ORIGINAL ARTICLE: GENETICS

Association of functional genetic variants of *CTLA4* with reduced serum CTLA4 protein levels and increased risk to idiopathic recurrent miscarriages

Q4 Maneesh Kumar Misra, Ph.D.,[a] Aditi Mishra, M.Tech.,[b] Shubha R. Phadke, D.M.,[c] and Suraksha Agrawal, Ph.D.[c]

[a] Department of Neurology, University of California–San Francisco, San Francisco, California; [b] Department of Computer Science and Engineering, Shri Ramswaroop Memorial University, Barabanki; and [c] Department of Medical Genetics, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, Uttar Pradesh, India

Objective: To determine whether idiopathic recurrent miscarriages (IRM) are associated with the alteration in serum CTLA4 protein levels and to evaluate their correlation with *CTLA4* tag single-nucleotide polymorphisms (SNPs).
Design: Retrospective case-control study.
Setting: Tertiary-care referral hospital.
Patient(s): Three hundred women with IRM (mean age: 28.6 ± 5.4 years) and 600 age-matched (mean age: 29.2 ± 6.8) control women.
Intervention(s): Detection of genetic variants of *CTLA4* markers rs231775, rs5742909, rs11571317, rs16840252, rs4553808, and rs3087243 by polymerase chain reaction followed by restriction fragment length polymorphism analysis and validated through DNA sequencing, and *CTLA4* serum levels measured by enzyme-linked immunosorbent assay.
Main Outcome Measure(s): Serum CTLA4 levels, genotypes, and haplotype frequencies compared in IRM cases versus controls.
Result(s): We observed statistically significantly higher occurrence of minor allele homozygous of rs231775 and rs3087243 tag-SNPs in IRM cases, which suggests a risk association. A statistically significantly reduced level of CTLA4 protein was seen for mutant genotypes of rs231775 and rs3087243 tag-SNPs in women with IRM, revealing a risk association. Serum CTLA4 levels were statistically significantly reduced in women with IRM as compared with the control women. The mutant haplotype carriers of six studied tag-SNPs showed 2.34-fold higher frequencies in IRM cases. In silico analyses strengthened our observations and suggested that variation in *CTLA4* gene content may influence the expression of this gene and directly or indirectly influence the function of other genes in the protein–protein interaction pathway.
Conclusion(s): These results suggest an effect of *CTLA4* gene variants, with reduced sCTLA4 secretion and an increased risk for IRM. Reduced CTLA4 secretion and specific *CTLA4* variants may contribute to the pathogenesis of IRM. (Fertil Steril® 2016;■:■–■. ©2016 by American Society for Reproductive Medicine.)
Key Words: *CTLA4*, haplotype, linkage disequilibrium, polymorphisms, recurrent miscarriage

Discuss: You can discuss this article with its authors and with other ASRM members at http://fertstertforum.com/misram-ctla4-variants-recurrent-miscarriage/

Recurrent miscarriage (OMIM: 614389), which is defined by the Society of Human Reproduction and Embryology (ESHRE) as three or more consecutive losses of clinically recognized pregnancies before 20 weeks of gestation, affects approximately 1% to 3% of couples attempting to conceive (1). The factors associated with known causes of recurrent miscarriage are parental chromosomal anomalies (genetic), maternal thrombophilias,

Received February 23, 2016; revised and accepted June 3, 2016.
M.K.M. has nothing to disclose. A.M. has nothing to disclose. S.R.P. has nothing to disclose. S.A. has nothing to disclose.
Supported in part by the Indian Council of Medical Research, Government of India, New Delhi, India [63/8/2010-BMS] and Department of Biotechnology, Government of India, New-Delhi, India, through the Bioinformatics Infrastructure Facility (BIF) research grant.
Reprint requests: Suraksha Agrawal, Ph.D., Department of Medical Genetics, Sanjay Gandhi Post-graduate Institute of Medical Sciences, Raebareli Road, Lucknow, Uttar Pradesh, India 226014 (E-mail: sur_ksha_agrawal@yahoo.co.in).

Fertility and Sterility® Vol. ■, No. ■, ■ 2016 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2016.06.011

anatomic, endocrine, and immunologic disorders (2). Thus, for recurrent miscarriage to be considered idiopathic, the factors associated with known causes of recurrent miscarriage must be ruled out (2). In 40% to 50% of recurrent miscarriage cases, the exact underlying pathophysiologic mechanisms remain obscure; these cases are considered idiopathic (3), thus encouraging the assessment of other risk factors.

Cytotoxic T-lymphocyte antigen 4 (CTLA4) is expressed constitutively on regulatory T cells (4). It has been suggested that *CTLA4* expressed on human placental regulatory T cells in decidual and peripheral dendritic cells may induce the expression of an immunosuppressive enzyme indoleamine 2,3-dioxygenase, particularly during early phases of pregnancy (5). Consecutively, expression of indoleamine 2,3-dioxygenase promotes maternal fetal tolerance (5). Further, in vivo studies have shown that expression of regulatory T cells and *CTLA4* in peripheral and decidual lymphocytes is down-regulated in human miscarriages (6). Single-nucleotide polymorphism (SNP) is the most common type of DNA variation in individuals (7). Genetic variations due to the SNP in a gene may affect its promoter activity (gene expression), messenger RNA (mRNA) conformation (stability), and subcellular localization of mRNAs and/or proteins and hence may be associated with human disease (7). Therefore, it may be hypothesized that the reduced number and/or functional deficiency of regulatory T cells due to the genetic variations in *CTLA4* gene may increase the risk of idiopathic recurrent miscarriage (IRM).

The *CTLA4* (Gene ID: 1493, MIM number: 123890) gene maps to band q33 of human chromosome 2, spans approximately 6.2 kilobases, and consists of four exons and three introns. The first exon encodes a leader peptide sequence, the second exon codes for an immunoglobulin domain, the third exon codes for the hydrophobic transmembrane domain, and the fourth exon codes for the cytoplasmic domain (8). The *CTLA4* heterogeneous nuclear RNA transcript undergoes alternative splicing, which results as three mRNA isoforms in humans: [1] the surface full-length CTLA4 (flCTLA4) including exons 1, 2, 3, and 4; [2] soluble CTLA4 (sCTLA4) lacking exon 3; and [3] a transcript lacking both exons 2 and 3. *CTLA4* acts as a safeguard during immune responses and helps in the maintenance of peripheral tolerance (9), inflammatory cytokine production (10), suppression of T-cell proliferation (10), and induction of apoptosis in activated T-cells (10).

Genomewide scans in affected sibling pairs with IRM have suggested a genetic linkage (11). The underlying genetic causes of IRM are complex, and it is currently unclear which genes are involved or how individual genetic variants contribute to IRM. There have been a number of candidate gene–associated studies in IRM, including both human leukocyte antigen (HLA) (12, 13) and non-HLA genes (14, 15), but none of these studies have been conclusive. Therefore, the identification of new potential candidate genes is needed for developing suitable biomarkers for IRM.

A genomewide association study conducted in autoimmune Grave's disease confirmed the association of *CTLA4* gene variants with disease susceptibility (16). Recently, the pedigree analysis of families with *CTLA4* mutations revealed its association with autosomal dominant immune dysregulation syndrome in humans (17). Further, it has been shown that the down-regulated expression of *CTLA4* shifts T-helper 2 (T_H2) to T_H1 type immune response at maternal–fetal interface, which leads to human miscarriages (18). Considering the critical role of *CTLA4* in T-cell mediated immunity at the maternal–fetal interface, variations in *CTLA4* gene content may influence the risk for IRM.

Several polymorphic markers have been reported in *CTLA4* regions. The most often reported polymorphic markers of *CTLA4* are present either in the upstream regions (rs5742909, rs11571317, rs16840252, and rs4553808 in the promoter region and rs231775 in exon 1) or in downstream regions (rs3087243) (19–22). Few studies have evaluated rs231775 tag-SNP in IRM (23, 24), and *CTLA4* tag-SNPs other than rs231775 have not been fully explored in IRM. Further, there are no studies on the soluble *CTLA4* (sCTLA4) levels in IRM and their correlation with *CTLA4* tag-SNPs. Our study fills this gap. We investigated the association of *CTLA4* rs231775, rs5742909, rs11571317, rs16840252, rs4553808, and rs3087243 polymorphic markers with IRM. We also evaluated the effect of *CTLA4* gene variants with altered sCTLA4 secretion and their impact on IRM.

MATERIALS AND METHODS

Study Participants and Inclusion/exclusion Criteria

All the samples were collected from women treated as outpatients for IRM in the Department of Medical Genetics, Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS), Lucknow (Uttar Pradesh), India. All the patients were registered during April 2011 to September 2015. The women treated for IRM were primary aborters with a history of at least three consecutive miscarriages. All recurrent miscarriages cases included in this study are of clinical intrauterine pregnancies. Our inclusion and exclusion criteria for the selection of IRMs and control cases are mentioned herein.

All the cases were screened for various known causes of miscarriages with appropriate investigations including karyotypes of the couple; day 21 progesterone levels, antiphospholipid antibodies including lupus anticoagulant (positive likelihood ratio 0.8–1.05) and anticardiolipin antibodies (IgG 0–12 IgG anticardiolipin units, immunoglobulin M 0–5 IgM anticardiolipin units), and prothrombotic risk factors including factor V Leiden and prothrombin mutations, prolactin level, glycemic curve, and thyroid hormone levels. Uterine causes and cervical incompetence were ruled out by history, ultrasonography, and appropriate investigations.

A well-designed study proforma was used to record the detailed clinical information and pregnancy history of both the women with IRM and the control cases before their inclusion in this study. Both maternal and paternal genetic information determine the development of the fetus and the placenta as well as the outcome of the pregnancy, so we tested the blood samples of both partners for chromosomal abnormalities and excluded couples who had any chromosomal abnormality. Because fetal aneuploidy is the most common causes of recurrent miscarriages, couples with aneuploid abortuses were excluded from the present study. Of the