CLINICAL STUDY

Analysis of extended human leukocyte antigen haplotype association with Addison's disease in three populations

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Abstract

Objective: Addison's disease is an organ-specific autoimmune disorder with a polygenic background. The aim of the study was to identify non-class II human leukocyte antigen (HLA) susceptibility genes for Addison's disease.

Design and methods: Addison's disease patients from three European populations were analysed for selected HLA–DR–DQ alleles and for 11 microsatellite markers covering ~4 Mb over the HLA region. Subjects were 69 patients with Addison's disease from Estonia (24), Finland (14) and Russia (31). Consecutively recruited healthy newborns from the same geographical regions were used as controls (269 Estonian, 1000 Finnish and 413 Russian). Association measures for HLA–DRB1, DQB1, DQA1 and 11 microsatellites between D6S273 and D6S2223 were taken. A low-resolution full-house typing was used for HLA class II genes, while microsatellite markers were studied using fluorescence-based DNA fragment sizing technology.

Results: We confirmed that the HLA–DR3–DQ2 and the DQB1*0302–DRB1*0404 haplotypes confer disease susceptibility. In Russian patients, we also found an increase of DRB1*0403 allele, combined with DQB1*0305 allele in three out of six cases ($P < 0.0001$). Analysis of 11 microsatellite markers including STR MICA confirmed the strong linkage in DR3–DQ2 haplotypes but DRB1*0404–DQB1*0302 haplotypes were diverse. MICA5.1 allele was found in 22 out of 24 Estonian patients, but results from Finnish and Russian patients did not support its independent role in disease susceptibility.

Conclusion: HLA–DRB1*0403 was identified as a novel susceptibility allele for Addison's disease. Additionally, we found no evidence of a non-class II HLA disease susceptibility locus; however, the HLA–DR3–DQ2 haplotype appeared more conserved in patient groups with high DR–DQ2 frequencies.

European Journal of Endocrinology 157 757–761

Introduction

Addison's disease is an organ-specific autoimmune disease characterized by the damage of adrenal cortex and insufficiency of corticosteroid production (1). Steroidogenic P450 cytochrome enzymes have been identified as target antigens of the autoimmune response (2, 3) and circulating antibodies against 21-hydroxylase are a sensitive diagnostic marker of the disease with a tendency to decrease in percentage when adrenal gland destruction progresses (4). The disease is often found as a part of polyendocrinopathy syndromes combining it with other organ-specific autoimmune diseases, thyroid disease and type 1 diabetes (T1D) being the most frequent associates (5).

Human leukocyte antigen (HLA) association of the disease with the common 'autoimmune' HLA–DR3 haplotype has been known for decades (6) and some

studies have also found an increased frequency of HLA–DR4 among patients (7, 8). An association with specific HLA–DR4-positive haplotype, HLA–DQB1*0302–DRB1*0404, was described among Addison's disease patients and diabetic children positive for 21-hydroxylase autoantibodies (9). More recently, analysis of microsatellites as well as some candidate genes within class I region in Addison's disease (10) and other autoimmune diseases (11) has given a basis to suggest that there might be other susceptibility genes outside of DR–DQ region. Among the candidates for association with Addison's disease is the major histocompatibility complex class I polypeptide-related sequence A (STR MICA), of which allele MICA5.1 was reported to be strongly associated with Addison's disease (12) or progression to Addison's disease among diabetic persons with antibodies against 21-hydroxylase (13).

To further explore the HLA-associated susceptibility, we analysed Addison's disease patients from three European populations for selected HLA-DRB1 and HLA-DQB1 alleles and for 11 microsatellite markers covering ~4 Mb over the HLA region.

Materials and methods

Sixty-nine Addison's disease patients, including 24 from Estonia, 14 from Finland and 31 from Russia, were studied. Most of the patients were adults (66 out of 69). None of these patients had type 1 polyendocrinopathy syndrome but type 2 autoimmune polyendocrinopathy syndrome was present in 20 out of 69 patients (29.0%). The 21-hydroxylase antibodies were measured by radiobinding assay as described elsewhere (14). Altogether, 70% of studied patients from Estonia and Finland were antibody positive, including seven out of eight patients with type 2 autoimmune polyendocrinopathy syndrome. We have further clinical information only from Finnish and Estonian patients. Autoimmune polyglandular syndromes were present in six Finnish and two Estonian patients. No reasonable further analyses can be based on this incomplete information. As a control we used background population frequencies of the class II haplotypes established in a previous study of the same populations (15).

For genotyping of the HLA class II genes HLA-DRB1, HLA-DQA1 and HLA-DQB1, we used a panel of oligonucleotide probes designed for low-resolution full-house typing, including a more detailed genotyping of the HLA-DR4 subtypes (16). Microsatellite markers (D6S273, TNFa, C12A, STR MICA, MIB, C125, C143, C245, C3211, MOGc and D6S2223) were studied using PCR primers labelled with fluorescent dyes (17) and products were identified by fluorescence-based automated DNA sequencing according to the manufacturer's instructions (MegaBace 1000). Commonly referred allele names were used for the TNFa and STR MICA microsatellites. For the other microsatellite loci, allele

numbers corresponded to the length of the PCR product (estimated by sequencing of homozygous samples).

Results

Table 1 shows haplotype frequencies among patients and controls in each population. The HLA-DR3 haplotype was associated with Addison's disease in all analysed populations but there was a considerable variation in the strength of this association. Eleven out of 14 (78.6%) of Finnish patients were positive for this haplotype when compared with 12 out of 24 (50.0%) Estonian and only 11 out of 31 (35.5%) Russian patients ($P=0.03$; $df=2$). In addition to the increased frequency of HLA-DR3 haplotype, we also found a significant increase in DQB1*0302-DRB1*0404 haplotype among Estonian and Russian patients ($P=0.0056$ and 0.0051 respectively). In the Finnish population, this haplotype was also more common among patients than controls, although the difference did not quite reach statistical significance.

Among Russian patients, we also found that the DRB1*0403 allele was significantly more common than among controls (6 out of 31 and 2 out of 413 respectively, $P<0.0001$). It was present in six patients, in three cases conventionally with DQB1*0302 and in three cases combined with the DQB1*0305 allele. Results of microsatellite analysis are shown in Fig. 1 together with individual HLA-DQB1 and HLA-DQA1 genotypes. Microsatellite markers present on both chromosomes are shown. Markers are ordered from centromere to telomere and sorted to demonstrate the DQB1*02-DQB1*05 (DR3-DQ2) haplotype and microsatellite markers characterizing it. The presentation also offers a way to analyse the possible contribution to disease risk by the other haplotype. The allele associated with the extended haplotype in each locus is marked using shading. We see the known strong conservation of 8.1 haplotype in the class III region but not in the telomeric class I region, which is most pronounced in Finnish samples. All 11 haplotypes share D6S273 and TNFa alleles, two differ at

Table 1 Distribution of Addison's disease-associated haplotypes of the HLA class II genes in Estonians, Finns and Russians.

Population	Haplotype	Patients N (%)	Controls N (%)	OR	P	95% CI
Finns	DR3, DQB1*02-DQA1*05	11 (78.6)	202 (20.2)	14.5	<0.0001	3.7-41.4
	DR4, DQB1*0302-DRB1*0401	2 (14.3)	124 (12.4)	-	NS	-
	DR4, DQB1*0302-DRB1*0404	3 (20.0)	62 (5.2)	4.1	0.078	0.9-16.5
	Total	14	1000			
Estonians	DR3, DQB1*02-DQA1*05	12 (50.0)	60 (22.3)	3.5	0.0056	1.4-8.8
	DR4, DQB1*0302-DRB1*0401	0 (0)	12 (4.5)	-	NS	-
	DR4, DQB1*0302-DRB1*0404	7 (29.2)	25 (9.2)	4.0	0.008	1.4-11.6
	Total	24	269			
Russians	DR3, DQB1*02-DQA1*05	11 (35.5)	65 (15.7)	2.9	0.0102	1.3-6.8
	DR4, DQB1*0302-DRB1*0401	3 (9.7)	31 (7.5)	-	NS	-
	DR4, DQB1*0302-DRB1*0404	7 (22.6)	28 (6.8)	4.0	0.0051	1.4-10.9
	Total	31	413			

P values were calculated by χ^2 -test with continuity correction.

DQA1	DQB1	D6S273	TNFA	C12A	STR MICA	MIB	C125	C143	C245	C3211	MOGc	D6S2223
Finns												
03, 05	02, 0302	137 141	2 11	242 256	5.1 5.1	336 350	194 198	417 449	436 436	195 199	130 148	170 170
03, 05	02, 0302	135 141	2 6	236 256	4 5.1	336 346	194 208	441 449	436 468	195 209	132 132	168 168
05	02, 0602	137 141	2 11	250 256	5.1 5.1	336 350	194 198	417 449	436 436	191 195	122 132	168 170
05	02, 02	141 141	2 2	256 256	5.1 5.1	350 350	194 194	445 449	436 436	207 217	132 148	170 172
05	02, 04	135 141	2 6	242 256	5.1 5.1	332 350	194 194	449 449	428 436	209 217	122 148	170 170
03, 05	02, 0302	137 141	2 6	250 256	5.1 5.1	332 350	194 200	425 449	436 436	207 217	130 148	170 170
05	02, 02	141 141	2 2	256 256	5.1 5.1	350 350	194 194	449 453	428 480	191 195	148 148	170 170
05	02, 0301	137 141	2 4	238 256	5.1 5.1	350 356	194 208	441 441	428 468	195 195	132 136	170 170
05	02, 0602	141 141	2 11	242 256	4 4	336 350	194 198	417 449	436 436	195 201	122 130	170 172
05	02, 02	135 141	2 2	238 242	5.1 5.1	350 350	194 194	449 453	436 436	195 201	148 148	170 170
05	02, 02	133 141	2 2	236 258	5.1 6	332 350	204 210	441 449	436 476	191 191	122 148	170 170
03	0303, 0501	131 135	5 11	236 256	4 9	350 352	210 218	441 445	428 480	187 191	130 132	170 170
03	0302, 0501	131 135	1 5	236 256	4 9	326 352	190 218	429 441	432 432	205 211	130 130	170 170
03	04, 0302	135 137	11 13	238 242	5.1 5.1	326 350	190 194	433 445	432 476	205 211	130 132	170 170
Estonians												
05	02, 04	135 141	2 13	238 256	5.1 5.1	326 350	194 204	433 449	432 436	195 201	136 148	170 170
05	02, 0602	137 141	2 11	242 256	5.1 5.1	336 350	194 198	417 449	436 436	195 199	130 148	170 170
03, 05	02, 0302	135 141	2 11	246 256	5.1 5.1	350 356	194 204	449 449	428 436	195 195	130 148	168 170
03, 05	02, 0302	135 141	2 10	254 256	5.1 9	332 350	194 208	421 449	436 436	195 211	122 148	170 170
03, 05	02, 0302	137 141	2 6	250 256	5.1 9	332 350	194 200	425 449	436 436	195 195	130 134	170 170
03, 05	02, 0302	135 139	2 6	250 256	5.1 9	350 356	194 200	425 449	436 436	195 195	132 134	170 172
05	02, 0602	135 141	2 11	242 256	5.1 51	336 350	194 198	417 449	436 436	195 195	128 130	170 170
03, 05	02, 0302	139 141	2 1	242 256	5.1 5.1	336 350	194 198	417 449	436 436	209 217	132 148	170 170
05	02, 04	135 141	2 2	250 256	5.1 5.1	350 350	194 208	445 453	428 436	189 195	130 130	170 170
05	02, 0602	131 141	2 11	236 256	4 5.1	350 350	198 198	445 449	436 480	195 201	130 132	170 170
05	02, 0301	135 141	2 12	238 254	5.1 9	348 350	194 212	445 449	428 436	195 211	130 144	170 172
03, 05	02, 0302	135 143	2 6	236 256	4 5.1	340 350	180 194	433 449	428 436	205 215	132 138	168 168
0201, 03	02, 0301	131 135	6 7	236 244	5.1 5.1	336 356	190 212	441 449	432 436	195 201	122 132	170 170
0201, 03	02, 0301	131 135	6 7	236 244	5.1 5.1	336 356	190 212	449 449	432 436	195 201	122 132	170 170
05	0301, 0602	137 137	4 11	238 242	5.1 5.1	336 356	194 210	417 449	436 472	205 209	132 136	170 172
05	0301, 0602	137 137	4 11	238 242	5.1 5.1	336 356	198 198	417 437	436 472	195 201	132 136	170 172
	0602, 0602	137 137	4 11	238 242	5.1 5.1	338 356	190 198	417 449	432 436	195 195	122 130	170 170
	0502, 0602	137 137	11 11	238 242	5.1 9	348 350	210 212	445 445	428 480	195 217	122 130	170 170
	0602, 0602	137 137	11 11	238 242	5.1 9	348 352	200 212	433 445	428 480	199 207	122 130	170 170
03	0301, 0602	135 139	6 11	236 242	5.1 5.1	336 356	188 198	417 441	432 436	195 195	130 148	170 172
03	0302, 0603	135 137	7 11	242 246	5.1 5.1	336 350	198 204	417 449	428 436	195 195	130 138	168 170
05	0301, 0301	131 135	5 7	254 254	5.1 6	326 352	200 29	417 433	432 432	205 207	132 132	168 168
03	0303, 0501	135 137	5 10	242 252	4 9	346 352	198 218	441 445	428 432	195 201	130 130	170 170
05	0301, 0301	135 135	10 13	238 238	5 9	332 342	180 208	421 437	428 480	195 199	132 138	170 172
Russians												
05	02, 0301	137 141	2 11	242 256	5.1 5.1	338 350	194 198	417 449	436 436	195 195	132 148	168 170
03, 05	02, 0302	137 141	2 11	242 256	5.1 5.1	336 350	194 198	417 449	436 436	195 226	130 148	170 170
03, 05	02, 0302	133 141	2 11	242 256	5.1 5.1	336 350	194 198	417 449	436 436	197 207	130 130	168 170
03, 05	02, 0302	135 141	2 11	248 256	5.1 5.1	350 356	194 204	449 449	428 436	211 217	122 148	170 170
03, 05	02, 0302	135 141	2 11	256 258	4 5.1	350 350	194 204	449 449	436 476	211 217	148 148	170 170
03, 05	02, 0302	137 141	2 11	242 256	5.1 5.1	336 350	194 198	417 445	436 436	195 226	130 148	168 170
05	02, 0503	137 141	2 2	256 258	4 5.1	352 352	196 196	449 449	436 480	195 217	130 148	170 170
03, 05	02, 0302	133 141	2 7	236 258	5.1 6	326 350	194 216	417 445	436 472	207 215	122 132	170 170
03, 05	02, 0305	135 141	6 13	238 238	6 6	326 326	206 206	433 437	480 480	195 195	144 144	170 170
05	02, 0301	129 131	5 7	236 254	6 6	326 342	212 218	417 437	432 432	195 195	122 132	168 170
03, 05	02, 0302	135 137	2 10	238 250	5.1 9	332 350	190 194	421 445	436 472	207 217	122 148	170 170
0201, 05	02, 0301	133 135	2 7	238 244	5 6	336 348	202 206	441 445	428 448	195 205	122 136	170 170
0201, 03	02, 0302	131 141	2 7	244 256	5.1 5.1	350 350	194 194	449 453	436 436	217 219	130 148	170 170
0201	02, 0501	131 133	2 7	244 252	5.1 9	336 344	196 202	445 445	436 436	195 201	134 144	168 170
0201	02, 0501	133 137	10 11	242 246	5.1 5.1	336 354	198 210	417 453	436 436	195 205	130 132	170 172
0201	02, 0502	135 135	7 11	238 256	5.1 6	336 342	198 202	417 433	436 476	209 217	130 136	170 170
0201, 03	02, 0302	131 135	7 11	244 254	5.1 6	336 348	196 212	445 449	432 436	195 211	122 132	170 172
0201	02, 0603	133 137	3 7	244 250	6 6	334 348	210 212	437 445	432 476	195 201	132 144	170 170
03	0302, 0501	135 141	2 6	242 250	6 6	332 348	190 200	441 465	436 476	191 195	122 132	170 170
	0501, 0602	135 137	2 11	242 250	5.1 5.1	328 336	198 200	417 449	428 436	195 197	130 132	170 170
03	0302, 0602	137 137	2 11	242 250	5 6	326 348	192 208	441 449	432 432	209 217	148 148	168 170
03	0303, 0303	129 133	2 6	256 256	5 9	342 346	192 26	429 449	428 476	197 203	130 144	170 170
	0501, 0601	133 135	2 10	236 258	6 9	332 332	190 208	421 437	476 476	195 203	122 130	170 172
03, 05	0301, 0305	135 135	4 10	238 242	4 5.1	346 356	190 198	437 437	428 472	203 215	122 132	172 172
05	0301, 0501	135 137	4 5	238 256	5.1 9	352 354	190 218	437 441	432 432	195 201	122 130	170 172
03	0302, 0502	135 137	11 11	242 246	5.1 5.1	336 356	198 204	417 449	428 436	189 195	122 130	170 170
	0503, 0603	137 137	6 11	238 244	5.1 6	332 338	198 200	417 425	436 436	205 211	130 132	168 170
03, 05	0301, 0305	135 137	10 13	238 260	4 6	344 350	196 212	441 445	428 436	195 226	122 136	170 170
05	0301, 0501	137 139	6 11	236 238	4 9	340 348	180 212	433 445	428 436	195 195	136 140	170 170
	0301, 0602	133 137	1 6	246 250	5 5	326 342	188 194	429 433	428 428	195 217	148 152	170 170
03	0302, 0302	135 135	6 10	236 254	9 9	332 348	208 212	437 437	428 472	195 207	122 130	168 170

Figure 1 Extended HLA haplotypes in Finnish, Estonian and Russian patients with Addison's disease. Conserved haplotype structures are marked in grey.

C12A and one more at STR MICA. MIB allele 350 is found again in 13 out of 14 cases but only three haplotypes seem to extend to C3211. A similar type of pattern is seen in the Estonian patients, conserved haplotypes extend even further to C3211 where 7 of the original 11 DR3 haplotypes may continue and 4 may be extended up to MOGc marker. In the Russian haplotypes, there is slightly more heterogeneity in loci adjacent to HLA-DQ, at D6S273 and TNFa locus, where 8 out of 11 patients share the common allele 2.

Of the markers found on the haplotypes other than the DR3 haplotype, MICA5.1 is conspicuous especially on Estonian samples, less so in Finnish and Russians. In the analysis of 166 haplotypes non-transmitted in Finnish families to a diabetic child and therefore representing a background Finnish population, MICA5.1 allele was the most common allele present on 51.2% of all haplotypes (unpublished results). These haplotypes represent the Finnish background population and the results demonstrate that the high frequency of MICA5.1 allele is to be expected. Similarly, the most telomeric D6S2223 marker is hardly informative due to the high frequency of 170 bp allele.

Similar analysis of DQB1*0302-DRB1*0404 haplotypes revealed very poor linkage disequilibrium in them. TNFa11 was the most common allele at that locus but it was found (together with linked D6S273 allele) only in one of the three Finnish, two of the seven Estonian and four of the seven Russian haplotypes. Absence of T1D-associated DQB1*0302-DRB1*0404-B1*39 haplotype (18) was also confirmed by the lack of HLA-B*39 allele except in one Finnish patient.

No association between any of studied haplotypes or microsatellite markers and antibodies against 21-hydroxylase or type 2 autoimmune polyendocrinopathy syndrome was revealed among patients.

Discussion

Our results confirm the association of both DQB1*02-DQA1*05 (DR3-DQ2) and DQB1*0302-(DQA1*03)-DRB1*0404 haplotypes with Addison's disease but there were remarkable differences between the populations. DR3-DQ2 association was very strong in the Finnish population, whereas the effect of the DRB1*0404 association was in fact stronger among Estonians and Russians when estimated by odds ratio.

These differences could not only be related to patient selection, but might also reflect genetic differences between two more mixed populations and the 'outlier' Finnish population characterized by genetic isolation and strong founder effects. Susceptibility gene alleles associated with Addison's disease might therefore also be more diverse in Estonians and Russians than among Finns. The difference in the frequency of the DR3 haplotype between Finnish and Russian patients cannot be explained by the frequency in the background populations as these are similar.

Haplotype analysis using microsatellite markers did not provide statistical support to the importance of HLA regions other than HLA-DR-DQ loci. However, we found that the HLA-DR3-DQ2 haplotype appears more conserved in Finns and Estonians, than among Russians.

MICA5.1 allele was very common among patients. In the Finnish cases, it was found in 11 of 14 cases but this is the same percentage as was positive for DQB1*02-DQA1*05 combination and the allele is in strong linkage with these DQ alleles and also found in more than 50% of random selection of haplotypes. Results of Estonian patients might be interpreted to support the role of MICA5.1 allele, as this was found in all but two cases. However, among Russians it was again found in about half of the DR3-negative ones and the linkage of it with DR-DQ was not particularly strong in DR3 haplotypes.

The diversity of DRB1*0404-DQB1*0302-positive haplotypes and the fact that DRB1*0401-DQB1*0302 haplotype does not show any association with the disease emphasizes the role of the DRB1*0404 molecule itself. In this respect, it is very interesting that DRB1*0403 was found increased among Russian patients. It was not found among Estonians and Finns but the frequency of DRB1*0403 is very low among these populations and only DRB1*0401 and DRB1*0404 are the common DR4 alleles found in DR4-DQ8 haplotypes. This is also the case in most populations where DRB1*0404 association of Addison's disease has been described (8, 9). Because families were not available, the origin of each allele cannot be deduced by certainty, but exclusion of the presence of markers in the haplotype can be done.

More data should be collected in populations where DRB1*0403 and/or the structurally similar allele DRB1*0406 as well as other DRB1 alleles *0402 and *0405 are common to test which of the DR4 subtypes in fact show a disease association. The identification of three DQB1*0305 alleles combined with DRB1*0403 was also intriguing. This haplotype has been mainly reported in individuals from Sardinia but there is probably a reporting bias as it is not necessarily detected by traditional probe panels (19).

In conclusion, for the first time, we found evidence that the HLA-DRB1*0403 allele confers susceptibility to Addison's disease. Additionally, we propose that on the DRB1*0404-DQB1*0302 haplotype the DRB1*0404 allele is more important in conferring disease susceptibility than the DQB1 allele. We found no evidence of a non-class II HLA disease susceptibility locus for Addison's disease; however, the HLA-DR3-DQ2 haplotype appeared more conserved in patient groups with high DR-DQ2 frequencies.

Acknowledgements

We would thank Mare Suigom, Toomas Podar and Ahti Kallikorm for their kind cooperation.

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Received 6 September 2007

Accepted 12 September 2007