Correlation between CTLA4 genetic polymorphisms, its serum protein level and the susceptibility to recurrent spontaneous abortion: A case–control study

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Abstract

Present study aimed to detect the influence of cytotoxic T-lymphocyte associated protein 4 (CTLA4) gene polymorphisms for the risk of recurrent spontaneous abortion (RSA), as well as the serum level of CTLA4 protein in RSA patients.

One hundred thirty-three RSA patients and 146 healthy persons were recruited in this case–control study. PCR-RFLP was used to genotype the CTLA4 gene polymorphisms both in case and control groups. Serum level of CTLA4 was detected by ELISA kit. Quantitative variables were compared by t test or Mann–Whitney U test between groups. Qualitative variables were evaluated by χ² test or Fisher exact test. Association strength was expressed by odds ratios (ORs) and 95% confidence intervals (95% CIs).

G allele of rs4553806 (P = 0.027, OR = 0.570, 95% CI = 0.345–0.942) and T allele of rs5742909 (P = 0.027, OR = 0.570, 95% CI = 0.345–0.942) were distinctly associated with reduced susceptibility of RSA. Distinctly negative association has been discovered between rs231775 AA genotype and RSA susceptibility (P = 0.040, OR = 0.427, 95% CI = 0.188–0.979). CTLA4 protein had significantly higher serum level in RSA patients than in healthy controls (P = 0.028). In RSA patients, AA genotype carriers had higher CTLA4 serum level than that GG genotype carriers (17.83 ± 6.35 ng/mL vs 10.41 ± 7.28 ng/mL, P = 0.039).

Minor alleles of CTLA4 polymorphisms might inhibit the RSA susceptibility via upregulated the protein expression level.

Abbreviations: 95% CIs = 95% confidence intervals, CTLA4 = cytotoxic T-lymphocyte associated protein 4, ELISA = enzyme-linked immunosorbent assay, HWE = Hardy–Weinberg equilibrium, IRM = idiopathic recurrent miscarriages, ORs = odds ratios, RPL = recurrent pregnancy loss, RSA = recurrent spontaneous abortion, SNP = single nucleotide polymorphism.

Keywords: CTLA4, polymorphisms, recurrent spontaneous abortion

1. Introduction

Recurrent spontaneous abortion (RSA), also known as recurrent pregnancy loss (RPL), is a complication of pregnancy. It is defined as ≥3 or more than ≥3 times consecutive pregnancy loss which occurred before 20 weeks of gestation with unexplained reason.[1] Morbidity of RSA was not very high in total pregnancies.[2] With the development of RSA, it will lead to complications which could threaten the life.[3] RSA might reduce the life quality of patients and bring higher mental and economic burden for the family.[3] Most part of RSA had no definite reason.

But various factors have been found to be associated with RSA development, such as immunologic, endocrine factors, infections, and genetic factors.[4–6] Normal embryo is not rejected by mater due to the immune tolerance, disorders on the immune tolerance at maternal–fetal interface will lead to the occurrence of RSA.[7] During recent years, many studies found that immune factors play a crucial role in RSA development.[8]

Cytotoxic T-lymphocyte associated protein 4 (CTLA4), also known as cluster of differentiation 152 (CD152), belongs to the immunoglobulin superfamily. CTLA4 located at the surface of T cells and transmits the inhibitory signal to T cells.[9,10] CTLA4 plays an important role in many immune diseases.[11,12] CTLA4 could inhibit the autoreactive T cells proliferation and regulate the antigen specific apoptosis. Imbalance of Th1/Th2 is significantly associated with the outcomes of pregnancy.[13] Th1/Th2 cytokines ratio was significantly elevated in RSA patients than that in normal pregnant women.[14] Polymorphisms in CTLA4 gene might alter the expression level of protein, then effects the cellular stimulation for T cells, especially the polymorphisms in the promoter and exon regions.[15,16] CTLA4 gene has 4 exons, and various single nucleotide polymorphisms (SNPs) have been identified for the gene. Rs733618 (c.-1722T/C), rs4553806 (c.-1661A/G), rs5742909 (c.-318C/T) are located at the promote region, while rs231775 (c.49A/G) is located in exon 1.[11] Minor allele frequencies of them were more than 0.1 in CHB (Chinese Han in Beijing) population. All of these SNPs were widely explored in different diseases.[17–19] However, the genetic association of these SNPs with RSA occurrence were rarely reported in Chinese Han population.

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In present study, we investigated the genetic effects of CTLA4 rs733618, rs4553808, rs5742909, rs231775 on RSA susceptibility and CTLA4 serum level in Chinese Han population.

2. Materials and methods

2.1. Study subjects

Ethnic committee of First affiliate hospital of Jinan university approved present study. Informed consent was signed by every subject after realized the study process. All participants were Chinese Han population. Controls were matched with cases in ages. Baseline characteristics were gathered by professional questionnaire. Women diagnosed with RSA between January 2014 and June 2016 were recruited as cases. RSA patients were diagnosed by 2 pathologists in First affiliate hospital of Jinan university. Females with the experience of normal pregnancy were enrolled as healthy controls. Individuals with the history of abortion and infectious diseases were excluded from this study.

2.2. Genotyping method

Peripheral blood was collected from the vein of every subject and put into the tubes with EDTA. Samples were centrifuged into leukocytes and serum, then stored in −80°C standby application. Genomic DNA was extracted from leukocytes using the DNA extraction kit (TIANGEN, Beijing).

CTLA4 SNPs were amplified by PCR using the primer sequences as shown in Table 1. PCR reaction included initial denaturation at 94°C for 4 minutes, 30 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 40 seconds, extension at 72°C for 1 minute, and the final extension at 72°C for 5 minutes. PCR products were digested by restriction enzymes and examined by agarose gel electrophoresis (AGE).

2.3. Analysis of CTLA4 serum level

Serum level of CTLA4 in subjects was detected by CTLA4 (Human) ELISA Kit (Abnova Corporation) following the operation instruction.

2.4. Statistical analysis

Hardy–Weinberg equilibrium (HWE) test was used to detect the representativeness of the subjects. Continuous variables were presented by mean ± SD and assessed by t test or Mann–Whitney U test. Categorical variables were evaluated by χ² test or Fisher exact test. Association strength was expressed by odds ratios (ORs) with corresponding 95% confidence intervals (CIs). Calculations were performed by PASW 18.0. Significant level was set to 0.05 (2-tailed) and corrected by Bonferroni method.

3. Results

3.1. Baseline characteristics

One hundred thirty-one RSA patients and 146 healthy individuals were enrolled in this study. Mean age of RSA patients was 32.38±8.35 years and mean age of healthy controls was 33.60±8.48 years, no significant difference existed in age difference between case and control groups.

3.2. Association between CTLA4 SNPs and RSA susceptibility

CTLA4 SNPs distributions were accorded with the HWE test both in case and controls (Table 2, P>0.05), suggesting that these subjects could represent the general population.

Higher frequencies of rs733618 TC and CC genotypes were discovered in RSA patients than that in controls, but the differences were not significant (Table 2, P>0.05). C allele frequency was respectively 32.44% in RSA patients and 38.36% in controls. This allele slightly associated with reduced RSA susceptibility (P=0.05). GG genotype of rs4553808 SNP has been discovered in 1 RSA patient (0.76%) and 4 healthy controls (2.74%). AG genotype had higher frequency in controls than that in RSA patients (28.08% vs 19.08%). No significant difference existed in the genotype distributions between case and control groups (P>0.05). Rs4553808 G allele was more frequently discovered in controls, indicating a distinct association with decreased RSA susceptibility (P=0.027, OR=0.570, 95% CI=0.345–0.942). CT and TT genotypes of rs5742909 SNP had lower frequencies in RSA patients, but these genotypes had no significant association with RSA susceptibility (P>0.05). T allele frequency had significantly difference between case and control groups (P=0.027), demonstrating that rs5742909 T allele significantly associated with decreased susceptibility for RSA (OR=0.570, 95% CI=0.345–0.942). Frequencies of rs231775 GG, GA and AA genotypes were 51.15%, 41.22%, 7.63% in RSA patients and 43.15%, 41.78%, 15.07% in controls, respectively. Significant difference existed in AA genotype frequency between cases and controls (P=0.040). AA genotype might significantly correlated with reduced RSA susceptibility (OR=0.427, 95% CI=0.188–0.973).

3.3. Influence of rs231775 genotypes for CTLA4 serum level

Due to genotypes of rs733618, rs4553808, and rs5742909 SNPs had no significant association with RSA susceptibility, we did not

| SNP      | Allele | Primer sequence          | Restriction enzyme | Genotype | Fragments (bp) |
|----------|--------|--------------------------|--------------------|----------|----------------|
| rs733618 | c.-17227/C | F: 5’-CTAAGAGCATCGCCCGTGGAGCCT-3’ | BbvI | TT | 486 |
|          |        | R: 5’-TTGGTGTTGACGACGGTAGT-3’ |                  | CC | 257,229 |
| rs4553808| c.-1661A/G | F: 5’-CTAAGAGCATCGCCCGTGGAGCCT-3’ | Msel | AA | 333,153 |
|          |        | R: 5’-TTGGTGTTGACGACGGTAGT-3’ |                  | GG | 496 |
| rs5742909| c.-318C/T | F: 5’-AAATGAATTGACAGGTGATG-3’ | Msel | CC | 226,21 |
|          |        | R: 5’-TTACGAGAACAGACGGTAGT-3’ |                  | TT | 130,06,21 |
| rs231775 | c.+490A/G | F: 5’-CCAGGGCTTTCCTTCTTGTGTA-3’ | BbvI | AA | 329 |
|          |        | R: 5’-AGTGTTGCTGGGTGGGCAG-3’ |                  | GG | 245,84 |

Note: CTLA4 = cytotoxic T-lymphocyte associated protein 4, F = forward, R = reverse, SNP = single nucleotide polymorphism.
explore the influences of these SNPs for CTLA4 serum level. Then we only detected the influence of rs231775 genotypes for CTLA4 serum levels. Serum levels of CTLA4 was significantly higher in RSA patients (12.86 ± 5.17 ng/mL) than that in healthy controls (6.54 ± 2.21 ng/mL) (P = .028). Serum CTLA4 levels were higher in RSA patients than in controls respectively in GG, GA, and AA genotype carriers. No significant difference exited in these groups except AA genotype (Fig. 1, 17.83 ± 5.86 ng/mL vs 8.57 ± 1.89 ng/mL, P = .016). Thus we suggested that rs231775 AA genotype significantly associated with RSA susceptibility via upregulated CTLA4 level. While, serum CTLA4 level had no significant difference between genotypes of rs733618, rs4553808, rs5742909, respectively (Fig. 1, P > .05).

### 4. Discussion

Previous study proved that CTLA4 gene played a crucial role in the immune tolerance of maternal–fetal interface which was the key factor for RSA development.[20] Very few studies focused on the association of CTLA4 polymorphisms and RSA susceptibility. Therefore, we carried out this study.

Various studies found that rs733618 SNP significantly correlated with many immune diseases. Niknam et al.[21] demonstrated that -1722C allele negatively associated with acute rejection in kidney transplant patients with active Cytomegalovirus infection. A potential of rs733618 SNP also has been discovered in autoimmune myasthenia gravis.[22] A meta-analysis study found a positive association between TT genotype of rs733618 and the susceptibility for systemic lupus erythematosus.[23] While, rs733618 SNP had no significant association with nonanterior uveitis.[17] However, in this study we found that heterozygote and homozygote of rs733618 T allele were mildly associated with decreased susceptibility for RSA. At the same time, no significant association has been discovered between rs733618 alleles and RSA susceptibility.

| Genotype/allele | Case, n = 131 (%) | Control, n = 146 (%) | P     | OR (95% CI) |
|-----------------|-------------------|----------------------|-------|-------------|
| rs733618        |                   |                      |       |             |
| TT              | 61 (46.56)        | 58 (39.73)           | —     | —           |
| TC              | 55 (41.98)        | 64 (43.84)           | .437  | 0.817 (0.491–1.359) |
| CC              | 15 (11.45)        | 24 (16.44)           | .165  | 0.594 (0.284–1.244) |
| T               | 177 (67.56)       | 180 (61.64)          | —     | —           |
| C               | 85 (32.44)        | 112 (38.36)          | .417  | 0.772 (0.544–1.095) |
| rs4553808       |                   |                      |       |             |
| AA              | 105 (80.15)       | 101 (69.18)          | —     | —           |
| AG              | 25 (19.08)        | 41 (28.08)           | .016  | 0.837 (0.333–1.034) |
| GG              | 1 (0.76)          | 4 (2.74)             | .212  | 0.240 (0.026–2.188) |
| A               | 235 (89.69)       | 243 (83.22)          | —     | —           |
| G               | 27 (10.31)        | 49 (16.78)           | .027  | 0.570 (0.345–0.942) |
| rs5742909       |                   |                      |       |             |
| CC              | 106 (60.92)       | 102 (69.86)          | —     | —           |
| CT              | 23 (17.56)        | 39 (26.71)           | .055  | 0.567 (0.317–1.016) |
| TT              | 2 (1.53)          | 5 (3.42)             | .280  | 0.385 (0.073–2.029) |
| C               | 235 (89.69)       | 243 (83.22)          | —     | —           |
| T               | 27 (10.31)        | 49 (16.78)           | .027  | 0.570 (0.345–0.942) |
| rs231775        |                   |                      |       |             |
| AA              | 67 (61.51)        | 63 (54.31)           | —     | —           |
| GA              | 54 (41.22)        | 61 (41.78)           | .474  | 0.832 (0.504–1.376) |
| AA              | 10 (2.63)         | 22 (15.07)           | .040  | 0.427 (0.188–0.973) |
| G               | 188 (71.76)       | 187 (64.04)          | —     | —           |
| A               | 74 (28.24)        | 105 (35.96)          | .053  | 0.701 (0.489–1.005) |

*Note: 95% CI = 95% confidence interval, CTLA4 = cytotoxic T-lymphocyte associated protein 4, HWE = Hardy–Weinberg equilibrium, OR = odds ratios, RSA = recurrent spontaneous abortion.*
found that rs231775 G allele carriers had higher susceptibility to RSA in an Iran population. This difference might be caused by the divergences in study population.

In order to certify the exact mechanism of $CTLA4$ gene for RSA, we researched the influence of rs231775 genotype for $CTLA4$ serum level. Then we found that serum level of $CTLA4$ was significantly increased in RSA patients than in healthy individuals. In different rs231775 genotype carriers, the serum level of $CTLA4$ also had difference. Individuals carrying heterozygote and homozygote of +49A allele had orderly increased $CTLA4$ serum level both in case and control groups. But only in RSA patients with +49AA genotype had significantly higher $CTLA4$ serum level than in +49GG genotype carriers. Similar result has been discovered in previous study. Misra et al found that mutant genotypes of rs231775 SNP significantly associated with reduced $CTLA4$ serum level in IRM patients. Rs231775 polymorphism might have the capacity to influence the transcription activity of the gene. Polymorphisms in promoter or exon regions might alter the expression or structure of the gene, these alterations usually induced the different physiological or pathological changes. However, effects of polymorphisms in $CTLA4$ gene for RSA development were not clear until now.

In summary, we suggested that SNPs in promoter region and exon 1 of $CTLA4$ gene might significantly associated with reduced RSA susceptibility via the upregulation of $CTLA4$. In spite of this, it should be accepted that several limitations existed in present study. Firstly, sample size was not large enough to obtain a higher test power. Secondly, application range for this study was limited by the ethnicity number. Thirdly, a variety of factors contribute to the development of RSA, but interactions between $CTLA4$ gene and other genes, as well as environmental factors were not detect in present study. Haplotype between SNPs might alter the effects of each SNP for RSA susceptibility independently. However, the haplotype analysis was not performed in this study because the correlation coefficient of linkage disequilibrium ($D'$ and $r^2$) was not high enough. Finally, current results were not adjusted by confounding factors that might affect the stability thereof. As a consequence, well designed studies with enlarged sample size are needed in the future so as to certify the pathogenesis of $CTLA4$ in RSA development.

**Author contributions**

**Conceptualization:** Li Li, Jia Liu.

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**Formal analysis:** Li Li, Jia Liu.

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**Figure 1.** Influence of rs231775 genotypes for $CTLA4$ serum level. In RSA patients, AA genotype carriers had higher $CTLA4$ serum level than that in GG genotype carriers (17.83 ± 5.86 ng/mL vs 10.41 ± 3.86 ng/mL, $P = .039$). Serum $CTLA4$ level was significantly higher in RSA patients than in controls respectively carrying AA genotype (17.83 ± 5.86 ng/mL vs 8.57 ± 1.89 ng/mL, $P = .016$). While, serum $CTLA4$ level had no significant difference between genotypes of rs733618, rs4553808, rs5742909, respectively ($P > .05$).
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