Original Article

Single Nucleotide Polymorphism Analysis of Protamine Genes in Infertile Men

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Abstract ———

Background: Single nucleotide polymorphism (SNPs) are considered as one of the underlying causes of male infertility. Proper sperm chromatin packaging which involves replacement of histones with protamines has profound effect on male fertility. Over 20 SNPs have been reported for the protamine 1 and 2.

Materials and Methods: The aim of this study was to evaluate the frequency of two previously reported SNPs using polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) approach in 35, 96 and 177 normal, oligozoospermic and azoospermic individuals. These SNPs are: 1. A base pair substitution (G) at position 197 instead of T in protamine type 1 Open reading frame (ORF) including untranslated region, which causes an Arg residue change to Ser residue in a highly conserved region. 2. cytidine nucleotide change to thymidine in position of 248 of protamine type 2 ORF which caused a nonsense point mutation.

Results: The two mentioned SNPs were not present in the studied population, thus concluding that these SNPs can not serves as molecular markers for male infertility diagnosis.

Conclusion: The results of our study reveal that in a selected Iranian population, the SNP G197T and C248T are completely absent and are not associated with male infertility and therefore these SNPs may not represent a molecular marker for genetic diagnosis of male infertility.

Keywords: Protamine, Single Nucleotide Polymorphism, Mutation, Infertility

Introduction

Male infertility affects about 10-15% of couples with a desire to have children (1). Environmental factors or infections contribute to infertility to some extent, but genetic factors also play a pivotal role in etiology of male infertility. In recent years, several genetic modifications have been identified. The most frequent causes are chromosomal abnormalities or microdeletions of the Y chromosome, while point mutations of essential genes for spermatogenesis seem to be rare. Mutations that severely change the biochemical characteristics of synthesized proteins are not compatible with reproduction and seem not to contribute significantly to male infertility. A novel concept is evolving that SNPs, potentially modify gene function, might be tolerated in reproduction when their effects are subtle and the frequency among the population is high. Indeed, a number of such SNPs have been reported

recently, some of these SNPs are associated with reproductive functions, such as sperm production or different hormone sensitivities (2, 3).

During spermiogenesis, protamine substitutes somatic cell histones, a process that results in a highly condensed transcriptionally silent chromatin (4, 5). Distur bances in sperm nuclear condensation are considered as a major cause of male infertility. During this process histones are replaced with sperm nucleus transition nuclear proteins called transition nuclear protein (*TNP1* and *TNP2*). The *TNPs* are subsequently replaced by proteins called protamine 1 and 2 (protamine gene: *PRM1* and *PRM2*) which results in sperm nuclear condensation (6-9).

In mammals protamines are formed in two types, *PRM1*, *PRM2* (10-12). *PRM1* is formed from a stretch of 50 amino acid residues which is highly

conserved in all vertebrates (10). *PRM2* comprises several subtypes (6, 7) and has been only is identified in human and mice. The *PRM2* family proteins are synthesized as precursors of 66-101 residues (4, 13, 14). Humans have one copy of the *PRM1* and *PRM2* gene per haploid genome, located on chromosome 16 (15-17). Both genes contain a single intron. The genomic sequences of the *PRM1* and *PRM2* genes are organized in the form of a loop domain together with the transition protein2 genes (*TNP2*) and a sequence called gene4 (18). Alteration in expression of *PRM1* and *PRM2* has been associated with male infertility (19-21). Since, protamines play critical roles in spermatid differentiation, thus aberrations in protamine expression or changes in protein structure may result in certain idiopathic human male infertility (22, 23). The etiology of sperm protamine deficiency in infertile men remains elusive. Protamine expression deregulation may occur at multiple points along the expression pathway, including mutations in the protamine genes, aberrant transcription regulation, unfaithful translation repression or activation, and incomplete post-translational processing. Recently SNPs have been recognized to be associated with altered expression of *PRM1* and *PRM2*. Two recent SNPs in *PRM1* and *PRM2* genes have been reported by Iguchi (24) and Tanaka (25) in the group of patients with a fairly normal sperm count but with a markedly altered sperm morphology and azospermic individuals respectively.

A

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   Primer P1A                                         +1
ccccctggcatctataacaggccgcagagctggcccctgactcacagcccacagagttc

cacctgctcacaggttggctggctcagccaaggtggtgccctgctctgagcattcagcca

agcccatcctgcaccatggccaggtacagatgctgtcgcagccagagccggagcagatat

                                    (G197T SNP)  ←  T
taccgccagagacaaagaagtcgcagacgaaggaggcggagctgccagacacggagGaga
gccatgagtaagtgggcccagctgagggtgggctggggctgaggctgggagctctcaggg
cccagccttcctctcaccacttttcttggtctcaccagggtgctgccgcccccaggtacag
accgagatgtagaagacactaattgcacaaaatagcacatccaccaaactcctgcctgag
aatgttaccagacttcaagatcctcttgccacatcttgaaaatgccaccatccaataaaa

atcaggagcctgctaaggaacaatgccgcctgtcaataaatgttgaaaagtcatcccact
cttctctccttgttcttga
   Primer P1B
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B

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+1                                              Primer P2A
aacagtaacaccaagggcaggtgggcaggcctccgccctcctcccctactccagggccca

ctgcagcctcagcccaggagccaccagatctcccaacaccatggtccgataccgcgtgag
gagcctgagcgaacgctcgcacgaggtgtacaggcagcagttgcatgggcaagagcaagg
acaccacggccaagaggagcaagggctgagcccggagcacgtcgaggtctacgagaggac

     T ⟶  (C248T SNP)
ccatggcCagtctcactataggcgcagacactgctctcgaaggaggctgcaccggatcca
caggcggcagcatcgctcctgcagaaggcgcaaaagacgctcctgcaggcaccggaggag
gcatcgcagagtctgcctgcgcccccgccttgccctgcatgtccctgaccaccccaggca
acggaggggaggcggggacccaccccacctgacaaaagctccagccccctaaaccccgtcc
ccacccagagtccctaggtgacccctcaaccagaacttttttcccaaaagggctgcaga
accaggaagagaacatgcagaaggcactaagcttcctgggcccctcacccccagctggaa

attaagaaaaagtcgcccgaaacaccaagtgaggccatagcaattc
                        Primer P2B
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Fig 1: Genomic DNA sequences of the protamine-1 (PRM1) (A) and -2 (PRM2) (B) genes (40), and location of the primers used for PCR amplification. The underlined primer sequences are: P1A and P1B for PRM1; and P2A and P2B for PRM2. The transcriptional start site and single nucleotide polymorphisms (SNPs) that were examined are shown in bold letters.

The SNP in *PRM1* gene results in one arginine residue substitution with serine at codon 34 in a highly conserved arginine cluster (Fig 1A) (24). The next SNP in the *PRM2* gene results in replacement of glutamate residue with a termination codon (nonsense mutation) at position 50. Thus, this SNP disrupts maturation of *PRM2* protein and results in azoospermia (Fig 1B) (25). To address the prevalence of the above SNPs in different population, we screened a population of infertile male patients referring to Isfahan fertility and infertility center and Royan institute using RFLP technique.

Materials and Methods
Specimen
After obtaining institutional review board approval of Royan Institute and Isfahan fertility and infertility center, semen samples were obtained from 273 patients referring to Isfahan fertility and infertility center and Royan Institute. Semen samples were also obtained from 35 volunteer fertile individuals. Semen samples were analyzed according to WHO criteria. Following consultation, physical examination, signing the consent form and questionnaires, 5 ml of blood were obtained from fertile, azoospermic and oligozoospermic individuals. Patients were considered as azoospermic, who had no spermatozoa in their ejaculates even after centrifugation and had at least three reports of being azoospermic. Obstructive azoospermic individuals were not included in this study. Patients were considered as oligozoospermic that their sperm concentrations were less than 5 million/ml.

PCR and Agarose gel electrophoresis
Genomic DNA was isolated from the blood samples (26). To analyze the protamine genes, PCR-RFLP technique was performed. Thus, primers pair used for protamine 1 amplification were: Primer sense was: (5´-cccctggcatctataacaggccgc-3´) from nucleotide -42 to nucleotide -19 upstream of ORF and primer anti-sense: (5´- tcaagaacaaggagagaagagtgg-3´) covering nucleotides 492 to 515 of the ORF including poly adenylation signal (Fig 1A). In order to amplify protamine 2 gene, the following primers were used: 1) Primer sense was: (5´-ctccagggcccactgcagcctcag- 3´) from nucleotide 49 to nucleotide 72 of ORF and 2) primer anti-sense: (5´-gaattgctatggcctcacttggtg-3´) covering nucleotides 624 to 647 of the ORF (Fig 1B).

Using above primer pairs, the fragments of 557 nucleotides of protamine1 gene locus and 599 nucleotides of protamine gene2 were amplified. The PCR condition to amplify protamine1 and 2 fragment were as follows: 1) 35 cycles of denaturation at 96°C for 45 seconds, annealing at 64°C for 45 seconds and extension at 72°C for 45 seconds and 2) 35 cycles of denaturation at 96°C for 45 seconds, annealing at 68°C for 45 seconds and extension at 72°C for 1 minutes. The resultant PCR products were applied for agarose gel electrophoresis and enzymatic digestion.

SNPs detection using RFLP approach
G197T detection in protamine gene 1
One single nucleotide polymorphism (G197T) was reported to present in protamine 1 gene. The G197T mutation disrupts the recognition site of the restriction enzyme *BSe*RI (changing GAG-GAG to GAG-TAG) (27). To develop a RFLP assay for this SNP, PCR products were digested with *BSe*RI for 2.5 h at 37°C and separated on a 1.5% agarose gel. The DNA fragments were visualized by ethidium bromide staining.

Fig 2: PCR restriction fragment length polymorphism of PCR products from infertile patients and fertile man which were analyzed for G197T SNP.M) Molecular marker, C1) show PCR product that was partially digested, C2) Undigested PCR product, C3) completely digested, A) Azoospermic, O) Oligospermic and F) Fertile individual.
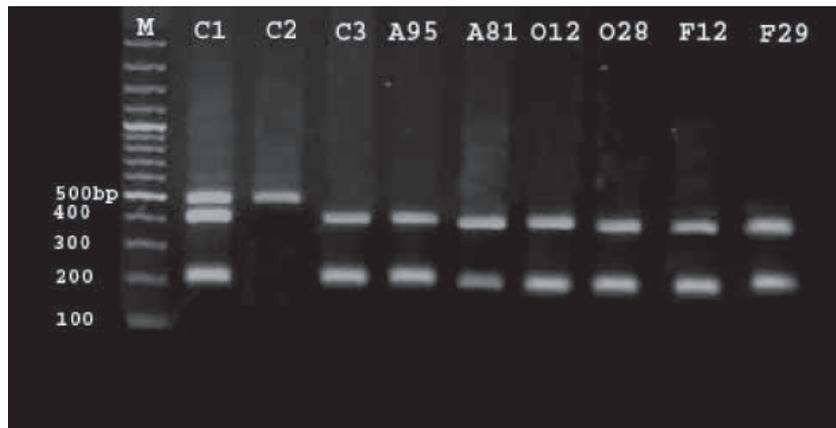
Fig 3: PCR restriction fragment length polymorphism of genomic DNA from infertile patients and fertile man which were analysed for C248T SNP. M) Molecular marker, C1) shows a PCR product that was partially digested, C2) Undigested PCR product, C3) completely digested, A) Azoospermic, O) oligospermic and F) Fertile individual.

C248T detection in protamine gene 2
Single nucleotide polymorphism which was previously identified in protamine gene 2 (C to T) causes disruption of the recognition site of the restriction enzyme *MSc*I (Isoschizomeric form of *Bal*I) (changing TGGCCA to TGGCTA) (28). PCR products of protamine 2 were digested with *MSc*I for 2.5 h at 37°C and separated on a 1.5% agarose gel.

Results
Analysis of G197T SNP of PRM1
All PCR products of *PRM1* genomic fragments (557 bp) were used for digestion with *BSe*RI. The absence of mentioned SNP results in full enzymatic digestion of the amplified fragment, which produces two fragments with different length (238 bp and 319 bp). The mentioned SNP was not identified in the population screened in the present study. In all 308 samples (273 infertile and 35 fertile individuals) both of fragments which were products after *BSe*RI digestion, suggesting that there is no G197T SNP of *PRM1* in the studied population (Fig 2).

Analysis of C248T SNP of PRM2
All PCR products of PRM2 genomic fragment (599bp) were used for digestion with *Msc*I. The absence of mentioned SNP causes full enzymatic digestion of the amplified fragment which produces two fragments with different length (197bp and 402bp). In all 308 samples of the present study, both fragments which were products of *Msc*I digestion were present. Therefore suggesting that, there is not C248T SNP of *PRM2* in the studied population (Fig 3).

Discussion
One of the important events during spermatogenesis is sperm nuclear chromatin condensation. During this event, replacement somatic histones with basic proteins including transition proteins and protamines, leads to normally condensed sperm nuclear genome. During initial steps of sperm chromatin condensation somatic histones are replaced with testis specific histones (29). Subsequently testis specific histones are substituted with transitional proteins. Finally, the transitional proteins are replaced with protamine1 and 2. Different studies have reported that aberrant protamine expression is related with male infertility (19, 21, 30-34). In particular, a number of studies have described infertile male populations with abnormal P1/P2 ratios (30, 31, 33). Among these studies, small populations of infertile men with complete selective absence of protamine2 protein (19, 21) and deregulated protamine1 expression (31) have been reported. Furthermore, literature studies have revealed that improper sperm chromatin condensation leads to different phenotypic expression including protamine deficiency, aberrant P1/P2 ratio and altered semen and even azoospermia. Genetic and molecular analyses have revealed that SNPs may results in such disorders and prone sperm to DNA fragmentation (35). De Yebra et al performed a preliminary mutational analysis of protamine genes in four patients with markedly altered P1/P2 ratios while no mutation was identified in protamine genes (21). Schlicker et al. also screened 36 infertile patients with chromatin anomalies, but they failed to identify any mutation in the gene encoding P1, P2 or TP1, suggesting that altered P1 and P2 expression may have other underlying mechanisms (36). In contrast to these studies, Aoki et al. screened a large patient population with known abnormal protamine ratios. They identified fifteen SNPs (three SNPs in P1, seven in P2, two in TP1 and three in TP2); however, the

frequencies of these SNPs were similar to infertile individuals without protamine deficiency (37). Tanaka et al. recently reported a C248T SNP in Protamine2 gene (25). They identified presence of this SNP among of 266 azoospermic individuals. This SNP results in appearance of a stop codon and premature termination of the protamine2 mRNA leading to azoospermia. Iguchi et al. also reported occurrence of G197T SNP in patients with a fairly normal sperm count but with a markedly altered morphology based upon Kruger's strict criteria with a frequency rate of 3 to 30 (24). The latter SNP converts the highly conserved arginine to a serine residue, which can serve as a potential phosphorylation site for the enzyme serine/arginine-rich protein specific kinase1 (SRPK1). Improper phosphorylation can substantially alter both DNA binding and protamine-to-protamine interaction ability of protamine1 in the sperm nucleus. The aim of this study was to evaluate the occurrence rate of these two SNPs in our study population. The results of SNP analysis in 273 infertile and 35 fertile individuals revealed absence of the two mentioned SNPs in our studied population. The 273 individuals included 96 oligozoospermic and 177 azoospermic individuals. Although the above two SNPs were reported in smaller population than the studied population, it is not uncommon to observed absence of previously reported SNPs. Indeed a more recent study, evaluating two SNPs in *PRM1* in 1195 individuals, one of which was the same as the SNP assessed in this study and they concluded that this SNP has no significant effect in male infertility (38). Even though considerable number of SNPs has been detected in *PRM1* and 2, but the overall conclusion derived from these study suggest that except the SNPs that lead to termination codons, these SNPs do not result in the aberrant expression of P1 or P2. Therefore, recent attention has been directed on the promoters of the *PRM1* and *PRM 2*. Indeed, Gazquez et al., recently, reported an SNP in promoter of *PRM1* which lead to aberrant ratio of P1/P2 and results in abnormal morphology (39,40). The frequency of the reported SNP was significantly different compared to normal fertile males, which suggest that such SNP may serve as a good molecular marker for genetic diagnosis of male infertility.

Conclusion

The results of our study reveal that in a selected Iranian population, the SNP G197T and C248T are completely absent and are not associated with male infertility with aforementioned oligozoospermic and azoospermic conditions and therefore these SNPs may not represent as a molecular marker for the diagnosis of genetic cause of male infertility in our studied population.

Acknowledgments

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