

Single-Nucleotide Polymorphisms and Mutation Analyses of the *TNP1* and *TNP2* Genes of Fertile and Infertile Human Male Populations

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ABSTRACT: Previously, we examined the relationship between protamine gene variations and human male infertility. In this study, we show specific variability in the transition nuclear protein genes (*TNPs*) of sterile male patients. Transition nuclear proteins (TPs) are major nuclear proteins that replace nuclear histones, leading to eventual substitution by protamines during human spermiogenesis. Analysis of the human *TNP1* and *TNP2* gene sequences in 282 sterile male patients and 270 (*TNP1*) and 266 (*TNP2*) proven-fertile male volunteers revealed 5 amino acid substitution—causing single

nucleotide polymorphisms (SNPs) in the open-reading frame of the *TNP2* gene. On the other hand, a deletion of 15 nucleotides, which encompassed the recognition site for the cAMP response element (CRE) transcription factor, was found in the 5'-promoter region of the *TNP1* gene in infertile men. This deletion reduces *TNP1* expression and may cause human male infertility.

Key words: Protamine, transition nuclear protein, sperm, male infertility, genome, promoter, SNPs.

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Approximately 15% of couples who attempt to conceive over a period of 2 years are unable to become pregnant (de Kretser and Baker, 1999). Recent technological developments in in vitro fertilization (IVF) have ensured that even when sperm activity is low, pregnancy and birth are possible. The molecular mechanisms behind infertility remain uncertain. Many genes have been implicated in male sterility experiments with mice (Matzuk and Lamb, 2002), and it is possible that mutations in these genes are also related to human infertility.

During spermiogenesis, round spermatids undergo complex morphologic, physiologic, and biochemical modifications that result in the formation of mature spermatozoa. These specific events are supported by spermiogenesis-specific gene products (Tanaka and Baba, 2005). The sperm nucleus undergoes marked rearrangement, which involves the removal of histones and their replacement by various nuclear proteins. Finally, the DNA of human sperm is highly condensed in the sperm head by highly positively charged protamines (PRMs; Tanaka and Baba, 2005). The replacement of histones and the depo-

sition of protamines is supported by different nuclear proteins, including the transition nuclear proteins (TPs), for major remodeling of the chromatin (Meistrich et al, 2003). Almost all of these nuclear basic proteins, including PRMs, are derived from histone H1 and undergo complex processes of modification in mammals (Lewis et al, 2004). The *PRM1*, *PRM2*, and *TNP2* genes of these nuclear proteins, which are expressed during spermiogenesis, are clustered on 16p13.13 (Martins et al, 2004). PRMs are highly charged, arginine rich, and bind to DNA in a nonspecific manner. However, the mechanism of condensation of sperm chromatin has not been resolved. On the other hand, the technique of gene targeting to produce knockout animals allows the study of gene function in vivo. Disruption of *PRM1* or *PRM2* in mice has shown that the PRM1 and PRM2 proteins are essential for fertility and that haploinsufficiency is caused by a mutation in 1 protamine allele (Cho et al, 2001). Moreover, when *PRM2* is disrupted, the resultant sperm nuclei are infertile, even via intracytoplasmic sperm injection (ICSI) (Cho et al, 2003). These results indicate that PRM2 is essential for the process of nuclear compaction during spermiogenesis. It has been reported that mouse null mutants for either *TNP1* or *TNP2* are subfertile (Yu et al, 2000; Zhao et al, 2001), while mice that lack both these *TNPs* are infertile (Zhao et al, 2004). These results indicate that these basic proteins are important players in nuclear formation during spermiogenesis. In previous studies of sin-

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gle-nucleotide polymorphisms (SNPs) in the open-reading frames (ORFs) of the *PRM1* and *PRM2* genes in the protamine gene cluster on 16p13.13, we found that a mutation leading to a single nucleotide replacement induced a non-sense mutation in the *PRM2* gene in a group of infertile patients (Tanaka et al, 2003). This mutation would be expected to cause male infertility, even in the hemizygous condition, because haploinsufficiency of either *PRM1* or *PRM2* is known to cause infertility in male mice.

In the present study, we assessed the prevalence of *TNP2* gene SNPs in the protamine gene cluster at 16p13.13. We also examined the prevalence of alterations in the gene for another transition nuclear protein, TP1. DNA samples were analyzed from 548 men: 282 infertile patients who were undergoing fertility evaluation and 270 (*TNP1*) and 266 (*TNP2*) proven-fertile volunteers. We discovered a deletion mutation of the recognition site for the cyclic adenosine monophosphate (cAMP) response element (CRE) transcription factor (Sassone-Corsi, 1998) in the promoter region of the *TNP1* transcription unit. The cAMP-responsive element modulator (CREM) plays an important role in regulating spermiogenesis by binding to the recognition site for the CRE transcription factor (Nantel et al, 1996). Mice that are CREM deficient have reduced testis weights and a complete lack of mature spermatozoa in the seminal fluid. The promoter regions of many genes expressed during spermiogenesis include the recognition site for the CRE transcription factor, which regulates gene expression in spermiogenesis (Sassone-Corsi, 1998). Deletion of the recognition site for the CRE transcription factor in *TNP1* dramatically decreases the expression of *TNP1* mRNA (Kistler et al, 1994). *TNP1* plays an important role in nuclear formation during spermiogenesis in mice (Yu et al, 2000). This deletion may be associated with human male infertility, although mouse null mutants for *TNP1* and *TNP2* are subfertile (Yu et al, 2000).

Materials and Methods

Participants

Japanese infertile subjects (N = 282) were divided into subgroups according to the degree of defective spermatogenesis: 192 (68%) of these patients had nonobstructive azoospermia, while 90 (32%) had severe oligospermia ($<5 \times 10^6$ cells/mL). The subjects had primary idiopathic infertility based on a genetic study (Birmingham, 2004). The control group of fertile males (N = 266) consisted of men who had fathered children born to pregnant women at the maternity clinic. The donors gave permission for their blood to be used for the analysis of genomic DNA in this study.

Identification of SNPs in the *TNP1* and *TNP2* Genes by Direct Sequencing of PCR-Amplified DNA

Genomic DNA was isolated from the blood samples using protease treatment and phenol extraction (Sambrook et al, 1989). Two polymerase chain reaction (PCR) primer sets, *TNP1A-TNP1B* and *TNP1C-TNP1D*, were designed to amplify the *TNP1* gene (Figure 1). The *TNP1A-TNP1B* primer set comprised *TNP1A* (5'-CACAGTATCTACTGTGTTTATCCTCCAC-3') from nucleotide (nt) -785 to nt -757 upstream of the transcription start site (Luerssen et al, 1990), and *TNP1B* (5'-GTGCAGCTCAAGGGCTGCCC-3') from nt -152 to nt -171 downstream of the transcription start site. The *TNP1C-TNP1D* primer set comprised *TNP1C* (5'-GGCTGGGATTCAAGTTTCTCAATAACACC-3') from nt -244 to nt -217 upstream of the transcription start site, and *TNP1D* (5'-TACGGTGGTGGGAGG-3') from nt 724 to nt 748 upstream. The following PCR conditions were used: 35 cycles of denaturation at 98°C for 10 seconds, annealing at 62°C for 30 seconds, and extension at 72°C for 1 minute for *TNP1A-TNP1B*; and 35 cycles of denaturation at 98°C for 10 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute for *TNP1C-TNP1D*. The PCR-amplified fragments were purified using the SUPREC PCR spin column (Takara, Shiga, Japan). The fragments of *TNP1A-TNP1B* and *TNP1C-TNP1D* DNA were independently sequenced from both ends using the same PCR primers with thermal-cycle sequencing kits purchased from Applied Biosystems (Foster City, Calif). The DNA sequences of *TNP1A* and *TNP1B* were compared based on the results of sequencing from both directions. The reaction products were analyzed using an ABI-PRISM 310 Genetic Analyzer (Applied Biosystems).

The *TNP2A-TNP2C* PCR primer set was designed to amplify the *TNP2* gene (Figure 2). This primer set comprised *TNP2A* (5'-ATAATCAGCCCCAACTATATAAC-3') from nt -62 to nt -40 upstream of the transcription start site (Kistler et al, 1994), and *TNP2C* (5'-CATTTTCAGCCCCCTGTGCAGGCC-3') from nt 1509 to nt 1532 upstream. The following PCR conditions were used for *TNP2A-TNP2C*: 40 cycles of denaturation at 98°C for 10 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 1 minute 45 seconds. The PCR-amplified fragments were purified using the SUPREC PCR spin column (Takara), and thermal-cycle sequencing (Applied Biosystems) was performed. The DNA sequences were determined using the same PCR primers and the 24-nucleotide *TNP2B* primer (5'-CCAAGGTCTGCTCTCCATCATCTG-3') from nt 486 to nt 509 upstream (Figure 2). The DNA sequences of *TNP2A* and *TNP2B* were compared based on the results of sequencing from both directions. The sequences of *TNP2B* and *TNP2C* were determined only from the 3' end of *TNP2C*. The PCR conditions used to sequence all the *TNP1* and *TNP2* fragments were as follows: 25 cycles of denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds, and extension at 60°C for 4 minutes.

Statistical Analysis

Differences between experimental and control conditions were compared using one-way analysis of variance with Fisher's protected least significant difference tests. Significant differences ($P < .05$) are discussed here.

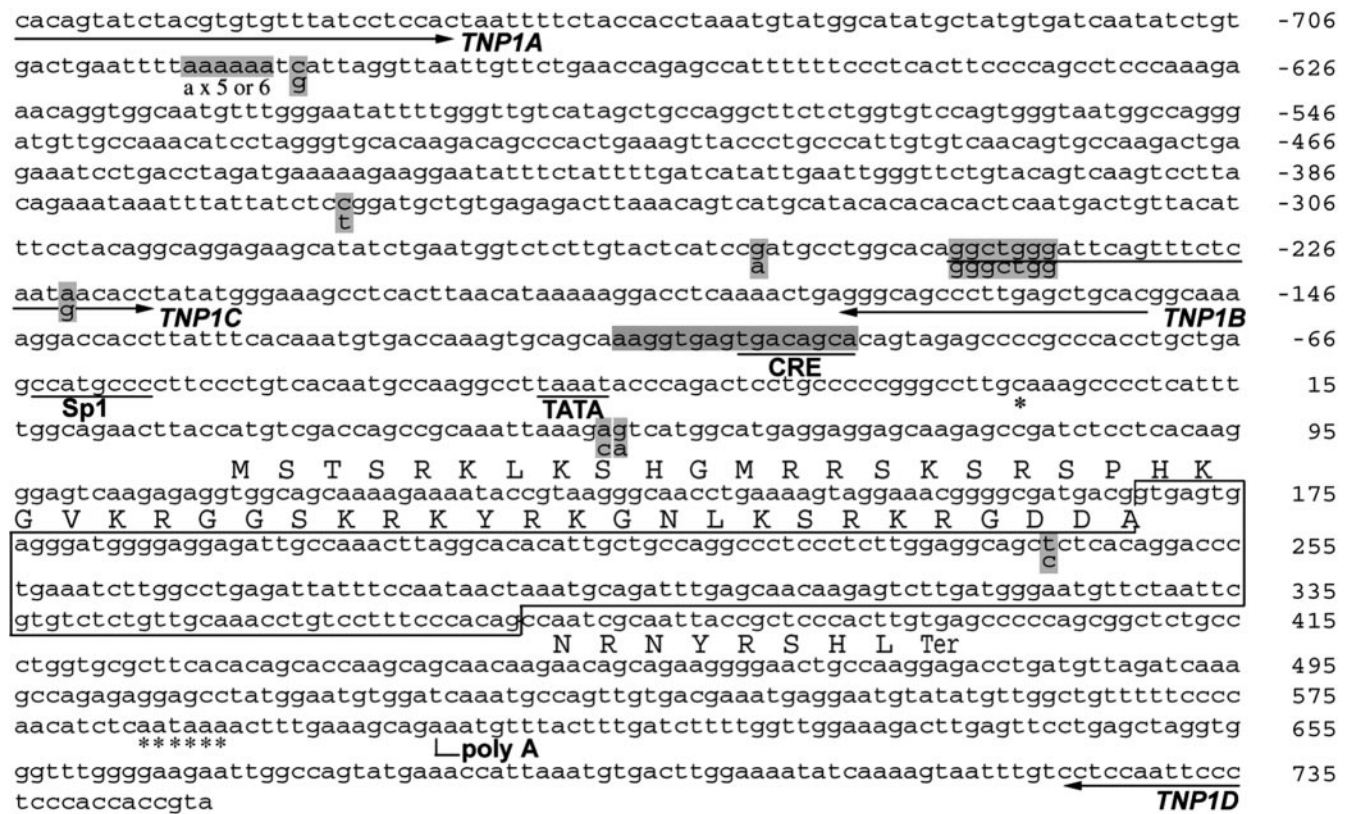


Figure 1. Genomic DNA sequences of the transition nuclear protein genes (*TNP1s*) and primers used for polymerase chain reaction (PCR) amplification and sequencing. The deduced amino acid sequences of the *TNP1* open-reading frames (ORFs) are shown below the DNA sequence (Luerssen et al, 1990). The recognition site for CRE, Sp1, and TATA nucleotide sequences are underlined. The single nucleotide polymorphisms (SNPs) are shown by shadowed letters, and minor alleles are indicated underneath the nucleotide. The numbers in the right margins indicate the nucleotide positions (with the transcription start site designated as +1); the arrows below the DNA sequence, the primers used for PCR amplification and sequencing; the stars below the DNA sequence, the transcription start site and polyA-additional signal (the polyA signal is added from the straight arrow to the *TNP1* mRNA); a box, the intron; and the shadowed sequence at nt -106 to -91, the region that is deleted found in the infertile male patients.

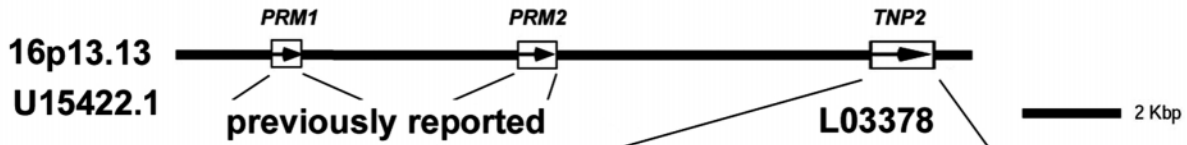
Results

Analysis of *TNP1* Gene SNPs

Two PCR primer sets were used to analyze the *TNP1* gene sequences (EMBL/DDBJ/GenBank accession number M29704; Keyeux et al, 1989) of the infertile and proven-fertile subjects. Direct sequencing of the PCR-amplified DNA was performed using genomic DNA from blood samples. The PCR-amplified 1533-bp DNA fragment included the promoter region and 200-nt intron of *TNP1* (Figure 1). Thus, we could identify SNPs that were located within 1480 bp of the primer sequence in the 1533-bp DNA fragment. The SNP prevalences were compared for infertile males and proven-fertile males. We found 5 SNPs or mutations in the promoter region at nt -694 to -689, nt -364, nt -258, and nt -222, a deletion of the nt -91 to -106 region in the recognition site for the CRE transcription factor, 2 SNPs in the 5'-untranslated region of the *TNP1* mRNA, and 1 SNP in the intron (Table 1; Figure 1). One of the SNPs registered in the NCBI dbSNPs

(rs1179733) was not found in this study. The nt -245 to -239 region in the sequence registered in GenBank (M59924) differed from that found in this study. None of these SNPs resulted in changes to the amino acid-coding region. The 3 SNPs found in this study, at -222A>G, 54A>C, and 55G>A (Figure 1), were either major homozygous or heterozygous SNPs; no minor homozygous SNPs were observed (Table 1). The SNPs did not show significantly higher prevalences in infertile patients than in proven-fertile volunteers (Table 1). Similarly, the SNPs of nucleotide sequence AAAAA (A × 5) or AAAAAA (A × 6) at nt -694 to -698, -364C>T, and -258G>A in the 3'-promoter region (Figure 1) did not show significantly higher prevalences in infertile patients than in proven-fertile volunteers (Table 1). The heterozygous deletion at nt -91 to -106, which introduced a deletion in the recognition site for the CRE transcription factor for haploid-specific expression of *TNP1*, was observed in only one of the azoospermic patients and was absent in the 266 fertile controls (Table 1). Because the recognition

a



b

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ataatcagccccaactatataaacagggtgggctgccagggcctctgtaaagctaggcctgctgggagaggatgaggagg 18
aggcctgccctccaaacgtggcctcctatggacaccagactcacagccttctatcaccacactcagctccatagc 98
      M D T Q T H S L P I T H T Q L H S
aactctcagccccaagccgcacctgcaaccggccattgccaaaccttcagccagagttgcagacagagccatcgtggcag 178
N S Q P Q S R T C T R C H C Q T F S Q S C R Q S H R G S
ccggagccagagctccagccagagcccggccagccaccgcaacccaactggagcccacagctcatccggccaccagagcc 258
R S Q/H S S S Q S P A S H R N P T G A H S S S S G/S H Q S Q
agagtcccaactagtccaccaccaaagcgccacaaaagactatgaactcccaccactctccatgctggccaccatc 338
S P N T S P P P K R H K K T M N S H H S P M R P T I
ctgcaactgcccgtgccccagaacagaaagaacttggaaaggcaagctgaaaaagaaaaaatggccaagaggatccagca 418
L H C R C P K/Q N R K N L E G K L K K K K M A K R I Q Q
gggtgtaaaaaaccaagacgctgggagctcaggtaccctttaaaggaggtggggaaggccaccgagccacagatgatggaga 498
V Y K T K T R/W S S G
gcagaccttgggggagctgagaggaaggctgcagccaggtcacaagggaaccacagggaagaagaggaggagaaagaga 578
aacaatggcagttggctagctgaatgtatgatacgttgacggaaagtctctttgaaattggatgggttgattaggaggat 658
ggaaagatggacagatagcagataagctagatgaaagcatgaatggagttgagaggttgggtggatgactgggtgggtaa 738
acaataaataggttatagaaggatagttggaagaatgcatggctgaatgataggaagtttggatacgattagctggat 818
ggatggataaatggatgaatgcactggctggctagttatttggttgggttaggttagatgatcagtttgaagattgtggttg 898
gtggatgaattgggttagaaatagagttaaataagttgtagaagttttgtaggggttgggttggattgggttaaataattatccta 978
atagagtaatatagagtaattgaataaacagagagaagaatagatatctagactaatgggatagaatgggaaagaaatgt 1058
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acataggtcaaaaggacactgacggtagtctaaactctatctatgtcccatattcaatcaaaatgagtagttgtaagac 1218
cttacaggaggtcaaggaggtcactgacttcatgaagtgtcagctatataaaggcttcttcccactcttatcccttagg 1298
atggaaatccaactaatgagaccgactccttggcttgttctgctgtgttcacccaaggagaaaatgctaggatgaag 1378
W K S N Ter
tcaatcttcttgcaggaacatggtactatgggtgatttctacgcaacactaatataagcttgtacctggaagactatccct 1458
gagtagtcatagtcattttgatttcactaataaagggtgtatgtgttttgggggctgcacaggggcagaaatg polyA 1532
      TNP2C ←
    
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Figure 2. Schematic representation of the protamine gene cluster and genomic DNA sequences of the *TNP2* genes. a) The *PRM1*, *PRM2*, and *TNP2* genes are clustered within approximately 10 kb of genomic DNA on 16p13.13 (Engel et al, 1992). Previously, we identified SNPs in the ORFs of *PRM1* and *PRM2* (Tanaka et al, 2003). The GenBank accession numbers are listed. b) The deduced amino acid sequences of the *TNP2* ORFs are shown below the DNA sequence (Nelson et al, 1993). Several of the SNPs (nt 613, 721, 816, 946, 947, and 950) that are registered in the NCBI dbSNPs were not found in this study (see Table 2). Comparisons with the DNA sequences registered in GenBank indicate deletions at nt 1036 and nt 1046 (see Table 2). The SNPs are shown as shadowed letters, and minor alleles are indicated below the nucleotide. The numbers in the right margins indicate the nucleotide positions (with the transcription start site designated as +1); the arrows below the DNA sequence, the primers used for PCR amplification and sequencing; the stars below the DNA sequence, the transcription start site; the bar below the DNA sequence; the polyA-additional signal (the polyA signal was added from the straight arrow to the *TNP1* mRNA); and a box, the intron.

Table 1. Prevalence of the polymorphisms in TNP1 in infertile or proven-fertile populations*

Position	Nucleotide		Number of SNPs (%)		Reference (NCBI dbSNP rs no.)
	Genotype		Infertile	Proven Fertile	
-694 to -689	AAAAAA		246 (87.2)	238 (88.0)	
	A×6/A×5		35 (12.4)	27 (10.0)	
	AAAAA		1 (0.4)	5 (2.0)	
-687	C/C		282 (100)	270 (100)	
	C/G		0 (0)	0 (0)	rs1179733
-364	C/C		112 (39.7)	105 (38.9)	
	C/T		132 (46.8)	127 (47.0)	rs1179734
	T/T		38 (13.5)	38 (14.1)	
-258	G/G		113 (40.0)	112 (41.5)	
	G/A		131 (46.5)	120 (44.4)	
	A/A		38 (13.5)	38 (14.1)	
-245 to -239	GGCTGGG		282 (100)	270 (100)	
	GGGCTGG		0 (0)	0 (0)	M59924 (GenBank)
-222	A/A		280 (99.3)	265 (98.1)	
	A/G		2 (0.7)	5 (1.9)	
-91 to -106	Deletion (CRE)		1 (0.4)	0 (0)	
	54	A/A	281 (99.6)	268 (98.1)	
55	A/C		1 (0.4)	2 (0.7)	
	G/G		281 (99.6)	269 (99.6)	
243	G/A		1 (0.4)	1 (0.4)	
	T/T		243 (86.2)	225 (83.3)	
	T/C		38 (13.5)	44 (16.3)	
	C/C		1 (0.4)	1 (0.4)	

* The numbers (%) of single nucleotide polymorphisms (SNPs) in the infertile and proven-fertile men are shown in association with data on the genotypes and genomic positions. The bold SNPs are registered in the database, but were not found in this study. TNP1 indicates transition nuclear protein gene 1; CRE, cAMP response element.

site for the CRE transcription factor is important for the expression of *TNP1*, this deletion may cause azoospermia, even in the hemizygous condition.

Analysis of TNP2 Gene SNPs

The *TNP2* gene (GenBank accession number L03378; Nelson et al, 1993) was sequenced in various infertile patients and proven-fertile volunteers. SNPs located within 1547 bp of the primer could be identified by direct-sequence analysis of the 1594-bp DNA fragments (Figure 2). We observed 7 SNPs in the 1547-bp region of the *TNP2* gene; 2 were in the intron and 5 were in the exon (Table 2; Figure 2). The guanines at nt 1036 and nt 1046 in the intron of the registered *TNP2* sequence (GenBank accession number L03378) were deleted. Eight SNPs have been registered for this DNA sequence in the NCBI dbSNPs. However, 6 SNPs (rs181695, rs2857758, rs11640282, rs3867172, rs3884056, and rs3867171) listed in the NCBI dbSNPs were not found in this study. All 5 SNPs in the exon induced amino acid substitutions. The SNPs at nt 613, nt 721, and nt 816 were not fully determined because of the condition of the samples. 129C>T (R28C), 188G>C (G47H), 246G>A (G67S), and 357A>C (K104Q) were approximately 99% homozygous major types. Homozygous minor types were not found in these SNPs. 246G>A (G67S) and 357A>C (K104Q) were not found in the infertile patients. In the case of

518C>T (R131W), 66.3% and 41.0% were homozygous major C/C types, 30.5% and 49.6% were heterozygous (C/T), and 3.2% and 9.4% were homozygous minor-type (T/T) SNPs in the infertile and fertile populations, respectively. Furthermore, we found 2 SNPs, 1019T>G and 1272G>C, in the *TNP2* intron. The 1019T>G SNP was heterozygous of the T/G type in 1 infertile subject. In the case of the 1272G/C SNP, 66.0% and 41.4% were homozygous for the major (G/G) type, 30.5% and 49.2% were heterozygous (G/C), and 3.5% and 9.4% were minor-type homozygous (C/C) in the infertile and fertile control populations, respectively. The prevalence of these SNPs in infertile males was not significantly different from that in proven-fertile volunteers (Table 2).

Discussion

During spermiogenesis, chromatin is changed by the replacement of the somatic-type histones with PRMs, which results in the sperm nucleus being compacted. Drastic alteration of chromatin during spermiogenesis is achieved through systematic expression of spermiogenesis-specific nuclear proteins (Tanaka and Baba, 2005). Because deficiencies in the genes that code these nuclear proteins lead to male infertility in mice, it appears that the essential event in chromatin transformation via the replacement of

Table 2. Prevalence of the SNPs in TNP2 in infertile or proven-fertile populations*

Position		Genotype	Number of SNPs (%)		Reference (NCBI dbSNP rs no.)
Nucleotide	Amino Acid		Infertile	Proven Fertile	
129	28 (R)	C/C	281 (99.6)	265 (99.6)	
	(R/C)	C/T	1 (0.4)	1 (0.4)	
188	47 (Q)	G/G	278 (98.6)	264 (99.2)	
	(G/H)	G/C	4 (1.4)	2 (0.8)	
246	67 (G)	G/G	282 (100)	265 (99.6)	
	(G/S)	G/A	0 (0)	1 (0.4)	
357	104 (K)	A/A	282 (100)	265 (99.6)	
	(K/Q)	A/C	0 (0)	1 (0.4)	
518	131 (R)	C/C	178 (66.3)	109 (41.0)	
	(R/W)	C/T	86 (30.5)	132 (49.6)	rs11640138
	(W/W)	T/T	9 (3.2)	25 (9.4)	
613		G/G		72 (100)	
		G/A		0 (0)	rs181695
		ND	282	194	
721		C/G		72 (100)	
		G/T		0 (0)	rs2857758
		ND	282	194	
		G/G		73 (100)	
816		g/t		0 (0)	rs11640282
		ND	282	193	
946		A/A	282 (100)	263 (100)	
		T/A	0 (0)	0 (0)	rs3867172
947		T/T	282 (100)	263 (100)	
		T/A	0 (0)	0 (0)	rs3884056
950		G/G	282 (100)	263 (100)	
		G/C	0 (0)	0 (0)	rs3867171
1019		T/T	281 (99.6)	266 (100)	
		T/A	1 (0.4)	0 (0)	
1036		G	282 (100)	266 (100)	
		Deletion	0 (0)	0 (0)	L03378 (GenBank)
1046		G	282 (100)	266 (100)	
		Deletion	0 (0)	0 (0)	L03378 (GenBank)
		G/G	186 (66.0)	110 (41.4)	
1272		G/C	86 (30.5)	131 (49.2)	rs8043625
		C/C	10 (3.5)	25 (9.4)	

* The numbers (%) of SNPs in the infertile and proven-fertile men are shown in association with data on the genotypes and genomic positions. The bold SNPs are registered in the database, but were not found in this study. TNP2 indicates transition nuclear protein gene 2; ND, not determined.

the somatic-type histones with PRMs is the systematic expression of spermiogenesis-specific nuclear proteins (Zhao et al, 2004). To examine *PRM* gene expression in relation to human male infertility, we previously assessed the prevalence of these SNPs in infertile patients and proven-fertile volunteers and found that the 248C>T mutation of the *PRM2* gene induced a nonsense codon under conditions of heterozygosity in 1 infertile patient (Tanaka et al, 2003). This nonsense mutation in *PRM2* may cause male infertility due to haploinsufficiency of *PRM2*. In the present study, we assessed the prevalence of *TNP* gene SNPs in male patients who were undergoing fertility evaluation.

Two TPs have been identified as abundantly expressed basic nuclear proteins that participate in chromatin condensation by the replacement of somatic-type histones with protamines during spermiogenesis (Meistrich et al,

2003). The TP1 amino acid sequence is highly conserved among various mammals (Kremling et al, 1989). In contrast, the TP2 sequence is poorly conserved (Alfonso and Kistler, 1993), and the levels of TP2 expression and TP2 protein vary among species (Steger et al, 1998). The relative molecular mass (Mr) of TP1 is approximately 6200, with about 20% arginine and 20% lysine distributed uniformly, and without cysteine residues (Kistler et al, 1975). TP2 (Mr 13000) is composed of about 10% arginine, 10% lysine, and 5% cysteine residues (Grimes et al, 1975). It has a highly basic C-terminal domain and an N-terminal domain that forms zinc fingers (Meetei et al, 2000). The *TNP2* gene is closely linked to the 2 *PRM* genes (Engel et al, 1992), which suggests that they arose by gene duplication and have retained common functions. In contrast, *TNP1* exists on a separate chromosome (Heidaran et al, 1989). Biochemical analyses have shown that

TP1 decreases the melting temperature of DNA (Akama et al, 1998). In contrast, TP2 increases the melting temperature of DNA and compacts the DNA into nucleosomal cores, which indicates that it is a DNA-condensing protein (Baskaran and Rao, 1990). These analyses of TPs show that these proteins play different roles in chromatin remodeling during spermiogenesis. TP2 contains cysteine residues, as do the PRMs, and it is duplicated in the protamine gene cluster, which suggests that it might have a similar role to the PRMs.

In general, the loss of the *TNP1* or *TNP2* gene does not lead to infertility in mice, although some *TNP1*-deficient mice are infertile (Yu et al, 2000). The loss of both *TNP1* and *TNP2* results in reduced litter sizes (Zhao et al, 2004). The idea that these 2 genes compensate each other functionally evolved from the biochemical characterization and expression kinetics of TP1 and TP2. However, individual *TNP1*-null mice cannot compensate completely for the TP1 deficiency with TP2 (Meistrich et al, 2003), which is probably due to differences in the expression levels of some other proteins. It is clear that TPs, especially TP1, play important roles in mouse spermiogenesis. However, the functions of TPs in human sperm formation are not fully understood. We need to study the roles of TPs in human sperm formation.

In this study, we found that the ORF of *TNP2* included 5 different SNPs. *TNP2* localizes to the protamine gene cluster, and the transcription of each of these genes is regulated by association with the nuclear matrix and attachment region on the genomic DNA (Martins et al, 2004). A previous study found 4 *PRM1* SNPs, which did not cause any amino acid substitutions, in an ORF of almost the same nucleotide length. The frequency of SNPs in the protamine gene cluster is almost the same (average, 1 SNP/190 bp; *PRM1*, 1 SNP/106 bp; *PRM2*, 1 SNP/299 bp; *TNP2*, 1 SNP/219 bp). However, the amino acid sequence of *PRM1* is more highly conserved than that of TP2. These results indicate that TP2 functions may not be stringent, in that a few amino acid changes are tolerated. Therefore, other proteins with similar characteristics might readily compensate for TP2.

SNPs in the region surrounding the *TNP1* gene, including the promoter unit, were analyzed. We noted a deletion of the recognition site for the CRE transcription factor in the promoter region of 1 of the infertile patients. The recognition site for the CRE transcription factor plays an important role in the transcription of *TNP1* (Kistler et al, 1994). The *TNP1* promoter region is conserved and is considered to be important in mammals (Kistler et al, 1994). This mutation decreases the expression of *TNP1* mRNA dramatically; otherwise, the mutational promoter that includes the recognition site for the Sp1 transcription factor might disturb the expression timing of *TNP1* during spermiogenesis. Moreover, it is possible that this mutation

influences spermiogenesis by modulating the expression of *TNP1* in somatic cells, such as Sertoli cells. In humans, the amino acid sequences of TP1 are highly conserved. TP1 may be more important for spermiogenesis in humans than in mice. The mutation in the recognition site of the CRE transcription factor of the promoter region of *TNP1* is thought to be a cause of male sterility. The prevalences of other SNPs in the region 5'-upstream of the transcription start site of *TNP1* did not differ for the infertile and fertile control populations. The A × 5 or A × 6 stretch was identified in the region 5'-upstream of the transcription start site. This region may not have a specific function, and the easily deleted adenine stretches or DNA structures of A × 5 and A × 6 are very similar to each other. Some of the SNPs that appear in the NCBI dbSNPs were not found in this study. These unidentified SNPs may reflect the ethnicity of the loci registered in the NCBI dbSNPs.

Previously, Schlicker et al (1994) did not detect any mutation in *PRM1*, *PRM2*, or *TNP1* in 36 infertile men whose spermatozoa showed the presence of histones in the nuclei. In our analysis of the *TNP1* DNA sequences of a total of 552 men, we detected a deletion of the recognition site for the CRE transcription factor in the *TNP1* promoter region in 282 idiopathic infertile men, but not in 270 fertile men. The human *TNP1* gene is highly conserved, as are the protamine genes. By contrast, the *TNP2* gene in the protamine gene cluster appears to have many more SNPs than the protamine genes. These results imply that TP1 has a more important role than TP2 in the formation of human sperm nuclei. Further investigations using a larger population of infertile cases and defined SNP pedigrees should confirm the causal link between *TNP* gene polymorphisms and male infertility.

References

- Akama K, Sato H, Hasegawa S, Shimada I, Nakano M. Transition protein 1 from boar late spermatid nuclei having DNA-melting activity is a dimeric protein. *Biochem Mol Biol Int.* 1998;44:315–323.
- Alfonso PJ, Kistler WS. Immunohistochemical localization of spermatid nuclear transition protein 2 in the testes of rats and mice. *Biol Reprod.* 1993;48:522–529.
- Baskaran R, Rao MR. Interaction of spermatid-specific protein TP2 with nucleic acids, in vitro: a comparative study with TP1. *J Biol Chem.* 1990;265:21 039–21 047.
- Birmingham A. Guideline for sperm donation. *Fertil Steril.* 2004; 82(suppl 1):S9–S12.
- Cho C, Jung-Ha H, Willis WD, Goulding EH, Stein P, Xu Z, Schultz RM, Hecht NB, Eddy EM. Protamine 2 deficiency leads to sperm DNA damage and embryo death in mice. *Biol Reprod.* 2003;69:211–217.
- Cho C, Willis WD, Goulding EH, Jung-Ha H, Choi YC, Hecht NB, Eddy EM. Haploinsufficiency of protamine-1 or -2 causes infertility in mice. *Nat Genet.* 2001;28:82–86.
- de Kretser DM, Baker HW. Infertility in men: recent advances and continuing controversies. *J Clin Endocrinol Metab.* 1999;84:3443–3450.

- Engel W, Keime S, Kremling H, Hameister H, Schluter G. The genes for protamine 1 and 2 (PRM1 and PRM2) and transition protein 2 (TNP2) are closely linked in the mammalian genome. *Cytogenet Cell Genet*. 1992;61:158–159.
- Grimes SR Jr, Platz RD, Meistrich ML, Hnilica LS. Partial characterization of a new basic nuclear protein from rat testis elongated spermatids. *Biochem Biophys Res Commun*. 1975;67:182–189.
- Heidaran MA, Kozak CA, Kistler WS. Nucleotide sequence of the Stp-1 gene coding for rat spermatid nuclear transition protein 1 (TP1): homology with protamine P1 and assignment of the mouse Stp-1 gene to chromosome 1. *Gene*. 1989;75:39–46.
- Keyeux G, Lefranc G, Lefranc MP. A multigene deletion in the human IGH constant region locus involves highly homologous hot spots of recombination. *Genomics*. 1989;5:431–441.
- Kistler MK, Sassone-Corsi P, Kistler WS. Identification of a functional cyclic adenosine 3',5'-monophosphate response element in the 5'-flanking region of the gene for transition protein 1 (TP1), a basic chromosomal protein of mammalian spermatids. *Biol Reprod*. 1994;51:1322–1329.
- Kistler WS, Noyes C, Hsu R, Heinrichson RL. The amino acid sequence of a testis-specific basic protein that is associated with spermatogenesis. *J Biol Chem*. 1975;250:1847–1853.
- Kremling H, Luerssen H, Adham IM, Klemm U, Tsaousidou S, Engel W. Nucleotide sequences and expression of cDNA clones for boar and bull transition protein 1 and its evolutionary conservation in mammals. *Differentiation*. 1989;40:184–190.
- Lewis JD, Saperas N, Song Y, Zamora MJ, Chiva M, Ausio J. Histone H1 and the origin of protamines. *Proc Natl Acad Sci U S A*. 2004;101:4148–4152.
- Luerssen H, Mattei MG, Schroter M, Grzeschik KH, Adham IM, Engel W. Nucleotide sequence of the gene for human transition protein 1 and its chromosomal localization on chromosome 2. *Genomics*. 1990;8:324–330.
- Martins RP, Ostermeier GC, Krawetz SA. Nuclear matrix interactions at the human protamine domain: a working model of potentiation. *J Biol Chem*. 2004;279:51862–51868.
- Matzuk MM, Lamb DJ. Genetic dissection of mammalian fertility pathways. *Nat Cell Biol*. 2002;4(suppl):S41–S49.
- Meetei AR, Ullas KS, Rao MR. Identification of two novel zinc finger modules and nuclear localization signal in rat spermatid protein TP2 by site-directed mutagenesis. *J Biol Chem*. 2000;275:38500–38507.
- Meistrich ML, Mohapatra B, Shirley CR, Zhao M. Roles of transition nuclear proteins in spermiogenesis. *Chromosoma*. 2003;111:483–488.
- Nantel F, Monaco L, Foulkes NS, Masquillier D, LeMeur M, Henriksen K, Dierich A, Parvinen M, Sassone-Corsi P. Spermiogenesis deficiency and germ-cell apoptosis in CREM-mutant mice. *Nature*. 1996;380:159–162.
- Nelson JE, Krawetz SA. Linkage of human spermatid-specific basic nuclear protein genes. Definition and evolution of the P1→P2→TP2 locus. *J Biol Chem*. 1993;268:2932–2936.
- Sambrook J, Fritsch E, Maniatis T. In: Molecular cloning. Nolan C, ed. *Isolation of DNA from Mammalian Cells*. New York: Cold Spring Harbor Press; 1989:9.16–9.21.
- Sassone-Corsi P. CREM: a master-switch governing male germ cells differentiation and apoptosis. *Semin Cell Dev Biol*. 1998;9:475–482.
- Schlicker M, Schnulle V, Schnepffel L, Vorob'ev VI, Engel W. Disturbances of nuclear condensation in human spermatozoa: search for mutations in the genes for protamine 1, protamine 2 and transition protein 1. *Hum Reprod*. 1994;9:2313–2317.
- Steger K, Klonisch T, Gavenis K, Drabent B, Doenecke D, Bergmann M. Expression of mRNA and protein of nucleoproteins during human spermiogenesis. *Mol Hum Reprod*. 1998;4:939–945.
- Tanaka H, Baba T. Gene expression in spermiogenesis. *Cell Mol Life Sci*. 2005;62:344–354.
- Tanaka H, Miyagawa Y, Tsujimura A, Matsumiya K, Okuyama A, Nishimune Y. Single nucleotide polymorphisms in the protamine-1 and -2 genes of fertile and infertile human male populations. *Mol Hum Reprod*. 2003;9:69–73.
- Yu YE, Zhang Y, Unni E, Shirley CR, Deng JM, Russell LD, Weil MM, Behringer RR, Meistrich ML. Abnormal spermatogenesis and reduced fertility in transition nuclear protein 1-deficient mice. *Proc Natl Acad Sci U S A*. 2000;97:4683–4688.
- Zhao M, Shirley CR, Hayashi S, Marcon L, Mohapatra B, Suganuma R, Behringer RR, Boissonneault G, Yanagimachi R, Meistrich ML. Transition nuclear proteins are required for normal chromatin condensation and functional sperm development. *Genesis*. 2004;38:200–213.
- Zhao M, Shirley CR, Yu YE, Mohapatra B, Zhang Y, Unni E, Deng JM, Arango NA, Terry NH, Weil MM, Russell LD, Behringer RR, Meistrich ML. Targeted disruption of the transition protein 2 gene affects sperm chromatin structure and reduces fertility in mice. *Mol Cell Biol*. 2001;21:7243–7255.