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Original Article

c.822+126T>G/C: a novel triallelic polymorphism of the *TSSK6* gene associated with spermatogenic impairment in a Chinese population

Dan Su[1], Wei Zhang[2], Yuan Yang[1], Hao Zhang[1], Yun-Qiang Liu[1], Gang Bai[1], Yong-Xin Ma[1], Yan Peng[1], Si-Zhong Zhang[1]

[1]*State Key Laboratory of Biotherapy, Department of Medical Genetics, West China Hospital, West China Medical School, Sichuan University, Chengdu 610041, China*
[2]*Department of Molecular Microbiology and Immunology, School of Medicine, University of Missouri, Columbia 65212, MO, USA*

Abstract

TSSK6 is a member of the testis-specific serine/threonine kinase family. Male *Tssk6* knockout mice are infertile owing to spermatogenic impairment, including sperm count reduction, a decrease in motile sperm number and motility rates, and an increase in the number of sperms with abnormal morphology. We investigated the possible association between variations of the *TSSK6* gene and spermatogenic impairment in humans. Mutation screening of *TSSK6* was carried out in 519 patients with azoospermia (n = 273) or severe oligozoospermia (n = 246) and in 359 controls with normozoospermia by denaturing high-performance liquid chromatography and DNA sequencing. The frequencies of alleles and genotypes of gene polymorphism were compared between patients and controls. A novel triallelic polymorphism in *TSSK6*, c.822+126T>G/C, was identified. The frequencies of genotype TT and allele T were increased dramatically in infertile patients compared with controls, whereas genotype TG, allele G and allele C frequencies were significantly higher in controls than in patients. Further study revealed that the allele C frequency of controls was remarkably higher than that of patients with oligospermia. Our findings, for the first time, suggested an association of c.822+126T>G/C in *TSSK6* with spermatogenic impairment in humans in which allele T may be a risk factor for male infertility, while alleles C and G may decrease susceptibility to male infertility.

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1 Introduction

Greater than 10% of couples are unable to achieve pregnancy, about half of which are due to male infertility, in which genetic defects, including gene mutations, chromosomal aberrations and azoospermic factor (AZF) microdeletions, are believed to have an important role [1–4]. However, few genetic defects have been identified in humans so far. In recent years, studies from a series of infertile mouse models revealed hundreds of candidate genes that are associated with male infertility, especially idiopathic spermatogenic impairment [1–3]. Now, it is known that not only gene mutations but also some gene polymorphisms may affect male infertility [5].

Spermatogenesis is a complex process that requires strict gene expression, in which protein phosphorylation

Correspondence to: Dr Si-Zhong Zhang, State Key Laboratory of Biotherapy, Department of Medical Genetics, West China Hospital, West China Medical School, Sichuan University, Gaopeng Street, Keyuan Road 4, Chengdu 610041, China.
Fax: +86-28-8516-4009 E-mail: szzhang@muwcums.com
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is a critical event in protein modification and signal conduction. Through phosphorylation of proteins, the testis-specific serine/threonine kinase (TSSK) family, which is expressed abundantly in the testis, is deeply involved in sperm differentiation, capacitation and fertilization [6–11]. Up to now, five members of this family have been identified, including TSSK1, TSSK2, TSSK3, TSSK4 and TSSK6. The expression of TSSK1 and TSSK2 is limited to meiotic and postmeiotic spermatogenic cells, respectively [7, 12]. TSSK3 presents exclusively in and may function in Leydig cells [8, 13]. TSSK4 phosphorylates the cAMP responsive element binding protein, which is important for germ cell differentiation [10, 14]. Our previous study proposed that some single-nucleotide polymorphisms (SNPs) of *TSSK4* may contribute to the susceptibility to idiopathic spermatogenic impairment [15].

TSSK6, also named small serine/threonine kinase, is expressed in elongating spermatids, and its coding gene, *TSSK6*, is mapped to 19p13.11, with only one exon encoding 273 amino acids [9, 16]. A further study proposed that TSSK6 can phosphorylate histones H1, H2A, H2AX and H3, and can associate with heat shock protein HSP90 and HSC70 [16]. Both histones and HSPs perform significant functions during spermatogenesis. More importantly, male mice with a targeted deletion of *Tssk6* are infertile, showing considerable reduction in sperm counts, impaired DNA condensation, abnormal morphology and impaired spermatozoa motility [16]. The above evidence strongly suggested that *TSSK6* is essential for spermatogenesis and male fertility. However, whether *TSSK6* is also associated with human male infertility still needs to be investigated. In the present study, we carried out mutation screening of the entire coding region, the 5' untranslated region (UTR) and 3' UTR of the *TSSK6* gene, in 519 patients with azoospermia or severe oligozoospermia and 359 controls, and compared the results between the two groups.

2 Materials and methods

2.1 Study participants

This study was approved by the Institutional Ethics Review Board of West China Hospital, Sichuan University, China. A total of 878 unrelated Chinese Han people were recruited from West China Hospital, Sichuan University. The 519 enrolled infertile patients, aged 25–40 years, included 273 with azoospermia and 246 with severe oligozoospermia (sperm concentration $< 5 \times 10^6$ sperm mL^{-1}). All of them underwent at least two semen analyses according to the World Health Organization guidelines (1999) [17]. In addition, these patients had been examined to exclude obstruction of the vas deferens, cryptorchidism, varicocele, chromosomal abnormalities and microdeletions of the AZF region on the Y chromosome according to karyotyping and STS analysis in the AZF region [18]. The control group consisted of 359 fertile men with normospermia aged 25–50 years. All participants gave their informed consent.

2.2 Polymerase chain reaction amplification

Genomic DNA was extracted from peripheral blood lymphocytes by standard phenol-chloroform methods [19]. Based on the mRNA sequence (GenBank No. NM_032037) and the genomic sequence, three pairs of primers were designed using PRIMER PREMIER 5.0 (Premier Biosoft International, Palo Alto, CA, USA) to amplify the entire coding region, 5' UTR and 3' UTR (Table 1). Polymerase chain reactions (PCRs) were carried out

Table 1. PCR-amplified regions, annealing temperatures, product sizes and melting temperatures of denaturing HPLC for mutation screening.

Amplified region	Primer sequence (5'–3')	Annealing temperature (ºC)	Product size (bp)	Melting temperature (ºC)
I	F1: 5'-TGAGTCACAAAGCAGGGAGG-3'	59.0	514	63.0
	R1: 5'-CGTTGCACACCTCGATGAA-3'			
II	F2: 5'-TCGTCAACAAGTTCCTGCCG-3'	60.7	550	64.7
	R2: 5'-TCGAGGCCTTCGGGATAGAG-3'			
III	F3: 5'-GGGTGCATGCCCTTCGACGA-3'	63.4	594	64.1
	R3: 5'-GCCAGGTGCGAGGAACAGCG-3'			

Abbreviations: HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction.

in a total volume of 50 μL containing 0.2 μg genomic DNA, 10 pmol of each primer, 10 pmol of dNTP and two units of Taq polymerase. The PCR cycling protocols included predenaturation at 94ºC for 5 min, followed by 35 amplification cycles of denaturation at 94ºC for 30 s, annealing at 59ºC and 63.4ºC for 30 s, extension at 72ºC for 1 min, and a final extension at 72ºC for 10 min. To genotype c.822+126T>G/C, primer mismatch PCRs were used to amplify a 302-bp fragment at an annealing temperature of 59ºC and to create a new site recognized by restriction endonucleases *Ava*II, *Ban*I and *Kpn*I (F: 5'-CTGCCG CGTGCTGCTGCACATGCGCTTTTTCGG-3' and R: 5'–GCCAGGTGCGAGGAACAGCGAGT-3'). Amplicons were resolved by 1.5% agarose gel electrophoresis to confirm the specific PCR products.

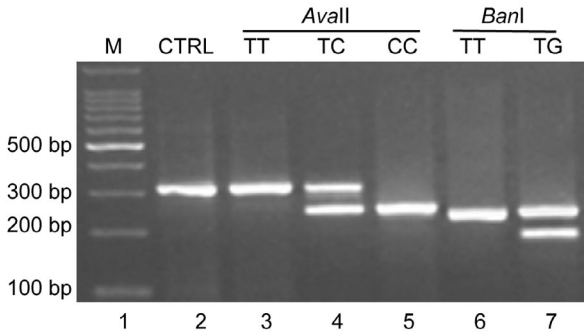

Figure 1. Polymerase chain reaction (PCR) product and enzyme digestion result. Lane 1: M, 100 bp DNA marker; Lane 2: CTRL, PCR product; Lane 3–5: Digestion result of *Ava*II. TT, wild homozygote; TC, heterozygote; CC, mutant homozygote; Lane 6–7: Digestion result of *Ban*I. TT, wild homozygote; TG, heterozygote.

2.3 Denaturing high-performance liquid chromatography analysis and DNA sequencing

Screening of *TSSK6* variations was carried out using denaturing high-performance liquid chromatography (DHPLC) on an automated WAVE system (Transgenomic, Omaha, Nebraska). WAVEMAKER 4.1 software was used to determine the optimal melting temperature for the tested fragments. Before DHPLC, the PCR products were denatured at 94ºC for 5 min and cooled at room temperature for 45 min. Then, 6 μL of each product was injected into a high-throughput DNASep column and eluted with a linear acetonitrile gradient of 2% per minute at a flow rate of 0.9 mL min^{-1}. The elution profiles of the heterozygous fragments were represented as aberrantly shaped peaks, whereas the homozygous fragments were represented as single peaks. After DHPLC, the heterozygous fragments were reamplified and purified with a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sent for direct sequencing in both directions using an ABI 3100 DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

2.4 Genotyping and statistical analysis

Genotyping for all identified variations, including SNPs, was carried out by PCR restriction fragment length polymorphism analysis (*Ava*II, *Ban*I, *Kpn*I). After endonuclease digestion, the products were electrophoresed on a 3% agarose gel and observed with a Gel Doc1000 system (Figure 1).

Hardy-Weinberg equilibrium (HWE) was tested using the HWE program. The differences in genotypic and allelic frequencies between the infertile patients and controls were assessed by χ^2 test using SPSS 11.0 software (SPSS Inc, Chicago, IL, USA).

3 Results

After screening of the entire coding region, the 5'UTR and 3'UTR of the *TSSK6* gene, in 519 infertile men with oligo-/azoospermia and 359 controls, we failed to find any SNPs registered in the NCBI dbSNP database, but we identified a novel single-nucleotide variation in both groups, which is a triallelic polymorphism located in the 3'UTR (Figure 2). According to the nomenclature recommendations of sequence variations by the Human Genome Variation Society (http://www.hgvs.org/mutnomen) [20], this polymorphism was named c.822+126T>G/C.

The genotype distributions c.822+126T>G/C did not deviate from HWE ($P > 0.05$) in patients and controls (data not shown). The genotype CC was detected only in one control, whereas the genotype CG was found in six fertile men and in one azoospermic patient, respectively. No genotype GG was detected in either group. The distributions of the genotype and allele frequencies of c.822+126T>G/C in the cases and controls are presented in Table 2.

Bonferroni's corrections were made for multiple comparisons of genotype and allele frequencies between the different groups [21–23]. The comparison results are summarized in Table 2. After applying Bonferroni's correction, the difference with the corrected $P < 0.0083$

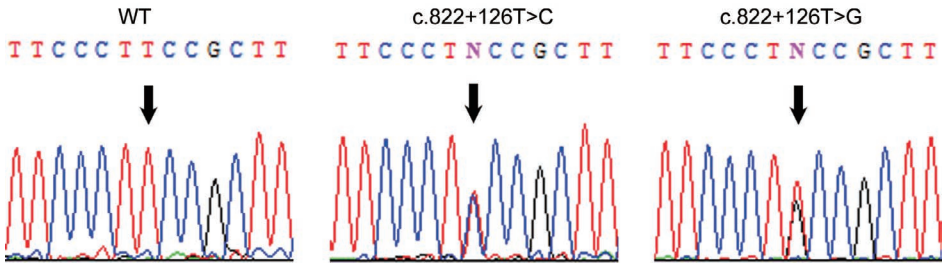

Figure 2. Nucleotide sequences of c.822+126T>G/C: the wild-type sequence (WT) and the mutant sequence. Arrows indicate the position of the variation. The nomenclature of variation follows the recommendations on the Human Genome Variation Society web site (http://www.hgvs.org/mutnomen/).

Table 2. Genotype and allele frequencies of the SNP in the *TSSK6* gene of infertile patients and controls.

SNP c.822+126T>G/C	Controls (n = 359)	Azoo + Oligo (n = 519)		Azoo (n = 273)		Oligo (n = 246)	
		n	P^*	n	P^*	n	P^*
Genotype frequency, n (%)							
TT	263 (73.26)	448 (86.32)	< 0.001	232 (84.98)	< 0.001	216 (87.80)	< 0.001
TC	67 (18.66)	65 (12.52)	NS	37 (13.55)	NS	28 (11.38)	NS
CC	1 (0.28)	0 (0)	NS	0 (0)	NS	0 (0)	NS
TG	22 (6.13)	5 (0.96)	< 0.001	3 (1.10)	0.001	2 (0.81)	0.001
CG	6 (1.67)	1 (0.19)	NS	1 (0.37)	NS	0 (0)	NS
GG	0 (0)	0 (0)	NS	0 (0)	NS	0 (0)	NS
TC + CC + TG + CG + GG	96 (26.74)	71 (13.68)	< 0.001	41 (15.02)	< 0.001	30 (12.20)	< 0.001
Allele frequency, n (%)							
T	615 (85.65)	966 (93.06)	< 0.001	504 (92.31)	< 0.001	462 (93.90)	< 0.001
C	75 (10.45)	66 (6.36)	0.002	38 (6.96)	NS	28 (5.69)	0.004
G	28 (3.90)	6 (0.58)	< 0.001	4 (0.73)	< 0.001	2 (0.41)	< 0.001

Abbreviations: Azoo, azoospermic patients; NS, not significant; Oligo, oligospermic patients; SNP, single-nucleotide polymorphism.
*Compared with controls.

was considered statistically significant. As shown in Table 2, among possible six genotypes of c.822+126T>G/C, four genotypes (TT, TC, TG and CG) with frequencies of over 1% were detected in controls. The frequency of genotype TT increased dramatically in patients compared with that in controls (86.32% *vs.* 73.26%, $P < 0.001$), whereas the frequency of genotype TG was significantly lower in patients than in controls (0.96% *vs.* 6.13%, $P < 0.001$). No obvious difference in the frequencies of genotypes TC and CG between patients and controls was observed. Both allele C and G frequencies of c.822+126T>G/C were significantly higher in controls than in patients (allele C: 10.45% *vs.* 6.36%, $P = 0.002$; allele G: 3.90% *vs.* 0.58%, $P < 0.001$).

After the infertile patients were classified into azoospermic and oligospermic sub-groups, the coincident frequency differences of both alleles and genotypes between either of the sub-groups and the controls still remained, except that the frequency of allele C had no statistical difference between the azoospermic patients and controls (Table 2). Moreover, we did not observe any differences in either alleles or genotypes between azoospermic and oligospermic patients (data not shown).

4 Discussion

Until now, the aetiology and pathogenesis of idiopathic male infertility were still not fully understood. Although a number of candidate genes related to male infertility were reported, most of them have not been tested and confirmed in humans. Gene mutation

screening in infertile patients with spermatogenic impairment is currently the most effective way to explore the possible genetic causes of infertility. Our group aimed to investigate the roles of the TSSK family in idiopathic human male infertility. We had performed mutation screening of *TSSK4* [15] and *TSSK2* (Communicated) in Chinese infertile patients.

It has been shown that TSSK6 can participate in the regulation of gene expression during spermatogenesis in several ways, including phosphorylating histones, H1, H2A, H2AX and H3. However, in our previous study in infertile men, we did not find any mutations or genetic risk factors of *H2AX* [24]. On the other hand, male mice with null *Tssk6* were infertile. Therefore, we hypothesized that the *TSSK6* may associate with human male infertility and carried out gene mutation screening in patients with azoospermia or oligospermia. After examining 878 subjects, only a novel triple polymorphism, c.822+126T>G/C, located in the 3'UTR was detected in both groups, and it was confirmed by DNA sequencing. Further association study disclosed that the frequencies of allele T and genotype TT were significantly higher in infertile patients than in controls, and that the differences still remained between either azoospermic or oligospermic patients and controls. These results suggest that allele T of c.822+126T>G/C may be a genetic risk factor for male infertility (OR: 2.247). However, its frequency in the control group was still high (85.65%), suggesting that the effect of allele T *per se* on reproductive fitness may be mild. Compared with individuals carrying allele T, carriers with allele G seem to have a lower risk for infertility, as the frequencies of allele G and genotype TG decreased significantly in azoospermic and/or oligospermic patients (OR: 0.143 and 0.149). For allele C, after the infertile patients were divided into azoospermic and oligospermic groups, a statistical difference remained only between oligospermic patients and controls (6.36% *vs.* 10.45%), indicating that allele C may have a protective effect against oligospermia (OR: 0.517).

Although our study failed to find gene mutations, it was based on a large sample size and suggested that the novel triple polymorphism c.822+126T>G/C of the *TSSK6* gene may be a genetic susceptibility factor for the development of spermatogenic impairment in humans. Recently, many reports have shown that the SNP in UTRs could have an impact on mRNA stability, translation efficiency and gene expression [25–28]. After searching the microRNA database, no target sequence was found. Whether this polymorphism influences mRNA stability and gene function warrants further investigation. Moreover, considering that the infertile *TSSK6* knockout mouse mainly presented sperm number reduction and abnormal sperm morphology and mobility, studying the mutation of the *TSSK6* gene in patients with oligospermia or teratozoospermia is an optional method to illuminate its possible contribution to spermatogenic impairment in the future.

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