Granulocyte colony-stimulating factor gene rs1042658 variant and susceptibility to idiopathic recurrent pregnancy loss: A case-control study

Mahboobeh Nasiri Ph.D., Kobra Jahangirzadeh M.Sc.

Department of Biology, Islamic Azad University, Arsanjan Branch, Arsanjan, Iran.

Abstract

Background: Granulocyte colony-in stimulating factor (G-CSF) gene can be a potential candidate gene implicated recurrent pregnancy loss (RPL), a common complication of pregnancy with the prevalence of 1-5% among women of reproductive age.

Objective: To investigate the association between rs1042658 polymorphism in the 3′ untranslated region (3′UTR) of G-CSF gene and the risk of unexplained RPL among Iranian women.

Materials and Methods: In total, 122 women with unexplained RPL and 140 healthy postmenopausal women as a control group were enrolled in this case-control study. Tetra-primer amplification refractory mutation system-polymerase chain reaction was performed to determine the rs1042658 genotypes in all subjects.

Results: Statistically significant differences were detected between the distribution frequencies of both heterozygote CT, and carriage of T allele (TT+CT) genotypes of the rs1042658 between case and control groups. Allelic association was not observed with RPL.

Conclusion: Regarding the results of the present study, G-CSF rs1042658 gene polymorphism could be considered as a probable risk factor for unexplained RPL among Iranian women.

Key words: Granulocyte colony-stimulating factor, Recurrent pregnancy loss, Polymorphism.

Introduction

Cytokines were proved to have a large impact on the transplantation tolerance, such as maintenance of semi-allograft fetus during pregnancy (1, 2).

Granulocyte colony-stimulating factor (G-CSF or CSF3) is stimulated in response to inflammatory conditions and injuries (3).

G-CSF is transducing the specific biological signals by binding to the cell surface receptor (G-CSFR) expressed mostly on hematopoietic cell lineages and some non-hematopoietic cells e.g. endothelial cells, placenta, trophoblastic cells, and granulosa luteinized cells (4).

Among G-CSF biological actions, a cascade of tolerogenic events are more prominent composing of; the blockade of pro-inflammatory cytokine production, up-regulation of the anti-inflammatory cytokines e.g. interleukin (IL)-4 and IL-10, reducing interferon γ production in lymphocytes, as well as, shifting the T-helper (Th)-1/Th-2 balance toward Th-2 responses, and accumulation of regulatory T cells (5, 6).

Recurrent pregnancy loss (RPL) is a complex disease, the cause of which is unknown for more than 50% of the cases, called idiopathic RPL (7). Defects in the balance regulation of Th-1/Th-2/Th-17 and regulatory T cells (Treg) function has been implicated in the pathobiology of RPL (8, 9).

The association of the G-CSF and several autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus was demonstrated in many studies, which both are Th-1/Th-17 diseases as RPL (10, 11). Those evidences encouraged us to assess a possible association between the G-CSF gene, mapped on chromosome 17q11.2-12, with RPL (12).

In this study, we investigated whether or not genotype and allele frequencies of the rs1042658 single nucleotide polymorphism (SNP) in the 3′ untranslated region of the G-CSF gene confer susceptibility to RPL in Iranian women.
Materials and methods

The case-control study consisted of women with the history of at least two unexplained miscarriages, which were screened for polymorphism of the G-CSF gene. The presence of anatomical uterine abnormalities detected by hysteroscopy, positive cultures for chlamydia and mycoplasma, paternal and maternal chromosomal aberration, and endocrine disorders were considered as exclusion criteria. As one of the main objectives of the study was to identify different RPL-related factors, we, therefore, collected detailed information including abortion type, the number of live children, and oral contraceptive use from all participants. A total of 122 RPL women as the case group and 140 healthy controls were recruited into the study. Pregnant women were excluded from the study.

Population-based controls were randomly sampled from postmenopausal women referred for an annual checkup to the Shafa Hospital Laboratory, Shiraz, Iran. They have never experienced any pregnancy complications even miscarriage but also have at least two live births.

DNA extraction and rs1042658 genotyping

Genomic DNA was extracted from the white blood cells of each sample using a standard salting out method (13). The quality of the extracted genomic DNA was evaluated on 1% agarose gel electrophoresis. The fast, simple, and cost-effective Tetra-primer amplification refractory mutation system-PCR (T-ARMS PCR) was used to determine different genotypes regarding the selected polymorphism using the following four primers: forward outer (FO), 5'-GATGAGCCGCCTGTAGACCCCTGGTGTTGAG-3'; reverse outer (RO), 5'-CAGACGAACCTGGAACCTTTCTCTCCTCCCC-3'; forward inner (FI), 5'-GTTCTGCTGCCATTTGCTCTGACAT-3'; and reverse inner (RI), 5'-TGCTCCCTCCCACTCCCCACAT-3'. The FO and RI primers were used to amplify T allele fragment (456bp), whereas FI and RO primers were used to amplify C allele fragment (335bp). FO and RO primers amplified a 738bp fragment as an internal control, which is genotype-independent amplicon. The reaction mixture was as follows: 6.25µl PCR master mix (Yekta Tajhiz Azma, Iran); 1µl FO primer (10µM); 1µl RO primer (10µM); 3µl FI (10µM); 3 µl RI primer (10µM), and 1.0µl DNA template. Double-distilled water was added to obtain a final reaction volume of 12.5µl. The reaction mixture was heated in a thermocycler (ABI, Perkin Elmer 9600) under the following condition: 95°C for 5 min, 30 cycles (95°C for 1 min; 70°C for 1 min), and 72°C for 1 min, and final extension at 72°C for 7 min. PCR products were resolved on 2% agarose gel electrophoresis and the bands were detected under UV light using UViSoft software (UViTeC Cambridge, UK) following staining by DNA safe stain dye.

Ethical consideration

This study was approved by the Islamic Azad University, Arsanjan Branch Ethics Committee. Written informed consent was obtained from all participants.

Statistical analysis

Student's t-test and Chi-square test were used to compare the continuous and nominal variables among RPL (cases) and control women, respectively. The X² test was also used to test Hardy-Weinberg equilibrium (HWE). Allelic and genotyping association of the selected SNP were evaluated using logistic regression and calculation of odds ratio (OR) with 95% confidence interval (CI). Considering the significant age difference between RPL patients and controls, all logistic regression analysis was adjusted for age. In order to assess the risk of confounding factors on the RPL susceptibility, the data were subjected to regression analysis considering RPL as the dependent variable and each of the risk factors as independent factors.

Statistical analysis was performed using the Statistical Package for Social Sciences (version 16; SPSS Inc., Chicago, IL). p value<0.05 was considered statistically significant.

Results

General characteristics

The general characteristics of the study participants are shown in Table I.

Genotypes and allelic distribution of rs1042658

Table II shows the genotypic and allelic distributions of the rs1042658 polymorphism in the cases and controls. The observed frequency of the genotypes in control group was in agreement with those expected based on Hardy-Weinberg equilibrium (X²=0.35; df=1; p=0.55).
The frequency of the heterozygote genotype CT was significantly higher in controls compared to cases (43% vs. 33%), then it showed a protective effect against RPL (OR: 0.42, 95%CI: 0.18-0.98, p=0.04). Distribution of the T-allele was not significantly differed between cases and controls (OR: 0.89, 95%CI: 0.6-1.31, p=0.55). Logistic regression analysis confirmed the association of the G-CSF rs1042658 gene variant, after adjusting for the RPL variables; age, oral contraceptive use and body mass index (Table III).

**Table I.** Demographic and clinical features of cases and controls

| Characteristics | Case group (n= 122) | Control group (n= 140) | p-value * |
|-----------------|---------------------|------------------------|-----------|
| Age (mean±SD)   | 35.5 ± 12.5         | 57.8 ± 6.8             | <0.001    |
| Abortion (mean±SD) | 3.3 ± 1.2    | 0.0 ± 0.0              | -         |
| Abortions       | 22                  | 0.0 ± 0.0              | -         |
| ≥3              | 100                 | 0.0 ± 0.0              | -         |
| Abortion type   |                     |                        |           |
| Primary         | 62 (51)             | 0.0 ± 0.0              | -         |
| Secondary       | 60 (49)             | 0.0 ± 0.0              | -         |
| Number of pregnancies | 4.61 ± 2.34     | 5.36 ± 2.3             | 0.01      |
| Number of children | 1.3 ± 2.22     | 5.36 ± 2.3             | 0.001     |
| Body mass index (BMI kg/m²) | 25.4 ± 2.9 | 26.9 ± 3.2             | <0.001    |

*p<0.05, Logistic regression |

**Table II.** Allelic and genotypic frequencies of G-CSF rs1042658 gene polymorphism

| Genotype  | Controls (n= 140) | Cases (n= 122) | p-value | OR | 95% CI |
|-----------|------------------|---------------|---------|----|--------|
| Co-dominant model |                   |               |         |    |        |
| CC        | 70 (50)          | 70 (57)       | -       | 1  | Reference |
| CT        | 60 (43)          | 40 (33)       | 0.04    | 0.42 | 0.18-0.98 |
| TT        | 10 (7)           | 12 (10)       | 0.45    | 0.56 | 0.12-2.5  |
| Dominant model (T) |             |               |         |    |        |
| TT+TC (T+) | 70 (50)        | 52 (43.3)     | 0.04    | 0.44 | 0.2-0.99 |
| Allele    |                   |               |         |    |        |
| C         | 200 (71)         | 180 (74)      | -       | 1  | Reference |
| T         | 80 (29)          | 64 (26)       | 0.55    | 0.89 | 0.6-1.31 |

*Adjusted for age; OR: Odds ratio; CI: Confidence interval|

**Table III.** Influence of rs1042658 variant on the risk of unexplained RPL after adjustment for potential variables

| Factors               | p-value | OR    | 95% CI |
|-----------------------|---------|-------|--------|
| Age                   | <0.001  | 0.83  | 0.80-0.87 |
| Oral contraceptive use| 0.23    | 1.66  | 0.72-2.8 |
| BMI                   | 0.004   | 0.82  | 0.72-0.94 |
| rs1042658 (T+)†       | 0.04    | 0.42  | 0.18-0.96 |

† Homozygote+ heterozygote carriers | BMI: Body mass index | Multivariate logistic regression

**Figure 1.** Schematic representation of the electrophoretic results of the T-ARMS PCR for rs1042658 variant. Heterozygote CT genotype is determined by the size fragments of 738 bp, 456 bp, and 335 bp. The TT homozygote is determined by 738 bp and 335 bp fragments. The CC homozygote is shown in the last line in right by fragments of 738 bp and 456 bp fragment in length. The 100 bp size marker ladder is loaded in first line of the left corner.

International Journal of Reproductive BioMedicine Vol. 16, No. 1, pp: 35-40, January 2018
Discussion

The key finding of the current study is that the rs1042658 genetic variant of G-CSF gene is associated with decreasing susceptibility to RPL in the dominant model for allele T, which this association is apparently independent of the confounding variables including age, oral contraceptive use, and body mass index.

G-CSF cytokine is a recently discovered molecular marker with potent therapeutic property and pivotal roles in curtailing pregnancy failure (14, 15). G-CSF guarantees the health of the pregnancy by enhancing embryo implantation and ovarian function (4) and therefore, by increasing the endometrial thickness contributes to reduced pregnancy loss (16). Expression profiling of the cells followed by the administration of recombinant human G-CSF (rhG-CSF) resulted in the significant increase in the genes involved in cell migration and embryo implantation (Integrin alpha-V/beta-3 and Plasminogen Activator Urokinase Receptor), angiogenesis (Thymidine Phosphorylase), and cell proliferation control (CD40 and CD40 Ligand) (17), and also an increase in regulatory T (Treg) cells and dendritic cells (17, 18).

The immunological factors are considered critical for embryo implantation, and from this point of view, it is proven that the G-CSF attenuated the production of pro-inflammatory cytokines including IL-1β, IL-12, interferon (IFN)-γ, IL-18, and tumor necrosis factor (TNF)-α, and in turn up-regulated anti-inflammatory cytokines, IL-4 and IL-10 (19, 20).

Pregnancy is known as a Th-2 phenomenon, which high and constitutive expression of IL-4 and IL-10 anti-inflammatory cytokines guarantee the maintenance of tolerogenic environment during pregnancy (21). IL-17 producing T (Th-17) cells have a high pathogenic potential by inducing inflammation (22). Previous studies demonstrated that increased Th-17 and hence, an elevated level of IL-17 in pregnancy decidua might be disadvantageous for maintenance of pregnancy. G-CSF influences the IL-17-associated immune responses by negatively regulation of IL-17 production (23, 24). Besides these two hypotheses, the role of Treg cells in the maintenance of pregnancy has come into scene recently (25). Reduced number of both circulating and decidua Treg is an obvious marker in women with miscarriage (26). Administration of G-CSF increases the number of Treg in decidua in pregnant women and therefore, reduces the risk of pregnancy failure (27, 28). Further evidence strongly supported the crucial role of IL-10 in G-CSF-Treg interplay (29).

We detected an association between RPL and variant in G-CSF, but the specific function of rs1042658T allele is unknown. We hypothesized that the substitution of the C allele with the T allele resulted in an increased expression of G-CSF by several processes including ribosome binding, initiation, and elongation of the translation. Consequently, the higher quantity of G-CSF up-regulates the expression of target immune modulation genes (like IL-10) and the increase the number of Treg during pregnancy which improves the implantation and protects the fetus to term.

Conclusion

In conclusion, our results showed that the polymorphic T allele of the G-CSF rs1042658 polymorphism is associated with decreased risk of RPL among Iranian women, which this association is independent of the profounding risk factors of the RPL.

Acknowledgments

The authors appreciate specialist Dr. Minoo Zolghadri (Gynecologist) and her staffs for collaborating in sampling. We also thank Nosaybe Jaafari and Najmeh Noruzi for technical assistance. This study was funded by Islamic Azad University, Arsanjan Branch.

Conflict of interest

The authors have no conflict of interest to declare.
G-CSF gene polymorphism and recurrent pregnancy loss

References

1. Tay SS, Plain KM, Bishop GA. Role of IL-4 and Th2 responses in allograft rejection and tolerance. Curr Opin Organ Transplant 2009; 14: 16-22.

2. Rasti Z, Nasiri M. Association of the G/G polymorphism of CTLA-4 gene with idiopathic recurrent spontaneous abortion in women in southwest of Iran. J Reprod Infertil 2016; 17: 151-156.

3. Martins A, Han J, Kim SO. The multifaceted effects of granulocyte colony-stimulating factor in immunomodulation and potential roles in intestinal immune homeostasis. IUBMB Life 2010; 62: 611-617.

4. Root RK, Dale DC. Granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor: comparisons and potential for use in the treatment of infections in nonneutropenic patients. J Infect Dis 1999; 179 (Suppl.): S342-352.

5. Mannon PJ, Leon F, Fuss IJ, Walter BA, Begnani M, Quezado M, et al. Successful granulocyte-colony stimulating factor treatment of Crohn’s disease is associated with the appearance of circulating interleukin-10-producing T cells and increased lamina propria plasmacytoid dendritic cells. Clin Exp Immunol 2009; 155: 447-456.

6. Boneberg EM, Hartung T. Granulocyte colony-stimulating factor attenuates LPS-stimulated IL-1β release via suppressed processing of proIL-1, whereas TNF-α release is inhibited on the level of proTNF-α formation. Eur J Immunol 2002; 32: 1717-1725.

7. Brigham SA, Conlon C, Farquharson RG. A longitudinal study of pregnancy outcome following idiopathic recurrent miscarriage. Hum Reprod 1999; 14: 2868-2871.

8. Halonen M, Lehman IC, Stern DA, Spangenberg A, Anderson D, Mobley S, et al. Th1/Th2 patterns and balance in cytokine production in the parents and infants of a large birth cohort. J Immunol 2009; 182: 3285-3293.

9. Saito S, Nakashima A, Shima T, Ito M. Th1/Th2/Th17 imbalance in peripheral and decidual lymphocytes of women with recurrent miscarriage: a randomised controlled trial. Int J Reprod Biomed 2016; 14: 737-742.

10. Cavalcante MB, Costa Fda S, Barini R, Araujo Júnio E. Granulocyte colony-stimulating factor and reproductive medicine: A review. Iran J Reprod Med 2013; 15: 195-202.

11. Barad DH, Yu Y, Kushnir VA, Shohat-Tal A, Lazzaroni E, Lee HJ, et al. A randomized clinical trial of endometrial perfusion with granulocyte colony-stimulating factor in in vitro fertilization cycles: impact on endometrial thickness and clinical pregnancy rates. Fertil Steril 2014; 101: 710-715.

12. Rahmati M, Petitbarat M, Dubanchet S, Bensussan A, Chauvat G, Ledee N. Granulocyte-colony stimulating factor related pathways tested on an endometrial ex-vivo model. PLoS One 2014; 9: e102286.

13. Würfel W, Santjohanser C, Hirv K, Bühler M, Meri O, Labourt I, et al. High pregnancy rates with administration of granulocyte colony-stimulating factor in ART-patients with repetitive implantation failure and lacking killer-cell immunoglobulin-like receptors. Hum Reprod 2010; 25: 2151-2152.

14. Boneberg EM, Hartung T. Molecular aspects of anti-inflammatory activity of G-CSF. Inflamm Res 2002; 51: 119-128.

15. Arpinati M, Green CL, Heimfeld S, Heuser JE, Anasetti C. Granulocyte-colony stimulating factor mobilizes T helper 2-inducing dendritic cells. Blood 2000; 95: 2484-2490.

16. Veenstra van Nieuwenhoven AL, Heineman MJ, Faas MM. The immunology of successful pregnancy. Hum Reprod Update 2003; 9: 347-357.

17. Singh RP, Hasan S, Sharma S, Nagra S, Yamaguchi DT, Wong DT, et al. Th17 cells in inflammation and autoimmunity. Autoimmun Rev 2014; 13: 1174-1181.

18. Martins AJ, Colquhoun P, Reid G, Kim SO. Reduced expression of basal and probiotic-inducible G-CSF in intestinal mononuclear cells is associated with inflammatory bowel disease. Inflamm Bowel Dis 2009; 15: 515-525.

19. Santner-Nanan B, Peek MJ, Khanam R, Richards L, Zhu E, Fazekas de St Groth B, et al. Systemic increase in the ratio between Foxp3+ and IL-17-producing CD4+ T cells in healthy pregnancy but not in preeclampsia. J Immunol 2009; 183: 7023-7030.

20. Sharma S. Natural killer cells and regulatory T cells in early pregnancy loss. Int J Dev Biol 2014; 58: 219-229.

21. Jin LP, Chen QY, Zhang T, Guo PF, Li DJ. The role of G-CSF in recurrent implantation failure: A randomized double blind placebo control trial. Int J Reprod Biomed 2016; 14: 708-7091.

22. Nasiri M, Rasti Z. CTLA-4 and IL-6 gene polymorphisms: risk factors for recurrent pregnancy loss. Hum Immunol 2016; 77: 1271-1274.

23. Davari-tanha F, Shahrokh Tehraninejad E, Ghazi M, Shahraki Z. The role of G-CSF in recurrent miscarriage: A randomized double blind placebo control trial. Int J Reprod Biomed 2016; 14: 737-742.
29. Morris ES, MacDonald KP, Rowe V, Johnson DH, Banovic T, Clouston AD, et al. Donor treatment with pegylated G-CSF augments the generation of IL-10-producing regulatory T cells and promotes transplantation tolerance. *Blood* 2004; 103: 3573-3581.