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The association of IL-33 and Foxp3 gene polymorphisms with recurrent pregnancy loss in Egyptian women

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ABSTRACT

Deregulated immunity is one of the most important factors implicated in recurrent pregnancy loss (RPL). The possible role of interleukin-33 (IL-33) and forkhead/winged helix transcription factor (Foxp3) in RPL have not been fully investigated. We aimed to evaluate IL-33 rs1929992 and Foxp3 rs2232365 single nucleotide polymorphisms (SNPs) and their serum levels in Egyptian RPL females.

Blood samples were collected from 142 RPL patients and 123 women as healthy controls. IL-33 rs1929992 SNP was determined by polymerase chain reaction restriction fragment length polymorphism and Foxp3 rs2232365 SNP was determined using allele specific polymerase chain reaction. The serum IL-33 and Foxp3 levels were measured by enzyme linked immunosorbent assay.

Foxp3 rs2232365 SNP showed statistically significant association with RPL. The risk of RPL was significantly higher in women carrying Foxp3 G allele than those carrying A allele. Lower serum levels of Foxp3 and IL-33 were observed in RPL patients than controls ($P < 0.001$). Foxp3 serum levels were much lower in carriers of G allele than those carrying A allele in all studied groups.

Foxp3 rs2232365 SNP could be considered as a risk factor for RPL. The lowered serum levels of IL-33 and Foxp3 in RPL patients suggested that they might have an important role in the pathogenesis of the disease. Therefore, we hypothesized that Foxp3 polymorphisms may be important in RPL pathogenesis.

1. Introduction

Recurrent pregnancy loss (RPL) is two or more consecutive pregnancy losses prior to twenty weeks from the last menstrual period. It occurs in about 1–5% of females during child bearing period [1]. Several factors have been detected in the occurrence of RPL, such as uterine anomalies, endocrine dysfunction, chromosomal abnormalities, infections and factors related to the life style. About 40–50% of cases of RPL are considered idiopathic [2]. Although many previous studies have been done to identify the possible cause of idiopathic RPL, its pathogenesis remains a matter of debate [3]. One of the main mechanisms underlying RPL is dysregulated immunity [4]. The balance between T regulatory cells (Tregs) and T helper cells (Th1-Th2-Th17) modulate maternal immune response [5]. Successful pregnancy depends on the balance between pro-inflammatory and anti-inflammatory cytokines [6]. There is an increase of Treg subset together with Th2 cytokines predominance in the normal pregnancy [7,8]. In contrast, disorders in pregnancy outcome are combined with over expression of pro-inflammatory Th1 cytokines [9].

Interleukin-33 (IL-33), the most recent member of the IL-1 family [10], is located in cytoplasm, also in the nucleus suggesting dual nuclear function through regulation of gene transcription and cytokine function through activation of the IL-33/ST2 pathway [10,11]. IL-33 is located on chromosome 9p24 [12] and mainly expressed in endothelial and smooth muscle cells in the placenta, chorioamniotic membranes, umbilical cord [13], epithelial, endothelial cells, activated Th2 cells and mast cells [14,15]. IL-33 can act as full-length 31-kDa protein or cleaved by caspase-1 into a shorter 18 kDa fragment, which is considered functionally inactive [16]. It can enhance T helper (Th) cell immune responses by combining with its receptor ST2 (suppression of tumorigenicity 2) which is encoded by interleukin 1 receptor like 1 (IL-1RL1) and co-receptor IL-1 receptor accessory protein (IL-1RAcP) (IL33/ST2 pathway) [17]. IL-33 has the ability to enhance both Th1, Th2 (IL-4, IL-5 and IL-13) cytokines [18], and Th17 immune responses [19]. Furthermore, It stimulates the activation of natural killer cells, eosinophils and basophils and induces IFN-γ production [20,21]. IL-33 promotes regulatory T (Treg) cells differentiation and facilitate their maintenance in inflamed tissues [22].

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Forkhead/winged helix transcription factor (Foxp3) is a member of the forkhead transcription factors. Foxp3 is expressed primarily by the immunosuppressive Treg cells [23]. Treg cells play an essential role in the maintenance of maternal tolerance to the fetus by contact-dependent suppression or producing anti-inflammatory cytokines [24]. Decreased levels of Treg cells in the deciduas and in peripheral blood were suggested in women with unexplained RPL. This results in reduction in the expression of Foxp3 as well as reduced suppressive capacity of Treg cells [25]. Therefore, it is hypothesized that SNPs of Foxp3 gene may predispose to RPL.

The association of polymorphisms of IL-33 and Foxp3 with idiopathic RPL is still unclear and needs further research to be proved. As there are differences in genotype and allele frequencies of cytokine genes regarding ethnicity and race as well as their critical role in the immune system regulation, this research was conducted for the first time to evaluate the association of the IL-33 and Foxp3 gene polymorphisms and their serum levels with RPL in Egyptian women. IL-33 rs1929992 and Foxp3 rs2232365 genes were selected for genotyping by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) and allele specific polymerase chain reaction (AS-PCR) respectively.

2. Materials and methods

2.1. Participants

This study included 142 RPL female patients attending the outpatient's clinics of Obstetrics and Gynecology Department, Zagazig University Hospitals. DNA extraction, Genotyping of gene polymorphisms and serum levels measurement were done in Medical Biochemistry and Molecular Biology Department, Faculty of Human Medicine, Zagazig University.

The Control group included 123 healthy age-matched women (mean age: 34.2 ± 4.3 years) with at least one full term pregnancy and with no history of pregnancy loss, or any other medical disorders. The mean age of 142 RPL female patients is 33.9 ± 5.7 years.

All patients were having no previous live births (primary miscarriage), and the average number of RPL was 3 losses ≤ 20 weeks of gestation. Patients with the following criteria were excluded; uterine and cervical congenital anomalies determined by pelvic ultrasound scans and hysteroscopy, chromosomal aberrations determined by karyotyping of both parents, luteal phase defects detected by endometrial biopsies, Rh incompatibility, older age ≥ 40 years at first miscarriage, endocrine diseases like diabetes, thyroid dysfunction and hyperprolactinemia, infectious disorders like chlamydia, abnormal liver functions, and thrombosis. Patients with autoimmune disorders (rheumatoid arthritis, systemic lupus erythematosus and anti-phospholipid syndrome) were also excluded.

All participants provided informed consent after approval of the study by the local ethical committee.

2.2. Blood sampling

After an overnight fasting, venous blood samples were collected from all subjects and divided into 2 portions: 2 mL of blood was collected in EDTA containing tubes, for genomic DNA extraction. Serum was separated immediately from the remaining part of the sample and stored at −20 °C till the time of analysis. Blood sampling from patients with RPL was performed from 4 to 6 months from the last miscarriage to exclude effect of pregnancy on IL-33 and Foxp3 levels.

2.3. DNA extraction

Genomic DNA was extracted using the QIAamp DNA blood mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's guidelines and stored at −20 °C for further use.

2.4. Genotyping of IL-33 (rs1929992) polymorphisms

Genotyping of IL-33 (rs1929992) was performed using PCR–RFLP method according to Jafarzadeh et al. [26]. The PCR for IL-33 (rs1929992) was performed in a total volume of 25 µl reaction mixture using 12.5 µl of PCR master mix (Fermentas, Burlington, Canada), 1 µl of genomic DNA, 2 µl of each primer and 7.5 µl ddH_2O. The amplified PCR product of SNP rs1929992 was digested with SspI restriction enzyme (New England Biolabs, England).

2.5. Genotyping of Foxp3 (rs2232365) polymorphisms

Allele-specific PCR was performed to detect the Foxp3 (rs2232365) gene polymorphism according to Metz et al. [27].

A "touch-down" procedure was applied as follows: an initial denaturation at 95 °C for 3 min. The PCR was done in a total volume of 20 µl reaction mixture with 10 µl PCR Master mix, 2µl (10 pmol) of each primer, 4µl Nuclease-Free Water and 2 µl (20 ng) of genomic DNA. Electrophoresis of the PCR products of IL-33 and Foxp3 was performed on a 2.5% agarose gel stained with ethidium bromide then visualized under ultraviolet transilluminator. The primers, PCR conditions, and DNA fragment sizes for IL-33 and Foxp3 were listed in Table 1.

Table 1
Primer sequence and PCR cycling conditions of IL-33 and Foxp3 genotyping.

SNP	Primer sequence	PCR cycling conditions	Fragment size (bp)
IL-33 rs1929992	F:5′-GAAGTCATCATCAACTTGGAACC-3′,R:5-GGATTGGAATCCCATGGTC-3	95 °C for 10 min, 35 cycles ;95 °C for 30 sec, 61 °C for 30 sec; 72 °C for 30 sec.	G 217 A 134 + 83
Foxp3 rs2232365 A Allele &G Allele	A Allele F: 5′- CCCAGCTCAAG AGACCCCA-3′ R:5′- GGGCTAGTGAGGAGGCTATTGTAAC-3′ G Allele F: 5′- CCAGCTCAAGAGACCCCG-3′ R:5′-GCTATTGTAACAGTCCTGGCAAGTG -3′	95 °C, 3 min; 95 °C, 30 sec; 66 °C, 45 sec; 72 °C, 50 sec; **5 cycle**; 95 °C, 30 sec; 61 °C, 50 sec; 72 °C, 50 sec; **15 cycle**; 95 °C, 50 sec; 61 °C, 1 min; 72 °C, 1.5 min; **15 cycle**; 72 °C, 7 min	A 442 G 427