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Original Article

Genetic Factors of Idiopathic Recurrent Miscarriage in Kazakh Population

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Abstract

Background: It seems that 50% of the possible causes of recurrent miscarriage do not have any explainable etiology and they require in-depth etiopathogenesis analysis. The purpose of this research was to study polymorphisms relationship of the immune response genes including Val249Ile CX3CR1 (rs3732379), CT60 G/A CTLA4 (rs3087243), and HLA DQA1, DQB1, DRB1 (major histocompatibility complex, class II) with development of idiopathic form of recurrent miscarriage (iRM) in Kazakh population.

Methods: TagMan genotyping for 302 patients with iRM and 300 women with normal reproduction was performed. Molecular genetic studies were carried out by the TaqMan method of unified site-specific amplification and real-time genotyping using test systems. Statistical tests and Chi Square were carried out using PLINK, STATA13 software and $p<0.05$ was considered statistically significant.

Results: It has been shown that carriage of unfavorable genotypes (Val/Ile, Val/Val) by the Val249Ile polymorphism of CX3CR1 gene increases the risk of developing iRM by 1.43 times. Search for associations of genes allelic variants of HLA class 2 complex with iRM revealed 501 allele in DQA1 locus, 0301 in DQB1 locus, 10, 12, 15, 16 alleles in DRB1 locus, which increase the risk of developing iRM in Kazakh population.

Conclusion: The highly significant associations of immune response genes with development of iRM in Kazakh population indicate the possible involvement of the immune system interaction of mother cells with syncytiotrophoblast, which is realized by vascular defects and defective embryo implantation, causing termination of pregnancy.

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Introduction

Recurrent miscarriage (RM), classically defined as two or more spontaneous miscarriages up to 20 weeks of pregnancy, is a heterogeneous disorder affecting up to 3% of couples in the reproductive period (1, 2). American Society for Reproductive Medicine (ASRM) considers that two consecutive pregnancy losses are sufficient for diagnosing RM so far as the recurrence rate and risk factors are similar to those observed after three losses (1, 3-6). The independent replication genotyping of genome-wide association studies (GWASs) associated with RM polymerphisms in the Kazakh population did not confirm the genetic contribution of coagulation and cardiovascular system, anti-inflammatory cytokines, apoptosis, and angiogenesis regulating gene polymorphisms in iRM development (7). Gene polymorphism affects blood pressure levels (8). This can lead to one of the most dangerous complications in pregnant women, *i.e*. eclampsia (9). Thus,

the probability of clots in the placenta and recurrent miscarriages at a later date would increase.

Many different researchers (4-6) point to violations of mother and fetus immune tolerance mechanisms in iRM, where the main protective role at the early stages of pregnancy is played by regulatory T cells of the mother's immune system. In the present study, the main objective was to assess the possible contribution of immune response genes for the development of iRM, such as CTLA4 (rs3087243, CT60 G/A), CX3CR1 (rs3732379, Va-l249Ile), HLA DQA1, DQB1, and DRB1 in Kazakh population. Cytotoxic T-Lymphocyte Associated Protein 4 (CTLA-4) is a key immunoregulatory molecule and is involved in intolerance and suppression of T-cell activation (5). One of the co-stimulatory molecules, CTLA-4, is expressed on CD+4 and CD+25 regulatory T cells, which is responsible for their activation and proliferation. It has been shown that decreased expression of CTLA4 in the placenta can weaken the inhibition of activated T-lymphocytes and impair immunity, which leads to RM development (5, 10, 11). Several studies have shown that CTLA4 gene rs3087243 mutant genotypes contributed to CTLA4 level decrease in the blood serum and led to RM (5, 10, 11).

It is known that fractalkine regulates the early stages of pregnancy, promotes blastocyst implantation, and participates in the remodeling of uteroplacental arteries. During the first week of pregnancy, immunohistochemical analysis of endometrium showed maximum expression of fractalkine in uterus glandular epithelium; it activates chemokine receptors and contributes to successful blastocyst implantation (12, 13). The carriage of mutant genotypes reduces production and binding of fractalkine to CX3CR1 receptor, which leads to adhesion and migration disruption of fetal and maternal cells, causing spontaneous abortion (12, 13).

HLA DQA1, DQB1, and DRB1 genes are within the histocompatibility complex, which encodes a large number of immunological proteins, including classic human leukocyte antigen (HLA). In recent years, the role of HLA has been widely studied in the genesis of recurrent miscarriage, which is represented by more than 150 antigens (14-18). Trophoblast antigenic composition is mainly represented by histocompatibility antigens class II (HLA DRB1, DQA1, DQB1), which allows them to be used as immunological markers of RM development (19). There are publications that HLA complex suppresses maternal immune response required for implantation and in the presence of homogeneous histocompatibility complex (common for the mother and father) in inbred populations, the frequency of RM increases (20). The choice of polymorphisms of CTLA4 (rs-3087243, CT60 G/A) and CX3CR1 (rs3732379, Val249Ile) and genes of HLA DQA1, DQB1, and DRB1 (class II major histocompatibility complex) for the analysis of immune processes in iRM is statistically determined by their participation in T-cell tolerance and suppression of significant associations with RM according to GWAS data (2).

Numerous scientific studies indicate possible causes of RM; the common reasons are fetal chromosomal abnormalities, infectious agents, adverse environmental factors, bad habits, anatomical defects, thrombophilic disorders, and thrombotic disorders. However, RM causes in 50% of cases remain unknown (2, 3). These RM cases do not have any explainable etiology and require in-depth etiopathogenesis investigation; thus, they are considered idiopathic RM (iRM). Evidence for a multifactorial genetic etiology of iRM will be increased by eliminating known clinical, laboratory, and environmental risk factors. The absence of large-scale GWASs devoted to iRM is due to several objective reasons; the first is lack of clear iRM definitions, and difficulties in recruitment and small sample size in conjunction with insufficient number of replication studies in ethnically similar populations are the second and third reasons (4-9). In this context, published GWA meta-analyses results were used for "candidate genes" of RM involved in the immune response, coagulation, metabolism, angiogenesis, placental function, and chromosomal segregation (7).

Methods

The current prospective study was carried out during 2019-2020 in the outpatient department of Scientific Center of Obstetrics, Gynecology, and Perinatology. The study group with iRM consisted of 302 women of Kazakh, aged 18-35, who had two or more miscarriages before 12 weeks of pregnancy. The control group was composed of 300 Kazakh women with normal reproductive function, with at least two term pregnancies and without any indication of spontaneous miscarriages (Table 1).

The inclusion criteria for study group with iRM were ethnic belonging and citizenship in Kazakhstan, age range of 18-35, having at least 2 earlier

Table 1. Characteristics of age groups

Age	Number of women (study group)	Number of women (control group)	Total number of people
18-20	22	18	40
21-25	63	67	130
26-30	95	92	187
31-35	122	123	245

spontaneous miscarriages, and confirmed pregnancies by ultrasound and/or pregnancy hormones.

Exclusion criteria were luteal phase abnormalities in the results of endometrial biopsy, anatomical abnormalities of the uterus diagnosed by 3D gynecological scan, hysteroscopy or sonohysteroscopy, balanced chromosomal abnormalities detected by karyotyping of both spouses, the presence of antiphospholipid syndrome, confirmed by anti-beta I2-glycoprotein analysis, anti-cardiolipin (IgG or IgM) antibodies, lupus anticoagulant, multiple pregnancies, confirmed by ultrasound, the presence of sexually transmitted infections, confirmed by two different analyses of various biological materials (enzyme-linked immunosorbent assay, PCR), and thyroid dysfunction according to TSH and thyroid antibodies.

Molecular genetic studies: DNA isolation was performed by separation of M-PVA magnetic particles on Prepitto automatic analyzer (PerkinElmer, USA) for the isolation of ChemagicPrepito nucleic acids (Wallac, Finland) using the PrepitoDNA-CytoPure reagent kit. Molecular genetic studies were carried out by the TaqMan method of unified site-specific amplification and real-time genotyping (Real-Time PCR) using test systems (TestGen, Russia) for molecular genetic studies.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and based on Declaration of Helsinki (1964) and its later amendments or comparable ethical standards. The study was approved by Scientific Center for Obstetrics, Gynecology, and Perinatology on October 23, 2020 (No: 1411-O).

Statistical analysis: Statistical tests and Chi Square were performed using PLINK, STATA13 software. The significance level was considered at $p<0.05$. Differences in allele and genotype frequencies were assessed using Chi Square with odds ratio (OR) (21).

Results

As shown in table 2, replication studies in Kazakh population revealed a significant association of alleles and genotypes (rs3087243) of CTLA4 gene polymorphism with the risk of iRM development ($p>0.05$). The frequency of allele A was quite high in groups (29.47% in study group and 33.83% in control group ($\chi^2=2.65$; $p>0.05$)). The noteworthy feature was the low frequency of the mutant AA genotype in study group which was 6.95% and the differences were not statistically significant ($\chi^2=3.47$; $p=0.06$).

Table 2. Frequency of alleles and genotypes of immune response genes in the group with iRM and control group

Gene/polymorphism	Genotypes/alleles	Study group (N=302) n (%)	Control group (N=300) n (%)	p-value
CTLA4 (CT60 G/A)				
	GG	145 (48.0%)	131 (43.7%)	0.285
	GA	136 (45.0%)	135 (45.0%)	0.994
	AA	21 (7.0%)	34 (11.3%)	0.063
	G	426 (70.5%)	397 (66.2%)	0.121
	A	178 (29.5%)	203 (33.8%)	0.104
CX3CR1 (Val249Ile)				
	Ile/Ile	181 (60.0%)	208 (69.3%)	0.016
	Val/Ile	109 (36.1%)	85 (28.3%)	0.042
	Val/Val	12 (3.9%)	7 (2.4%)	0.250
	Ile	471 (78.0%)	501 (83.5%)	0.015
	Val	133 (22.0%)	99 (16.5%)	0.016

Table 3. Allelic frequencies of HLA DQA1, DQB1, and DRB1 genes in the study and control groups

Gene/polymorphism	Genotypes/alleles	Study group n (%)	Control group n (%)	p<0.05	OR DI 95%
HLA DQA1					
	0101	92 (15.2%)	90 (15.0%)	0.911	1.02 (0.74-1.39)
	0102	80 (13.3%)	87 (14.5%)	0.529	0.90 (0.65-1.25)
	0103	60 (9.9%)	75 (12.5%)	0.159	0.77 (0.54-1.11)
	0201	87 (14.4%)	97 (16.2%)	0.396	0.87 (0.64-1.20)
	0301	87 (14.4%)	81 (13.5%)	0.651	1.08 (0.78-1.49)
	0401	20 (3.3%)	22 (3.7%)	0.737	0.90 (0.49-1.67)
	0501	166 (27.5%)	132 (22.0%)	0.028	1.34 (1.03-1.75)
	0601	12 (2.0%)	16 (2.6%)	0.434	0.74 (0.35-1.58)
HLA DQB1					
	02	123 (20.4%)	120 (20.0%)	0.875	1.02 (0.77-1.36)
	03	8 (1.3%)	22 (3.7%)	0.010	2.90 (0.16-0.80)
	0301	135 (22.4%)	103 (17.2%)	0.024	1.39 (1.04-1.85)
	0302	40 (6.6%)	54 (9.0%)	0.125	0.72 (0.47-1.10)
	0303	28 (4.6%)	29 (4.8%)	0.872	0.96 (0.56-1.63)
	0304	4 (0.7%)	2 (0.3%)	0.418	1.99 (0.36-10.9)
	0305	3 (0.5%)	3 (0.5%)	0.994	0.99 (0.20-4.94)
	0401/0402	31 (5.1%)	32 (5.3%)	0.876	0.96 (0.58-1.59)
	05	11 (1.8%)	14 (2.3%)	0.534	0.78 (0.35-1.72)
	0501	47 (7.8%)	48 (8.0%)	0.889	0.97 (0.64-1.48)
	0502/0504	27 (4.5%)	17 (2.8%)	0.131	1.61 (0.87-2.98)
	0503	19 (3.2%)	25 (4.2%)	0.346	0.75 (0.41-1.37)
	0601	24 (3.9%)	19 (3.2%)	0.451	1.27 (0.69-2.34)
	0602-8	104 (17.2%)	112 (18.7%)	0.513	0.91 (0.68-1.22)
HLA DRB1					
	01	29 (4.8%)	43 (7.2%)	0.840	0.65 (0.40-1.06)
	03	57 (9.4%)	51 (8.5%)	0.570	1.12 (0.76-1.67)
	04	87 (14.4%)	94 (15.7%)	0.540	0.91 (0.66-1.24)
	07	86 (14.2%)	84 (14.0%)	0.906	1.02 (0.74-1.41)
	08	32 (5.3%)	35 (5.8%)	0.686	0.90 (0.55-1.48)
	09	31 (5.1%)	34 (5.7%)	0.682	0.90 (0.55-1.49)
	10	15 (2.5%)	5 (0.8%)	0.026	3.03 (1.09-8.39)
	11	55 (9.1%)	53 (8.8%)	0.869	1.03 (0.69-1.54)
	12	37 (6.1%)	21 (3.5%)	0.034	1.80 (1.04-3.11)
	13	65 (10.8%)	86 (14.3%)	0.062	0.72 (0.51-1.02)
	14	38 (6.3%)	51 (8.5%)	0.144	0.72 (0.47-1.12)
	15	63 (10.5%)	41 (6.9%)	0.027	1.59 (1.05-2.39)
	16	9 (1.5%)	2 (0.3%)	0.035	4.52 (0.97-21.2)

As shown in table 2, statistically significant differences were obtained in frequency of alleles and genotypes of Val249Ile fractalkine receptor 1 CX3CR1 polymorphism in the compared groups (p<0.05). The frequency of the minor Val allele in iRM group was significantly higher, equal to 22.02% (χ^2=5.89; p=0.02). Accordingly, homozygous wild-type Ile/Ile genotype was found in the group of women with iRM (59.93%), which was significantly lower than the control group (69.93%, p=0.02). Significant differences were not found in unfavorable homozygous Val/Val genotype frequency (χ^2=1.33; p=0.25), which is probably due to its low frequency in both compared groups (3.97 and 2.33, respectively). As shown in table 2, carriage of unfavorable genotypes (Val/Ile, Val/Val) for the Val249Ile CX3CR1 gene polymorphism increases the risk of iRM development by

1.43 times (OR=1.43; 95% CI=1.03-2.02).

Statistical analysis using PLINK includes the calculation of associations based on various models. The results of tests performed on our sample data, including genotypic (GENO), additive (TREND), allelic (ALLELIC), and dominant (DOM) models, revealed significant associations with iRM, which imply a specific relationship between genotype and phenotype for the CX3CR1 gene immune response (rs3732379, Val249Ile) (χ^2= 6.15; 6.11, 5.89, and 5.82; p<0.00001, according to the models). Table 3 shows the results of the possible contribution analysis for the multi-allelic loci of HLA DQA1, DQB1, DRB1 in development of iRM among Kazakh population.

Pairwise comparison analysis of variant allele frequencies in HLA DQA1 gene revealed a significant excess of 0501 allele carriage in study group (27.48%) and in control group (22.00%, χ^2=4.86; p=0.03). There was a significant association between HLA DQA1 gene 0501 allele with the risk of iRM development in the Kazakh population (OR=1.34; 95% CI=1.03-1.75.) Table 3 shows that the most frequent HLA DQB1 gene allelic variant in iRM group was allele 0301, which was 22.35% and significantly higher than the same indicator in the control group (17.17%, χ^2=5.10; p=0.02). As shown by our calculations, the carriage of allele 0301 increases the risk of iRM development in the Kazakh population by 1.4 times (OR=1.43; 95% CI=1.04-1.85). The frequency of protective HLA DQB1 gene allele 03 was 1.32%, which turned out to be significantly lower in the group of women with normal reproduction (3.67%, χ^2=6.80; p=0.01). Carriage of the protective allele 03 of the main histocompatibility complex class II, HLA DQB1 gene, reduces the risk of iRM development by 2.9 times (OR=2.90; 95% CI=1.27-6.64) (Table 3). In the HLA DRB1 gene, four allelic variants were found; they were reliably associated with iRM development (10, 12, 15, 16). Frequency of carriage in study group was significantly higher than similar values in control group (p<0.05). Carriage of allele 10 increases the risk of iRM development by 3 times, allele 12 about 1.8 times, and allele 15 about 1.6 times. The highest risk was found with the rare 16 allele, which increases the risk of iRM development by 4.5 times (Table 3).

Discussion

In the available scientific literature, only one population-based retrospective study, conducted in North India, was found which revealed highly significant associations of CX3CR1 gene polymorphism (rs3732379) with iRM development (22). Moreover, significant differences with a high carriage frequency in unfavorable genotypes, CX3CR1 rs3732379 gene, were found in iRM group. It may indicate the involvement of the immune response system in the process of implantation through an insufficient expression of CX3CR1 receptor in invasive trophoblast cells. This, in turn, disrupts the immune processes of maternal cells' interaction with syncytiotrophoblast and leads to termination of pregnancy. Also, only one large study conducted by Wang et al. (2005) in the Chinese population revealed a significant contribution of the studied polymorphism to the immunopathogenesis of iRM (23).

Scientific literature demonstrates the role of carriage of homozygous genotypes for the risk alleles of HLA class II, HLA DQA1, DQB1, DRB1 genes, in the development of RM, which was confirmed by studies on several other diseases (24-26). In Gordeeva et al.'s (27) study, occurrence of homozygotes was significantly higher for the HLA DRB1 04 allele frequency in women with iRM, which suggests the contribution of homozygosity for risk alleles in incidence of reproductive losses. The frequency for homozygous genotypes, 0501/0501 HLA DQA1 gene, was 10.92% in study group and 6.67% in control group, but the differences were not statistically significant (p>0.05). Small number of homozygous genotypes for the rare alleles 10, 12, 16 of the HLA DRB1 gene was found in study and control groups. Significant differences were observed in homozygous genotype of the HLA DQB1 0301/0301 gene (7.28% in women with iRM and 3.00% (χ^2=5.66; p= 0.018) in control group). The results obtained suggest an increased iRM development with the carriage of two risk alleles. The complexity of the immunological relationship between mother and fetus during pregnancy dictates the need to continue research and study additional SNP genes of the immune response.

The results obtained by Tkach et al. (28) during genotyping of Ukrainian women with RM (HLA DQB1 0301 and DQA1 0501) were similar to Kazakh population. Iskhakov et al. (29) showed a significant contribution of HLA DRB1 gene 15 allele to the increased risk of RM development. It should be noted that in several large studies of Russian women with iRM, contribution of HLA class II genotypes, alleles, and genotypes was

identified which did not coincide with our results (29, 30).

Conclusion

This study confirms the need to conduct independent replication studies in each separate population since genetic polymorphisms are associated with the geographical, ethnic, and historical characteristics of each group. It is impossible to extrapolate the highly significant associations of even large-scale GWA analyses and use them as genetic markers for iRM without replication genotyping, as this would lead to false-positive results. The independent replication genotyping in GWAS excluded the genetic contribution of coagulation and cardiovascular system, anti-inflammatory cytokines, apoptosis, and angiogenesis genes in iRM development among Kazakh population. For the first time, the current research was conducted to determine the etiology of idiopathic forms of RM as disorders of the immune interaction of maternal cells with syncytiotrophoblast, as well as the carriage of HLA class II complex risk alleles.

Conflict of Interest

Authors declare no conflict of interest.

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