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# Studies on the relationship of CTLA-4 +49A/G gene with Recurrent Miscarriage in Northwest of Iran

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## Abstract

**Background**: Immunological factors are important in pregnancy loss because of the interaction between mother and fetus. T-regulatory cells as the component of humeral immune response play important role in the fetu-maternal interface. One of the regulatory mechanisms for these cells is mediated by antigen independent co-stimulatory signals and interaction of Cytotoxic T-Lymphocyte Antigen 4 (B7/CTLA-4) is one of these signals. The CTLA-4 which down regulates the activation and proliferation of T-cells occurs in a competitive interaction with CD28 to bind to B7. The aim of this study was to find out the relationship of CTLA-4 +49A/G gene with Recurrent Miscarriage in a group of Iranian women.

**Methods**: In the present study, 60 women with the history of two or more pregnancy loss were selected and considered as the case group. A group of women (n=60) with at least two live births without any previous history of pregnancy loss and autoimmune diseases were taken as control group. Genomic DNA was extracted from whole blood using standard protocols. The CTLA-4 +49 A/G were detected using polymerase chain reaction-restriction fragment length polymorphisms assay.

**Results**: The results showed that CTLA-4 +49 A/G polymorphisms were not significantly different in women with the history of two or more pregnancy loss compared to normal individuals. The frequency of G-allele polymorphism was 39.16% and 35.83% in patients and controls respectively.

**Conclusions**: The data presented may suggest that the CTLA-4 is not associated with recurrent miscarriage in an Iranian population in Northwest region.

**Keywords**: Recurrent miscarriage, CTLA-4, immune system

## Introduction

Recurrent miscarriage (RM) is basically defined as three or more consecutive losses of the conceptus before 20th week of gestation. Studies show that approximately one woman in 100 experience recurrent miscarriage (Gupta et al., 2012).There are several etiological factors including genetics, anatomy, endocrine, infectious, immunology, and lifestyle, which are considered to be involve in recurrent miscarriage (Mojarad et al., 2013; Ford et al., 2009).

A successful pregnancy belongs to the

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interaction between mother and fetus (Kristiina Rull, 2012). This interaction is vital since the fetus is not genetically the same as the mother. Several mechanisms have been found to establish this fetu-maternal interaction. Among these, immunological mechanisms are one of the important factors involving in tolerance toward semi-allogeneic fetal by maternal immune system (Christiansen, 2013; Molazadeh et al., 2014).

Regulatory T cells (Treg) of mother Immune system plays a main protective role in maternal tolerance during the early stages of pregnancy (Kumiko et al., 2013).

There are two different ways for T cell regulation, one is via interactions between T cell receptors and specific peptides expressed on the surface of antigen presenting cells (APCs), and the other one is through the interactions between co-stimulatory receptors and their ligands (Xipeng et al., 2006).

One of the co-stimulatory molecules is Cytotoxic T lymphocyte antigen-4 (CTLA-4) expressed onCD+4, CD+25 regulatory T cells (Saito et al., 2005). CTLA-4 is a 33 -37 kD, trans-membrane glycoprotein receptor of immunoglobulin superfamily, which is presented on the surface of T-cells as a down regulator of activation and proliferation (Dagmar Quandt, 2007; Rui et al., 2014).

In addition, CTLA-4 and its homologue (CD28), in a competitive interaction binds to the B7, which is a cell surface molecule on APCs. The affinity of CTLA-4 is greater than that of CD28 for B7, which results in the inactivation of T-cells with CTLA-4 (Rui et al., 2014; Ahmadi et al., 2013).

The interaction of CTLA-4 and B7 reduces the proliferation of several lymphokines, such as IL-2, and results in extreme decrease of T cells proliferation (Krummel and Allison, 1996). IL-2, which is known as a T-cell growth factor plays a key role in response to antigens. On the other hands, when CTLA-4 binds to B7, it commences the JNK (Jun N-terminal kinase) pathway to increase the localization of transcription factor Foxo3, which leads to prevention of IL-6 production (Elia and Hunte, 2009).

*CTLA-4* gene, which is located in 2q33 of human chromosome, has 4 exons and the +49 A/G polymorphism is located at +49 position of exon 1 (Rui et al., 2014).

Several studies have been carried out to investigate the association of CTLA-4 polymorphism with several autoimmune diseases, such as diabetes, Graves' disease, etc. (Almasi et al., 2006). However the relationship between +49 A/G CTLA-4 polymorphisms with recurrent miscarriage in Iranian has not been reported.

## Materials and Methods

In this study, 60 women with the history of

two or more pregnancy loss were selected and considered as the case group. A group of ethnically-matched women (n=60) with at least two live births without any previous history of pregnancy loss and autoimmune diseases were taken as control group.

All participants were informed about the study and written informed consent was obtained before blood collection. Genomic DNA was extracted from peripheral leukocytes in whole-blood samples using standard salting out technique as described previously (Bonyai et al., 2011).

The target region of CTLA-4 gene was amplified by PCR using appropriate primers previously described (Ahmadi et al., 2013). The PCR condition reaction was started with initial denaturation at 94˚C for 3 min, followed by 30 cycles of denaturation at 94˚C for 30″, annealing at 58˚C for 30″ and extension at 72˚C for 15″. Finally the reaction was followed by a final extension at 72˚C for 3 min. Next, the amplicons were digested by BbvI through RFLP procedure. The digestion of wild type allele produced a single fragment with 162 bp length whereas the polymorphic allele produced two fragments of 72 bp and 90 bp (Figure 1).

The PCR products and restriction enzyme-digested fragments were separated on agarose and polyacrylamide gel respectively by electrophoresis technique and visualized with ethidium bromide.

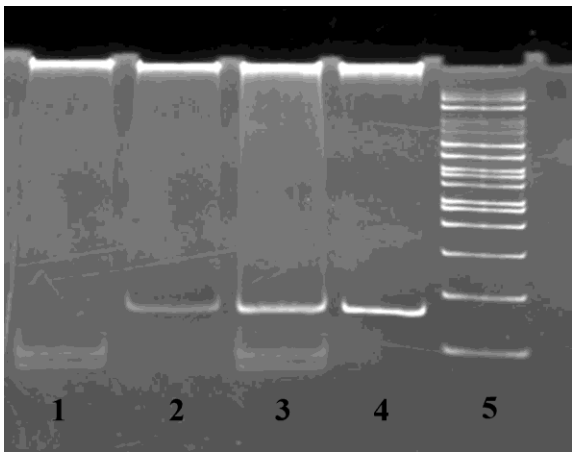


**Figure 1** Allelic distribution of +49 A/G CTLA-4 in Recurrent Miscarriage patients and controls. The DNA sample and products were separated on polyacrylamide gel and electrophoresed. 1- Homozygote (GG), 2- Homozygote (AA), 3- Heterozygote (AG), 4- PCR product, and 5- DNA Ladder

## Statistical analysis

Comparison of alleles and genotype frequencies between patients and controls was carried out using chi-square test with Yates' correction or Fisher's exact test, where appropriate. Hardy–Weinberg equilibrium (HWE) for the genotype frequencies was also verified by the chi-square test. Probability values of 0.05 or less were regarded as statistically significant. The odds ratio (OR) and confidence intervals (CI) at 95% significant level were estimated for all data.

## Results

DNA samples from patients with recurrent miscarriage history and sixty healthy controls were genotyped for *CTLA-4 +49 A/G* polymorphism. The age range of patients was

between 19-39 years. The demographic data and genotype frequencies of both patients and controls are mentioned in Ttable-1. The data analysis was done using Fisher test. The frequencies of AA, AG and GG genotypes were 33%, 55% and 11.66% in control group and 40%, 48.33% and 11.66% in patients respectively. The results showed no significant differences among patients and controls.

**Table 1** Genotype and allelic distribution of +49 A/G CTLA-4 in Recurrent Miscarriage patients and control individuals

| Genotype | Cases n (F) | Control n (F) | OR | P |
|---|---|---|---|---|
| **AA** | 20 (33%) | 24 (40%) | 0.739 | 0.189 |
| **AG** | 33 (55%) | 29 (48.33%) | 1.307 | 0.185 |
| **GG** | 7 (11.66%) | 7 (11.66) | 1 | - |
| **Allele** | | | | |
| **A** | 73 (60.83%) | 77 (64.16%) | 0.867 | 0.279 |
| **G** | 47 (39.16) | 43 (35.83%) | 1.153 | 0.279 |

## Discussion

Pregnancy is a unique condition in women's reproductive life. When three or more pregnancy losses happen before the 20$^{th}$ weeks of gestation, it is called recurrent miscarriage (Ford et al., 2009; Molazadeh et al., 2014). There are many factors which contribute to recurrent miscarriage including immune system and its components which could play an important role in maternal tolerance during pregnancy (Christiansen, 2013).

The regulation of Regulatory T cells (Treg), as a crucial component of humeral immune response, is mediated by antigen independent co-stimulatory signals, which act on surface of Treg cells (Gupta et al., 2012). The expression of CTL-4 (cytotoxic T lymphocyte antigen 4) could induce nuclear localization of Foxo3, leading to its downregulation by activated T cells (Tait and Hunte, 2009). Based on this it was assumed the existence of an association between the *CTLA-4 A49G* gene polymorphism and recurrent miscarriage among women from Northwest Iran. Analysis of the polymorphism in CTLA-4A49G gene revealed that there is no significant difference in the frequency of genotypes and alleles in control and patient samples.

There are only few studies, which have investigated the polymorphism of *CTLA-4* gene in maternal tolerance during pregnancy; for instance, a recent study performed by Gupta and co-workers (2012) on a North Indian population showed no significant association between the *CTLA-4 49 A/G* (G allele) and recurrent miscarriage in that population. Likewise, study carried out on Chinese women with unexplained recurrent spontaneous abortion (RSA) showed a lack of association between CTLA-*4* and genotypes of

RSA/SA in RSA women. The results of current study are in consistent with the previous reports and suggest that *+49 A/G CTLA-4* polymorphic region may not be effective in recurrent miscarriage.

Although no association was observed between *+49 A/G CTLA-4* gene polymorphism with recurrent miscarriage in Chinese and Indian populations, the involvement of other genes, which have had association with *CTLA-4 has not* been ruled out. Hence, verification of the results in these genes in an Iranian population with different ethnical background was undertaken. This would also indicate some important modifier genes which may influence on special polymorphisms of *CTLA-4* in a different population. Overall results showed that perhaps the frequency of G allele of *CTLA-4* gene in population from Northwest of Iran is higher than that of other previously reported populations. However, the CTLA-4 polymorphism was not associated with recurrent miscarriage in this population.

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**References**

[1] Ahmadi S, Rostamzadeh J, Khosravi D, Shariati P, Shakiba N. 2013. Association of CTLA-4 Gene 49 A/G Polymorphism with the Incidence of Type 1 Diabetes Mellitus in the Iranian Kurdish Population. Pakistan Journal Biology, 16: 1929-35.

[2] Almasi S, Erfani N, Mojtahedi Z, Rajaee A, Ghaderi A. 2006. Association of CTLA-4 gene promoter polymorphisms with systemic sclerosis in Iranian population. Genes and Immunity, 6: 401-6.

[3] Bonyadi M, Fotouhi N, Esmaeili M. 2011. Prevalence of IVS1+1G>A mutation among Iranian Azeri Turkish patients with autosomal recessive non-syndromic hearing loss (ARNSHL). International Journal of Pediatric Otorhinolaryngology, 75: 1612-5.

[4] Christiansen OB. 2013. Reproductive Immunology. Molecular Immunology, 55: 8-15.

[5] Ford HB, Schust DJ. 2009. Recurrent Pregnancy Loss. Etiology, Diagnosis, and Therapy. Review in Obstetrics & Gynecology, 2: 76-83.

[6] Gupta R, Prakash S, Parveen F, Suraksha A. 2012. Association of CTLA-4 and TNF-α polymorphism with recurrent miscarriage among North Indian women. Cytokine, 60: 456-62.

[7] Krummel ME, Allison JP. 1996. CTLA-4 Engagement Inhibits IL-2 Accumulation and Cell Cycle Progression upon Activation of Resting T Cells. The Journal of Experimental Medicine, 183: 2533-40.

[8] Kumiko I, Tomoko S, Akitoshi N, Koji A,

Mika I, et al. 2013. Characterization of regulatory T cells in decidua of miscarriage cases with abnormal or normal fetal chromosomal content. Journal of Reproductive Immunology, 97: 104-11.

[9] Mojarrad M, Hassanzadeh-Nazarabadi M, Tafazoli N.2013. Polymorphism of Genes and Implantation Failure. International Journal of Molecular and Cellular Medicine, 2: 1-8.

[10] Molazadeh M, Karimzadeh H, Azizi M. 2014. Prevalence and clinical significance of antinuclear antibodies in Iranian women with unexplained recurrent miscarriage. Iranian Journal of Reproductive Medicine, 12: 221-6.

[11] Quandt D, Hoff H, Rudolph M, Fillatreau S, Brunner-Weinzier M. 2007. A New Role of CTLA-4 on B Cells in Thymus-Dependent Immune Responses in Vivo. The Journal of Immunology, 179: 7316-24.

[12] Rui G, Fanglong S, Xiao Y, Peng S, Junzheng H, et al. 2014. Association between cytotoxic T lymphocyte antigen-4 +49A/G, −1722T/C, and −1661A/G polymorphisms and cancer risk: a meta-analysis. Tumour Biology, 35: 3627-39.

[13] Rull K, Nagirnaja L, Laan M. 2012. Genetics of recurrent miscarriage: challenges, current knowledge, future directions. Frontiers in Genetics, 3: 1-13.

[14] Saito S, Sasaki Y, Sakai M. 2005. CD4+CD25 high regulatory T cells in human pregnancy. Journal of Reproductive Immunology, 65: 111-20.

[15] Tait DE, Hunte AC. 2009. The Foxo and the hound: chasing the in vivo regulation of T cell populations during infection. Nature Immunology, 10: 457-8.

[16] Xipeng W, Zhengwen M, Yan H, Peihua L, Qide L. 2006. Expression of CD28 and cytotoxic T lymphocyte antigen 4 at the maternal-fetal interface in women with unexplained pregnancy loss. International Journal of Gynecology and Obstetrics, 93: 123-9.