Mutation screening of the *FKBP6* gene and its association study with spermatogenic impairment in idiopathic infertile men

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Abstract

Fkbp6 has been proved to be a new component of synaptonemal complexes and be involved in homologous chromosomes pairing and male infertility in mice. To explore the possible association between variations in the *FKBP6* gene and impaired spermatogenesis in human, mutation screening of all the eight exons and the intron/exon boundaries of the gene was performed in 323 patients with azoospermia or severe oligozoospermia and 205 fertile controls by denatured HPLC and DNA sequencing. As a result, four novel and one known single nucleotide transitions were identified, including c.58-2A>G, c.111C>T, c.156G>T, c.594G>A, and c.216C>A (rs3750075). The frequencies of genotype CA, allele A of c.216C>A and haplotype 'GAG' consisting of c.156G>T, c.216C>A, and c.594G>A were significantly lower in infertile patients than those in controls. These findings suggest that the *FKBP6* gene may play a role in modifying the susceptibility to idiopathic spermatogenic impairment in human and propose that the allele A of c.216C>A seems to be a protective factor for the development of male infertility.

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Introduction

As a major health problem, infertility occurs in approximately 15% of couples worldwide and about half of it is due to male factors (de Kretser 1997, Matzuk & Lamb 2002). It is generally accepted that genetic defects play an important role in etiology of idiopathic male infertility, and in genetically modified or mutant animal models, hundreds of candidate genes related to spermatogenic impairment have been identified (de Rooij & de Boer 2003, Scherthan 2003, Vogt 2004, O'Bryan & de Krester 2006). However, to date very few have been confirmed in human (de Kretser 1997, Matzuk & Lamb 2002). Therefore, it is reasonable to postulate that at least some of these genes may also play a role in impaired spermatogenesis in human.

Synaptonemal complexes (SCs) are elaborate meiosis-specific supramolecular proteinaceous structures involving in pairing and recombination of homologous chromosomes during meiosis (Heyting 1996, Zickler & Kleckner 1999). Genetic variations in SC genes may contribute to human male infertility (Page & Hawley 2004). For example, Miyamoto *et al.* (2003) found a heterozygous deletion of one base of synaptonemal complex protein 3 (SCP3) gene in two patients with azoospermia, which resulted in a premature stop codon

and truncation of SCP3, and proposed that the mutation was associated with non-obstructive azoospermia in human. Other two research groups also reported some abnormalities of SCP, probably in SCP1, in patients with azoospermia (Judis *et al.* 2004, Sun *et al.* 2004). In addition, polymorphisms of certain meiosis-related genes were reported to be associated with spermatogenic impairment in human (Sato *et al.* 2006, Zhoucun *et al.* 2006).

Recently, *Fkbp6* has been proved to be a new component of SCs (Crackower *et al.* 2003). In *Fkbp6* gene knockout mice, the male mice are infertile with azoospermia and absence of normal pachytene spermatocyte, but female mice show no apparent abnormalities in all aspects, including fertility (Crackower *et al.* 2003). In human, the *FKBP6* gene is located on chromosome 7q11.23 and expresses in various tissues with the highest expression in testis. The gene product, FK-506-binding protein 6 (FKBP6), belongs to the immunophilins FKBP family. It contains a three-unit tetratricopeptide repeat motif and has peptidyl-prolyl *cis-trans* isomerase activity (Meng *et al.* 1998).

With the essential role of the *Fkbp6* gene and SCPs in male reproduction in mind, we postulated that variations in human *FKBP6* gene might be associated with impaired spermatogenesis, and performed a mutation

screening of the *FKBP6* gene in 323 infertile patients with azoospermia or severe oligozoospermia and compared the results with those of 205 controls.

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 total 323 patients, aged from 23 to 38 years, included 218 infertile men with idiopathic azoospermia and 105 infertile men with severe oligozoospermia (sperm concentration $<5 \times 10^6$ sperm/ml). All of them underwent at least twice semen analyses according to the World Health Organization Guidelines (1999) and their chromosomal abnormalities, including microdeletions of azoospermia factor (AZF) region on Y chromosome were excluded by cytogenetic and corresponding molecular examinations (Simonim *et al.* 1999). The control group comprised 205 fertile men, aged from 26 to 45 years, who had fathered at least one child without any assisting reproductive techniques. This study was approved by the Institutional Ethic Review Board of West China Hospital, Sichuan University and informed consents were obtained from all participants.

PCR amplification

Genomic DNA was extracted from peripheral blood lymphocytes using standard phenol–chloroform procedures. To amplify all the eight exons, the intron/exon boundaries and the non-coding region upstream of exon 1, eight pairs of primers were designed according to the mRNA sequence (GenBank Accession no. NM_003602) and human genomic sequence using software Primer

Premier 5.0 (Table 1). PCRs were carried out in a total volume of 30 μ l containing 0.1 μ g genomic DNA, 7 pmol each primer, 6 pmol dNTP, 1.5 units Taq polymerase (TaKaRa, Shiga, Japan), and standard PCR buffer. After pre-denaturation at 94 °C for 5 min, 35 amplification cycles were performed with a temperature profile consisting of denaturation at 94 °C for 30 s, annealing at a temperature between 52 and 62 °C for 30 s, and extension at 72 °C for 1 min, followed by an extra final extension at 72 °C for 5 min. The annealing temperatures for amplifying each DNA fragment were shown in Table 1.

Denatured HPLC (DHPLC) analysis and DNA sequencing

The automated WAVE 3500HT Nucleic Acid Fragment Analysis System (Transgenomic, Inc., Omaha, NE, USA) was applied to screen variations in the *FKBP6* gene. The WAVEMAKER4.1 (Transgenomic) software was used to determine the optimal melting temperatures for the amplified fragments (Table 1). Prior to DHPLC analysis, PCR products were denatured at 94 °C for 5 min and cooled at room temperature over 45 min. Then, 5 μ l products were injected into a high-throughput DNasep column (Transgenomic) and eluted with a linear acetonitrile gradient of 2% per minute at a flow rate of 0.9 ml/min. The elution profiles of heterozygous fragments were represented as multiple peaks and/or aberrant shaped peak. In contrast, homozygous fragments showed a single peak. After DHPLC analysis, heterozygous fragments were reamplified and purified using QIAquick PCR purification Kit (Qiagen) and then underwent direct sequencing in both directions on ABI377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). Several PCR products were cloned into pGEM-T Easy Vector System (Promega) and sequenced (Fig. 1).

Table 1 The PCR amplified regions, primer sequences, annealing temperatures, 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)
Exon 1	F: AATGCCGCCGTCGGTAGGGG R: ACGTCTGGGCCCTCACCTGG	62	200	63.1
Exon 2	F: CGTAGACGCTGAGGGGTGGC R: TCTTCCCAACAGAGTGCGATGA	60	240	62.3
Exon 3	F: TCCTGTACCTCGTGGTGTCGG R: GGTGCCTCATCTCGCTCCTAAAC	55	281	58.1
Exon 4	F: GGGGAAGAACTGATAGCATTA R: ACACCAACCCATTTGTCTCTCTA	55	329	58.7
Exon 5	F: CACTTCCATTGTGGCATT R: TTCAAAGCATTCTCACCT	52	200	58.3
Exon 6	F: TAGTTTTGCGTGTGGATTCTTTA R: CCTTTCTCTGGGTGTTTATTC	55	320	59.9
Exon 7	F: CACCGTGCCAGCCAAATGATA R: GGGGGCGGCCTCCTAACA	56	296	59.6
Exon 8	F: TCACCTCCTCCAAACACAGACC R: TGCAGACAGCCACCATCACT	55	235	59.8

All PCRs were carried out in 1.5 mM MgCl₂ with exception of exon 2 in 2 mM MgCl₂.

Figure 1 Detection and confirmation of the four novel single nucleotide transitions in the *FKBP6* gene by DHPLC and DNA sequencing. (A) DHPLC elution profiles: homozygous fragments with a single peak and heterozygous fragments with an aberrant peak. (B) DNA sequences of the four variations: mutant sequences (upper panel) and wide-type sequences (lower panel). Arrows indicate the positions of the variations and codons are underlined by dark horizontal lines. The nomenclature of variations follows to the recommendations in website <http://www.hgvs.org/mutnomen/>.

Genotyping

Genotyping for the single nucleotide polymorphisms (SNPs) identified in this study was carried out by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis with corresponding restriction enzymes (*Hha*I, *Taq*I, and *Alw*NI; NEB, Beverly, MA, USA). After restriction enzyme digestion, the products were electrophoresed on a 2% agarose gel and observed with Gel Doc1000 system (Bio-Rad).

Statistical analysis

Hardy–Weinberg equilibrium (HWE) program was used to test the deviation from HWE for alleles. Differences in genotypic and allelic frequencies of SNPs between infertile patients and controls were assessed by χ^2 test using SPSS12.0 software. Pairwise linkage disequilibrium (LD) coefficients (D') were calculated using 2LD program (<http://www.mrc-epid.cam.ac.uk/Personal/jin-guazhao/software>; Zhao 2004). Program PHASE 2.1.1 using Bayesian algorithm (Stephens *et al.* 2001, Stephens & Donnelly 2003) was utilized to reconstruct haplotypes from genotype data and to evaluate their possible association with impaired spermatogenesis. The frequency differences of individual haplotypes between patients and controls were also assessed by χ^2 -test using SPSS12.0 software.

Results

By screening all the eight exons, the intron/exon boundaries and the 5' UTR of the *FKBP6* gene in 323 infertile men with oligo-/azoospermia and 205 controls, five single nucleotide transitions were detected and then confirmed by DNA sequencing. The nomenclature of these sequence variations followed the recommendations in the website <http://www.hgvs.org/mutnomen>. Among them, c.216C>A was the only non-synonymous variation resulting in substitution of Phe by Leu at codon 72 (p.Phe72Leu) and had been listed in the NCBI dbSNP database (ID: rs3750075). The other four were novel, including three synonymous variations (c.111C>T, c.156G>T, and c.594G>A) and one variation in the consensus splice site (c.58-2A>G).

Both c.111C>T and c.58-2A>G were observed only in one patient each. By PCR-RFLP analysis, we discovered that the frequencies of all the three minor alleles of c.156G>T, c.216C>A, and c.594G>A were over 1% in both infertile patients and controls, so they were SNPs (Table 2). The genotype distributions of the three SNPs were in HWE in both groups (data not shown). As shown in Table 2, the frequencies of allele A and genotype CA of c.216C>A were significantly lower in patients with oligo-/azoospermia than those in controls ($P<0.001$) that was consistent with our previous study on 177 patients with azoospermia

Table 2 Distributions of genotypic and allelic frequencies of three single nucleotide polymorphisms in the *FKBP6* gene of infertile patients and controls.

SNP	Genotype allele	Genotype and allele frequency		P value
		Patients (n=323)	Controls (n=205)	
c.156G>T	GG/GT/TT	294/29/0	189/16/0	NS
	G/T	617(95.5)/29(4.5)	394(96.1)/16(3.9)	NS
c.216C>A	CC/CA/AA	312/11/0	183/22/0	0.0006
	C/A	635(98.3)/11(1.7)	388(94.6)/22(5.4)	0.0007
c.594G>A	GG/GA/AA	276/46/1	169/36/0	NS
	G/A	598(92.6)/48(7.4)	374(91.2)/36(8.8)	NS

Data of genotype and allele frequency are presented as 'n' and 'n (%)' respectively. NS, not significant.

(Zhang *et al.* 2005). Moreover, the allele A of c.216C>A showed a protective effect against male infertility in human (by allele A: OR=0.306, 95%CI: 0.147–0.737; by genotype CA: OR=0.293, 95%CI: 0.139–0.619). No significant differences in genotypic or allelic frequencies of both c.156G>T and c.594G>A between patients and controls were observed.

The coefficients (D') of pairwise LD in the control group presented in Table 3 indicated that there was no complete LD among the three SNPs, c.156G>T, c.216C>A, and c.594G>A. In total, seven haplotypes consisting of these SNPs were observed and their frequencies in both patients and controls were estimated by PHASE2.1.1 program. As shown in Table 4, four haplotypes with frequency of more than 1% accounted for over 95% of the total haplotypes. The PHASE case-control global test revealed remarkable difference of haplotype distribution between infertile patients and controls (global $P=0.01$), which suggested a significant association between the haplotypes and human male infertility. Comparison of individual haplotypes between the two groups showed that the frequency of haplotype 'GAG' consisting of c.156G>T, c.216C>A, and c.594G>A was significantly decreased in infertile patients compared with controls (1.66 vs 4.86%, $P=0.0029$; Table 4).

Discussion

In order to explore the genetic causes of male infertility in human, mutation screening in patients with spermatogenic impairment may be the most effective approach at present time. Therefore, we screened the *FKBP6* gene and performed association study of the gene with male infertility. As the result, five single nucleotide transitions were detected, including three SNPs and two rare variations. Except the c.216C>A (rs3750075), other four transitions were newly discovered. Since the distributions of two novel SNPs, c.156G>T and c.594G>A, were similar between patients and controls ($P>0.05$), they might be normal polymorphisms. The c.111C>T was a synonymous variation with very scarce frequency,

and it might not be a disease-associated variation. Although c.58-2A>G changed the consensus splice site, its effect on spermatogenesis needed further research, because it was found in only one azoospermic man.

The frequencies of allele A and genotype CA of c.216C>A were significantly lower in patients with oligo-/azoospermia than those in controls ($P<0.001$). In addition, the odds ratios (ORs) suggested that the carriers with allele A had lower risk compared with those with allele C. Therefore, the allele A seemed to have a protective effect against development of male infertility. Besides, we further used the web-based program ESEfinder (Exonic Splicing Enhancer (ESE); <http://rulai.cshl.edu/tools/ESE/>; Cartegni *et al.* 2003) to predict the effects of c.216C>A on activity of pre-mRNA splicing. It was shown that the substitution of C by A of SNP c.216C>A created a new exonic splicing enhancer consensus sequence (GACCCTTA), and that the consensus sequence could be recognized and bound by the splicing factor SC35, which was an important member of serine/arginine-rich protein family playing a key role in pre-mRNA splicing (Cartegni *et al.* 2003, Sanford *et al.* 2005). Therefore, the allele A of c.216C>A may enhance the activity of pre-mRNA splicing and further increase the expression of the *FKBP6* gene compared with allele C. So the SNP c.216C>A may be a functional polymorphism in the *FKBP6* gene and it is worthy of attention in the functional studies of the gene.

Since no complete LD ($D'=1$) was observed among the three SNPs, c.156G>T, c.216C>A, and c.594G>A, we performed further haplotype analysis of them in the patients and controls and found a significant association

Table 3 D' values of pairwise linkage disequilibrium among the three single nucleotide polymorphisms (SNPs) in the *FKBP6* gene.

SNP	c.156G>T	c.216C>A
c.216C>A	0.701	
c.594G>A	0.884	0.245

Linkage disequilibrium was calculated for the control group.

Table 4 Frequencies of the haplotype consisting of three single nucleotide polymorphisms (SNPs) in the *FKBP6* gene estimated using PHASE2.1.1 program and comparisons between infertile patients and controls.

Haplotypes	Control <i>F</i>	Patient <i>F</i>	<i>P</i> value
GCG	0.826736	0.868998	NS
GCA	0.081137	0.069454	NS
GAG	0.048592	0.016656	0.0029
GAA	0.004510	0.000000	NS
TCG	0.036311	0.039671	NS
TCA	0.002157	0.004849	NS
TAG	0.000556	0.000372	NS

Three SNPs constituting haplotypes: c.156G>T, c.216C>A, and c.594G>A. *P*=0.01 for PHASE case-control global test. Control *F*, haplotype frequency estimated from controls; patient *F*, haplotype frequency estimated from patients. NS, not significant.

of haplotypes with male infertility using PHASE case-control global test (global *P*=0.01). Comparison of individual haplotypes showed that the frequency of haplotype 'GAG' consisting of the three SNPs was significantly lower in patients than that in controls (1.66 vs 4.86%, *P*=0.0029). All of these suggested that the *FKBP6* gene might modify the human susceptibility to spermatogenic impairment, and also supported the protective effect of allele A of SNP c.216C>A. Alternatively, it could not be excluded that the c.216C>A might be in LD with another functional locus nearby.

Meanwhile, a recent study reported that the haploinsufficiency for the *FKBP6* gene did not cause azoospermia (Metcalf *et al.* 2005). Another research on 51 men with non-obstructive azoospermia failed to find mutations in the *FKBP6* gene including the c.216C>A and proposed that the gene mutations were not a common cause of azoospermia (Westerveld *et al.* 2005). Our study was based on a larger sample size with 528 subjects. Although no apparent mutations leading to oligo-/azoospermia were detected, the results of the association studies strongly suggested a role of the *FKBP6* gene in modifying the susceptibility to idiopathic spermatogenic impairment in human and proposed that the allele A of c.216C>A seemed to be a protective factor for development of male infertility. Therefore, further more in-depth researches with larger samples in more populations and the functional studies of some variations in the *FKBP6* gene are desirable.

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