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Genetic Variation in the Testis-Specific Poly(A) Polymerase Beta (*PAPOLB*) Gene Among Japanese Males

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Abstract: The testis-specific poly(A) polymerase beta (*PAPOLB*) gene was first identified as the intronless gene *TPAP* in mice. In TPAP-deficient mice, spermiogenesis is arrested due to altered post-transcriptional gene regulation, including translational activation via the cytoplasmic polyadenylation of mRNAs. To investigate the possible association between variations in *PAPOLB* and impaired spermatogenesis in Japanese males, we screened for genetic variations in *PAPOLB* using DNA from 282 sterile male patients and 96 proven-fertile male volunteers using direct sequencing methods on blood samples. Seven single nucleotide polymorphisms in the coding sequence and one DNA insertion in the 5' untranslated region of *PAPOLB* were found. These genetic variations were not present at statistically significant levels; however, the results of this study may be useful in future large-scale analyses of the association between *PAPOLB* and male infertility.

Keywords: Sperm, male infertility, genome, spermatogenesis, fertilization, SNP.

INTRODUCTION

Several genes are specifically expressed during spermatogenesis, including many intronless genes [1], and the disruption of these male germ cell-specific genes can cause infertility in males [2]. Testis-specific poly(A) polymerase beta (*PAPOLB/TPAP*) is a male germ cell-specific and intronless gene in mice [3]. Some mRNAs transcribed in round spermatids are not immediately translated; instead, they are held in protein complexes until the right time for translation [4]. *TPAP*-disrupted mice are male-infertile due to azoospermia caused by insufficient translation of specific mRNAs during spermiogenesis; heterozygous mutant mice show normal fertility [5]. Moreover, expression of the *TPAP* transgene in *TPAP*-disrupted mice complements the deficit in spermatogenesis and *TPAP*-overexpressing transgenic mice show normal spermatogenesis [6]. Germ cell-specific gene 1 (GSG1) [7] was identified as a TPAP interaction partner based on the results of yeast two-hybrid analysis [8]. GSG1 is exclusively localized in the endoplasmic reticulum (ER) of mouse testis, where TPAP is also present. These results indicate that TPAP plays an essential role in post-transcriptional processing of specific mRNAs during spermiogenesis in complex with other proteins.

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In humans, defects in *PAPOLB* may be related to male infertility. Mutations in this gene have no effect in females, but they may be passed to male offspring via females. To examine whether *PAPOLB* is a hereditary cause of male infertility, nucleotide polymorphisms in the *PAPOLB* coding region were assessed by the direct sequencing of polymerase chain reaction (PCR)-amplified DNA from male patients.

MATERIALS AND METHODS

Participants

Japanese male subjects with non-obstructive infertility (n = 282) were divided into subgroups according to the degree of defective spermatogenesis: 192 (68%) of these patients had non-obstructive azoospermia, while 90 (32%) had severe oligospermia ($<5\times10^6$ cells/mL). All patients displayed idiopathic infertility and had no history of prior medical conditions, including, but not limited to, cryptorchidism, recurrent infections, trauma, orchitis, or varicocele. All subjects were diagnosed with primary idiopathic infertility based on a cytogenetic analysis. The control group consisted of fertile males who had fathered children born at a maternity clinic (n=96).

All donors were informed for the purpose of the study and gave permission for their blood to be used for genomic DNA analysis. This study was carried out with the approval of the institutional review board and independent ethics committee of Osaka University (Osaka, Japan).

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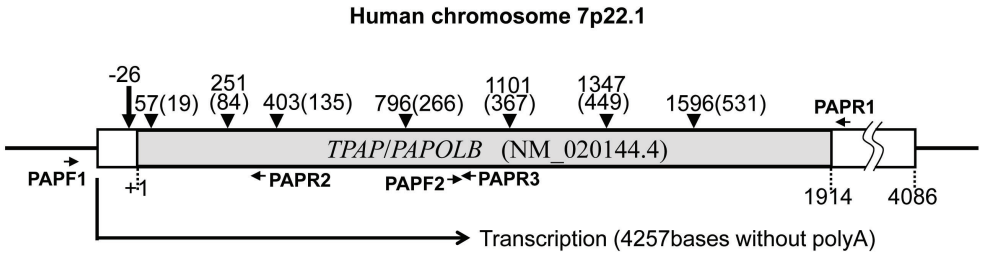

Fig. (1). Schematic representation of *TPAP/PAPOLB*. The intronless gene *TPAP/PAPOLB* is localized at 7p22.1. The numbers in the figure are nucleic acid sequence positions relative to the first nucleotide of the start codon. The numbers in parentheses indicate amino acid sequence positions. Horizontal arrows indicate the primers used for PCR and sequencing. The gray box indicates the CDS. The striped boxes indicate the putative central domain of poly(A) polymerase (amino acid sequence positions 21-366) and RNA-binding domain (amino acid sequence positions 367–504). The vertical arrowheads and the arrow indicate the positions of the SNPs.

Identification of Genetic Variation in *PAPOLB* by the Direct Sequencing of PCR-Amplified DNA

DNA was extracted from blood leukocytes. Genomic DNA was isolated from the blood samples by protease treatment and phenol extraction according to standard procedures. PCR was performed using the primers PAPTF1 (5'-CCGCGCGTGTACGGTAGGTTCTGG-3'; from nucleotide [nt] –255 to nt –232) and PAPTR1 (5'-CTTCCGTTTTGGTTTTCTTGGTCC-3'; from nt 1954 to nt 1977 downstream of the first methionine) (Fig. **1**). PCR was carried out in the manufacturer's recommended reaction buffer (50 µl) containing 0.05 µg of human genomic DNA; 0.2 µM each primer; 2.5µMeach of dGTP, dATP, dCTP, and dTTP; and EX Taq polymerase (Takara Bio Inc., Otsu, Japan). The cycling conditions were: 96ºC for 2 minutes, followed by 40 cycles of denaturation at 96ºC for 45 s, annealing at 66ºC for 45 s, and extension at 72ºC for 120 s. The PCR-amplified fragments were purified using AMPure® (Agencourt Bioscience Corp., Beverly, MA) then sequenced using the PCR primers PAPF2 (5'-CAGTGATAGGTACCATCTTATGCC-3'; from nt 921 to nt 944), PAPR2 (5'-GTCGCTTCGATCCACATGAC-3'; from nt 987 to nt 1010), and PAPR3 (5'-ACCATCCTGGTTGAAATAGACACG-3'; from nt 987 to nt 1010 downstream of the first methionine) with a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) (Fig. **1**). The reaction products were purified using BigDye® XTerminator and analyzed with an ABI-PRISM 3730xl Genetic Analyzer (Applied Biosystems). Screening for variations in the sequences was performed with SeqScape® software (Applied Biosystems).

Statistical Analysis

An analysis of differences between the experimental and control conditions and within-group comparisons were made using Student's *t*-tests. Significant differences ($P<0.01$) are discussed here.

RESULTS AND DISCUSSION

In Japan, more than 20% of married couples undergo infertility treatment (http://www.ipss.go.jp/ps-doukou/j/doukou14/doukou14.pdf: 14th birth trend survey, National Institute of Population of Social Security Research, Japan). Infertility may be due to problems with the female, male, or both [9]. More than half of all cases of infertility involve a problem with the male, and in about half of all cases of male infertility the cause is idiopathic [9]. To understand infertility, research has focused on identifying genetic mutations involved in human male infertility. We assessed the prevalence of single nucleotide polymorphisms (SNPs) in germ cell-specific genes by the direct sequencing of PCR-amplified DNA from male patients undergoing fertility evaluation [10]. Current data indicate that some SNPs related with male infertility exist in germ cell-specific genes [11, 12]; however, many of the SNPs in these genes are not associated with male infertility [13-15].

TPAP/PAPOLB is a candidate gene associated with human male infertility because *TPAP*-disrupted mice are male-infertile. We screened for *TPAP/PAPOLB* genetic variants affecting male fertility in this case-control study of fertile and infertile men. Our analysis identified seven SNPs in the coding sequence (CDS) of *TPAP/PAPOLB* and a nucleotide insertion in the 5' untranslated region in Japanese males (Fig. **1** and Table **1**). PCR products obtained using blood-derived DNA as the templates were sequenced, although the signal of g.251A/G (rs3750010) was too weak to distinguish a/a from a/g. Two SNPs in these polymorphisms were not found in the SNP database of the National Center for Biotechnology Information (NCBI; Table **1**), and two SNPs may be Japanese-specific. Four SNPs, g.57T/G (rs3750009), g.251A/G (rs3750010), g.403T/C, and g.1593A/T, induced amino acid substitutions. g.57T/G (rs3750009) was found outside of the N-terminal region of the central domain of poly(A) polymerase. g.251A/G (rs3750010) induced a K or R amino acid change, both of which showed similar characteristics. These SNPs were not associated with male infertility as no significant differences in frequency were observed. g.403T/C induced a Y to H amino acid substitution in the putative central domain of poly(A) polymerase, which was only observed in the infertile group. g.1593A/T induced an E to D amino acid substitution outside of the C-terminal region of the putative RNA binding domain, and was observed only in the infertile group. Experiments in *TPAP*-disrupted mice showed that regulation of the poly(A) length on mRNAs in spermatids was sufficient for spermiogenesis. It is possible that these nucleic acid substitutions induced male infertility in a

Table 1. Prevalence of single nucleotide polymorphisms (SNPs) in *PAPOLB*/*TPAP*in infertile or proven fertile populations.

Position				Genotype	Number (%) of SNP						Reference (NCBI dbSNP rs#)
	Nucleotide	Amino acid			Infertile		Proven fertile		Total		
PAPOLB/ TPAP	-25				83	(29.4)	26	(27.1)	109	(28.8)	rs71911252
				/insertion	141	(50.0)	41	(42.7)	182	(48.2)	
				insertion	58	(20.6)	29	(30.2)	87	(23.0)	
				(gaaggtcgcggtggtgggggacgt)							
	57	19	N	t/t	81	(28.7)	26	(27.1)	107	(28.3)	rs3750009
			N/K	t/g	140	(49.6)	41	(42.7)	181	(47.9)	
			K	g/g	61	(21.6)	29	(30.2)	90	(23.8)	
	251	84	K	a/a	*108	(38.3)	71	(74.0)	*179	(47.3)	rs3750010
			K/R	a/g	*172	(61.0)	23	(24.0)	*195	(51.6)	
			R	g/g	2	(0.7)	2	(2.1)	4	(1.1)	
	403	135	Y	t/t	281	(99.6)	96	(100)	377	(99.7)	
			Y/H	t/c	1	(0.4)	0	(0)	1	(0.3)	
			H	c/c	0	(0)	0	(0)	0	(0)	
	798	266	A	g/g	281	(99.6)	96	(100)	377	(99.7)	rs112213840
				g/a	1	(0.4)	0	(0)	1	(0.3)	
				a/a	0	(0)	0	(0)	0	(0)	
	1101	367	S	c/c	115	(40.8)	31	(32.3)	146	(38.6)	rs17135247
				c/t	127	(45.0)	43	(44.8)	170	(45.0)	
				t/t	40	(14.2)	22	(22.9)	62	(16.4)	
	1347	449	N	t/t	83	(29.4)	26	(27.1)	109	(28.8)	rs1553960
				t/c	141	(50.0)	42	(43.8)	183	(48.4)	
				c/c	58	(20.6)	28	(29.2)	86	(22.8)	
	1593	531	E	a/a	281	(99.6)	96	(100)	377	(99.7)	
			E/D	a/t	1	(0.4)	0	(0)	1	(0.3)	
			D	t/t	0	(0)	0	(0)	0	(0)	
Total					282		96			378	

* Numbers in a/a and a/g genotypes were obscure because these positions had very week signals to distinct the genotypes.

dominant negative manner, although the presence of these polymorphisms did not result in significant differences between the fertile and infertile groups in this study. The clinical characteristics of each idiopathic infertility patient were not examined in this study. The other SNPs, g.798G/A (rs112213840), g.1101C/T (rs17135247), and g.1347T/C (rs1553960), were silent mutations. The presence of these polymorphisms did not result in significant differences between the fertile and infertile groups.

TPAP/PAPOLB, which functions in spermiogenesis, is encoded by a conserved intronless gene in various mammals. Homozygous deletion of *TPAP/PAPOLB* was shown to cause infertility due to defective sperm, but it did not cause embryonic lethality [5]. To date, SNP analyses have been conducted for several intronless genes with a function similar to that of *TPAP/PAPOLB* (Table **2**) [11-15]. The numbers of SNPs observed in > 1% of humans are: four in *TPAP/PAPOLB* (CDS: 1941 nt), three in *SCOTT/OXCT2* (CDS: 1554 nt), one in *PGAM4* (CDS: 768 nt), three in *HANP1* (CDS: 765 nt), two in *CETN1* (CDS: 519 nt), and zero in *GSG3/CAPZA3* (CDS: 900 nt). These results indicate that one SNP from nt 250 to nt 750 was conserved in each

Table 2. Number of single nucleotide polymorphisms (SNPs) in the coding sequence (CDS) of each gene.

Gene	Function	Length of the CDS (base)	Number of SNPs*	Number of bp/SNP	Reference
TPAP/PAPOLB	Gene expression	1914	4 (2)**	479 (957)**	This study
SCOTT/OXCT2	Energy metabolism	1554	3 (3)**	518 (518)**	11
HANP1/H1FNT	Chromatin assembly	768	3 (3)**	256 (256)**	13
CETN1	Cytoskeletal regulation	519	2 (1)**	260 (519)**	14
PGAM4	Energy metabolism	765	1 (1)**	765 (765)**	12
GSG3/CAPZA3	Cytoskeletal organization	900	0	-	15
Average		**1070**	**2.2 (1.7)****	**456 (603)****	

*Number of SNPs (incidence of >1% in the population) in the CDS
** ( ) indicates the number of SNPs with amino acid substitutions.

CDS except for *GSG3/CAPZA3*. A meaningful numerical value was not obtained, even when SNPs causing amino acid substitutions were compared (Table **2**). *GSG3/CAPZA3* may be genetically more stable than other germ cell-specific intronless genes. These results may be useful for future large-scale genomic analyses.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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