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Original Article

Evaluation of *GSTP1* Gene Polymorphisms in Infertile Men with Azoospermia in Southern Iran

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Introduction: Approximately 30% of infertile men experience unexplained infertility, a condition categorized as idiopathic. Several studies have reported a correlation between specific genetic polymorphisms and impaired spermatogenesis in infertile men with idiopathic oligoasthenoteratozoospermia. Genetic factors contribute to 60% of cases categorized as idiopathic. Assessing semen quality serves as a crucial criterion in evaluating male fertility.

Materials and Methods: Sampling was conducted at the infertility centre, involving 60 azoospermic patients and 60 healthy individuals. The objective was to investigate and compare the polymorphism status of both groups using polymerase chain reaction and restriction fragment length polymorphism (RFLP) Technique.

Results: In the control group, the highest frequency of *GSTP1* gene polymorphisms was observed in individuals with the AA genotype (healthy homozygous) at a rate of 50%. Conversely, the lowest frequency of *GSTP1* gene polymorphism in the control group was associated with the G/G genotype (patient homozygous) at a rate of 15 %. Regarding the patient group, the frequency of *GSTP1* gene polymorphisms was found to be related to the A/A genotype (healthy homozygous) at 50%. The lowest frequency of *GSTP1* gene polymorphism in the patient group was observed in individuals with the G/G genotype (patient homozygous) at 16.6%. Additionally, within this group, the frequency of patient heterozygotes (A/G) was determined to be 33.4%.

Conclusion: The polymorphism study showed no significant disparity in the frequency of two dominant alleles and mutant alleles between the patient and control groups within the population of infertile Iranian men.

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Introduction

Despite significant advancements in medical and reproductive sciences, infertility remains a significant concern for many young couples. Typically, around 80-85% of couples who engage in unprotected sexual intercourse achieve pregnancy. However, it is estimated that approximately 15% of couples experience difficulties conceiving when attempting to do so for the first time. Infertility is a complex issue influenced by various factors, including genetics, environment, and anatomical conditions. Studies have shown that approximately 50% of infertility cases are attributed to male factors [1, 2]. Furthermore, about 30% of infertile men face the challenge of identifying the cause of their infertility, categorized as idiopathic [3, 4].

This article aims to shed light on factors affecting male infertility. Azoospermia, the absence of sperm in ejaculation, is one potential cause of infertility, affecting approximately 1% of the male population [5, 6]. Comprehensive laboratory analysis of semen allows for identifying clues related to various infertility problems. Semen analysis, a relatively simple and cost-effective test, provides crucial information such as sperm count, motility, morphology [7], volume, appearance, pH, and viability, collectively determining sperm function and fertility potential [6]. Parameters such as the number of motile sperm and the quality of motility are vital in achieving pregnancy [8].

Other factors contributing to male infertility include hypogonadotropic hypogonadism, characterized by a lack of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion, which can be attributed to genetic or acquired factors [9]. Varicocele, a condition involving enlarged veins within the scrotum, is another significant factor, affecting approximately one-third of infertile men compared to an incidence of 15% in the general population [10]. Environmental factors, such as stress [11], smoking, alcohol consumption, obesity, nutrition [12], oxidative stress [13], exposure to heavy metals [14], heat [15], and infection [16], also play a role in semen quality and fertility.

Genetic factors contribute to approximately 15 to 30% of infertility [17]. Examples of genetic factors include Kleinfelter syndrome (the most common cause of male infertility and hypogonadism resulting from an extra X chromosome) [18], aneuploidy (the most common chromosomal cause involving an incorrect number of chromosomes) [19], and Jacob syndrome (characterized the presence of an additional Y chromosome in sperm (47 XYY), due to the lack of postzygotic mitotic division [20].

Polymorphisms, or genetic variations, arise from differences in DNA sequences among individuals and populations. They can result from various factors, including deletions, insertions, recombinations, and external influences such as electromagnetic radiation. Genetic mutations represent a type of polymorphism associated with diseases and are found in a small population [21-23].

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Polymorphism in the *Glutathione S-Transferase Pi 1 (GSTP1)* gene occurs in two forms within its sequence. The first type involves the replacement of the nucleotide A with G, while the second type involves the placement of the nucleotide C with T. This substitution results in amino acid valine replacing alanine in codon 114, specifically in exon 6 during translation [24-26].

The primary objective of this article is to investigate *GSTP1* gene polymorphisms in infertile men with azoospermia, alongside exploring the aforementioned genetic and environmental factors.

Materials and Methods

Study design: The enrolled samples were divided into two groups: control and patient, as described below:

Control group: This group comprised 60 volunteers with no history of infertility and their first-degree relatives.

Patient group: This group consisted of 60 individuals with azoospermic infertility registered at infertility centres in Gerash, Jahrom, and Bandar Abbas counties. After confirmation of infertility through consultation with a doctor, these individuals were included in the study.

Blood samples were collected from the brachial area and veins in the arm region using a 5 ml syringe. The collected blood sample was then transferred to tubes containing anticoagulants ethylenediaminetetraacetic acid (EDTA) and transported to the Gerash University of Medical Sciences Research Center research laboratory. Subsequently, the samples were frozen at -20 °C for genetic analysis.

In this study, DNA was extracted from the white blood cells of infertile patients with azoospermia to facilitate the polymerase chain reaction (PCR) technique. Specific primers for *GSTP1* were employed to extract and amplify the desired genes.

DNA extraction and GSTP1 polymorphisms analysis

The blood sample was collected using EDTA as an anticoagulant, and total DNA was extracted from peripheral blood mononuclear cells using the salting-out method. The quality of the extracted DNA was assessed using NanodropTM densitometry (Thermo Fisher Scientific, USA) [27]. Additionally, a subset of randomly selected PCR-amplified samples underwent Sanger sequencing to validate the results obtained from PCR-restriction fragment length polymorphism (RFLP). Table 1 summarises the primer sequences, lengths of digested fragments, and the cycling conditions.

***GSTP1* genome detection**

Amplication of the *GSTP1* gene was performed on samples that tested positive for the β-globin gene, as previously described. The PCR reaction was conducted in a total volume of 25 µL. The reaction mixture included one µL of MgCl2 (CinnaGene, Iran), 0.5 µL of dNTPs (CinnaGene, Iran), 5 µL of reaction buffer (CinnaGene, Iran), 0.5 µL of Taq DNA polymerase (CinnaGene, Iran),1 µL of each specific primer (Table 1), 11 µL of water, and 5 µL of DNA template.

The PCR program for amplifying the *GSTP1* region of the genome consisted of an initial

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denaturation at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 60 seconds, annealing at 62 °C for 60 seconds, and extension at 72 °C for 60 seconds. A final extension step was performed at 72 °C for 10 minutes. The PCR products were then electrophoresed on a 1.5% agarose gel and visualized under UV light.

RFLP-PCR

RFLP refers to the non-uniform patterns observed when restriction enzymes enzymatically digest a specific region of DNA. These patterns indicate the presence or absence of specific restriction enzyme recognition sites, which result in DNA differences. Restriction enzymes, also known as endonucleases, are enzymes that recognize and cleave specific base sequences within a double-stranded DNA molecule.

In the RFLP method, a DNA sample is first digested with a specific restriction enzyme, generating multiple fragments of varying lengths. These fragments are then separated using agarose gel electrophoresis. Probes can be used to identify and detect specific fragments of interest.

In the present study, the PCR product was subjected to digestion with the Tth111 restriction enzyme under specific conditions. The PCR product was treated with Tth111 at 37 °C for 24 hours. After the incubation period, the contents of the reaction tubes were electrophoresed on a 2.5% gel. Finally, the gel was placed in a gel dock and photographed for further analysis.

Diagnosis of polymorphism after RFLP

Upon digestion with the restriction enzyme, samples displaying both genes being cleaved resulted in the observation of 250 and 150 bp bands. Different polymorphisms caused by mutations may lead to the observation of either a single band or three bands. A single bar indicates a change in both genes (mutated homozygous) where the restriction enzyme failed to produce cleavage. On the other hand, the presence of three bands (heterozygous) suggests the presence of a healthy gene and a modified gene. After determining the genotype in both the control and patient samples, the frequencies of the 5A/5A, 6A/6A and 5A/6A genotypes were selected in the two groups. The distribution of these genotypes was then compared.

Statistical analysis

The results were analyzed using various statistical tests, including Chi-square (X^2) and T-test. Microsoft Excel was used to generate tables for data visualization and analysis.

Table 1. The sequences and other characteristics of primers used in this study

Locus	5′ to 3′ Sequence	Reference
GSTP1	F: 5′ -TCCTTCCACGCACATCCTCT-3′	[28]
	R: 5′ - AGCCCCTTTCTTTGTTCAGC-3′	
β-globin	5'- ACACAACTGTGTTCACTAGC-3'	[29]
	5'- CAACTTCATCCACGTTCACC-3'	

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Results

Investigation of gene polymorphism and extraction of *GSTP1*

The present study used the PCR and RFLP methods to investigate gene polymorphism and extract the desired *GSTP1* gene. The findings are interpreted as follows.

Gene Polymorphism and *GSTP1* Extraction Investigation: In this study, the PCR and RFLP methods were employed to investigate gene polymorphism and extract the desired *GSTP1* gene. The findings of these methods are interpreted as follows.

Results of identification and extraction of the *GSTP1* gene using the PCR technique

Using specific primers and the PCR technique, it was determined that the *GSTP1* gene was present in both the patient and control groups. Gel electrophoresis and fluorescent microscopy analysis revealed a bandwidth of 400 bp for the *GSTP1* gene, as shown in Figure 1.

Evaluation of *GSTP1* polymorphism using the RFLP technique in azoospermic and normal groups

Studies have shown that genetic mutations occur naturally, leading to variations in an organism's genome's linear order of bases. These natural changes contribute to the diversity observed among individuals in a population. Using the RFLP technique and restriction enzymes to identify genetic polymorphisms, it was observed that the patient group exhibited heterozygous polymorphisms (5A/6A), healthy individuals showed healthy homozygotes (5A/6A), and the patient group displayed homozygotes in the case of 5A/6A. Figures 1 and 2 depict the presence of a single band, indicating a mutation in both genes (mutated homozygous), where the restriction enzyme failed to cleave. The presence of three bands in the RFLP analysis indicates a heterozygous genotype, indicating the presence of both a healthy gene and a modified gene.

Results on the frequency of polymorphisms in the study groups

Based on the findings presented in Table 2, the patient group exhibited a frequency of 50% for the A/A genotype (healthy homozygous), while the lowest frequency of *GSTP1* gene polymorphism in the control group was observed for the G/G genotype (patient homozygous) at 16.6%. Additionally, the patient heterozygotes (A/G) had a frequency of 33.4% in this group.

Table 3 displays the results for the control group, where the highest frequency of *GSTP1* gene polymorphisms was associated with the AA genotype (healthy homozygous) at 50%. Conversely, the lowest frequency of *GSTP1* gene polymorphism in the control group was observed for the G/G genotype (patient homozygous) at 15 %.

Results from the T-test shown in Table 4 showed a significant difference between A/G and A/A polymorphisms at 0.05. This suggests a higher frequency of the G gene as a defective and diseased gene in these particular polymorphisms. No significant differences

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were observed in the other polymorphisms at the 0.05 significance level.

Final findings of the research

Based on the p-value, there is no significant difference in the frequency of the two dominant alleles and mutants of this polymorphism in the population of Iranian infertile men between the patient and control groups. The observed polymorphism had a p-value greater than 0.05, indicating a lack of significant correlation. All samples related to the control group are shown in Figure 3. All samples related to the patient group are shown in Figure 4. In the columns where three bands (400-250-150 bp) were observed, the restriction enzyme cleaved one of the paternal or maternal genes, while the other gene remained mutated and uncut. Therefore, these columns represent a heterozygous genotype (Het). For example, the first column exhibited this pattern. The columns where two bands (250-150 bp) were present indicate that the restriction enzyme cleaved both the paternal and maternal genes. This suggests a dominant homozygous genotype (Hem D). The second column exemplifies this pattern. In the columns where only one band was observed, it indicates that both the paternal and maternal genes remained mutated and uncut. These columns represent a recessive homozygous genotype (Hem R), as shown in the third column.

Table 2. Frequency of polymorphism in the patient group

Polymorphism	Number	Percent
Patient Heterozygotes (A/G)	20.60	33.4
Healthy Homozygous (A/A)	30.60	50
Patient Homozygous (G/G)	10.60	16.6

Table 3. Frequency of polymorphism in the control group

Polymorphism	Number	Percent
Patient Heterozygotes (A/G)	21.60	35
Healthy Homozygous (A/A)	30.60	50
Patient Homozygous (G/G)	9.60	15

Table 4. Comparison of means of different polymorphisms based on the T-test at 95% confidence level

Sig. (2-tailed)	T	Upper	Lower	SD	Means	
۰,۳۷۸	٤.١	۳۰۳,۰٦	۳۸۳,۰٦	38.18	٤۰	A/G- G/G
۰,۰۹	۷	۲۹,٥	۸,٥	2.12	۱۰,٥	A/G- A/A
۰,۲۹۸	۱,۹	۲۷۳,٥	۳۷٤,٥	36.06	٥۰,٥	A/A- G/G

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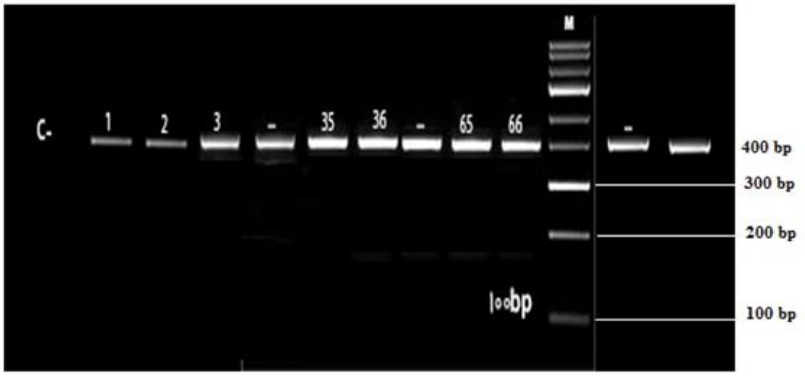

Fig. 1. Genome extraction of 100 samples (patient and control) was amplified by RFLP to study the *GSTP1* gene. Based on the designed primer, the gene length is 400 bp.

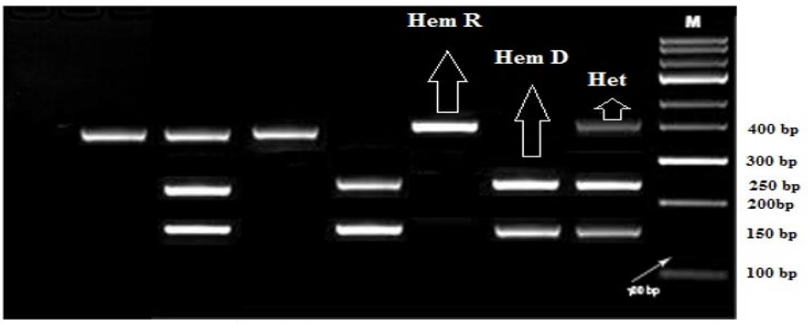

Fig. 2. Detection of gene polymorphisms by the RFLP technique and the presence of gene bands

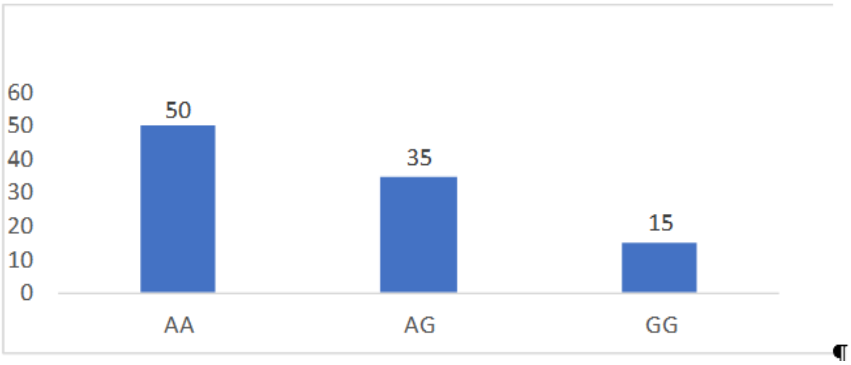

Fig. 3. Polymorphism: all samples related to the control group

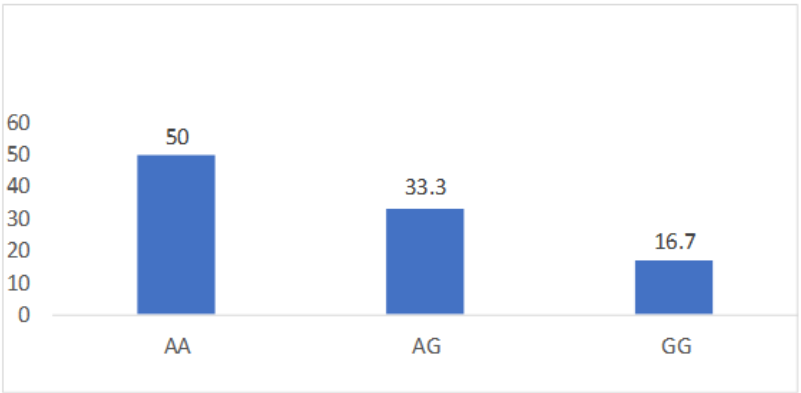

Fig. 4. Polymorphism: all samples related to the patient group

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Discussion

Infertility is a significant healthcare issue that has profound effects on individuals and society as a whole. According to the World Health Organization, approximately 80 million couples experience infertility each year, with prevalence rates varying from 5% to 30% across different countries [30]. Male factors contribute to around half of all infertility cases, and statistics indicate an annual incidence of at least 2 million male infertility cases. Clinical manifestations of male infertility include abnormalities in sperm count, motility, and morphology [31].

Extensive research on male infertility has identified various factors that can impact sperm quality, quantity, and the occurrence of infertility. These factors encompass genetic disorders, acquired conditions, and environmental factors. Lifestyle and habits also play a role in impairing spermatogenesis and can contribute to infertility. Examples include obesity, smoking, alcohol consumption, exposure to high temperatures, and stress.

Chromosomal abnormalities such as Klinefelter syndrome, genetic defects in the Y chromosome, as well as medical conditions such as diabetes, varicocele, obesity, genital tract infections, and hormonal imbalances, are recognized causes of male infertility [32]. However, there is a subgroup of men for whom the cause of infertility remains unknown, termed idiopathic infertility [33].

There has been growing interest in studying the role of genetic polymorphisms and allelic variants in male infertility in recent years. Genetic variability and epigenetic factors have been found to influence human reproduction and fertility throughout the process, from gametogenesis to birth. However, it is important to note that these genetic changes are likely not the sole causes of infertility but rather cofactors that interact with other infertility-causing factors, exacerbating their effects [34]. One gene family that has garnered attention among researchers is the glutathione S-transferases gene family, specifically the *GSTP1* gene polymorphism, whose products are important in the metabolism of oxidative stress and environmental toxins.

The investigation of the *GSTP1* gene polymorphism in the population of infertile Iranian men revealed no significant difference in the frequency of dominant alleles and mutants between the patient and control groups. Therefore, these polymorphisms are not associated with idiopathic infertility caused by azoospermia in Iranian men. This finding is consistent with previous studies conducted by Xiong et al. in northwest China, which also showed no significant difference in *GSTM1*, *GSTT1*, and *GSTP1* genotypes between infertile men and controls [24]. Similarly, a study by Huang et al. in 2017 found no significant relationship between *GSTP1* gene polymorphism and idiopathic infertility [35].

However, it is important to note that contrasting results have been reported in other studies. Xiong et al. reported a significant

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association between the *GSTP1* gene polymorphism and idiopathic infertility in a population of 361 men with azoospermia [24]. Additionally, Feng et al. found a significant relationship between the *GSTP1* gene polymorphism and idiopathic infertility [36]. These studies indicate the complexity of the genetic factors involved in male infertility and highlight the need for further comprehensive and controlled research to understand these factors better.

Conclusion

The present study evaluated the association between *GSTP1* gene polymorphisms and azoospermia in infertile men from southern Iran, showing no significant difference in the distribution of A/A, A/G, and G/G genotypes between patients and fertile controls. Therefore, this polymorphism alone does not appear to be a major determinant of idiopathic azoospermia in this population and may act only as a potential modifying factor. Considering the multifactorial nature of male infertility, future studies should explore additional genes, gene–gene, and gene–environment interactions. Limitations of this study include the relatively small sample size and focus on a single genetic locus, highlighting the need for larger studies to clarify the cumulative role of detoxification pathway genes in male infertility.

Ethical Considerations

This study was approved by the Ethics Committee of Gerash University of Medical Sciences (Approval Code: IR.GERUMS.REC.1399.020).

Funding Statement

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Conflict of Interest

The authors declare no conflicts of interest..

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Data Availability Statement

The data supporting this study’s findings are available from the corresponding author upon reasonable request..

Authors' Contributions

MM and SAM designed the study. ME, AA and AP analyzed the data. SAM, ME, MGJ and AP prepared the draft of the manuscript.MM and SAM reviewed and approved the final manuscript.

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