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HLA Alleles and Type 1 Diabetes Mellitus in Low Disease Incidence Populations of Southern Europe: A Comparison of Greeks and Albanians

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ABSTRACT

Type 1 diabetes mellitus (DM1) is caused by environmental factors acting on genetically susceptible individuals. HLA-DQA1 and -DQB1 are major genetic determinants of the disease. Greece and Albania represent the low DM1 incidence countries of South-Eastern Europe. The HLA-DQA1 and -DQB1 associations with DM1 were investigated in these two groups, as reference for comparisons to the high-risk populations of Northern Europe. One hundred and thirty Greeks and 64 Albanians with DM1 were studied; 1,842 Greeks and 186 Albanians were analysed as controls. The samples were typed for six HLA-DQB1 alleles, using time-resolved fluorometry to detect the hybridisation of lanthanide labelled oligonucleotides with PCR products. Individuals positive for DQB1*0201 were selectively typed for three DQA1 alleles. In both populations DQB1*0201 increased the risk for DM1 while DQB1*0301 was protective. DQB1*0302 was associated with lower risk than *0201, while *0602 and *0603 were protective in Greeks but not in Albanians. It was also shown that DQA1 has a modifying effect, altering the risk conferred by the susceptible DQB1*0201. The low incidence of DM1 in these two countries correlates with the high frequency of the protective allele DQB1*0301 and the low impact of the susceptible DQB1*0302.

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INTRODUCTION

Type 1 diabetes mellitus (DM1) is a multifactorial, multigenic disorder associated with severe complications and significant costs. It is caused by the autoimmune destruction of the insulin-secreting β-cells of the pancreas, eventually resulting in total exogenous insulin dependency[1,2].

Genetic susceptibility to the disease is complex, consisting of an unknown number of interacting loci. Three major genome scans[3-5], using microsatellite markers spaced at 10-20 cM across the entire genome and hundreds of affected sib-pairs, have indicated the existence of more than 20 loci possibly linked to DM1. Definite linkage to the disease, however, has only been confirmed for two of these loci; the HLA class II region and a VNTR locus at the 5′-end of the insulin gene[6-9]. The HLA region is the major genetic determinant of DM1, accounting for about 42% of the familial clustering of the disease[4], a relative risk (RR) greater by far than that attributed to any other single locus. HLA-DQ heterodimer molecules encoded by HLA-DQA1 and -DQB1 genes are believed to play the most important role, while the DR molecules and -DRB1 genes may have an independent effect and might in some cases modify the risk for the disease[10]. HLA-DQB1 genes *0201 and *0302, encoding for a non-Asp57 DQβ-chain, increase the risk for the development of DM1, while alleles *0301, *0602 and *0603 have a protective effect[6].

On the other hand, a DQα-chain with arginine at residue 52, which is encoded by HLA-DQA1 alleles *0301 and *0501, is associated with the development of DM1, while alleles DQA1*0201, *0101, *0102 and *0103 are protective[11].

The strong linkage disequilibrium observed between the HLA-DQA1, -DQB1 and -DRB1 loci simplifies in most cases the complicated analysis of HLA genotypes. One can often deduce the alleles of other loci based on the determination of the alleles in one locus, so that the whole haplotype is known. It has been shown that the susceptibility allele HLA-DQB1*0302 is associated with DQA1 *0301, which also increases the risk for the disease[12]. HLA-DQB1*0602 is almost always found with the protective DQA1*0102, while DQB1*0301 is always protective regardless of the DQA1 allele in the haplotype[12]. However, typing for HLA-DQA1 becomes very informative for individuals carrying DQB1*0201. When associated with DQA1 alleles *0301 or *0501 they confer a strong predisposition to DM1, although they are protective or neutral when found with DQA1*0201[12].

Although the role of genes in DM1 is essential, the penetrance of the predisposition or protection alleles is determined by yet unidentified environmental factors. The remarkable north to south gradient of the incidence of the disease in Europe is considered an indication of the important role of the environment in the pathogenesis of DM1, although it may also be due to the distribution of genetic factors[13]. The incidence of DM1 in Europe (expressed as new cases/100,000 children under the age of 14 years) is highest in Finland (50/100,000/year), and it decreases moving south[14]. Variation in the incidence of the disease may also be observed within ethnic groups. The neighboring countries of Greece and Albania represent the low DM1 incidence countries of Southeastern Europe. The incidence of the disease in Albania is 3/100,000/year[15]. In Greece, the mean incidence rate of DM1 is approximately 7/100,000/year. Nevertheless, it varies across the country. There is an impressive clustering of new cases observed in the Athens metropolitan area, where the incidence of the disease is 9-10/100,000/year, compared with rural and semi-rural areas (6.2/100,000/year in Northern Greece and 4.6/100,000/year in Crete)[16,17].

The associations of HLA-DQA1 and -DQB1 to DM1 were studied for the first time in the Albanian population and on the largest scale until now for Greece. The hypothesis of a genetic basis for the low incidence of DM1 in these two countries was investigated, and the data can serve as a reference for comparisons to high-risk populations.

PARTICIPANTS AND METHODS

The ethics committees of the university hospitals involved approved the study and informed consent was acquired from all participating individuals or their parents. Blood samples were collected from 130 Greeks and 64 Albanians with DM1. Reference samples for the Greek population were 1,842 cord bloods of consecutive births in a large maternity hospital in Athens. Blood samples were also collected from 186 healthy Albanians from Tirana. The analysis was performed directly on a drop of blood dried on filter paper.

All samples were typed for five HLA-DQB1 alleles, proven to be associated with increased risk for the development of DM1 (*0201, *0302) or protection from it (*0301, *0602, *0603). In addition, allele *0604, which is either positively or neutrally associated with the disease, was also detected[18,19]. The method used is based on time-resolved fluorometry (TRF) and has been described in detail previously[20,21]. In brief, 158 base pairs of the second exon of the DQB1 gene from each sample were amplified by polymerase chain reaction (PCR) using a primer pair with a biotinylated 3′ primer (5′-GCATGTGCTACTTCA CCAACG and 3′-CCTTCTGGCTGTTCCAGTACT). The amplification product was bound to streptavidin-coated microtitration plates and denatured with NaOH. After washing, bound DNA was hybridised with allele specific oligonucleotides (ASOs), each labelled with one of the three fluorescent lanthanides, europium, samarium or terbium[22]. The unique properties of the lanthanides allow simultaneous typing of all six detected alleles, using a combination of just two different hybridisation solutions. The probe hybridisation is measured using TRF (EG&G Wallac). The different emission wavelengths and delay times were used to distinguish the

signals of each lanthanide label[23] and the results were assessed by the Multicalc software.

The samples positive for DQB1*0201 were further typed for DQA1 alleles *0201, *0301 and *0501. This assay was based on the same principle as the one used for DQB1 genotyping and has been described elsewhere[24].

Statistical evaluation was performed using the χ^2 test. RR was estimated according to the formula RR = a(b + d) / b(a + c), in which a and b are the numbers of patients who were positive and negative for the marker, respectively, and c and d the respective numbers for control subjects.

RESULTS

HLA-DQB1 is strongly associated with both the development of DM1 and protection from it, in both populations studied (Tables 1, 2). HLA-DQB1*0201 confers a similar degree of risk for DM1 in both Greeks and Albanians, while *0301 is very frequent in both reference populations and protects strongly against the disease. Furthermore, DQB1*0302 increases the risk for DM1 in Greeks but not in Albanians. Nevertheless, the frequency of this allele in Albanian controls (11.29%) was almost half the frequency observed in the individuals with DM1 (20.31%). Alleles DQB1*0602 and

TABLE 1

Association of HLA-DQB1 alleles with type 1 diabetes mellitus (DM1) in Greece

HLA-DQB1* alleles	DM1		Background population		OR	RR	p
	n	%	n	%			
0201	86	66.15	539	29.26	4.72	4.21	<0.001
0301	15	11.54	1056	57.33	0.09	0.11	<0.001
0302	41	31.54	247	13.41	2.97	2.69	<0.001
0602-0603	3	2.31	323	17.54	0.11	0.12	<0.001
0604	9	6.92	108	5.86	1.19	1.18	NS
Total	130		1842				

OR = odds ratio; RR = relative risk; NS = non-significant.

TABLE 2

Association of HLA-DQB1 alleles with type 1 diabetes mellitus (DM1) in Albania

HLA-DQB1* alleles	DM1		Controls		OR	RR	p
	n	%	n	%			
0201	44	68.75	47	25.27	6.50	3.84	<0.0005
0301	9	14.06	85	45.70	0.19	0.27	<0.0005
0302	13	20.31	21	11.29	2.00	1.62	0.090
0602-0603	8	12.50	40	21.51	0.52	0.60	NS
0604	1	1.56	8	4.30	0.35	0.43	NS
Total	64		186				

OR = odds ratio; RR = relative risk; NS = non-significant.

*0603 appear to have a protective effect in Greeks but not in Albanians. The frequency in controls from Tirana (21.51%) was almost double that observed in individuals with DM1 (12.50%), but again this difference did not reach the level of statistical significance.

The distribution of the HLA-DQB1 alleles in genotypes revealed interesting information about the existing interaction between different alleles (Tables 3, 4). Thus, in Greece, the highest risk for the development of DM1 is associated with heterozygosity of the DQB1 alleles *0201 and *0302 (Table 3). This genotype is found in only 2% of the general populations of Greece and Albania (Tables 3, 4). Although its frequency is almost four times greater in Albanian individuals with DM1 (7.81%), it appears to have a statistically neutral effect in this population. An increased risk in both populations is detected with allele HLA-DQB1*0201 alone. Almost 50% of the patients in both populations carry this genotype, in contrast to 13.30% of the control population in Greece and 12.37% in Albania.

The difference in the degree of risk conferred by the DQB1 genotype *0302/y (where y represents *0302 or a non-defined allele) between Greeks and Albanians seems of particular interest. This genotype is associated with a high risk for the disease in Greeks. It is present in 11.45% of affected individuals versus 5.27% of the controls. In Albania, however, there is no difference in the frequency of this genotype between patients and controls (6.25% versus 5.38%).

The DQB1 genotype *0301/z (where z represents *0301 or a non-defined allele) protects strongly against the disease in both populations. It is found in 34.42% of the background population in Greece and in 28.49% in Albania (Tables 3, 4). It also provides a dominantly protective effect over the susceptibility allele *0201 in the Greek population (Table 3). Alleles *0602-*0603 alone are protective in Greeks (Table 3). They have a similar effect in Albanians only when they are found in combination with *0301 (Table 4).

Since DQB1*0201 alleles are at high frequency in both populations, further typing for DQA1 alleles *0201, *0301 and *0501 was performed to evaluate the importance of the actual risk conferred by the corresponding haplotype (Tables 5, 6). In both Greeks and Albanians, the haplotype HLA-DQA1*0501-DQB1*0201 was strongly associated with DM1. HLA-DQA1*0301-DQB1*0201 also increased the risk for the disease in Greeks, and was present almost twice as often in Albanians with DM1 (7.81%) than in controls (3.76%). On the other hand, the DQA1 allele *0201 offers protection to Greek carriers of the DQB1 allele *0201, while it has a neutral effect in Albanians.

Comparison of the detected allele frequencies and haplotypes between the two populations showed that the protective DQB1 allele *0301 is more frequent in the general population of Greece than in the population of Albania (Tables 7, 8). Furthermore, Greeks positive for HLA-DQB1*0201 carry the allele DQA1*0201 more frequently than Albanians, thus having a DM1 protective haplotype. The rest of the frequencies were comparable in the two populations.

DISCUSSION

It is still unclear whether the striking regional variations in the incidence of DM1 in Europe are due to environmental factors, genetic factors, or a combination of both. The effort to disentangle the relative contribution of genes and environment to the pathogenesis of DM1 is a difficult task, mainly because the environmental factors involved as well as many of the genetic factors remain obscure. The present study provides information on the DM1 association of the major genetic determinants of the disease (HLA-DQA1 and -DQB1) in two populations of South-Eastern Europe with very low incidence. Studies in low-risk populations can serve as a reference when compared to the high-risk populations of Northern Europe. Furthermore, our study provides data on the molecular typing of patients with DM1 in Albania for the first time. It is also the largest study of this kind ever performed on the Greek population.

Our results from the Greek sample are in concordance with similar studies in other European Caucasian populations and also with two small studies previously reported about the Greek population[25,26]. More specifically, HLA-DQB1 alleles *0201 and *0302 increase the risk for the

TABLE 3

Distribution of HLA-DQB1 genotypes in association with type 1 diabetes mellitus (DM1) in Greece

DQB1* genotype	DM1		Background population		OR	RR	P
	n	%	n	%			
0201/0302	20	15.27	36	1.95	9.12	6.22	<0.001
0302/y	15	11.45	97	5.27	2.34	2.17	<0.01
0301/0302	5	3.82	93	5.05	0.75	0.76	NS
0201/0301	4	3.05	193	10.48	0.27	0.29	<0.025
0201/x	62	47.33	245	13.30	5.94	4.94	<0.001
0302/0602-0603	0	0	13	0.71	0	0	NS
Others	8	6.11	119	6.46	0.95	0.95	NS
0301/z	5	3.82	634	34.42	0.07	0.08	<0.001
0201/0602-0603	1	0.76	44	2.39	0.32	0.33	NS
0301/0602-0603	0	0	109	5.92	0	0	<0.01
0602-0603/p	2	1.53	152	8.25	0.17	0.18	<0.01
0604/q	7	5.34	49	2.66	0.208	1.95	NS
Total	130		1842				

OR = odds ratio; RR = relative risk; NS = non-significant. x other than HLA-DQB1 *0302, *0301, *0602, *0603, *0604; y other than HLA-DQB1 *0201, *0301, *0602, *0603, *0604; z other than HLA-DQB1 *0201, *0302, *0602, *0603, *0604 p other than HLA-DQB1 *0201, *0301, *0302; q other than HLA-DQB1 *0201, *0301, *0302, *0602, *0603

TABLE 4

Distribution of HLA-DQB1 genotypes in association with type 1 diabetes mellitus (DM1) in Albania

DQB1* genotype	DM1		Controls		OR	RR	P
	n	%	n	%			
0201/0302	5	7.81	4	2.15	3.85	2.27	NS
0302/y	4	6.25	10	5.38	1.17	1.12	NS
0301/0302	2	3.13	3	1.61	1.96	1.58	NS
0201/0301	2	3.13	13	6.99	0.43	0.51	NS
0201/x	34	53.13	23	12.37	8.03	3.84	<0.001
0302/0602-0603	2	3.13	3	1.61	1.96	1.58	NS
Others	3	4.69	32	17.20	0.23	0.30	<0.01
0301/z	5	7.81	53	28.49	0.21	0.28	<0.001
0201/0602-0603	2	3.13	7	3.76	0.82	0.86	NS
0301/0602-0603	0	0.00	13	6.99	0	0	<0.025
0602-0603/p	4	6.25	17	9.14	0.66	1.73	NS
0604/q	0	0.00	4	2.15	0	0	NS
Total	64		186				

OR = odds ratio; RR = relative risk; NS = non-significant. x other than HLA-DQB1 *0302, *0301, *0602, *0603, *0604 y other than HLA-DQB1 *0201, *0301, *0602, *0603, *0604; z other than HLA-DQB1 *0201, *0302, *0602, *0603, *0604 p other than HLA-DQB1 *0201, *0301, *0302; q other than HLA-DQB1 *0201, *0301, *0302, *0602, *0603

TABLE 5

Association of HLA-DQA1 alleles with type 1 diabetes mellitus (DM1) in Greeks positive for HLA-DQB1*0201

HLA-DQA1* alleles	DM1		Background population		OR	RR	P
	n	%	n	%			
	130		1842				
0201	5	3.82	208	11.29	0.31	0.33	<0.001
0301	35	26.72	86	4.67	7.52	5.75	<0.001
0501	72	54.96	357	19.38	5.16	4.78	<0.01

OR = odds ratio; RR = relative risk.

TABLE 6

Association of HLA-DQA1 alleles with type 1 diabetes mellitus (DM1) in Albanians positive for HLA-DQB1*0201

HLA-DQA1* alleles	DM1		Controls		OR	RR	p
	n	%	n	%			
	64		186				
0201	6	9.38	11	5.91	1.64	1.42	NS
0301	5	7.81	7	3.76	2.16	1.68	NS
0501	33	51.56	34	18.28	4.75	2.91	<0.001

OR = odds ratio; RR = relative risk; NS = non-significant.

development of DM1, while *0301, *0602 and *0603 are protective. The genotype distribution of the DQB1 alleles shows that the highest risk for the disease in Greeks is associated with the heterozygous genotype DQB1*0201/*0302.

In Albanians, DQB1*0201 increases the risk for the disease while *0301 is strongly protective. On the other hand, DQB1*0302, *0602 and *0603 seem to provide a neutral effect, although their frequencies show that *0302 is found twice as often in individuals with DM1 than in controls, and *0602 and *0603 are found more often in controls than in patients. The genotype distribution of the DQB1 alleles of our Albanian sample is quite interesting. The genotype DQB1*0201/*0302 is not associated with DM1, although it is found four times more often in individuals with the disease

We cannot exclude the lack of association with the disease being due to the small numbers of the Albanian patients studied. In addition, the susceptible HLA DQB1 genotype *0302/y has almost the same frequency in individuals with DM1 and controls, conferring a neutral effect for the disease. Although the sample size is small we consider this effect more likely to be the result of linkage disequilibrium between the DQB1 and DRB1 locus. Different studies have shown that DR4 can have a modifying effect on the DQB1 *0302 haplotypes[10,27-31]. DQB1*0302 can be associated with the susceptible DRB1 alleles (*0401, *0402, *0405, *0404) or the protective DRB1*0403[10,27-31]. Therefore, it is possible that the neutral effect of the DQB1*0302 allele in the Albanian population may be due to the association

TABLE 7

Comparison of the frequencies of type 1 diabetes mellitus-associated HLA-DQB1 alleles in the general populations of Greece and Albania

HLA-DQB1* alleles	Albanians		Greeks		p
	n	%	n	%	
	186		1842		
0201	47	25.27	539	29.26	NS
0301	85	45.70	1056	57.33	0.002
0302	21	11.29	247	13.41	NS
0602-0603	40	21.51	323	17.54	NS
0604	8	4.30	108	5.86	NS

NS = non-significant.

TABLE 8

Comparison of the frequencies of HLA-DQA1 alleles in DQB1*0201 positive haplotypes in Greece and Albania

HLA-DQA1* alleles	Albanians		Greeks		p
	n	%	n	%	
	186		1842		
0201	11	5.91	208	11.29	0.041
0301	7	3.76	86	4.67	NS
0501	34	18.28	357	19.38	NS

NS = non-significant.

of this allele with another protective DRB1 allele, such as *0403. However, this speculation needs further investigation.

Our study also shows an independent effect of the DQA1 locus on the DQB1*0201 haplotype. DQA1*0501 is strongly associated with the development of DM1 in both Greek and Albanian carriers of DQB1*0201. This has also been shown by studies in other populations[32]. DQA1*0301 in Greeks has a similar effect to *0501, while DQA1 *0201 seems to be strongly protective.

Previous studies have shown a correlation to some extent between the frequencies of HLA alleles and incidence variation in different ethnic groups[31,33,34]. This study confirms to some extent the hypothesis of a genetic basis for the low incidence of DM1 in Greece and Albania. In both populations the protective allele DQB1*0301 is found in almost 50% of the general population. In comparison, it is only found in 20% of Finns[12], the population with the highest DM1 incidence in the world. Furthermore, in the case of Greece there seems to exist an extra protection from allele DQA1*0201, which is neutral in Finns[12]. This relationship, however, is not so simple, since the protective alleles DQB1*0602 and *0603 are found much more often in Finns than in the populations of the present study[12].

A recent study has emphasised the heterogeneity of HLA associations with DM1 within European Caucasians[13]. They have proposed that DQB1 *0302 is most important as a susceptibility factor in northern European countries, while DQB1*0201 is most important in southern Europe[13]. The importance of DQB1*0201 has been confirmed by our study. DQB1*0201 is the most commonly observed allele within our samples of individuals with DM1 from both Greece and Albania, while *0302 is much rarer and has a neutral effect in Albanians. In both South-Eastern European countries that we studied, what seems to be of particular importance for the susceptibility to DM1 is the linkage disequilibrium between DQB1 and DQA1.

Despite the indications of correlation between genetic susceptibility and the incidence of DM1 in different populations, one should consider the importance of environmental factors. The dramatic increase in the incidence of this disease in already high-risk populations of Europe over the past few years is unlikely to be due to changes in the genetic pool of the populations[35]. In a similar fashion the clustering of new cases of DM1 in the region of Athens as opposed to rural and semi-rural areas cannot be attributed to genetic factors, since the population of Athens is heterogeneous, consisting of Greeks from different parts of the country. Furthermore, according to these results, the protective DQB1*0301 is found somewhat more frequently in Greeks than in Albanians, when in fact the incidence of the disease is higher in Greece.

One limitation of our study is that we performed only selective HLA-DQB1 and -DQA1 typing and no -DRB1 typing. Thus we cannot exclude the possible contribution of other HLA loci not included in this protocol. Since DQB1*0302/x has a low frequency in both populations, further typing for DRB1 was not considered worthwhile in terms of cost/benefit for the initial screening of the population. However, DRB1 typing would be interesting in order to clarify its possible protective effect in the Albanian population.

During the last decade a considerable number of Albanians has entered Greece, seeking work in both rural and urban areas, and particularly in the Athens region. A follow-up study of the incidence of DM1 in Albanian immigrants living in Athens would be of particular interest. A study of immigrants in the UK showed that the incidence of DM1 after migration converged with that of the native population[36]. Other studies, however, suggest that the incidence remained similar to that in the country of origin[37,38]. Such studies could be very useful in determining environmental and genetic factors important to the pathogenesis of DM1.

In conclusion, DQB1*0201 is the main risk allele in Greeks and Albanians, while DQBI*0301 is protective in both populations. DQB1*0302,y and DQB1*0201,*0302 genotypes confer susceptibility to the disease in the Greek population. DQB1*0201,*0302 is four times more common in patients compared with controls in the Albanian population, but this difference is not statistically significant, possibly due to the small number of subjects examined. Furthermore, DQA1 alleles modify the risk of the DQB1*0201 allele, mainly in the Greek population; DQA1*0201 is strongly protective in a significant percentage of individuals carrying the high risk allele.

Further comparisons between low-disease incidence and high-risk populations will shed more light into the complex imbalance between environmental triggers and genetic predisposition that leads to the onset of DM1. A better understanding of the disease will ultimately result in the development of prediction and prevention strategies.

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