CLINICAL STUDY

Early onset of polyglandular failure is associated with HLA-DRB1*03

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Abstract

Objectives: Polyglandular failure or autoimmunity (PGA) involves at least two endocrine diseases. Several genes may play a role in its etiology. This study analyzed 1) whether HLA-DRB1, HLA-DQB1, and MHC class I chain-related gene A (MICA) polymorphisms are associated in PGA and 2) whether PGA patients display stronger associations with these immune genes than patients with monoglandular autoimmunity (MGA).

Design: Association study.

Methods: HLA-DRB1, HLA-DQB1, and MICA alleles were analyzed in 73 patients with PGA, 283 with MGA, and 206 healthy controls. The HLA-DRB1 and HLA-DQB1 polymorphisms were determined with PCR-amplified DNA being hybridized with PCR-sequence-specific oligonucleotide probes. MICA microsatellites were typed by PCR amplification and fragment size analysis on a DNA sequencer.

Results: HLA-DRB1*03 was strongly increased in patients with PGA (50.7%) versus both controls (21.8%, $P_c < 0.0001$; $RR = 2.32$, 95% $CI = 1.62–3.33$) and MGA (11.4%, $P_c < 0.0001$). HLA-DRB1*03 was highly prevalent in PGA patients with early versus late disease onset ($P < 0.05$, logistic regression analysis). HLA-DRB1*04 allele carriers were more present in PGA versus controls (53.4% vs 22.4%, $P_c < 0.0001$, $RR = 2.38$, 95% $CI = 1.68–3.38$). Further, HLA-DQB1*02 was increased in PGA versus controls ($P_c < 0.01$), whereas HLA-DQB1*06 was decreased ($P_c < 0.001$). Patients with PGA showed more MIC A5.1 and less MIC A6 allele carriers than controls (NS). Presence of the MIC A5.1 allele was not associated with the HLA-DRB1*03 or HLA-DQB1 alleles.

Conclusions: HLA-DRB1*03 is a stronger genetic marker in PGA than in MGA, foremost in those with early disease onset.

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Introduction

Polyglandular failure or autoimmunity (PGA) is characterized by autoimmune-induced dysfunction of at least two glands (1–3). In contrast to juvenile PGA, which is a monogenic disease (4), several genes may be involved in the etiology of adult PGA. Susceptibility genes for PGA may overlap with known susceptibility genes for monoglandular autoimmune diseases (MGA), for example, autoimmune thyroid disease (AITD).

The HLA genes are strongly involved in glandular autoimmunity (5). Both the HLA-DRB1*03 (6, 7) and HLA-DQB1 alleles (8–10) have been associated with MGA. But data are missing with respect to PGA. Linkage and family-based associations (transmission disequilibrium test) showed that the HLA class II locus contributes to a shared risk for AITD and type 1 diabetes mellitus (DM) (11). In the vicinity of the HLA genes on chromosome 6, the MHC class I chain-related gene A (MICA) is situated. It is a putative susceptibility gene and has been shown to be associated with DM and AITD

(12–17). The MICA gene has a trinucleotide (GCT) repeat microsatellite in the exon 5 encoding alanine residues. It is characterized by five common alleles (MIC A4, MIC A5, MIC A5.1, MIC A6, and MIC A9); allele A5.1 shows an additional 1 bp insertion (GGCT) (18). Exon 5 of the MICA gene corresponds to the transmembrane region of the MICA protein. Previous studies have analyzed the MICA polymorphism in MGA, but studies in PGA are still missing.

Comparisons of the distribution of these immune genes between PGA and MGA of same ethnic background have not yet been performed. Therefore, our objectives were twofold. (1) We hypothesized that these immune genes are more strongly associated with PGA than with MGA. For this, we performed a comparative analysis of HLA-DRB1, HLA-DQB1, and MICA genes in PGA and MGA. (2) Since the MICA microsatellite polymorphism has not yet been investigated in PGA, we analyzed whether MICA is involved in the genetic susceptibility to PGA and whether it interacts with HLA-DRB1 and HLA-DQB1 genes.

Subjects and methods

Subjects

In total, 73 patients with adult PGA type II (principal disease components: AITD and DM) and 283 patients with MGA (228 with AITD and 55 with DM) were investigated. Patients with AITD included 150 with Graves' disease (GD) and 78 with Hashimoto's thyroiditis (HT). Controls were 206 unrelated subjects living in the same area as the patients. Mean age $\pm$ s.d. for patients with PGA, AITD, DM, and healthy controls was 48.2 ± 14.5 , 45.1 ± 14.8 , 24.5 ± 16.6 , and 34.5 ± 12.9 years respectively. Male/female ratios were 26/47, 39/189, 31/24, and 103/103 respectively. The study protocol has been approved by the local ethics committee. Written informed consent was obtained from each subject.

HLA-DRB1 and HLA-DQB1 genotyping

Genomic DNA was prepared from whole EDTA blood using the QIAamp Blood Mini Kit protocol (Qiagen). PGA patients were typed for HLA-DRB1 using a PCR-based reverse dot blot technique (DynaL RELI SSO HLA-DRB typing kit, including HLA-DRB1*03/*11/*13/*14 typing kit; Dynal Biotech, Hamburg, Germany) following the manufacturer's protocol. In the first step, the samples were tested with the HLA-DRB typing kit. For most of the allele combinations, no further testing was required. For some allele combinations including the HLA-DRB1*03/*11/*13/*14 alleles, the second element of the kit was used. After PCR target amplification, the product was hybridized to array to immobilized sequence-specific oligonucleotide probes. The analysis was completed with the detection of the probe-bound amplified product by color formation. One hundred and ninety-five patients (all 73 PGA, 44 DM, 78 AITD = 39 GD and 39 HT) were typed for HLA-DQB1 by PCR-sequence-specific oligonucleotide (PCR-SSO) using the Dynal RELI SSO HLA-DQB1 typing kit; Dynal Biotech), as described in the manufacturer's protocol. Data from 174 German unrelated subjects were considered as controls (18).

MICA genotyping

MICA microsatellites were amplified by PCR. The forward primer was 5'-end labeled with a fluorescent dye (6-Fam, Roth, Karlsruhe, Germany). Forward (F) and reverse (R) primers were F-5'-Fam-CCCTTTTTCAGGGAAAGTGC and R-5'-CCTTACCATCTCCAGAAACTGC (modified after 19; PCR product size: 180–195 bp). The PCR was performed in a 25 μ l reaction volume comprising 0.5 μ l DNA, 0.5 μ l of each of the primers (10 μ M), 12.5 μ l of 2 $\times$ Qiagen Multiplex PCR MasterMix (Qiagen), and 10 μ l HPLC water. DNA amplification was done in a Primus 96 thermocycler (PiqLab, Erlangen, Germany)

using a pre-treatment temperature of 94 $^{\circ}$ C for 15 min, followed by 37 cycles consisting each of 1 min for denaturation at 94 $^{\circ}$ C, 1 min for annealing at 57 $^{\circ}$ C, and extension for 2 min at 72 $^{\circ}$ C. Post-treatment was performed for 10 min at 72 $^{\circ}$ C. For genotyping of samples, a MICA allelic ladder was generated from known genotypes. Fragment sizes of the PCR products were read by capillary electrophoresis using an ABI PRISM 310 DNA sequencer. Before capillary electrophoresis, the amplified PCR products were denaturated for 4 min at 96 $^{\circ}$ C, cooled to a temperature of 4 $^{\circ}$ C, and 4 μ l (or 6 μ l allelic ladder) were mixed with 24 μ l formamide (Sigma) and 2 μ l ILS-600 internal length standard (Promega). The number of microsatellite repeats was determined with the GeneScan Software release 3.1.2 (Applied Biosystems, Foster City, USA) with the standard size marker of ILS-600.

Statistical analysis

Allele and genotype frequencies were calculated by direct counting. The Hardy–Weinberg equilibrium was evaluated for each group and system by comparing observed and expected heterozygotes and homozygotes, as well as observed and expected genotype frequencies using the SAS package V 9.1, procedure 'Proc Allele'. The other statistical analyses were performed with the SPSS/PC software package for MS Windows, release 11.0.1 (SPSS, Inc., Chicago, IL, USA). Differences in specific allele carriers (an allele carrier showed the respective allele in heterozygous or homozygous form) between patients and controls were compared by the χ^2 method. *P* values were calculated using Fisher's exact probability. Relative risks (RRs) and 95% confidence intervals (95% CIs) were calculated by MedCalc software for MS Windows, release 7.2.0.2. Because of multiple comparisons made, the probability values were corrected (*P*_c) after Bonferroni–Holm for the number of comparisons made. The dependency of frequencies of HLA-DRB1, HLA-DQB1, or MICA alleles on sex and age at clinical onset of disease was evaluated in patients with PGA by logistic regression analysis. A *P* or *P*_c value < 0.05 (two sided) was considered statistically significant.

Results

HLA-DRB1, HLA-DQB1, and MICA genotyping

Allele frequencies for HLA-DRB1, HLA-DQB1, and MICA are shown in Table 1. For HLA-DRB1, 13 different alleles were noted (*01, *03, *04, and *07–*16). Most frequent HLA-DRB1 alleles were *03, *04, and *11 in patients, but *13 and *15 in controls. For HLA-DQB1, the alleles *02, *03, *04, *05, and *06 were observed in all groups. For MICA, sequence determination identified the five most common alleles as A4, A5, A5.1, A6, and A9. The MIC A5.1 allele occurred most frequently in patients and

Table 1 Frequencies of HLA-DRB1, HLA-DQB1 alleles, and MHC class I chain-related gene A (MICA) microsatellite alleles in patients with PGA, MGA (AITD or DM), and healthy controls.

Alleles	Allele frequencies			
	PGA n* (%)	AITD n* (%)	DM n* (%)	Controls n* (%)
HLA-DRB1				
*01	8 (5.5)	9 (5.8)	7 (8.0)	42 (12.3)
*03	41 (28.1)	28 (17.9)	17 (19.3)	38 (11.5)
*04	40 (27.4)	22 (14.1)	36 (40.9)	39 (11.3)
*07	9 (6.2)	19 (12.2)	8 (9.1)	29 (9.2)
*08	1 (0.7)	6 (3.8)	3 (3.4)	13 (3.8)
*09	0 (0.0)	1 (0.6)	1 (1.1)	5 (1.7)
*10	0 (0.0)	4 (2.6)	0 (0.0)	1 (0.3)
*11	11 (7.5)	25 (16.0)	4 (4.5)	34 (10.1)
*12	3 (2.1)	4 (2.6)	1 (1.1)	11 (3.2)
*13	19 (13.0)	13 (8.3)	8 (9.1)	46 (13.3)
*14	2 (1.4)	6 (3.8)	0 (0.0)	11 (3.2)
*15	7 (4.8)	15 (9.6)	1 (1.1)	61 (18.1)
*16	5 (3.4)	4 (2.6)	2 (2.3)	9 (2.6)
HLA-DQB1				
*02	50 (34.2)	46 (29.5)	25 (28.4)	59 (19.3)
*03	58 (39.7)	55 (35.3)	42 (47.7)	93 (29.4)
*04	1 (0.7)	4 (2.6)	3 (3.4)	10 (2.9)
*05	15 (10.3)	25 (16.0)	9 (10.2)	64 (19.0)
*06	22 (15.1)	26 (16.7)	9 (10.2)	101 (29.7)
MICA				
A4	16 (11.0)	57 (12.5)	16 (14.5)	53 (12.9)
A5	18 (12.3)	62 (13.6)	19 (17.3)	62 (15.0)
A5.1	77 (52.7)	192 (42.1)	41 (37.3)	156 (37.9)
A6	17 (11.6)	74 (16.2)	18 (16.4)	89 (21.6)
A9	18 (12.3)	71 (15.6)	16 (14.5)	52 (12.6)

*Number of chromosomes. AITD, autoimmune thyroid disease; DM, type 1 diabetes; MGA, monoglandular autoimmunity; and PGA, polyglandular autoimmunity.

controls. The MICA genotypes did not significantly deviate from Hardy–Weinberg (H–W) equilibrium in patients with PGA ($\chi^2=9.3$, $df=10$, $P_{\text{exact}}=0.4797$) and controls ($\chi^2=6.9$, $df=10$, $P_{\text{exact}}=0.7555$).

HLA-DRB1

The HLA-DRB1*03 allele carriers were strongly increased in patients with PGA compared with controls (50.7% vs 21.8%, $P<0.0001$, $P_c<0.001$, $RR=2.32$,

95% $CI=1.62\text{--}3.33$; Fig. 1). It was also increased in patients with PGA compared with those with MGA (AITD: 11.4%, $P=1.7\times 10^{-11}$; $P_c<0.0001$; DM: 29.1%, $P=0.018$, $P_c=NS$). In contrast to PGA, patients with MGA did not show increased frequencies of HLA-DRB1*03 when compared with controls (AITD: 11.4%, $P=0.006$, $P_c=NS$; DM: 29.1%, $P=0.278$).

HLA-DRB1*04 allele carriers were increased both in patients with PGA (53.4% vs 22.4%, $P<0.00001$, $P_c<0.0001$, $RR=2.38$, 95% $CI=1.68\text{--}3.38$) and with DM, compared with controls (60.0% vs 22.4%, $P=0.000001$, $P_c<0.0001$, $RR=2.68$, 95% $CI=1.89\text{--}3.80$), but were decreased in patients with AITD (9.6% vs 22.4%, $P=4.3\times 10^{-14}$, $P_c<10^{-11}$, $RR=0.43$, 95% $CI=0.27\text{--}0.70$). Analysis of the two groups with AITD separately gave prevalence of 6% for GD and 16.7% for HT; both were below the control values of 22%. There were no significant differences between GD and HT in the number of allele carriers for the different HLA-DRB1 alleles after Bonferroni correction for multiple testing.

The frequency of HLA-DRB1*13 allele carriers did not differ between patients with PGA (24.7%) and controls (26.4%), but was decreased in patients with AITD (5.7%, $P<0.000001$, $P_c<0.0001$, $RR=0.22$, 95% $CI=0.12\text{--}0.39$). When compared with controls (35.1%), the HLA-DRB1*15 allele was underrepresented in patients with PGA (9.6%, $P=0.00002$, $P_c<0.001$, $RR=0.27$, 95% $CI=0.13\text{--}0.57$), AITD (6.1%, $P<0.000001$, $P_c<0.0001$, $RR=0.18$, 95% $CI=0.10\text{--}0.30$), and DM (1.8%, $P<0.000001$, $P_c<0.0001$, $RR=0.05$, 95% $CI=0.01\text{--}0.37$).

HLA-DQB1

Patients with PGA showed more HLA-DQB1*02 allele carriers than controls (58.9% vs 33.9%, $P=0.0004$, $P_c<0.01$, $RR=1.73$, 95% $CI=1.31\text{--}2.30$), more HLA-DQB1*03 allele carriers (68.5% vs 53.4%, $P=0.034$, $P_c=NS$, $RR=1.28$, 95% $CI=1.04\text{--}1.58$), but less HLA-DQB1*05 carriers (20.5% vs 36.8%, $P=0.016$, $P_c=NS$, $RR=0.56$, 95% $CI=0.34\text{--}0.91$) and less HLA-DQB1*06 carriers (28.8% vs 58.0%, $P=0.00003$,

Figure 1 Percentages of allele carriers for HLA-DRB1 alleles, separately shown for patients with polyglandular autoimmunity (PGA), autoimmune thyroid disease (AITD), type 1 diabetes mellitus (DM), and healthy controls. Patients with PGA had significantly more HLA-DRB1*03 allele carriers than controls, whereas patients with MGA (DM or AITD) did not. Both patients with PGA and DM had significantly more HLA-DRB1*04 allele carriers than controls. HLA-DRB1*15 allele carriers were significantly decreased in patients with PGA and MGA, compared with controls.

$P_c < 0.001$, $RR = 0.50$, 95% $CI = 0.34-0.73$). Also, when compared with controls, patients with AITD and DM showed increased DQB1*02 and DQB1*03 carriers and less DQB1*05 and DQB1*06 carriers (Fig. 2). Separate analysis of HLA-DQB1 allele carriers in AITD showed more HLA-DQB1*03 carriers in HT versus GD (79.5% vs 48.7%, $P = 0.009$, $P_c = NS$).

Association of polymorphisms with clinical onset of disease in PGA

In patients with PGA, mean age at clinical onset of the first autoimmune endocrine disease was 31.0 ± 15.7 years. The HLA-DRB1*03 allele was more prevalent in those patients with early versus late onset of disease ($P = 0.045$, logistic regression analysis; Fig. 3). The same holds true for the HLA-DQB1*02 allele ($P = 0.042$; Fig. 3). By contrast, MICA alleles were not associated with disease onset. Gender was not an influencing factor on presence of alleles, neither for HLA-DRB1 and HLA-DQB1 nor for MICA.

MICA

MIC A5.1 allele carriers were more frequently observed in patients with PGA than in controls (Fig. 4), but differences were not significant after Bonferroni correction (76.7% vs 62.1%, $P = 0.031$, $P_c = NS$). Also, the A5.1/A5.1 genotype was increased in PGA in comparison with controls, but non-significant after correcting for multiple testing (28.8% vs 13.6%, $RR = 2.12$, 95% $CI = 1.29-3.49$, $P = 0.007$, $P_c = NS$). This genotype occurs less frequently in patients with MGA (AITD: 21.5% and DM: 14.5%). MIC A5.1 allele carriers were not increased in patients with AITD (62.3%, $P > 0.05$) and DM (61.8%, $P > 0.05$), compared with controls. MIC A6 allele carriers were less common in patients

Figure 3 Percentage of PGA patients with the presence of HLA-DRB1*03 allele and HLA-DQB1*02 allele decreases significantly with age at clinical onset of disease ($P = 0.045$ and 0.042 respectively).

with PGA (23.3%), AITD (29.4%), and DM (29.1%) than in controls (36.9%, NS). The separate analysis of AITD patients showed no significant differences between HT and GD patients in MICA allele carriers after Bonferroni correction.

Figure 2 Percentages of allele carriers for HLA-DQB1 alleles, separately shown for patients with polyglandular autoimmunity (PGA), autoimmune thyroid disease (AITD), type 1 diabetes (DM), and healthy controls. Patients with PGA had significantly more HLA-DQB1*02 allele carriers than controls, but less HLA-DQB1*06 carriers. HLA-DQB1*03 was significantly increased in patients with DM.

Figure 4 Percentages of allele carriers for MICA alleles, separately shown for patients with polyglandular autoimmunity (PGA), autoimmune thyroid disease (AITD), type 1 diabetes (DM), and healthy controls. Patients with PGA tended to have more MIC A5.1 allele carriers and less MIC A6 allele carriers than controls. Patients with MGA (DM or AITD) did not differ from controls.

Associations between HLA-DRB1, HLA-DQB1, and MICA polymorphisms

When the PGA group was stratified for the presence or absence of HLA-DRB1*03, the allele MIC A5 was decreased in DRB1*03 + patients (13%) when compared with DRB1*03– patients (36%, $P=0.032$, $P_c=NS$, Table 2). There was no association between HLA-DRB1*03 carriers and the presence of MIC A5.1 allele ($P=0.417$) or absence of MIC A6 allele in patients with PGA ($P=1.000$). The MIC A6 allele was decreased in HLA-DQB1*06 + patients when compared with HLA-DQB1*06– patients (4.8% vs 30.8%, $P=0.017$, $P_c=NS$).

Discussion

In this controlled study, for the first time, we did compare HLA-DRB1, HLA-DQB1, and MICA polymorphisms in patients with PGA versus MGA and healthy controls. We did find that especially HLA-DRB1*03 was more strongly associated with PGA than with MGA. Also, this association seems even more closed than the one between the DQB1*02 allele and PGA. Furthermore, foremost HLA-DRB1*03 and also HLA-DQB1*02 alleles were associated with early onset of PGA. On the other hand, the increased number of HLA-DRB1*04 allele carriers in PGA could be attributed to the disease-specific increase in patients with DM. Finally, there was no interaction between MIC A5.1 and HLA-DRB1*03 or HLA-DQB1 alleles in conferring risk for PGA.

HLA-DRB1

We found that the HLA-DRB1*03 and HLA-DRB1*04 alleles were significantly increased in patients with PGA, compared with controls. The very high HLA-DRB1*03 frequency in patients with PGA suggests that the allele HLA-DRB1*03 might be a risk factor for PGA. Also, patients with PGA had higher frequencies for HLA-DRB1*03 than those with MGA. A positive association of DRB1*03 with Graves' disease has been reported whereas the association with autoimmune hypothyroidism has been shown to be weaker (6–7, 20–21). For HLA-DRB1*04, both patients with PGA and DM had significantly more allele carriers than

controls. This indicates that the high frequency of PGA might be due to the component disease of DM. Since HLA-DRB1*04 was not increased in patients with AITD, there was no disease-specific effect of AITD on the high frequency in patients with PGA.

Since HLA-DRB1*03 was increased in PGA, it appeared a risk marker for PGA. Therefore, we analyzed whether patients at early onset of disease had higher allele frequencies than patients with late onset of disease. As anticipated, the presence of HLA-DRB1*03 was associated with early clinical onset of disease in patients with PGA. Allele carriers occurred more frequently in those patients with early versus late onset of disease. This indicates that HLA-DRB1*03 might be a stronger genetic marker in patients younger than 20 years in contrast to those older than 40 years. Moreover, this suggests that there may be a distinct genetic background in PGA patients with earlier disease onset in contrast.

HLA-DQB1

With respect to HLA-DQB1, the allele *02 was most prevalent in PGA compared with controls, whereas no significant differences were noted between MGA and controls. This allele occurred in PGA more frequently in patients with early onset of disease than in patients with late onset. The DQB1*06 allele was less prevalent in both patients with PGA and MGA, when compared with controls. Therefore, it seems to play a protective role regarding the development of PGA and MGA. This indicates that the allele DQB1*06 is protective not only for DM but also for PGA. In addition, the allele HLA-DQB1*03 was more prevalent in DM than in PGA, compared with controls. These observations are in line with findings that the HLA-DR3-DQB1*0201 haplotype may be associated with multiple component diseases of PGA, whereas the HLA DR4-DQB1*0302 haplotype is implicated in β -cell autoimmunity only (8).

MICA

In patients with PGA, an increased frequency of MIC A5.1 allele carriers and a reduced frequency of MIC A6 carriers were observed. Also, the A5.1/A5.1 genotype was increased in patients with PGA, but not in patients with MGA. However, these associations did not hold after correcting for the number of tests. There might be an immunogenetic relevance of MIC A5.1 in patients with PGA. The MICA molecule, a membrane-bound glycoprotein, binds to natural killer G2D (NKG2D), which is an activating receptor of NK cells (22). The G insertion in the MIC A5.1 allele results in a frameshift mutation accompanied by a premature stop codon. This particular allele encodes a MICA protein with a shortened transmembrane segment lacking its cytoplasmic tail and representing a soluble, secreted form of

Table 2 Frequencies of MHC class I chain-related gene A (MICA) allele carriers in DRB1*03 patients with polyglandular autoimmunity (PGA).

MICA allele	DRB1*03+ patients	DRB1*03– patients	P	P _{corr}
A4	8 (21.6%)	7 (19.4%)	NS	NS
A5	5 (13.5%)	13 (36.1%)	0.032	NS
A5.1	30 (81.1%)	26 (72.2%)	NS	NS
A6	9 (24.3%)	8 (22.2%)	NS	NS
A9	9 (24.3%)	7 (19.4%)	NS	NS

NS, not significant.

the MICA molecule. Thus, the MIC A5.1/5.1 genotype might negatively influence immune reactions.

The frequency of A5.1 allele carriers was not significantly increased in patients with MGA (AITD or DM). This agrees with findings in Chinese patients with AITD as well as Italian patients with DM (13, 16). There might be a protective effect of the MIC A6 allele. We observed a slightly negative association of MGA and a more pronounced negative association of PGA with MIC A6, but results were not statistically significant after correction for multiple testing. In comparison, a lowered frequency of MIC A6 has also previously been reported for patients with DM and Addison's disease (12, 15).

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