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Single nucleotide polymorphisms in the protamine-1 and -2 genes of fertile and infertile human male populations

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Although various genetic factors have been implicated in human male infertility, the causative genes for the different types of idiopathic male infertility have not been elucidated. Protamines, which are the major DNA-binding proteins in the sperm nucleus, package the DNA into the sperm head. Analysis of the human protamine-1 (PRM1) and -2 (PRM2) gene sequences in 226 sterile male patients and in 270 proven-fertile male volunteers revealed four single nucleotide polymorphisms (SNPs) in the PRM1 coding region, which did not cause any amino acid substitutions, and one SNP in the PRM2 gene, which produced translation termination. We also observed one SNP in the 3′ non-coding region of the PRM1 gene, and two SNPs within the intron of the PRM2 gene. The prevalence of these SNPs was similar in both infertile patients and in proven-fertile volunteers, except that the c248t alteration in the PRM2 gene induced a nonsense codon under conditions of heterozygosity in one infertile patient. Although the PRM1 and PRM2 genes are highly conserved, the single SNP in the PRM2 gene that induces translation termination may result in male infertility due to haploinsufficiency of PRM2.

Key words: male infertility/mutation/protamine/SNPs/sperm

Introduction

Approximately 10% of couples are known to experience some form of infertility, and about half of these cases are due to male factors. Although several causes have been proposed for impaired male fertility (Cram *et al*., 2001), the majority of cases, which involve either inadequate spermatogenesis or defective sperm, remain to be elucidated (Thielemans *et al*., 1998).

During spermatogenesis, the sperm nucleus undergoes a marked rearrangement, which involves the removal of histones and their replacement by various nuclear proteins, including highly positively charged protamines (Wouters-Tyrou *et al*., 1998; Sassone-Corsi, 2002). The DNA of human sperm is highly condensed in the sperm head by protamine-1 (PRM1) and -2 (PRM2). Although PRM1 is a single polypeptide of 50 amino acids, PRM2 can have at least two different forms, i.e. proteins of 57 and 54 amino acids (McKay *et al*., 1986). The PRM2 family proteins are synthesized as precursors of 66–101 residues from a single-copy gene on chromosome 16 (Krawetz *et al*., 1989; Reeves *et al*., 1989).

It has been suggested that disturbances in nuclear condensation might result in male infertility. Premature translation of PRM1 mRNA causes precocious nuclear condensation and arrests spermatid differentiation in mice (Lee *et al*., 1995). A reduction in PRM2 content has been reported in various studies on infertile patients (Balhorn *et al*., 1988; Belokopytova *et al*., 1993), and some infertile male patients were shown to have a complete selective absence of PRM2 in the nuclei of their sperm (de Yebra *et al*., 1993). However, subsequent sequencing of the PRM2 genes from these patients eliminated mutation as the cause of the observed reduction in PRM2 (de Yebra *et al*., 1993; Schlicker *et al*., 1994). In addition, it has been proposed that incomplete processing of the PRM2 precursor molecule might reduce the PRM2 levels in certain infertile patients (de Yebra *et al*., 1998).

In the present study, we assessed the prevalence of protamine gene single nucleotide polymorphisms (SNPs) by direct sequencing of PCR-amplified DNA from male patients who were undergoing fertility evaluation. DNA samples were analysed from 496 men: 226 infertile patients and 270 proven-fertile volunteers. SNPs that caused amino acid substitutions in the PRM1 gene were not identified in this analysis. One SNP in the PRM2 gene that induced translation termination was observed in only one infertile patient. This SNP would be expected to cause male infertility even in the hemizygous condition, since haploinsufficiency of PRM1 or PRM2 is known to cause infertility in male mice (Cho *et al*., 2001).

Materials and methods

Participants

Infertile patients ($n = 226$) were divided into subgroups according to the degree of defective spermatogenesis. One hundred and fifty-three (68%) of these patients had non-obstructive azoospermia, and 73 (32%) had severe oligospermia ($<5 \times 10^6$ cells/ml). The control group of fertile males ($n = 270$) comprised men who had fathered children borne by women at the maternity clinic. DNA samples were extracted from the blood leukocytes of infertile and proven-fertile males who gave permission for their blood to be used for the analysis of genomic DNA in this research.

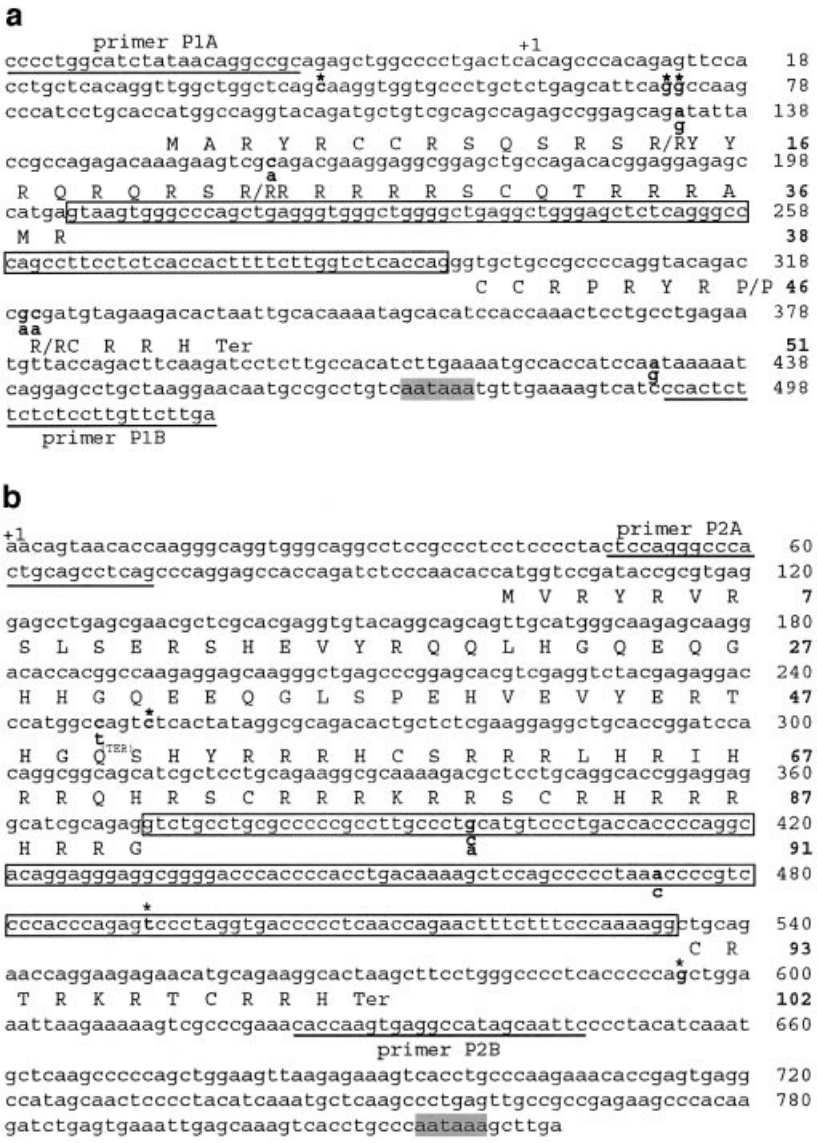

Figure 1. Genomic DNA sequences of the protamine-1 (PRM1) (**A**) and -2 (PRM2) (**B**) genes, and location of the primers used for PCR amplification and sequencing. The transcriptional start site, the intron and the canonical polyA-addition signal are indicated as nucleotide +1, the open box and shaded region respectively (Domenjoud *et al.*, 1990). The underlined primer sequences are: P1A and P1B for PRM1; and P2A and P2B for PRM2. The amino acid sequence of each protein is shown in capital letters under the nucleotide sequence. The numbers in the right margin indicate the positions of the nucleotide and amino acid (bold) sequences. Single nucleotide polymorphisms (SNPs) that we observed are shown in bold letters. Stars indicate nucleotide differences in all 496 human males, which included both infertile and proven-fertile populations, from the sequences obtained from DNA sequence databases (EMBL/DDBJ/GenBank accession nos Y00443, M29706, M60331, M60332 and U15422). c of nucleotide 44 in PRM1 included double c in our results.

Identification of SNPs in the PRM1 and PRM2 genes by direct sequencing of PCR-amplified DNA

Genomic DNA was isolated from blood samples using protease and phenol purification (Sambrook *et al*., 1989). Two primer pairs of PCR were designed from the 5′ and 3′ flanking regions to amplify the two protamine genes. The 24-nucleotide 5′ end primer P1A (5′-cccctggcatctataacaggccgc-3′) from nucleotides –42 to –19 upstream of the transcription start site, and the 24-nucleotide 3′ end primer P1B (5′-tcaagaacaaggagagaagagtgg-3′) from nucleotides 492 to 515 downstream of the canonical polyA-addition signal (AATAAA) were used to amplify the PRM1 gene (Domenjoud *et al*., 1990). The 24-nucleotide 5′ end primer P2A (5′-ctccagggcccactgcagcctcag-3′) from nucleotides 49 to 72, and the 24-nucleotide 3′ end primer P2B (5′-gaattgctatggcctcacttggtg-3′) from nucleotides 624 to 647 were used to amplify the PRM2 gene (Domenjoud *et al*., 1990). Using these primers, we were able to amplify fragments of 557 nucleotides (from nucleotides –42 to 515) and 599 nucleotides (from nucleotides 49 to 648) of the PRM1 and PRM2 genes respectively (Figure 1). The PCR conditions were as follows: 40 cycles of denaturation at 96°C for 45 s, annealing at 66°C for 45 s, and extension at 72°C for 1 min for PRM1; and 40 cycles of denaturation at 98°C for 10 s, annealing at 68°C for 45 s, and extension at 72°C for 45 s for PRM2. The PCR-amplified fragments were purified using the SUPREC PCR spin column (Takara, Shiga, Japan) and thermal cycle sequencing (ABI, WI, USA) was performed. All DNA sequences were determined using the same PCR primers in two directions to confirm that the sequences were consistent.

Results

Analysis of SNPs of PRM1

PCR primers from both ends of the gene were used to check the PRM1 gene sequences (EMBL/DDBJ/GenBank accession nos Y00443,

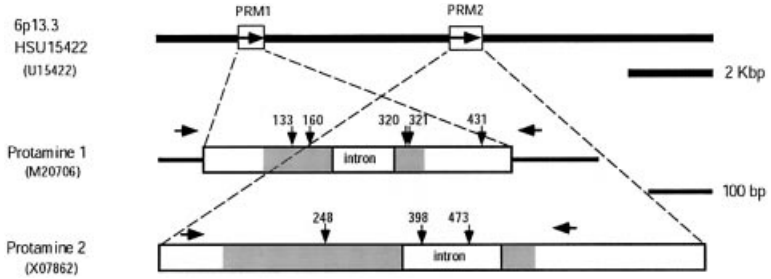

Figure 2. Schematic representation of the PRM1 and PRM2 genes and proteins, together with the positions of the SNPs. PCR-amplified DNA of the PRM1 and PRM2 genes was sequenced directly using two pairs of primers (horizontal arrows). PCR primer sets were designed from the 5′ and 3′ flanking regions of the genome and of the coding region to amplify the PRM1 and PRM2 genes respectively. The sequences of the PCR-amplified fragments were determined by direct sequencing. The upper row shows the arrangement of the two protamine genes, and the unit (open bar) and direction (arrows) of transcription. The lower two rows show the transcriptional units (open boxes) of the two protamine genes. The shaded regions indicate the open reading frames of the two protamine genes. Bold lines indicate the 5′ and 3′ flanking genomic regions. Vertical arrows indicate SNP positions, and the numbers indicate the nucleotide distances from the transcriptional initiation site of each protamine gene.

Table I. Prevalence of single nucleotide polymorphisms (SNPs) in protamine-1 and -2 in infertile or proven fertile populations

	Position			Genotype	Number (%) of SNP				Reference (NCBI dbSNP rs#)
	Nucleotide	Amino acid			Infertile		Proven fertile		
Protamine-1	133	14	(R)	a/a	220	(97.3)	268	(99.3)	
				a/g	6	(2.7)	2	(0.7)	
	160	23	(R)	c/c	226	(100)	269	(99.6)	
				c/a	0	(0)	1	(0.4)	
	320	46	(R)	g/g	225	(99.6)	270	(100)	
				g/a	1	(0.4)	0	(0)	
	321	47	(R)	c/c	125	(55.3)	129	(47.8)	
				c/a	86	(38.1)	117	(43.3)	allele c or a
				a/a	15	(6.6)	24	(8.9)	rs737008****
	431*			a/a	225	(99.6)	269	(99)	
				a/g	1	(0.4)	1	(1)	
Protamine-2	248	50	(Q)	c/c	225	(99.6)	270	(100)	
			(Ter)***	c/t	1	(0.4)	0	(0)	
	398**			g/g	127	(56.2)	127	(47.0)	
				g/c	80	(35.4)	118	(43.7)	
				g/a	0	(0)	1	(0.4)	allele g or c
				c/c	19	(8.4)	24	(8.9)	rs1646022
	473**			a/a	125	(55.3)	127	(47.0)	
				a/c	82	(36.3)	118	(43.7)	allele a or c
				c/c	19	(8.4)	25	(9.3)	rs2070923
Total					226		270		

*3' non coding region.
**intron.
***Ter: termination codon (tag).
****NCBI SNP cluster ID.

M29706 and M60331; Lee *et al*., 1987; Krawetz *et al*., 1989; Domenjoud *et al*., 1990) of both the infertile and proven-fertile subjects. Direct sequencing of the PCR-amplified DNA was performed using genomic DNA from blood samples. The PCR-amplified 557 bp DNA fragment included an intron of 91 nucleotides from nucleotides 204 to 294 (Figure 1A). Thus, we can identify SNPs, if they are located within 509 bp inside the primer sequence in the 557 bp DNA fragment. The primer sequences should not have contained SNPs, since all of the DNA samples were amplified to approximately the same extent, as assessed by agarose gel electrophoresis (data not shown). The SNP prevalences were compared in infertile males and proven-fertile males. We found SNPs at five different loci (four in the coding region at nucleotides 133, 160, 320 and 321, and one at nucleotide 431 in the 3′ untranslated region) in a total of 496 human males, which included 226 infertile subjects and 270 fertile subjects (Figure 2 and Table I). None of these SNPs resulted in amino acid changes. The three SNPs at a133g, c160a and g320a; which corresponded to amino acids 14, 23 and 46 respectively (Figure 1A), were either major homozygous or heterozygous SNPs; no minor homozygous SNPs were observed (Table I). In terms of the c321a SNP at amino acid 47, 55.3% (125) and 47.8% (129) were homozygous major c/c types, 38.1% (86) and 43.3% (117) were heterozygous (c/a), and 6.6% (15) and 8.9% (24) were homozygous minor-type (a/a) SNPs in the infertile and fertile populations respectively. Similar to the a431g SNP in the 3′ non-coding region, these SNPs did not show a higher prevalence in infertile patients than in proven-fertile volunteers and they caused no amino acid changes (Table I).

Analysis of SNPs in the PRM2 gene

The genomic DNA of the PRM2 gene (EMBL/DDBJ/GenBank accession nos M60332 and U15422; Domenjoud *et al*., 1990; Nelson and Krawetz, 1994) was also sequenced in various infertile patients and proven-fertile volunteers. SNPs can be identified, if they are located within 551 bp inside of the primer, by direct sequence analysis

of the 599 bp DNA fragments (Figure 1B). The primer sequences should not have contained SNPs since the PCR amplified approximately the same amount of DNA from each sample (data not shown). We observed three SNPs of 599 nucleotides of the PRM2 gene; one was in the exon and two were in the intron (Figure 2). The heterozygous SNP at nucleotide 248, which introduced a c to t change and thus altered the glutamine residue to a stop codon, was observed in only one of the 153 azoospermic patients, and was absent in the 270 fertile controls (Table I). This SNP might cause azoospermia, even in the hemizygous condition (see the Discussion section). Furthermore, we found two SNPs, g398c and a473c in the intron; g398c 56.2% (127) and 47.0% (127) were homozygous of the major (g/g) type, 35.4% (80) and 43.7% (118) were heterozygous (g/c), and 8.4% (19) and 8.9% (24) were minor-type homozygous (c/c) in infertile and fertile control populations respectively. In addition, we also found another heterozygous SNP of the g/a type at nucleotide 398 in one fertile volunteer. With respect to the a473c SNP, 55.3% (125) and 47.0% (127) were major homozygotes of the a type, 36.3% (82) and 43.7% (118) were heterozygotes, and 8.4% (19) and 9.3% (25) were minor homozygotes of the c type in the infertile and fertile populations respectively. The prevalence of these intron SNPs in infertile males was no different from that in proven-fertile volunteers (Table I).

The clinical features of the infertile patients in this study were idiopathic and limited to severe impairment of spermatogenesis. The majority of the infertile males displayed azoospermia, and the c248t PRM2 mutation was observed in this group. Further investigations using a larger population of infertile cases and defined SNP pedigrees should confirm the causal link between protamine gene polymorphisms and male infertility.

Discussion

Several genetic factors have been associated with defects in human sperm production, including deletion of Y-chromosomal regions (de Vries *et al*., 2002), gene mutations in methylene tetrahydrofolate reductase (Bezold *et al*., 2001) or in the mitochondrial DNA polymerase locus (Rovio *et al*., 2001), certain molecular variants of LH (Ramanujam *et al*., 2000), human leukocyte antigen (HLA) gene defects (van der Ven *et al*., 2000), a polymorphism of the cytochrome P450-1A1 gene (Fritsche *et al*., 1998), and CAG repeats in the androgen receptor (Dowsing *et al*., 1999; De Meyts *et al*., 2002). None of these factors represents an unambiguous causative agent of infertility or explains all idiopathic male infertility, and the debate continues as to their significance (Dowsing *et al*., 1999; De Meyts *et al*., 2002).

During spermiogenesis, the successive replacement of somatic histones by basic proteins, i.e. transition proteins and protamines, allows normal nuclear condensation in sperm. It has been suggested that disturbances in nuclear condensation result in male infertility. This notion has been supported by the observation that infertile individuals have decreased or null levels of male, sperm-specific, chromatin-packaging protamines (Belokopytova *et al*., 1993; de Yebra *et al*., 1993). Biochemical analyses of pooled human sperm from proven-fertile and infertile males suggest that the relative proportion of PRM1 and PRM2 that is bound to DNA is an important factor in fertility (Bench *et al*., 1998). Sperm samples from infertile individuals that produce only sperm with large, rounded heads (round-headed sperm syndrome) have been shown to be deficient in PRM2 (Balhorn *et al*., 1988). While the above studies have correlated protamine defects in sperm with infertility, the conclusions have been drawn from analyses of whole semen or pools of millions of cells. These investigators could not rule out the possibility that the observed deficiency might reflect the presence of a sub-population of supporting testicular cells, highly abnormal sperm, or spermatids that were arrested at a particular point in their development. Furthermore, the prospect that a significant number of the sperm that were produced by these males might have been biochemically normal was not addressed. Light microscopic studies have indicated that many infertile human males produce some sperm that appear morphologically normal. In addition, the degree of maturation of sperm cells that are present in the semen of some infertile human males can vary with time (Bench *et al*., 1998). Furthermore, no correlation has been documented between the absence of protamine proteins and mutations within the coding regions of the protamine genes (de Yebra *et al*., 1993; Schlicker *et al*., 1994). In the present study, we found three PRM1 SNPs that did not cause amino acid substitutions and that were equally prevalent in male infertility cases and proven-fertile volunteers. The SNP database at NCBI revealed a SNP at nucleotide 321 in the translated region of PRM1 (NCBI SNP CLUSTER ID rs 737008), which is in agreement with our data (Table I). We also found differences among the registered sequences at nucleotide 44 (Lee *et al*., 1987; Domenjoud *et al*., 1990), and at nucleotides 72 and 73 (Krawetz *et al*., 1989; Domenjoud *et al*., 1990). Nevertheless, our data showed a common sequence (Figure 1) for all 496 human males from both the infertile and proven-fertile populations. These results may be due to racial differences between the Japanese subjects in our studies and the ethnic groups who are represented in the sequence databases. Alternatively, rare SNPs may have been used for DNA sequencing and thus were registered.

We also found sequence differences within the intron of the PRM2 gene: one base (t) was deleted at nucleotide 492 in the PRM2 gene sequences (EMBL/DDBJ/GenBank accession no. M60332) of all 496 samples from both infertile and proven-fertile males. Furthermore, we found one SNP within the coding region and two SNPs in the intron of the PRM2 gene. The c248t SNP, which changes glutamine to a stop codon, was found in the middle of the PRM2 coding region. Although we call this SNP a mutation, since it produces severe changes in the PRM2 protein, it was observed only once in 153 azoospermia patients in the hemizygous condition. If protamine haploinsufficiency causes infertility in humans, as it does in mice (Cho *et al*., 2001), this mutation should be an important cause of infertility in this single patient and should have been inherited from his mother. An investigation into the genetic pedigree of this patient might confirm this notion. The other two SNPs in g398c and a473c did not show different prevalences among the proven-fertile and infertile populations. We also found a g/a heterozygote at nucleotide 398 in the fertile controls. These SNPs did not affect the function of the PRM2 protein. Although the SNP database at NCBI showed SNP at c252t that induced an amino acid change from Ser to Phe (NCBI dbSNP rs 3177008) and at c595g (NCBI dbSNP rs 452495), we did not find these SNPs in any of the 496 Japanese males from both infertile and proven-fertile populations. The conservation of protamines appears to be crucial, and even a slight change in the protamine gene may lead to male infertility. Furthermore, the introns were highly conserved in both the PRM1 and PRM2 genes, which indicates that they play important roles in the expression of protamine genes or in maintaining the stability of gene expression.

Ca++/calmodulin-dependent protein kinase IV (CamK4), which is a multifunctional serine/threonine protein kinase with limited tissue distribution, is necessary for spermiogenesis and for the exchange of basic nuclear proteins to protamines. Male Camk4 KO mice are infertile due to the impairment of spermiogenesis in late-elongating spermatids. A specific loss of PRM2 without concomitant loss of phosphorylation would disrupt the sequential deposition of sperm basic nuclear proteins on chromatin (Wu *et al*., 2000). Another protein kinase, casein kinase II, which is a cyclic-AMP and calcium-

independent serine-threonine kinase, is also necessary for spermiogenesis. Casein kinase 2a2 (Csnk2a2) is expressed preferentially in the late stages of spermatogenesis, and male mice in which Csnk2a2 has been disrupted are infertile, in that they display oligospermia and globozoospermia (round-headed spermatozoa). This phenomenon may be caused by abnormal chromatin condensation (Xu *et al*., 1999). The specific histone acetyltransferase encoded by chromodomain of Y (CDY), which is a human Y-chromosomal gene family that is located in the AZFc region, was demonstrated to play an important role in the displacement of histone to protamine in spermiogenesis (Lahn *et al*., 2002). A major reduction in total CDY protein might interfere with the histone-to-protamine transition during spermiogenesis, thus contributing to overall spermatogenic failure. The low incidence of protamine gene variation or SNPs causing amino acid substitution in fertile men indicates the strong requirement for conserved protamine function during normal fertilization.

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