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Association between idiopathic recurrent pregnancy loss and genetic polymorphisms in cytokine and matrix metalloproteinase genes

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ABSTRACT

Objectives: Recurrent reproductive loss (RPL) is a global health issue affecting a significant number of women. Approximately half of miscarriages have an unexplained etiology. Familial aggregation and twins studies prove that some cases of the RPL could have a genetic background. Recent evidences suggest that cytokines (*e.g.* IL-6, TNF alpha or TGF beta) and matrix metalloproteinases (MMP) are important for maintenance of pregnancy. Single gene polymorphisms (SNP), affecting these proteins production or their function may predispose to the loss of the pregnancy. The aim of this study was to evaluate the association between the following polymorphisms of *IL6* (rs1800795), *TNF* (rs1800629), *TGFB1* (rs1800471), *MMP1* (rs1799750), *MMP2* (rs2285053 and rs243865), *MMP3* (rs35068180), *MMP9* (rs3918242) and the recurrent pregnancy loss in polish population.

Material and methods: Study subjects comprised of 67 patients with a history of recurrent pregnancy loss (≥ 2 miscarriages in history) and 75 controls. The distribution of genotypes for selected polymorphisms were determined by RFLP-PCR.

Results: Maternal genotypes *GG TNF*, or *5A/5A MMP3* may be associated with the recurrent pregnancy loss. No association between the *IL6*, *TGFB1*, *MMP1*, *MMP2*, or *MMP9* studied polymorphisms and the predisposition to miscarriage was found.

Conclusions: This study demonstrated a possible association between rs1800629 *TNF*, rs35068180 *MMP3* polymorphisms and recurrent pregnancy loss.

Key words: recurrent pregnancy loss (RPL); genetic polymorphisms; cytokines; matrix metalloproteinases (MMP)

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INTRODUCTION

Recurrent pregnancy loss (RPL) is a reproductive disorder, which affects approximately 1–5% of couples [1, 2]. Miscarriages occur in 10–15% and even up to 30% of pregnancies [3, 4]. Repeatability of recurrent reproductive wastage in a certain number of couples shows that the phenomenon is not random and urges to define a cause. Genetic variation may have a high impact on reproductive failure and thus delineating of specific genetic factors is of great importance for genetic counselling.

RPL is traditionally defined as the occurrence of three or more (≥ 3) consecutive pregnancy losses before 20 weeks of gestation. However, due to the growing problem of infertility, global and European scientific societies including the American Society of Reproductive Medicine (ASRM) and European Society of Human Reproduction (ESRE), has recently redefined RPL as two or more pregnancy losses [5, 6]. The aetiology of the disease comprises of different factors, such as autoimmune diseases (20%), endocrinological disorders (17–20%), uterine alterations (10–15%), genetic factors such as chromosome abnormalities in the parents (2–5%) and infections (0.5–5%) [7]. Nevertheless, approximately 50% of RPL cases remain unexplained and defined as idiopathic [3, 4].

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Familial predisposition to RPL is described [8, 9]. Clinical data suggest from two to sevenfold increased prevalence of RPL among first-degree blood relatives compared to the general population [9]. Population-based register studies showed that overall frequency of miscarriage among the siblings of idiopathic RPL is approximately doubled compared to general population [10, 11]. A genome-wide linkage scan using sibling pairs with idiopathic RPL confirmed heterogeneity of contributing genetic factors [11, 12]. Moreover, some studies emphasize that not only maternally, but also paternally inherited genetic factors might influence the miscarriages [12–14].

A successful pregnancy is the result of a number of processes including implantation, decidual tissue and vessel remodeling and maternal-fetus immune tolerance [15]. Each of these phenomena may be genetically determined. Recent research results show that cytokines and extracellular matrix metalloproteinases (MMPs) are important determinants of the opening of the implantation window and the proper invasion of trophoblast into the uterine wall and maintenance of pregnancy [16–19].

The maintenance of pregnancy depends on the balance between Th1 and Th2 cells [20]. It was proven that domination of the anti-inflammatory Th2 cytokine pattern is associated with gestational success/normal pregnancy, whereas a pro-inflammatory Th1 cytokine profile is related to the pregnancy failure [20].

Communication between trophoblastic and decidual cells is mediated by cytokines *e.g.* IL6, TNFα, TGFβ [21]. The cytokines production undergo the genetic control, and the genetic polymorphisms might influence the modulation of their expression therefore it may be at least partially responsible for the incidence of unexplained recurrent pregnancy losses [22].

Metalloproteinases belong to a large family of zinc-dependent endopeptidases that include collagenases (MMP1, MMP8, and MMP13), gelatinases (MMP2, MMP9), stromelysins (MMP3, MMP10), matrilysins (MMP7, MMP26), and transmembrane metalloproteinases types I and II. MMPs are crucial regulators of vascular and uterine remodeling and are involved in spiral artery formation and adhesion [23].

Changes in the nucleotide sequences in the binding sites of transcription factors or transcription repressors alter the regulation of MMP gene expression and the level of their protein products. The imbalance in the MMPs level disurbes the process of implantation and placenation [24].

Objectives

The aim of this study is to evaluate the association between the following polymorphisms: rs1800795 (*IL6* gene), rs1800629 (*TNF* gene), rs1800471 (*TGFB1* gene), rs1799750 (*MMP1* gene), rs2285053 and rs243865 (*MMP2* gene), rs35068180 (*MMP3* gene), rs3918242 (*MMP9* gene) and the recurrent pregnancy loss in case and control groups.

MATERIAL AND METHODS

Blood samples were collected from 67 women with a history of two or more consecutive spontaneous abortions (mean age of 32.85 ± 5.53 years old) and 75 healthy women who had a history of successful pregnancy (mean age of 35.11 ± 3.98 years old). Case groups were enrolled between September 2016 and June 2018 in Department of Genetics, Polish Mother's Memorial Hospital Research Institute in Łódź and Department of Obstetrics and Perinatology, Jagiellonian Medical University of Cracow, Poland.

All members of the study and control groups were Caucasians and residents of Poland, with no immunological diseases, weight disorders [obesity body mass index (BMI) < 30 kg/m^2], hypertension, diabetes or coagulation disorders.

The study was positively evaluated by the Bioethics Committee at the Polish Mother's Memorial Hospital Research Institute in Łódź. All participants were informed of the study protocol and completed a consent form before participating to the study.

DNA extraction

Peripheral venous blood samples (3–5 mL) from patients with RPL and controls were collected into EDTA-coated vacutainers. Genomic DNA was isolated from peripheral blood leukocytes by standard procedures using a commercially available kits Blood Mini Kit and Genomic Midi AX (A&A Biotechnology, Poland). The concentration and quality of the DNA were examined by optical density in a spectrophotometer NanoDrop 2000 (ThermoFisher Scientific, USA).

Genotyping

The above-mentioned polymorphic variants were genotyped by polymerase chain reaction — restriction fragment length polymorphism (PCR-RFLP). PCR reaction conditions was optimized for each polymorphism. Characteristic of the polymorphisms and the specific primer sequences are shown in Table 1.

Statistical Analyses

Data analysis was performed using Statistica v12 (StatSoft, Tulsa, OK). The Hardy–Weinberg equilibrium was tested in control group. Comparisons of variables with a categorizing distribution were made using the *chi*^{2} test or the Yates corrected *chi*^{2} test or the Fisher bilateral test. Variables relevant for single-factor comparisons were introduced to the regression model. To multifactor analysis (logistic regression) those parameters were introduced, which in univariate analyses obtained the significance

Table 1. Primer sequences, annealing temperatures, and restriction enzymes of the polymorphisms

SNP-ID	Polymorphism	Primer sequences 5′→3′	Annealing temperature	Restriction enzyme	Restriction products (bp)
MMP1 rs1799750	-16071G>2G	F: GAGTATATCTGCCACTCCTTGAC R: CTTGGATTGATTTGAGATAAGTCATA	53°C	AluI	*G1/G1*-288 *G1/G2*-262, 288 *G2/G2*-262
MMP2 rs2285053	-735C>T	F: GGTGGGTGCTTCCTTTAACATG R: GTAAAATGAGGCTGAGACCTGC	60°C	HinfI	*CC*-247 *CT*-203, 247 *TT*-203
MMP2 rs243865	-1306C>T	F: CTTCCTAGGCTGGTCCTTACTG R: GCTGAGACCTGAAGAGCCA	56°C	BstXI	*CC*-194 *CT*-170, 194 *TT*-170
MMP3 rs35068180	-11715A>6A	F: CATTCCTTTGATGGGGGGAAAGA R: GAAGGAATTAGAGCTGCCACAGC	60°C	Tth111I	*6A/6A*-194 *6A/5A*-170, 194 *5A/5A*-170
MMP9 rs3918242	-1562C>T	F:GCAGATCACTTGAGTCAGAAGTTC R: GGGAAAAACCTGCTAACAACTC	63°C	SphI	*CC*-286 *CT*-188, 286 *T T*-188
IL6 rs1800795	-174G>C	F: GTCAAGACATGCCAAAGTGCT R: GAGGGGCTGATTGGAAACC	60°C	NlaIII	*GG*-173, 11 *GC*-173, 122, 51, 11 *CC*-122, 51, 11
TNF *alpha* rs1800629	-308G>A	F: GGCAATAGGTTTTGAGGGCCA R: CCTTCTGTCTCGGTTTCTTCTCC	60°C	NcoI	*GG*-177, 19 *GA*-197, 177, 19 *AA*-197, 19
TGFB1 rs1800471	915C>G Arg25Pro	F: CACACCAGCCCTGTTCGC R: CTTCCGCTTCACCAGCTCCAT	65°C	BglI	*CC*-142, 103, 60 *CG*-163, 142, 103, 60 *GG*-163, 142

level $p < 0.15$. Multivariate analysis was performed with backward stepwise logistic regression. Results for which p was < 0.05 were considered statistically significant. For allele carriers and genotypes whose frequencies differed between groups, the odds ratio (OR) was calculated with a 95% confidence interval (CI).

RESULTS

The distribution of genotypes and alleles for the eight investigated polymorphisms and deviation from Hardy–Weinberg equilibrium in RPL cases and in control group are shown in Table 2.

Among the 8 polymorphisms, only 3: rs1800629 (*TNF* gene), rs243865 (*MMP2* gene) and rs35068180 (*MMP3* gene) demonstrated a significant association with risk of recurrent pregnancy loss. Significant statistically relevant maternal genotypes were included in the multivariable analysis. The results of this analysis are presented in Table 3.

These analyses revealed that *GG* homozygosity in *TNF* rs1800629 increases over 2.5 times the risk of RPL (OR = 2.56, 95% CI: 1.23–5.32; p = 0,0002). We also observed *MMP3* rs35068180 homozygosity *5A/5A* decreases the risk of RPL 0.24-fold (OR = 0.24, 95% CI: 0.11–0.52).

Based on univariate analysis, the statistical significance of the data for the analysis of the rs243865 polymorphic variant of the *MMP2* gene was not confirmed.

No associations between occurrence of recurrent pregnancy loss and the distribution of genotypes or alleles of studied *IL6, TGFB1, MMP1, MMP9* and *MMP2* rs2285053 gene polymorphisms were observed.

DISCUSSION

The regulation of cytokine and metalloproteinase secretion in the maternal-fetal interface plays a pivotal role in the process of trophoblast invasion and placentation.

The present study examines whether the occurrence of eight single-nucleotide polymorphisms (SNPs) in the *IL6, TNF, TGFB1, MMP1, MMP2, MMP3* and *MMP9* genes is related to the recurrent pregnancy loss. We observed that the three following polymorphisms: *TNF* rs1800629, *MMP2* rs243865 and *MMP3* rs35068180 are associated with the recurrent pregnancy loss.

Tumor necrosis factor α (TNF alpha) is a cytokine associated with the regulation of a wide spectrum of biological processes, including inflammation, cell proliferation and apoptosis. This multifunctional proinflammatory cytokine is produced mainly by the active monocytes and macrophages and by other cells (adipocytes, keratinocytes, fibroblasts, neutrophils, mast cells and some lymphocytes).

The *TNF* gene is located on chromosome 6p21.33. Changes in the nucleotide sequence of the gene in its promoter part are very important because the expression of the *TNF*

Table 2. Distribution of genotypes between cases and controls in compliance with Hardy-Weinberg law

Polymorphism SNP-ID	Genotypes alleles	Cases n = 67	Controls n = 75	pHWE	p
***MMP1* rs1799750**	1G/1G	21	22	0.05	0.794
	1G/2G	26	29		0.986
	2G/2G	20	24		0.782
	2G	66	77		0.849
	1G	68	73		0.849
***MMP2* rs2285053**	CC	51	60	0.233	0.576
	CT	16	13		0.339
	TT	0	2		0.526
	T	16	17		0.812
	C	118	133		0.812
***MMP2* rs243865**	CC	7	1	0.000	0.044
	CT	60	70		0.593
	TT	0	4		0.162
	T	60	78		0.428
	C	74	72		0.428
***MMP3* rs35068180**	5A/5A	16	43	0.000	0.00005
	5A/6A	34	15		0.0001
	6A/6A	17	17		0.706
	5A	66	101		0.002
	6A	68	49		0.002
***MMP9* rs3918242**	CC	49	58	0.268	0.562
	CT	14	17		0.799
	TT	4	0		0.101
	T	22	17		0.576
	C	112	133		0.576
***IL6* rs1800795**	CC	17	22	0.662	0.597
	CG	37	39		0.700
	GG	13	14		0.911
	G	63	67		0.743
	C	71	83		0.743
***TNF alpha* rs1800629**	GG	46	33	0.003	0.003
	GA	20	41		0.003
	AA	1	1		0.526
	A	22	43		0.014
	G	112	107		0.014
***TGFB1* rs1800471**	CC	56	65	0.536	0.779
	CG	10	10		0.975
	GG	1	0		0.954
	G	12	10		0.494
	C	122	140		0.494

For p value analysis, the chi2 or Yates' corrected chi2 tests were used; HWE — Hardy–Weinberg equilibrium

Table 3. Logistic regression results determining the chance of a miscarriage

Parameter	OR	95% CI	p
5A/5A MMP3	0.24	0.11 – 0.52	0.0002
GG TNF alpha	2.56	1.23 – 5.32	0.011

OR — odds ratio; CI — confidence interval

gene is mainly regulated at the transcription level. The *TNF* gene polymorphism of greatest interest is the transition of the guanine into the adenine at the position of –308. This polymorphism identified as rs1800629 is associated with increased expression of *TNF*, probably by changing the binding efficiency of transcription factor AP 2 [25].

Among the three polymorphisms in cytokine genes investigated in this study, only *TNF* rs1800629 was found to be significantly associated with an increased risk of recurrent pregnancy loss. The analysis shows that patients with *GG* genotype for rs1800629 polymorphism have a higher risk of miscarriage according to controls (OR = 2.56, 95% CI: 1.23–5.32).

A meta-analysis performed by a group from Iran showed a correlation of the rs1800629 polymorphism of the *TNF* gene promoter region with an increased risk of reproductive failure [6]. Moreover, it suggests that the investigated polymorphic variant is more significant in Asians than in Caucasians.

One of the purposes of this study was also to verify if the occurrence of five SNPs in the matrix metalloproteinase genes is related to the miscarriage predisposition.

Gelatinases A (MMP2) and B (MMP9) digests collagen type IV and V, elastin and other extracellular matrix proteins (ECM), which indicates their important role in the metabolism of vessel basement membrane.

MMP2 is encoded by the matrix metalloproteinase 2 gene (*MMP2*) located on chromosome 16q12.2. Two polymorphic variants of this gene rs2285053 and rs243865 are located in the promoter region and are responsible for regulating the expression of the *MMP2* gene and thus may affect the amount of synthesized protein. The presence of thymidine at positions –735 and –1306 in the *MMP2* gene promoter region prevents binding to the transcriptional factor Sp-1, thus reducing the activity of the *MMP2* promoter [26].

We did not find an association between the studied polymorphism of *MMP2* gene (rs2285053) and risk of recurrent pregnancy loss. These observations were similar to the results presented by Li et al., and. Behforouz et al. [19, 23]. However, not all studies are in consistency with results of the present study. Pereza et al., indicated that polymorphism rs2285053 was associated with recurrent miscarriage risk [27].

Results of our study referring to rs243865 *MMP2* gene polymorphism after univariate analysis did not identify any significant relationship for genotype nor for allele distribution between cases and control. They agrees with the results of Behforouz et al. [23], Pereza et al. [27], and Ramu et al. [28]. In contrast Li et al. [19], showed that the polymorphism rs243865 were significantly associated with an increased susceptibility to recurrent miscarriages.

Stromielizines, which include MMP3 (stromielizine-1), digest basement membrane collagen, proteoglycans and extracellular matrix glycoproteins. The stromielzine-1 gene is located on chromosome 11q22.3. The polymorphism rs35068180 of the *MMP3* gene occurs at –1171 in the promoter region and is associated with increased transcription and local expression of the *MMP3* gene [29]. This common polymorphism, which was identified by Ye et al. [30], in 1996, has one allele with a sequence of six adenosine (*6A*) and another five adenosine (*5A*). In vitro studies have shown that the *5A* allele is associated with a higher expression of the *MMP3* gene compared to the *6A* allele [29, 30].

Our results show that the maternal polymorphism rs35068180 *MMP3* gene occur significantly more frequently in RPL cases. Multifactorial analysis showed that patients with the *5A/5A* genotype for the rs35068180 polymorphism of the *MMP3* gene are about 0.24 less likely to experience a miscarriage OR (95% CI) = 0.24 (0.11–0.52). This relationship may result from the fact that *5A* allele carriers, associated with higher transcriptional activity of *MMP3* gene, are characterized by higher degradation of the extracellular matrix. As a result, *5A* allele predisposes to the successful of implantation and consequently reduces the risk of pregnancy loss.

Our findings confirm the results of Behforouz et al. [23]. They have found a significant association between rs35068180 polymorphism of *MMP3* gene and the pregnancy loss. The findings of this study are in keeping with the reports of Balci and Özdemir [31].

CONCLUSIONS

In the present study, we have for the first time investigated an association between recurrent pregnancy loss and the profile of eight selected single nucleotide polymorphisms in cytokine and metalloproteinase genes in women in the polish population.

In conclusion, this work has demonstrated an association between *TNF* rs1800629 and *MMP3* rs35068180 gene polymorphisms and recurrent pregnancy loss. Our results show that the maternal *GG TNF* and *5A/5A MMP3* gene genotypes occur significantly more frequently in cases with repeated miscarriages.

Limitations of the Study

The study included a relatively small number of patients, and the findings need to be confirmed in a larger population.

Conflicts of Interest

The authors declare no conflict of interests.

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