Correlation of Single Nucleotide Polymorphisms of PRM1, PRM2, PYGO2, and DAZL Genes with Male Infertility in North West of Iran

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ABSTRACT

Objective: Almost half of infertility is related to male factors. Although the effect of genetic factors on male infertility is identified, about 30%-50% still has no proven cause and is classified as idiopathic infertility. This study was performed to investigate the correlation of some single nucleotide polymorphisms of PYGO2, DAZL, PRM1, and PRM2 genes with male infertility in idiopathic cases among the Iranian population.

Material and methods: In this case-control study, 120 idiopathic azoospermia or severe oligospermia men in the range of 25-45 years and 120 fertile men in the same age range were recruited as case and control groups, respectively. Eight different single nucleotide polymorphisms including PRM1 rs737008, PRM1 rs423668, PRM2 rs1646022, PRM2 rs11645592, PYGO2 rs341722381, PYGO2 rs141722381, PYGO2 rs61758741, DAZL rs75931701, and DAZL rs188506466 were genotyped by using amplification-refractory mutation system polymerase chain reaction methods. Hardy-Weinberg was calculated by using online website. Statistical Package for Social Sciences software was applied for statistical analysis. P value <0.05 was considered significant. Thirty percent of the samples were regenotyped to confirm the obtained results.

Results: The obtained results showed a significant correlation between PYGO2 rs141722381 in the heterozygote form (odds ratio: 2.803, 95% CI: 1.397-5.626). Heterozygote over-dominance was also observed in this variant (odds ratio: 2.637, 95%CI: 1.321-5.264). There was no significant association between other studied single nucleotide polymorphisms and male infertility.

Conclusion: This study proposed a novel single nucleotide polymorphism as a predisposition of male infertility among the Iranian population, but more studies in larger populations are needed to confirm the results.

Keywords: Male infertility, DAZL, PYGO2, PRM1, PRM2

Introduction

Male infertility affects about 7% of the male population, a figure that is increasing.1 Male infertility is usually associated with decreased sperm count.2 The role of genetic factors in male infertility is well-known, such as Klinefelter syndrome, Y-chromosome microdeletions, and cystic fibrosis. Genetic factors are reported in about 4% of infertile men, but the vast majority of infertility in men (50%-60%) have no distinct causes yet and are classified as idiopathic infertility.1 The sperm production process which is called spermatogenesis is a complex process involving spermatogonia stem cell proliferation, meiosis, and spermatid differentiation. Genetic diversity involved in this process may play an important role in spermatogenesis defects and male infertility.3,4 Pygo2, DAZL, and PRM1 are some genes involved in spermatogenesis which are reported to play the main role in male infertility.5 Human PYGO2 protein is localized in the nucleus and composed of 2 domains: the zing finger motif (PHD) and the N-terminal homology domain (NHD).6 PYGO2 acts as a co-activator of the Wnt pathway. N-terminal homology domain of this gene, with the help of beta-catenin, BCL9, and LEP/TCF transcription factors, forms the transcription complex and leads to the expression of some specific target genes.7 N-terminal homology domain also plays an important role in histone methylation.8 Pygo2 gene is located on chromosome 1q21.3 and is expressed...
during sperm chromatin remodeling. The effects of pygo2 targeted mutations in mice have shown that the limited activity of pygo2 during spermatogenesis leads to a selective decrease in the expression of essential post-meiotic genes for chromatin condensation including protamine (P1 and P2), transfer protein 2 (TNP2), and H1fnt genes which may lead to infertility. It has also been shown that decreased pygo2 activity in elongated spermatids leads to disruption of histone H3 acetylation suggesting that pygo2 recruit HAT facilitates H3 acetylation and further histone-to-protamine transitions.

DNA in the sperm head is highly compacted, which is vital to maintain the hydrodynamic shape of the sperm and the genome's integrity. At the last stage of the spermatogenesis process, histones are replaced with protamines to provide this level of chromatin condensation. There are 2 types of protamines (PRM1 and PRM2) in humans that are located on chromosome 16. PRMs are highly basic and rich in arginine residue, which provides strong bindings to negatively charged DNA. More than 20 single nucleotide polymorphisms (SNPs) are reported in PRM1 and PRM2 genes. Various studies have reported an association between PRM2 and male infertility. Jiang et al released a report indicating PRM2 rs1646022 polymorphisms and the risk of male infertility. Iguchi et al have reported a correlation of PRM1 c.197G>T polymorphism and male infertility. It was also suggested that PRM1 c.-190C>A polymorphism may lead to male infertility due to abnormal sperm morphology.

Another gene that plays an important role in spermatogenesis is DAZL. DAZL is expressed in primordial germ cells (PGCs) and produces an RNA-binding protein that controls the growth, differentiation, and maturation of germ cells. DAZL protein acts as an activator of transcription and is present in the nucleus of gonocytes and spermatogonia. Lin et al reported that dazl transcripts were lower in the testes of azoospermia cases than in fertile men. Also, it is suggested that approximately 10% of patients with azoospermia and oligospermia show deletion in the DAZ family gene.

In this study, we tried to find the association of some SNPs of PYGO2, PRM1, PRM2, and DAZL genes with male infertility. Single nucleotide polymorphisms were selected for the study which lead to a stop codon or missenses that alter the nature of the amino acids which in turn can produce truncated proteins or proteins with improper function.

Materials and Methods

In this case–control study, the association of the studied SNPs and their majority or minority effect on male infertility was investigated to identify biomarkers that can be used for diagnosis and prognosis of male infertility in medical centers.

Participants: A total of 120 case samples were selected among infertile men referred to the assisted reproductive technology center of the Valiasr Hospital (Tabriz, Iran) between 2019 and 2020. Control subjects were 120 fertile healthy men with normal spermogram who had at least 1 child at the time of study. All the participants were in the range of 30-45 years old. Case samples were idiopathic azoospermia or oligospermia. To exclude samples that have a possible cause, all cases were subjected to physical examination, and necessary hormonal (Follicle-stimulating hormone (FSH), Thyroid stimulating hormone (TSH), luteinizing hormone (LH), testosterone, and prolactin) and genetic tests (karyotyping and microdeletions of Y chromosome, CFTR, etc.) were taken. Semen analysis was performed twice for all participants using microscopic methods and according to World Health Organization criteria (WHO, 2010). The sperm count of infertile patients was <5x10⁶. All the parameters for control subjects were in the normal range.

Sample size influences random sampling error. In order to control type I and type II errors, the sample size was obtained using the statistical sample size calculation formula. The ratio of the case to control (r) was considered 1. The P value was considered similar to the standard deviation (0.29). The probability of exposure in case (P2) and control groups (P1) was 0.38 and 0.2, respectively. The minimum sample size was 100 individuals, but more (120) were recruited to increase power calculation.

Ethical Considerations

This study has been carried out in compliance with all the rules and instructions related to medical research in Iran. Written consent was obtained from all participants in this project. The project was approved by the Science and Research Branch of
DNA Extraction: 3 mL of venous blood was collected from all participants in the ethylenediaminetetraacetic acid (EDTA) containing venoject tubes. DNA was extracted using PCR Bio Rapid Extract kit (PCR Biosystems, London, N6 4ER, UK). The yield and purity of DNA samples (OD 260/280) were measured by using a nanodrop (Denovix Ds-11 spectrophotometer).

Genotyping: Amplification refractory mutation system method was used for genotyping the studied polymorphisms. Primers were designed by Primer 3 software. Oligoanalyzer and primer blast softwares were used to check the quality and specificity of the primers. Polymerase chain reaction products were visualized on 2% agarose gel electrophoresis by using Novel juice stain (Cat.No.LD001-1000). The obtained results were confirmed by regenotyping 30% of samples.

Primers and Cycling Condition: Polymerase chain reaction was performed in a final volume of 25 µL. Ready to use mastermix (PCR Biosystems Ltd) was used to amplify the target gene. Cycling condition for all the reactions was 95°C for 5 minutes for the first cycle, followed by denaturation at 94°C for 45 seconds, annealing temperature according to optimum Tm of each reaction (Table 1) for 40 seconds, extension at 72°C for 40 seconds for 29-30 cycle and then final extension at 72°C for 7 minutes. The sequence of primers is summarized in Table 1.

Statistical Analysis
In order to find a significant difference between the case and control groups in relation to each of the studied SNPs, minor allele frequencies (MAFs) were estimated by using excel software and compared between the 2 groups.

**Table 1. Sequence of Primers and Annealing Temperatures**

| Primer Type | Primer Sequence | Annealing Tm (°C) | Product Size (bp) |
|-------------|-----------------|------------------|------------------|
| PRM1 rs737008G>A | Wild type forward primer 5’-AAACGCTGTCACCATTGTGTCG-3′ | 55.5°C for 45 seconds | 189 |
| | Mutant type forward primer 5’-GAACGCTGTCACCATTGTGTA-3′ | | |
| | Common reverse primer 5’-TCTTCTTGGGTGATTGTGTTG-3′ | | |
| PRM1 rs423668 C>T | Wild type forward primer 5’-TTAGCCAGGTGTGGTGGC-3′ | 58.5°C for 45 seconds | 193 |
| | Mutant type forward primer 5’-TTAGCCAGGTGTGGTGTT-3′ | | |
| | Common reverse primer 5’-ACAGTGTGCACTGCTGGT-3′ | | |
| PRM2 rs1646022 C>G | Wild type forward primer 5’-GGTTGTGTCAGGACATAC-3′ | 55°C for 30 seconds | 171 |
| | Mutant type forward primer 5’-GGTTGTGTCAGGACATAG-3′ | | |
| | Common reverse primer 5’-GGGACTTCTCAGTATAGG-3′ | | |
| PRM2 rs11645592 G>A | Wild type reverse primer 5′-GGAGCAATGAGCATATTGAG-3’ | 56°C for 30 seconds | |
| | Mutant type reverse primer 5′-GGAGCAATGAGCATATTGAG-3’ | | |
| | Common forward primer 5′-CAGGAGATGATTGAGTTGAG-3‘ | | |
| PYGO2 rs14172238 T>A | Wild type forward primer 5’-CCAGGAAAGGGATTTTCG-3′ | 56°C for 40 seconds | 233 |
| | Mutant type forward primer 5’-CCAGGAAAGGGATTTTCG-3′ | | |
| | Common reverse primer 5′-CAGGAGATGATTGAGTTGAG-3‘ | | |
| PYGO2 rs61758741 T>C | Wild type forward primer 5′-CAGGTGATCATGAGGGTTGATC-3′ | 57°C for 40 seconds | 171 |
| | Mutant type forward primer 5′-CAGGTGATCATGAGGGTTGATC-3′ | | |
| | Common reverse primer 5′-CAGGAGATGATTGAGTTGAG-3‘ | | |
| DAZL rs75931701 T>G | Wild type forward primer 5′-ACGTGGGCTAGATGCAGATG-3′ | 55°C for 35 seconds | 218 |
| | Mutant type forward primer 5′-ACGTGGGCTAGATGCAGATG-3′ | | |
| | Common reverse primer 5′-ACGTGGGCTAGATGCAGATG-3′ | | |
| DAZL rs188506466 G>A | Wild type forward primer 5′-TGGTGGCGATCAGAGG-3′ | 59.5°C for 45 seconds | 549 |
| | Mutant type forward primer 5′-TGGTGGCGATCAGAGG-3′ | | |
| | Common reverse primer 5′-TGGTGGCGATCAGAGG-3′ | | |
To determine the statistical analysis pattern, Hardy Weinberg’s equilibrium (HWE) and chi-square were calculated. Obtained $\chi^2$ was compared with standard statistical table (3.8). If it was smaller than 3.8, the group is in HWE otherwise HWE is disturbed in that group. The status of HWE in the case and control group was obtained using online software (https://wpcalc.com/en/equilibrium-hardy-weinberg/). If both the case and control groups were in HWE, the multiplicative model was used for statistical analysis. If the control group was under HWE but the case group was not, dominant/recessive model was used; otherwise, additive model was performed. The association of genotypes with male infertility was analyzed by calculating odds ratios (ORs) with 95% CIs via logistic regression. Statistical analysis was performed IBM SPSS Statistics v.22 (IBM SPSS Corp.; Armonk, NY, USA). $P < .05$ was considered significant.

Results

Semen analysis parameters are summarized in Table 2. The concentration of extracted DNA was in the range of 77-297 ng/µL and obtained OD 260/280 was in the range of 1.79-1.9. The frequency of extracted DNA was in the range of 77-297 ng/µL and concentration of extracted DNA was in the range of 1.79-1.9. The frequency of the studied genotypes and their association with male infertility are summarized in Table 3. There was an association between PYGO2 rs141722381 and male infertility, but no relationship was found between the other studied SNPs and male infertility.

The obtained $P$ values comparing MAF between case and control groups showed a significant difference between MAF of infertile and controls in the case of PYGO2 rs141722381 ($P$ value = .007), but there was no such a difference between case and control groups in the other studied SNPs. The results are summarized in Table 3.

Table 2. Semen Characteristic

| Semen Characteristic | Fertile Controls (n = 120) | Infertile Cases (n = 120) | Lower Reference Limit (WHO 2010) |
|----------------------|---------------------------|--------------------------|---------------------------------|
| Volume, ml           | 2.85                      | 1.8                      | 1.5                             |
| Total sperm count in ejaculate ($x10^9/mL$) | 50-120 | <10 39 |
| Sperm count $x10^9/mL$ | 35-95 | 0-5 | 15 |
| Total motility, %    | 45-85 | – | 42 (38-42) |
| Progressive motility (PR), % | 42-80 | – | 32 (31-34) |
| Vitality, %          | 55-65 | – | 58 (55-63) |
| Normal morphology %  | 4.5 | 0.1 | 3-4 |
| PH                   | 7.2-8                      | 7.2-7.6                  | >7.2                            |
| Viscosity            | N                          | N-M-H’N                  | Smooth & watery                 |
| Liquefaion time      | 20-35 min                  | 20-45 min                | <60 min                         |
| Round cells $x10^9/mL$ | 0-4 | 0-4 | <5 |
| Sperm agglutination  | 0-1                       | 0-1                      | <2                              |

*N, Normal; M, Moderate; H, High.

Table 3. Allele Frequency and Distribution of the PRM2, PYGO2, and DAZL Genotypes in Infertile Patients and Controls

| Genotype             | Cases (n = 120) | Controls (n = 120) | MAF* (Case Group) | MAF* (Control Group) | P*  |
|----------------------|----------------|-------------------|-------------------|----------------------|-----|
| PRM2 1646022         | 12 (10.0)      | 20 (16.7)         | 55                | 50                   | .292|
| PRM2 1646022 CC      | 84 (70.0)      | 80 (66.7)         |                   |                      |     |
| PRM2 1646022 CG      | 24 (20)        | 20 (16.7)         |                   |                      |     |
| PRM2 1646022 GG      | 57 (47.5)      | 68 (56.7)         | 36.25             | 29.16                | .338|
| PRM2 1164592 GG      | 39 (32.5)      | 34 (28.3)         |                   |                      |     |
| PRM2 1164592 GA      | 24 (20)        | 18 (15.0)         |                   |                      |     |
| PRM2 1164592 AA      | 79 (65.8)      | 80 (66.7)         | 24.58             | 25.41                | .734|
| PRM1 737008GG        | 23 (19.2)      | 19 (15.8)         |                   |                      |     |
| PRM1 737008GA        | 18 (15.0)      | 21 (17.5)         |                   |                      |     |
| PRM1 737008AA        | 52 (43.3)      | 56 (46.7)         | 43.75             | 38.33                | .413|
| PRM1 423668 CC       | 31 (25.8)      | 36 (30.0)         |                   |                      |     |
| PRM1 423668 CT       | 37 (30.8)      | 28 (23.3)         |                   |                      |     |
| PRM1 423668 TT       | 7 (5.8)        | 10 (8.3)          | 52.5              | 48.75                | .305|
| PYGO2 61758741 TT    | 100 (83.3)     | 103 (85.8)        |                   |                      |     |
| PYGO2 61758741 TG    | 13 (10.8)      | 7 (5.8)           |                   |                      |     |
| PYGO2 61758741GG     | 79 (65.8)      | 100 (83.3)        | 21.25             | 10.83                | .007|
| PYGO2 141722381TT    | 9 (7.5)        | 7 (5.8)           |                   |                      |     |
| PYGO2 141722381TA    | 10 (8.3)       | 6 (5)             |                   |                      |     |
| DAZL 75931701        | 89 (74.2)      | 98 (81.7)         | 17.5              | 12.5                 | .374|
| DAZL 75931701 TT     | 20 (16.7)      | 14 (11.7)         |                   |                      |     |
| DAZL 75931701 TG     | 11 (9.2)       | 8 (6.7)           |                   |                      |     |
| DAZL 188506466 GG    | 45 (37.5)      | 53 (44.2)         | 46.25             | 39.16                | .377|
| DAZL 188506466 GA    | 39 (32.5)      | 40 (33.3)         |                   |                      |     |
| DAZL 188506466 AA    | 36 (30.0)      | 27 (22.5)         |                   |                      |     |

P values were obtained from $\chi^2$ test.

*MAF, minor allele frequency in studied case and control group.
In all studied genotypes, obtained $\chi^2$ indicated neither the case group nor the control group was in HWE (Table 4). Since the additive model is independent of HWE and our samples showed an imbalance, the additive model was used for association analysis. There was a significant association between PYGO2 rs141722381 and male infertility in the heterozygote form (OR: 2.803, CI: 1.397-5.626). Heterozygote over-dominance was also observed in this polymorphism (OR: 2.637, CI: 1.321-5.264). No significant association was found between the other studied polymorphism and male infertility. The association of each genotype with male infertility is summarized in Table 5. Heterozygote over-dominance was not observed in other studied SNPs (Table 5).

**Discussion**

Spermatogenesis is a complex process of mitosis and meiosis in tandem to produce haploid sperm, in which at least 150 different genes play a key role and any defect of these genes may affect the production of healthy and functional sperm. Since almost half of the male infertility has no definite cause and is classified as idiopathic infertility, analysis of the possible association of genes involved in the spermatogenesis process with male infertility remains an area of active investigation. In this study, we investigated the correlation of 6 different SNPs involved in spermatogenesis with male infertility in idiopathic cases, which was not studied before.

Chromatin condensation during sperm remodeling is one of the important procedures through spermatogenesis. Histones are first replaced with TNP1 and TNP2 proteins, and then these proteins are replaced with PRM1 and PRM2 proteins. This causes maximum compression of the chromatin to be placed in the sperm head. Pygo2 gene is also essential for chromatin remodeling and regulate TNP2and PRM gene expression via histone methylation changes. In the present research, there was no association between studied SNPs of PRM genes (PRM2 rs1646022, PRM1 rs737008, and PRM2 rs423668) and male infertility among the Iranian population. But results revealed a critical correlation between PYGO2 rs141722381 in the heterozygote form (OR: 2.803, 95%CI: 1.397-5.626) and male infertility. There was also no association between PYGO2 rs141722381 leads to damage to the tertiary structure of protein and rs61758741 is a missense mutation that replaces a basic amino acid with an acidic amino acid. It is reported that 2 SNP mutations in PYGO2 (rs61758740 and male infertility in the heterozygote form) and male infertility (OR: 2.637, CI: 1.321-5.264) and male infertility. There was also no association between PYGO2 rs141722381 leads to damage to the tertiary structure of protein and rs61758741 is a missense mutation that replaces a basic amino acid with an acidic amino acid. It is reported that 2 SNP mutations in PYGO2 (rs61758740 and male infertility in the heterozygote form) and male infertility (OR: 2.637, CI: 1.321-5.264) and male infertility. There was also no association between PYGO2 rs141722381 leads to damage to the tertiary structure of protein and rs61758741 is a missense mutation that replaces a basic amino acid with an acidic amino acid. It is reported that 2 SNP mutations in PYGO2 (rs61758740 and male infertility in the heterozygote form) and male infertility (OR: 2.637, CI: 1.321-5.264) and male infertility. 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Two separate studies among the Spanish and Japanese populations showed an association between the PRM1 gene (c.-190C>A) and
Microdeletions of DAZ gene family on the chromosome Y are one of the well-characterized causes of infertility in azoospermia and oligospermia patients. DAZ-like (DAZL), an autosomal homolog of DAZ, is expressed in germ cells and is essential for spermatogenesis. According to the meta-analysis review in 2016, there is no correlation between SNP260 (rs121918346) of DAZL and azoospermia while SNP386 (rs121918346) of this gene is related to male infertility only among the Chinese Han population. A significant association is also reported between T54A polymorphism and male infertility in only Asian population. An important SNP N109T (rs75931701) in the coding sequence of the gene was predicted to be deleterious by computational methods which may affect the structure and/or function of DAZL significantly, but contrary to expectations in the present study, no significant difference was observed among the allele frequency of case and control group. There was also no association between DAZLrs188506466 and male infertility among the Iranian population. As of our knowledge, this was also the first study that investigated the effect of this SNP on male infertility. Confounders such as smoking, alcohol consumption, some environmental factors, high-risk jobs, and so on were not included in the study which was the limitation of this study.

In conclusion, the results of the study revealed an important association between PYGO2rs141722381 and male infertility that suggests a novel risk factor for idiopathic cases. The obtained results can be used by urologists in infertility treatment centers as a predisposition of male infertility in idiopathic cases, but the results should be validated by different populations first.

**Informed Consent:** Written informed consent was obtained from all participants who participated in this study.

**Peer-review:** Externally peer-reviewed.

**Author Contributions:** Concept – S.A.A.; Design – S.A.A., E.G.; Supervision – E.G., S.A.A.; Funding – E.G., A.G.; Materials – M.R.M.; Data Collection and/or Processing – E.G., S.A.A., B.B.; Analysis and/or Interpretation – B.B., S.A.A., E.G., Literature Review – A.G., M.R.M.; Writing Manuscript – A.G., E.G.; Critical Review – E.G., S.A.A.

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