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RESEARCH COMMUNICATION

Analysis of sperm nuclear protein gene polymorphisms and DNA integrity in infertile men

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Sperm nuclear proteins, the protamines (PRM) and transition nuclear proteins (TNP) play a crucial role in sperm nuclear condensation. The compact packaging of sperm DNA by protamines maintains sperm genome integrity, which is prerequisite for normal sperm function. However the effect of nucleotide variations in *PRM* and *TNP* genes on sperm DNA integrity and male fertility is not clear. This case-control study was planned to analyze *PRM* and *TNP* gene nucleotide variations and sperm DNA integrity in 100 oligozoospermic infertile men and 100 fertile controls. Protamine and *TNP* genes were amplified by polymerase chain reaction and sequenced. Flow cytometry-sperm chromatin structure assay (FC-SCSA) was applied to measure the DNA fragmentation index (DFI) in sperm. Semen analysis was performed as per WHO [1999] guidelines with slight modification. In total, 7 nucleotide variations including two novel changes, a non-synonymous mutation in the exon-2 of *PRM2* gene (c.443C > A) and a novel insertion of T (c.396_397InsT) at the 3′ UTR region of *TNP1* were detected. None of the nucleotide changes were observed with increased risk frequency in the oligozoospermic infertile men compared to the controls. Though overall DFI was significantly ($p < 0.0001$) higher in infertile men compared to controls (36.31 ± 7.25 vs. 26.49 ± 2.78) irrespective of nucleotide changes, no such difference was observed between 100 infertile men or pooled population of 200 with and without mutations. However it was observed that two cases with novel nucleotide changes *PRM2* c.443C > A and *TNP1* c.396_397InsT had higher DFI value of 34.82% and 43.85%, respectively. In conclusion, our pilot study for the first time in the Indian population revealed two rare novel mutations in sperm nuclear protein genes that are perhaps associated with higher sperm DNA fragmentation.

Keywords male infertility; mutation; protamine; sperm DNA integrity; transition nuclear proteins

Abbreviations PRM: protamine; TNP: transition nuclear protein; SCSA: sperm chromatin structure assay; DFI: DNA fragmentation index; UTR: untranslated region; WHO: World Health Organization.

Introduction

Couples at higher risk for infertility are increasing in number, and majority of cases are idiopathic. Approximately half of these cases are due to male factor [Poongothai et al. 2009]. Men with idiopathic infertility often present with compromised semen quality. Several genetic factors such as Y chromosome genes (*USP9Y, RBMY*, and *DAZ)*, androgen receptor (AR) gene, and genes regulating pituitary gonadal axis, LH and FSH are involved in the regulation of spermatogenesis, but their role in impaired semen quality is still not clearly understood [Dada et al. 2004; Huynh et al. 2002]. Spermiogenesis is a crucial event in the maturation of sperm where sperm nuclear proteins tend to play an important role in chromatin condensation. Moreover, compact packaging of sperm chromatin is essential for maintaining sperm DNA integrity, which is associated with many physiological events like sperm motility, capacitation, acrosome reaction, normal embryogenesis, and birth of healthy offspring [Castillo et al. 2010; Giwercman et al. 2003; Moskovtsev et al. 2005]. Recent studies have focused on the role of sperm nuclear proteins, the protamines (PRM) and transition nuclear proteins (TNP) on sperm function [Garcia-Peiro et al. 2011; Hammadeh et al. 2010; Hammoud et al. 2009; Haraguchi et al. 2009; Tavalaee et al. 2009; Tseden et al. 2007; Zhao et al. 2001]. PRM1 and PRM2 are present in equal proportions but unlike PRM1, PRM2 gene is translationally regulated in a species specific manner [Bianchi et al. 1992] and unlike TNP1, TNP2 amino acids are highly conserved among various mammals [Kremling et al. 1989]. Altered ratios of PRM1/PRM2 resulting in varied levels of

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chromatin condensation in sperm have also been reported as one of the important causes of male infertility [de Mateo et al. 2007; Mengual et al. 2003; Zhang et al. 2006]. The reason for this altered ratio may reflect the lack of post translational modification or a mutation in the *PRM/TNP* genes. On one hand various population studies have shown that mutations in these genes leading to infertility were not common and on the other hand, these studies have also reported novel mutations and high frequency of single nucleotide polymorphisms (SNPs) in infertile men [Adham et al. 2001; Carrell and Liu 2001; Cho et al. 2003; Cho et al. 2001; Jodar et al. 2010; Mengual, et al. 2003]. This pilot study is the first to report the effect of both *PRM* and *TNP* DNA sequence variations on sperm DNA integrity in the Indian population.

Results

The cDNA nucleotide positions were numbered according to their position in the genomic sequence from the start ATG codon, where A is considered as +1. In the *PRM1 and PRM2* protamine genes, a total of four nucleotide changes were identified as summarized in Figure 1. As summarized in Table 1, this includes one synonymous variant c.230 C > A in exon 2 of *PRM1* and two variants (c.298 G > C, c.373 C > A) in intron 1 of *PRM2* and one novel non-synonymus change (c. 443C > A - Gene accession ID-GU190363) in exon 2 of *PRM2* as shown in Figure 2. PolyPhen prediction analysis identified a novel c. 443C > A mutation as benign. An amino acid change from polar threonine to polar asparagine (p.T94N) was noted. This patient was an oligo-asthenozoospermic male with a sperm count of 17 million/ml that contained only 10% motile sperm. No difference in the frequency of *PRM* nucleotide changes between infertile men and controls was observed (Table 1).

In the *TNP* genes, a total of 3 variants and one novel insertion were identified (Fig. 3). A variant c.214T > C in the intron 1 of *TNP1* was observed with no increase in risk frequency in the infertile men as compared to controls (Table 1). A novel insertion of nucleotide T between 396 and 397 in 3′UTR [T (c.396_397InsT) - Gene accession ID- GU190364] of *TNP1* exon 1 (Fig. 4) was identified in one patient with severe oligozoospermia (sperm count of 2.3 million/ml). Another variant c.391C > T changing amino acid polar arginine (R) to non-polar tryptophan (W) (p.R131W) in *TNP2* was identified in both controls and infertile men. PolyPhen prediction analysis suggested that this change was also benign. All the nucleotide changes in the *TNP* gene were found with no increased risk frequency in infertile men compared to controls.

Sperm chromatin structure assay revealed that the overall DFI in infertile men was significantly ($p < 0.0001$) higher as compared to controls (36.31 ± 7.25 vs. 26.49 ± 2.78). The lowest DFI in the study population was found in controls (19.82%) and the highest DFI was found in an infertile patient (43.85%). When the cases and controls were pooled together (200) and classified as with and without variations (31.27 ± 7.32 vs. 31.99 ± 5.62) no significant ($p = 0.592$) difference in the DFI was observed between subjects with and without variations. Similarly non significant difference ($p = 0.751$) in the DFI was observed between infertile men with and without nucleotide variations (36.19 ± 7.22 vs. 36.83 ± 4.57). However, a case with novel 443C > A mutation in the *PRM2* gene showed a DFI - 34.82 % (Fig. 5), whereas the second case with novel insertion in the non-coding 3'UTR of (c.396_397InsT) *TNP1* gene showed the highest DFI (43.85%) in the study (Fig. 6).

Discussion

Sperm has the unique function of transmitting the paternal genome to the egg. The integrity of sperm genome is a prerequisite for normal embryogenesis and fetal well being. Gradual replacement of histones by the transition proteins followed by protamines plays a crucial role in sperm nuclear condensation and maintaining nuclear DNA integrity [Lewis et al. 2004; Oliva 2006]. However, studies have shown altered PRM1/PRM2 levels in infertile men compared to normal men [Balhorn et al. 1988; de Yebra et al. 1993; Garcia-Peiro et al. 2011; Steger et al. 2008]. These altered levels could be either due to pathogenic mutations in the *PRM* and *TNP* genes or aberrant post translational modification of the protamines, and/or transition protein mRNAs or their products. Mutations in the *PRM* and *TNP* genes and their effect, if any, on male infertility remains controversial. However, studies are lacking in showing the effect of such mutations on sperm DNA integrity.

This study, which was the first in an Indian population has assessed both the nucleotide changes in the *PRM* and *TNP* genes as well as the corresponding changes in the DFI. In this study, the previously reported synonymous SNP c.230C > A (rs737008) in the exon 2 of the *PRM1* gene was identified but with no increase in risk frequency. Other SNPs reported by Ravel et al. [2007] such as c.102G > T and c.119G > A, Aoki et al. [2006] (c. 230C > A), Gázquez et al. [2008] (c.-190 C > A), Imken et al. [2009] (c.65G > A), and Tanaka et al. [2003] (133A > G, 160C > A, 320G > A, 321C > A, 431A > G) in the *PRM1*gene were not identified in this population which may be due to different geographical origin and ethnicity [Aoki et al. 2006; Gázquez et al. 2008; Imken et al. 2009; Ravel et al. 2007; Tanaka et al. 2003]. Among the nucleotide changes observed in the *PRM2* gene, the heterozygous c. 373C > A and the homozygous c.298G > C in *PRM2* gene which has been previously reported were observed but with no increase in risk frequency in the study population. Moreover none of the previously reported *PRM2* SNPs (−67C > T, 398G > A) and translation termination induced 248C > T were identified in this study [Imken, et al. 2009; Tanaka et al. 2003]. But, a novel non-synonymous mutation (c.443C > A) in exon 2 of *PRM2* was observed in an oligo-asthenozoospermic man with a high DFI (34.82%). This is consistent with the notion that this may reflect an amino acid change that altered PRM2 protein conformation leading to deficient protamine packaging. However, this requires functional

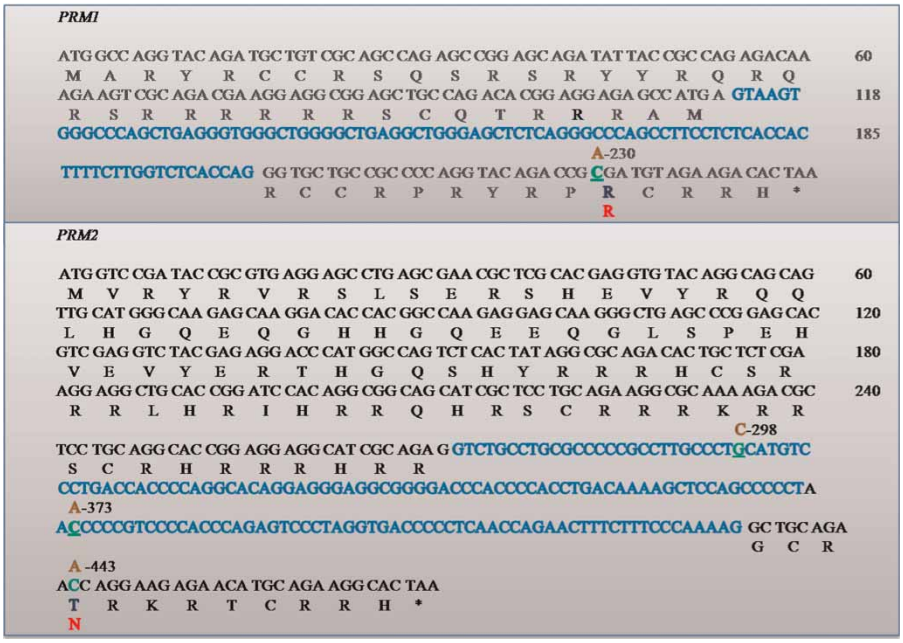

Figure 1. Schematic representation of *PRM1* and *PRM2* gene nucleotide sequence. The blue colored regions are introns. Nucleotide changes are green and the variant is shown just above in brown with their respective position. The corresponding amino acid change is shown below the wild type in red.

confirmation. This case had normal seminal ROS levels (data not shown) and thus oxidative stress causing DNA damage was excluded. This may require the screening of a large number of cases with functional studies to explore pathogenicity, though computational PolyPhen analysis predicted a non-pathogenic change with no alteration in the polarity of the amino acid. Therefore the *PRM* gene associated SNPs identified in this study which correspond to highly conserved amino acids may not contribute to male infertility in this study population.

Since TNPs are the intermediates of sperm chromatin condensation, disruption in TNP expression and binding may also impair sperm DNA integrity. Most of the studies have screened only *PRM* genes for SNPs [Iguchi et al. 2006; Kichine et al. 2008; Ravel et al. 2007] and very few have screened the *TNP* genes [Adham et al. 2001; Miyagawa

Table 1. Sperm *PRM* and *TNP* genotypic and allelic frequency in oligozoospermic infertile and control men.

						Infertile patient (n = 100)			Controls (n = 100)		
Gene	Nt position/ Nt change	Region	NCB ID	Codon change	Amino acid change	Allele frequency (%)	Genotype frequency (%)	P[a]	Allele frequency (%)	Genotype frequency (%)	P[b]
PRM1	230 C → A	Exon 2	rs737008	CGA → AGA	R47R	C: 0.66 A:0.34	CC-0.56 CA-0.20 AA-0.24	NS	C:0.60 A:0.40	CC-0.48 CA-0.24 AA-0.28	NS
PRM2	298 G → C	Intron 1	rs1646022	None	None	G:1.0 C: 0.0	GG- 1.0 GC-0.0 CC-0.0	NS	G:0.98 C:0.02	GG-0.98 GC- 0.00 CC-0.02	NS
PRM2	373 C → A	Intron 1	rs2070923	None	None	C:0.65 A:0.35	CC- 0.55 CA-0.20 AA- 0.25	NS	C:0.60 A:0.40	CC- 0.60 CA-0.00 AA-0.40	NS
PRM2	443 C → A	Exon 2	Novel[c]	ACC → AAC	T94N	C:0.99 A:0.01	CC-0.99 CA-0.00 AA-0.01	NS	C:1.0 A:0.0	CC-1.0 CA- 0.0 AA- 0.0	NS
TNP1	214 T → C	Intron	rs62180545	None	None	T:0.93 C:0.07	TT-0.85 TC-0.15 CC-0.00	NS	T:1.0 C:0.0	TT-1.0 TC-0.0 CC-0.0	< 0.05
TNP1	Ins T 396-397 3′UTR	UTR	Novel[d]	-	-	-	-	-	-	-	-
TNP2	391 C → T	Exon-1	rs11640138	CGC → TGC	R101C	C:0.40 T:0.60	CC-0.40 CT-0.00 TT- 0.60	NS	C:0.43 T:0.57	CC-0.43 CT-0.00 TT-0.57	NS

P[a]: significance of allelic frequency between patients and controls
P[b]: significance of genoptype frequency between patients and controls
[c]: Gene bank ID- GU190363; [d]:- Gene bank ID- GU190364

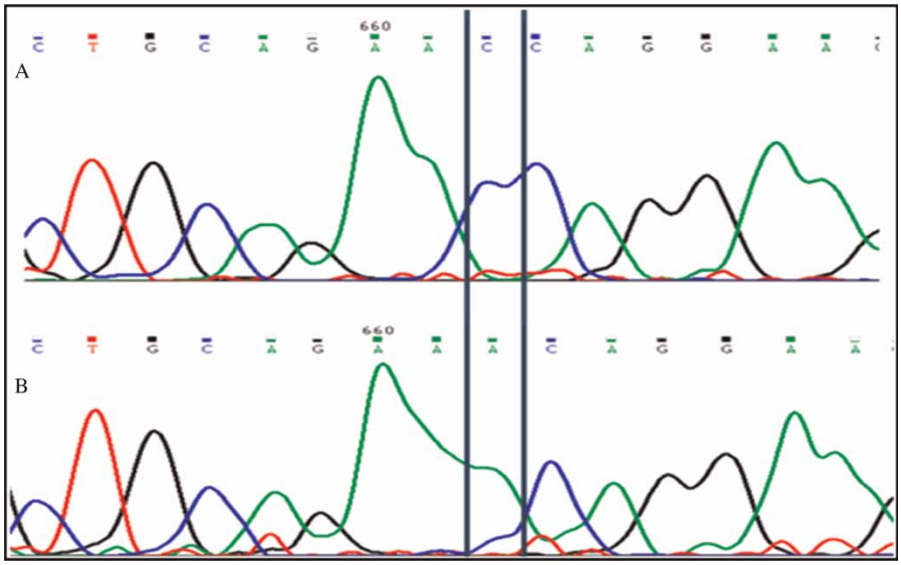

Figure 2. Sequence electrophoreogram of the SNP variant. The nucleotide variant SNP c443C > A in *PRM2* gene is resolved. A) represents wild type sequence (C/C); B) represents sequence showing homozygous mutant (A/A).

et al. 2005]. Studies in mice have shown a lack of *TNP* or null mutant for *TNP* genes and reduced fertility [Yu et al. 2000; Zhao et al. 2004; Zhao et al. 2001]. In the present study, a previously reported heterozygous SNP c.214T > C (rs62180545) in intron 1 of the *TNP1* gene was observed but with no increase in risk frequency of infertile when compared to the control population. However the novel insertion of T between 396 and 397 in the 3′UTR region of the *TNP1* gene in a severe oligozoospermic man (sperm count-2.3 million/ml) showed a higher DFI (44%). The change in the non-coding region (5′ and 3′ UTR) may have a strong effect on protein expression due to disruption of their regulatory mechanism [Hammoud et al. 2007]. This mutation in 3′UTR may alter mRNA stability, localization, and translational efficiency during expression. A previously reported homozygous c.391C > T change in exon 1 of the *TNP1* gene was observed at equal frequency in both infertile men and controls which is in accordance with Aoki et al.

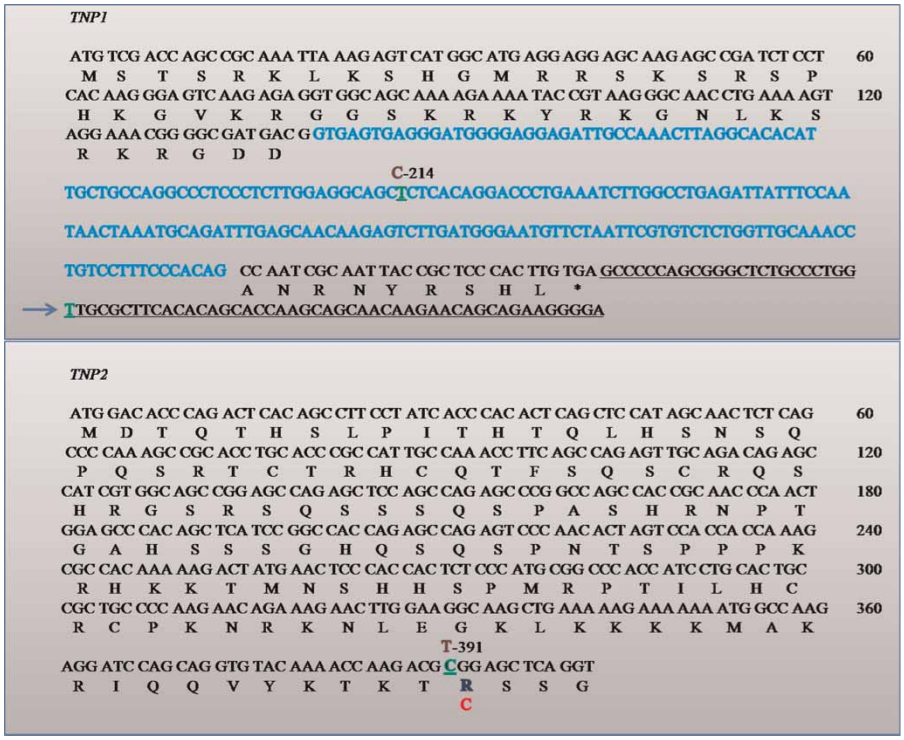

Figure 3. Schematic representations of *TNP1* and *TNP2* genes showing the location of the SNPs. Blue-colored regions are introns. Nucleotide changes are green and the variant is shown just above in brown with their respective position. The corresponding amino acid change is shown below the wild type in red. The non coding exon region is shown underlined.

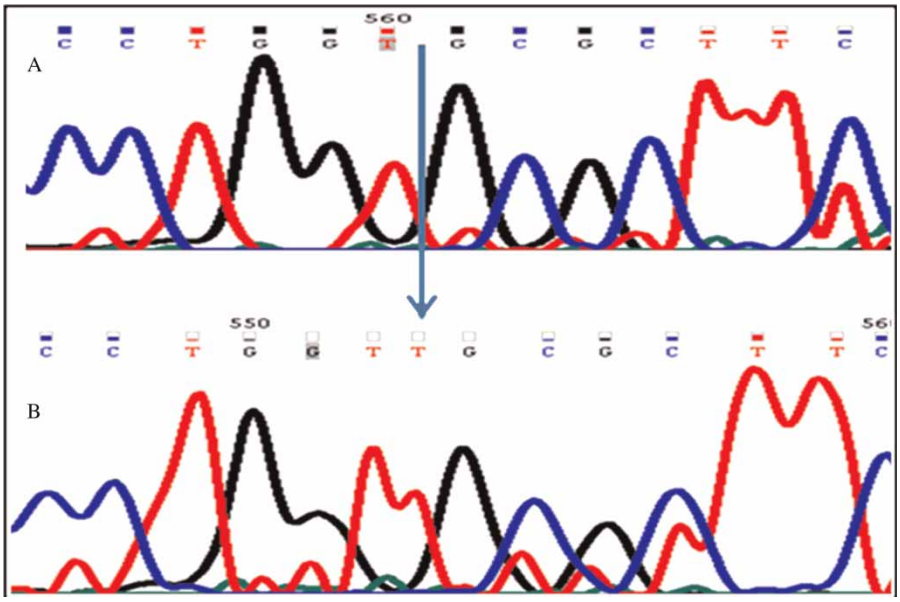

Figure 4. Sequence electrophoreogram of the SNP variant. The insertion c. Ins T 396-397 3′UTR *TNP1* gene is highlighted. A) represents sequence showing homozygous wild-type (GGTG); B) represents sequence showing insertion (GGTTG).

[2006]. Therefore similar to *PRM* genes, *TNP* gene nucleotide variants also had no role in disrupting spermatogenesis in this study population. Though the effect of these SNPs on the gene expression and translation is not well known, the coordinate control of *PRM1, PRM2*, and *TNP2* genes in the protamine cluster are essential during spermatogenesis [Wykes and Krawetz 2003]. Similar to most of the previous reports, there is no increase in risk frequency of sperm nuclear protein gene nucleotide changes associated with male infertility in our population [Aoki et al. 2006; Jodar et al. 2010]. However, the two novel mutations require further screening in a large number of cases and different populations.

Infertile men with protamine deficiency may have poor sperm DNA packaging that makes the sperm genome highly susceptible to toxic environmental stimulus such as mutagenic agents, chemical and mechanical stimuli, and more susceptible to free radical induced damage and higher DNA fragmentation [Nili et al. 2009; Venkatesh

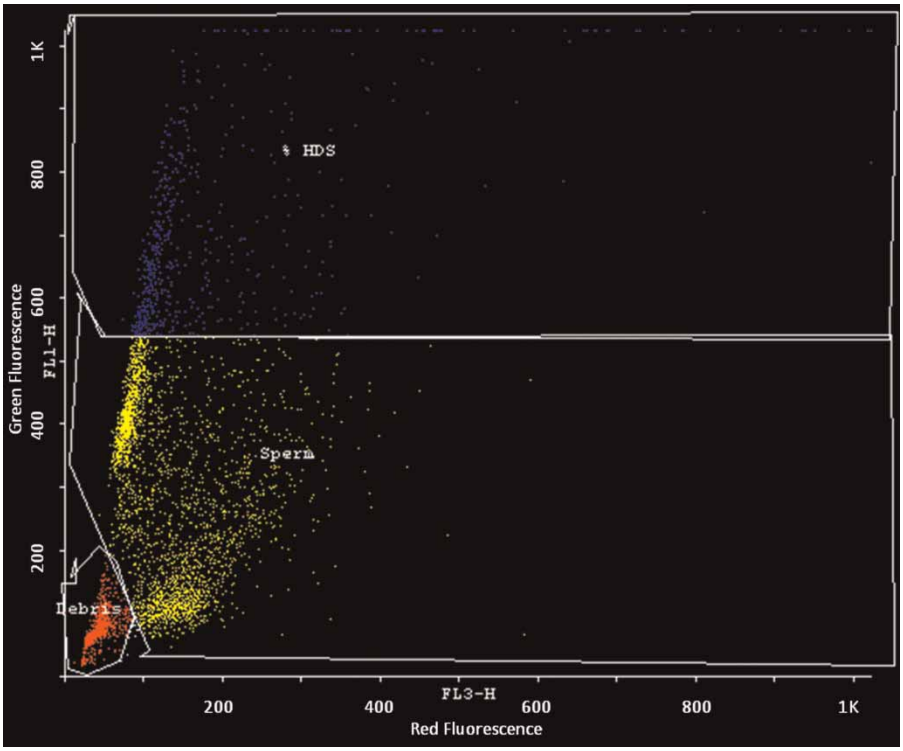

Figure 5. SCSA pseudocolor dot plot of semen sample presenting with c443C > A mutation. FL 3-H -axis represents fragmented DNA and FL 1-H-axis represents native DNA. HDS: high DNA stainability cells in the semen.

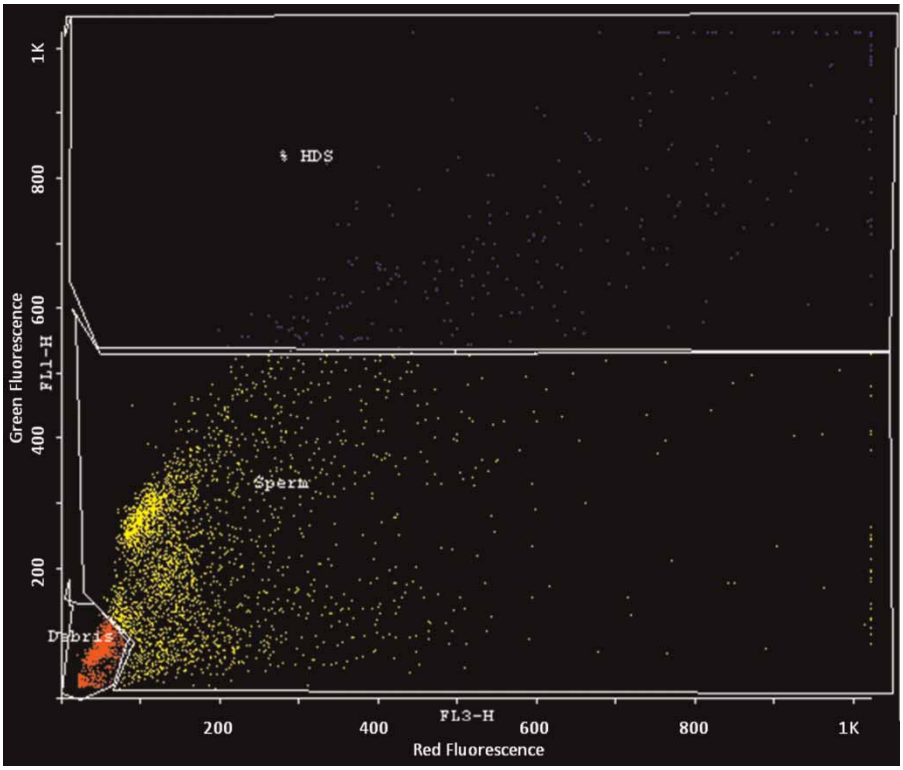

Figure 6. SCSA pseudocolor dot plot of semen sample presenting with c.396_397InsT mutation. FL 3-H -axis represents fragmented DNA and FL 1-H- axis represents native DNA. HDS: high DNA stainability cells in the semen.

et al. 2009]. When the pooled population of 200 were grouped into men with and without nucleotide changes, our results failed to support the effect of the observed mutations on sperm DNA integrity. Similar results were achieved when infertile men were grouped into men with and without mutations. However it is important to note that two cases with novel mutations c.443C > A and 3′UTR c.396_397InsT had a higher degree of sperm DNA fragmentation of DFI 34.82% and 43.85%, respectively. The DFI of the oligoasthenozoospermic case with c.443C > A is higher than the average controls (26.49%) and the effect of amino acid change on the protein structure cannot be overruled. This case also harbored the *PRM1* c.230 C > A silent mutation. In the second case that presented with a novel 3′UTR c.396_397InsT in the *TNP1* gene, the sperm showed the highest DFI (43.85%). The case also harbored the previously reported *PRM2* intronic c.373 C > A variant. As TNP2 proteins are necessary to initiate the protamine binding to the sperm DNA, mutation in the 3'UTR may alter the mRNA stability and subsequently result in aberrant translation. Both novel variants were not observed in the control population. It would be of interest to screen these novel variants in a large number of samples. It is well known that compact packaging of sperm chromatin is essential for maintaining the sperm DNA integrity, which is essential for normal sperm motility, capacitation, acrosome reaction, successful fertilization, placentation, and embryogenesis as sperm are not mere vectors of paternal DNA but are important determinants of the developmental competence of the embryo [Wykes and Krawetz 2003]. Moreover, in our study the variants observed in the intronic regions such as c.298G > C, c.373C > A of *PRM2* and c.391C > T, c.396_397InsT of the *TNP1* gene are in the highly conserved region of eutherian mammals. Altered nucleotide changes in conserved regions may have a significant role in impaired gene expression and translation. Though protamine deficiency may lead to aberrant DNA packaging in the sperm, polymorphisms in the sperm nuclear protein gene may not have significant effect on sperm DNA integrity. An altered epigenetic mechanism and impaired post translational modification of the *PRM* and *TNP* genes should be considered to resolve the 92% of infertile men in the current study that presented with a high DFI (>30%). It is necessary to elucidate other factors involved in the maintenance of genomic integrity.

Materials and Methods

Study population

After approval from the institute ethical committee (IEC), AIIMS, informed consent was obtained from the subjects enrolled in the study. The study included 100 oligozoospermic men with an average duration of infertility of 5.60 ± 3.77 years and 100 controls (fathered a child in last one year). After thorough examination and questionnaire evaluation, oligozoospermic men (< 20 million sperm/ml) with normal 46, XY chromosomal complement, absence of Yq microdeletion, no varicocele, and no hydrocele, were only included in the study. After 4 days of sexual abstinence, semen samples were collected in a wide mouth sterile plastic container and

delivered to the laboratory immediately. Semen was allowed to liquefy at room temperature and the semen parameters were analyzed as per WHO [1999] guidelines. For morphological evaluation, a 10 µl of semen smear was prepared on a clean microscope slide and fixed with 90% ethanol and stained with Giemsa. A minimum of 100 sperms per sample were evaluated for the morphological defects.

Separation of spermatozoa and sperm DNA isolation

After semen analysis, the sample was layered in isolate sperm separation medium, (Irvine Scientific Co., Santa Ana, CA, USA) and centrifuged at 300 rpm for 10 min to separate sperm cells from other cells like leukocytes, epithelial cells, and debris. After confirming the absence of other cells by examining the smear under microscope, sperm cells were washed with sperm washing media (Irvine Scientific Co.). The washed cells were subjected to DNA isolation [Laird et al. 1991]. Briefly, the sperm pellet was incubated at 55°C overnight with the sperm lysis medium (60 mM DTT, 4% SDS and 350µg/ml protienase K made in lysis buffer). After complete digestion, sperm DNA was precipitated by adding an equal amount of chilled isopropanol. The DNA pellet was then washed with 70% ethanol, dried at 37°C, and dissolved in Tris-EDTA (TE) buffer.

Identification of SNPs in the *PRM* and *TNP* genes

Four primer pairs were used and a 25 µl PCR reaction mixture was prepared as described by Aoki et al. [2006] with slight modification: 94°C for 4 min followed by 35 cycles of 94°C for 30s; annealing temperature for 30s [*PRM1*-62.5°C, *PRM2*-88.5°C, *TNP1*-55.5°C, *TNP2*-59°C], extension of 72°C [*PRM1*-60s, *PRM2*-80s, *TNP1*-50s, *TNP2*-70s], and a final hold for 5 min at 72°C. The amplified PCR products were confirmed by using a 2 µl aliquot of the reaction products as resolved on a 2% agarose gel. The remaining amplified products were then purified by guanidium-HCl method using a gel/PCR DNA fragments extraction kit (Geneaid, Biotech Ltd., Sijhih City, Taiwan). The purified products were directly sequenced after the samples were dissolved in 10 µl of 50% deionized formamide and sequenced in an automated DNA analyzer (Applied Biosystems, Foster City, CA, USA). The obtained sequences were analyzed using chromas software and compared with the reference sequence to identify nucleotide changes using Genebee multiple alignment (http://www.genebee.msu.su).

Sperm chromatin structure (SCSA) assay

Preparation of samples

SCSA was performed [Evenson et al. 2002] with slight modification. The aliquot from each ejaculate was thawed in a water bath at 37°C for 30s and diluted to a concentration of 2×10^6 sperm/ml in TNE (Tris-50mM (pH 7.5), NaCl-140mM, EDTA-5mM) buffer to a total of 200 µl in a Falcon tube. Immediately, 0.4 ml of acid detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1% v/v Triton X-100, pH 1.2) was added to the Falcon tube. After exactly 30s, 1.2 ml of Acridine Orange (AO)-staining solution (6 µg AO (chromatographically purified) (Polysciences Inc. PA, USA) per ml citrate buffer (0.037 M citric acid, 0.126 M Na_2HPO_4, 1.1 mM EDTA disodium, 0.15 M NaCl, pH 6.0) was added. For every six test samples, one reference sample was analyzed to ensure instrument stability.

Flow cytometric measurements

The samples were analyzed using a FAC Scan flow cytometer (BD Biosciences, CA, USA), with an air-cooled argon laser operated at 488 nm and a power of 15 mW. The green fluorescence (FL1) was collected through a 515–545 nm bandpass filter, and the red fluorescence (FL3) was collected through a 650 nm longpass filter. The sheath/sample was set on 'low', adjusted to a flow rate of 200 events/s when analyzing a sample containing 2×10^6 sperm/ml. Immediately after the addition of the AO staining solution, the sample was placed in the flow cytometer and run through the flow system. After complete analysis of the sample, the X-mean (red fluorescence) and Y-mean (green fluorescence) values were recorded manually after selecting gate for sperm cells using Cyflogic software version 1.2.1 (CyFlo Ltd, Finland).

DFI calculation

The sperm cells are gated after excluding debris and high DNA stainability (HDS) cells. The DNA fragmentation index (DFI) was calculated by the ratio of mean red fluorescence to the sum of mean red and mean green fluorescence. The study population including both infertile men and controls were categorized into groups with one or more mutation and without mutation. DFI was compared between these two groups to find the effect of these mutations on sperm DNA integrity.

Computational assessment of missense mutations

The homology-based program PolyPhen (polymorphism phenotyping; Division of Genetics, Department of Medicine, Brigham and Women's Hospital/Harvard Medical School, Boston, MA) was used in this study to predict the functional impact of missense mutation. PolyPhen scores of > 2.0 indicate the polymorphism is probably damaging protein function. Scores of 1.5 – 2.0 are possibly damaging, and scores of < 1.5 are likely benign.

Statistical analysis

DFI between infertile men and controls were compared by Mann-Whitney test. Genotypic and allelic frequency between infertile men and controls were analyzed by Fischer's exact test. Statistical analyses were performed using MedCalc trial version for Windows, (MedCalc Software, Mariakerke, Belgium).

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