

## Some single nucleotide polymorphisms of the *TSSK2* gene may be associated with the human spermatogenesis impairment

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### Abstract:

*Tssk2*, a member of the testis specific serine/threonine kinase (TSSK) family, is expressed predominantly in the testis and crucial for the formation and function of the sperm cells in mouse. Targeted deletion of *Tssk1* and *2* in male chimeric mice caused infertility due to haploinsufficiency of the genes. Therefore it is reasonable to postulate that mutations in its human homologue *TSSK2* gene may also play a role in impaired spermatogenesis in humans. To explore the possible association between mutations in the *TSSK2* gene and idiopathic infertility in human, mutation screening of the gene in 494 patients with azoospermia or severe oligozoospermia and 357 fertile controls was performed by DHPLC and DNA sequencing. As a result, four single nucleotide transitions were identified, including c.80 A>G(rs3747052), c.774 C>T(rs1052756), c.839 C>T(rs1052763), c.1026 G>A(rs1052773). Among them, significant differences in polymorphism frequencies were observed of c.80 A>G (rs3747052) and c.774 C>T (rs1052756) between the patients and controls, the allele G of c.80 A>G (rs3747052) and allele T of c.774 C>T (rs1052756) seem to be risk factors for the development of spermatogenic impairment, suggesting that the *TSSK2* gene may be associated with male idiopathic infertility in humans.

Key words: male infertility, single nucleotide polymorphism, TSSK gene

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## Introduction

Infertility is one of the major health problems which occur in approximately 15% of couples worldwide and about half of it is due to male factors (de Kretser, 1997; Matzuk & Lamb, 2002). Although some genetic factors such as numerical and structural chromosomal abnormalities, microdeletions of azoospermia factor (AZF) region on the Y-chromosome and mutations of few known spermatogenesis related genes may contribute to the male idiopathic infertility, they account for only 15% of the cases and the majority of genetic causes including gene mutations still remain to be investigated (Foresta et al, 2002).

Protein phosphorylation is the most common posttranslational protein modification in eukaryotes and it controls nearly all of the cellular processes, including spermatogenesis, directly or indirectly (Hanks et al, 1988, Sasson-Corsi, 1997). As a protein kinase family, the testis-specific serine/threonine kinases (TSSK) may play an important role in spermatogenesis since they are expressed mainly or specifically in the testis. To date, five members of the *Tssk* gene family, including *Tssk1*, *Tssk2*, *Tssk3*, *Tssk4*, and *Tssk6*, have been identified in mouse (Bielke et al, 1994; Kueng et al, 1997; Zuercher et al, 2000; Hao et al, 2004; Chen et al, 2005; Xu et al, 2007<sup>b</sup>). Among them, the *Tssk3* gene is expressed specifically in the testis of sexually mature mice and functions in the differentiated Leydig cells (Zuercher et al, 2000). Also the *Tssk4* gene is expressed exclusively in the testis and involved in the early stages of spermatid differentiation (Don et al, 2002; Chen et al, 2005). The *Tssk6* gene, also known as small serine/threonine kinase (*Sstk*), is expressed largely in the elongating spermatids and required for sperm-egg fusion in the mouse (Spiridonov et al, 2005). Our previously study shows that some variations in its human homologue gene may be associated with male infertility (Su et al, 2008).

The mouse *Tssk2* has first been identified in 1997 and may be functionally redundant with *Tssk1*, because of their high sequence similarity and the same kinase substrate, the testis specific kinase substrate (*TSKS*) (Kueng et al, 1997). In human, the *TSSK2* gene is mapped to 22q11.21. It consists of only one exon encoding a protein of 358 amino acids and containing a ser/thr protein kinase domain at its N terminus. Previously research reveals that the *TSSK2* phosphorylates several flagellar proteins in the central apparatus of the sperm axoneme, such as the SPAG16L and the *TSKS* (Hao et al, 2004; Xu et al, 2008<sup>a</sup>; Xu et al, 2008<sup>b</sup>, Zhang et al, 2008). Since the expression of *TSSK2* persists in the flagella of both human and mouse and is in good correlation with the formation of microtubule structures during spermatogenesis (Xu et al, 2008<sup>a</sup>), it is suggested that *TSSK2* may be crucial for spermatid production in both human and mouse. Further, the targeted deletion of *Tssk1* and *2* interrupts spermiogenesis and results in the failure of formation of elongated spermatids in the male chimeric mice (Xu et al, 2008<sup>b</sup>). So, it is reasonable for us to speculate that *TSSK2* may be associated with male spermatogenic impairment in human.

Present study is aimed to investigate the possible relationship between the variations in the *TSSK2* gene and idiopathic male infertility. For this we performed a mutation screening of the gene in 494 infertile patients with azoospermia or severe oligozoospermia and compared the results with those in 357 fertile controls to explore the possible association of the gene with impaired human spermatogenesis.

## Materials and Methods

### *Patients and controls*

All patients and controls participating in this study were genetically unrelated and recruited from West China Hospital, Sichuan University. The 494 patients included 266 infertile men with idiopathic azoospermia and 228 infertile men with severe oligozoospermia (sperm concentration < 5 × 10<sup>6</sup> sperm/ml) aged from 23 to 38 years. All of them underwent at least twice semen analyses according to the World Health Organization Guidelines (1999). To exclude possible chromosomal abnormalities and microdeletions of azoospermia factor (AZF) region on Y chromosome of them, cytogenetic and corresponding molecular examinations were also performed (Simonim et al, 1999). The control group

consisted of 357 fertile men aged from 26 to 45 years who had fathered at least one child without any assisting reproductive techniques. All participants in the study were of Han nationality that made up more than 90% of the Chinese population and informed consent was obtained from all of them.

#### *PCR amplification*

Genomic DNA was extracted from peripheral blood lymphocytes using standard phenol–chloroform procedures. Four pairs of primers were designed using PRIMER PREMIER 5.0 (Premier Biosoft International, Palo Alto, California) to amplify the *TSSK2* gene according to its mRNA sequence (GenBank Accession No. NM\_053006) and its genomic sequence (Table 1). PCRs were carried out in a total volume of 50  $\mu$ l containing 0.1  $\mu$ g genomic DNA, 10 pmol of each primer, 10 pmol dNTP, 3 units of Taq polymerase (TaKaRa, Shiga, Japan), 1  $\mu$ l of dimethyl sulfoxide (DMSO) and standard PCR buffer. The PCR profile was predenatured at 94 °C for 5 min, then followed by 94 °C for 30 s, 55–60 °C for 30 s, and 72 °C for 40 s for 35 cycles with a final extension at 72 °C for 5 min. In order to genotype the c.774 C>T, mismatch PCR was used to amplify a 126bp fragment at an annealing temperature of 61.5 °C for 15 seconds and to create a new recognition site for the restriction endonuclease Tsp 45I. The sequences of primers used are F: 5'-GCGTATCCAGAAGGAGCACCGTGTG-3' and R: 5'-GGATCTCATCGATGTGGAGCCGGTG-3'. The annealing temperatures for the amplification of all DNA fragment are also shown in Table 1. In order to confirm the presence of specific PCR products, every amplicon was resolved by 1.5% agarose gel electrophoresis.

#### *Denatured HPLC (DHPLC) analysis and DNA sequencing*

Mutation screening in the *TSSK2* gene was carried out with the automated WAVE 3500HT Nucleic Acid Fragment Analysis System (Transgenomic, Inc., Omaha, NE, USA). The WAVEMAKER4.1 software was used to determine the optimal melting temperatures for the PCR amplified fragments (Table 1). Prior to DHPLC analysis, the PCR products were denatured at 94°C for 5min and cooled at room temperature over 45 min. Then, 8  $\mu$ l of the products were injected into a high-throughput DNASep column and eluted with a linear acetonitrile gradient at a flow rate of 0.9 ml/min. The elution profiles of homozygous fragments were represented as a single peak while heterozygous fragments showed multiple peaks and/or aberrant shaped peak. The heterozygous samples detected by the DHPLC analysis were reamplified and purified using E.Z.N.A Gel Extraction Kit (Omega) and then sequenced in both directions on ABI 3100 DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

#### *Genotyping*

PCR restriction fragment length polymorphism (PCR-RFLP) analysis with corresponding restriction endonucleases enzymes Bgl II, Nla III, Tsp 45I and Sau96 I (NEB, Beverly, MA, USA) was carried out to genotype the single nucleotide polymorphisms (SNPs) identified in this study. The products were electrophoresed on a 3% agarose gel and observed with Gel Doc1000 system (Bio-Rad) after restriction enzyme digestion.

#### *Statistical analysis*

The allele and genotype frequencies of the patient and control groups were calculated by counting. The software HWE was used to evaluate the Hardy–Weinberg equilibrium of the SNPs. Differences in genotypic and allelic frequencies of the SNPs between infertile patients and controls were assessed by  $\chi^2$  test using software SPSS11.0.

## **Results**

By screening the 5' UTR, 3' UTR and the exon sequence of the *TSSK2* gene in 494 infertile men with oligo-/azoospermia and 357 controls, 4 single nucleotide transitions, all located in the coding region, were detected and then confirmed by DNA sequencing. They were c.80A>G, c.774C>T, c.839C>T and

c.1026G>A according to the nomenclature recommendations (<http://www.hgvs.org/mutnomen>). Both c.80A>G and c.839C>T were missense mutations which result in substitution of Lys by Arg and Thr by Met respectively. The other two transitions were synonymous variations.

The frequencies of minor alleles of all the 4 transitions were over 1% in both infertile patients and controls, so they were SNPs and their genotypes were in Hardy-Weinberg equilibrium (Table 2). At c.80A>G, both the frequencies of allele G (P=0.027, odds ratio (OR) =2.515, 95% confidence interval (CI) 1.077-5.868) and the genotype AG (P=0.026, OR=2.553, 95% CI 1.088-5.993) were significantly greater in patients than those in controls. Also, the frequencies of allele T of c.774C>T (P=0.003, OR=1.403, 95% CI 1.125-1.750) and carriers with allele T (CT+TT) (P<0.001, OR=1.571, 95% CI 1.194-2.067) were significantly higher in patients than those in controls. However, for the other two SNPs no obvious differences in either allele or genotype frequencies were found between the two groups.

## Discussion

It is suggested that many genetic factors may be involved in idiopathic male infertility. However, to date, only a few genetic defects have been confirmed to result in abnormal spermatogenesis (Matzuk and Lamb, 2002). In recent years, hundreds of candidate genes related to spermatogenesis have been identified in animals with gene targeting techniques that disrupt specific genes in animal models (Scherthan, 2003). As to humans, mutation screening of these genes in male infertile patients may be the most effective way to investigate the genetic causes of idiopathic male infertility.

Previous researches have revealed that *Tssk2* plays an important role in the spermatogenesis in mouse (Kueng et al, 1997; Hao et al, 2004; Xu et al, 2008; Xu et al, 2008, Zhang et al, 2008). To explore the possible association of its human homolog with male infertility in humans, we performed mutation screening of the *TSSK2* gene in a group Chinese patient with idiopathic infertility. As the result, 4 SNPs, c.80A>G, c.774C>T, c.839C>T, c.1026G>A, were identified. These SNPs had been already registered in the National Center for Biotechnology Information dbSNP database before and the frequencies of the minor alleles of c.80A>G, c.774C>T and c.1026G>A are similar to that reported in dbSNP. However the frequency of allele T of c.839C>T is significantly lower than that presented in the database.

The c.839C>T and c.1026G>A may be just polymorphisms without obvious disease-related effect since their distributions were similar between patients and controls. In contrast, the frequencies of allele G and genotype AG of c.80A>G site were significantly higher in patient group with oligo-/azoospermia than those in controls, suggesting that the allele G may be a risk factor for the development of idiopathic male infertility. This A>G mutation result in a substitution of amino acid Lys by Arg at 27th amino acid. Analysis with the on-line software PROSITE (<http://www.expasy.ch/prosite/>, Hofmann et al, 1999) reveals that the 18th to the 41st amino acids is the ATP binding domain and the 18th to the 26th amino acids is the nucleotide phosphate-binding region of TSSK. These suggest that this substitution is located closely to the nucleotide phosphate binding site in the ATP binding region of TSSK2. So, it is reasonable to speculate that the amino acid substitution may influence the activity of TSSK2. Of course, more functional study is needed to confirm this.

The statistical analysis show that the allele T of c.774C>T might also be a risk factor for the spermatogenic impairment. Analysis of the possible influence of the c.774C>T on the gene expression with program Consite (<http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite>, Sandelin et al, 2004) showed that the substitution created a consensus sequence (GTCAGTCA), which could be recognized by the transcription factor *c-FOS*, a member of the *FOS* family of transcription factors that can be rapidly induced by acute challenges (Justin, 1998; Krisztina, 1998). Therefore, the allele T of c.774C>T may influence the expression level of TSSK2. However, since the c-FOS is not constitutively expressed and functions mainly in the central neuron, it is also possible that this SNP is in linkage disequilibrium with some other loci which are associated with idiopathic male infertility.

To our knowledge, present study is the first systematic mutation analysis of the *TSSK2* gene in human infertility with azoospermia or severe oligozoospermia. Our findings indicate that some nucleotide

variations in the *TSSK2* gene may be associated with impaired spermatogenesis and male infertility. Of course, more studies in different populations as well as further investigation including functional analysis of the variants are needed in order to elucidate the role and its mechanisms of the *TSSK2* gene in human spermatogenesis and its pathology.

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Table 1 The PCR amplified regions, primer sequences, annealing temperature, product size, and the melting temperature of denaturing HPLC (DHPLC) for mutation screening.

Amplified region	Primer sequence(5'-3')	Annealing Temperature(°C)	Product Size(bp)	Melting Temperature(°C)
5' UTR+Exon 1-1	F:CAATGCTGAGTGTTCACCCCT G R:TGTAGATCCGTCCGTCAGAGG TC	59.3	407	60.4
Exon1-2	F:ATCTTTGAGACCTCTGACGGAC G R:CTGATGTCGGAGTCGTCATAG GG	60.2	434	62.5
Exon 1-3	F:ATGCCCTATGACGACTCCGA R: TGCTAGGTGCTTGCTTTCCC	60.4	440	63.2
Exon 1-4+3' UTR	F:AGAACGAGAACAGGATGGAG R:TGCTGCTAAGTGGAAGAAAG	55.8	272	58.1

Abbreviations: PCR, polymerase chain reaction; DHPLC, denatured high-performance liquid chromatography; UTR, untranslated region

Table 2 Distributions of genotype and allele frequencies of four SNPs in *TSSK2* of infertile patients and controls.

	Restriction Endonuclease	Genotype Allele	Genotype and Allele Frequency		P value
			Control(n=357)	Patients(n=494)	
c.80 A>G (rs3747052)	Bgl II	AA/AG/GG	350/7/0	470/24/0	0.026
		A/G	707/7	964/24	0.027
c.774C>T (rs1052756)	Tsp 45I*	CC/CT/TT	217/124/21	241/215/38	<0.001
		C/T	558/166	697/291	0.003
c.839C>T (rs1052763)	Nal III	CC/CT/TT	310/44/3	420/69/5	NS
		C/T	664/50	909/79	NS
c.1026G>A (rs1052773)	Sau 96I	GG/GA/AA	255/89/13	346/135/13	NS
		G/A	599/115	827/161	NS

\*The restriction site is created by primer mismatch polymerase chain reaction.