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ORIGINAL ARTICLE

The first genome-wide association study identifying new susceptibility loci for obstetric antiphospholipid syndrome

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Antiphospholipid syndrome (APS) is the most important treatable cause of recurrent pregnancy loss. The live birth rate is limited to only 70–80% in patients with APS undergoing established anticoagulant therapy. Lupus anticoagulant (LA), but not anticardiolipin antibody (aCL), was found to predict adverse pregnancy outcome. Recent genome-wide association studies (GWAS) of APS focusing on aCL have shown that several molecules may be involved. This is the first GWAS for obstetric APS focusing on LA. A GWAS was performed to compare 115 Japanese patients with obstetric APS, diagnosed according to criteria of the International Congress on APS, and 419 healthy individuals. Allele or genotype frequencies were compared in a total of 426 344 single-nucleotide polymorphisms (SNPs). Imputation analyses were also performed for the candidate regions detected by the GWAS. One SNP (rs2288493) located on the 3′-UTR of *TSHR* showed an experiment-wide significant APS association ($P = 7.85E-08$, OR = 6.18) under a recessive model after Bonferroni correction considering the number of analyzed SNPs. Another SNP (rs79154414) located around the *C1D* showed a genome-wide significant APS association ($P = 4.84E-08$, OR = 6.20) under an allelic model after applying the SNP imputation. Our findings demonstrate that a specific genotype of *TSHR* and *C1D* genes can be a risk factor for obstetric APS.
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INTRODUCTION

Antiphospholipid syndrome (APS) is the most important treatable etiology in recurrent pregnancy loss (RPL).[1,2] The main clinical manifestations of APS are arterial/venous thrombosis and obstetric complications such as RPL (recurrent early miscarriage and intrauterine fetal death), preeclampsia and placental insufficiency.[3] APS, as a thrombotic disorder, is exclusively diagnosed by measuring lupus anticoagulant (LA), by means of the activated partial thromboplastin time (aPTT) and Russell's viper venom time (RVVT) and/or anticardiolipin antibodies (aCLs) and/or anti-β2glycoprotein I (β2GPI) antibodies. When these levels remain elevated for 12 weeks, the case is diagnosed as APS, as these conditions meet the classification criteria established by the International Congress on APL.[3] The other established causes of RPL include uterine anomalies, abnormal chromosomes, particularly translocations, in either partner, or abnormal embryonic karyotypes.[4–6]

Clinical features of APS include stroke, transient ischemic attack, venous thromboembolism, thrombocytopenia, heart valve disease, coronary artery disease, myelopathy, vascular dementia, chorea, migraine, epilepsy, osteonecrosis and nephropathy, suggesting that APS is a systemic and heterogeneous syndrome.[7] Up to 40% of patients with systemic lupus erythematosus (SLE) have APS as a complication, although APS is rarely found in patients with other autoimmune diseases including rheumatoid arthritis.[7]

A number of genome-wide association studies (GWAS) on SLE have identified several susceptible genes involved in the pathophysiology of B-cell/T-cell responses and the nuclear factor-κB signaling pathway in different ethnic backgrounds, including *STAT4,*

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HLA-DRB1, TNFAIP3, BLK, HIP1, ETS1 and *AFF1* in the Japanese lupus population.[8–11] Candidate gene approaches have revealed that *STAT4* and *BLK* are also associated with primary APS.[12,13]

The first GWAS for antiphospholipid antibodies (aPLs) compared allele frequencies of cases positive and negative for aCL, LA by RVVT and anti-β2GPI IgG, and results suggested some loci with a $P<1.00E-05$.[14] That study was unable to reveal thrombotic or obstetric APS-susceptible genes due to a limitation in its study design since it included patients with a low aPL titer. The recent GWAS for APS revealed significant association of anti-β2GPI IgG and *APOH* and anti-domain I of β2GPI and *MACROD2*.[15] That study focused on aCL.

Even though the live birth rate is limited to only 70–80% in patients undergoing low-dose aspirin and heparin combined therapy during pregnancy, this is currently the most established treatment in general use.[16,17] LA, but not aCL, was found to predict adverse pregnancy outcome.[18,19] The addition of pravastatin or hydroxychloroquine to conventional treatment was associated with a higher rate of live births and a lower prevalence of pregnancy morbidity.[20,21] Despite the large efforts of clinical scientists in this field, the results of clinical research have not been optimistic, basically due to the lack of understanding of the mechanisms involved in obstetric APS. Little is known as to why aPL causes or is related to recurrent early miscarriage and early onset preeclampsia.

Looking for ways to advance the treatment of obstetric APS patients, we conducted the first GWAS in patients with APS focusing on LA.

MATERIALS AND METHODS

Patients and controls

Samples from 154 Japanese patients were collected at Nagoya City University Hospital, Hokkaido University Hospital, Fujita Clinic, National Center for Child Health and Development, Juntendo University Hospital, Kanazawa University Hospital and National Cerebral and Cardiovascular Center, RPL Research Project Team of the Japanese Ministry of Health and Welfare, between November 2009 and March 2014. Most of the cases and control samples were collected from the mainland of Japan Honshu and the neighboring island of Hokkaido.

All patients with RPL underwent conventional examinations, such as hysterosalpingography, chromosomal analysis of both partners, and determination of aPLs. Patients with uterine or chromosomal abnormalities in either partner were excluded.

APS was diagnosed according to the criteria of the International Congress on Antiphospholipid Antibodies.[3] Several patients with two miscarriage were included because the prevalence of abnormal results did not differ among women with two and three or more miscarriages.[22] β2GPI-dependent aCL (Yamasa kit; Yamasa Corp., Choshi, Japan), LA by diluted RVVT (Gradipore Ltd, Pyrmont, NSW, Australia) and LA by diluted aPTT (PTT-LA test and StaClot LA; Diagnostica Stago, Paris, France) were examined and their persistency confirmed.[23]

A total of 419 healthy controls for GWAS analysis had been independently recruited from the Tokyo area and used in previous GWAS reports.[24,25] We included male controls because the sample size of the controls should be large considering the rarity of this disease. The study was conducted with the approval of the Research Ethics Committee of Nagoya City University Medical School, the University of Tokyo and other collaborating hospitals. Written informed consent was obtained from all subjects. The methods were carried out in accordance with the approved guidelines.

Genotyping and quality control

DNA was extracted from whole peripheral blood with a QIAamp DNA Blood Midi Kit (Qiagen, Tokyo, Japan). For the GWAS, we genotyped 573 samples (154 Japanese APS cases and 419 Japanese healthy controls) using an Affymetrix Axiom Genome-Wide ASI 1 Array to interrogate 600 307 SNPs according to the manufacturer's instructions. SNPs in chromosome X were excluded since male controls were included. After excluding three APS samples with a Dish QC of <0.82 (recommended sample quality control (QC) metric for the Axiom array), we recalled the remaining samples using Genotyping Console v4.2 software. Of the 600 307 SNPs embedded in the array, two samples with an overall call rate of $<97\%$ were also excluded. As a result, 149 cases and 419 controls were subjected to further analysis. All samples used for GWAS passed a heterozygosity check and three duplicated or related samples were identified by descent testing. Remaining sample pairs showed the PI-HAT value <0.07, indicating that remaining samples are unrelated individuals. Principal component analysis found no outliers to be excluded via the Smirnov–Grubbs test (Supplementary Figure S1).

We then applied the following thresholds for SNP quality control: an SNP call rate $\geqslant 95\%$, a minor allele frequency (MAF) $\geqslant 5\%$ in APS cases and healthy controls, and a Hardy–Weinberg equilibrium P-value $\geqslant 0.001$ in healthy controls. Of the 582 050 SNPs on autosomal chromosomes, the SNP number excluded by an SNP call rate threshold, an MAF threshold and a Hardy–Weinberg equilibrium P-value threshold were 9677, 143 679 and 1890, respectively. All cluster plots for SNPs with P-values $<1.00E-04$ from a χ^2 test of the allele frequency model, dominant model or recessive model were checked by visual inspection and 460 SNPs with ambiguous genotype calls were excluded. In total, 426 344 SNPs passed the quality control filters and were used for the association analysis. Candidate regions for further analysis were selected using a P-value threshold of 1.00E-04 (Supplementary Table S1).

SNP imputation analysis and validation experiment

An imputation method was used to estimate genotypes in 103 candidate regions to increase the chances of uncovering novel and significant associations without typing by estimating the haplotypes through a genotyped panel, which included denser SNPs. In this study, IMPUTE2 was used to predict the genotypes of untyped or missing SNPs.[26] Haplotype data were obtained from the 1000 Genomes Project (Phase III, http://www.1000genomes.org/) and used in reference panels. To apply IMPUTE2, GTOOL was first used to convert the genotyped file from PLINK format to IMPUTE2 input format.[27] A 1 Mb window size was applied for each candidate region detected by GWAS. After performing genotype imputation, association tests for the allelic model, dominant model or recessive model were performed and regional association plots were constructed by LocusZoom.[28] For quality control of the imputation data, the imputation probability threshold of 0.9 recommended by the developer was applied, and SNPs with more than 1% un-imputed genotype data and an MAF $<1\%$ and a Hardy–Weinberg equilibrium P-value <0.0001 were eliminated. The detected association of imputed SNPs with a P-value $<5.00E-06$ was confirmed through a validation experiment using the TaqMan SNP Genotyping method.

HLA imputation analysis

An imputation analysis of *HLA* alleles in APS samples was performed based on data of the SNP genotyping for the GWAS. We established a high-accuracy imputation method that can infer *HLA* alleles (*HLA-A, -B, -DPB1, -DQB1, -DRB1*) using a HIBAG R package and only Japanese subject data as a reference material.[29] HIBAG R is based on the attribute bagging method utilizing unphased genotype data and is robust for populations with complex linkage disequilibrium blocks by assuming minimal Hardy–Weinberg equilibrium. For the imputed data, we applied the posterior probability threshold of 0.5 recommended by the developer of this method. Rates of samples remaining after quality control were 96.5% for *HLA-A*, 87.0% for *HLA-B*, 98.2% for *HLA-DPB1*, 98.2% for *HLA-DQB1* and 93.0% for *HLA-DRB1* (Supplementary Table S2).

Statistical analysis

For the GWAS and imputation analysis, a χ^2 test was applied to a two-by-two contingency table in an allele frequency model, a dominant model or a recessive model. Fisher's exact test was applied if the number of tested genotypes in cases or controls was <5. We applied the term 'experiment-wide significance' for SNPs that passed Bonferroni's correction defined by the number of SNPs

Table 1 Characteristics of 115 patients with obstetric antiphospholipid syndrome

Characteristics	
Age at sampling, mean (s.d.)	40.7 (9.7)
Clinical features	
Recurrent pregnancy loss, mean (s.d.) number and range	1.24 (1.71) and 0–8
Presence % (*n*)	53.0% (61)
Absence % (*n*)	47.0% (54)
Intrauterine fetal death, mean (s.d.) number and range	0.85 (1.08) and 0–6
Presence % (*n*)	54.8% (63)
Absence % (*n*)	45.2% (52)
Complication of SLE	
Presence % (*n*)	43.5% (50)
Absence % (*n*)	55.7% (64)
Thrombosis	
Presence % (*n*)	45.2% (52)
Absence % (*n*)	53.9% (62)
Laboratory criteria	
β2glycoprotein I dependent anticardiolipin antibody, mean (s.d.) values	97.01 (151.17) IU
Lupus anticoagulant by aPTT, mean (s.d.) values	27.76 (16.37) seconds
Lupus anticoagulant by RVVT, mean (s.d.) values	1.66 (0.30)

Abbreviations: aPTT, activated partial thromboplastin time; RVVT, Russells' viper venom time; SLE, systematic lupus erythematodus.

analyzed at $P=1.17E-07$ (0.05/426,344). On the other hand, we applied the genome-wide significance threshold at 5.00E-08 and SNPs with a *P*-value $<5.00E-06$ were considered to have a suggested association. For each *HLA* association analysis, a χ^2 test was applied and we considered the number of alleles with more than 1% frequency in cases or controls in order to correct the multiple comparisons. If the frequency of one allele was $<1\%$ in both cases and controls, the allele was grouped as 'others.' Fisher's exact test was applied if the number of tested genotypes in cases or controls was <5. In *HLA-DQB1* analysis, its significance level was 0.00385 (0.05/13).

RESULTS

Among the 154 patients recruited in this study, we successfully genotyped 146 samples with an overall call rate of more than 97%. We decided to exclude 31 patients for the following reasons. Seven had a single positive for aCL. The remaining 24 had a single low level of LA-aPTT as LA was not confirmed with both clotting times (i.e. only by dRVVT or aPTT) and LA is known to be important in obstetric APS.[18,19] Finally, a principal component analysis showed that all APS cases ($n=115$) and healthy controls ($n=419$) clustered together with the HapMap JPT (Japanese in Tokyo from the CEPH collection), but not with the CHB (Han Chinese in Beijing), samples (Supplementary Figure S1). The average overall call rates of the remaining 115 APS cases and 419 controls were 99.38% (97.45–99.79) and 99.44% (97.33–99.81), respectively.

A total of 115 Japanese patients with obstetric APS were analyzed in the present study. Characteristics of the 115 patients are shown in Table 1. Mean (s.d.) values of β2GPI-dependent aCL, LA-RVVT and LA-aPTT were 97.01 (157.17), 1.66 (0.3) and 27.76 (16.37), respectively. The mean age of these patients at sampling was 40.7 (9.7) years (range 27–70).

Case–control association analysis

A total of 426 344 autosomal SNPs remained after applying quality control thresholds (SNP call rate $\geqslant 95\%$, MAF $\geqslant 5\%$ and a Hardy–Weinberg equilibrium *P*-value $\geqslant 0.001$ for controls). In the association analysis, we calculated the minimum *P*-value under three genetic models (allelic, dominant and recessive). A quantile–quantile plot of the distribution of test statistics for comparing genotype frequencies in APS cases and healthy controls showed that the inflation factor lambda for all the SNPs tested under the allelic, dominant and recessive model were 1.053, 1.054 and 1.123, respectively (Supplementary Figure S2). This result indicates that the effect of population stratification was negligible under the allelic and dominant model. On the other hand, substantial inflation was observed under the recessive model compared with the other models. This might imply the presence of some biases, although we have excluded ambiguous genotype calls by visual inspection of cluster plots for SNPs with *P*-values $<1.00E-04$.

A Manhattan plot of the GWAS showed that one SNP located in the *TSHR* region (rs2288493: odds ratio (OR) = 6.18, 95% confidence interval (CI) = 2.95–12.95, $P=7.85E-08$, under a recessive model) reached the experiment-wide significance threshold (Figure 1). The GWAS for APS revealed SNPs from *TSHR* (chromosome 14, rs2288493 and rs17630128), *SYCP2L* (chromosome 6, rs1225763 and rs1225731), *HLA-DRA* (chromosome 6, rs4959098 and rs2395166), *GATA3* (chromosome 10, rs2577963 and rs1020096), *FRMD4A* (chromosome 10, rs12570849), *RGS10* (chromosome 10, rs10886503), *PTPRO* (chromosome 12, rs1024843) and *MRPS23* (chromosome 17, rs1443267) regions at a value of $P<5.00E-06$ under the three genetic models (Supplementary Table S1). In addition, 161 SNPs in 103 regions showed *P*-values lower than 1.00E-04.

SNP imputation analysis

We focused on 103 candidate regions with SNPs showing a $P<1.00E-04$ in the GWAS for further imputation analysis. We also performed a validation experiment of imputed SNP genotypes with the *P*-value $<5.00E-06$ to confirm the associations. One SNP (rs2288493) in the *TSHR* region still showed a significant association after imputation analysis (Table 2 and Figure 2). In addition, the analysis revealed an association with rs79154414 located at 145kbp 3′ of the *C1D* gene, which also reached the genome-wide significance threshold (*P*-value $<5.00E-08$) (OR = 6.20, 95% CI = 2.96–13.0, $P=4.64E-08$, under an allelic model) (Table 2 and Figure 3). The association of rs79154414 after the genotyping experiment became more significant than that seen with imputation analysis due to the complementing of genotypes missing in the imputed data. Among the imputed SNPs, six other loci had a *P*-value of $<5.00E-06$ and were considered to have a suggested association with APS (Table 2). In summary, the imputation analysis revealed two additional regions with a significant or suggested association located near *C1D* (chromosome 2) and *NGF* (chromosome 1) besides the candidate regions found before imputation analysis.

HLA imputation analysis

In our imputation analysis, rs2395166 located near the *HLA-DRA* gene showed evidence suggestive of an association (OR = 0.39, 95% CI = 0.26–0.59, $P=3.33E-06$, under an allelic model). As the *HLA* region on chromosome 6 shows a high linkage disequilibrium and *HLA-DRA* is known to be a non-polymorphic gene, we further assessed the association of *HLA* alleles.[30] We performed *HLA* imputation analysis to clarify the association of several *HLA* alleles (*-A, -B, -DPB1, -DRB1, -DQB1*) with APS at 4-digit resolution.

We found that no *HLA*-allele showed a significant association with APS susceptibility after applying a Bonferroni correction by the allele count of each *HLA* gene (Supplementary Tables S3–S7). The

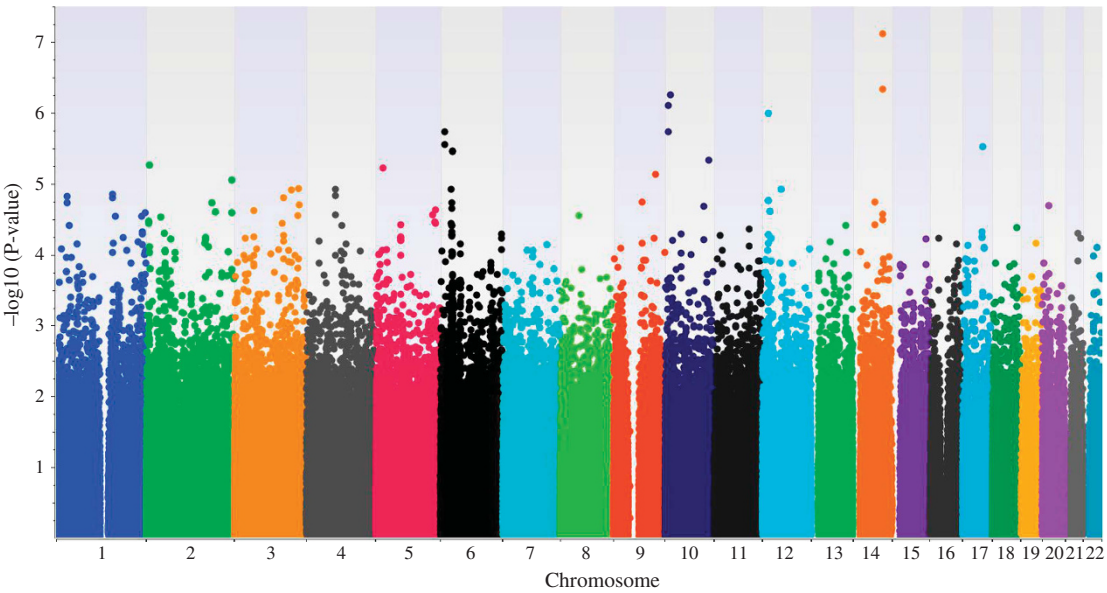

Figure 1 The minimum *P*-values under three genetic models (allelic, recessive and dominant) were obtained from 534 samples (115 Japanese APS cases and 419 Japanese healthy controls). The *y* axis shows –log10 *P*-values of 426,344 SNPs, and the *x* axis shows their chromosomal positions. The Bonferroni-corrected significance threshold is 1.17E-07.

minimum *P*-value was observed in the *HLA-DQB1*03:02* allele (OR = 1.98, 95% CI = 1.23–3.20, *P* = 5.10E-03, under a dominant model), but this allele showed a higher *P*-value than rs2395166. This result suggests that 4-digit *HLA* alleles cannot explain the association observed in the *HLA* region which exceeds that of SNP rs2395166.

DISCUSSION

In this first GWAS of obstetric APS, *TSHR* and *C1D* were found to be associated with this disorder, a finding supported by means of a previous GWAS and subsequent imputation analysis.

TSHR SNPs or variants are associated with Grave's disease, subclinical hypothyroidism and disturbances in serum levels of thyroid-stimulating hormone.[31,32] To our knowledge, there has been no report concerning an association between APS and *TSHR*. The prevalence of a *TSHR*-risk genotype in subjects who were homozygous for a risk allele of rs2288493 was 16.5% in patients and 3.1% in healthy controls in this study. Only limited information has been available on the relationship between thyroid hormone and obstetric APS; De Carvalho *et al.*[33] found that hypothyroidism was present in 22% of primary APS patients and thyroid autoantibodies were detected in 18% of them.

The C1D nuclear receptor corepressor (*C1D*) gene encodes a DNA-binding and apoptosis-inducing protein, and is also a Rac3-interacting protein that acts as a corepressor for the thyroid hormone receptor.[34] Both *TSHR* and *C1D*, susceptibility loci for APS, were associated with thyroid function. Thyroid function increases during pregnancy due to the presence of human chorionic gonadotropin and hyperestrogenemia.[35] TSHRs are widely expressed in granular cells, oocytes, endometrium, glandular epithelium and luminal epithelium during implantation. Both the endometrium and the trophoblast might be influenced by thyroid function either directly or through the action of implantation-mediating molecules. An impaired response to increased thyroid function might lead to implantation failure or miscarriage. Clinical and subclinical hypothyroidism is related to infertility and pregnancy loss. Thyroid autoantibodies are strongly associated with recurrent miscarriage and preterm delivery.[36]

C1D interacts with condensin which plays a major role in mitotic chromosome condensation, DNA repair and replication checkpoint control.[37] The DNA double-strand break repair protein, C1D, has roles in RNA processing and DNA damage repair.[38] C1D maintains genomic stability at highly transcribed gene loci by coordinating these processes through the timely recruitment of relevant regulatory factors. When the damage is beyond repair, C1D induces apoptosis in a p53-dependent manner. The prevalence of a *C1D*-risk allele in this study was 8.3% in patients and 1.4% in healthy controls. There has been no report concerning an association between *C1D* and reproduction. Quenby *et al.*[39] showed that aPLs directly prevent extravillous trophoblast differentiation in some early miscarriages. *C1D* might have a role in controlling trophoblast cell proliferation and migration, and dysregulated *C1D* might be a possible cause of pregnancy complications in APS.

Our results suggest that genetic polymorphisms in *TSHR* and *C1D* represent risk factors for obstetric APS, indicating an absolute need for accelerated research in this area. We found that the risk-allele homozygous genotype rs288493 tended to decrease the expression of the*TSHR*gene in whole blood in the GTEx database which registered expression quantitative trait (eQTL) loci in the European population, suggesting the functional effect of this SNP (Supplementary Figure S3).[40] The findings was consistent with the detection of a rs2288493 risk under the recessive model. Unfortunately, rs79154414 around *C1D* was not registered with the GTEx database probably due to the low frequency of this SNP in the European population. Further study is needed to evaluate the functional effect of rs79154414 especially in Asian individuals.

The *HLA* region included in eight regions suggested as associated with APS after imputation analysis. We performed *HLA* imputation analysis at 4-digit allele resolution but could not find the significant association of *HLA* alleles after applying a Bonferroni correction. The previous study in Caucasian populations suggested that *HLA-DQB1*03:01* was a risk factor for LA, especially phosphatidylserine-dependent antiprothrombin antibody and *HLA-DQB1*05:01* was present at a lower frequency in patients with

Table 2 Significantly or suggestively associated genes under three models in 115 APS patients and 419 controls after the SNP imputation analysis

Gene	Top SNP	Chr	Position (hg19)	Distance	Minor/Major [D/d]	Model	P in imputation	Data type (G/I)	Case (genotyped)				Control (genotyped)				Odds ratio (95% CI)	P after genotyping
									DD	Dd	dd	MAF	DD	Dd	dd	MAF		
TSHR	rs2288493	14	81 611 606	3′-UTR	[T/C]	Recessive	7.85E-08	G	19	41	55	0.34	13	178	228	0.24	6.18 (2.95–13.0)	7.85E-08
C1D	rs79154414	2	68 124 206	145kbp 3′	[T/C]	Allelic	4.53E-07	I	1	17	97	0.08	0	12	407	0.01	6.20 (2.96–13.0)	4.64E-08
NGF	rs145365907	1	115 958 423	78kbp 5′	[T/A]	Allelic	3.50E-06	I	0	14	101	0.06	0	9	410	0.01	5.97 (2.55–14.0)	3.50E-06
SYCP2L	rs2788869	6	10 918 513	Intron	[A/G]	Allelic	9.92E-07	I	1	30	84	0.14	0	40	379	0.05	3.22 (1.97–5.26)	9.74E-07
HLA-DRA	rs2395166	6	32 388 275	19kbp 5′	[C/T]	Allelic	4.47E-06	G	2	27	86	0.14	36	167	216	0.29	0.39 (0.26–0.59)	3.33E-06
GATA3	rs1020096	10	9 266 868	1.15mbp 3′	[A/G]	Recessive	7.53E-07	G	34	40	41	0.47	46	209	163	0.36	3.39 (2.05–5.62)	8.01E-07
FRMD4A	rs12570849	10	14 189 600	Intron	[C/T]	Allelic	5.68E-07	G	3	27	85	0.14	1	39	379	0.05	3.26 (2.01–5.28)	5.68E-07
PTPRO	rs2300291	12	15 735 682	Intron	[A/G]	Dominant	5.00E-07	I	4	28	83	0.16	29	192	198	0.30	0.35 (0.22–0.54)	2.13E-06

Abbreviations: CI, confidence interval; CID, C1D nuclear receptor corepressor; FRMD4A, FERM domain containing 4A; GATA3, GATA binding protein 3; HLA-DRA, major histocompatibility complex, class II, DR alpha; MAF, minor allele frequency; NG, nerve growth factor (beta polypeptide); PTPRO, protein tyrosine phosphatase, receptor type, O; *TSHR*, thyroid-stimulating hormone receptor; SYCP2L, synaptonemal complex protein 2-like. *P*-value of $\chi 2$ test from the two-by-two crosstable under the allelic, dominant or recessive model.
Data type: G: genotyped, I: imputd. Gene information from Refseq.

LA (7.1%) than in healthy controls (13.2%), although the association was not statistically significant.[41] We observed the risk of *DQB1*03:02* for APS (OR = 1.69, 95% CI = 1.09–2.61, OR = 0.019) but could not find the risk of *DQB1*03:01* in the present study (Supplementary Table S6). We could confirm the protective effect of *HLA-DQB1*05:01* in our Japanese data set (2.2% in obstetric APS cases and 7.5% in controls (OR = 0.28, 95% CI = 0.11–0.70, $P = 0.0065$)). Further interethnic studies could confirm our finding that the *HLA-DQB1* alleles was associated with APS.

Among the regions suggested in our GWAS, one SNP of the *GATA3* region appeared to be associated with APS (Table 2). *GATA3* is a master transcription factor for the differentiation of T helper 2 cells, and is important at earlier stages of hematopoietic and lymphoid-cell development.[42] T helper 2 cytokines inhibit a response by T helper 1 cytokines during implantation and T helper 2 works to keep an embryo viable as a semi-allograft and to maintain a normal pregnancy.[43] Homozygous mutant mouse embryos die between days 11 and 12 postcoitum and display massive internal bleeding, marked growth retardation, severe deformities of the brain and spinal cord, and gross aberrations in fetal liver hematopoiesis.[44]

One SNP of *STAT4* (rs10168266, OR = 1.90, 95% CI = 1.41–2.57, $P = 2.56E\text{-}05$, allelic model) was included in 161 SNPs with a *P*-value $< 1.00E\text{-}04$ (Supplementary Table S1). Among the SLE-related loci that showed an association ($P < 1.00E\text{-}04$) in the previous GWAS for SLE in Japanese subjects, *STAT4, TNFAIP3, HIP1* and *ETS1* showed an association in our APS samples ($P < 1.00E\text{-}03$) (Supplementary Table S8). In contrast, *TNFSF4, PRDM1, IKZF1, IRF5* and *ELF1* had a *P*-value of more than 0.05, suggesting shared and unique genetic risk factors between APS and SLE. *TSHR* and *C1D* have not been registered with the INSIDEGEN-LUPUS database, suggesting that these genes are unique to APS.[45] Only 43.5% of the patients in the present study had SLE as a complication. Further study is necessary to confirm that they are susceptibility genes shared with SLE.[46–48]

A significant association with rs2288493 in the *TSHR* region was found only under the recessive model and rs2288493 showed a weak association under the allelic model ($P = 2.32E\text{-}03$) or additive model ($P = 1.77E\text{-}03$). Given the receptor function controlled by the *TSHR* gene, it is speculated that there might be a signaling threshold under TSHR to maintain pregnancy and that the risk-allele homozygous genotype of rs2288493 drops the TSHR signal below the threshold. In this study, the R^2 value between the *P*-values under the allelic model and those under the dominant model was 0.7138, whereas that between the *P*-values under the allelic model and those under the recessive model was 0.2507, indicating low correlation coefficiency of the results under the recessive model compared with those under the allelic model (Supplementary Figure S4). SNPs with a *P*-value $< 1.00E\text{-}05$ under the recessive model showed *P*-value more than 1.00E-03 under the allelic model. These results indicate that analysis by means of a recessive model was effective in this study for detecting genetic factors that are hidden under an allelic model.

Another significant association with rs79154414 around the *C1D* region was found only after imputation analysis and the lowest *P*-value in the upper and lower 500kbp region before imputation analysis was 4.69E-04 from rs1503243 under the allelic model. As the association of rs79154414 was confirmed by our validation experiment, this supports the effectiveness of imputation analysis to detect genetic factors that are not covered by SNP arrays.

In the present study, not only *TSHR* and *C1D* but also other genes suggestive of APS have supplied us with clues to account for the unexplained mechanisms in early miscarriage, fetal loss, preeclampsia and many features of this disease. One limitation of our study is the

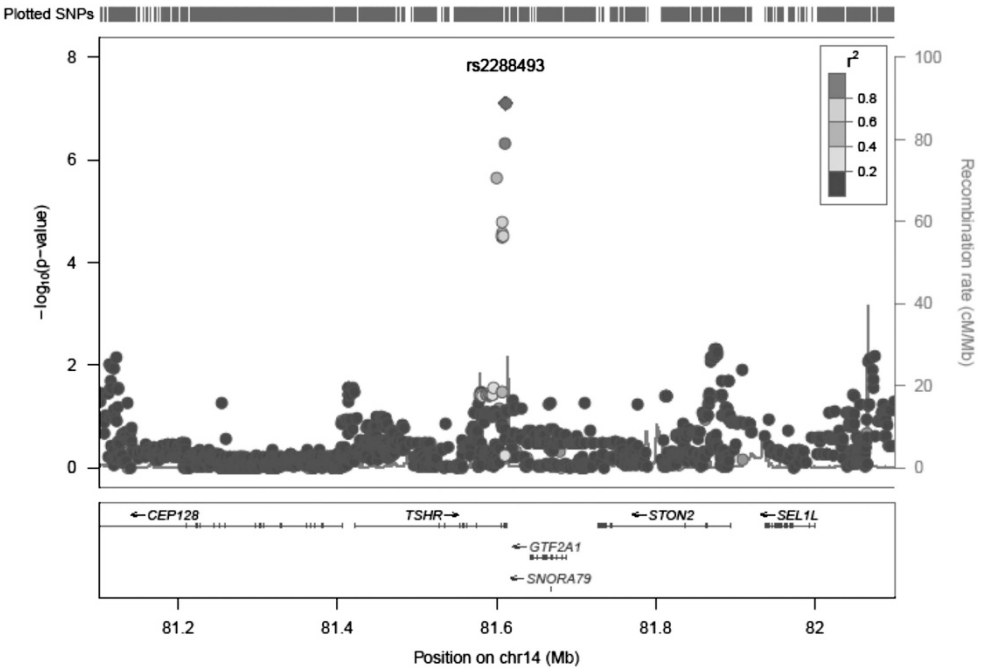

Figure 2 rs2288493 ($P=7.85E-08$) is located in the 3′-UTR of the *TSHR* (thyroid-stimulating hormone receptor) gene. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

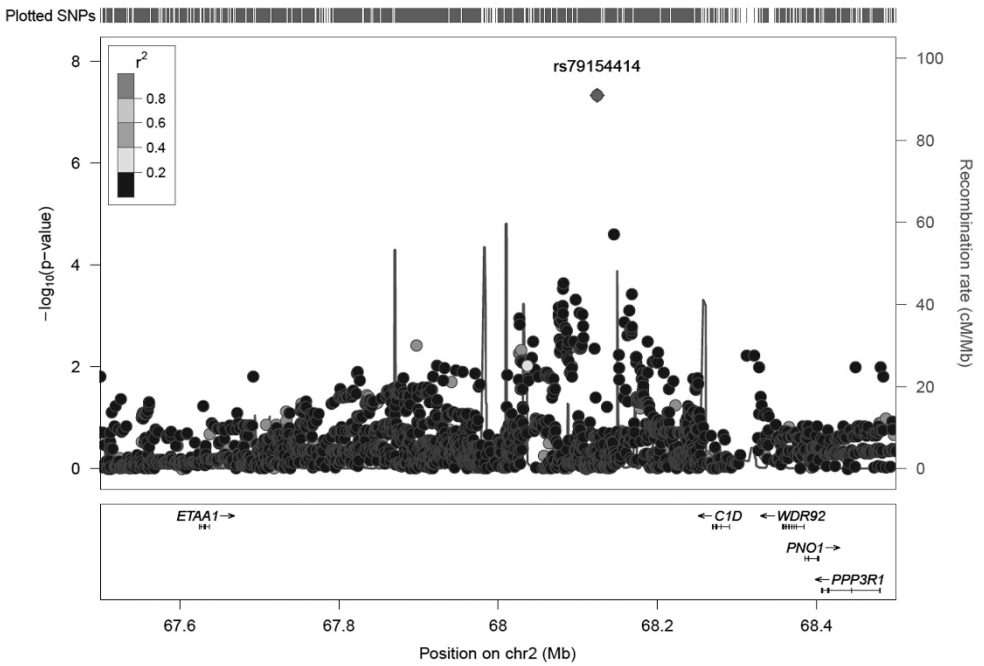

Figure 3 rs79154414 ($P=4.64E-08$) is located at 145kbp 3′ of the *C1D* (C1D nuclear receptor corepressor) gene. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

small sample size due to the rarity of this disease. We could not replicate the associations of *TSHR* and *C1D* in an independent Japanese data set. The prevalence of RPL was 4.2% in our previous study. The prevalence of APS is 1–2% according to criteria of the International Congress on APS in patients with RPL. Thus, the prevalence of APS is speculated to be 4–8/10 000 in women who have been pregnant. However, we focused on cases that were strongly positive for LA which is associated with a poor pregnancy prognosis. The prevalence of APS is not rare but in patients with a strongly positive LA it is extremely rare.

Another limitation of our analysis was the increased load of multiple testing corrections through considering multiple genetic models. We detected several SNPs with suggestive evidence under the recessive or dominant model; however, application of a multiple mode of testing can increase the risk of detecting false-positive associations. If we set the strict genome-wide significance threshold

considering the number of tested genetic models, no SNP passed the threshold of 1.66E-08 (5.00E-08/3). Thus, further interethnic replication studies in a world-wide research collaboration will be necessary to confirm our finding and to reveal the biological significance of the genes implicated in our GWAS for obstetric APS. Identification of genetic risk factors for obstetric APS will ultimately result in specific treatment to improve the pregnancy outcome of the affected patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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