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Frequency distribution of six cytokine gene polymorphisms in North- and South-Italy -Italy

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Short Communications

Population Study

Frequency distribution of six cytokine gene polymorphisms in North- and South-Italy

Running Head: Cytokine gene polymorphisms in an Italian sample

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Summary

Allelic and genotype frequencies of four cytokine genes were obtained from 738 subjects from North- and South-Italy. Populations were in Hardy-Weinberg equilibrium for all genes but significantly differed in the frequency of all SNPs and three haplotypes. In the MDS graph, they were plotted in separate positions close to Europeans and an Ivorian population, respectively.

Introduction

Cytokines are small regulatory proteins mainly secreted by active immune cells in response to different stimuli such as infection and tissue damage. These molecules play a crucial role in regulating all aspects of immune and inflammatory responses and represent key components in the pathogenesis of many diseases like cancer, metabolic disorders, infectious and autoimmune diseases (for reviews see: Bidwell *et al*., 1999, 2001; Haukim *et al*., 2002; Hollegaard & Bidwell, 2006).

Inter-individual differences in the related cytokines serum levels were observed (Hoffmann et al., 2002). These differences in cytokine production and, consequently, in the individual response to various antigens were attributed to a different factors, including gene polymorphisms. Indeed, certain cytokine polymorphisms, mostly single nucleotide polymorphisms (SNPs) located within in the promoter or coding regions, have been shown to affect the overall expression and secretion of the gene products (Hoffmann et al., 2001).

At individual level, this different cytokines expression could explain the different susceptibility to various diseases including autoimmune, infectious or cancer diseases observed among individual belonging to the same population. At population level, it was found that the distribution of cytokine polymorphisms significantly varies among different ethnic groups, mainly as a result of founder effects and geographically localized selective forces (Santovito *et al*., 2012; Hollegaard & Bidwell, 2006; Hoffmann *et al*., 2002). For this reason, the association of cytokine gene polymorphisms with a particular disease cannot be extrapolated from a specific population to other populations with

different genetic background. On the other hand, data concerning distribution of cytokine polymorphisms in healthy populations are important in order to investigate the possible associations of these polymorphisms with a particular disease, especially in case-control studies.

Finally, the analysis of cytokine gene polymorphisms in different populations has been used in anthropological studies in order to establish possible gradient in the distribution of the genetic variation among human populations.

Italy has received the passage of multiple human groups in prehistoric and historic times: Phoenician, Greek, Carthaginian, Roman, Arabic, Norman and Barbaric populations contributed to the present genetic composition of Italy (Rickards et al., 1998). Genome wide association studies evidenced a genetic structure of the Italian population strongly influenced by the geographical distance, with certain degree of genetic substructure between Southern Italians, Northern Italy and other European populations (Nelis et al. 2009; Di Gaetano et al., 2012). In particular, studies based on mtDNA and Y-chromosome variability (Barbujani et al., 1995; Capelli et al., 2007) identified a North-South gradient of the genetic variation within the peninsula. Differential Neolithic/Mesolithic contributes in the two regions as well as local drift and founder effects were invoked to explain the observed genetic variation distribution (Cappelli et al., 2007; Di Giacomo et al., 2003).

In this scenario, the aim of the present study was to evaluate the alleles, genotypes and haplotypes frequencies of selected cytokine gene polymorphisms in South and North Italian populations, and to compare these allele frequencies with those already published for other populations worldwide distributed.

Materials and Methods

Subjects

The study was conducted on a cohort of 738 unrelated healthy Italian subjects of Caucasian origin collected specifically for this epidemiological study and not for diseases studies: 635 from North

Italy (359 females and 276 males, mean age 51.04±0.87, 341 from Piedmont, 198 from Lombardy and 96 from Aosta regions) and 103 from South Italy (Sicily region, 53 females and 50 males, mean age 49.63±1.65, 62 from Trapani and 41 from Pantelleria Island). All the subjects were randomly chosen healthy volunteers, received detailed information about the study, and gave their informed consent prior the analyses.

The study was approved by the University of Turin ethics committee and was performed in agreement with the ethical standards laid down in the 1964 Declaration of Helsinki.

DNA extraction and Genotyping

Peripheral blood samples (5-10 ml obtained by venipuncture) were collected in heparinized vacutainers and stored at -20°C. DNA extraction was conducted using a Chelex solution, according to the following protocol: 10 µL of peripheral blood was diluted in 1 mL of sterile distilled water for 15 min at room temperature. After centrifugation at 14,000 rpm for 1 min, the pellet was re-suspended in 200 µL of 5% Chelex solution in Tris-EDTA at pH 8, heated to 56°C for 15 min and, after vortex for 10 sec, at 100°C in boiler water for 8 min. For PCR reactions we used 19 of the 200 µL of this solution, containing about 10 ng of DNA as indicated by the spectrophotometric analysis. PCR-based genotyping was performed for the genes encoding *TNFα* (G/A -308), *IL10* (G/A -1082, C/T -819), *TGFβ* (C/T codon 10, G/C codon 25), *IL6* (G/C -174). The sequence polymorphisms were determined by SSP-PCR methodology, using primers described in Perrey et al., (1999). PCR reactions were performed in a 25 µL volume, with a final concentration of 1X Reaction Buffer, 1.5 mM of MgCl2, 5% of DMSO, 250 µM of dNTPs, 0.5 µM of each primer, and 1 U/sample of Taq DNA polymerase (Fischer, U.S.). Cycles were set as follows: 35 cycles, 1 min at 95°C, 1 min at 60°C, 1 min at 72°C, and a final extension step 10 min at 72 °C. Amplification products were detected by ethidium bromide staining after 2.5% agarose gel electrophoresis. To verify the genotyping results, about 10% of the total sample (n = 80) were also analysed by another investigator. The two analyses showed identical results.

Statistical Analysis

Allele, genotype and haplotype frequencies of the six SNPs were calculated by direct counting, dividing by the number of subjects (to produce genotype frequency) or chromosomes (to produce allele and haplotype frequency). The Pearson chi-square test (χ^2-test), with a 95% confidence interval, as implemented in SPSS statistical package program (v23.0, IBM, Chicago, IL), was used to analyse possible statistical differences in allele and haplotype frequencies between studied populations, as well as to test the Hardy-Weinberg equilibrium (HWE). Allele frequencies found in our populations were compared with published data for other worldwide distributed populations. Genetic relationships among these populations were analysed via non-metric multidimensional scaling (MDS) analysis of allele frequencies, as implemented in SPSS, and by plotting the positions of the populations using the two principal dimensions. We computed the Euclidean distance for each pair of populations using cytokine allele frequencies. This dissimilarity matrix was used to generate a MDS plot of population variation in two dimensions.

Results and Discussion

Allele and genotype frequencies of North and South Italian populations were reported in Table 1. In both studied populations the genotype frequency distribution of all polymorphic cytokine genes did not show a significant deviation from HWE, indicating a random distribution and the absence of evolutionary forces acting in shaping the frequencies of these gene polymorphisms. However, when we compared the two populations, we found significant differences for all SNPs of the cytokine polymorphisms tested. Therefore, we considered these two Italian populations as two separate groups when compared with other populations.

We further calculated the haplotype frequencies of *IL10 (-1082G/A, -819C/T)* and *TGFβ1 (cod.10 C/G, cod. 25 C/T)* (Table 2). For *IL10 (-1082G/A, -819C/T)*, a total of 4 haplotypes (for both North- and South-Italy, samples) was found, with the GC haplotype showing the higher frequency in both

populations (0.796 and 0.602 for North- and South-Italy, respectively), followed by the GT haplotype (0.090 and 0.199 for North- South-Italy, respectively). *IL10* (-1082G/A, -819C/T) GG/CC was the most common genotype found, with a frequency of about 0.613 and 0.359 for North- and South-Italian populations, respectively, while the least common genotype was GA/TT for North-Italy (0.002) and AA/CC for South-Italy where it resulted absent.

The most common *TGFβ1 (cod.10 C/G, cod. 25 C/T)* haplotype was CG for both populations (with frequencies of 0.765 and 0.840 for North- and South-Italy, respectively), followed by TG haplotype found at 0.162 and 0.082 in North- and South-Italian populations, respectively. The most common *TGFβ1 (cod.10 C/G, cod. 25 C/T)* genotype was CC/GG, with a frequency of 0.586 in North-Italy and 0.728 in South-Italy, while the least common was CC/CC (0.003) for North-Italy and TT/GG (0.010) for South-Italy. Significant differences were found between North- and South-Italy in the frequency of *IL10 (-1082G/A, -819C/T)* GC ($P = 0.021$), GT ($P < 0.001$) and AT ($P = 0.002$) haplotypes as well as in the frequency of *TGFβ1 (cod.10 C/G, cod. 25 C/T)* TG haplotype ($P = 0.008$).

In order to further assess population relationships, a MDS analysis based on the cytokine allele frequencies was carried out comparing our studied populations with some worldwide distributed populations data set typed for the same loci (Nancy et al., 2004; Louie et al., 2005; Mihailova et al., 2005; Trajkov et al., 2005; Kubistova et al., 2006; Skorpil et al., 2007; Javor et al., 2007; Sodsai et al., 2011; Visentainer et al., 2008; Norhalifah et al., 2015; Santovito et al., 2012; Kaur et al., 2007; Costeas et al., 2003) (Fig. 1). MDS can be considered to be an alternative to factor analysis that allows to analyse any kind of similarity or dissimilarity matrix, in addition to correlation matrices. In this case, the two dimensions account for 99% of the observed variance and were used to plot the positions of the populations.

In the MDS plot, the studied Italian populations were separated from the major group: North-Italy was plotted in the same quadrant of other European populations, such as Slovak, Czech, Germany, Netherland and Macedonian populations, although separated from them. South-Italy, *vice versa*,

was well separated from all the other populations and plotted in the same quadrant of the Ivory Coast population.

In general, when compared to other European populations, the Northern Italian population was genetically close to North-European populations, such as the French population, whereas the Southern Italians had some similarities with other Mediterranean populations, as well as with those from Middle East (Di Gaetano et al., 2012).

Our results seem to be concordant with data obtained by other authors with more polymorphic markers, showing for Italy a differential genetic diversity pattern between North- and South-Italy (Boattini et al., 2013). This picture probably reflects the different genetic history of Sicily with respect to Northern Italy. Indeed, because to its central geographic location in the Mediterranean domain, Sicily has long been the meeting place of ancient civilizations and cultures and hosted various human groups in both prehistoric and historic times (Sarno et al., 2014). As consequence, various populations have contributed to the genetic structure of this island. Since the arrival of the first human groups (Sicani, Siculi, Elymi), the island has been subjected to numerous migratory flows: Greeks, Phoenicians, Etruscans, Romans, Vandals, Goths, Byzantines, Arabs, Aragoneses and Normans contribute to the strong heterogeneity observed in the genetic structure of Sicilian populations (Rickards et al. 1998; Cerutti et al., 2004).

For other populations, such as Asian and African populations, the observed differences could be explained by founder effects and/or local selective pressures imposed by host-pathogen interactions on specific geographic populations (Hollegaard & Bidwell, 2006; Santovito et al., 2012). Finally, for the European populations differences could be probably explained with the different sample size and possible stochastic factors, although also for these populations micro-evolutionary forces cannot be excluded.

Conclusion

In conclusion, in this study we found that the frequencies of analyzed cytokine alleles showed a significant different distribution between North- and South-Italy, as also evidenced in the MDS plot. Because of the ethnical and local differences in the distribution of cytokine gene polymorphisms, the population data from healthy individuals are of relevant interest for the evaluation of the role of these polymorphisms in the differential response to various immunological diseases and in the occurrence of those diseases influenced by variations of cytokines production.

Ethics

Samples included in present study were collected after informed consent.

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Disclosures

None of the authors have a conflict of interest to declare in relation to this work.

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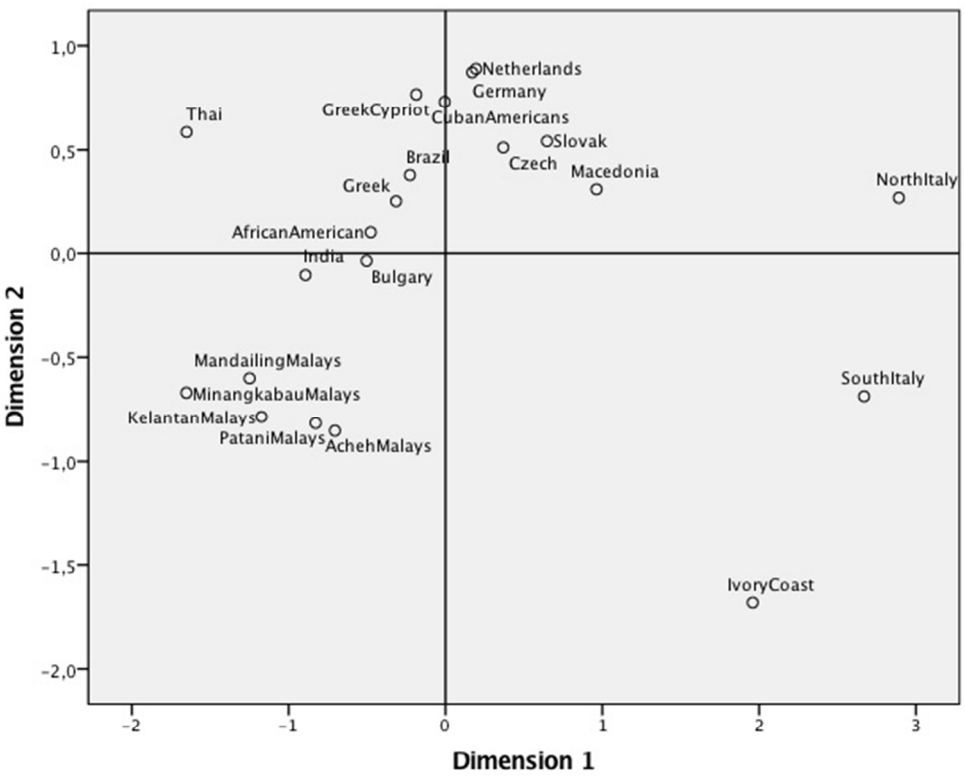
Figure 1 - Nonmetric MDS applied in R matrix based on six cytokine gene polymorphisms analyzing the genetic relationships among some populations worldwide distributed. The two axes explain about 99% of the variation in allele frequencies.



223x184mm (72 x 72 DPI)

Table 1 – Allele and Genotype Frequencies of six cytokine gene polymorphisms In North- (n = 635) and South-Italy (n = 103)

Cytokine polymorphisms	Allele	N	Frequency	Genotype	N	Frequency	HWE *P*-value
TNFa-308							
North-Italy	A	155	0.122	AA	6	0.009	0.440
	G	1115	0.878	AG	143	0.225	
				GG	486	0.765	
South-Italy	A	59	0.286	AA	6	0.058	0.498
	G	147	0.714	AG	47	0.456	
				GG	50	0.485	
IL6 -174							
North-Italy	C	114	0.090	CC	5	0.008	0.998
	G	1156	0.910	CG	104	0.164	
				GG	526	0.828	
South-Italy	C	48	0.233	CC	9	0.621	0.171
	G	158	0.767	CG	30	0.291	
				GG	64	0.087	
IL10 -1082							
North-Italy	A	144	0.133	AA	6	0.009	0.694
	G	1126	0.887	AG	132	0.208	
				GG	497	0.783	
South-Italy	A	41	0.199	AA	4	0.039	0.999
	G	165	0.801	AG	33	0.320	
				GG	66	0.641	
IL10 -819							
North-Italy	T	155	0.122	TT	8	0.013	0.864
	C	1115	0.878	CT	139	0.219	
				CC	488	0.769	
South-Italy	T	58	0.282	TT	12	0.117	0.175
	C	148	0.718	CT	34	0.330	
				CC	57	0.533	
TGFB-codon 10							
North-Italy	T	233	0.183	CC	28	0.044	0.214
	C	1037	0.817	CT	177	0.279	
				TT	430	0.677	
South-Italy	T	24	0.117	CC	3	0.029	0.309
	C	182	0.883	CT	18	0.175	
				TT	82	0.796	
TGFB-codon 25							
North-Italy	C	93	0.073	CC	2	0.003	0.714
	G	1177	0.927	CG	89	0.140	
				GG	544	0.857	
South-Italy	C	16	0.078	CC	2	0.019	0.166
	G	190	0.922	CG	12	0.117	
				GG	89	0.864	

HWE = Hardy-Weinberg Equilibrium. Significant differences for all SNPs of the cytokine polymorphisms tested were found between North- and South-Italy.

Table 2 – *IL10 (-1082G/A, -819C/T)* and *TGFβ1 (cod.10 C/G, cod. 25 C/T)* genotype and haplotype frequency in the studied North- (n = 635) and South- (n = 103) Italian populations.

Cytokine	North-Italy N (frequency)	South-Italy N (frequency)	*P* χ^2 _ test
IL10 (-1082G/A, -819C/T) **Genotype**			
GG/CC	389 (0.613)	37 (0.359)	0.009
GG/CT	101 (0.159)	21 (0.204)	0.333
GG/TT	7 (0.011)	8 (0.078)	0.001
GA/CC	95 (0.150)	20 0.194)	0.320
GA/CT	36 (0.057)	9 (0.087)	0.272
GA/TT	1 (0.002)	4 (0.039)	0.002
AA/CC	4 (0.006)	0 (0.000)	1.000
AA/CT	2 (0.003)	4 (0.039)	0.005
IL10 (-1082G/A, -819C/T) ***Haplotypes***			
GC	1010 (0.796)	24 (0.602)	0.021
GT	114 (0.090)	41 (0.199)	<0.001
AC	105 (0.083)	24 (0.116)	0.151
AT	39 (0.031)	17 (0.083)	0.002
TGFβ1 (cod.10 C/G, cod. 25 C/T) **Genotype**			
CC/GG	372 (0.586)	75 (0.728)	0.208
CC/GC	50 (0.079)	5 (0.049)	0.415
CC/CC	2 (0.003)	2 (0.019)	0.098
CT/GG	143 (0.225)	13 (0.126)	0.066
CT/GC	34 (0.054)	5 (0.049)	1.000
TT/GG	29 (0.046)	1 (0.010)	0.107
TT/GC	5 (0.008)	2 (0.019)	0.258
TGFβ1 (cod.10 C/G, cod. 25 C/T) **Haplotype**			
CG	971 (0.765)	173 (0.840)	0.402
TG	206 (0.162)	17 (0.082)	0.008
CC	54 (0.042)	9 (0.044)	0.854
TC	39 (0.031)	7 (0.034)	0.828

P χ^2 = Probability from Chi-square test between North and South Italy
Significant results are highlighted in gray

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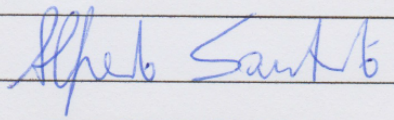
Contributor's signature  Date 02-02-2017

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