

Association of genetic variants of *PD1* with recurrent pregnancy loss

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Abstract

Purpose: Programmed cell death 1 (*PD1*) and PD ligand 1 (*PDL1*) are speculated to have an important role in maintaining a normal pregnancy and there are also a few reports of an association between the single-nucleotide polymorphisms (SNPs) of *PD1* or *PDL1* and diseases in humans. The association was examined between the polymorphisms of *PD1*, *PDL1*, *CTLA4* and recurrent pregnancy loss (RPL).

Methods: The study group consisted of 243 women with two or more pregnancy losses and 176 parous women. The frequency of three SNPs of *PD1*, two of *PDL1*, and four of *CTLA4* were compared between the patients and controls. Next, the subsequent live birth rates in the patients with RPL with and without risk alleles were examined.

Results: Two SNPs (rs36084323 and rs3481962) of *PD1* were found to occur at significantly higher frequencies in the patient group than in the control group. The subsequent live birth rate of the patients with vs. without the risk alleles of rs36084323 and rs3481962 were 83.3% vs. 83.3% and 81.6% vs. 84.0%, respectively.

Conclusion: Variations of the *PD1* gene were identified as risk factors for RPL. However, the presence or absence of the *PD1* risk alleles had no predictive effect on the subsequent pregnancy outcome.

KEYWORDS

CTLA4, *PD1*, *PDL1*, recurrent pregnancy loss, single-nucleotide polymorphism

1 | INTRODUCTION

Established causes of recurrent pregnancy loss (RPL) include antiphospholipid syndrome (APS), uterine anomalies, and abnormal parental and embryonic chromosomes.¹⁻⁵ In over one-half of the cases, the cause remains unexplained, even after conventional examinations have been conducted to identify the cause.⁵ The authors found that an abnormal embryonic karyotype was the most frequent cause of RPL, accounting for as high as 41% of the cases and that the prevalence of true RPL without an identifiable cause is around 25%.⁴

Many factors have been proposed to be involved in fetomaternal tolerance, including particular major histocompatibility class I molecules, hormones, complement regulatory proteins, several immunoregulatory molecules (indolamine 2,3-dioxygenase, Fas and Fas ligand), regulatory T (Treg) cells, interleukin (IL)-10, regulatory macrophages, and growth factors that are expressed at the placental-decidual interface.⁶ The fetus is a semi-allograft for the maternal host and the maternal immune system is activated by fetal antigens at the fetomaternal interface. In the early stage of pregnancy, maternal tolerance of the fetus is vital for the pregnancy to continue.

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However, the exact mechanisms by which the maternal immune system shows tolerance to a semi-allogeneic fetus without immunological rejection remains poorly understood.

It is well known that maternal Treg cells suppress an aggressive allogeneic response that is directed against the fetus and that the early human decidua contains an abundance of CD4⁺CD25^{bright}T cells, which show high expression levels of cytotoxic T lymphocyte-associated antigen 4 (CTLA4).^{7,8} Several studies have reported an association between the SNPs of *CTLA4* and RPL.^{9–11} According to one study, a decreased number of Treg cells was present in cases of pre-eclampsia, which appeared to break the maternal tolerance to the fetus.¹²

However, programmed cell death ligand 1 (PDL1) has been identified as a new member of the B7 family¹³ and is a ligand for programmed cell death 1 (PD1), which is expressed on activated T and B cells.¹⁴ Engagement between PD1 with PDL1 typically generates an inhibitory signal, which results in the suppression of T cell activation. The interaction between PD1 and PDL1 also has been found to negatively costimulate and attenuate the activities of auto-reactive T cells through the inhibition of cytokine production and cell cycle arrest in the G₀/G₁ phase.¹⁵ A deficiency in *PD1* gene expression has been demonstrated in vitro to result in inadequate auto-reactive lymphocyte removal and auto-antibody production.¹⁶ These findings represent evidence that suggest that the PD1–PDL1 pathway is pivotal for maintaining peripheral self-tolerance and preventing autoimmune diseases. These findings were further investigated in human autoimmune diseases and SNPs on the human *PD1* gene were found to be linked with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and type I diabetes.¹⁷ The haplotypes of *PD1* have been shown to be associated with RA,^{18,19} SLE,^{20,21} and subacute sclerosing panencephalitis (SSPE) in the Japanese population.²²

PDL1 is expressed abundantly in the placenta, syncytiotrophoblast, and extravillous cytotrophoblast, both of which lie in direct contact with the maternal blood and tissue.²³ PDL1 also has been shown to be expressed on human decidual stromal cells and to inhibit the downstream effects of human leukocyte antigen–antigen D-related interactions.²⁴ Thus, PD1 and PDL1 are speculated to have an important role in maintaining a normal pregnancy. There are few reports of an association between the SNPs of *PD1* or *PDL1* and miscarriage.

In this study was examined the association with RPL of three SNPs of the *PD1* gene, two SNPs of the *PDL1* gene, and four SNPs of the *CTLA4* gene by a univariate analysis in a case-control study of Japanese women. Those SNPs that have been reported to date as showing an association with some diseases were chosen.^{18–22} As data on the combined effect of maternal age and genetic risk factors are still lacking, a cohort study also was conducted in order to examine, in relation to several factors, whether a subsequent pregnancy would result in further loss or a normal delivery. Furthermore, the expression levels of PDL1 in the decidual cells in cases of miscarriage were evaluated by immunohistochemistry and the association between *PD1* SNPs and the expression level of the *PD1* gene by expression quantitative trait locus (eQTL) analysis. This is the first

study to investigate *PD1* gene polymorphism as a molecular marker of RPL.

2 | MATERIALS AND METHODS

2.1 | Patients and controls

The data of 243 Japanese women with a history of unexplained RPL (defined as a history of two or more pregnancy losses) who were recruited from Nagoya City University Hospital between June, 2007 and November, 2012, were examined. All the patients underwent systematic examinations, including a hysterosalpingography, chromosome analysis of both partners, determination of the antiphospholipid antibodies, including lupus anticoagulant, by the diluted partial thromboplastin time, diluted Russell viper venom time, and β 2 glycoprotein I-dependent anticardiolipin antibody,²⁵ and blood tests for hypothyroidism and diabetes mellitus, before the subsequent pregnancy. Those patients with APS, uterine anomalies, abnormal chromosomes in either partner, hypothyroidism, and diabetes mellitus were excluded from the analysis.²⁶ Patients with a history of pre-eclampsia or abruptio placentae also were excluded.

The subsequent pregnancies of all the patients were followed up until February, 2013. The gestational age was calculated from the basal body temperature charts. Ultrasonography was performed once per week from 4 weeks to 8 weeks of gestation. A dilatation and curettage was performed on the patients who were diagnosed as having experienced a miscarriage. A portion of the villi was cultured and the cells were harvested after 6–22 days of cultivation for chromosome analysis. A total of 49 aborted conceptuses could be karyotyped by using the standard G-banding technique.

Furthermore, 176 women with at least one child and no history of infertility or miscarriage were examined as the controls. The controls were recruited from Nagoya City University Hospital between January and April, 2012 and they had no history of pre-eclampsia and abruptio placentae.

The genotypic frequencies of the three SNPs of the *PD1* gene, two SNPs of the *PDL1* gene, and four SNPs of the *CTLA4* gene were compared between the patients and controls. The subsequent pregnancy outcomes were compared between the women with and without the risk alleles among the 243 patients.

This study was conducted with the approval of the Research Ethics Committee of Nagoya City University Graduate School of Medical Sciences. Each patient provided written consent after being provided with a full explanation about the purpose and methods of the study.

2.2 | DNA analysis

Genomic DNA was extracted from peripheral blood samples by using the Midi Blood DNA Extraction kit (Qiagen, Tokyo, Japan). In all, nine SNPs were analyzed: three SNPs of *PD1* were rs36084323 (–606G > A at promoter), rs35933396 (+91C > T at intron 1), and rs34819629 (+6371G > A at intron 2), four SNPs of *CTLA4* were

TABLE 1 Patient's background

Characteristic	Median (range)		P-value ^a
	Patients	Controls	
Age (years)	34 (21-45)	35 (18-61)	.057
Gravidity	3 (2-9)	2 (1-6)	<.001
Miscarriage	3 (2-9)	0 (0)	<.001
Parity	0 (0-2)	2 (1-4)	<.001

^aMann-Whitney *U*-test.

rs231775 (+49A > G at exon 1), rs733618 (-1722T > C at promoter), rs3087243 (+6254G > A at 3'UTR), and rs231779 (+1822C > T at intron 1), while two SNPs of *PDL1* were rs822342 (-2141T > C at intron 1) and rs2297137 (+9619G > A at intron 5). All the genotyping was carried out by using polymerase chain reaction (PCR) assays (TaqMan; Applied Biosystems, Warrington, UK) in 96-well arrays that included two blank wells as negative controls, according to the manufacturer's instructions. TaqMan Pre-Designed SNP Genotyping assay and TaqMan minor groove binder probes were used. The TaqMan PCR and genotyping analyses were carried out on the Applied Biosystems 7500 Fast Real-Time PCR System. The reaction mixtures were amplified in 1 μ L of template DNA (10 ng/ μ L), 12.5 μ L of 2X TaqMan Universal Master Mix, .625 μ L of 20X primer/probe mix, and 10.875 μ L of double-distilled water in a volume of the mixture of 25 μ L. The cycling conditions were as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, and 58°C for 1 minute. The results were automatically analyzed on the Applied Biosystems 7500 Real Time PCR System by using an allelic discrimination assay program.²⁷

2.3 | Immunohistochemistry

In order to evaluate the expression levels of *PDL1* in the decidual cells of the patients with RPL, the products of conception (decidua and villi) were tested by immunohistochemistry in the 22 cases with miscarriage with a normal embryonic karyotype in the subsequent pregnancy, using a rabbit polyclonal antibody that is directed against *PDL1* (Abcam). Immunostaining was performed by $\times 100$ diluted citric acid buffer activation. Then, the absorbance values of the immunostained decidual stromal cells were measured to estimate if there were any differences between cases with and without the risk alleles. First measured was the absorbance of the *PDL1*-stained part of the decidual stromal cells, the interstitial non-stained part, and the tissue-free part (as the blank). Next was subtracted the absorbance value of the blank part from those of the *PDL1*-stained part and the interstitial-unstained part. Using the absorbance value that had been corrected in this manner, the ratio (the absorbance at the *PDL1*-stained part)/(the absorbance at the non-stained part) was determined. This ratio was compared between those with and those without the risk allele of *PD1*. An absorbance analysis was carried out by microscopy (BZ-9000; Keyence Company, Osaka, Japan).

2.4 | Statistical analysis

Departure from the Hardy-Weinberg equilibrium (HWE) for the nine SNPs was tested by using an exact test.²⁸ As previous studies have shown that the mainland Japanese population is genetically similar, the population substructure in this sample was not examined or corrected.^{29,30}

For individual SNP association analyses, a univariate logistic regression analysis was performed with dominant, recessive, and log-additive models, using RPL as the dependent variable. In order to avoid multiple comparisons by fitting three genetic models and determining the best-fitting model among them, the max-statistic approach was used.³¹

To characterize the linkage disequilibrium (LD) pattern, the r^2 -values for all pairs of SNPs were estimated. Then, the haplotype frequencies were estimated by using the expectation-maximization algorithm.³¹ In order to evaluate the association between the haplotypes and the risk of pregnancy loss, logistic regression models were used. The haplotype analyses were conducted, based on the best-supported model in the max-statistic analysis.

The logistic regression analyses were performed in the cohort study to examine the association of the haplotypes of each gene with the subsequent miscarriage by using age as a covariate. These statistical analyses were conducted using R software,³² including the SNPassoc³³ and haplo.stat³⁴ packages.

Furthermore, the differences in the expression levels of the *PD1* and *PDL1* genes were analyzed in the endometrium of the luteal phase between the patients with RPL and the controls by Wilcoxon's rank sum test using a public database, GSE65099,³⁵ which includes the data of endometrial biopsies from 10 infertile patients and 10 patients with RPL. The biopsies were timed between six and 10 days after the pre-ovulatory luteinizing hormone surge. The causes of infertility were male factors, tubal disease, endometriosis, polycystic ovary syndrome, and unexplained. The downloaded data of the transcript per million were used for the statistical examination.

An eQTL analysis was performed by using the public database, E-MATB-264,³⁶ for the expression data of *PD1* and the 1000-genomes JPT (72 Japanese) for the data on SNPs. The associations between the SNPs and the expression of *PD1* in the lymphoblastoid cell lines was investigated by a linear regression analysis. The log₂ normalized value was used as an expression level. The recessive model of the SNP was assessed. A statistical analysis of the results of the eQTL was conducted by using PLINK (v.1.07;).

3 | RESULTS

The medians of the age (range) of the patients with RPL and the controls were 34 (21-45) and 35 (18-61) years, respectively. The medians of previous miscarriages were 3 (2-9) and 0, respectively. The medians of gravida were 3 (2-9) and 2 (1-6), respectively. The medians of previous live births were 0 (0-2) and 2 (1-4), respectively (Table 1).

TABLE 2 Results of the analysis of the associations of individual single-nucleotide polymorphisms (SNPs), based on the max-statistic approach

SNP	Allele ^a	MAF ^b	HWE ^c	Genetic models			P-value
				Dominant	Recessive	Log-additive	
PD1 rs36084323	G/A	0.49	0.92	0.01	5.51	2.10	.042
PD1 rs35933396	C/T	0.50	0.85	4.79	0.01	1.94	.062
PD1 rs34819629	G/A	0.48	0.70	0.52	5.78	3.48	.036
CTLA4 rs231775	G/A	0.36	0.09	0.10	0.08	0.01	.934
CTLA4 rs733618	T/C	0.42	0.62	0.02	0.82	0.34	.595
CTLA4 rs3087243	G/A	0.24	0.18	0.57	0.01	0.32	.695
CTLA4 rs231779	C/T	0.36	0.06	0.33	0.00	0.16	.807
PDL1 rs822342	T/C	0.43	0.43	0.83	0.02	0.47	.591
PDL1 rs2297137	G/A	0.49	0.77	0.00	0.11	0.04	.932

The bold type represents the max-statistic among the three genetic models in each SNP.

^aAlleles on the right side are minor alleles.

^bMinor allele frequency.

^cP-values of the exact test for the Hardy–Weinberg equilibrium.

The genotypic frequencies of all 9 SNPs were found to be in the HWE ($P > .05$). A univariate logistic regression analysis was conducted for each SNP by using the max-statistic and it was found that 2 SNPs in *PD1*; namely, rs36084323 and rs34819629, were significantly associated with RPL and the best-fitting genetic models were the recessive models for both SNPs ($P = .042$, odds ratio [OR] = 1.75 and $P = .036$, OR = 1.78, based on the recessive models for rs36084323 and rs34819629, respectively; Table 2).

Next was estimated the LD among all three SNPs of *PD1* and there was found to be a strong LD among the three SNPs (Figure 1). Then, a univariate logistic regression analysis was conducted in order to examine the association of the haplotypes of *PD1* with RPL and there was found to be a significant association between haplotype “A-T-A” and RPL, based on the recessive model that was inferred from the max-statistic test described above ($P = .014$; Table 3). No significant difference in the haplotypes of *CTLA4* or *PDL1* were found between the patients with RPL and the controls (data not shown).

Of the 204 patients with RPL who became pregnant during the follow-up, 170 patients had live births, while the remaining 34 patients again had a miscarriage (note that 12 cases of biochemical pregnancy and 27 cases with an abnormal karyotype were excluded from the 73 miscarried products) (Table 4). For the two SNPs of *PD1* that showed significant differences in the case-control study, the subsequent live birth rate was calculated with or without the risk allele (Figure 2). Excluding miscarriage cases with an abnormal embryonic karyotype and chemical pregnancy, the subsequent live birth rate due to the presence or absence of the risk allele was equal to 83.3% in rs36084323 and 81.6% and 84.0% in rs34819629, respectively. A logistic regression analysis was conducted in this cohort to examine the association between the subsequent miscarriage and the haplotypes of *PD1*, using age as a covariate, and no significant association of any haplotype of *PD1* was associated with

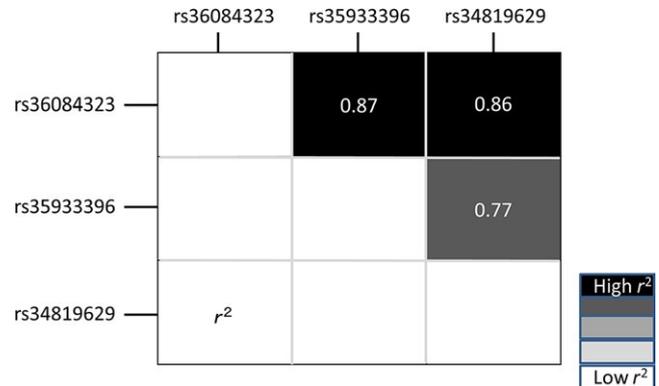


FIGURE 1 Linkage disequilibrium of the three *PD1* single-nucleotide polymorphisms (SNPs). Pairwise linkage disequilibrium r^2 values (white to black scales, low to high) for the three studied SNPs of *PD1*. The rs36084323 polymorphisms and rs35933396 or rs34819629 showed a high linkage disequilibrium ($r^2 > .8$)

the subsequent miscarriage, based on the recessive model (Table 3). A haplotype analysis of the two other genes; namely, *CTLA4* and *PDL1*, was not conducted for the risk of RPL or miscarriage in a subsequent pregnancy because no significant association with any SNPs of these genes was found in the individual SNP analyses using the max-statistic test.

Although no significant difference in the *PD1* gene expression levels in the endometrium of the luteal phase was found between the controls (10 infertile patients) and the patients with RPL, significant differences between the two groups were found in the *PDL1* gene expression levels in the Wilcoxon's rank sum test using GSE65099 ($P = .03$; Figure 3).

In the eQTL analysis of *PD1*, no significant association was found between the expression levels of *PD1* in the lymphoblastoid cell lines and rs36084323 or rs34819629 ($P = .88$ and $P = .55$, respectively).

TABLE 3 Results of the haplotype analysis, based on the best-supported (recessive) model in the max-statistic analysis

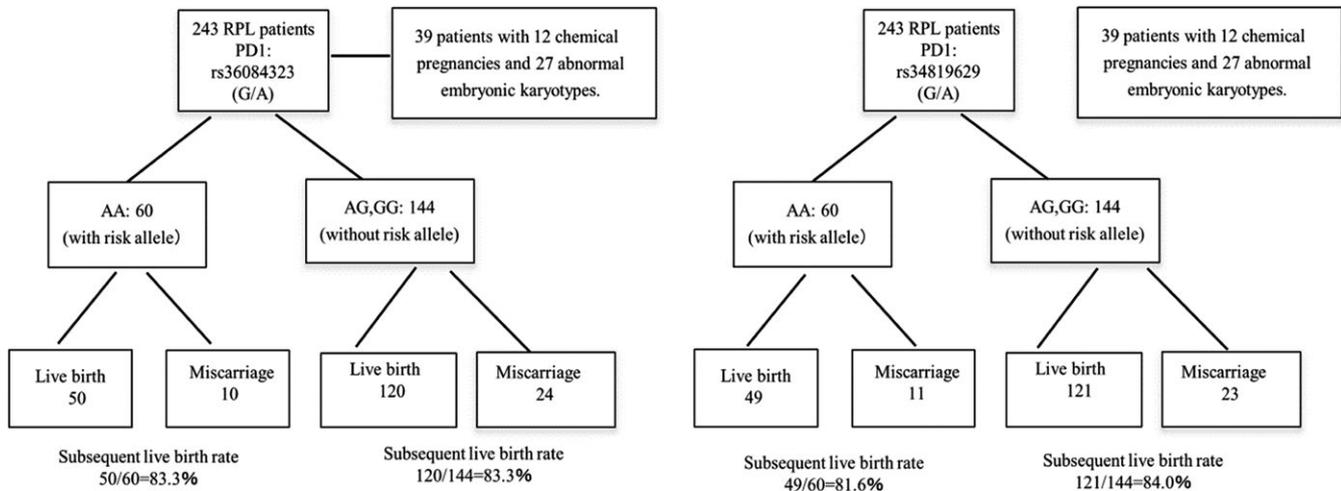
Variable	rs36084323	rs35933396	rs34819629	Haplotype frequency	P-value
RPL vs. control	A	T	A	0.457	.014
	G	C	G	0.495	
Miscarriage vs. live birth on subsequent pregnancy in patients with RPL	A	T	A	0.483	.944
	G	C	G	0.473	

RPL, recurrent pregnancy loss.

Haplotypes occurring at a frequency of >5% are shown.

TABLE 4 Subsequent live birth rate for every genotype

Genotype	rs36084323		rs34819629	
	AA	GG, AG	AA	GG, AG
Subsequent live birth rate in 243 patients	73.5% (50/68)	68.6% (120/175)	73.1% (49/67)	68.8% (121/176)
Live birth rate after excluding miscarriage cases with an abnormal embryonic karyotype or chemical pregnancy (n = 204)	83.3% (50/60)	83.3% (120/144)	81.6% (49/60)	84.0% (121/144)

**FIGURE 2** Analysis of 204 patients, excluding 39 cases, of which 12 cases were of chemical pregnancy and 27 cases were of an abnormal embryonic karyotype. For the two single-nucleotide polymorphisms of *PD1* that showed significant differences in the case control study, the subsequent pregnancy outcome in the patient group was examined as a cohort study. Except for the cases where the result of the pregnancy was miscarriage due to a fetal abnormal karyotype or biochemical pregnancy, the subsequent live birth rate was compared by grouping with or without risk allele. RPL, recurrent pregnancy loss

The immunohistochemistry showed the presence of PDL1 in the decidual stromal cells in cases of miscarriage (Figure 4). There were six cases with risk alleles and 14 cases without risk alleles and two cases were excluded because of the poor quality of the specimens. The absorbance values in the cases with and without risk alleles were similar (16.0 ± 7.95 vs 17.6 ± 6.6 , $P = .637$).

4 | DISCUSSION

This case-control study demonstrated a significantly higher frequency of the A/A genotype of rs36084323 (-606G>A) and

rs34819629 (+6371G>A) of the *PD1* gene in the RPL group, as compared to the controls, for the first time. Furthermore, the use of the max-statistic revealed the new finding that the inheritance models of these SNPs of *PD1* were represented by the recessive model.

It has been observed that Treg cells are essential for promoting fetal survival, as they allow the recognition of the paternal semi-allogeneic tissues by the maternal immune system to be avoided. Several functional studies have shown that miscarriage is often associated with a decrease of the Treg cell number or function, while in a normal pregnancy, the accumulation of maternal forkhead-box-P3⁺ (FoxP3⁺) CD4⁺ Treg cells with fetal specificity is selectively stimulated.³⁷ The number of FoxP3⁺ CD4⁺ Treg cells in the decidua basalis

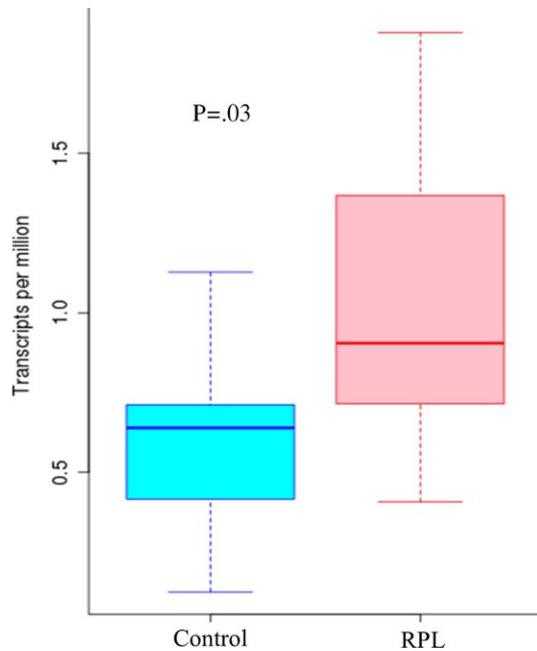
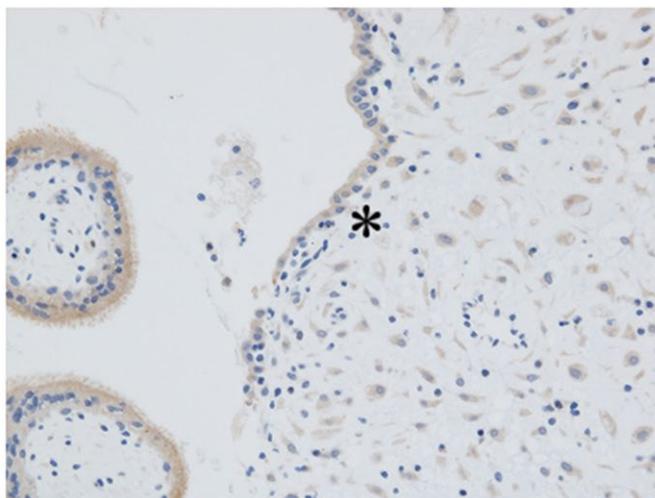


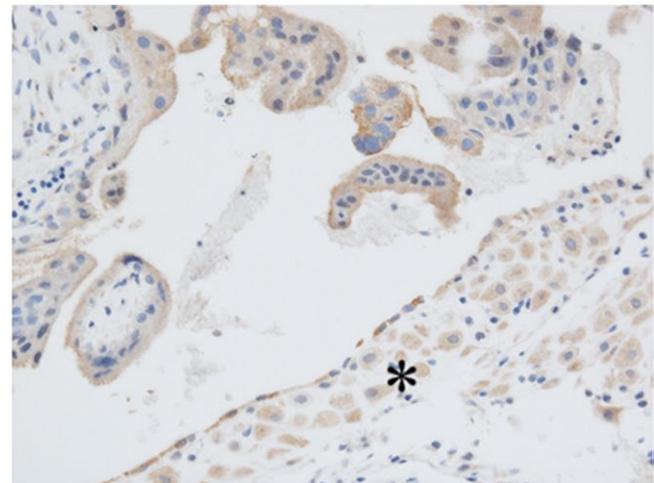
FIGURE 3 The *PDL1* gene expression in the controls and the patients with recurrent pregnancy loss (RPL). Differences in the expression levels of the *PDL1* gene in the endometrium of the luteal phase between patients with RPL and the controls (infertile patients) were analyzed by Wilcoxon's rank sum test, using the public database, GSE65099. Significant differences in the *PDL1* gene expression levels in the endometrium were found between the controls and the patients with RPL

in cases of miscarriage with a normal embryonic karyotype was significantly lower than that in normally progressing pregnancies and fetuses with an abnormal embryonic karyotype.³⁸

PD1 is a negative regulator of the Treg cell-mediated immune response. Inhibition of IL-2 production by the PD1/PDL1 pathway causes immune tolerance and the SNPs of *PD1* have been shown to be related to the susceptibility to some diseases. In SLE, the A allele of rs11568821 (7146G/A) was significantly less frequent in Spanish female patients²⁰ and the C allele of rs2227981 (7785C/T) was significantly higher in Indian SLE patients and Malay controls.²¹ In regard to RA susceptibility, the A allele of rs36084323 was associated with a decreased risk of developing RA.¹⁸ In the present study, in contrast to the aforementioned effect on RA susceptibility, the A alleles of rs36084323 and rs34819629 were identified as risk factors for RPL. One of the limitations of the present study is that functional analyses of the SNPs were not performed. Instead, an eQTL analysis was performed. It could not show any significant association between the two SNPs under study and the expression levels of *PD1* in the lymphoblastoid cell lines. As the control system for gene expression varies according to the organ and sample, the possibility that these SNPs represented the eQTL of *PD1* could not be ruled out entirely. Actually, a previous study on SSPE suggested that the promoter activity was significantly lower in the construct with the *PD1* -606A allele than in that with the -606G allele.²² The patients with RA who were carrying the GG genotype of rs36084323 exhibited increased messenger (m)RNA expression levels of PD1, as compared to those with the AA genotype.¹⁹ These data suggest that the SNP at rs36084323 might cause functional alterations of *PD1* and also reduce the expression levels of this gene. Combined targeting of the PD1 and T-cell immunoglobulin mucin-3 (Tim-3) pathways results in a decreased production of Th2-type cytokines by the decidual CD4+ T cells and increased fetal resorption in murine models of a normal pregnancy.³⁹ It is conceivable that a lower promoter activity might



genotype AG
(rs36084323 and rs34819629)
*decidua



genotype AA
(rs36084323 and rs34819629)
*decidua

FIGURE 4 Immunohistochemistry of the deciduas. The products of conception (decidua and villi) were examined by immunohistochemistry in 22 cases of miscarriage with a normal embryonic karyotype of the subsequent pregnancy by using the rabbit polyclonal antibody that is directed against PDL1. The results revealed the existence of PDL1 in the decidual cells in these cases

result in lower expression levels of *PD1*, which could be associated with dysfunction of the feto-maternal tolerance.

It has been suggested that measurement of the peripheral blood Treg cell count could be useful to predict the risk of miscarriage in newly pregnant women, as the number of circulating Treg cells was higher in patients with a successful ongoing pregnancy than in patients who miscarried in the first trimester of pregnancy.⁴⁰ Furthermore, decreased numbers of Treg cells in the decidua were reported to be correlated with waning levels of Treg cells in the peripheral blood.⁴¹ Another study reported that the Tim-3⁺ PD1⁺ CD8⁺ T cells in the decidua greatly outnumbered those in the peripheral blood during human early pregnancy and that the numbers and functions of the Tim-3 PD1⁺ CD8⁺ T cells in the decidua were significantly reduced in cases of miscarriage.⁴²

In one reported study, while the expression level of *PDL1* mRNA was decreased in the decidua in cases of recurrent miscarriage, no significant difference was found in the expression level of *PD1* mRNA.⁴³ In this study, the immunohistochemistry revealed the presence of *PDL1* in the decidual stromal cells in cases of miscarriage and a differential expression analysis using public data showed that the expression level of *PDL1* in the endometrium in the luteal period was significantly higher in the patients with RPL than in the infertile patients. It was not clear in this study whether the expression level of *PDL1* in the endometrium of the luteal phase was higher in the patients with RPL than in the normal controls. Furthermore, this study did not test the products of a normal pregnancy; thus, the absorbance between the cases with RPL and the controls could not be compared. There was also no denying the possibility that the interstitial cells contain extravillous trophoblasts. Further studies are required to clarify the expression levels of *PDL1* in the decidua.

No significant difference was detected in the frequency of the SNPs of *CTLA4* and *PDL1*, although recent reports have shown associations between the SNPs of *CTLA4* (rs231775) and with RPL.⁹⁻¹¹ The max-statistic was carried out in order to detect the heredity model of four SNPs of *CTLA4*, whereas previous studies used only the allelic model to examine the frequency of the SNPs. The current results revealed no significant difference among the four models (dominant, codominant, recessive, log-additive). As there were racial differences among the polymorphisms, there was a possibility that this might be one of the reasons why this study did not show an association between *CTLA4* and RPL.

In the present study, two SNPs of the *PD1* gene were identified as risk factors for RPL for the first time and it was demonstrated that the "A-T-A" haplotype occurred at a significantly higher frequency in the patients with RPL than in the controls. However, the subsequent live birth rates were found to be similar in the cases with and without both risk alleles after the exclusion of the cases with an abnormal embryonic karyotype and chemical pregnancy. The SNPs of >120 genes have been reported to be associated with RPL. The genome-wide association study proved that the effect of the one of many kinds of SNPs that are associated with a common disease is very small when the OR is relatively small.⁴⁴ The adequate selection of patients, based on an analysis of several kinds of SNPs,

might improve therapeutic effectiveness. Further study is necessary in order to examine the mechanism underlying the influence of the variations of *PD1* on RPL.

DISCLOSURES

Conflict of interest: The authors declare no conflict of interest.
Human Rights Statement and Informed Consent: This study has been approved by a suitably constituted Ethics Committee of the institution.
Animal studies: This article does not contain any study with animal participants that have been performed by any of the authors.

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