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ORIGINAL ARTICLE

The early onset of type 1 autoimmune hepatitis has a strong genetic influence: role of HLA and KIR genes

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We have previously reported a strong association between HLA-DRB1*1301 and type 1 pediatric autoimmune hepatitis (PAH) and between HLA-DR*0405 and adult autoimmune hepatitis (AAH). Because human killer cell immunoglobulin-like receptors are known to be associated with susceptibility to autoimmune diseases, we investigated the frequencies of HLA-A, B, C, DRB1 and KIR genes in 144 type 1 PAH and 86 AAH patients, which were compared with 273 healthy controls. We demonstrated in PAH the increased frequency of the functional form of KIR2DS4-Full Length (KIR2DS4-FL), which in combination with HLA-DRB1*1301 revealed a strong synergistic effect (odds ratio = 36.5). PAH-KIR2DS4-FL+ subjects have shown an increased frequency of their putative HLA-C*02, 04 and 06 ligands. KIR analysis of PAH also revealed a decreased frequency of KIR2DL2 gene and its ligand. In contrast, AAH cases have shown a weaker increased frequency of KIR2DS4-FL, a lack of synergistic effect with HLA class II antigens and a moderate association with HLA-DRB1*0405. Of note, we demonstrated that liver T cells have a unique pattern of KIR expression. These results show a KIR gene involved in autoimmune hepatitis and suggest a stronger genetic influence for the early onset type I autoimmune hepatitis.

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INTRODUCTION

Autoimmune hepatitis (AH) is a progressive liver disease characterized by the presence of circulating autoantibodies, hypergammaglobulinemia and the response to immunosuppressive treatments. Different disease subtypes are defined according to the autoantibodies present in patient serum; antinuclear antigens and anti-smooth muscle antibodies (SMAs) define type 1 AH.[1–3]

Although the etiology of AH is unknown, susceptibility is determined in part by a gene linked to the class II region of the major histocompatibility complex. We previously described for the first time a strong association between the pediatric form of AH pediatric autoimmune hepatitis (PAH) and *HLA-DRB1*1301*,[4,5] and this association was further confirmed in groups from different populations.[6–8] The relationship between susceptibility to the adult form of AH adult autoimmune hepatitis (AAH) and HLA appears to be more complex; both a primary association with *HLA-DR3* and a secondary association with *HLA-DR* have been described in Britain,[9] specifically demonstrating an association between *HLA-DRB1*0301* in younger patients and poor outcomes and an association between *HLA-DRB1*0401* and a better prognosis.[10] In Argentina[11] and Japan,[12] AAH has been associated with *HLA-DRB1*0405*. Genetic and clinical differences led us to postulate that PAH and AAH may represent different clinical entities.[5]

Killer cell immunoglobulin-like receptors (KIRs) are expressed on natural killer (NK) cells and subsets of T cells. KIRs comprise 14 gene and 2 pseudogene receptors that exhibit extensive haplotype variation in terms of the number and identity of genes as well as allelic polymorphisms for individual genes. NK cell function is tightly regulated by a balance between positive and negative signals transmitted by activating and inhibitory receptors, respectively.[13] NK cells express KIRs in a varied manner, with NK cells expressing between 0 and 7 KIRs.[14]

The present study was designed to confirm the differences between the pathogenic mechanisms of PAH and AAH with regard to the combined effects of the HLA and KIR genes and the roles of innate and adaptive immunity.

RESULTS

KIR genotypes of PAH patients

We examined the gene frequency of the KIRs 2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 3DL1, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5 and 3DS1 in 273 healthy controls and 144 PAH patients (Table 1). The analysis revealed decreased frequencies of KIR2DS2 ($P = 0.02$), KIR2DL2 ($P = 0.003$), KIR2DS3 ($P = 0.009$) and KIR2DL5 ($P = 0.03$). After applying the Bonferroni correction, only KIR2DL2 remained significantly decreased in PAH patients (Pc = 0.03; Table 1).

The most prevalent KIR2DS is KIR2DS4,[15] which is represented by a variable balance between the 'full-length' (FL) and 'deleted' forms (KIR1D). As we have previously described,[16] and as observed with other Caucasian populations,[17] the KIR2DS4-FL allele was only detected in 42% of the Argentinean Caucasian population among the individuals carrying the KIR2DS4 gene. In contrast, KIR2DS4-FL was present in 70% of PAH patients ($P < 0.0001$, odds ratio (OR) = 3.2, 95% CI: 2.1–5.1; Table 2). KIR2DS4 is the only activating receptor present in the A haplotype. Although PAH

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Table 1. Distribution of KIR gene frequency in healthy controls and PAH patients

	2DS1	*2DS2*	*2DS3*	*2DS4*	*2DS5*	*3DS1*	*2DL1*	*2DL2*	*2DL3*	*2DL4*	*2DL5*	*3DL1*
Controls (%), n = 273	45	58	29	95	35	40	95	61	85	100	55	95
PAH (%), n = 144	40	45	17	90	29	35	93	45*	83	100	43	92

Abbreviation: PAH, pediatric autoimmune hepatitis. The KIR phenotypical frequencies in healthy controls and PAH are shown. Pc (= 0.03) is the *P*-value corrected using the Bonferroni inequality method.

Table 2. Distribution of KIR2DS4-FL alleles in PAH and AAH patients in comparison with healthy controls

	Patients with AH		*Controls* (n = *259*)		P	*OR*	*95% CI*
	n	*%*	n	*%*			
PAH (n = *130*)							
HLA-DRB1*1301+	71	54.6	30	11.6	< 0.001	9.2	5.5–15.4
HLA-C*02+	22	17.2	25	9.6	0.05	1.9	1.03–3.5
2DS4 FL+	91	70.0	108	41.7	< 0.001	3.2	2.1–5.1
2DS4 FL −	39	30.0	151	58.3			
KIR2DS4 expanded genotype							
2DS4 FL+ homozygous	43	33.1	46	17.8	0.001	2.3	1.4–3.7
2DS4 FL+/1D heterozygous	48	36.9	62	23.9	0.004	1.9	1.2–2.9
KIR1D homozygous	39	30.0	151	58.3	< 0.001	0.3	0.2–05
AAH (n = *78*)							
HLA-DRB1*1301+	14	17.9	30	11.6	NS		
HLA-DRB1*0405+	9	11.5	3	1.2	0.002	11.1	2.9–42.2
2DS4 FL+	45	57.7	108	41.7	0.01	1.9	1.1–3.2
2DS4 FL −	33	42.3	151	58.3			

Abbreviations: AAH, adult autoimmune hepatitis; NS, non significant; PAH, pediatric autoimmune hepatitis.

patients exhibited an increased trend for the KIR A haplotype, only the presence of KIR2DS4-FL was found to be significantly increased. In addition, homozygous KIR2DS4-FL was detected in 43 (33%) of the PAH patients compared with 46 (18%) of the controls ($P = 0.001$; Table 2). In contrast, the presence of homozygous KIR1D was associated with a protective effect (OR = 0.3, $P < 0.0001$, 95% CI: 0.2–0.5; Table 2).

To establish a relationship between the functional and deleted forms of the gene, all subsequent studies were performed using patients and controls carrying the KIR2DS4 gene.

Combined effects of HLA-DRB1 and KIR2DS4-FL on susceptibility to PAH

An increased frequency of *HLA-DRB1*1301* was confirmed in PAH patients: 54.6% in PAH vs 11.6% in the control subjects ($P < 0.0001$, OR = 9.2, 95% CI: 5.5–15.4; Table 2).

Having identified *HLA-DRB1*1301* and the KIR2DS4-FL allele as two factors that are associated with susceptibility to PAH, we used the method of Svejgaard and Ryder[18] to investigate whether these two factors act synergistically (Table 3). The first two comparisons indicated that *HLA-DRB1*1301* (factor A) confers susceptibility to the disease in samples that were positive for factor B (*KIR2DS4*-FL) (1) or negative for factor B (2). Similarly, the second two comparisons indicated that factor B confers susceptibility to the disease within samples that were positive (3) or negative (4) for factor A. When factors A and B were analyzed in combination (5), we observed a strong synergistic effect: the combined presence of *DRB1*1301* and KIR2DS4-FL generated an OR of 36.5, which is greater than the product from multiplication of the individual ORs for factors A and B.

Table 3. Two-factor analysis identifying a strong synergistic effect between HLA-DRB1*1301 and KIR2DS4-FL

*Factor A (DRB1 * 1301)*	*Factor B (KIR2DS4-FL)*	*PAH,* n = *130*	*Controls,* n = *259*
+	+	46	13
+	−	26	17
−	+	45	95
−	−	13	134

Comparisons between patients with factors AB vs *Controls AB*

(AB vs *AB)*	*OR*	a	b	c	d	P	*95% CI*
(1) ++ vs − +	7.3	46	45	13	95	< 0.001	3.4–14.8
(2) + − vs − −	15.8	26	13	17	134	< 0.001	6.8–36.4
(3) ++ vs + −		46	26	13	17	NS	
(4) − + vs − −	4.9	45	13	95	134	< 0.001	2.5–9.6
(5) ++ vs − −	36.5	46	13	13	134	< 0.001	15.7–84.4

Abbreviations: PAH, pediatric autoimmune hepatitis; NS, non significant. Note: *a*, *b*, *c* and *d* refer to the data entry of a 2x2 table where *a* and *b* are patients and *c* and *d* are controls, with the purpose of obtaining the OR for each comparison. The first two comparisons detect whether factor A (DRB1*1301) confers susceptibility to the disease within the sample positive for factor B (KIR2DS4-FL) (1) or within the negative sample for factor B (2). The second two comparisons analyze whether factor B confers susceptibility to the disease, as in the first two, but within the positive or negative sample for factor A, respectively. The last one (5) tests whether the two factors act in a synergistic way. DRB1*1301 and KIR2DS4-FL have an association, with the disease showing a strong synergistic effect, but when combined (OR = 36.5).

Does genetic susceptibility association have any clinical relevance?

From a clinical point of view, we have found of interest that seven out of nine patients who required an orthotopic liver transplantation showed the combined presence of DRB1*1301 and KIR2DS4-FL. In contrast, the combined presence of DRB1*1301 and KIR2DS4-FL was detected at diagnosis in only 12% of PAH patients with an Ishak fibrosis score of 0–2. Those with an Ishak score of 3–4 and 5–6 were detected in 29% and 30% of PAH patients, respectively. A more extensive study will be required to confirm the relationship of the combined presence of DRB1*1301 and KIR2DS4-FL and the different disease phenotypes of the PAH patients.

HLA-C ligands to KIR NK receptors in PAH patients

Among inhibitory KIRs, KIR2DL1 recognizes HLA-C2 allotypes containing a lysine at position 80 of the HLA-Cα_1 domain and KIR2DL2/3 recognize HLA-C1 alleles containing Ser77/Asp80.[19,20] However, KIR2DL2, and to some extent also KIR2DL3, interacts with some C2 alleles as well, although much weaker than KIR2DL1 does.[21,22] In addition to its diminished frequency, PAH KIR2DL2+ patients exhibited an increased frequency of HLA-C2/C2 (29% vs 14% in controls; $P = 0.02$), a finding not detected when we compared PAH KIR2DL3+ or KIR2DL1+ patients and controls.

This increase was more robust in those KIR2DL2+ patients who were also KIR2DS4-FL$^+$ homozygous (44% vs 5% among control subjects; $P = 0.01$, OR = 15, 95% CI: 1.5–151.4; Table 4).

It has been suggested that KIR2DS4 binds HLA-C*02, *C*04*, *06 and HLA-A*1102.[23,24] In preliminary studies, we detected an increased frequency of HLA-C2 in PAH patients, without any increase in HLA-A*1102. Thus, we investigated the frequency of HLA-C alleles belonging to the HLA-C2 group as putative ligands for KIR2DS4. The combined presence of HLA-C*02, C*04 and C*06 was detected in 71 PAH patients (54%) and 117 control subjects (45%). Strikingly, the combined presence of HLA-C*02, C*04 and C*06 was detected in 64% of PAH-KIR2DS4-FL$^+$ patients vs 44% of controls ($P < 0.003$, OR = 2.4, 95% CI: 1.3–4.2).

KIR genotype of AAH patients

In comparison with healthy controls, the analysis of KIR gene frequencies in AAH patients did not show statistical differences (data not shown). KIR2DL2 has a clear decreased trend, but did not resist Bonferroni correction (46% vs 61% in controls). However, the analysis of all AH (AAH plus PAH) showed that the decrease of KIR2DL2 was highly significant and survives Bonferroni correction (45% vs 61% in the controls; $P < 0.001$, OR = 1.9, 95% CI: 1.3–2.7), suggesting that decreased frequency of KIR2DL2 may affect all AH patients. Similar to PAH, the frequency of KIR2DS4-FL was also increased in AAH (58% vs 42% in the controls; $P = 0.01$, OR = 1.9, 95% CI: 1.1–3.2) (Table 2).

Table 4. Distribution of HLA-C groups among PAH patients in comparison with healthy controls within the KIR2DL2+ population

	2DL2+				*2DS4 FL+ Homozygous/ 2DL2+*			
	Controls		*PAH*		*Controls*		*PAH*	
	n	%	n	%	n	%	n	%
C1C1	68	43	14	24*	10	45	1	11
C1C2	68	43	27	46	11	50	6	56
C2C2	23	14	17	29*	1	5	5	44**

Abbreviation: PAH, pediatric autoimmune hepatitis. The table shows the increased frequency of C2C2 in PAH patients. *$P < 0.05$. This increase was more robust in those KIR2DL2+ patients who were also KIR2DS4-FL$^+$ homozygous (**$P = 0.01$, Fisher's exact test).

HLA-C ligands to KIR NK receptors in AAH patients

Whereas the frequency of KIR2DS4-FL was increased in adult patients, we did not detect any statistical difference in the frequencies of its putative HLA-A*1102, HLA-C*02, C*04 or C*06 ligands, both in the general AAH population and in those carrying the KIR2DS4-FL gene (data not shown).

The present study also confirmed our previous report[5] demonstrating that the *HLA-DRB1*0405* allele confers susceptibility to AAH (11.5% vs 1.2% in control subjects; $P = 0.0002$, OR = 11.1, 95% CI: 2.9–42.2), whereas no association was found for the *HLA-DRB1*1301* allele (Table 2). We investigated the role of the combined presence of the two factors associated with AAH susceptibility, and we did not detect a synergistic effect (data not shown). Additionally, in contrast to the results obtained from PAH patients, the comparison of AAH patients with control subjects did not reveal differences in the frequencies of HLA-C alleles or the HLA-C1 and HLA-C2 genotypes.

Differential expression of KIR genes in liver and peripheral blood

Once an increased frequency of the functional form of KIR2DS4 gene was detected, we were interested in establishing the expression of KIR2DS4 and other activating and inhibitory KIR genes in the liver. As illustrated in Figure 1, KIR2DS4 is well represented in liver and peripheral blood NK^{dim} cells, but the expression on T cells is almost restricted to liver T cells. Similarly, the expression of KIR3DL2, KIR2DL3, KIR2DS1 and KIR2DS3 was only detected in liver T cells, with a negligible expression on peripheral blood T cells. These results indicate that liver T cells have a unique pattern of KIR expression.

DISCUSSION

In the present study, we confirmed the previously reported association of type I PAH with *HLA-DRB1*1301*. We also detected a high frequency of the functional form of KIR2DS4 in these patients and demonstrated for the first time a strong combined effect of these two genetic systems.

The high frequency of the functional alleles of KIR2DS4 in PAH contrasted with the predominant frequency of KIR1D, present in most other healthy Caucasian populations studied.[16] Several studies have found an association between the genes for *KIR* activation and autoimmune disease susceptibility. For instance, KIR2DS1 has been reported to be associated with psoriasis,[25–27] KIR2DS2 with scleroderma,[28] KIR2DS2 and KIR2DS4-FL with rheumatoid arthritis.[29,30] KIR2DS1 and KIR2DS4 recipients of transplants from donors lacking C1 ligands for the inhibitory KIR2DL2/3 genes demonstrated worse graft survival.[31]

The genotyping of PAH patients have also revealed a diminished frequency of KIR2DL2. Furthermore, PAH-KIR2DL2+ subjects have diminished frequency of the HLA-C1 ligand, pointing out towards an altered inhibitory mechanism.

In the same line, we showed that the C2/C2 frequency within KIR2DS4-FL$^+$ homozygous/KIR2DL2$^+$ individuals was also increased; thus, a high frequency of these individuals lacked inhibitory signals transmitted by KIR2DL2, supporting a connection between KIR2DL2 and KIR2DS4-FL in PAH susceptibility. The increase in C2/C2 could also result in a greater inhibition capacity for KIR2DL1, but KIR2DL1$^+$ PAH patients did not exhibit an increased frequency of C2/C2 ligands (data not shown).

At this point, we cannot exclude alternative inhibitory mechanisms such as those mediated by NKG2A, which can be expressed on the same NK cell.

The identity of the ligand for KIR2DS4 is controversial, and several HLA-C ligands have been suggested;[23,32,33] we find of

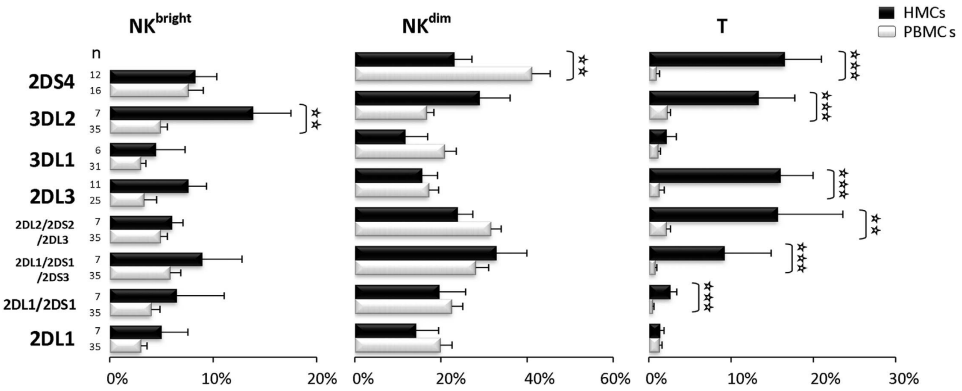

Figure 1. KIR expression on NK and T cells in HMCs and PBMCs. Expression of KIR receptors on gated NK^{bright}, NK^{dim} and T cells on PBMCs and HMCs. Results are expressed as mean values obtained from individuals (*n*) who have been previously typed and showed the presence of the gene receptor. Mann–Whitney *U*-test, $^{**}P < 0.01$, $^{***}P < 0.001$.

interest that PAH-KIR2DS4-FL$^+$ subjects exhibited an increased frequency of the combined presence of the putative ligands *HLA-C*02*, *C*04* and *C*06*.

The present study also supports our first report suggesting that pediatric and adult forms of type 1 AH may involve different pathogenic mechanisms.[5] AAH showed a weaker increased frequency of KIR2DS4-FL, but not associated with their ligands. KIR2DL2 was found to be decreased when all AH patients were analyzed together suggesting that this decrease is not restricted to PAH patients. Nevertheless, none of the interactions we described between KIR2DL2 and KIR2DS4 or with their putative ligands in PAH were detected in AAH patients. However, the interaction between KIR2DL2 and KIR2DS4 if any, is not completely elucidated, and further studies will be required to get insight into this putative interaction. The association with HLA class II molecules has a high OR, but only includes a small number of AAH patients (11% of AAH patients). Similarly, adult patients showed no combined effect between KIR2DS4-FL and HLA class II antigens. Altogether, these results support not only that pediatric and adult forms of AH represent different clinical entities, but also that genetic systems have a stronger impact in the early onset of this disease.

The mechanisms of liver damage in AH are not completely elucidated.[34] Naive CD4+ cells that recognize the antigenic peptide contained within DRB1*1301 expressed in several liver antigen presenting cell (APC) may allow *in situ* antigen presentation without requiring trafficking to regional lymphoid tissue.[35] After cell activation, CD4+ cells become effector Th1 cells, which secrete IFN-γ, the main cytokine mediating liver tissue damage. IFN-γ can also enhance the expression of HLA class I molecules on hepatocytes, which become the target for CD8+ cells. NK cells account for 25–40% of total intrahepatic human lymphocytes. Given that there is no functional evidence of KIR-class II interaction, we cannot exclude that combined adaptive and innate mechanisms acting independently may be responsible for the very strong synergistic effect detected when we analyze both genetic systems together. In this context, the role of liver T cells that express a high frequency of activating KIR genes, in particular KIR2DS4, for which PAH patients showed an increased frequency of their putative ligands, deserves further consideration.

PATIENTS AND METHODS

A total of 144 PAH patients and 86 AAH patients were included in this study, all of whom were diagnosed according to the criteria established by the International Group for the Study of Autoimmune Hepatitis.[3] Clinical, laboratory and immunological findings in terms of the differences between PAH and AAH were described in detail in our previous report[5] and justifies the classification of all patients as having type 1 AH. Patients who experienced disease onset after 17 years of age were considered to have AAH. The patients and healthy controls ($n = 273$) were gender and ethnicity-matched with patients. They belong to a homogeneous Latin American Caucasoid population that is primarily composed of second or third Argentine generations, most of whom have a Spanish or Italian background. A small number of Amerindians and black population are living in Argentina, but were not detected in the present study. All patients were examined and found to be negative for HIV, hepatitis B and hepatitis C infections. All were positive for antinuclear and/or anti-actin antibodies. Liver biopsy specimens were obtained from patients, and histology was interpreted in accordance with published criteria.[36] The study was approved by the Investigation and Ethics Committee of the Hospital de Clínicas José de San Martín and the Hospital Nacional de Pediatría J. P. Garrahan, in accordance with the Declaration of Helsinki.

Mononuclear cell isolation

Liver perfusion collection was obtained from transplant donors according to the technique described by Kelly *et al.*[37] Peripheral blood mononuclear cells (PBMCs) from 35 adult healthy controls and hepatocyte mononuclear cells (HMCs) from 12 healthy cadaveric donors were obtained using a Ficoll-Hypaque density gradient (GE Healthcare Bio-Sciences, Uppsala, Sweden).

Monoclonal antibodies and flow cytometry

PBMCs and HMCs were stained with antibodies against KIR2DL3-FITC, KIR2DL1-FITC, KIR2DS4.PE, KIR2DL3-PE, IgG1/IgM/IgG2b/IgG2a, KIR2DS4 (R&D Systems, Minneapolis, MN, USA), CD3-PerCP, CD56-APC, CD3-FITC, KIR3DL1-FITC, IgG1-FITC, IgG2a-PE, CD45-APCH7 and their respective isotype controls (BD Biosciences, San Diego, CA, USA). 3DL1/3DL2 (5133 from M.Colonna), 2DL1/2DS1/2DS3 (HP3E4) and 2DL1/2DS1(HPMA from M.Lopez-Botet), 2DL2/2DS2/2DL3 (CHL from S.Ferrini) and FITC or PE-labeled F(ab)2 rabbit anti-mouse Ig (Dako, Glostrup, Denmark) were used as secondary antibodies.

The samples were acquired using a FACSAria II cell sorter (Becton Dickinson, San Jose, CA, USA), and the data were analyzed with FlowJo 7.6.2 software (Tree Star, Inc., Ashland, OR, USA). Statistical analyses of particular populations of interest were based on at least 100 000 gated events.

Immunofluorescence studies

The presence of antibodies directed against nuclear antigens, SMAs and type 1 liver-kidney microsomes was detected as previously described.[5]

KIR and HLA-A, B, C and DRB1 typing by PCR sequence-specific oligonucleotide probing

The conditions used to identify the presence or absence of each KIR gene have been previously documented.[38] Briefly, two PCR amplifications were performed: PCR-1 amplified the combined domains D1 and D2, and PCR-2 amplified the transmembrane and cytoplasmic regions. Nineteen 5´-digoxigenin-labeled probes were used in the sequence-specific oligonucleotide probing (SSOP) approach, with 13 for PCR-1 and 6 for PCR-2. The KIR gene content for each individual was inferred after the combined analysis of all probes.

The KIR2DS4 gene was amplified by PCR with primers previously described[39] to analyze the cell membrane-anchored receptor (designated KIR2DS4-FL or FL) and a truncated soluble protein (designated KIR1D), which is produced when exon 5 contains a 22-bp deletion.

HLA-A, B and C genotyping was performed for sequences from exons 2 and 3. The primers and conditions for PCR amplification were the same as those described by Cereb *et al.*[40] The comparison of HLA-A and B typing between PAH and AAH with healthy controls did not reveal significant differences, and the data are not presented in this report. HLA class II DNA typing was performed as previously reported,[5] following protocols from the 12th International Histocompatibility Workshop. HLA-C SSOP typing also utilized two probes (5´-digoxigenin label); one of the probes (5´-TGACCGAGTGAACCTGC-3´) was specific for the HLA-C alleles that belong to the C1 group (Asn 80), and the other probe (sequence: 5´-ACCGAGTGAGCCTGCG-3´) anneals with the HLA-C alleles that belong to the C2 group (Lys 80).

Statistical analysis

The HLA allele frequencies in the patients and controls were compared to evaluate significant differences using Fisher´s exact test analysis, and the *P*-value was corrected with the Bonferroni method when appropriate. The strength of the association was estimated by calculating the OR. The method described by Svejgaard and Ryde[18] was used to identify whether the factors HLA-DRB1*1301 and KIR2DS4 had a synergistic or combined effect. The Student's *t*-test or Mann–Whitney *U*-test were used to compare independent groups.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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