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Genetic Evaluation of Thrombophilia Panel in Recurrent Miscarriage at Karami Hospital of Karaj

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Abstract--- *Molecular evaluation of thrombophilia panel is one of the practical evaluations in recurrent miscarriage. Clotting and disruption in uterine placental circulatory system result in miscarriage, intrauterine growth retardation, and preeclampsia. In Iranian population, it is possible to identify new genetic disorders due to the high rate of kinship marriages and different gene storage. The present study was conducted with the aim of investigating the new mutations in the thrombophilia panel related to recurrent miscarriage. This is a case-control study. The research population consisted of the patients with recurrent miscarriage referred to Kamali Hospital in Karaj. A total of 100 women with recurrent miscarriage (at least 2 miscarriages) and 100 women with at least one successful pregnancy (more than 2 miscarriages) were selected through convenience sampling method. DNA amplification was performed by PCR. Mutation analysis was performed using chromes software, and then, it was analyzed by SPSS software. In the case group, 10 patients (10%) with PAI-1 homozygous gene (G / 4G4) and in the control group, 2 patients (2%) were PAI-1 homozygous gene (G / 4G4) were identified, and this difference was statistically significant. Moreover, 20 patients (20%) in the case group and 3 patients (3%) in the control group were positive in terms of MTHFR gene polymorphism, which the difference between two groups being statistically significant. The results of this study revealed that the presence of PAI-1 homozygous gene and MTHFR polymorphism gene increases the risk of recurrent miscarriage. Thus, thrombophilia panel screening is recommended in patients with recurrent miscarriage.*

Keywords--- *Gene Mutation, Thrombophilia Panel, Recurrent Miscarriage, PAI-1 Homozygous Gene, MTHFR Polymorphism.*

I. INTRODUCTION

Recurrent miscarriage (repeated abortion) refers to the occurrence of three or more miscarriages in a clinically known pregnancy before week 20. Clinical pregnancy is confirmed by ultrasound or histopathology evaluation and does not include chemical pregnancy. The rate of recurrent miscarriage (less than 20 weeks) is 10-15% [1]. The etiology of recurrent miscarriage includes anatomical, immunological, genetic, endocrine, infectious, thrombophilia, and environmental factors [2]. Unfortunately, the cause of recurrent abortion is identified only in 50% of patients and remains unclear in the rest cases [3]. Parental chromosomal testing is a part of the recurrent miscarriage evaluation. Its aim is identifying Robertson or balance chromosomal abnormalities or identifying mosaicism states

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that can be transmitted to fetus [4]. Approximately, half of translocation abnormalities are balance, one-fourth of them are Robertson, and one-tenth of them are mosaicism of sex chromosomes in women and rest of them are inversions and balance disorders.

The karyotype of miscarriage products is also crucial. Normal karyotype usually indicates environmental factors involved in miscarriage; while, abnormal karyotype is a sufficient reason for miscarriage. Chromosome structural abnormalities can be hereditary or sporadic. Parents who have chromosomal abnormalities are screened by using their fetal amniocentesis or CVS, and legal abortion is permitted if the chromosomal abnormality is diagnosed. Moreover, IVF along with PGD is another strategy to prevent the birth of a disabled child or recurrent miscarriage due to chromosomal abnormalities [5-6]. Ahangari et al. examined the genetic variants of hereditary thrombophilia in recurrent miscarriage in Iran. Mutant alleles of MTHFR C677T and A1298C were significantly higher in the patients than those in control group subjects. However, the genetic variants of F2G20210A and F5 G1691A were not significantly different between the two groups. Allele frequency for the genotypes evaluated in this study was consistent with the data obtained for other countries. Significant effects of MTHFR C677T and A1298C were observed among the participants. Due to the relatively high prevalence of these variants, genetic tests for women with RPL were recommended before making a treatment decision [7].

Ocak et al. examined the relationship between recurrent pregnancy loss and inherited chromosomal and thrombophilia abnormalities in Pakistan. Parental chromosomal abnormalities were observed in 28 cases (2.8% of cases, 5.7% of couples) that most of them (92.9%) were structural abnormalities. All structural abnormalities were balanced chromosomal translocation. Chromosomal analysis of miscarriage cases showed major chromosomal abnormalities in 31.9% of cases. The most common change observed in heritable thrombophilia genes was heterozygous mutation for MTHFR C677T polymorphism in 55 people [8]. Karata et al. examined hereditary thrombophilia, anti-beta 2-IgA glycoprotein 1, and anti-Annexin V antibodies in recurrent miscarriage. Homozygous mutation of methylene tetrahydrofolate reductase C677T C677T was observed in 28.5% of the case group and 14.2% of the control group and its difference was significant ($P < 0.001$). Heterozygous mutation of this gene was found in 64.3% of the study population and 38.1% in the control group and the difference in the frequency of heterozygous mutation was also significant ($P < 0.001$). None of the homozygous and heterozygous mutations of PT G20210A and factor V G1691A was significantly different between the two groups. No significant difference was found between two groups in terms of anti-ANA and 2-gp 1 levels. The results of this study revealed that both homozygous and heterozygous mutations of MTHFR C677T were associated with RPL in Caucasian women [9].

In a retrospective cohort study, Lund et al. examined a hereditary thrombophilia and recurrent miscarriage using the outcome of pregnancy and delivery complications in Denmark. Pregnancy outcomes were recorded in a retrospective cohort group consisting of 363 women with at least three consecutive miscarriages (preterm miscarriage, late miscarriage and delivery) who were not treated with anticoagulant. Out of 363 women, 29 were carriers of the FVL mutation and 6 were carriers of the PT mutation. Analysis showed that FVL and PT mutations had a negative predictive effect on the live birth rate in women with RPL. However, the results no longer showed statistical differences when adjusting for the variables. No conclusion can be made about the association between labor complications and hereditary thrombophilia in this study. Large-scale randomized controlled trials are needed

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in order to know whether anticoagulation therapy can improve the prognosis in women with RPL and FVL / PT mutations [10]. In a prospective follow-up study, Vossen et al. examined the relationship between hereditary thrombophilia and miscarriage. The results of the above-mentioned study revealed that women with thrombophilia are at increased risk of miscarriage, although the probability of a positive outcome in both women with thrombophilia and control groups is high [11]. Arch et al. evaluated the relationship between hereditary thrombophilia and recurrent miscarriage in a meta-analysis. A systematic review of the studies was performed. The results were confirmed using fixed and random effects meta-analysis models. The combined odds ratios for the relationship between RPL and FVL and between RPL and G20210A were 2.0 (95% confidence interval, 1.5-2.7% P <.001) and 2.0 (95% confidence interval, 1.0-4.0% P = 0.03), respectively. Similar results were found by logistic regression and both fixed and random effects meta-analysis models. The results of the mentioned study revealed that FVL or prothrombin gene mutation carrier doubled the risk of two or more miscarriages compared to the women without thrombophilia. Hereditary thrombophilia may be an unknown cause of RPL [12]. It will be possible to prevent the birth of a disabled child and miscarriage, if structural abnormalities are identified. Molecular analysis of thrombophilia panel is one of the applied analyses of recurrent miscarriage. Clotting and disorder in uterine placental circulatory system lead into miscarriage, intrauterine growth retardation, and preeclampsia. Finally, families that are negative in thrombophilia panel and chromosomal tests (no abnormality is seen) are candidates for the whole exome assessment. It is possible to identify new genetic disorders due to the high rate of kinship marriages as well as the different gene storage in Iranian population. Hence, the question of the present study is what are the new mutations in the thrombophilia panel related to recurrent miscarriage at Karaj Kamali Hospital?

II. METHODOLOGY

This is a case-control study. The research population included the patients with recurrent miscarriage referred to Kamali hospital in Karaj. A total of 100 women with recurrent miscarriage (at least 2 miscarriages) and 100 women with at least one successful pregnancy (more than 2 miscarriages) at the age group of 18-40 were selected by convenience sampling method. The inclusion criteria included two or more miscarriages, no genetic diseases, and willingness to participate in the study. The data were collected through demographic information questionnaire, genetic analysis of aborted samples and genetic analysis (karyotype) of blood samples taken from the parents using information software (http: / /www.progenygenetics.com) and family tree of the patients. The thrombophilia panel (including factor 2, 5, 13, and the MTHFR gene) was molecularly analyzed. QIAamp DNA Mini and Blood Mini Kit (cat no. 51306) were used for genomic DNA extraction. Two conventional methods of nanodrop and electrophoresis measurements were used to assess the quality and quantity of the extracted DNA. For DNA quality assessment, after preparation of 1% agarose gel, it was placed in a tank containing buffer. 1µL of DNA product and 0.3µL of loading buffer were run inside the wells and the voltage of the power generator was set to the desired voltage (depending on the length of the gel).

After 30 minutes, a photo was taken from the gel using ethidium bromide staining solution though gel doc system. Electrophoresis is a simple, fast and highly sensitive method used as a separation technique to study the properties of pregnant molecules. The optical absorption ratio of the DNA solution was measured at 260 to 280

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wavelengths and sometimes 260 to 230 nm to determine the DNA concentration and to evaluate its purity. As this ratio is closer to 1.8-2, the level of contamination with protein and RNA will be lower, and if it is greater than 2, it will indicate the presence of RNA contamination, and if it is lower than 2, it will indicate contamination with protein. The concentration of DNA products was measured using a nanodrop device (ND2000). After quantitative and qualitative examination of DNA in the studied samples, the samples with lower quantitative characteristics (lack of DNA electrophoresis band detection or evidence of its degradation or low ratio of 260 to 280) were extracted. DNA was amplified by PCR method. DNA amplification was performed using ABI thermo-cycler (2720). (Table 1) presents the values used for thrombophilia panel PCR.

Table 1: The Values of the Compounds Used in PCR Reaction Mixture

substance	*concentration*	*volume*
H_2O		19.7
10 ×PCR buffer		2.5
$MgCl_2$	50mM	0.5
dNTP	10mM	0.5
Primer (Forward, Reverse)	10 pM/ µL	1
DNA	20-50 ng/µl	0.7
Taq-DNA polymerase	5U/ µL	0.1
Total volume		25

The product derived from PCR underwent electrophoresis on 1.5-2% agarose gel at a voltage of 100-180 ° C for 1 hour and then stained with ethidium bromide and the bands were observed with gel doc system so that the presence of the band with the desired size to be proven. In cases where additional bands were observed, PCR touch-down was used or the PCR conditions were changed to remove additional bands. The sequencing results were obtained through PCR products along with the forward primer and reverse primer. The sequences of the genes were compared with the published standard sequence of the standard using Chromas software (Version 2.33) and any nucleotide change was recorded. The desired genes in the panel included:(Factor V Leiden G1691A, Factor V H1299R, Factor II G20210A, Factor XIII V34L, PAI-I -675 4G/5G, FGB -455G/A ,MTHFR C677T, MTHFR A1298C, MTR A2756G, and MTRR A66G) .

Data were analyzed through SPSS 25 software by using Wilcoxon statistical test and mean and standard deviation. The P-values less than 5% were considered significant.

III. RESULTS

Descriptive Results

The mean age of the patients was 29.92 with a standard deviation of 5.90 in the case group. The youngest of them was 18 and the oldest of them was 40 years of old (Chart 1).

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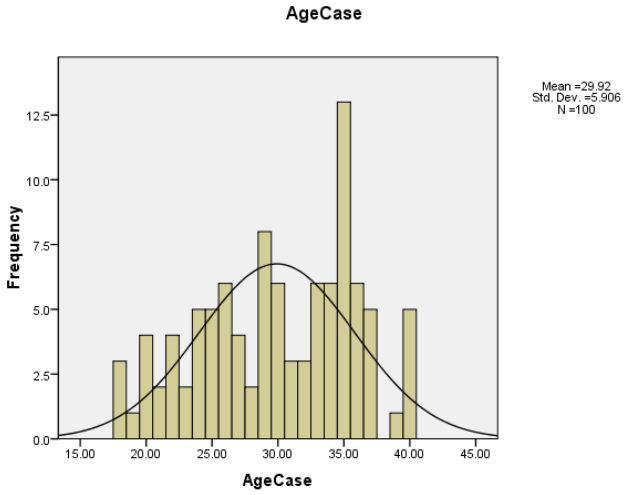

Chart 1: Age Frequency of the Patients in Case Group

The mean age in the control group was 28.54 with a standard deviation of 6.10. The youngest of them was 18 years of old and the oldest of them was 40 years of old (Chart 2).

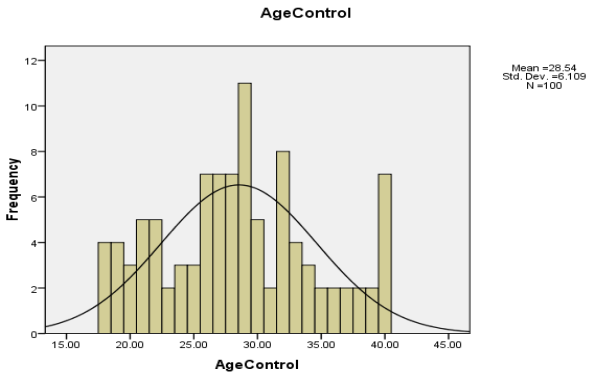

Chart 2: Age Frequency of the Control Group

In the case group, 10 patients (10%) were homozygous PAI-1 gene (G / 4G[4]) (Chart 3).

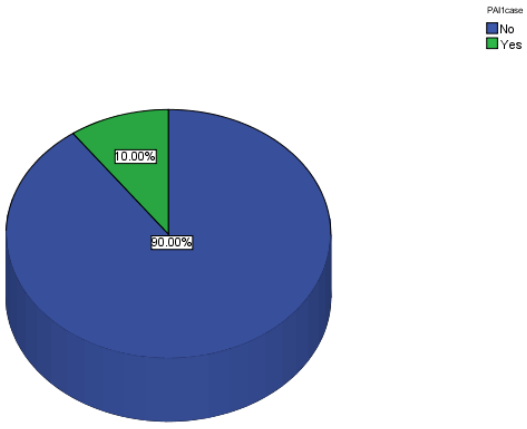

Chart 3: Frequency of Homozygous PAI-1 Gene (G / 4G[4]) in Case Group

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In the control group, 2 patients (2%) had homozygous PAI-1 gene (G / $4G^4$) (Chart 4).

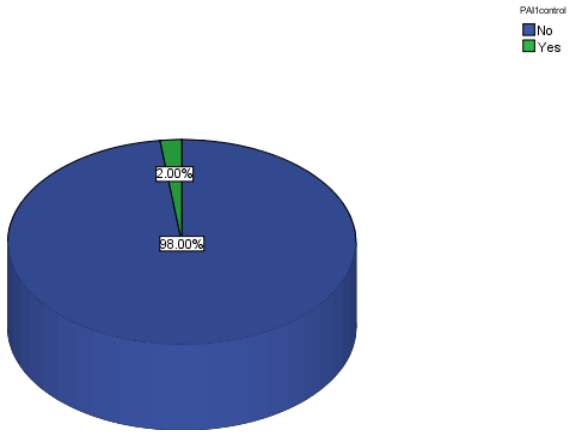

Chart 4: Frequency of Homozygous PAI-1 Gene (G / $4G^4$) in the Control Grou.

In the case group, 20 patients (20%) were positive in terms of MTHFR gene polymorphism (Chart 5).

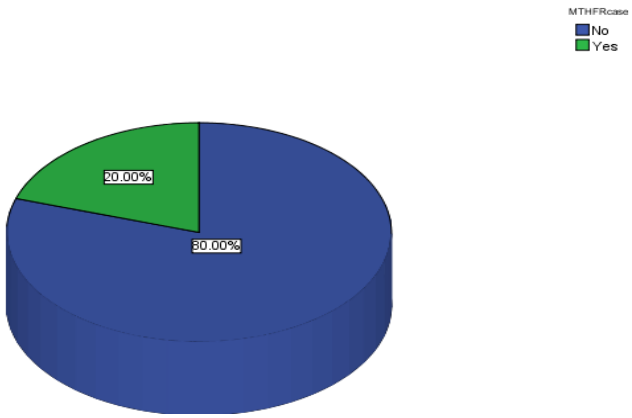

Chart 5: Frequency of MTHFR Gene Polymorphisms in Case Grou.

In the control group, 3 patients (3%) were positive in terms of MTHFR gene polymorphism (Chart 6).

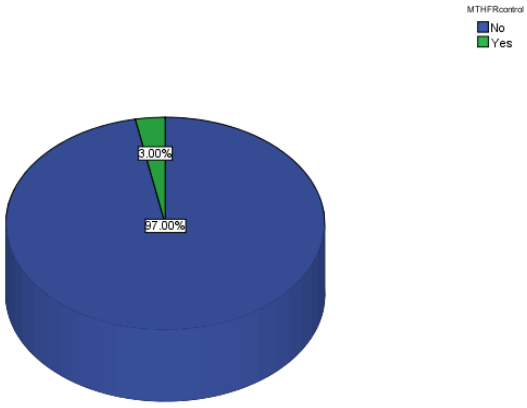

Chart 6: Frequency of MTHFR Gene Polymorphisms in the Control Group

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Analytical Results

The Wilcoxon test was used to compare the mean age of the patients in the two groups. Comparison of the mean age of the patients in the two groups (Table 2) revealed that there was no significant difference in the Wilcoxon test (P> 0.05).

Table 2: Comparison of the Mean Age of the Patients in Two Groups

Variable	*Case group*	*Control group*	*P value*
Mean age (SD)	29.92 (5.90)	28.54 (6.10)	0.083

Then, the frequency of homozygous PAI-1 gene was compared in two groups (Table 3). Based on Fisher's exact, there is no statistically significant difference between them (P <0.05).

Table 3: Comparison of Frequency of Homozygote PAI-1 Gene in the Patients of Two Groups

gene PAI-1	*Case group*	*Control group*	*P value*
yes	10 (10%)	2 (2%)	0.021
no	90 (90%)	98 (98%)	

The frequency of MTHFR gene polymorphism was compared in two groups (Table 4). Based on the Fisher exact test, no statistically significant difference was seen between two groups (P <0.05).

Table 4: Comparison of the Frequency of MTHFR Gene Polymorphisms in the Patients of Two Groups

gene MTHFR	*Case group*	*Control group*	*P value*
yes	20 (20%)	3 (3%)	0.000
no	80 (80%)	97 (97%)	

The odds ratio (OR) for the homozygous PAI-1 gene and recurrent miscarriage was 5.44 (95% confidence interval, 1.16 to 25.52) and P value was 0.03. Moreover, the odds ratio (OR) for MTHFR gene polymorphism and recurrent miscarriage was 8.08 (95% confidence interval, 2.31 to 28.18) and P value was 0.001.

IV. DISCUSSION

In the present study, 100 patients were investigated in the case group and 100 patients in the control group. In the case group, 10 patients (10%) were homozygous for PAI-1 gene (G / $4G^4$) and in the control group, 2 patients (2%) were homozygous for PAI-1 gene (G / $4G^4$). The odds ratio (OR) for the homozygous PAI-1 gene and recurrent miscarriage was 5.44 (95% confidence interval, 1.16 to 25.52). However, in a study conducted in 2012 in Iran, no significant relationship was found between homozygous PAI-1 (G / $4G^4$) and recurrent miscarriage in Iran. The difference in the results could be due to the sample size limitation in this study (30 patients in the case group and 10 patients in the control group) [13]. Additionally, in a similar study conducted in 2005, the odds ratio (OR) for the homozygous PAI-1 gene and recurrent miscarriage was 4.8 (95% confidence interval, 2.33 to 10.25) [14], confirming the results of the present study. In addition, in another study conducted in 2013, the odds ratio (OR) for the homozygous PAI-1 gene and recurrent miscarriage was 5.00 (95% confidence interval, 2.44 to 10.24) [15], which is in line with the results of the present study. In the present study, 20 patients (20%) were positive for MTHFR gene polymorphism in the case group and 3 patients (3%) were positive for MTHFR gene polymorphism in the control group. The difference between two groups was statistically significant. The odds ratio (OR) for MTHFR gene polymorphism and recurrent miscarriage was 8.08 (95% confidence interval, 2.31 to 28.18). In this regard, in a similar meta-analysis conducted in 2019, the odds ratio (OR) for MTHFR gene polymorphism and recurrent

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miscarriage was 1.34 (95% confidence interval, 1.20 to 1.50) in developing countries [16]. It is consistent with the results of the present study. Moreover, in another meta-analysis conducted in 2019, the odds ratio (OR) for MTHFR gene polymorphism and recurrent miscarriage was 4.99 (95% confidence interval, 1.65 to 15.12) [17], which is consistent with the results of the present study.

V. CONCLUSION

The present study results revealed that the presence of PAI-1 homozygous gene and MTHFR polymorphism increases the risk of recurrent miscarriage. Hence, thrombophilia panel screening is recommended for patients with recurrent miscarriage.

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