Novel mutations in ubiquitin-specific protease 26 gene might cause spermatogenesis impairment and male infertility

Jie Zhang1, Shu-Dong Qiu1,2, Sheng-Bin Li1, Dang-Xia Zhou1, Hong Tian1,2, Yong-Wei Huo1,2, Ling Ge1, Qiu-Yang Zhang1,2

1Department of Anatomy, Histology and Embryology, 2Research Center of Reproductive Medicine, 3Department of Forensic Medicine, 4Department of Pathology, Medical College of Xi’an Jiaotong University, Xi’an 710061, China

Abstract

Aim: To study the incidence of single nucleotide polymorphisms in ubiquitin-specific protease 26 (USP26) gene and its involvement in idiopathic male infertility in China. Methods: Routine semen analysis was performed. Infertility factors such as immunological, infectious and biochemical disorders were examined to select patients with idiopathic infertility. DNA was isolated from peripheral blood of the selected patients and control population, which were examined for mutations using polymerase chain reaction-single strand conformation polymorphism analysis. Furthermore, nucleotide sequences were sequenced in some patients and controls. Results: Of 41 infertile men, 9 (22.0%, \( P = 0.01 \)) had changes in USP26 gene on the X chromosome. A compound mutation (364insACA; 460G→A) was detected in 8 patients (19.5%, \( P = 0.01 \)) and a 1044T→A substitution was found in 1 patient (2.4%, \( P > 0.05 \)). All three variations led to changes in the coding amino acids. Two substitutions predict some changes: 460G→A changes a valine into an isoleucine, and 1044T→A substitutes a leucine for a phenylalanine. Another insertion of three nucleotides ACA causes an insertion of threonine. No other changes were found in the remaining patients and fertile controls. Conclusion: The USP26 gene might be of importance in male reproduction. Mutations in this gene might be associated with male infertility, and might negatively affect testicular function. Further research on this issue is in progress. (Asian J Androl 2007 Nov; 9: 809–814)

Keywords: male; infertility; deubiquitination enzymes; ubiquitin-specific protease 26

1 Introduction

Infertility affects 10%–15% of couples, with 20% of such cases being caused by pure male factor infertility [1]. Unfortunately, in nearly 50% of infertile men it is not possible to find a cause of infertility and this situation has been defined as unexplained or idiopathic, although some causes of male infertility have been determined. Recent studies suggest that impaired spermatogenesis is an essential etiology of male infertility and that genetic disorders affecting spermatogenesis might
be responsible for many cases of idiopathic infertility [2]. Other than Y chromosome microdeletion and mutation of autosome genes being associated with male infertility, X chromosomes are also closely related to male fertility; however, the molecular mechanisms responsible are not known [2, 3]. Nishimune et al. [4] observed many genes on the X chromosome that are related to male infertility. One of those genes is ubiquitin-specific protease 26 (USP26), which was first identified by Wang et al. [5], who confirmed expression of USP26 mRNA in mice. USP26 belongs to a family of deubiquitination enzymes (DUB). Recent findings provide strong support for the concept that the ubiquitin-proteasome pathway is regulated by both ubiquitination and DUB, many of which are localized to subcellular structures or to form molecular complexes [6]. DUB are involved in numerous biologically important processes, including control of growth, differentiation, oncogenesis and genome integrity. Because of the importance of DUB, as well as testis-specific expression of this gene, it was decided to choose USP26 as a novel candidate gene for the study of male infertility [4, 5]. Preliminary data indicates increased number of mutations in the USP26 gene in men with severe male factor infertility.

Ubiquitin-specific protease 26 is located on the X chromosome, at Xq26.2. The mRNA sequence of the USP26 gene is 2794 bp long and comprises a single exon. The protein consists of 913 amino acids (Genbank: NM_031907.1).

We have analyzed 44 idiopathic infertility patients and 56 fertile controls for the presence of mutations in USP26.

2 Materials and methods

2.1 Patient selection

First, 56 fertile men (with normal sperm parameters) and 150 infertile patients were examined. Furthermore, some factors of anatomical defects were excluded.

Then, 44 patients of the above 150 patients were selected for idiopathic infertility samples using semen routines and infertility factors, such as immune (antispermatzoal antibody IgG, A), infection (mycoplasma, chlamydia) and biochemical abnormality (α1-4 glucosidase, acid phosphatase, fructose). The protocol was fully approved by the Clinical Research Ethical Committee of Xi’an Jiaotong University (Xi’an, China).

Idiopathic infertility was divided into groups: azoospermatism, oligozoospermatism, teratozoospermatism and asthenozoospermatism. Besides sperm abnormality, others are normal.

World Health Organization (WHO) criteria [7] were used to define normozoospermia. Motility was graded using the qualitative system also proposed by the WHO: grade A, rapid and linear; grade B, slow or nonlinear; grade C, nonprogressive; and grade D, nonmotile. Sperm was considered as asthenozoospermia when more than 50% of the spermatza had A + B type motility or less than 25% had A type motility during the first hour after ejaculation. Teratozoospermic semen was diagnosed when more than 85% of spermatozoa from an ejaculate had abnormal shape, and semen was considered necrozoospermia when more than 50% of spermatozoa were dead [7]. Dead spermatozoa were determined by eosin staining.

Relating the above infertility factors, mycoplasma was detected by improved cultivation of semen; immune factors were examined from patients sera by enzyme-linked immunosorbent assay (ELISA), using kits from Xindi Company (Nanjing, China); and α1-4 glucosidase, acid phosphatase, and fructose were observed by biochemical reaction.

2.2 Polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) and sequence analysis

Total genomic DNA was extracted from peripheral blood using DNA extraction kit (A004-1) (Dinguo, Beijing, China) and stored at –20ºC. USP26 sequence (NC_000023) was obtained from the NCBI website: http://www.ncbi.nlm.nih.gov (date of accession: 3 March 2006). According to the mutation site confirmed in Paduch et al. [8], primers were designed using Primer 5 to amplify and sequence the related region of the USP26 gene (Table 1). Each fragment was amplified in a single PCR using Bio-RAD Mycycler thermal cycler 580BR 3007 (Bio-RAD Laboratories, Hercules, CA, USA). A short fragment of the USP 26 gene was selected as described in Table 1. PCR was carried out in 13 μL reaction volumes (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. Amplify condition is listed in Table 1. PCR product of the three fragments were analyzed on a 2% agarose gel (Figure 1).

To obtain SSCP phenotypes, PCR product (3 μL) was added to 3 μL of loading buffer (99% formamide, 1 mmol/L NaOH, 0.2% w/v bromophenol blue and xylene cyanol), denatured for 10 min at 95°C and...
placed directly in ice for 10 min. Samples were loaded onto an 8% polyacrylamide (49:1 acrylamide-
bisacrylamide) gel (32 cm long, 0.5 mm thick) containing 5 × TBE, and run at 150 V for 24 h at 4ºC
using 2297 MACRODRIVE 5 Constant Power Supply electrophoresis apparatus (LKB, BROMMA,
Sweden). SSCP products were visualized using the silver staining method.

2.3 Sequence analysis

The nine samples were different from others detected by SSCP, hence the nine samples and controls
were sequenced. First, PCR was carried out in 50 µL reactions volumes (10× Taq Buffer with (NH₄)₂SO₄,
0.2 mmol/L dNTP, 1.5 mmol/L MgCl₂, 0.5 µmol/L of each primer, 10 U Taq DNA polymerase [All Applied
Fermentas, Lithuania]), and approximately 200 ng DNA template). Then, PCR products were purified and
sequenced by Shanghai AuGCT Biotechnology using ABI3730. For each individual, sequencing reactions
were performed using both forward and reverse primers.

2.4 Statistical analysis

All statistical analyses were carried out using SPSS statistical software (version 13.0, SPSS, Chicago, IL,
USA). All data were expressed as mean ± SD. The mutation rate was analyzed using the χ²-test and Fisher
exact test. P < 0.05 was considered significant.

3 Results

3.1 Semen analysis

In the first part of the present study, 44 patients were diagnosed with idiopathic male infertility after semen
analysis and examination of numbers of infertility factors. The above results indicate that there was low sperm count
and motility in the patients with idiopathic infertility (Table 2).

3.2 Mutation detection

Sequence changes with amino acid changes or insertion were found in nine (22.0%, P = 0.01) out of 44
patients screened. Among those, a compound mutation (364insACA; 460G→A) was detected in 8 patients
(19.5%, P = 0.01) and a 1044T→A substitution was found in another patient (2.4%, P > 0.05) (Table 3).
Moreover, no other changes were detected in the fertile
USP26 gene and male infertility

For detailed discussion of identified mutations, please see below.

Using PCR-single strand conformation polymorphism analysis, different SSCP band patterns of fragments 1 and 2 were observed (Figure 2). For the former, band patterns of eight patients were not the same as those of the remaining patients and fertile controls. For the latter, the band pattern of one patient is distinct.

The sequence from Genbank (NC_000023) was used as the reference sequence. In total, three differences with the published sequence were observed. Two of these are single-nucleotide substitutions, while another change was an insertion of three nucleotides (Figure 3).

Two substitutions predict an amino acid alteration: 460G→A changes a valine into a isoleucine, while 1044T→A substitutes a leucine for a phenylalanine. The insertion of three nucleotides ACA causes an insertion of a threonine (Table 3).

4 Discussion

In this report, we present evidence that USP26 might be important in male infertility and testicular dysfunction. USP26 is a member of the DUB family. Recent studies have demonstrated that not only ubiquitination but also deubiquitination play crucial roles in regulating protein stability and activity. According to some analyses, DUB enzymes have several possible functions. First, these enzymes process the products of ubiquitin genes. Second, DUB can also remove esters and amides from ubiquitin to produce free monomeric ubiquitin in the cell. Third, ubiquitin-dependent protein degradation requires attachment of at least one ubiquitin to a target protein via an isopeptide bond between the carboxy-terminal glycine of ubiquitin and the e amino group of the side chain of a lysine residue on the target protein. Finally, DUB might counteract the effects of ubiquitin conjugating enzyme and ubiquitin protein ligase mediated conjugation by competitively removing the polyubiquitin chain from the conjugated protein [9]. The above functions of DUB are reflected in biologically important processes, which include control of growth, differentiation, oncogenesis and genome integrity. According to structure character of deubiquitinating enzymes, which can be grouped into two

Table 2. Related details of nine patients’ information and their semen analysis. A, grade A, rapid and linear; B, grade B, slow or nonlinear.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Occupation</th>
<th>Sperm count (10⁹/L)</th>
<th>A Grade spermatozoa ratio (%)</th>
<th>Motility (A + B) (%)</th>
<th>Other abnormality (immune/ infection/ biochemistry)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Computer manipulator</td>
<td>20</td>
<td>0</td>
<td>30</td>
<td>no</td>
</tr>
<tr>
<td>2</td>
<td>Driver</td>
<td>9</td>
<td>0</td>
<td>30</td>
<td>no</td>
</tr>
<tr>
<td>3</td>
<td>Driver</td>
<td>12</td>
<td>0</td>
<td>20</td>
<td>no</td>
</tr>
<tr>
<td>4</td>
<td>Computer manipulator</td>
<td>16</td>
<td>0</td>
<td>25</td>
<td>no</td>
</tr>
<tr>
<td>5</td>
<td>Contacting flour</td>
<td>19</td>
<td>0</td>
<td>20</td>
<td>no</td>
</tr>
<tr>
<td>6</td>
<td>Farmer</td>
<td>14</td>
<td>0</td>
<td>25</td>
<td>no</td>
</tr>
<tr>
<td>7</td>
<td>Driver</td>
<td>12</td>
<td>0</td>
<td>20</td>
<td>no</td>
</tr>
<tr>
<td>8</td>
<td>Driver</td>
<td>5</td>
<td>0</td>
<td>20</td>
<td>no</td>
</tr>
<tr>
<td>9</td>
<td>Computer manipulator</td>
<td>2</td>
<td>0</td>
<td>30</td>
<td>no</td>
</tr>
</tbody>
</table>

Figure 2. Silver stained polyacrylamide gel showing the eight single strand conformation polymorphism (SSCP) variants detected in fragment 1 of the idiopathic infertility (A: lanes 1–8). Another SSCP change was shown in fragment 2 (B: lane 1). These abnormal bands suggest the possibility of gene mutation.

http://www.asiaandro.com; aja@sibs.ac.cn
The ubiquitin-specific processing proteases (UBP/USP) was first identified by Hochstrasser et al. and Pellman [11–13]. Members of the USP family of deubiquitinating enzymes possess a core region containing six highly conserved sequence motifs, including the presumptive active-site cysteine and histidine [12]. Among them, USP26 was first identified by Wang et al. [5], and it is believed to be expressed only in testis. Stouffs et al. [14] and Paduch et al. [8] reported data on mutation analysis in the USP26 gene about patients with infertility. They report one insertion of 363insACA causing a threonine insertion and two substitutions of 460G→A and 1423C→T, which change leucine into serine and histidine into tyrosine, respectively. In their studies, those patients were diagnosed with Sertoli cell-only syndrome and no spermatozoa were detected in the majority.

In our study, nine out of 41 (22.0%, $P = 0.01$) infertile men had changes in USP26 genes on X chromosomes. A compound mutation (364insACA; 460G→A) was detected in eight patients (19.5%, $P = 0.01$) and a 1044T→A substitution was found in another patient (2.4%, $P > 0.05$). Moreover, no other changes were detected in the fertile controls. 1044T→A substitution has no conspicuous statistical significance, but it is worthy of some attention. The above two single-nucleotide substitutions and another insertion of three nucleotides can produce amino acid alteration. Two substitutions predict some changes: 460G→A changes a valine into a isoleucine, whereas 1044T→A substitutes a leucine for a leucine. The insertion of three nucleotides ACA causes an insert of threonine. Semen analysis of the nine patients indicated low sperm count and motility. The study indicates that the mutation of USP26 gene is observed in the azoospermia patients as well as in the oligozoospermia and asthenozoospermia ones. In review of Cavallini [15], he suggests that idiopathic oligoastheno-atozoospermia affects approximately 30% of all infertile men. Considering the results above, we should pay close attention to the USP26 gene because of the high muta-

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Nucleotide change</th>
<th>Amino-acid alteration</th>
<th>No. of patient</th>
<th>Frequency (%)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>363-364 insACA</td>
<td>T121ins</td>
<td>8</td>
<td>19.5</td>
<td>0.01</td>
</tr>
<tr>
<td>2</td>
<td>1044T→A</td>
<td>F348L</td>
<td>1</td>
<td>2.4</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>3</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3. Normal and altered sequence. Parts of fragment 1, showing the insert of ACA (insACA) and G→A substitution in eight patients and part of fragment 2, showing T→A.
tion rate in the gene and patients’ conspicuous clinical manifestation, which are closely related. Therefore, the USP26 gene might become a hot research topic in male infertility and contraception studies in the future.

Assisted reproduction technologies, particularly intracytoplasmic sperm injection (ICSI) procedures are quite susceptible to genetic risks in clinical practice. Abnormalities of the X-chromosome have significantly affect the quality of ICSI, because man have only one X chromosome and the changes in any genes are not compensated [16]. Combining our study results, it is, therefore, evident that mutation of the USP26 gene can also be used as a detection index before ICSI procedure.

In the studies by Paduch et al. [8] and Stouffs et al. [14], the ethnic origins of the selected patients include Arabic and Caucasian. However, all patients of our study are Chinese. Obviously, the mutation positions of USP26 in Chinese are different from the above ethnic origins. Structure analysis using DNA star shows that there is disparity in secondary structure in USP26 with and without mutation.

In conclusion, our results indicate that mutations in USP26 might cause male infertility. Previous studies have testified that USP26 mRNA or protein was expressed predominantly in the testis; however, within the testis, its spatial localization is unclear. It is possible that USP26 might be expressed in either spermatogenic epithelium or Leydig or Sertoli cells as well. It is hoped that this report will stimulate further research on the expression of USP26 and its effect on male fertility.

Acknowledgment

We thank the laboratory, clinical and paramedical staff of the center of Reproductive Medicine, and the Department of Forensic Medicine, Pathology for their assistance. We especially thank Dr Sheng-Bin Li for practical support. This study was supported by National Natural Science Foundation of China (No. 30471735) and Science & Technique Research Intensive Project of Education Ministry of China (No.105157) and Sci-Technical Development Project of Shaanxi Province, China (2005K15-G2, 2006K15-G4).

References

Edited by Dr Sidney Grimes

http://www.asiaandro.com; aja@sibs.ac.cn