polymorphic genes responsible for the cytokines and their receptors [19-21]. Therefore we can expect that cytokine gene polymorphisms will have a great impact in the ethiopathogenesis of the Hyperimmuno-globulinemia E Syndrome.

The aim if this study was to determine if there is an association between genetic polymorphisms of the cytokine genes and Hyperimmunoglobulinemia E.

Patients and Methods

Groups

The total studied sample consisted of 329 examinees, divided into healthy individuals, and patients with Hyperimmunoglobulinemia E.

Healthy individuals. All of the healthy individuals included in this study attended the Institute of Immunobiology and Human Genetics for donation of DNA and signed written consent to participate in the study. There were 301 unrelated individuals. Individuals with family history of allergies and atopy were excluded from the investigation.

Hyperimmunoglobulinemia E. Twenty eight hyperimmunoglobulinemia E patients aged 4 months to 21 years participated in this study that took place during 2006 - 2010 at the Institute of Immunobiology and Human Genetics. The 20 male and 8 female patients exhibited at least three-fold higher IgE levels than normal.

All individuals were of Macedonian origin and nationality, and residents of different regions of the Republic of Macedonia. Each individual was interviewed on a one-to-one basis, his/her genealogy was recorded for the past three generations, and a signed consent was obtained. All of the patients and normal individuals included in this study signed a written consent to participate in the study which was approved by the Committee of the Ministry of Education and Science from Republic of Macedonia (No 087405), and Ethical Committee of the Medical Faculty in Skopje (No 03-5325/2).

Genomic DNA Isolation and Storage

Ten millilitres of blood samples were collected after the signing of the written consent and with the usage of phenol-chlorophorm extraction method or BioRobot EZ1 workstation (QIAGEN) the DNA was isolated [22]. The quality and quantity of DNA were analyzed by GeneQuant (Pharmacia Biotech, Uppsala, Sweden). The DNA samples were stored in the anthropology project field of the Macedonian Human DNA Bank (hDNAMKD) [23].

Typing Methods

For cytokine genotyping we used commercially available PCR-SSP kit (Heidelberg kit, Cytokine genotyping Tray, *Invitrogen,* GmbH, Karlsruhe, Germany). Fourteen cytokine genes with 22 single nucleotide polymorphisms (SNP) were typed: *IL-1alpha -889, IL-1beta -511, IL-1beta +3962, IL-1R psti1970, IL-1RA mspa11100, IL-4Ralpha +1902, IL-12 -1188, IFNgamma utr5644, TGF-beta1 cdn10, TGF-beta1 cdn25, TNF-alpha -308, TNF-alpha -238, IL-2 -330, IL-2 +166, IL-4 -1098, IL-4 -590, IL-4 -33, IL-6 -174, IL-6 565, IL-10 -1082, IL-10 -819,* and *IL-10 -592*. Briefly, PCR-SSP typing Heidelberg kit consists of 48 PCR primer mixes aliquoted in 96-well PCR trays (two typing per tray). Master mix, which was supplied along with the reagents and consisted of $MgCl_2$, buffer, dNTP's, and glycerol was mixed with 1.2-3.0 μg DNA and 20 U Taq polymerase and dispensed in 48 wells [24]. Agarose gel

electrophoresis on a 2% gel revealed a positive or negative signal for specific amplification in each well. Subsequently, the results were analysed according to the interpretation scheme provided with the kit.

Statistical Analysis

For analysis of the data we used the population genetics analysis package, PyPop, developed by the Biostatistics Core for the Workshop [25]. Allele frequencies and expected Hardy Weinberg proportions (HWP) for each single nucleotide polymorphism (SNP) were determined [25]. The exact test for genotype frequency deviation from HWP was calculated using the Arlequin implementation accessed via PyPop [26]. Those SNPs that did not fit HWP were evaluated to determine whether there was an excess of homozygotes or heterozygotes, or if any particular genotypes significantly differed from the expected frequencies by the chi square test [27]. Comparisons of frequencies for two groups were tested by the $\chi 2$ test. Crude odds ratios (OR) (as estimates of the relative risk) were calculated with 95% confidence interval (CI).

Results

Table 1: Cytokine allele frequency, Pearson's p-value, Odds ratio and Wald's 95% confidence interval in patients with HIES and normal Macedonian population.

Cytokine Polymorphism	Allele	HIES (n=28)		Control (n=301)		Pearson's p-value	Odds ratio	Wald' 95% CI
		N	F	N	F			
IL-1 alpha -889	*C*	34	0.607	482	0.814	0.002*	0.352	0.199-0.627
	T	22	0.393	110	0.186		2.835	1.596-5.038
IL-1 beta -511	*C*	36	0.643	404	0.671	0.667	0.882	0.497-1.563
	T	20	0.357	198	0.329		1.133	0.639-2.009
IL-1 beta +3962	*C*	40	0.715	439	0.729	0.810	0.982	0.505-1.703
	T	16	0.285	163	0.270		1.077	0.587-1.976
IL-1R pstI1970	*C*	33	0.589	399	0.662	0.267	0.733	0.417-1.276
	T	23	0.411	203	0.337		1.370	0.783-2.394
IL-1RA mspa11100	*T*	35	0.625	420	0.698	0.260	0.722	0.409-1.274
	C	21	0.375	182	0.302		1.384	0.784-2.444
IL-4R alpha +1902	*A*	47	0.839	502	0.834	0.917	1.040	0.494-2.190
	G	9	0.161	100	0.166		0.961	0.456-2.024
IL-12 -1188	*A*	24	0.755	433	0.744	0.940	1.032	0.454-2.347
	C	8	0.255	149	0.256		0.968	0.426-2.202
IFNgamma +874	*T*	32	0.572	259	0.520	0.465	1.230	0.704-2.150
	A	24	0.428	239	0.480		0.812	0.465-1.119
TGF-beta1 cdn10	*T*	26	0.525	282	0.502	0.804	1.075	0.602-1.920
	C	24	0.485	280	0.498		0.929	0.521-1.658
TGF-beta1 cdn25	*G*	42	0.845	532	0.947	0.003*	0.296	0.128-0.686
	C	8	0.165	30	0.053		3.378	1.457-7.830
TNF-alpha -308	*A*	9	0.161	74	0.123	0.415	1.366	0.643-2.902
	G	47	0.840	528	0.877		0.731	0.344-1.554
TNF-alpha -238	*A*	6	0.107	27	0.045	0.041*	2.556	1.007-6.481
	G	50	0.893	575	0.955		0.391	0.154-0.992
IL-2 -330	*G*	14	0.731	191	0.332	0.350	0.738	0.400-1.400
	T	38	0.654	383	0.667		1.353	0.716-2.560
IL-2 +166	*G*	34	0.346	422	0.735	0.206	0.680	0.373-1.240
	T	18	0.255	152	0.264		1.470	0.806-2.680
IL-4 -1098	*G*	6	0.755	176	0.308	0.574	0.750	0.292-1.921
	T	18	0.708	396	0.692		1.333	0.520-3.416
IL-4 -590	*C*	17	0.292	377	0.659	0.617	1.256	0.512-3.080
	T	7	0.458	195	0.341		0.796	0.324-1.952
IL-4 -33	*C*	11	0.542	479	0.837	<0.001*	0.164	0.071-0.377
	T	13	0.233	93	0.163		6.087	2.646-14.00
IL-6 -174	*C*	13	0.767	182	0.302	0.217	0.697	0.366-1.328
	G	43	0.258	420	0.698		1.433	0.752-2.733
IL-6 nt565	*A*	14	0.250	173	0.287	0.553	0.826	0.440-1.552
	G	42	0.750	429	0.713		1.210	0.644-2.271
IL-10 -1082	*A*	29	0.483	352	0.589	0.304	0.750	0.433-1.300
	G	27	0.733	246	0.411		1.332	0.769-2.306
IL-10 -819	*C*	41	0.267	435	0.727	0.940	1.024	0.551-1.900
	T	15	0.375	163	0.272		0.976	0.526-1.811
IL-10 -592	*A*	21	0.625	173	0.289	0.180	1.474	0.834-2.604
	C	35	0.375	425	0.710		0.678	0.384-1.198

N = total number; F = frequency; CI = Confidence Interval; * = statistically significant.

Cytokine Alleles

Cytokine allele frequency, Pearson's p-value, Odds ratio and Wald's 95% confidence interval in patients with Hyperimmunoglobulinemia E and normal Macedonian population are shown in Table 1. In the region of *IL-1* gene cluster, *IL-1 alpha -889/T* (*P*=0.002, OR=2.835, Wald's 95% CI between 1.596 and 5.038) we found positive (susceptible) association with Hyperimminoglobulinemia E, and negative (protective) association for HIES was found in *IL-1 alpha -889/C* (*P*=0.006, OR=0.352, Wald's 95% CI between 0.199 and 0.627).

In the group of proinflammatory cytokines positive (susceptible) association was found also for *TNF-alpha -238/G* (*P*=0.041, OR=0.391, Wald's 95% CI between 0.154 and 0.992). This means that patients with *TNF-alpha -238/A* have 2.556 times higer risk (possibility) to develop Hyperimmunoglobulinemia E (*P*=0.041, Wald's 95% CI between 1.007 and 6.481) in comparison with those who have *TNF-alpha -238/A*.

In the gruop of anti-inflammatory cytokines, positive (susceptible) association with HIES was found for *IL-4 -33/T* (*P*<0.001, OR=6.087, Wald's 95% CI between 2.646 and 14.00), whereas *IL-4 -33/G* has shown negative (protective) association (*P*<0.001, OR=0.164, Wald's 95% CI between 0.071 and 0.377).

During the analyzes for the gene polymorphisms for *TGF-beta1 cdn25*, we found that *C* allele is positively associated with HIES (*P*=0.003, OR=3.378, Wald's 95% CI between 1.457 and 7.830), and for the G allele we found negative association (*P*=0.003, OR=0.296, Wald's 95% CI between 0.128 and 0.686) (Table 1).

Cytokine Genotypes

Cytokine genotype frequency in patients with HIES and normal Macedonian population are shown in Table 2. From all genotypes which belonging to *IL-1* gene cluster we found that only homozygous *C:C* genotype in *IL-1 alpha -889* is with negative (protective) association with HIES (*P*<0.001, OR=0.158, Wald's 95% CI between 0.065 and 0.385), while the heterozygous genotype showed positive association (*P*<0.001, OR=7.668, Wald's 95% CI between 3.242 and 18.13). We found positive (susceptible) association between patients with HIES and heterozygous genotypes of *IL-1 beta -511* and *IL-1 beta +3962* (*P*=0.009, OR=3.877, Wald's 95% CI between 1.653 and 9.088, and *P*=0.031, OR=2.307, Wald's 95% CI between 1.057 and 5.037). Apart from that, in the IL12/IFN gamma axis, we found that only heterozygous genotype of *IFN gamma utr5644* showed positive association with HIES (*P*=0.005, OR=5.991, Wald's 95% CI between 1.924 and 18.65).

Table 2: Cytokine genotype frequency, Pearson's p-value, Odds ratio and Wald's 95% confidence interval in patients with Hyperimmunoglobulinemia E and normal Macedonian population.

Cytokine polymorphism	Geno-type	HIES (n=28)		Control (n=301)		Pearson's p-value	Odds ratio	Wald' 95% CI
		N	F	N	F			
IL-1alpha -889	*C:C*	7	0.250	204	0.689	<0.001*	0.158	0.065-0.385
	C:T	20	0.714	74	0.250	<0.001*	7.668	3.242-18.13
	T:T	1	0.036	18	0.061	0.601	0.582	0.074-4.532
IL-1beta -511	*C:C*	8	0.285	143	0.475	0.054	0.442	0.188-1.034
	C:T	20	0.714	118	0.392	0.009*	3.877	1.653-9.088
	T:T	0	0	40	0.133	§	§	§
IL-1 beta +3962	*C:C*	13	0.465	174	0.578	0.244	0.632	0.290-1.376
	C:T	14	0.500	91	0.302	0.031*	2.307	1.057-5.037
	T:T	1	0.036	36	0.120	0.178	0.272	0.035-2.067
IL-1R pstI1970	*C:C*	9	0.321	133	0.442	0.589	0.874	0.262-0.251
	C:T	15	0.535	133	0.442	0.340	0.874	0.670-3.170
	T:T	4	0.142	35	0.116	0.677	1.688	0.415-3.865
IL-1RA mspa11100	*C:C*	4	0.442	30	0.100	0.472	1.505	0.489-4.613
	C:T	13	0.464	122	0.405	0.544*	1.271	0.584-2.767
	T:T	11	0.392	149	0.495	0.300*	0.606	0.299-1.456
IL-4Ralpha +1902	*A:A*	19	0.680	212	0.704	0.775	0.886	0.386-2.034
	A:G	9	0.321	78	0.259	0.474	1.354	0.588-3.118
	G:G	0	0	11	0.037	§	§	§
IL-12 -1188	*A:A*	10	0.625	160	0.550	0.470	1.468	0.520-4.143
	A:C	4	0.250	113	0.388	0.311	0.554	0.174-1.760
	C:C	2	0.125	18	0.062	0.300	2.246	0.473-10.65
IFNgamma +874	*A:A*	5	0.178	64	0.257	0.672	0.805	0.300-2.201
	A:T	14	0.500	111	0.446	0.005*	5.991	1.924-18.65
	T:T	9	0.321	74	0.297	0.380	1.453	0.630-3.350
TGF-beta1 cdn10	*C:C*	6	0.240	65	0.231	0.309	1.675	0.613-4.580
	C:T	12	0.480	150	0.534	0.260	1.725	0.661-4.503
	T:T	7	0.280	66	0.235	0.132	2.077	0.786-5.486
TGF-beta1 cdn25	*C:G*	8	0.320	30	0.107	0.001*	5.560	2.132-14.49
	G:G	17	0.680	251	0.893	0.053	0.423	0.173-1.034
	C:C	0	/	0	/	§	§	§
TNF-alpha -308	*A:G*	9	0.321	66	0.219	0.220	1.686	0.729-3.902
	G:G	19	0.678	231	0.768	0.292	0.640	0.277-1.477
	A:A	0	0	4	0.013	0.540	/	/
TNF-alpha -238	*A:G*	6	0.214	23	0.076	0.013*	3.296	1.215-8.941
	G:G	22	0.785	276	0.917	0.223	0.500	0.159-1.560
	A:A	0	/	2	0.007	§	§	§
IL-2 -330	*G:G*	0	/	27	0.094	§	§	§
	G:T	14	0.538	137	0.477	0.413	1.400	0.625-3.120
	T:T	12	0.461	123	0.429	0.600	1.240	0.554-2.773
IL-2 +166	*G:G*	11	0.423	162	0.565	0.260	0.629	0.280-1.414
	G:T	12	0.461	98	0.341	0.160	1.775	0.790-3.982
	T:T	5	0.115	27	0.094	0.091	2.416	0.843-6.921
IL-4 -1098	*G:T*	4	0.333	174	0.608	0.093	0.364	0.107-1.238
	T:T	7	0.583	111	0.388	0.132	2.396	0.742-7.731
	G:G	1	0.083	1	0.004	0.006*	27.272	1.600-465.1
IL-4 -590	*C:C*	5	0.416	95	0.332	0.461	1.548	0.480-5.006
	C:T	7	0.583	187	0.654	0.846	0.890	0.276-2.871
	T:T	0	/	4	0.014	§	§	§
IL-4 -33	*C:C*	5	0.416	209	0.731	0.042*	0.314	0.097-1.016
	C:T	1	0.083	61	0.213	0.309	0.357	0.045-2.824
	T:T	6	0.500	16	0.056	<0.001*	17.812	5.161-61.46
IL-6 -174	*C:C*	2	0.095	25	0.083	0.845	1.162	0.255-5.278
	C:G	9	0.428	132	0.439	0.929	0.960	0.400-2.347
	G:G	17	0.772	144	0.478	0.033*	4.633	1.523-14.09
IL-6 nt565	*A:A*	2	0.071	25	0.083	0.830	0.849	0.190-3.788
	A:G	10	0.357	123	0.409	0.595	0.804	0.359-1.801
	G:G	16	0.572	153	0.508	0.523	1.290	0.590-2.819
IL-10 -1082	*A:A*	2	0.071	70	0.234	0.045*	0.040	0.058-1.096
	A:G	25	0.892	212	0.709	0.007*	3.706	1.333-10.305
	G:G	1	0.035	17	0.057	0.670	0.700	0.080-4.830
IL-10 -819	*C:C*	13	0.464	155	0.518	0.607	0.607	0.380-1.774
	C:T	15	0.535	125	0.418	0.217	0.217	0.750-3.535
	T:T	0	/	19	0.064	§	§	§
IL-10 -592	*A:A*	5	0.178	28	0.094	0.149	0.150	0.747-6.011
	A:C	11	0.392	117	0.391	0.965	0.965	0.460-2.249
	C:C	12	0.428	154	0.515	0.400	0.400	0.327-1.564

N = absolute number; F = frequency; CI = Confidence Interval; &, cannot be calculated because expected <5, $\chi 2$ test; *, statistically significant.

For the *IL-4* gene, we found positive association for the homozygous genotypes *IL-4 -1098/G:G* (*P*=0.006, OR=27.272, Wald's 95% CI between 1.600 and 465.1) and *IL-4 -33/T:T* (*P*<0.001, OR=17.812, Wald's 95% CI between 5.161 and 61.46) with HIES, and for the homozygous variant of *IL-4 -33/C:C* (*P*=0.042, OR=0.314, Wald's 95% CI between 0.097 and 1.016) we found negative association with HIES.

We found that patients with *IL-10 -1082/A:G* have positive association with HIES (*P*=0.007, OR=3.706, Wald's 95% CI between 1.333 and 10.305), while *IL-10 -1082/G:G* genotype is with negative association (*P*=0.033, OR=0.033, Wald's 95% CI between 1.117 and 5.831) in comparison to patients who have the rest of the *IL-10* genotypes. We also found that patients with genotype *IL-6 -174/G:G* have 4.633 times higher possibility to develop HIES (*P*=0.033, OR=2.552, Wald's 95% CI between 1.523-14.09) than the patients with the others *IL-6* genotypes.

Positive association with HIES was also found for the heterzygous genotypes of *TGF-beta1 cdn25* and *TNF-alpha -238* (*P*=0.001, OR=5.560, Wald's 95% CI between 2.132 and 14.49, *P*=0.013, OR=3.296, Wald's 95% CI between 1.215-8.941).

Cytokine Haplotypes

Cytokine haplotypes frequency, Pearson's p-value, crude odds ratio and Wald's 95% confidence interval in the patients with hyperimmunoglobulinemia E and normal Macedonian population are presented in Table 3. With the Heidelberg kit it was possible to analyze haplotypes for *TGFbeta1, TNFalpha, IL-2, IL-4, IL-6* and *IL-10*.

Table 3: Haplotype frequency of cytokine polymorphism, Pearson's p-value, Odds ratio and Wald's 95% confidence interval in patients with hyperimmunoglobulinemia E and normal Macedonian population.

Cytokine polymorphism	Haplotype	HIES (n=28)		Control (n=301)		Pearson's p-value	Odds ratio	Wald' 95% CI
		N	F	N	F			
TGF-beta1	*CC*	6	0.120	30	0.053	0.038*	2.595	1.021-6.662
	CG	18	0.360	250	0.445	0.411	0.774	0.420-1.427
	TG	24	0.480	282	0.502	0.907	1.036	0.571-1.880
	TC	2	0.040	/	/	/	/	/
TNF-alpha	*AG*	7	0.125	74	0.123	0.963	1.019	0.445-2.334
	GA	6	0.107	26	0.043	0.033*	2.658	1.045-6.671
	GG	42	0.750	502	0.834	0.112	0.600	0.314-1.135
	AA	1	0.018	/	/	/	/	/
IL-2	*GG*	12	0.230	178	0.310	0.233	0.667	0.341-1.302
	GT	2	0.038	14	0.024	0.538	1.600	0.353-7.239
	TG	22	0.423	244	0.425	0.977	0.991	0.558-1.761
	TT	16	0.307	138	0.240	0.280	1.404	0.755-2.608
IL-4	*GCC*	2	0.083	163	0.285	0.026*	0.220	0.051-0.950
	GCT	1	0.041	8	0.014	0.260	3.065	0.367-25.54
	GTC	1	0.041	4	0.007	0.068	6.173	0.663-57.45
	GTT	2	0.083	1	0.002	<0.001*	51.90	4.533-594.3
	TCC	7	0.291	202	0.353	0.536	0.754	0.307-1.850
	TCT	6	0.250	4	0.007	<0.001*	47.33	12.27-182.4
	TTC	1	0.041	110	0.192	0.063	0.182	0.024-1.366
	TTT	4	0.166	80	0.140	0.711	1.230	0.409-3.692
IL-6	*CA*	13	0.232	172	0.286	0.393	0.755	0.400-1.440
	CG	/	/	9	0.150	/	/	/
	GG	42	0.750	420	0.698	0.412	1.300	0.692-2.439
	GA	1	0.017	1	0.002	0.035*	10.92	0.674-177.1
IL-10	*ACA*	3	0.053	12	0.020	0.109	2.764	0.756-10.10
	ACC	11	0.196	177	0.296	0.115	0.581	0.293-1.150
	ATA	15	0.267	161	0.269	0.964	0.986	0.531-1.830
	ATC	/	/	2	0.003	/	/	/
	GCC	24	0.428	246	0.411	0.802	1.073	0.616-1.867
	GCA	3	0.053	/	/	/	/	/

N = absolute number; F = frequency; CI = Confidence Interval; &, cannot be calculated because expected <5, $\chi 2$ test; *, statistically significant.

We have found significant association with Hyperimmunoglobulinemia E for three haplotypes of *IL-4*, one haplotype of *IL-6,* one haplotype of *TNF-alpha* and one haplotype of *TGF-beta1.* We found positive association (according the level of susceptibility) for *IL-4/GTT* (*P*<0.001), odds ratio 51.90 (4.533-594.3); *IL-4/TCT* (*P*<0.001), odds ratio 47.33 (12.27-182.4); *IL-6/GA* (*P*=0.035), odds ratio 10.92 (0.674-177.1); *TNF-alpha/GA* (*P*=0.033), odds ratio 2.658 (1.045-6.671) and *TGF-beta1/CC* (*P*=0.038), odds ratio 2.595 (1.021-6.662). We found negative (protective) association with hyperimmunoglobulinemia E only for *IL-4/GCC* (p=0.026), odds ratio 0.220 (0.051-0.950).

Susceptible and protective cytokine polymorphisms for hyperimmuno-globulinemia E

Summary of all susceptible and protective cytokine polymorphisms for hyperimmunoglobulinemia E in Macedonian population are presented in Table 4.

We can see that most of the cytokine genotypes (ten of them) and cytokine haplotypes (five of them) have shown positive association with hyperimmunoglobulinemia E, but only three cytokine genotypes and only one cytokine haplotype have negative association. Positive association was found for four cytokines alleles. Negative association was also found in four cytokines alleles.

Tabele 4: Summary of all susceptible and protective cytokine polymorphisms for hyperimmunoglobulinemia E in Macedonian population.

	Susceptible			Protective		
	Polymorphism	p	Odds ratio	Polymorphism	p	Odds ratio
Cytokine Alleles	*IL-4 -33/T*	<0.001	6.087	*IL-4 -33/C*	<0.001	0.164
	TGF-beta1 cdn25/C	0.002	3.377	*TGF-beta1 cdn25/G*	0.002	0.296
	IL-1 alpha -889/T	0.002	2.835	*IL-1 alpha -889/C*	0.002	0.352
	TNF-alpha -238/A	0.041	2.556	*TNF-alpha -238/G*	0.041	0.391
Cytokine Genotypes	*IL4 -1098/G:G*	0.006	27.272	*IL-10 -1082/A:A*	0.045	0.040
	IL4 -33/T:T	<0.001	17.812	*IL-1 alpha -889/C:C*	<0.001	0.158
	IL-1 alpha -889/C :T	<0.001	7.668	*IL4 -33/C:C*	0.042	0.314
	IFN gamma +874/A:T	0.005	5.991			
	TGF-beta1 cdn25/C:G	0.001	5.560			
	IL-6 -174/G:G	0.033	4.663			
	IL-1 beta -511/C:T	0.009	3.877			
	IL-10 -1082/A:G	0.007	3.706			
	TNF alpha -238/A:G	0.013	3.296			
	IL-1 beta +3962/C:T	0.031	2.307			
Cytokine Haplotypes	*IL-4/GTT*	<0.001	51.90	*IL-4/GCC*	0.026	0.220
	IL-4/TCT	<0.001	47.33			
	IL-6/TCC	0.035	10.92			
	TNF-alpha/GA	0.033	2.658			
	TGF-beta1/CC	0.038	2.595			

Discussion

Previous studies of the cytokine polymorphisms in Macedonian population have shown association with a number of diseases [28-32]. In this paper we present our results of 22 cytokine polymorphisms in patients with HIES and in normal Macedonian population. To our knowledge, these are first results about the cytokine polymorphism in the world.

We examined the role of two single nucleotide polymorphisms of the *IL-1 beta* gene *(-511 C/T* and *+3962 C/T*), one polymorphism of *IL-1 alpha* gene (*-889 C/T*), one polymorphism of *IL-1R* gene (*psti1970 C/T*) and one polymorphism of the *IL-1RA* gene (*mspa11100 T/C*) in the pathogenesis of hyperimmunoglobulinemia E. The results have shown that the genes from the IL-1 gene cluster are associated with the hyperimmunoglobulinemia E, meaning that persons who have *IL-1 alpha -889/T* allele have three times higher susceptibility for hyperimmunoglobulinemia E than those who have *IL-1 alpha -889/C* allele. Regarding the genotypes, persons who are heterozygotes for *IL-1 alpha -889* have eight times higher possibility to develop hyperimmunoglobulinemia E unlike the heterozygotes for *IL-1 beta -511* and *IL-1 beta +3962* where the susceptibility is 3.8 or 2.3 times. Our investigation didn't show any association between hyperimmunoglobulinemia E and *IL-1R* and *IL-1RA* polymorphisms (alleles or genotypes).

IL- 12, as part of the IL-12/IFN gamma axis, plays an important role in the host defence from the intracellular pathogens [33, 34]. The results from the polymorphisms of IL- 12 and their role is still unclear. It was shown that additionally to the polymorphisms located in the promoter region also the polymorphism in the 3'UTR is in correlation with the intensity of the secretion of the protein [35, 36].

We examined the possible role of polymorphisms in the 3'UTR of the *IL-12B* gene in the pathogenesis of hyperimmunoglobulinemia E. The results showed that there is no significant difference in allele frequencies or in the genotypes, suggesting that 3'UTR polymorphisms have none or negligible effect in the pathogenesis of hyperimmunoglobulinemia E in the Macedonian population. A possibility remains that there is some role of 3'UTR polymorphisms in the pathogenesis of hyperimmunoglobulinemia E through its association with another functional polymorphism in the *IL-12/IFN gamma* axis [37, 38].

IFN gamma is also a participant in the IL-12/IFN gamma axis, in addition to IL- 12, IL-12R and the receptor for IFN gamma. He is one of the most important Th1 cytokines that participate in the host defense established through the activation of macrophages [39]. IFN gamma plays a key role in defense against viruses and intracellular organisms. The analysis of the data found significant differences only for *IFN gamma +874 / A: T* genotype. Individuals who are heterozygous for this polymorphism have almost 6 times higher chances to develop hyperimmunoglobulinemia E. The possible significance of this polymorphism arises from the fact that this polymorphism is in significantly unbalanced relationship with the other two polymorphisms of the *IFN gamma* gene, and it is known that they are associated with certain diseases [40].

It is believed that the role of TGF-beta1 is associated with its anti - inflammatory and profibrotic activities, such as regulation of growth and differentiation of cells, release of cytokines and angiogenesis, production of extra- cellular matrix and restoration of tissues [41, 42] and the creation of elastin [43, 44]. The results showed an association with hyperimmunoglobulinemia E only for *TGF-beta1 cdn 25* polymorphism. We found protective association only for *TGF-beta1 cdn 25/G* allele, while the */C, /C:G* genotype and */CC* haplotype showed negative association with hyperimmunoglobulinemia E.

Tumor - necrosis factor alpha (TNF-alpha) is a proinflammatory cytokine that acts synergistically with IFN gamma in the activation of the macrophages [45]. TNF-alpha has a role in airway remodeling and alters the function of smooth muscle cells [46], and because of that it is considered as a major factor in the genesis and the maintenance of the lung damage in patients with certain respiratory system diseases [47, 48]. Our results showed association between *TNF-alpha -238 A/G* polymorphism and hyperimmunoglobulinemia E. *TNF-alpha -238/A* allele,

/A:G genotype and */GA* haplotype showed negative association with hyperimmunoglobulinemia E, while *TNF-alpha -238/G* showed protective association. The question remains whether this association represents an independent effect of *TNF-alpha* or it is a result of his association with the HLA-A1, B17 and DR7 [49].

Interleukin 2 is a cytokine produced by T cells during an immune response. It can be also produced by eosinophils and epithelial cells from the respiratory system. He stimulates the growth, differentiation and survival of number of immune cells [50]. In our study we did not find any significant association between allele frequencies, genotypes and haplotypes in the *IL-2 -330* and *IL-2 +160* polymorphisms and hyperimmunoglobulinemia E.

Interleukin - 4 (IL-4) is a multifunctional Th2 cytokine, which reduces Th1 cell response. IL-4 may also stimulate the production of mucus, as well as hyperplasia of the goblet cells in the bronhial submucosa [51]. In this study we examined alleles frequency, genotypes and haplotypes in three polymorphisms in the IL-4 gene at positions *-1098* , *-590* and *-33*. Results showed positive association between *IL-4 -33/C* allel, */C:C* genotype and */GCC* and hyperimmunoglobulinemia E. Analysis of the frequencies showed negative associaton between *IL-4 -33/T* allel and hyperimmunoglobulinemia E, which increases from 6 times to 17.8 times in homozygous *T* genotype, to 47.3 times in *TCT* haplotype and 52 times in *GTT* haplotype. Only */G:G* genotype from *IL-4 -1098* polymorphism showed significant association. People who have this genotype have 27.2 times greater chance to develop hyperimmunoglobulinemia E in comparison with the individuals who are carriers of the other genotypes. For the alleles, genotypes and haplotypes in *IL-4 -590* and *IL-4R alpha +1902* polymorphisms with didn't find any associations with hyperimmunoglobulinemia E .

It is known that IL-6 plays an important role in the initial phase of the innate immune response [52, 53]. We investigated the association of two polymorphisms in the *IL-6*, *-174C/G* and *nt565 A/G*. The results showed that *IL-6 -174/G:G* genotype and *IL-4/GA* haplotype are associated with hyperimmunoglobulinemia E (postitive).

IL-10 is a possible anti-inflammatory Th2 cytokine which inhibits the replication of on macrophages/ monocytes and T lymphocytes, reduces the production of the proinflammatory cytokines in the inflammatory responses [54-57] and inhibits the expression of the co-stimulatory molecules and the moleculs of class 2 of MHC on the macrophages surfeces [58]. We examined the association between 3 single nucleotide polymorphisms in the region of the gene promotor for IL-10 (*-1082 A/G*, *-819 C/T* and *-592 A/C*) and hyperimmunoglobulinemia E. We analyzed the polymorphisms and we did not find any significant association between the investigated alleles and hyperimmunoglobulinemia E. But the analysis of the genotypes showed positive association between *IL-10 -1082/A:A* genotype (homozygous for the A allele) and the heterozygous genotype of the same polymorphism with hyperimmunoglobulinaemia E .

Although the mechanisms which are associated with polymorphisms which makes changes in the gene expression are still not well known, today we known that hyperimmunoglobulinemia E is partly under polygenic control. The role inn the pathogenesis of hyperimmunoglobulinemia E is played not only by the large number of alleles located on different genes and even on the chromosomes, but also by the interaction between the genes and surrounding. We still don't know whether the polymorphisms alone by themself contribute for protection/susceptibility of hyperimmunoglobulinaemia E. Because of this it is possible that various ethnic groups will show different associations with cytokine polymorphisms.

The number of patients in our study is very small. In the association studies, there are possibilities that some positive results might be spurious and some negative findings might be a consequence of low statistical power. It could be due to their small sample size or methodological shortcomings, such as the selection of an appropriate control group. It is necessary to investigate cytokine gene polymorphisms in our population in well defined subgroups of phenotypes with bigger number of participants in order to have more precise conclusions for genetic background of development of hyperimmunoglobulinemia E in Macedonians. However, multicentre studies and/or meta-analysis of the patients with hyperimmunoglobulinaemia E and association with cytokine polymorphisms should be very useful, as it was shown before with our published data about cytokine polymorphism associations [59-61].

The results of this study for the association of the cytokine polymorphisms with hyperimmuno-globulinaemia E in the Macedonians showed positive association with four alleles, 10 genotypes and 5 haplotypes and negative associations with four alleles, 3 genotypes and 1 haplotype. The results can be used for future meta analyses of the association of cytokine polymorphisms with patients with hyperimmun-oglobulinemia E.

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