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Glutathione S-transferase polymorphisms and their role in recurrent pregnancy loss: A genetic risk assessment

Glutatyon S-transferaz polimorfizmleri ve bunların tekrarlayan gebelik kaybındaki rolü: Genetik bir risk değerlendirmesi

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Abstract

Objective: The frequency of recurrent pregnancy loss in society is 3-5%. Experts suggest that genetics account for over 80% of unexplained recurrent pregnancy loss. Glutathione S-transferase (GST) enzymes, regulated by GST genes, facilitate the detoxification of a variety of naturally occurring metabolites as well as environmentally derived chemicals. This research aimed to investigate *GST* gene polymorphisms as a potential risk factor in recurrent pregnancy loss etiology in the Turkish population.

Materials and Methods: This study involved 107 recurrent pregnancy loss patients who sought treatment at the Sivas Cumhuriyet University Faculty of Medicine, Department of Medical Genetics, along with a control group of 107 individuals who had a successful birth and no previous history of miscarriage. The multiplex polymerase chain reaction and restriction fragment length polymorphism techniques were employed to analyze *GSTM1, GSTT1 and GSTP1* gene polymorphisms in these cases.

Results: GSTT1 null genotype (X^2=4.74; p=0.029) and GSTT1/GSM1 null genotype (X^2=3.333; p=0.047) were associated with statistically significant differences between the study groups. No statistical significance was detected when considering the GSTM1 null genotype (X^2=3.326; p=0.068) or the *GSTM1/GSTP1* and *GSTT1/GSTP1* gene polymorphisms.

Conclusion: A statistically significant association was observed between the GSTT1 null genotype and the diseased group. Our research demonstrated a substantial increase in the risk of recurrent pregnancy loss in the Turkish population, specifically among individuals with the GSTM1-null genotype. No statistical correlation was found between the *GSTM1* and *GSTP1* gene polymorphisms and recurrent pregnancy loss. Furthermore, no statistical significance was observed when they were assessed together.

Keywords: Glutathione S-transferase, GSTM1, GSTT1, GSTP1, recurrent pregnancy loss

Öz

Amaç: Toplumda tekrarlayan gebelik kaybı sıklığı %3-5'tir. Uzmanlar, açıklanamayan tekrarlayan gebelik kayıplarının %80'inden fazlasının genetiğe bağlı olduğunu ileri sürmektedir. GST genleri tarafından kodlanan glutatyon s-transferaz (GST) enzimleri, çevresel olarak türetilen kimyasalların yanı sıra çeşitli doğal olarak oluşan metabolitlerin detoksifikasyonundan sorumludur. Bu çalışma, Türk toplumunda tekrarlayan gebelik kayıplarının etiyolojisinde bir risk faktörü olarak *GST* gen polimorfizmlerini araştırmayı amaçlamaktadır.

PRECIS: In the investigation of GSTM1, GSTT1, GSTP1 polymorphisms of the Glutathione S-Transferase (GST) genes in Patients with Recurrent Pregnancy Loss, statistically significant differences were detected indicating an increased risk ratio in the groups with GSTT1 and GSTT1/GSM1 null genotype.

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Gereç ve Yöntemler: Bu çalışmaya Sivas Cumhuriyet Üniversitesi Tıp Fakültesi, Tıbbi Genetik Bölümü'nde tedavi gören 107 tekrarlayan gebelik kaybı hastası ve daha önce düşük yapmamış ve başarılı bir doğum yapmış 107 kişiden oluşan bir kontrol grubu dahil edildi. Bu olgulardaki *GSTM1, GSTT1* ve *GSTP1* gen polimorfizmlerini multipleks polimeraz zincir reaksiyonu ve kısıtlama parça uzunluğu polimorfizmi yöntemi kullanarak araştırdık.

Bulgular: GSTT1 null genotipi (X^2=4,74; p=0,029) ve GSTT1/GSM1 null genotipi (X^2=3.333; p=0,047) çalışma grupları arasında istatistiksel olarak anlamlıydı. GSTM1 null genotipi (X^2=3.326; p=0,068), ***GSTT1/GSTP1*** ve ***GSTM1/GSTP1*** gen polimorfizmleri dikkate alındığında istatistiksel olarak anlamlı bir fark saptanmadı.

Sonuç: GSTT1 null genotipi hasta grubunda istatistiksel olarak anlamlıydı. Araştırmamız Türk popülasyonunda, özellikle GSTM1 ve null genotiplere sahip bireyler arasında tekrarlayan gebelik kaybı riskinde önemli bir artış olduğunu gösterdi. ***GSTM1*** ve ***GSTP1*** genlerinin polimorfizmleri ile tekrarlayan gebelik kaybı arasında istatistiksel bir korelasyon bulunamadı. Ayrıca, birlikte değerlendirildiğinde istatistiksel olarak anlamlı bir fark saptanamadı.

Anahtar kelimeler: Glutatyon S-transferaz, GSTM1, GSTT1, GSTP1, tekrarlayan gebelik kaybı

Introduction

Recurrent pregnancy loss is characterized by two or more antecedent pregnancy losses before 24 weeks gestation[1,2]. The most commonly identified cause of explained pregnancy loss is fetal chromosomal abnormalities, which are responsible for about 70% of spontaneous abortions and 30-50% of recurrent losses[1-6].

In at least 50% of recurrent pregnancy loss, the reason cannot be determined by any diagnostic test and is thought to be idiopathic. Environmental and lifestyle-related risk factors cause genetic susceptibility to recurrent pregnancy loss[3-6]. Despite the proposal of numerous etiological factors, the etiology of recurrent pregnancy loss remains elusive. In recurrent pregnancy loss, the balance between phase I and II enzyme systems in response to endogenous and exogenous substances is influenced by the genetic variability of individual metabolic detoxification activation[7]. Many genetic studies have recently discovered a link between genetic polymorphisms relating to metabolic enzymes and recurrent pregnancy loss. Phase I enzymes like cytochrome P450-1A1 (CYP-1A1) activate many potentially toxic compounds, including those found in cigarettes, coffee, and alcohol, to form the final reactive compound[8,9]. Phase II enzymes, particularly glutathione-S-transferases (GST), have a key impact on detoxifying these active forms. Activation of toxins by phase I enzymes increases oxidative stress, and these toxins are then eliminated by the phase II detoxification system utilizing glutathione. Cytosolic GST is categorized into four primary classes: pi (P), alpha (A), mu (M), and theta (T)[10]. Each consists of one or more isoenzymes. Genetic differences in the GST and CYP-1A1 enzymes may impact the equilibrium between the phase I and II biotransformation pathways. This could explain why some people are more or less likely to get diseases linked to smoking, alcohol, coffee, or other toxins[7]. In this regard, phase I and II biotransformation enzymes are believed to be relevant in individuals with recurrent pregnancy loss. GSTT1 and GSTM1, glutathione detoxification pathway enzymes, shield the embryo against oxidative stress[8].

Recurrent pregnancy loss is a multifactorial disease affected by various epidemiological risk factors such as diabetes, genetic mutations, uterine structural anomalies, genital infections, maternal age, coffee, smoking, alcohol, and chemical use. An abnormal placenta causes the production of reactive oxygen products and results in harmful effects which invade the embryo. It is hypothesized that oxidative stress contributes to the etiopathogenesis of abortion[10]. This study aimed to determine if *GST* gene polymorphisms increase the risk of recurrent pregnancy loss in Turkish females.

Materials and Methods

Study Population

This prospective cohort study recruited 214 participants from January 15 to March 2015. The study group included 107 patients who had been admitted to Sivas Cumhuriyet University Faculty of Medicine, Department of Medical Genetics. They experienced three or more consecutive miscarriages. The control group comprised 107 healthy female controls with no history of miscarriage or infertility. Patients with two or more of the unexplained recurrent pregnancy loss diagnoses were included in the study. Female patients with less than 2 abortions and those patients with abortions due to anatomical or endocrine reasons were excluded from the study. The healthy control group included women with no history of abortion.

Patients with anatomical, infectious, or systemic diseases that could contribute to recurrent pregnancy loss, those with chromosomal abnormalities in themselves or their spouses, and those who either declined participation or did not provide written informed consent were excluded from the study.

This study design was based on the principles of the Declaration of Helsinki. The study received ethical approval from the Sivas Cumhuriyet University Research Ethics Committee (approval number: 2013-09/15, date: 24.09.2013). Verbal and written consent of all participants were obtained.

DNA Extraction

Restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR) techniques were used to analyze deletions in the *GSTM1* and *GSTT1* genes and the exon 5 Ile105Val polymorphism in the *GSTP1* gene. Peripheral venous blood samples of 3 mL were collected from all volunteers in ethylenediaminetetraacetic acid tubes and stored at -20 °C

until analysis. For total genomic DNA isolation, the UltraClean BloodSpin DNA (Mo Bio Laboratories, Carlsbad, CA, USA) kit was used.

Polymerase Chain Reaction and Genotyping

The PCR mixture prepared for these genes contained 100 ng template DNA, 2.5 mmol/L dNTP, 0.4 µmol/L GSTM1 primer, 0.8 µmol/L GSTT1 primer, 0.8 µmol/L albumin primer, 5 µL 10x buffer (Complete, Bioron GmbH, Ludwigshafen, Germany), 5 U DNA Taq polymerase (New England BioLabs, Ipswich, USA), and dH_2O in a total reaction volume of 25 µL. Then, the conditions of the thermal cycler (2720, Applied Biosystems, Foster City, USA) were programmed as 15 minutes at 95 °C, 35 cycles of 60 seconds at 94 °C, 60 seconds at 58 °C, and 60 seconds at 72 °C, followed by 10 min at 72 °C. The PCR amplification product was confirmed by 2% agarose gel electrophoresis. Band lengths of the PCR products obtained for *GSTT1*, *GSTM1,* and *albumin* were 459 bp, 219 bp, and 350 bp, respectively. The primer sequences used for the *GSTM1*, *GSTT1* and *GSTP1* genes are shown in Table 1.

GSTP1 Ile105Val polymorphism was determined by the PCR-RFLP method. Then 25 µL PCR reaction mixture consisted of: 100 ng template DNA, 5 µL of 10x buffer (Complete, Bioron GmbH, Ludwigshafen, Germany), 2.5 mmol/L dNTP, 10 U Taq polymerase (New England Biolabs, Ipswich, MA, USA), 0.3 µM GSTP1 primers (Table 1), and dH_2O. Then, the conditions of the thermal cycler were programmed as follows: for 5 minutes at 94 °C, 5 cycles of 30 seconds at 94 °C, 30 seconds at 64 °C, and 30 seconds at 72 °C, (annealing temperature decreased 1 °C in each cycle) and 25 cycles of 30 seconds at 94 °C, 30 seconds at 59 °C, and 30 seconds at 72 °C. The length of the PCR products obtained was 433 bp. The PCR product was incubated for 16 hours at 37 °C with 5 units of BsmAI (Fermentas, Lithuania) restriction endonuclease. After the restriction, 2 bands formed in the sizes of 328 and 105 bp in the wild type (AA), 4 bands in the sizes of 328, 222, 106, and 105 bp in the heterozygous genotype (AG), and 3 bands in the sizes of 222, 106, and 105 bp in the homozygous mutant genotype.

Table 1. Primer sequences of GST

Gene	Primer sequences (5'->3')
GST T1- forward	TTCCTTACTGGTCCTCACATCTC
GST T1- reverse	TCACCGGATCATGGCCAGCA
GST M1- forward	GAACTCCCTGAAAAGCTAAAGC
GST M1- reverse	GTTGGGCTCAAATATACGGTGG
Albumin- forward	GCCCTCTGCTAACAAGTCCTAC
Albumin- reverse	GCCCTAAAAAGAAAATCCCCAATC
GST P1, Ile105Val forward	GTAGTTTGCCCAAGGTCAAG
GST P1, Ile105Val reverse	AGCCACCTGAGGGGTAAG

GST: Glutathione S-transferase

Statistical Analysis

The SPSS 22.0 software package (SPSS Inc., Chicago, IL, USA) was employed. The chi-square test for 2x2 tables and the chi-square test for multi-way contingency tables were used to analyze categorical variables, while the independent samples t-test was used to compare group means. Statistical significance was defined as a p-value of 0.05.

Results

The patient group included 107 female recurrent pregnancy loss patients who had never given birth and had experienced two or more abortions. The control group comprised 107 women who were in good health and had successfully given birth without any previous instances of abortion. The patient group had a mean age of 26.54±6.67, whereas the control group's mean age was 38.32±9.23. We examined the GST polymorphisms in diseased and control groups. Individuals in the patient and control groups did not engage in smoking or alcohol consumption.

The number of patients with GSTT1 null genotype is 35 (32.7%). The number of individuals with null genotype in the control group is 21 (19.6%). The number of patients with GSTT1 positive genotype is 72 (67.3%). GSTT1 positive genotype was determined in 80.4% (n=86) of the control group. Our results showed a significant difference between the study groups (X^2=4.74; p=0.029) (Figure 1).

There were 48 patients with the GSTM1 null genotype (44.9%), and 35 individuals with the null genotype in the control group (32.7%); 59 patients had the GSTM1 positive genotype (55.1%), and 72 individuals in the control group had the positive genotype (67.3%). There was no significant difference between the study groups (X^2=3.326; p=0.068) (Figure 2).

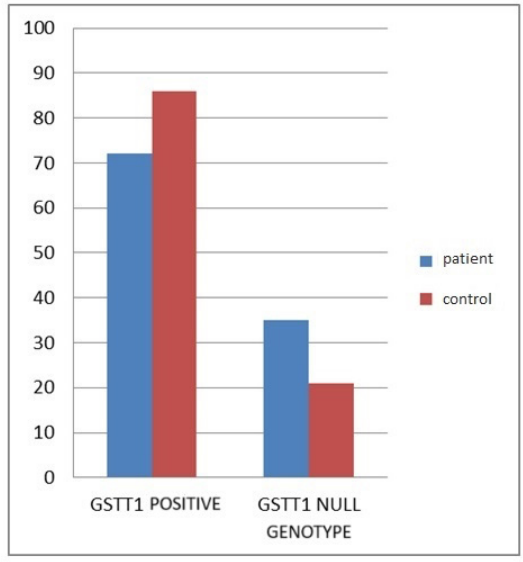

Figure 1. GSTT1 genotype in patient and control groups

The genotype frequency of GSTP1 exon 5 I105V polymorphism was found to be I allele 165, V allele 49 in the patient group; and I allele 166 and V allele 48 in the control group (X^2=0.013; p>0.90).

The GSTP1 wild genotype was 64 (59.5%) in the diseased group and 65 (60.7%) in the healthy group. The heterozygous genotype was 37 (34.6%) among patients and 36 (33.6%) in healthy individuals. The homozygous genotype was 6 (5.6%) in the patient group and 6 (5.6%) in the control group (X^2=0.021; p=0.989) (Table 1). The difference between the groups in terms of gene polymorphisms was not statistically significant (Figure 3).

The patient group exhibited significantly higher null genotypes for GSTM1 and GSTT1 positive and null genotypes combined (X^2=5.57; p=0.018) (Figure 4).

The comparison of the *GSTP1* and *GSTM1* gene polymorphisms across the study groups revealed no statistically significant difference (X^2=4.07, p=0.131).

GSTP1 and *GSTT1* gene polymorphisms were not significantly different between the study groups (chi-squared statistic, X^2=5.02, p=0.081).

GSTT1 null and GSTT1/GSTM1 genotypes are considered risk factors because they were significantly higher in recurrent pregnancy loss patients than in the healthy group.

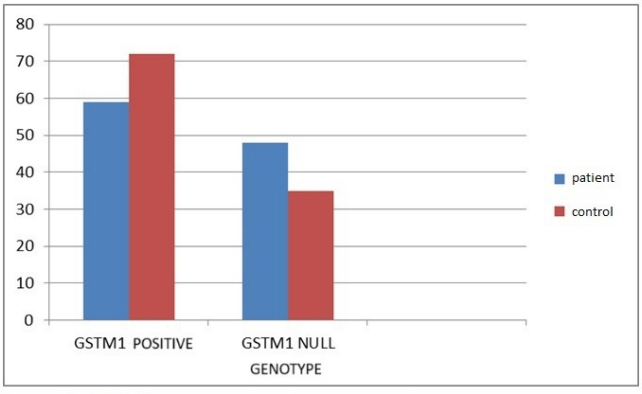

Figure 2. GSTM1 genotype in patient and control groups

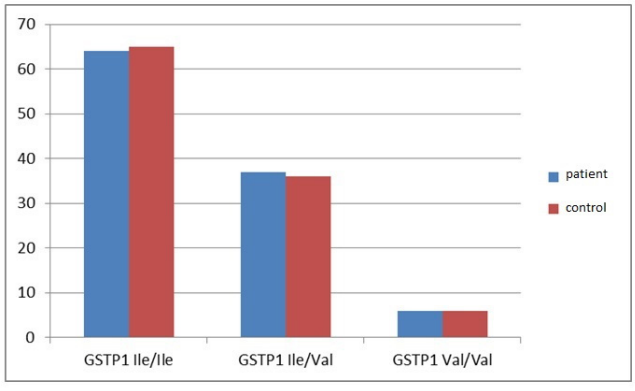

Figure 3. *GSTP1* gene polymorphism in patient and control groups

Discussion

GSTT1 and GSTT1/GSM1 null genotypes were significantly more frequent in recurrent pregnancy loss patients compared to the healthy group. Our study suggests that GSTT1 and GSTT1/GSTM1 null genotypes may increase the risk of recurrent pregnancy loss.

The oxidative state of cells plays a critical role in embryonic growth and endometrial differentiation by inducing angiogenesis. Oxidative stress causes functional changes in idiopathic recurrent pregnancy loss. This situation causes cellular and endometrial damage and destroys placental vascularization[11]. We investigated the GST gene polymorphism in recurrent pregnancy loss for the first time in our society.

According to epidemiological studies, recurrent pregnancy loss, a common pregnancy complication, is a multifactorial disease with a genetic predisposition[12]. The reason for recurrent pregnancy loss has been identified in only 30-50% of the cases[13]. The true cause of recurrent pregnancy loss is controversial, and the pathophysiological and etiological mechanisms are not fully understood. Oxidative stress increases the risk of recurrent pregnancy loss. An elevated oxidative load on the placenta supports the process of embryonic differentiation and development during pregnancy. An imbalance in the oxidant-antioxidant system can lead to an excessive oxidative load or an inadequate antioxidant defense to clear the oxidative load[14]. Phase I and II metabolic enzymes, which eliminate the destructive agents of oxidative stress, primarily determine a cell's ability to manage oxidative stress. According to several studies, genetic polymorphisms of antioxidant enzymes are implicated in an elevated risk of oxidative stress-linked diseases[15,16]. GST is an enzyme in the phase II detoxification system. The GST system and glutathione represent two of the most critical mechanisms in detoxifying and metabolizing carcinogens, xenobiotics, and reactive oxygen products[17].

In studies investigating the involvement of GST genes in recurrent pregnancy loss pathology, researchers have analyzed three different functional variants of GST. We examined the

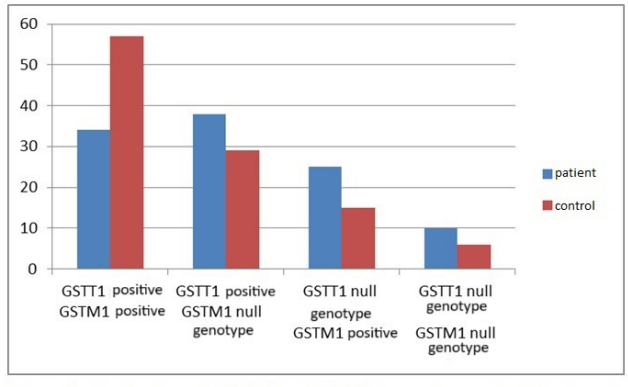

Figure 4. Evaluation of GSTT1 and GSTM1 genotypes together in patient and control groups

I105V substitution in the *GSTP1* gene to distinguish individuals with GSTT1 positive genotypes from those with positive/null genotypes of GSTM1, GSTT1, and null genotypes; as well as to distinguish non-identical carriers[17-21].

Research studies have suggested a potential association between genetic polymorphisms in metabolic enzymes and recurrent pregnancy loss[17-19]. In particular, GSTM1 null genotype may pose a risk for recurrent pregnancy loss, study in Japan suggests[20]. Conversely, an Indian study revealed a potential association between the GSTT1 null genotype and recurrent pregnancy loss[17-21]. A study conducted in the Netherlands observed a significant relationship between GSTP1 I105V polymorphism and the risk of recurrent pregnancy loss[21]. In light of the above, GST enzymes may be considered crucial for maintaining antioxidant defense, especially during pregnancy. It has been shown that a decrease in the activity and expression of enzymes can increase the risk of experiencing recurrent pregnancy loss by increasing sensitivity to oxidative stress. GST genes may provide a new perspective on recurrent pregnancy loss genetics. However, further investigation is required to validate this hypothesis[12].

Sata et al.[20], in their study of the Japanese population in 2003, found a higher rate of GSTM1 null genotype in the recurrent pregnancy loss group than in the control group [65.2%, 45.6%; odds ratio (OR)=2.23, 95% confidence interval (CI)=1.36-3.66]. The GSTM1 null genotype frequency in both groups with primary and secondary recurrent pregnancy loss was significantly high in comparison with the control group. Females with a history of three or more recurrent pregnancy losses were found to have a significant increase in the risk of recurrent pregnancy loss associated with the GSTM1 null genotype (OR=2.90, 95% CI=1.58-5.34). The International Project on Genetic Susceptibility to Environmental Carcinogens data indicates that the GSTM1 null genotype is 47.5% prevalent in the Japanese population, with a higher rate in Caucasians, 54.3% in America, and 50.4% in the Netherlands. In the Japanese population, the lower frequency of the GSTM1 null genotype makes the recurrent pregnancy loss risk even more statistically significant.

In the study conducted by Nair et al.[22] in 2013, the GSTT1 null genotype was compared between early, recurrent pregnancy loss and control groups. Individuals with pregnancy loss demonstrated a significantly higher frequency of GSTT1 null genotype compared to the other groups (10.92%, p=0.004; 10.77%, p=0.006). The GSTM1 null allele frequency was 37.36% among early pregnancy loss (EPL) individuals, 36.15% in recurrent pregnancy loss, and 28.89% in the control group. Nevertheless, no significant relationship was detected between the GSTM1 null genotype and recurrent pregnancy loss and EPL. When analyzed together, the combined GSTT1 and GSTM1 null genotypes had a 4.74-fold higher risk for EPL and a 5.67-fold higher risk for recurrent pregnancy loss.

In 2010, Parveen et al.[23] conducted a study on northern Indian women. They observed that the recurrent pregnancy loss group exhibited a significant increase in the GSTT1 null genotype (26%) compared to the controls (15%) (p=0.0034, OR=1.99, CI=1.27-3.12). The analysis of Phase II genes revealed a 4-fold increase in disease risk among north Indian women who had either the GSTP1 variant alleles or the GSTM1 null genotype. The fact that the risk goes up seven times when GSTM1 and GSTT1 null genotypes are combined with GSTP1 variant alleles suggests a strong link with recurrent pregnancy loss.

In 2011, Nonaka et al.[9] reported the presence of a statistically significant elevated frequency of GSTM1 null genotype in patients who consumed coffee daily compared to healthy individuals (61%; OR=2.25; 95% CI=1.13-4.49; p=0.025). It is estimated that increased embryonic exposure to endogenous or exogenous toxins due to the deficiency in the decidual and placental detoxification systems significantly contributes to the pathophysiology of recurrent pregnancy loss. GST catalyzes the transport and binding of many harmful substances, as well as the detoxification of oxygen radicals, by attaching a wide range of electrophilic compounds to the sulfhydryl group of glutathione[23]. Importantly, placental GSTs are essential for both fetal and maternal detoxification. Previous research in the Japanese population has documented the presence of a link between the GSTM1 null genotype and impaired caffeine detoxification in individuals experiencing recurrent pregnancy loss[7].

In 2004, Ada et al.[24] conducted a study on Turkish society and documented that the GSTM1 null genotype prevalence was 51.9%, whereas the GSTT1 null genotype prevalence was 17.3%. In 2001, Törüner et al.[25] found that the frequencies of GSTM1 and GSTT1 null genotypes in Turkish society were 45.5% and 17.4% respectively. According to Aktas et al.[26], the GSTM1 null genotype frequency was 34.7%. While in a study by Pinarbasi et al.[27], the GSTM1 null genotype frequency was 16%. Karaca et al.[28] found that the GSTM1 and GSTT1 null genotype frequencies were 52% and 23% respectively.

Factors such as genetic heterogeneity between societies, differences in susceptibility to some diseases, differences in exposure to toxins, selection based on different lifestyles, and differences in the evolutionary history of each society explain the distribution difference in the GSTT1 and GSTM1 null phenotype frequencies[29].

Study Limitations

In our study, the statistically significant difference observed between patients with the GSTT1 null genotype and the control group shows that we have identified it as a risk factor in the diagnosis of recurrent pregnancy loss. However, to obtain more accurate results about other variants, comprehensive studies on these genes should be conducted by expanding the sample size.

Conclusion

We genotyped the GSTM1, GSTT1, and GSTP1 biotransformation enzymes in both recurrent pregnancy

loss and healthy individuals. The GSTT1 null genotype was statistically significant in the recurrent pregnancy loss group, which shows how important biotransformation enzymes are in etiopathogenesis. It is thought that genetic polymorphism studies on biotransformation enzymes may shed light on the recurrent pregnancy loss pathogenesis. The link between GSTT1 and GSTT1/GSM1 null genotypes and recurrent pregnancy loss as risk factors should be supported by studies on larger patient groups and functional studies.

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Ethics

Ethics Committee Approval: This study design was based on the principles of the Declaration of Helsinki. The study received ethical approval from the Sivas Cumhuriyet University Research Ethics Committee (approval number: 2013-09/15, date: 24.09.2013).

Informed Consent: Verbal and written consent of all participants were obtained.

Footnotes

Authorship Contributions

Surgical and Medical Practices: N.A., H.K.K., Concept: N.A., H.K.K., Design: N.A., H.K.K., Data Collection or Processing: N.A., H.K.K., Analysis or Interpretation: N.A., H.K.K., Literature Search: N.A., H.K.K., Writing: N.A., H.K.K.

Conflict of Interest: No conflict of interest was declared by the authors.

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