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GENETICS

An association study of SPO11 gene single nucleotide polymorphisms with idiopathic male infertility in Chinese Han population

Jing Zhang · Dang-xia Zhou · Hai-xu Wang · Zhao Tian

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Abstract
Purpose To investigate the incidence of single nucleotide polymorphisms in SPO11 and its influence in idiopathic male infertility in China.
Methods Infertility factors such as anatomical, immunological and infectious disorders were examined in selecting patients with idiopathic male infertility. Routine semen analysis was performed. DNA was isolated from peripheral blood of the selected patients and control group, and five SNP loci of SPO11 were genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. Furthermore, nucleotide sequences were sequenced.
Results SNP5 (*rs28368082*) in the exon7 of SPO11 was identified to be associated with idiopathic male infertility (P=0.037 for differences across genotypes). A transversion (C5679T) was detected in eight patients (11.0%), which led arginine change into tryptophan. And this variant was not found in the remaining patients and controls.
Conclusion A SPO11 SNP was associated with idiopathic male reproduction, suggested that SPO11 might has an effect on premorbid functioning, which increase susceptibility for idiopathic male reproduction. Further research on this issue is still necessary.

Keywords Idiopathic male infertility · Single nucleotide polymorphisms (SNP) · Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) · SPO11

Capsule In this study, we performed a case–control study using SNPs to examine the association of SPO11 with idiopathic male infertility and detected a SNP that was significantly over-represented in the cases compared with the controls in Chinese Han population.

J. Zhang · D.-x. Zhou (✉)
Department of Pathology, Medical School,
Xi'an Jiaotong University,
Xi'an 710061, China
e-mail: dangxia75@163.com

H.-x. Wang
Reproductive Medical Center, Xi'an Jiaotong University,
Xi'an 710061, China

Z. Tian
Department of Forensic Science, Medical School,
Xi'an Jiaotong University,
Xi'an 710061, China

D.-x. Zhou
Key Laboratory of Environment and Genes Related to Diseases,
Ministry of Education,
Xi' an 710061, China

Introduction

Infertility affects 10–15% of couples wideworld, and male factors account for nearly half of all infertility cases [1]. Although modern diagnostic methods identified much more pathogenesis of infertility, unfortunately, approximately 50% of infertility cases are still unexplained or idiopathic [2]. As we all known, impaired spermatogenesis is an important etiology of male infertility. Spermatogenesis is a complex physiological process, regulated by multiple genes. With the development of molecular biology, many infertility-related genes have been identified [3]. Studies have shown that genetic abnormalities account for approximately 5% of infertility in males, and the proportion increases to 15% in azoospermia males [4].

Besides Y chromosome micro deletion, many studies have suggested that polymorphism of genes in autosomal chromosomes may also play an important role in male infertility [4], such as CFTR [5], BRCA2 [6] and TSPYL1 [7], which are

closely associated with male infertility. Understanding the molecular mechanism of abnormal spermatogenesis and the genes involved is important in developing both diagnostic tools and treatment strategies for male infertility [8].

Meiosis, producing haploid cells from diploid progenitors, is an essential process in gametogenesis [9]. The SPO11 gene is one of critical genes involved in meiosis [10–13], The SPO11 gene is locatedin 20q13.2-13.3, contains 13 exons encoding two different type of proteins (Fig. 1) [14], which plays an important role in initiating meiotic recombination by generating double-stranded breaks in DNA through its topoisomerase activity. When recombination fails to occur, chromosomal disjunction also frequently fails, disrupting the normal production of gametes. For example, when the SPO11 gene was disrupted in mice by homologous recombination, there was a generalized arrest of spermatogenesis in spermatocytes before the pachytene stage, resulting in male infertility [15]. The recent study of Greg et al. found that mutations and polymorphisms of the SPO11 gene in azoospermia individuals of American population [16]. Prompted by these findings, we were interested in investigesting whether the SPO11 gene is associated with idiopathic male infertility in Chinese population.

China is one of the most populous areas in the world. However, to the best by our knowledge, there are still no study concerning the association of SPO11 gene and male infertility in Chinese population. Therefore, this study was designed to investigate the single nucleotide polymorphisms (SNP) of SPO11 gene using the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method in idiopathic male population of China, whichmight provide useful information for male infertility studies.

Materials and methods

Subjects selection

The study was approved by the Institutional Review Board and the National Medical Ethics Committee. All subjects were randomly selected from Chinese Han population in the Shaanxi Province, China.

The infertile patients were selected from couples attending the infertility clinic who had a history of infertility above 12 months. The risks and benefits of participating in the study were explained. After obtaining the informed consent of patients, a detailed medical and reproductive history was obtained from all subjects, including reproductive history and infertility evaluation of the female partner. Semen routine was examined for all subjects. A male-infertility specialist performed the genital examination of the male partner. Patients who havenormal female mates, with normal reproductive history, ovulation (by follicular ultrasound scan, serum hormones levels, and endometrial biopsy) and tubal patency (hysterosalpingogram) were eligible for the study. After excluded the anatomical, immune, infectious abnormality, according to the semen parameters on repeated analysis, 73 patients were finally selected as idiopathic infertile case group). One hundred seventeen healthy fertile male volunteers with the same genetic background served as the control group.

DNA isolation, SNP selection and genotyping

After obtaining written informed consent of all subjects, 2 ml venous blood was taken from each individual and cryopreserved in tubes containing anticoagulant Sodium citrate in −20°C.

Genomic DNA was extracted from peripheral white blood cells, using DNA extraction kit (A004-1) (Dinguo, Beijing, China).

On the basis of previous studies, five SNPs, namely SNP1 (*rs28368062*), SNP2 (*rs28368064*), SNP3 (*rs79564060*), SNP4 (*rs23736832*), and SNP5 (*rs28368082*) were selected from the dbSNP database at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/SNP) (Fig. 1).

The genotyping was performed using polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP). Oligonucleotide primers were designed using

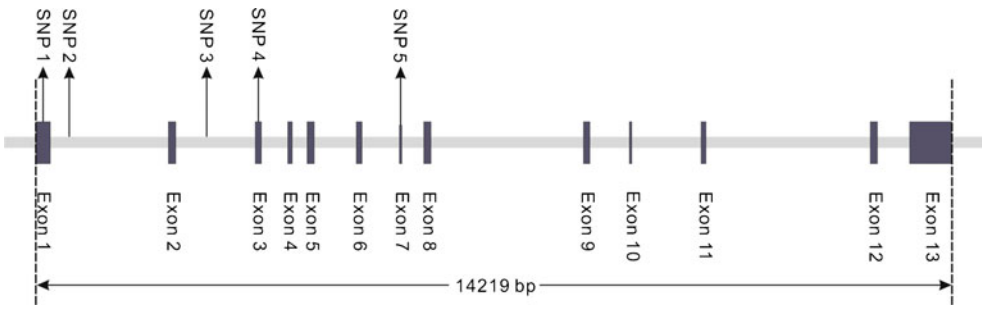

Fig. 1 SPO11 Gene structure. Gene structure and five novel coding single-nucleotide-polymorphism (cSNP) sites in the human SPO11 gene which consists of 13 exons. Arrows indicate five novel SNPs that SNP1 (rs28368062), SNP2 (rs28368064), SNP3 (rs79564060), SNP4 (rs23736832), andSNP5 (rs28368082)

Table 1 The amplified SNP sites, primer sequences, product size, amplify conditions of PCR

SNP ID	Fragment size (bp)	Primers(5′–3′)	Amplify conditions (°C)
rs28368062	331	F: TACGCATACGCCTGTCCA R:CGCCCGACTTTTCTACAGAG	95°C4′,95°C30″, 56°C30″,72°C45″,35cycles, 72°C10′
rs3736832	272	F:TTATCTTAGTGCCTCTGTATGTC R: TTTTGATCTTTCTGGTGG	95°C4′,95°C30″, 52°C30″,72°C45″,35cycles, 72°C10′
rs28368082	330	F: TACCCCTGTTGGGAGAAT R:CTTACACATTATGAAGGCTTGG	95°C4′,95°C30″, 58°C30″,72°C45″,35cycles, 72°C10′
rs79564060	305	F:GTGAAAGATGAGGCAAAGGA R: TTTAGCAGGGGCAGAAGAT	95°C4′,95°C30″, 58°C30″,72°C45″,35cycles, 72°C10′
rs28368064	262	F: TCTTCGACGTTTTGGACC R: CAAAGCAGGTCACGGGTA	95°C4′,95°C30″, 58°C30″,72°C45″,35cycles, 72°C10′

Primer Premier 5.0 (Table 1). Polymerase chain reaction (PCR) was performed in 20 μl containing 10 μl of PCR Mix (10× Taq Buffer with (NH4)2SO4, 0.2 mmol/L dNTP, 1.5 mmol/L MgCl2, 0.5 μmol/L of each primer, 2.5 U Taq DNA polymerase [All Applied Fermentas, Lithuania]), and approximately 50 ng DNA template), 1.5 μl of each primer, 5 μl ddH_2O, and 2 μl of genomic DNA, the PCR cycling was performed using the MyCycler Thermal Cycler (Bio-Rad) with conditions shown in Table 1, and the products were detected on 1.5% agarose gel (Fig. 2).

Restriction maps of the DNA sequences were obtained using the software Webcutter 2.0. The enzyme selection was determined by their ability to generate characteristic restriction profiles for each species with band sizes easily distinguishable on agarose gels. Three microlitre of PCR product was digested with 0.3 μl of the selected enzyme [All Applied Fermentas, Lithuania], incubated at 37°C for 16 h (Table 2). The fragments of PCR-RFLP were detected on 2% agarose gels with 50–500 bp DNA ladders (DL500, Takara) (Fig. 3). Finally, genotypes of randomly selected subjects, as determined by PCR-RFLP, were confirmed by direct resequencing in an ABI3100 autosequencer. All the oligonucleotides were synthesized and sequenced by Augct Inc. (Beijing, China).

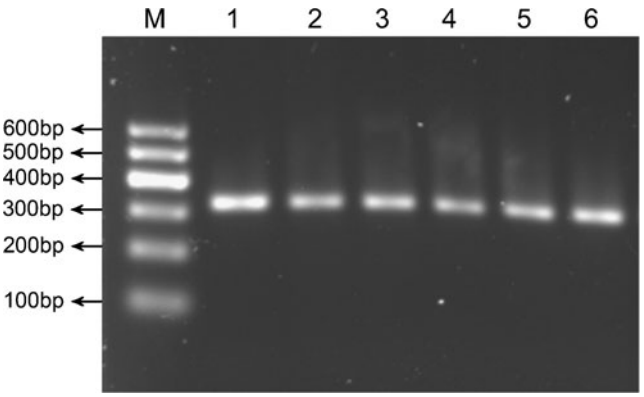

Fig. 2 PCR product. Fragments were analyzed on a 1.5% agarose gel with ethidium bromide. Lengths of two fragments are approximately 330 bp. (lanes 1–6)

Sequence analysis

In total, the eight samples were different from others detected by RFLP, hence the eight samples and controls were sequenced. Detailed method as following, firstly, PCR was carried out in 50 uL reaction volumes (10× Taq Buffer with $(NH_4)_2SO_4$, 0.2 mmol/L dNTP, 1.5 mmol/L $MgCl_2$, 0.5 umol/L of each primer, 10U Taq DNA polymerase [All applied Fermentas, Lithuania]), and approximately 200 ng DNA template. Then PCR products were purified and sequenced by Beijing AuGCT Biotechnology. For each individual, sequencing reactions were performed using both forward and reverse primers.

Statistical analysis

Allele frequencies of SNPs and Hardy–Weinberg equilibrium (HWE) were performed using Modified-Powerstates (Promega Corporation, USA). Difference in the distributions between groups was tested with Chi-squared tests for individual SNPs using SPSS statistical software (version 13.0, SPSS, Chicago, IL, USA), Fisher's exact test was applied when one of expected numbers was less than 5All data were expressed as mean ± SD., and $P<0.05$ was considered to be significant.

Table 2 Reaction conditions of Restriction enzyme

SNP ID	SNP	Restriction enzyme	Reaction temperature	Reaction time
rs28368062	A/G	AluI	37°C	16 h
rs3736832	A/G	Hpy188I	37°C	16 h
rs28368082	C/T	EcoRI	37°C	16 h
rs79564060	A/G	BspHI	37°C	16 h
rs28368064	A/G	HaeII	37°C	16 h

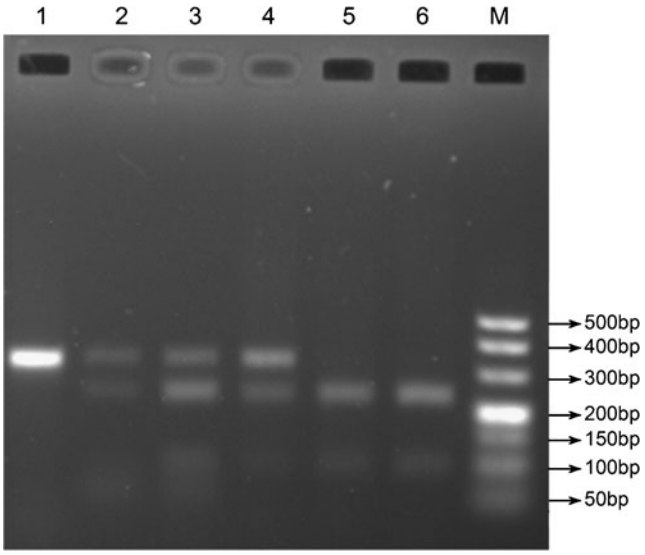

Fig. 3 Restriction enzyme reaction results. two percent agarose gel with ethidium bromide showing the restriction fragment length polymorphism (RFLP) variants detected in fragment of the idiopathic infertility (A: lanes 2–4). These abnormal bands suggest the possibility of gene mutation. Lanes 1 is PCR product and lanes 5 and 6 are homozygous

Results

Subjects

In the first part, 73 patients were diagnosed as idiopathic infertility samples after semen analysis and examination of infertility factors. The genotyping data of all subjects were included for analysis.

The mean ages of controls and idiopathic male infertile patients were 30.26±3.44 and 30.40±3.77 years, respectively, and there was no significant difference between two groups (P=0.792). All cases (χ^2= 0.02187136, df=1, P=0.88242984, respectively) and controls(χ^2=0.00022877, df=1,P=0.98793238, respectively) were in compliance with Hardy-Weinberg equilibrium at the SPO11 gene loci.

Table 3 Distributions of genotypic frequencies of SNP5 (rs28368082) in the SPO11 gene of infertile patients and controls

SNP ID	Genotype	Frequency		*P*-value*
		Patients (n=73)	Controls (n=117)	
rs28368082	CC	(65/73)89.0%	(114/117)97.4%	0.023[a]
	CT	(8/73)11.0%	(3/117)2.56%	
	TT	0%	0%	

[a] Statistically significant

*Fisher's exact test

Table 4 Distributions of allelic frequencies of SNP5 (rs28368082) in the SPO11 gene of infertile patients and controls

SNP ID	Allele	Frequency		*P*-value*
		Patients	Controls	
rs28368082	C	94.5%	98.7%	0.025[a]
	T	5.5%	1.3%	

[a] Statistically significant

*Fisher's exact test

Mutation detection

In our study, no polymorphism has been found in SNP1(AA), SNP2(GG), SNP3(AA) and SNP4 (AA) in both groups. Distributions of genotypic and allelic frequencies of SNP5 in a total of 190 samples are listed in Tables 3 and 4.

However, amino acid sequence changes were found in eight (10.96%) out of 73 patients screened and in three (2.56%) out of 117 control subjects in SNP5(*rs28368082*). A 5679C→T transversion was detected in those population (Fig. 4). Moreover, no other changes were detected in the remaining patients and controls.

Comparing the genotype distribution and allele distribution of two groups, the significant difference in the SNP (*rs28368082*) between cases and controls has been found. The substitutions predict an amino acid alteration: 5679C→T changes a arginine into a tryptophan.

Discussion

Male reproductive activities are the range of complex physiological processes, including spermatogenesis, maturation, release and gaining energy in the female reproductive tract until impregnating. Pathogenic factors on any one part of the interference may lead to infertility. Many factors are now clearly can cause male infertility, such as

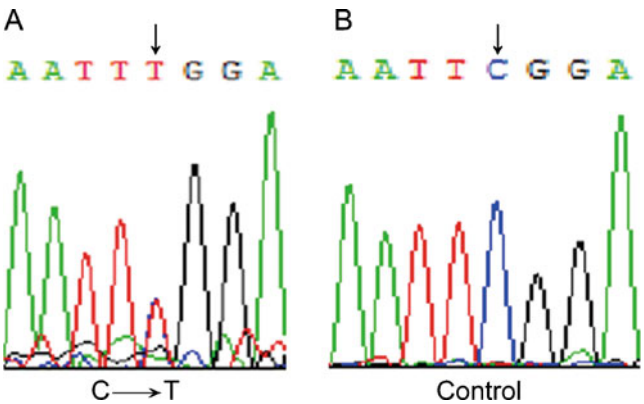

Fig. 4 Normal and altered sequence. Parts of fragment, showing 5679C→T substitution in eight patients

varicocele, cryptorchidism, genital infection, hypogonadism, etc. But there is still about 50% of the male infertility of unknown etiology, referred to as idiopathic male infertility. Because of its etiology is unknown, it brings great difficulties on the clinical diagnosis and treatment, currently there is a fundamental lack of effective to control measures [17].

Male fertility is a highly complex process which requires an estimated 2,000 genes that function in numerous pathways controlling spermatogenesis as well as development and maintenance of the testis [18]. Recent reports showed that genetic disorders affecting spermatogenesis might be responsible for many cases of idiopathic male infertility [19]. In our present study, A 5679C→T transversion was detected in eight (10.96%) out of 73 patients screened and in three (2.56%) out of 117 control subjects in SNP5 (*rs28368082*) of SPO11 gene, and this transversion predicts an amino acid alteration: 5679C→T changes a arginine into a tryptophan. Comparison of the genotype distribution and allele distribution in two groups, there is significant difference in the SNP5 (*rs28368082*) between patients and controls with idiopathic male infertility. These results demonstrated potential association of the SPO11 gene with idiopathic male infertility, suggesting that the SPO11 gene may be a risk gene of idiopathic male infertility in our sample population.

Meiosis is an essential process in gametogenesis and SPO11 gene is one of important genes which involved in meiosis. Studies showed that SPO11 plays an important role in initiating meiotic recombination by generating double-stranded breaks in DNA through its topoisomerase activity [11]. The study of Peter et al. found that the dependence of normal synapsis on recombination is central to the deficiency seen in SPO11—deleted spermatocytes. Either the failure to initiate recombination signals an arrest and normal synapsis does not occur, or the lack of recombination results in a subsequent failure to complete synapsis that triggers the arrest [9]. Therefore, SPO11 might be important in male infertility and testicular dysfunction.

Besides animal experiments, the association between the SPO11 gene antigens and idiopathic male infertility has been examined in American population. In the study of Christensen et al. [16], 48 azoospermic and 48 severely oligospermic patients was enrolled. Finally, two azoospermic individuals were identified with single, heterozygous, missense mutations that changed a glutamic acid residue in exon 1 to lysine (E9K) and an aspartic acid residue in exon 9 to glutamic acid (D277E). An additional 16 single nucleotide polymor-phisms (SNPs) were identified in the intronic regions adjacent to the exons, five of which fell in the 3′untranslated region (UTR). However, in our study, we found that a transversion C5679→T changes a arginine into a tryptophan in exon7 of the SPO11 gene, and no other changes were detected in the remaining patients and controls. These inconsistent results might be attributed to differences in ethnic background and geographic variations. Although different, the results of these two studies demonstrate that mutations in human SPO11 gene may be linked to the susceptibility of idiopathic male infertility. SPO11 gene might be an important research topic in male infertility and contraception studies in the future.

Intracytoplasmic sperm injection (ICSI) is one of important assisted reproduction technologies. Although ICSI can be very effective in by passying the normal reproductive constraints on infertile or subfertile men, it might also be transmitting genetic abnormalities to offspring produced by the technique [18]. Identifying which genes have the most clinical relevance to infertility will help in implementing the development of newer technologies. Combining our research results, the mutation of the SPO11 gene can also be used as a detection index before ICSI procedure.

In conclusion, our studies indicate that mutations in SPO11 gene might associated with male infertility. With the development of molecular biology techniques, the precise role of SPO11 gene in the pathophysiology of idiopathic male infertility will be clarified. It is hoped that this report will stimulate further research on the expression of SPO11 and its effect on male fertility.

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