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ARTICLE

Association of *PDCD1* polymorphisms with childhood-onset systemic lupus erythematosus

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A regulatory single nucleotide polymorphism (SNP) PD1.3G/A located on programmed cell death 1 (*PDCD1*) gene, was shown to be involved in susceptibility to systemic lupus erythematosus (SLE) in Swedish, European American, and Mexican cases. However, association to childhood-onset SLE has not been analyzed. The aim of this study was to investigate the association of *PDCD1* polymorphisms and haplotypes with susceptibility to childhood-onset SLE in Mexican population. Three *PDCD1* SNPs, PD1.3G/A, PD1.5C/T, PD1.6G/A, were analyzed in 250 childhood-onset SLE Mexican patients and 355 healthy controls in a case–control association study. Polymorphisms were genotyped by TaqMan technology. Stratification analysis was performed on the SLE cohort to investigate the SNP association with renal disorder. In addition, haplotypes were constructed with these three SNPs. The PD1.3A allele was significantly associated to childhood-onset SLE ($P = 0.0019$, odds ratio (OR) 2.73, 95% confidence interval (95% CI) 1.35–5.56). The other *PDCD1* SNPs did not show association. A total of 155 patients (62%) had nephritis, and no association was observed with *PDCD1* SNPs. The ACG haplotype (PD1.3A, PD1.5C, PD1.6G) included almost all PD1.3A alleles, and it was more frequent in SLE patients (5.5%) than in controls (2.1%) ($P = 0.003$; OR 2.73, 95% CI 1.37–5.46). The haplotype structure in Mexican controls was significantly different from those reported in Spanish and Swedish. Our results support association of the PD1.3A SNP to susceptibility of childhood-onset SLE in Mexican population and does not show association to lupus nephritis in this age group.
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Introduction

Systemic lupus erythematosus (SLE) is a complex autoimmune disorder that predominantly affects women during childbearing age.[1] However, 15–17% of all SLE patients present in children younger than age 16.[2] Although clinical manifestations and immunological findings of pediatric SLE are similar to those seen in adults with SLE, in childhood-onset SLE the initial symptoms tend to be more severe and it has a more aggressive clinical course.[3–5]

Susceptibility to SLE has been attributed to complex interaction between genetic and environmental factors. In

recent years, several potential SLE susceptibility *loci* have been identified by nine complete genome scans using family collections that differ greatly in ethnic compositions and geographic locations, although only eight of these have been significantly linked to SLE. These map to 1q23, 1q25–31, 1q41–42, 2q35–37, 4p16–15.2, 6p11–21, 12q24 and 16q12.[6] Recently, Prokunina *et al* found the programmed cell death gene (*PDCD1*) within the 2q37 region as a good candidate risk gene for SLE.[7] *PDCD1* encodes an inhibitory immune receptor (PD-1), member of the immunoglobulin superfamily that carries an immune tyrosine-based inhibitory motif (ITIM), and plays a pivotal role in maintaining peripheral immune tolerance.[8] C57BL/6 background mice deficient in PD-1 expression develop a lupus-like syndrome, with glomerulonephritis and arthritis.[9] Prokunina *et al* identified seven single nucleotide polymorphisms (SNPs) within the *PDCD1* gene. One of them (PD1.3 G/A), a regulatory SNP located in intron 4, showed to be involved in susceptibility to SLE in Swedish, European American, and Mexican families and in sporadic cases.[7] However, other studies that included sporadic SLE patients from northern Sweden and Denmark did not find association to PD1.3A SNP.[10,11] Moreover, the PD1.3A allele has been associated to renal manifestations in Swedish patients with SLE, but not in female patients from the United States with European American descent.[10,12] In contrast, analysis of a large Spanish cohort showed that the PD1.3G allele is associated with susceptibility to SLE as opposed to the observed for other populations.[13]

We performed a case–control study to test the association between SNPs on *PDCD1* gene and susceptibility to childhood-onset SLE in a cohort of Mexican patients.

Patients and methods

Patients

We studied 250 unrelated patients with childhood-onset SLE, consisting of 228 cases with sporadic SLE and 22 cases with familial SLE who were recruited from four pediatric hospitals in Mexico City: Hospital de Pediatría Centro Médico Nacional Siglo XXI, Instituto Nacional de Pediatría, Hospital Infantil de México, and Hospital General Centro Médico 'La Raza'. All patients were <16 years of age at onset of disease and fulfilled the American College of Rheumatology (ACR) criteria for the diagnosis of SLE.[14] Of these, 214 were female (85.6%) and 36 were male (14.4%), with a mean $\pm$SD age at onset of 11.62 ± 2.46 years in the whole group. All patients were born in Mexico like their parents, grandparents and great-grandparents. Additionally, 355 blood bank donors were included as healthy control group. The controls were drawn from Mexico City and sex matched, consisting of 305 female subjects (86%) and 50 male subjects (14%). This study was approved by the respective local ethics and research committees and all individuals signed an informed consent.

Study design

We performed a case–control association study to investigate whether the *PDCD1* polymorphisms were associated with childhood-onset SLE. We studied three of the seven *PDCD1* SNPs previously described by Prokunina *et al* not fully linked to each other: PD1.3 (7,146G/A), PD1.5 (7,785C/T), and PD1.6 (8,738G/A).[7] We also constructed haplotypes with the alleles of these three SNPs.

Stratification analysis was performed in the SLE cohort to investigate the SNP association with renal disorder. One hundred and fifty five patients (62%) were considered as having lupus nephritis according to the ACR criteria and were confirmed by kidney biopsy in 104 (67%). Seventeen were Class II (16%), seven Class III (7%), seventy six Class IV (73%), three Class V (3%), and one Class VI (1%), according to the World Health Organization (WHO) classification.[15]

Genotyping

Genomic DNA from patients and controls was extracted from peripheral blood leukocytes using the conventional technique of phenol chloroform[16] or the GenePure Genomic Blood DNA Purification kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. PD1.3, PD1.5, and PD1.6 polymorphisms were genotyped using the TaqMan system 5′-allele discrimination Assay-By-Design method (Applied Biosystems, Foster City, CA, USA). The primers and probes used for identification of each allele are shown in Table 1.

Polymerase chain reaction (PCR) was carried out by using TaqMan Universal Master Mix (Applied Byosystems) in 5 μl final volume reactions, using 20 ng of genomic DNA.

Table 1 The 5′ nuclease assays (TaqMan) for *PDCD1* polymorphisms

PD1.3	
Forward-primer	5′-CCTCAATCCCTAAAGCCATGATCTG-3′
Reverse-primer	5′-CAGGCAGGCACACACATG-3′
G-allele probe	5′-ACCTGCGGTCTCC-MGB[a]-NFQ[b]-3′
A-allele probe	5′-ACCTGCAGTCTCC-MGB[a]-NFQ[b]-3′
PD1.5	
Forward-primer	5′-GCGGAATGGGCACCTCAT-3′
Reverse-primer	5′-TCCATCCTCAGGCCTCAGT-3′
C-allele probe	5′-CGTCGGCTGAGCC-MGB[a]-NFQ[b]-3′
T-allele probe	5′-CCGTCAGCTGAGCC -MGB[a]-NFQ[b]-3′
PD1.6	
Forward-primer	5′-GCAGAAGTGCAGGCACCTA-3′
Reverse-primer	5′-GAATGGGTTCCAAGGAGAGCT-3′
G-allele probe	5′-CCCCATGTGCCCAC-MGB[a]-NFQ[b]-3′
A-allele probe	5′-CCCCCATATGCCCAC-MGB[a]-NFQ[b]-3′

Primers and probes were designed using Assays-by-Design (Applied Biosystems).
The probes were labeled at their 5′ ends with FAM™ (the first allele) and VIC™ (the second allele) and the 3′ ends contained quenchers.
[a]Minor Groove Binder.
[b]Quencher.

Thermal cycling conditions were 95°C for 10 min, followed by 40 cycles of 92°C for 15 s and 60°C for 1 min. The genotype was automatically attributed in each sample measuring the allele-specific fluorescence in the ABI Prism 7900 HT Sequence Detection System, using the SDS 2.2.2 software for allele discrimination (Applied Biosystems). The overall genotype call rate by TaqMan was 98%; five samples from a total of 255 cases and five samples from a total of 360 controls were discarded from the analysis because genotyping was not successful in one or more of the SNPs analyzed. The assay reproducibility was 100% in the 250 repeated samples from patients and the 355 samples from controls.

To confirm the genotype obtained by the TaqMan 5′-allele discrimination assay, PCR-restriction fragment length polymorphism (PCR-RFLP) was performed in 201 samples from cases (80%), and 260 samples from controls (73%). The PD1.5 and PD1.6 SNPs were genotyped by this technique as previously described.[13] The PCR-RFLP on PD1.3 was performed in a 25 μl reaction mixture containing 100 ng of genomic DNA, 100 μM of dNTPs, 0.1 μM of each primer, 5 U of *Amplitaq Gold* DNA polymerase (Applied Biosystems, USA), 1.5 mM of $MgCl_2$ and 1 × PCR buffer. Thermal cycling conditions were 95°C for 10 min, followed by 35 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 15 s. A final extension at 72°C for 5 min was carried out. The 180-pb PCR product was digested with 5 U of *Pst I* (Invitrogen, CA, USA) at 37°C overnight and was resolved on a 15% polyacrylamide gel with ethidium bromide. The A allele include a *Pst*I site that is not present in the G allele. Therefore, digestion resulted in 130- and 50-bp fragments for the A allele or 180-bp fragments for the G allele. The primers sequences are 5′-CCCCAGGCAG CAACCTCAAT-3′ and reverse primer: 5′-GACCGCAGG CAGGCACATAT-3′, (conditions provided by Alarcon-Riquelme ME).

We also confirmed the genotype of PD1.3 SNP by sequencing on representative samples from each genotype. The system used for sequencing PD1.3 was the BigDye Ready Reaction Kit (Applied Biosystems) in an ABI Prism 3130 xL Genetic Analyzer (Applied Biosystems). The genotype obtained by TaqMan was confirmed by PCR-RFLP and by sequencing in 100% of the samples tested.

Statistical analysis

We used χ^2 analysis to evaluate the significance of differences in genotype and allele frequencies between cases and controls. Also, allele frequencies for cases and controls were used to calculate the odds ratio (OR) and the 95% confidence interval [95% CI]. The χ^2 value was calculated from 2×2 contingency tables, and *P*-values were determined using one degree of freedom. The software used for all statistical calculations was the StatCalc program (Epi Info 2005 v3.3.2; Centers of Disease Control and Prevention, Atlanta, GA, USA). Genotype distributions in patients and controls were evaluated for departure from Hardy–Weinberg equilibrium by the FINETTI program using the exact test (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl).

In the case–control haplotype analysis, Haploview v3.2 software was used to estimate haplotypes frequencies, which uses an implementation of the expectation-maximization algorithm.[17] We tested association with χ^2 analysis for each possible combination of haplotypes derived from the three SNPs (PD1.3, PD1.5 and PD1.6). In order to obtain a measure of significance corrected for multiple testing bias, we ran 10 000 permutations to compute *P*-values using the Haploview program. *P*-values less than 0.05 were considered statistically significant.

Results

Genotype frequencies of PD1.3, PD1.5 and PD1.6 SNPs were found to be in Hardy–Weinberg equilibrium in cases ($P = 1.0$, 0.58 and 1.0) and controls ($P = 1.0$, 0.36 and 0.33), respectively. When genotype frequencies were compared between cases and controls only PD1.3 SNP showed statistically significant association, the heterozygous genotype GA was more frequent in patients with SLE than in controls (10.4 *versus* 4%; $P = 0.0016$; OR 2.83, 95% CI 1.38–5.84) (Table 2). The PD1.3A allele frequency was 5.2% in SLE patients while in controls it was 2% ($P = 0.0019$, OR 2.73, 95% CI 1.35–5.56) (Table 3). When we analyzed only female cases and female controls as reported by Prokunina *et al*,[7] the association with PD1.3A allele was still significant ($P = 0.0052$, OR 2.53, 95% CI 1.29–4.95) (data not shown). The frequency of PD1.3A allele was lower in male SLE patients than female cases (2.8 *versus* 5.6%), and in male controls it was 0%, whereas in female controls it was 2.3%. Nevertheless, these differences could be due to the small number of male patients with SLE and male controls analyzed (36 and 50 respectively). Allele frequencies of PD1.5C and of PD1.6A polymorphisms did not differ significantly between cases and controls (Table 3).

A total of 155 patients (62%) had nephritis according to biopsy and/or for ACR criteria. When association was evaluated between the PD1.3A SNP and glomerulonephritis, no significant differences were observed between patients with and those without lupus nephritis (5.48 and 4.73% respectively, $\chi^2 = 0.133$, $P = 0.71$). Neither PD1.5C nor PD1.6A showed association to glomerulonephritis (data not shown).

Haplotype frequencies were estimated in female cases and in female controls using the three SNPs of *PDCD1*. A total of six haplotypes were identified, but only five had a frequency greater than 1% (Table 4). The ACG haplotype (PD1.3A, PD1.5C, PD1.6G) included almost all PD1.3A alleles, and it was more frequent in SLE patients (5.5%) than in controls (2.1%) ($P = 0.003$; OR 2.73, 95% CI 1.37–5.46) (Table 4). Haplotype frequencies obtained in

Table 2 Genotype distribution of *PDCD1* polymorphisms in Mexican childhood-onset SLE

Genotype	*SLE* n = *250 (%)*	*Controls* n = *355 (%)*	P[a]	*OR (95% CI)*
PD1.3GG	224 (89.6)	341 (96)	0.0016	0.35 (0.17–0.72)
PD1.3GA	26 (10.4)	14 (4)	0.0016	2.83 (1.38–5.84)
PD1.3AA	0 (0)	0 (0)		
PD1.5CC	102 (40.8)	150 (42)	0.72	0.94 (0.67–1.33)
PD1.5CT	119 (47.6)	155 (43.7)	0.34	1.17 (0.84–1.64)
PD1.5TT	29 (11.6)	50 (14.1)	0.37	0.80 (0.48–1.34)
PD1.6GG	55 (22)	72 (20.3)	0.61	1.11 (0.73–1.68)
PD1.6GA	125 (50)	165 (46.5)	0.39	1.15 (0.82–1.61)
PD1.6AA	70 (28)	118 (33.2)	0.17	0.78 (0.54–1.13)

SLE = systemic lupus erythematosus; OR = odds ratio; 95% CI = 95% confidence interval.
[a]*P*-value uncorrected for multiple tests, 1 degree of freedom.

Table 3 Distribution of alleles of SNPs in *PDCD1* gene in Mexican childhood-onset SLE

Allele	*SLE n/N*[a] *(%)*	*Controls n/N*[a] *(%)*	P[b]	*OR (95% CI)*
PD1.3A	26/500 (5.2)	14/710 (2.0)	0.0019[c]	2.73 (1.35–5.56)
PD1.5C	323/500 (64.6)	455/710 (64)	0.85	1.02 (0.88–1.35)
PD1.6A	265/500 (53)	401/710 (56.5)	0.23	0.87 (0.69–1.10)

[a]n/N = number of alleles out of total number of chromosomes. SLE = systemic lupus erythematosus; OR = odds ratio; 95% CI = 95% confidence interval.
[b]*P*-value uncorrected for multiple tests, 1 degree of freedom.
[c]$P = 0.0089$ after correcting the *P*-value for multiple testing by Haploview program using 10 000 permutations.

Table 4 Haplotype analysis of *PDCD1* polymorphisms in Mexican SLE female cases and female controls

Haplotype number	*PD1.3*	*PD1.5*	*PD1.6*	*Cases* n[a] = *428*	*Frequency*	*Controls* n[a] = *610*	*Frequency*
1	G	C	A	123	0.29	193	0.32
2	G	C	G	125	0.29	179	0.29
3	G	T	A	106	0.25	167	0.27
4	G	T	G	50	0.12	57	0.094
5[b]	A	C	G	24	0.055	13	0.021
6	A	C	A	0.3	0.0007	1.2	0.002

[a]Number of studied chromosomes.
[b]$P = 0.003$, uncorrected for multiple tests; odds ratio = 2.73; 95% confidence interval: 1.37−5.46. $P = 0.045$ after correcting the *P*-value for multiple testing by Haploview program using 10 000 permutations.

female Mexican controls were compared to those previously reported in female controls from Spain and Sweden.[7,13] We observed significant differences between these populations and the Mexican (Figure 1). Interestingly, the GCA haplotype, which was the least frequent in Spanish (1%) and Swedish controls (2%), resulted the most frequent in Mexican controls (32%) ($P < 1.0 \times 10^{-7}$ and $P < 4.0 \times 10^{-7}$, respectively). The GTA haplotype showed to be more frequent in Mexican (27%) than in Spanish (9.67%) or Swedish populations (19%), however, this difference showed significance only when compared to Spanish ($P < 1.0 \times 10^{-7}$). On the other hand, haplotype frequencies of GCG, GTG, and ACG were lower in Mexican population than in Spanish or Swedish female controls. These differences were statistically significant, except for the ACG haplotype between Mexican and Swedish (Figure 1).

Discussion

The genetic basis in childhood-onset SLE is currently unclear. Although several new insights have been obtained in the past decade using linkage and association studies of multicases SLE families as well as case–control studies, most of these studies have involved mainly patients with adult-onset SLE. Recently, we have replicated the observed association of the *PTPN22* gene with adult-onset SLE in childhood-onset SLE in Mexican population,[18] and to our knowledge there are only two other genetic studies with a small sample size on pediatric SLE.[19,20] One was performed in 46 Chinese children showing association between SLE and a SNP in *RANTES* gene.[19] The other one found an association of polymorphisms in the estrogen receptor α gene in 41 Korean with childhood-onset SLE.[20]

We performed a case–control study to test association between SNPs on the *PDCD1* gene to childhood-onset SLE

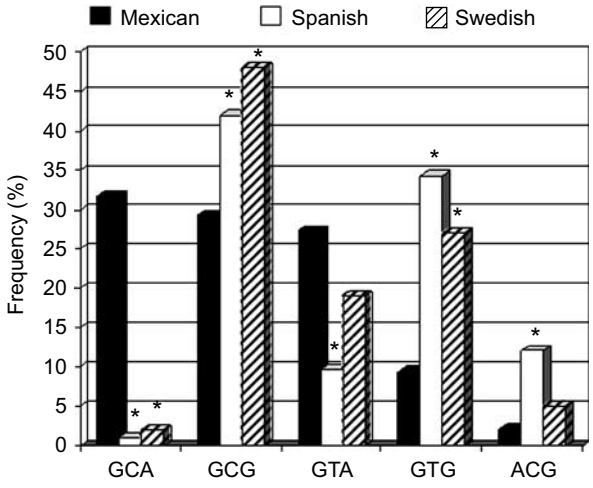

Figure 1 Haplotype frequencies of *PDCD1* derived from PD1.3, PD1.5, and PD1.6 SNPs in female controls from Mexico, Spain and Sweden. Haplotype frequencies for the Mexican female controls were estimated from 610 chromosomes. Haplotype frequencies for the Spanish female controls and Swedish female controls were obtained from Figure 1 of reference.[13] $*P<0.05$ *versus* Mexican female controls.

in a large cohort of Mexican patients. Prokunina *et al*,[7] previously reported an association of the PD1.3A allele with SLE in adults from three different populations, including Mexicans. This SNP disrupts the predicted DNA-binding site for the runt-related transcription factor 1 (RUNX1) located in an intronic enhancer, suggesting a mechanism through which it can contribute to the development of SLE in humans.[7] Our results show that the PD1.3A allele increases genetic risk to childhood-onset SLE in Mexicans. However, we found a weaker association of this SNP with childhood-onset SLE female patients ($P=0.0052$, OR 2.53, 95% CI 1.29–4.95) compared with that reported by Prokunina *et al*,[7] in Mexican female adults with SLE ($P=0.0023$, OR 3.23, 95% CI 1.46–7.16; data calculated from Prokunina *et al*[7]).

As renal disease is extremely common in childhood-onset SLE, and it has been previously associated with the PD1.3A allele in adult SLE Swedish population, we explored the possible association between this SNP with lupus nephritis in Mexican children with SLE. Despite a high frequency of lupus nephritis (62%) in our sample, we could not confirm association of PD1.3A allele with the phenotype. No association with renal disorder was also found in female patients with SLE from the United States of European American descent.[12] This discrepancy could be explained by genetic heterogeneity.

Pediatric SLE is phenotypically similar to adult SLE, however childhood-onset SLE has a higher incidence of renal, neurological, hematological, cardiac and pulmonary disease, resulting in to more severe presentation and aggressive clinical course.[3–5]

The earlier-onset and the higher severity of the disease in childhood-onset than in adult-onset SLE, could be explained because of the presence of different polymorphisms or combination of them, or because there is a greater genetic background of susceptibility alleles in the childhood-onset SLE.

Mexican mestizos are an admixed population with a complex genetic structure, where Native American genes account for 51%, European genes for 45.4%, and African genes for 3.7%.[21] This could explain the observed difference in the distribution of haplotype frequencies between Mexican population and Spanish and Swedish populations. The high frequency of the GCA haplotype in Mexican population compared with Spanish and Swedes (32 *versus* 1 and 2%, respectively) suggests that in our population this haplotype could be provided from Amerindian ancestors. However, further studies on Mexican Amerindians are needed to confirm this asseveration.

In conclusion, our results support the involvement of the PD1.3A SNP as a genetic risk factor for susceptibility in childhood-onset SLE in Mexican population but not for lupus nephritis. Further studies comparing the genetic background between childhood-onset and adult-onset SLE patients in ethnically homogeneous populations would allow us to explain the phenotypical differences between these populations.

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