ORGINAL ARTICLE
Iran J Allergy Asthma Immunol
March 2009; 8(1): 31-42

Association of Cytokine Gene Polymorphisms with Chronic Obstructive Pulmonary Disease in Macedonians

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Received: 10 April 2008; Accepted: 13 July 2008

ABSTRACT

The aim of this study was to examine the association of 22 cytokine gene polymorphism in Macedonians with chronic obstructive pulmonary disease (COPD).

The sample of the population comprised of 301 normal respondents and 62 patients with COPD. Cytokine genotyping was performed by polymerase chain reaction with sequence-specific priming (PCR-SSP).

Positive (susceptible) association was found between patient with COPD and *IL-1a -889/C* allele; where as negative (protective) association among was found for the following alleles *IL-1β +3962/C; IL-12B -1188/A; IFNγ +874/T; IL-2 -330/G; IL-4 -1098/G* and *IL-4-33/C*. We found positive (susceptible) association between patients with COPD and following genotypes: *IL4 -33/T:T; IFNγ +874/A:A; IL-4 -1098/T:T ; IL-1a -889/C:C; IL-1β +3962/C:T; IL-12B -1188/C:C; IL-4Ra +1902/G:G; IL-10 -1082/G:G; IL-2 -330/T:T; IL-4 -590/C:C*; and *IL-1a -889/C:T*. Negative (protective) association between patients with COPD and following genotypes was found: *IFNγ +874/A:T; IL-4 -33/C:T; IL-4 -1098/G:T; IL-2 -330/G:T; IL-1β +3962/C:T; IL-4 -590/C:T; IL-10 -1082/A:G*; and *IL-4 -33/C:C*. Positive (susceptible) association between patients with COPD and following haplotypes was found: *IL-4/TCT; IL-10/ATC; and IL-2/TG*, and negative (protective) association was found between the patients with COPD and haplotypes for: *IL-4/TTC; and IL-4/GCC*.

It could be concluded that several cytokine polymorphisms are positively (susceptible), or negatively (protective) associated with COPD in Macedonians.

Key words: Chronic obstructive pulmonary disease; Cytokine polymorphism; Macedonians

INTRODUCTION

Chronic obstructive pulmonary disease (COPD), which includes chronic pulmonary emphysema (CPE) and chronic bronchitis,[1] represents a significant prob-

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lem in more developed countries.[2,3] Although basically it is a benign disease characterized with chronic progression and irreversible obstruction of the airways, it has a poor prognosis. Because of the high death rate it is predicted that by year 2020, in more developed countries COPD will take the third place of death causing diseases.[4,5] Smoking is the most important risk factor in the ethiopathogenesis of COPD, but only 10-20% smokers will develop a disease.[2,6] The main factors causing COPD development are still unknown, but a genetic basis is considered contributes to in the development of COPD.[7,8] Today it is very well known that COPD is a multifactorial disease whereby both environmental and genetic factors contribute to the aetiology and/or clinical severity. Genetics of COPD is complex, involving multiple genes, and Mendelian patterns of inheritance do not follow.[9-12] Autosomal 10-cM genome wide scan of short tandem repeat polymorphic markers in 72 pedigrees (585 individuals) ascertained through probands with severe early-onset COPD was performed.[13]

Observations provided significant evidence for an early-onset COPD-susceptibility locus on chromosome 2 and suggestive evidence for linkage of spirometry-related phenotypes to several other genomic regions. Later it was concluded that there is significant linkage of airflow obstruction susceptibility to loci on chromosomes 2q and 8p and that postbronchodilator spirometric measures may be optimal phenotypes for COPD genetic studies.[14] By integrating results from microarray studies of murine lung development and microarray studies of human COPD gene expression with human COPD linkage results on 2q, *SERPINA2* (177010) was identified as a positional candidate susceptibility gene for COPD.[15]

An association of a SNP in the matrix metalloproteinase-1 gene (MMP1, 120353.0001) with the rate of decline of lung function in COPD was found.[16] The most widely recognized candidate gene in COPD is *SERPINA1* (107400), although it has been suggested that *SERPINA3* (107280) may play a role.[17]

Cytokine gene polymorphisms are integral parts of the immune response stimulated by antigen presentation in the context of Human Leukocyte Antigen (HLA). Published data show contradictory results in relationship between cytokine polymorphism and COPD, while some authors show positive association of certain cytokine polymorphisms and COPD, others report quite the opposite results.[18-20] We published preliminary results of cytokine gene polymorphisms in 125 healthy Macedonians.[21] There are no data about the possible associations of cytokine polymorphisms and COPD in the Republic of Macedonia. The aim of this study was to examine the association of cytokine gene polymorphisms in Macedonians with COPD.

PATIENTS AND METHODS

Participants

The total study sample consisted of 363 participants, divided into two different groups as follows: normal individuals and patients with COPD. Normal individuals were 301 age and sex non-matched normal individuals (aged 20-45 years) who attended Institute of Immunobiology and Human Genetics for DNA donation between May 5, 2003 and April 25, 2004 and agreed to take part in this study as a control group. Individuals with family history of chronic obstructive pulmonary diseases were excluded from the investigation.

Chronic obstructive pulmonary disease (COPD)

There were 44 males and 18 females (n=62) fulfilling the American Thoracic Society standards for the diagnosis of patients with chronic obstructive pulmonary disease.[1] All patients were smokers, except for four. They were 35-50 years old patients hospitalized in the Clinic for Pulmoallergology, between May, 5 2003 and April 5, 2004. All individuals were of Macedonian origin and residents of different geographical areas of the Republic of Macedonia. Each individual was interviewed on a one-to-one basis, his/her genealogy was recorded for the last three generations, and mongrel, if any, was recorded for each individual. Individuals with only one Macedonian parent were excluded from the study. All patients and normal individuals included in this study signed a written consent to participate in the study which was approved by the Ethics Committee of the Ministry of Education and Science of the Republic of Macedonia (No 087405).

Genomic DNA Isolation and Storage

DNA was isolated from peripheral blood leukocytes by the phenol-chlorophorm extraction method or with BioRobot EZ1 workstation (QIAGEN).[22] The quality and quantity of DNA was analyzed by GeneQuant (Pharmacia). Isolated DNA samples were stored in Macedonian Human DNA Bank (hDNAMKD).[23]

Cytokine Genotyping

Cytokine genotyping was performed by polymerase chain reaction with sequence-specific priming (PCR-SSP) using the Cytokine Genotyping Kit (Dynal Biotech, Invitrogen Corporation, Brown Deer, WI, USA) allowing us to determine allels, genotypes, haplotypes and diplotypes of these 22 single nucleotide polymorphism within 13 cytokine genes: *IL-1α –889 (T/C), IL-1β –511 (T/C), IL-1β +3962 (T/C), IL-1R pst1 1970 (T/C), IL-1RA mspa1 11100 (T/C), IL-4Rα +1902 (G/A), IL-12 –1188 (C/A), IFN-γ +874(A/T) [previously IFN-γ UTR5644(A/T)], TGF-β codon 10 (C/T), TGF-β codon 25 (G/C), TNF-α –308 (A/G), TNF-α –238 (A/G), IL-2 +166 (G/T), IL-2 –330 (T/G), IL-4 –1098 (T/G), IL-4 -590 (T/C), IL-4 –33 (T/C), IL-6 –174 (C/G), IL-6 nt565 (G/A), IL-10 –1082 (G/A), IL-10 –819 (C/T)* and *IL-10 -592 (A/C)*. The lyophilized primer mixes and reagents contained in the kit were developed by University of Heidelberg for the cytokine polymorphism component of the 13th International Histocompatibility Workshop (Seattle, USA, 2002). PCR amplification was carried out exactly according to manufacturer's manual using a PTC-100 Thermal Cycler (MJ Research, Inc., Waltham, MA, USA). Briefly, PCR-SSP typing consisted of 48 PCR primer mixes aliquotted in 96 well PCR trays (two typings 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 the 48 wells. Agarose gel electrophoresis on a 2% agarose gel revealed either a positive or a negative specific amplification for each well.[24] Subsequently, the results were entered in the Cytokine-SCORE software[25] and analyzed automatically. Manual interpretation was also possible according to the interpretation scheme provided along with the kit.

Statistical Analysis

The population genetics analysis package, PyPop, developed by the Biostatistics Core for the Workshop,[26-28] was used for analysis of the cytokine data for this report. Allele frequencies and expected Hardy Weinberg proportions (HWP) for each SNP were determined.[29] The exact test for genotype frequency deviation from HWP was calculated using the Arlequin implementation accessed via PyPop.[30] 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. Comparisons of frequencies for two groups were tested by the χ^2 test. Pearson P-values, crude odds ratio (OR) and Wald's 95% confidence intervals (CI) were calculated to test the associations between cytokine polymorphisms and chronic obstructive pulmonary disease with GraphPad QuickCalcs: free statistical calculators (http://www. graphpad.com/quickcalcs/). P values less than 0.05 were taken as significant.

RESULTS

Cytokine Alleles

The frequencies of polymorphic cytokine alleles in COPD patients and normal Macedonian population are shown (Table 1).

The highest positive (susceptible) odds ratio was found for *IL-1α -889/C* (*P*<0.001, OR=3.619, CI=1.640-7.985), meaning that people with IL-1α-889/C allele have 3.6 times higher risk to develop COPD in comparison to others with *IL-1α -889/T* allele. Negative (protective) association for COPD was found for the following alleles: *IL-1β +3962/C* (*P*=0.002, OR=0.523, CI=0.348-0.786); *IL-12B -1188/A* (*P*=0.022, OR=0.616, CI=0.406-0.935); *IFNγ +874T* (*P*<0.001, OR=0.260, CI=0.157-0.431); *IL-2 -330/G* (*P*<0.001, OR=0.367, CI=0.213-0.632); *IL-4 -1098/G* (*P*<0.001, OR=0.211, CI=0.090-0.496) and *IL-4 -33/C* (*P*<0.001, OR=0.275, CI=0.162-0.464) (Table 1).

Cytokine Genotypes

The different cytokine genotypes found in our study are summarized (Table 2).

We found positive (susceptible) association between patients with COPD and following genotypes (according the levels of susceptibility): *IL4 -33/T:T* (*P*<0.001, OR=91.607, CI= 46.120-181.958); *IFNγ +874/A:A* (*P*<0.001, OR=9.154, CI=4.507-18.591); *IL-4 -1098/T:T* (*P*<0.001, OR=7.620, CI=3.065-18.943); *IL-1α -889/C:C* (*P*<0.001, OR=4.871, CI=1.886-12.578); *IL-1β +3962/C:T* (*P*<0.004, OR=4.086, CI=2.152-7.689); *IL-12B -1188/C:C* (*P*=0.006, OR=3.033, CI=1.323-6.955); *IL-4Rα +1902/G:G* (*P*=0.034, OR=2.929, CI=1.039-8.257); *IL-10 -1082/G:G* (*P*=0.03, OR=2.602, CI=1.067-6.347); *IL-2 -330/T:T* (*P*<0.001, OR=2.407, CI=1.114-5.202); *IL-4 -590/C:C* (*P*=0.014, OR=2.386, CI=1.175-4.852); and *IL-1α -889/C:T* (*P*<0.001, OR=1.161, CI=0.049-0.529) (Table 2).

Table 1. Cytokine allele frequency, Fisher exact p-value, Odds ratio and Wald's 95% CI in COPD patients and normal Macedonians*

Cytokine Polymorphism	Allele	COPD (n=62)		Control (n=301)		Pearson P-value	Odds ratio	Wald's 95% CI
		N	F	N	F			
IL-1α -889	*C*	111	0.941	482	0.814	<0.001	3.619	1.640-7.985
	T	7	0.059	110	0.186			
IL-1β -511	*C*	85	0.708	404	0.671	0.426	1.190	0.775-1.828
	T	35	0.292	198	0.329			
IL-1β +3962	*C*	69	0.585	439	0.729	0.002	0.523	0.348-0.786
	T	49	0.415	163	0.270			
IL-1R pst1 1970	*C*	84	0.700	399	0.662	0.429	1.187	0.776-1.817
	T	36	0.300	203	0.337			
IL-1RA mspa1 11100	*T*	84	0.700	420	0.698	0.960	1.011	0.659-1.550
	C	36	0.300	182	0.302			
IL-4Rα +1902	*A*	95	0.792	502	0.834	0.264	0.757	0.464-1.236
	G	25	0.208	100	0.166			
IL-12B -1188	*A*	77	0.642	433	0.744	0.022	0.616	0.406-0.935
	C	43	0.358	149	0.256			
IFNγ +874	*T*	22	0.780	259	0.520	<0.001	0.260	0.157-0.431
	A	78	0.220	239	0.480			
TGF-β1 cdn10	*T*	64	0.467	282	0.502	0.530	1.135	0.766-1.684
	C	56	0.533	280	0.498			
TGF-β1 cdn25	*G*	112	0.067	532	0.947	0.565	0.790	0.353-1.768
	C	8	0.933	30	0.053			
TNF-α -308	*A*	16	0.133	74	0.123	0.723	1.098	0.615-1.960
	G	104	0.867	528	0.877			
TNF-α -238	*A*	7	0.058	27	0.045	0.524	1.319	0.561-3.103
	G	113	0.942	575	0.955			
IL-2 -330	*G*	17	0.155	191	0.332	<0.001	0.367	0.213-0.632
	T	93	0.845	383	0.667			
IL-2 +166	*G*	76	0.691	422	0.735	0.339	0.805	0.516-1.256
	T	34	0.309	152	0.264			
IL-4 -1098	*G*	6	0.086	176	0.308	<0.001	0.211	0.090-0.496
	T	64	0.914	396	0.692			
IL-4 -590	*C*	53	0.757	377	0.659	0.100	1.613	0.909-2.860
	T	17	0.243	195	0.341			
IL-4 -33	*C*	41	0.586	479	0.837	<0.001	0.275	0.162-0.464
	T	29	0.414	93	0.163			
IL-6 -174	*C*	28	0.233	182	0.302	0.129	0.702	0.445-1.110
	G	92	0.767	420	0.698			
IL-6 nt565	*A*	27	0.225	173	0.287	0.163	0.720	0.453-1.144
	G	93	0.775	429	0.713			
IL-10 -1082	*A*	71	0.602	352	0.589	0.792	1.056	0.706-1.580
	G	47	0.398	246	0.411			
IL-10 -819	*C*	88	0.746	435	0.727	0.682	1.099	0.700-1.727
	T	30	0.254	163	0.272			
IL-10 -592	*A*	29	0.246	173	0.289	0.337	0.801	0.508-1.262
	C	89	0.754	425	0.710			

*Abbreviations: N= absolute number; F=frequency; CI=Confidence Interval; COPD, chronic obstructive pulmonary disease.

Table 2. Cytokine genotype frequency, Pearson's P-value, Odds ratio and Wald's 95% CI in COPD patients and normal Macedonians*

Polymorphism	Genotype	COPD (n=62)		Controls (n=301)		Pearson's P-value	Odds ratio	Wald's 95% CI
		N	F	N	F			
	C:C	54	0.915	204	0.689	<0.001	4.871	1.886-12.578
IL-1α -889	*C:T*	3	0.051	74	0.250	<0.001	1.161	0.049-0.529
	T:T	2	0.034	18	0.061	0.420	0.542	0.122-2.401
	C:C	30	0.500	143	0.475	0.724	1.105	0.635-1.923
IL-1β 511	*C:T*	25	0.417	118	0.392	0.722	1.107	0.631-1.945
	T:T	5	0.083	40	0.133	0.289	0.593	0.224-1.571
	C:C	31	0.525	174	0.578	0.455	0.808	0.462-1.415
IL-1β +3962	*C:T*	7	0.119	91	0.302	0.004	0.311	0.136-0.710
	T:T	21	0.356	36	0.120	<0.001	4.086	2.152-7.689
	C:C	30	0.500	133	0.442	0.409	1.263	0.725-2.200
IL-1R pst1 1970	*C:T*	24	0.400	133	0.442	0.550	0.842	0.479-1.481
	T:T	6	0.100	35	0.116	0.717	0.844	0.339-2.107
	C:C	7	0.117	30	0.100	0.692	1.193	0.498-2.859
IL-1RA mspa1 11100	*C:T*	22	0.367	122	0.405	0.577	0.850	0.479-1.507
	T:T	31	0.516	149	0.495	0.759	1.091	0.626-1.899
	A:A	41	0.683	212	0.704	0.746	0.906	0.498-1.647
IL-4Rα +1902	*A:G*	13	0.217	78	0.259	0.489	0.791	0.406-1.539
	G:G	6	0.100	11	0.037	0.034	2.929	1.039-8.257
	A:A	27	0.450	160	0.550	0.158	0.670	0.383-1.734
IL-12B -1188	*A:C*	23	0.383	113	0.388	0.942	0.979	0.553-1.734
	C:C	10	0.167	18	0.062	0.006	3.033	1.323-6.955
	A:A	38	0.760	64	0.257	<0.001	9.154	4.507-18.591
IFNγ +874	*A:T*	2	0.040	111	0.446	<0.001	0.052	0.012-0.218
	T:T	10	0.200	74	0.297	0.163	0.591	0.281-1.245
	C:C	11	0.183	65	0.231	0.418	0.746	0.367-1.518
TGF-β1 cdn10	*C:T*	34	0.567	150	0.534	0.643	1.142	0.651-2.003
	T:T	15	0.250	66	0.235	0.803	1.086	0.569-2.072
	C:G	6	0.100	30	0.107	0.877	0.930	0.369-2.343
TGF-β1 cdn25	*G:G*	53	0.883	251	0.893	0.823	0.905	0.376-2.170
	C:C	1	0.017	0	0	†	†	†
	A:G	14	0.233	66	0.219	0.811	1.084	0.561-2.092
TNF-α -308	*G:G*	45	0.750	231	0.768	0.771	0.909	0.478-1.729
	A:A	1	0.017	4	0.013	0.838	1.259	0.139-11.461
	A:G	7	0.117	23	0.076	0.302	1.596	0.652-3.909
TNF-α -238	*G:G*	53	0.883	276	0.917	0.403	0.686	0.282-1.667
	A:A	0	0	2	0.007	†	†	†
	G:G	4	0.073	27	0.094	0.062	0.363	0.121-1.090
IL-2 -330	*G:T*	9	0.164	137	0.477	<0.001	0.270	0.128-0.570
	T:T	42	0.763	123	0.429	<0.001	6.829	3.537-13.187
	G:G	32	0.582	162	0.565	0.812	1.074	0.598-1.926
IL-2 +166	*G:T*	12	0.218	98	0.341	0.073	0.538	0.271-1.068
	T:T	11	0.200	27	0.094	0.022	2.407	1.114-5.202
	G:T	6	0.171	174	0.608	<0.001	0.133	0.054-0.331
IL-4 -1098	*T:T*	29	0.829	111	0.388	<0.001	7.620	3.065-18.943
	G:G	0	0	1	0.004	†	†	†
	C:C	19	0.543	95	0.332	0.014	2.386	1.175-4.852
IL-4 -590	*C:T*	15	0.428	187	0.654	0.009	0.397	0.195-0.810
	T:T	1	0.029	4	0.014	0.511	2.074	0.225-19.093

Table 2. continued

	C:C	20	0.571	209	0.731	0.049	0.491	0.239-1.008
IL-4 -33	*C:T*	1	0.029	61	0.213	0.009	0.109	0.015-0.809
	T:T	14	0.400	16	0.056	<0.001	91.607	46.120-181.958
	C:C	2	0.033	25	0.083	0.181	0.381	0.088-1.652
IL-6 -174	*C:G*	24	0.400	132	0.439	0.582	0.834	0.485-1.501
	G:G	34	0.567	144	0.478	0.212	1.426	0.816-2.492
	A:A	2	0.033	25	0.083	0.181	0.381	0.088-1.652
IL-6 nt565	*A:G*	23	0.383	123	0.409	0.715	0.900	0.509-1.589
	G:G	35	0.584	153	0.508	0.288	1.354	0.773-2.373
	A:A	20	0.339	70	0.234	0.090	1.678	0.919-3.063
IL-10 -1082	*A:G*	31	0.525	212	0.709	0.006	0.454	0.257-0.802
	G:G	8	0.136	17	0.057	0.030	2.602	1.067-6.347
	C:C	33	0.559	155	0.518	0.565	1.179	0.672-2.068
IL-10 -819	*C:T*	22	0.373	125	0.418	0.519	0.828	0.466-1.472
	T:T	4	0.068	19	0.064	0.903	1.072	0.351-3.273
	A:A	6	0.102	28	0.094	0.847	1.096	0.433-2.776
IL-10 -592	*A:C*	17	0.288	117	0.391	0.135	0.630	0.342-1.158
	C:C	36	0.610	154	0.515	0.181	1.474	0.833-2.607

*Abbreviations: N= absolute number; F=frequency; CI=Confidence Interval; †, cannot be calculated because expected <5, $\chi 2$ test; COPD, chronic obstructive pulmonary disease.

Negative (protective) association between patients with COPD and following genotypes (according the levels of protectivity) was found for: *IFNγ +874/A:T* (*P* <0.001, OR=0.052, CI=0.012-0.218); *IL-4 -33/C:T* (*P*=0.009, OR=0.109, CI=0.015-0.809); *IL-4 -1098/G:T* (*P*<0.001, OR=0.133, CI=0.054-0.331); *IL-2 -330/G:T* (*P*<0.001, OR=0.270, CI=0.128-0.570); *IL-1β +3962/C:T* (*P*=004, OR=0.311, CI=0.136-0710); *IL-4 -590/C:T* (*P*=009, OR=0.397, CI=0.195-0.810); *IL-10 -1082/A:G* (*P*=0.006, OR=0.454, CI=0.257-0.802); and *IL-4 -33/C:C* (*P*=0.049, OR=0.491, CI=0.239-1.008) (Table 2).

Cytokine Haplotypes

Cytokine haplotypes frequency, Pearson P-value, crude odds ratio and Wald's 95% CI in the COPD patients and normal Macedonians are shown (Table 3). With the Heidelberg kit it is possible to analyse haplotypes for *TGF-β1, TNF-α, IL-2, IL-4, IL-6* and *IL-10*.

Positive (susceptible) association between the patients with COPD and following haplotypes was found (according the levels of susceptibility): *IL-4/TCT* (*P*<0.001, OR=35.500, CI=11.30-111.515); *IL-10/ATC* (*P*=0.008, OR=7.774, CI= 1.285-47.045); and *IL-2/TG* (*P*=0.031, OR=1.565, CI=1.039-2.357). Negative (protective) association was found between the patients with COPD and haplotypes for: *IL-4/TTC* (*P*<0.001, OR=0.061, CI=0.008-0.443); and *IL-4/GCC* (*P*<0.001, OR=0.193, CI=0.076-0.488). Haplotypes *IL-2/GT*, *IL-4/GCT*, *IL-4/GTC*, *IL-4/GTT* and *IL-6/GA* were present only in normal Macedonians, while only patients with COPD had *TGF-β1/TC* and *TNF-α/AA* haplotypes (Table 3).

Cytokine Diplotypes (haplotype zygosity)

Cytokine diplotypes (haplotype zygosity), Pearson P-value, crude odds ratio and Wald's 95% CI for each SNP in the COPD patients and healthy Macedonian population are shown (Table 4).

Positive (susceptible) association between the patients with COPD and following diplotypes was found (according the levels of susceptibility): *IL-4/TCT:TTT* (*P*<0.001, OR=47.000, CI=14.207-155.484); *IL-10/ATC:GCC* (*P*=0.008, OR=7.955, CI=1.300-7.354); *IL-10/GCC:GCC* (*P*=0.010, OR=2.986, CI=1.261-7.071); *IL-2/TG:TG* (*P*<0.001, OR=2.928, CI=1.569-5.462); *IL-2/TT:TT* (*P*=0.012, OR=2.620, CI=1.204-5.703); and *IL-4/TCC:TCC* (*P*=0.015, OR=2.404, CI=1.167-4.953) (Table 4).

Negative (protective) association between patients with COPD and following genotypes (according the levels of protectivity) was found for: *IL-4/GCC:TTC* (*P*<0.001, OR=0.052, CI=0.007-0.387); *IL-2/GG:TG* (*P*=0.042, OR=0.247, CI=0.058-1.057); *IL-2/GG:TG* (*P*=0.010, OR=0.347, CI=0.151-0.797); and I*L-10/ATA:GCC* (*P*=0.028, OR=0.452, CI=0.219-0.931) (Table 4).

Table 3. Haplotype frequency of cytokine polymorphism, Pearson's P-value, Odds ratio and Wald's 95% CI in COPD patients and normal Macedonians*

Polymorphism	Haplotype	COPD (n=62)		Control (n=301)		Pearson's P-value	Odds ratio	Wald's 95% CI
		Number	Frequency	Number	Frequency			
TGF-β1	*CC*	7	0.058	30	0.053	0.828	1.099	0.471-2.564
	CG	49	0.408	250	0.445	0.464	0.861	0.577-1.285
	TG	63	0.525	282	0.502	0.644	1.097	0.740-1.628
	TC	1	0.008	0	0	†	†	†
TNF-α	*AG*	13	0.108	74	0.123	0.654	0.867	0.464-1.620
	GA	4	0.033	26	0.043	0.621	0.764	0.262-2.230
	GG	100	0.833	502	0.834	0.988	0.996	0.589-1.685
	AA	2	0.025	0	0	†	†	†
IL-2	*GG*	17	0.155	178	0.310	0.798	0.928	0.524-1.643
	GT	0	0	14	0.024	†	†	†
	TG	59	0.536	244	0.425	0.031	1.565	1.039-2.357
	TT	34	0.309	138	0.240	0.128	1.413	0.904-2.211
IL-4	*GCC*	5	0.071	163	0.285	<0.001	0.193	0.076-0.488
	GCT	0	0	8	0.014	†	†	†
	GTC	0	0	4	0.007	†	†	†
	GTT	0	0	1	0.002	†	†	†
	TCC	34	0.486	202	0.353	0.030	1.730	1.05-2.850
	TCT	14	0.200	4	0.007	<0.001	35.500	11.30-111.515
	TTC	1	0.014	110	0.192	<0.001	0.061	0.008-0.443
	TTT	16	0.229	80	0.140	0.049	1.822	0.944-3.340
IL-6	*CA*	27	0.225	172	0.286	0.174	0.726	0.457-1.154
	CG	1	0.008	9	0.150	0.571	0.554	0.070-4.412
	GG	92	0.767	420	0.698	0.129	1.424	0.901-2.250
	GA	0	0	1	0.002	†	†	†
IL-10	*ACA*	2	0.017	12	0.020	0.823	0.842	0.186-3.812
	ACC	38	0.322	177	0.296	0.573	1.130	0.739-1.727
	ATA	27	0.229	161	0.269	0.362	0.805	0.505-1.283
	ATC	3	0.025	2	0.003	0.008	7.774	1.285-47-045
	GCC	48	0.407	246	0.411	0.926	0.981	0.657-1.467

*Abbreviations: CI=Confidence Interval; †, cannot be calculated because expected <5, $\chi 2$ test; COPD, chronic obstructive pulmonary disease.

Diplotypes *TGF-β1/CC:CC, TGF-β1/CG:TC* and *TNF-α/GG:AA* were present in COPD patients only, while diplotypes *TNF-α/GA:GA, IL-2/GT:TG, IL-2/GT:GG, IL-2/GT:TT, IL-4/GCC:GCC, IL-4/GCC:TTC, IL-4/GCC:TTT, IL-4/TCC:TTC, IL-4/TCC:TTT, IL-4/GCT:TTT, IL-4/GTC:TTC, IL-4/GTT:TTC, IL-6/GA:GG* and *IL-10/ACA:GCC* were found only in normal Macedonians.

Summary of all susceptible and protective cytokine polymorphisms for bronchial asthma in Macedonian population are presented (Table 5). If the odds ratio has a significant value above 1.000 we could say that positive or susceptible association exists, and if the odds ratio has a significant value below 1.000 then negative or protective association could exist. From the Table 5 we can see that the highest number of cytokine genotypes (10 of them) are susceptible for bronchial asthma with highest odds ratio of 7.155 for *IL-4 -1098/T:T*, and more than three times higher risk (P<0.001) for *TNF-α -238/A:G* (6.944), *IL-4 -590/C:C* (5.608), *IL-2 +166/T:T* (4.430), and *IL-2 -330/T:T* (3.353). Six cytokine diplotypes, four cytokine haplotypes, and two cytokine alleles were found to be positively (susceptible) associated with bronchial asthma (Table 5). Moreover protective cytokine polymorphisms for bronchial asthma in seven cytokine genotypes, six cytokine diplotypes, four cytokine haplotypes, and four cytokine alleles were found. Most of the negative (protective) associations with bronchial asthma were with very high protective level (P<0.001) (Table 5).

Table 4. Cytokine diplotypes (haplotype zygotes), Pearson's P-value, Odds ratio and Wald's 95% CI in COPD patients and normal Macedonians*

Polymorphism	Diplotype	COPD (n=62)		Control (n=301)		Pearson's P-value	Odds ratio	Wald's 95% CI
		Number	Frequency	Number	Frequency			
TGF-β1	*CC:CG*	2	0.033	16	0.057	0.458	0.571	0.128-2.552
	CC:TG	3	0.050	14	0.050	0.995	1.004	0.279-3.608
	CG:CG	8	0.133	49	0.174	0.439	0.728	0.326-1.630
	CG:TG	30	0.500	136	0.484	0.822	1.066	0.611-1.862
	TG:TG	15	0.250	66	0.235	0.803	1.086	0.569-2.072
	CC:CC	1	0.017	0	0	†	†	†
	CG:TC	1	0.017	0	0	†	†	†
TNF-α	*AG:GG*	11	0.183	66	0.219	0.535	0.799	0.394-1.624
	GA:GG	4	0.067	24	0.080	0.730	0.824	0.275-2.469
	GG:GG	41	0.683	206	0.684	0.987	0.995	0.584-1.806
	AG:AG	1	0.017	4	0.013	0.838	1.259	0.138-11.461
	GG:AA	3	0.050	0	0	†	†	†
	GA:GA	0	0	1	0.004	†	†	†
IL-2	*GG:GG*	4	0.073	27	0.094	0.613	0.755	0.253-2.251
	GG:TG	7	0.127	85	0.296	0.010	0.347	0.151-0.797
	GG:TT	2	0.036	38	0.133	0.042	0.247	0.058-1.057
	GT:TG	0	0	11	0.058	†	†	†
	TG:TG	21	0.382	50	0.174	<0.001	2.928	1.569-5.462
	TG:TT	10	0.182	48	0.168	0.792	1.107	0.522-2.347
	TT:TT	11	0.200	25	0.087	0.012	2.620	1.204-5.703
	GT:GG	0	0	1	0.003	†	†	†
	GT:TT	0	0	2	0.007	†	†	†
IL-4	*GCC:GCC*	0	0	1	0.003	†	†	†
	GCC:TCC	4	0.118	26	0.091	0.654	1.290	0.422-3.941
	GCC:TTC	0	0	103	0.360	<0.001	0.052	0.007-0.387
	GCC:TTT	0	0	32	0.112	†	†	†
	TCC:TCC	15	0.441	68	0.238	0.015	2.404	1.167-4.953
	TCC:TTC	0	0	7	0.025	†	†	†
	TCC:TTT	0	0	28	0.098	†	†	†
	TTT:TTT	1	0.029	4	0.014	0.511	2.074	0.225-19.093
	GCT:TTT	0	0	8	0.028	†	†	†
	GTC:TTC	0	0	4	0.014	†	†	†
	TCT:TTT	14	0.412	4	0.014	<0.001	47.000	14.207-155.484
	GTT:TTC	0	0	1	0.003	†	†	†
IL-6	*CA:CA*	2	0.033	25	0.083	0.181	0.381	0.088-1.652
	CA:GG	23	0.383	122	0.405	0.751	0.912	0.516-1.611
	CG:GG	1	0.017	9	0.030	0.568	0.550	0.068-4.423
	GG:GG	34	0.567	144	0.479	0.218	1.426	0.816-2.492
	GA:GG	0	0	1	0.003	†	†	†
IL-10	*ACC:ACC*	7	0.119	21	0.070	0.206	1.782	0.721-4.406
	ACC:ATA	7	0.119	21	0.070	0.206	1.782	0.721-4.406
	ACC:GCC	17	0.288	114	0.381	0.175	0.657	0.357-1.209
	ATA:ATA	4	0.068	19	0.064	0.903	1.072	0.351-3.273
	ATA:GCC	10	0.169	93	0.311	0.028	0.452	0.219-0.931
	GCC:GCC	9	0.152	17	0.057	0.010	2.986	1.261-7.071
	ACA :GCC	0	0	3	0.010	†	†	†
	ACA :ATA	2	0.034	9	0.030	0.877	1.131	0.238-5.371
	ATC :GCC	3	0.051	2	0.007	0.008	7.955	1.300-7.354

*Abbreviations: CI=Confidence Interval; †, cannot be calculated because expected <5, $\chi 2$ test; COPD, chronic obstructive pulmonary disease.

Table 5. Summary of all positive (susceptible) and negative (protective) cytokine polymorphisms for COPD in Macedonians

cytokine	Positive (susceptible)			Negative (protective)		
	Polymorphism	P-Value	Odds ratio	Polymorphism	P-value	Odds ratio
Cytokine Alleles	*IL-1α -889/C*	<0.001	3.619	*IL-4 -1098/G*	<0.001	0.211
				IFNγ +874/A	<0.001	0.260
				IL-4 -33/C	<0.001	0.275
				IL-2 -330/G	<0.001	0.367
				IL-1β +3962/C	0.002	0.523
				IL-12B -1188/A	0.022	0.616
Cytokine Genotypes	*IL4 -33/T:T*	<0.001	91.607	*IFNγ +874/A:T*	<0.001	0.052
	IFNγ +874/A:A	<0.001	9.154	*IL-4 -33/C:T*	0.009	0.109
	IL-4 -1098/T:T	<0.001	7.620	*IL-4 -1098/G:T*	<0.001	0.133
	IL-2 -330/T:T	<0.001	6.829	*IL-2 -330/G:T*	<0.001	0.270
	IL-1α -889/C:C	<0.001	4.871	*IL-1β +3962/C:T*	0.004	0.311
	IL-1β +3962/T:T	<0.001	4.086	*IL-4 -590/C:T*	0.009	0.397
	IL-12B -1188/C:C	0.006	3.033	*IL-10 -1082/A:G*	0.006	0.454
	IL-4Rα +1902/G:G	0.034	2.929	*IL-4 -33/C:C*	0.049	0.491
	IL-10 -1082/G:G	0.030	2.602			
	IL-2 +166/T:T	0.022	2.407			
	IL-4 -590/C:C	0.014	2.386			
	IL-1α -889/C:T	<0.001	1.161			
Cytokine Haplotypes	*IL-4/TCT*	<0.001	35.500	*IL-4/TTC*	<0.001	0.061
	IL-10/ATC	0.008	7.774	*IL-4/GCC*	<0.001	0.193
	IL-4/TCC	0.030	1.730			
	IL-2/TG	0.031	1.565			
Cytokine Diplotypes (Haplotype Zygosity)	*IL-4/TCT:TTT*	<0.001	47.000	*IL-4/GCC:TTC*	<0.001	0.052
	IL-10/ATC:GCC	0.008	7.955	*IL-2/GG:TT*	0.042	0.247
	IL-10/GCC:GCC	0.010	2.986	*IL-2/GG:TG*	0.010	0.347
	IL-2/TG:TG	<0.001	2.928	*IL-10/ATA:GCC*	0.028	0.452
	IL-2/TT:TT	0.012	2.620			
	IL-4/TCC:TCC	0.015	2.404			

DISCUSSION

This report summarizes the results of 22 cytokine polymorphisms in patients with COPD and in normal Macedonians. To our knowledge, this is the first study analyzing comprehensive group of 22 cytokine polymorphisms and their associations with COPD.

We found that *IL-1α -889* allele is positively associated with COPD patients: *C:C* genotype is associated with odds ratio of 4.871; *C* allele is associated with odds ratio of 3.619; and *C:T* genotype is associated with smallest odds ratio of 1.161. Our results are the first published results about the association of *IL-1α -889* allele and patients with COPD.

Contrary to the previously reported results,[31] we could not confirm the association of *IL-1β -511* polymorphism and COPD, which is similar to that of other reports.[32] Unlike other studies,[33] we demonstrated significant association of *IL-1β +3962* polymorphisms with COPD in this study. *IL-1β +3962/C* allele was protectively associated with COPD, and *IL-1β +3962/C:T* genotype, but *IL-1β +3962/T:T* genotype was susceptible for COPD.

Our data showed that *IL-12B -1188/A* allele was protective for COPD, but frequency analysis of genotypes showed that *IL-12B -1188/C:C* was susceptible for COPD.

Our results demonstrated protective effect of *IFNγ +874/T* allele, as well as *IFNγ +874/A:T* genotype. However, *IFNγ +874/A:A* genotype was strongly susceptible for COPD. We could say that patients with *IFNγ +874/A:A* (homozygous A allele) are around 9 times more susceptible for COPD than those with *IFNγ +874/A:T* (heterozygous for *A* allele).

Several studies have analyzed the association between polymorphisms of transforming growth factor-β1

(TGFB1) gene and COPD.[34-37] Unlike the results of several authors,[34-36] we did not find any significant association in TGFB1 codon 10 and codon 25 frequencies of alleles, genotypes, haplotypes, or diplotypes. Our results are in agreement with others.[37]

Many authors investigated the role of TNF-α in the pathogenesis of COPD. Some of them found that the TNF-α polymorphisms is a risk factor for COPD[38,39] and the others did not find.[36,40,41] Most of the data are about the connection of *TNF-α -308A/G* polymorphism and COPD.[32,38,40,42-44] Only few investigated the role of other *TNF-α* polymorphisms. It was demonstrated that only *TNF-α +489A* allele was significantly more frequent in COPD group.[45] Other polymorphisms, analysed in the same study (*-376/G:A, -308/G:A and -238/G:A*), showed no association with the COPD. We did not find any significant association between COPD and *TNF-α* frequencies of alleles, genotypes, haplotypes, or diplotypes.

To our knowledge, this is the first study analyzing the association between IL-2 polymorphisms and COPD. In our study, we have demonstrated that there was protective association between the COPD and *IL-2 -330/G* allele, *IL-2 -330/G:T* genotype, *IL-2/GG:TG*, and *IL-2/GG:TT*. We also found positive (susceptible) association between COPD and: *IL-2 -330/T:T* genotype, *IL-2 +260/T:T* genotype, *IL-2/TG* haplotype, *IL-2/TG:TG* diplotype, and *IL-2/TT:TT* diplotype. The question remains whether observed associations are solely the effects of IL-2 polymorphisms or they could be in linkage disequilibrium with some other genes nearby.

In this study, we investigated alleles and genotypes of three polymorphisms of *IL-4* (at the positions *-1098*, *-590*, and *-33*), as well as haplotypes and diplotypes of investigated polymorphisms. Several authors showed no associations between *IL-4* allele and genotype frequency and COPD.[46] Our results of *IL-4* haplotypes are in agreement with others, who also found significant differences in frequencies of *IL-4 -59*0 and *IL-4 -33* haplotypes between COPD patients and controls.[47]

Interleukin 4 mediates its activity through IL-4 receptor (*IL-4Rα*). When we analyzed the *IL-4Rα +1902* polymorphism, our results showed no significant differences of frequency at allele level, but *IL-4Rα +1902/G:G* genotype was significantly susceptible associated with COPD (odds ratio=2.929).

We did not find any association between the *IL-10* alleles (at the positions *-1082, -819,* and *-590*) and COPD. Analysis of genotypes showed significant negative (protective) association between COPD and *IL-10 -1082/A:G* haplotype, and positive (susceptible) association with *IL-10 -1082/G:G* genotype. *IL-10* genotypes at the locations *-819* and *-590* were not significantly associated with COPD. Only one *IL-10/ATC* haplotype was positively associated with COPD (odds ratio=7.774). One haplotype combination of *IL-10* (diplotype or haplotype zygozity) was negatively associated with COPD (*IL-10/ATA:GCC*), and two haplotype combinations were positively associated with COPD (*IL-10/GCC;GCC*, and *IL-10/ATC:GCC*). It was found that only *IL-10 -1082* polymorphism seemed to be associated with COPD.[42] In contrast, other authors[48] found that not *IL-10 -1082G/A*, but *IL-10 -819C/T* polymorphism seemed to be associated with COPD.

We published association of 22 cytokine gene polymorphisms with bronchial asthma (BA) in population of ethnic Macedonians. Susceptible cytokine polymorphisms for BA for ten genotypes, six diplotypes, four haplotypes, and two alleles were found. Protective cytokine polymorphisms for BA for seven cytokine genotypes, six cytokine diplotypes, four cytokine haplotypes, and four cytokine alleles were found.[49] We need analysis of 22 cytokine gene polymorphisms in more patients in groups of bronchial asthma and chronic obstructive pulmonary disease, in order to compare the similarities and differences between two entities.

There are several advantages for the use of Heidelberg kit for cytokine polymorphism: i) we can investigate 22 polymorphisms in one run; ii) we can investigate haplotypes of *TGFβ1, TNF-α, IL-2, IL-4, IL-6,* and *IL-10*; and iii) we can use data as a part of the International Project Cytokine Polymorphism Component (CPC), 13th IHWC. However, there are some drawbacks for the general use of Heidelberg kit for cytokine polymorphism: i) several cytokine polymorphisms important for inflammatory processes are not included in the kit (*IL-13, IL-18*); ii) several cytokine clusters are not completely included (*IL-1, TNF-α*).

In conclusion, we confirmed that some cytokine polymorphisms are associated with COPD in the population of ethnic Macedonians. Since COPD is a complex-trait of widely heterogeneous diseases, it involves multiple alleles located on different genes and even on different chromosomes, which are in linkage disequilibrium with selected human leukocyte antigen alleles. Inconsistent results obtained from various authors highlight the genetic role among different ethnic groups. Because the complex-trait diseases, like COPD, are influenced not only by genetic factor but by gene-environment interac-

tion as well, it is possible that different ethnic groups will show association with different cytokine polymorphisms. Only one meta analysis about the *TNF-α* polymorphism and association with COPD has been published.[50] Meta analysis of all cytokine polymorphisms and association with COPD are needed in order to identify the pathogenesis of the disease.

ACKNOWLEDGMENT

This research is part of the project "Molecular analysis of cytokine gene polymorphisms in the Republic of Macedonia" supported by the Ministry of Education and Science from Republic of Macedonia (Project No. 087405). We would like to gratefully acknowledge Prof. G. Opelz and Dr. J. Mytilineos from the Institute of Immunology, Department of Transplantation Immunology, University of Heidelberg, Heidelberg, Germany for kindly supplying the Heidelberg PCR-SSP kit reagents for this project. For sample collection, technical support, and laboratory direction, we thank Elena Zaharieva.

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