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Distribution of 22 Cytokine Gene Polymorphisms in Roma from the Republic of Macedonia

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ABSTRACT

The aim of this study was to analyze 22 cytokine polymorphisms in the Roma population from the Republic of Macedonia. The Roma population consists of 77 healthy unrelated individuals, residents of different geographical regions of the Republic of Macedonia (Skopje, Gostivar, and Kochani).

Blood samples were collected after obtaining written consent. DNA was isolated from peripheral blood and 22 polymorphisms were typed: *IL1A -889, IL1B -511, IL1B +3962, IL1R pst1 1970, IL1RN mspa11100, IL4RA +1902, IL12 -1188, IFNG utr5644, TGF-β1 cdn10, TGF-β1 cdn25, TNF-a -308, TNF-a -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*. Cytokine genotyping was performed by PCR-SSP. The population genetics analysis package, PyPop, was used for analysis of the cytokine data.

F_{nd} was negative and significantly different from 0 for *IL-4 -590* (*p* of F=0.006), *IL-10 -1082* (*p* of F=0.010), *IFNγ utr5644* (*p* of F=0.024), *IL-4 -1098* (*p* of F=0.026) and *TGF-β1 cdn25* (*p* of F=0.001) alleles, as well as for *IL-2* haplotypes (p=0.025). Several SNPs (*IL-12B -1188, IL-2 -330, IL-4 -1098, IL-4 -590,* and *IL-10 -1082*) were not in HWP (p<0.05). A few SNPs (*IL-12B -1188, IL-2 -330, IL-4 -1098, IL-4 -590*, and *IL-10 -1082*) and several observed frequencies of cytokine diplotypes (*IL-2/GG:TG, IL-2/TG:TG, IL-4/GCC:GCC, IL-4/TTC:TTC, IL-4/TTT:TTC, IL-10/GCC:GCC, IL-10/ATA:GCC, IL-10/ACC:GCC,* and *IL-10/ACC:ATA*) were not in HWP and were significantly different from the expectations. Hardy Weinberg proportion could not be calculated for TNFα genotypes and diplotypes because nearly all genotypes and diplotypes belong to GG genotype or *GG:GG* diplotype.

The results of cytokine polymorphisms in Roma population can be used for characterization of the current genetic profile of the Gypsies, anthropological comparisons, as well as for the association studies with different diseases.

Keywords: Cytokine; DNA; Genetic polymorphism

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INTRODUCTION

The Roma are a distinct ethnic minority whose origins began on the Indian subcontinent over one

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thousand years ago. Because recorded history of the Roma prior to their first documented appearances in Europe in the early 15th century is non-existent, there has been much debate as to their origins and early migration. They began their migration to Europe and North Africa via the Iranian plateau about 1000 years ago, and by the 14th century, the Roma had reached the Balkans.[1,2] Although it is very hard to reveal their true numbers because many Roma often for a variety of reasons choose not to register their ethnic identity in official censuses for fear of discrimination, today estimated population of Roma is at least 15 million in the whole world. Eight million of them reside in Europe.[2] There is no one group that can call themselves the one, "true" Roma. Because Romani culture is diverse with many traditions and customs and all groups around the world have their own individual beliefs and tenets, data provided by the social sciences as well as genetic research suggest that Roma who live in Europe today are best described as a conglomerate of genetically isolated founder populations. There is no universal culture per se, but there are attributes common to all Roma, where the Group is the primary unit, and the boundaries, demographic history and biological relatedness of the diverse founder populations appears complex and has not been addressed by population genetic studies. The available incomplete epidemiological data suggest a non-random distribution of disease-causing mutations among Roma groups.[3]

All today immunogenetic investigations in Roma people are about variation in mtDNA, Y chromosome,[4-6] one autosomal locus,[7] HLA distribution and associations with some diseases.[8-15]

According to the 2002 census, in the Republic of Macedonia, Macedonians are majority representing 64.1% of the population. Although some unofficial estimates indicate that in Republic of Macedonia live more than 260000 Roma individuals, officially 2.66% have identified themselves as Roma.[16]

Until now, we have published data for the cytokine polymorphisms in healthy Macedonian population[17-19] and for the possible association of cytokine polymorphism with bronchial asthma,[20] chronic obstructive pulmonary disease,[21] tuberculosis,[22] dilated cardiomyopathy,[23] rheumatoid arthritis,[24] and chronic periodontitis.[25] To our knowledge, this is the first study dealing with cytokine gene polymorphism in Roma individuals form the Republic of Macedonia and in the world. The aim of this study was to present the data of 22 cytokines gene polymorphism in 77 healthy Roma which can be used as a part of an anthropology report, and as a base for the studies of disease association.

MATERIALS AND METHODS

Population Samples

The Roma population included in this study consisted of 78 healthy unrelated individuals, age 20-35. All individuals were of Roma origin and nationality, born in different parts of Macedonia and residents of the geographical areas of different regions of the Republic of Macedonia (Skopje, Gostivar, and Kochani). Each individual was interviewed on the one-to-one basis, his/her genealogy was recorded for the last three generations and a written consent was obtained. Admixture, if any, was recorded for each individual. Individuals with only one Roma parent were excluded from the study. All of the healthy individuals included in this study attended the Institute of Immunobiology and Human Genetics for DNA donation and signed written consent to participate in the study which was approved by the Committee of the Macedonian Ministry of Education and Science (No.13-874/3-05) and the Ethical Committee of the Medical Faculty in Skopje.

Genomic DNA Isolation and Storage

Blood samples were collected and DNA was isolated from peripheral blood leukocytes using the phenol-chlorophorm extraction method or BioRobot EZ1 workstation (QIAGEN).[26] The quality and quantity of DNA were analysed by GeneQuant (Pharmacia Biotech, Uppsala, Sweden). Isolated DNA samples were stored in the anthropology project field of the Macedonian Human DNA Bank (hDNAMKD).[27]

Typing Methods

For cytokine genotyping commercially available PCR-SSP kit (Heidelberg kit, Cytokine genotyping Tray, *Invitrogen,* GmbH, Karlsruhe, Germany) was used. Fourteen cytokine genes with 22 single nucleotide polymorphisms (SNP) were typed: *IL-1α -889, IL-1β -511, IL-1β +3962, IL-1R psti1970, IL-1RA mspa11100, IL-4Rα +1902, IL-12 -1188, IFNγutr5644, TGF-β1 cdn10, TGF-β1 cdn25, TNF-α -308, TNF-α -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 typings per tray). Master mix, which was supplied along with the reagents and consisted of MgCL2, buffer, dNTP's, and glycerol was mixed with 1.2-3.0 μg DNA and 20 U Taq polymerase and dispensed in 48 wells. [28] 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 Methods

The population genetics analysis package, PyPop, developed by the Biostatistics Core for the Workshop,[29-31] was used for analysis of the cytokine data in this study. Allele frequencies and expected Hardy Weinberg proportions (HWP) for each single nucleotide polymorphisms (SNP) were determined.[32] The exact test for genotype frequency deviation from HWP was calculated using the Arlequin implementation accessed via PyPop.[33] Those SNPs that did not fit HWP were evaluated to determine whether there was an excess of homozygotes or heterozygotes, or if any particular genotype frequencies were significantly different from the expected frequencies by the chi square test.

The Ewens-Watterson homozygozity test of neutrality[34] with Slatkins *p*-values [35,36] were used to indicate any deviations from the hypothesis of neutral selection for each locus. The Heidelberg kit allowed SNP haplotypes in *TGF-β1, TNF$-\alpha$, IL-2, IL-4, IL-6*, and *IL-10* to be detected.

RESULTS

Cytokine Alleles

Frequencies of polymorphic alleles, test of neutrality with F_{nd} statistic [Ewens-Watterson test of neutrality (EWN)], and Slatkin's Exact P. Value (SEPV) with p of F statistics in Roma population are shown in the Table 1. The frequency of alleles for some single nucleotide polymorphisms (SNPs) varies from 0.987 for *TNF-α 238/A*, 0.974 for *TNF-α 308/A*, 0.942 for *TGF-β1 cdn25/C*, 0.904 for *IL-4Rα +1902/A*, followed by 0.857 for *IL-RA mspa11100/C*, and 0.818 for *IL-4 -33/C* indicating common "wild type" allele in those cytokines. The frequency ranges spanned 50 % for each allele of *IFNγ utr5644, TGF-β1 cdn10, IL-1β -511, IL-2 -330, IL-4 -1098, IL-4 -590*, and *IL-10 -1082*, indicating no common "wild type" allele in those cytokines (Table 1).

For the majority of SNPs, test of neutrality showed a negative value for F_{nd} statistic (Ewens-Watterson test of neutrality), which indicates a balancing selection operating on the alleles at that locus. F_{nd} was negative and significantly different from 0 for *IL-4 -590* (*p* of F=0.006), *IL-10 -1082* (*p* of F=0.010), *IFNγ utr5644* (*p* of F=0.024), *IL-4 -1098* (*p* of F=0.026) and *TGF-β1 cdn25* (*p* of F=0.001). Only 4 SNPs (*TGF-β1 cdn25, IL-4Rα +1902, TNF-α -308*, and *TNF-α -238*) showed positive value for F_{nd} statistic, but without significant *P* values (Table 1).

Cytokine Genotypes

The observed versus the expected cytokine genotypes for each SNP, Hardy Weinberg proportion (HWP), and Guo and Thompson Hardy Weinberg Output (GTHWO) in Roma population is given in the Table 2. Several observed frequencies of cytokine genotypes were significantly different from the expectations: *IL-12B -1188/A:C* (0.038), genotypes of *IL-4 -1098/G:T* (p<0.001), *IL-4 -1098/T:T* (p=0.001), *IL-4 -1098/G:G* (p<0.001), genotypes of *IL-4 -590/C:C* (p<0.001), *IL-4 -590/C:T* (p<0.001), *IL-4 -590/T:T* (p<0.001), and genotypes of *IL-10 -1082/A:A* (p<0.001), *IL-10 -1082/A:G* (p<0.001), and *IL-10 -1082/G:G* (p<0.001) polymorphisms. In some instances, test cannot be calculated because the expected frequency was smaller than 5 (*IL-1α -889/T:T, IL-1β +3962/T:T, IL-1RA mspa11100/C:C, IL-4Rα +1902/G:G, TGF-β1 cdn25/C:C, TNF-α -308/A:A, TNF-α -308/A:G, TNF-α -238/A:A, TNF-α -238/A:G, IL-4 -33/T:T*, and *IL-6 nt565/A:A*). Most of SNPs showed a good fit with HWP expectations. A few SNPs (*IL-12B -1188, IL-2 -330, IL-4 -1098, IL-4 -590*, and *IL-10 -1082*) were not in HWP (p=0.002, 0.017, <0.001, <0.001, and <0.001, respectively), and Guo and Thompson Hardy Weinberg Output (GTHWO) was significant (p=0.003, 0.022, <0.001, <0.001, and <0.001, respectively) (Table 2). Hardy Weinberg proportion could not be calculated for both *TNFα* genotypes, because all Roma individuals were *G:G* homozygotes.

Table 1. Frequencies of polymorphic alleles, test of neutrality with F_{nd} statistic (Ewens-Watterson test of neutrality [EWN]) and Slatkin's Exact *p* Value (SEPV) with *p* of F statistics in Roma population.

Cytokine polymorphism	Alleles			Test of neutrality	
				EWN	SEPV
	Allele	Number	Frequency	F_{nd}	p of F
IL1A -889	*C*	117	0.760	-1.127	0.21
	T	37	0.240		
IL1B -511	*C*	93	0.596	-1.825	0.072
	T	63	0.404		
IL1B +3962	*C*	122	0.782	-0.985	0.229
	T	34	0.218		
IL1R pst1 1970	*C*	108	0.692	-1.493	0.146
	T	48	0.308		
IL1RA mspa 11100	*C*	132	0.857	-0.410	0.322
	T	22	0.143		
IL4RA +1902	*A*	141	0.904	0.013	0.399
	G	15	0.096		
IL12B -1188	*A*	98	0.636	-1.711	0.102
	C	56	0.364		
IFNG UTR5644	*A*	82	0.532	-1.920	0.024*
	T	72	0.468		
TGFB1 cdn10	*C*	85	0.552	-1.900	0.040*
	T	69	0.448		
TGFB1 cdn25	*C*	145	0.942	0.396	0.505
	G	9	0.058		
TNFA -308	*A*	152	0.974	0.752	0.668
	G	4	0.026		
TNFA -238	*A*	154	0.987	0.899	0.824
	G	2	0.013		
IL2 -330	*G*	66	0.429	-1.872	0.055
	T	88	0.571		
IL2 +160	*G*	114	0.740	-1.243	0.192
	T	40	0.260		
IL4 -1098	*G*	79	0.534	-1.912	0.026*
	T	69	0.466		
IL4 -590	*C*	75	0.507	-1.9251	0.006*
	T	73	0.493		
IL4 -33	*C*	121	0.818	-0.721	0.273
	T	27	0.182		
IL6 -174	*C*	116	0.744	-1.226	0.194
	G	40	0.256		
IL6 nt565	*A*	123	0.788	-0.941	0.235
	G	33	0.212		
IL10 -1082	*A*	80	0.513	-1.933	0.010*
	G	76	0.487		
IL10 -819	*C*	115	0.737	-1.263	0.188
	T	41	0.263		
IL10 -590	*A*	111	0.712	-1.401	0.162
	C	45	0.288		

Statistically significant $F_{nd} < 0$ indicates a balancing selection; significant $F_{nd} > 0$ indicates a directional selection. * Statisticaly significant.

Table 2. The observed vs. expected cytokine genotypes for each SNP, Hardy Weinberg proportions (HWP), and Guo and Thompson Hardy Weinberg output (GTHWO) in Roma population.

Polymorphism	Genotype	Observed	Observed frequency	Expected	*p*-value	HWP *p*-value	GTHWO *p*-value
	C:C	44	0.571	44.4	0.947		
IL1A -889	*C:T*	29	0.377	28.1	0.867	0.857	1.000
	T:T	4	0.052	4.4	§		
	C:C	29	0.372	29.7	0.808		
IL1B -511	*C:T*	35	0.449	37.6	0.676	0.548	0.639
	T:T	14	0.179	12.7	0.720		
	C:C	46	0.590	47.7	0.805		
IL1B +3962	*C:T*	30	0.385	26.6	0.508	0.480	0.339
	T:T	2	0.025	3.7	§		
	C:C	39	0.500	37.4	0.792		
IL1R pst1 1970	*C:T*	30	0.385	33.2	0.575	0.391	0.427
	T:T	9	0.115	7.4	0.552		
	C:C	3	0.039	1.6	§		
IL1RA mspa11100	*C:T*	16	0.208	18.9	0.511	0.494	0.169
	T:T	58	0.753	56.6	0.849		
	A:A	64	0.820	63.7	0.972		
IL4RA +1902	*A:G*	13	0.167	13.6	0.879	0.877	0.527
	G:G	1	0.013	0.7	§		
	A:A	25	0.325	31.2	0.268		
IL12B -1188	*A:C*	48	0.623	35.6	0.038*	0.002*	0.003*
	C:C	4	0.052	10.2	0.053		
	A:A	21	0.273	21.8	0.859		
IFNG UTR5644	*A:T*	40	0.519	38.3	0.788	0.704	0.821
	T:T	16	0.208	16.8	0.840		
	C:C	24	0.312	23.5	0.911		
TGFB1 cdn10	*C:T*	37	0.480	38.1	0.861	0.803	0.820
	T:T	16	0.208	15.5	0.890		
	C:G	9	0.117	8.5	0.857		
TGFB1 cdn25	*G:G*	68	0.883	68.3	0.975	0.854	1.000
	C:C	0	§	0.3	§		
	A:G	4	0.051	3.9	§		
TNFA -308	*G:G*	74	0.949	74.1	0.995	§	1.000
	A:A	0	§	0.1	§		
	A:G	2	0.026	2.0	§		
TNFA -238	*G:G*	76	0.974	76.0	0.999	§	1.000
	A:A	0	§	0.0	§		
	G:G	9	0.117	14.1	0.172		
IL2 -330	*G:T*	48	0.623	37.7	0.094	0.017*	0.022*
	T:T	20	0.260	25.1	0.305		
	G:G	44	0.571	42.2	0.781		
IL2 +160	*G:T*	26	0.338	29.6	0.507	0.285	0.370
	T:T	7	0.091	5.2	0.428		
IL4 -1098	*G:T*	67	0.905	36.8	<0.001*	<0.001*	<0.001*
	T:T	6	0.081	21.1	0.001*		

	G:G	1	0.014	16.1	<0.001*		
	C:C	1	0.014	19.0	<0.001*		
IL4 -590	*C:T*	73	0.986	37.0	<0.001*	<0.001*	<0.001*
	T:T	0	§	18.0	<0.001*		
	C:C	54	0.730	49.5	0.519		
IL4 -33	*C:T*	13	0.176	22.1	0.053	0.0417*	0.0015*
	T:T	7	0.094	2.5	§		
	C:C	4	0.051	5.1	0.618		
IL6 -174	*C:G*	32	0.410	29.7	0.679	0.503	0.766
	G:G	42	0.539	43.1	0.864		
	A:A	1	0.013	3.5	§		
IL6 nt565	*A:G*	31	0.397	26.0	0.329	0.298	0.168
	G:G	46	0.590	48.5	0.721		
	A:A	5	0.064	20.5	<0.001*		
IL10 -1082	*A:G*	70	0.897	39.0	<0.001*	<0.001*	<0.001*
	G:G	3	0.039	18.5	<0.001*		
	C:C	39	0.500	42.4	0.603		
IL10 -819	*C:T*	37	0.474	30.2	0.218	0.0477*	0.077
	T:T	2	0.026	5.4	0.144		
	A:A	5	0.064	6.5	0.559		
IL10 -590	*A:C*	35	0.449	32.0	0.598	0.411	0.584
	C:C	38	0.487	39.5	0.813		

§ cannot be calculated because expected <5, χ^2 test, *, Statisticaly significant.

Cytokine Haplotypes

The cytokine haplotypes frequency and test of neutrality with F_{nd} statistic (Ewens-Watterson test of neutrality (EWN)), and Slatkin's Exact *P*. Value (SEPV) with p of F statistics in Roma population is shown in the Table 3.

For several genes with multiple SNPs per gene (*TGF-β1, TNF-α, IL-2, IL-4, IL-6, IL-10*), the Heidelberg PCR-SSP kit was able to detect true haplotypes. The most frequent haplotypes for *TGF-β1* are *TGF-β1/CG* (0.494), and TGF-β1/TG (0.448) with the absence of *TGF-β1/TC* haplotype in Roma population. The most frequent *TNF−α* haplotype was *TNF-α/GG* (0.834), following with *TNF-α/AG* (0.026), and TNF-α/GA (0.013). *IL-2* haplotypes in Roma population are nearly equally distributed between the *IL-2/GG* (0.377), *IL-2/TG* (0.334), and *IL-2/TT* (0.208), with the smallest frequency of *IL-2/GT* (0.052) haplotype. The most frequent *IL-4* haplotype is *IL-4/GCC* (0.385), followed with *IL-4/TTC* (0.324), and *IL-4/TTT* (0.135) haplotypes. The rest of *IL-4* haplotypes have a very small frequency (*IL-4/TCC, IL-4/GTC*, and *IL-4/GCT*). The most frequent *IL-6* haplotype is *IL-6/GG* (0.744), followed with *IL-6/CA* (0.212), and *IL-6/CG* (0.045) with the absence of *IL-6/GA* haplotype in Roma population. Several *IL-10* haplotypes are equally distributed in Roma population [*IL-10/GCC* (0.474), *IL-10/ACC* (0.218), and *IL-10/ATA* (0.256)], and three of them with a very small frequency [*IL-10/ACA* (0.026), *IL-10/GCA* (0.013) and *IL-10/ATC* (0.013)]. Test of neutrality showed a negative value for F_{nd} statistic (Ewens-Watterson test of neutrality), which indicates a balancing selection operating on the haplotypes at that locus, except for the *TNF-α* haplotypes, where it showed a positive value for F_{nd} statistic, but without significant *P* of F statistic. F_{nd} was negative and significantly different from 0 for *IL-2* haplotypes (*p*=0.025) (Table 3).

Cytokine Diplotypes

The cytokine diplotypes, observed vs. expected genotype frequency for each SNP, Hardy Weinberg proportions (HWP), Guo and Thompson Hardy Weinberg output (GTHWO), and Linkage Disequilibrium in Roma population is shown in the Table 6.

Table 3. The haplotypes frequency and test of neutrality with F_{nd} statistic (Ewens-Watterson test of neutrality [EWN]), and Slatkin's Exact *P* Value (SEPV) with *p* of F statistics in Roma population.

Polymorphism	Haplotypes			Test of neutrality	
	Haplotype	Number	Frequency	EWN F_{nd}	SEPV p of F
TGF-β1	*CC*	9	0.058	-1.325	0.091
	CG	76	0.494		
	TC	0	0.000		
	TG	69	0.448		
TNF-α	*AG*	4	0.026	1.237	0.852
	GA	2	0.013		
	GG	150	0.962		
IL-2	*GG*	58	0.377	-1.522	0.025*
	GT	8	0.052		
	TG	56	0.364		
	TT	32	0.208		
IL-4	*GCC*	57	0.385	-1.122	0.096
	GCT	6	0.041		
	GTC	6	0.041		
	TCC	11	0.074		
	TTC	48	0.324		
	TTT	20	0.135		
IL-6	*CA*	33	0.212	-0.511	0.377
	CG	7	0.045		
	GG	116	0.744		
IL-10	*ACC*	34	0.218	-0.756	0.258
	ACA	4	0.026		
	ATC	2	0.013		
	ATA	40	0.256		
	GCC	74	0.474		
	GCA	2	0.013		

Statistically significant F_{nd} <0 indicates a balancing selection; significant F_{nd} >0 indicates a directional selection, *, Statistically significant.

Table 4. The observed vs. expected diplotype frequency, Hardy Weinberg proportions (HWP), and Guo and Thompson Hardy Weinberg output (GTHWO), and Linkage Disequilibrium in Roma population.

Polymorphism	Genotype	Observed	Expected	*p*-value	HWP *p*-value	GTHWO *p*-value
TGF-β1	*CG:CG*	21	18.8	0.604	0.454	0.506
	CG:TG	31	34.1	0.601		
	CG:CC	3	4.4	§		
	TG:TG	16	15.5	0.890		
	CC:TG	6	4.0	§		
	CC:CC	0	0.3	§		
TNF-α	*GG:GG*	72	72.1	0.989	§	1.000
	GG:AG	4	3.8	§		
	GG:GA	2	1.9	§		
	AG:AG	0	0.1	§		
	GA:AG	0	0.1	§		
	GA:GA	0	0.0	§		
IL-2	*GG:GG*	9	10.9	0.561	0.002*	<0.001*
	GG:TG	32	21.1	0.018*		
	GG:TT	8	12.1	0.243		
	GG:GT	0	3.0	§		

	TG:TG	3	10.2	0.024*		
	TT:TG	10	11.6	0.631		
	TT:TT	7	3.3	§		
	GT:TG	8	2.9	§		
	GT:TT	0	1.7	§		
	GT:GT	0	0.2	§		
	GCC:GCC	0	11.0	<0.001*	<0.001*****	<0.001*****
	GCC:TTC	45	18.5	<0.001*		
	GCC:TTT	10	7.7	0.408		
	GCC:TCC	1	4.2	§		
	GCC:GCT	0	2.3	§		
	GCC:GTC	1	2.3	§		
	TTC:TTC	0	7. 8	0.005**		
	TTT:TTC	0	6.5	0.011*		
	TTT:TTT	0	1.4	§		
	TCC:TTC	3	3.6	§		
IL-4	*TCC:TTT*	3	1.5	§		
	TCC:TCC	0	0.4	§		
	GCT:TTC	0	1.9	§		
	GCT:TTT	6	0.8	§		
	GCT:TCC	0	0.4	§		
	GCT:GCT	0	0.1	§		
	GTC:TTC	0	1.9	§		
	GTC:TTT	1	0.8	§		
	GTC:TCC	4	0.4	§		
	GTC:GCT	0	0.2	§		
	GTC:GTC	0	0.1	§		
	CA:GG	28	24.5	0.485	0.372	0.204
	CG:GG	4	5.2	0.597		
IL-6	*GG:GG*	42	43.1	0.864		
	CA:CA	1	3.5	§		
	CG:CA	3	1.5	§		
	CG:CG	0	0.2	§		
	GCC:GCC	3	17.6	<0.001*	<0.001*	<0.001*
	ATA:GCC	31	19.0	0.006*		
	ACC:GCC	32	16.1	<0.001*		
	GCC:ACA	3	1.9	§		
	GCC:ATC	2	0.9	§		
	GCC:GCA	0	0.9	§		
	ATA:ATA	2	5.1	0.167		
	ACC:ATA	2	8.7	0.023*		
	ACC:ACC	0	3.7	§		
	ACA:ATA	1	1.0	§		
IL-10	*ACA:ACC*	0	0.9	§		
	ACA:ACA	0	0.1	§		
	ATC:ATA	0	0.5	§		
	ATC:ACC	0	0.4	§		
	ATC:ACA	0	0.1	§		
	ATC:ATC	0	0.0	§		
	GCA:ATA	2	0.5	§		
	GCA:ACC	0	0.4	§		
	GCA:ACA	0	0.1	§		
	GCA:ATC	0	0.0	§		
	GCA:GCA	0	0.0	§		

§ cannot be calculated because expected <5, χ^2 test, *, statistically significant.

The cytokine diplotypes (or haplotype zygozity) are combinations of haplotypes from both parents. They showed a good fit with HWP expectations for *TGF-β1* and *Il-6*. Several observed frequencies of cytokine diplotypes were significantly different from the expectations: *IL-2/GG:TG* (p=0.018), *IL-2/TG:TG* (p=0.024), *IL-4/GCC:GCC* (p<0.001), *IL-4/TTC:TTC* (p=0.005), *IL-4/TTT:TTC* (p=0.011), *IL-10/GCC:GCC* (p<0.001), *IL-10/ ATA:GCC* (p=0.006), *IL-10/ACC:GCC* (p<0.001), and *IL-10/ACC:ATA* (p=0.023). Several frequencies of diplotypes were less than 5 and chi-square test was not calculated. Hardy-Weinberg proportion and Guo and Thompson Hardy Weinberg output were statistically significant for *IL-2* (p=0.002 and p<0.001), *IL-4* (p<0.001), and *IL-10* (p<0.001) (Table 4). Because all Roma individuals have *TNFα/GG:GG* diplotype, Hardy Weinberg proportions could not be calculated for this polymorphism.

DISCUSSION

Unlike other founder populations, whose history, genealogy and genetic epidemiology have been extensively documented,[37-40] Roma do not have their own written history, church records and historical research of their own; therefore, theories about their origins and migrations are based on legends or on linguistics and cultural anthropology. Anthropological studies such as the present report are fundamental for the studies of disease association in members of this population group.

In this study, the detailed characterization of 22 cytokines gene polymorphism was done. To our knowledge, this is the first systematic investigation of cytokine polymorphism in a sample of Roma population.

We used F statistic for the Ewens-Watterson homozygosity test of neutrality. If the normal deviate of homozygosity (F_{nd}) is significantly less than 0, it indicates that balancing selection is operating on the alleles at that locus; significant $F_{nd}>0$ indicates directional selection,[36] provided the assumptions of the model are met. P values lower than 0.025 or higher than 0.975 were considered significant for this 2-tailed test at the 0.05 level. Although for the majority of SNPs, test of neutrality showed a negative value for F_{nd} statistic (Ewens-Watterson test of neutrality), which indicates a balancing selection operating on the alleles and haplotypes at that locus, F_{nd} was negative and significantly different from 0 for *IL-4 -590* (p of F=0.006), *IL-10 -1082* (p of F=0.010), *IFNγ utr5644* (p of F=0.024), *IL-4 -1098* (p of F=0.026) and *TGF-β1 cdn25* (p of F=0.001) alleles, as well as for *IL-2* haplotypes (p=0.025). Only 4 SNPs (*TGF-β1 cdn25, IL-4Rα +1902, TNF-α -308*, and *TNF-α -238*) and *TNF-α* haplotypes showed positive value for F_{nd} statistic, but without significant P values.

Most of cytokine genotypes and *TGF-β1* and *IL-6* diplotypes showed a good fit with HWP expectations. A few SNPs (*IL-12B -1188, IL-2 -330, IL-4 -1098, IL-4 -590*, and *IL-10 -1082*) and several observed frequencies of cytokine diplotypes (*IL-2/GG:TG, IL-2/TG:TG, IL-4/GCC:GCC, IL-4/TTC:TTC, IL-4/TTT:TTC, IL-10/GCC:GCC, IL-10/ ATA:GCC, IL-10/ACC:GCC*, and *IL-10/ ACC:ATA*) were not in HWP and were significantly different from the expectations. Hardy Weinberg proportion could not be calculated for *TNFα* genotypes and diplotypes because nearly all genotypes and diplotypes belong to *GG* genotype or *GG: GG* diplotype.

Inter-population discrepancies in allele frequencies particularly between Caucasian and non-Caucasian sample cohorts are often large.[41] Significant differences in allelic frequencies among ethnic groups were reported.[42] The *IL-1RN* allele 2 was very rare in Koreans (frequency, 0.060). In addition, it was also found a significant difference for the *IL-1A* (-889) and *IL-1B* (+3953) polymorphisms in Koreans compared with Caucasians.[43] The frequency of *IL-1RN* 2-repeat allele was significantly lower in Taiwanese than in Caucasians. In contrast, the frequencies of the pro-inflammatory *IL-1B –511T* allele and *+3954C* allele were significantly higher among Taiwanese compared with Caucasians.[44]

Gypsies as a nomadic people, widely persecuted throughout Europe and often used as slaves until the abolition of slavery in the 19th century, have lived in scattered populations in different countries throughout the world. This divergent Gypsy groups have been shown to share unique Mendelian disorders and founder mutations,[45-49] as well as ancestral Y chromosome and mtDNA lineages.[50,51]

Because Gypsy groups are separated from each other by strict rules of endogamy and intermarriage between neighbouring communities in the same small town is often proscribed, they are a transnational genetic isolate. They have reduced genetic diversity

and increased linkage disequilibrium, and therefore present a particular genetic disease spectrum, with some prevalent diseases almost absent, others specific to Gypsies, and others with private Roma mutations.[3] There are nine known Mendelian disorders caused by private "Romani" mutations, [45,46,52,53-55] and a number of previously known but rare disorders have been identified and shown to be caused by novel private mutations. [47,49,56,57]

Our results can be explained partly by reduced genetic diversity, increased linkage disequilibrium and small number of participants in this report. Additional investigations of cytokine polymorphisms are necessary in Roma people from neighbouring countries to verify our results.

In summary, the results of cytokine alleles, genotypes, haplotypes, haplotype zygosity and linkage disequilibrium in the sample from the Roma population can be used for characterization of the current genetic profile of the Gypsies, anthropological comparisons, as well as for association studies with different diseases.

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