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·*Original Article*·

Differential expression of *VASA* gene in ejaculated spermatozoa from normozoospermic men and patients with oligozoospermia

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Abstract

Aim: To detect the expression of *VASA* in human ejaculated spermatozoa, and to compare the expression of *VASA* between normozoospermic men and patients with oligozoospermia. **Methods:** Ejaculated spermatozoa were collected from normozoospermic men and patients with oligozoospermia by masturbation, and subsequently segregated through a discontinuous gradient of Percoll to obtain the spermatozoa. Reverse transcription polymerase chain reaction (RT-PCR), quantitative RT-PCR (QRT-PCR), immunoflurescence and Western blotting were used to detect the expression of VASA in mRNA and protein levels. **Results:** *VASA* mRNA was expressed in the ejaculated spermatozoa. QRT-PCR analysis showed that *VASA* mRNA level was approximately 5-fold higher in normozoospermic men than that in oligozoospermic men. Immunofluorescence and Western blotting analysis showed that VASA protein was located on the cytoplasmic membrane of heads and tails of spermatozoa, and its expression was significantly decreased in oligozoospermic men, which is similar to the result of QRT-PCR. **Conclusion:** The expression of *VASA* mRNA and protein was significantly decreased in the sperm of oligozoospermic men, which suggested the lower expression of the *VASA* gene might be associated with pathogenesis in some subtypes of male infertility and *VASA* could be used as a molecular marker for the diagnosis of male infertility. *(Asian J Androl 2007 May; 9: 339–344)*

Keywords: *VASA*; ejaculated spermatozoa; oligozoospermia; male infertility; spermatogenesis

1 Introduction

Spermatogenesis is a unique process of cellular differentiation in which diploid testicular stem cells differentiate into haploid spermatozoa. A disorder of the process results in male infertility. The main causes of male infertility are oligozoospermia, asthenozoospermia, teratozoospermia and azoospermia, which account for 20–25% of cases [1]. The molecular mechanisms of spermatogenesis are beginning to be understood. It is estimated that about 2 000 genes regulate the process, and most of them are present on the autosomes, with approximately 30 genes on the Y chromosome [2]. With gene knockout technology, it has been shown that about 200 genes are indispensable in mammalian reproduction

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[3, 4]. Some recent studies have also shown that BGR-like and *NORPEG* genes are specifically expressed in the testis and functionally involved in spermatogenesis [5, 6].

Vasa, originally identified from *Drosophila*, is a member of the DEAD-box family of genes encoding an ATP-dependent RNA helicase [7–9]. The mouse *Vasa* homolog (*Mvh*) gene exhibits specific expression in developing germ cells [10]. It has been reported that male mice homozygous for a targeted mutation of *Mvh* exhibited a reproductive deficiency, and produced no sperm in the testes, where premeiotic germ cells cease differentiation by the zygotene stage and undergo apoptotic death [11]. In humans, the gene is mapped to human chromosome 5q, and its expression is restricted to the ovary and testis, and is undetectable in somatic tissues [7]. However, there are no reports about the expression of *VASA* in human ejaculated spermatozoa. The aim of the present study was to detect the expression of *VASA* in human ejaculated spermatozoa, and to compare the expression of *VASA* between normozoospermic men and patients with oligozoospermia.

2 Materials and methods

2.1 Sperm samples

Sperm samples were obtained from normozoospermic men and infertile patients from the Center for Reproductive Medicine, Peking University Shenzhen Hospital (Shenzhen, China). Semen was collected by masturbation after 3 days of sexual abstinence and allowed to liquefy for 30–60 min at room temperature. A semen analysis was carried out according to World Health Organization guidelines (1999) [12]. Eosin–nigrosin staining was used for assessing the viability of selected sperm and the samples with more than 5% dead spermatozoa were excluded from the study. Diff–Quik staining was used to evaluate the sperm morphology. Normozoospermic semen (sperm concentration $\geq 20 \times 10^6$/mL, total of motility grades A and B $\geq$ 50%, normal sperm morphology $\geq$ 30%, n = 15) and oligozoospermic semen (sperm concentration $< 20 \times 10^6$/mL, total of motility grades A and B $\geq$ 50%, normal sperm morphology $\geq$ 30%, n = 15) were selected for the study. The samples were purified by a discontinuous gradient technique of 30% and 90% Percoll. After centrifugation (20 min at 400 × g, 18°C), the mature spermatozoa were collected from the under layer of 90% to be used for making smears or stored at –80°C for RNA analysis. The present study was approved by the ethical committee of the hospital and all participants signed the consent form, permitting the use of their sperm samples in the study.

2.2 Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA from mature spermatozoa was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed with Reverse Transcription System (Promega, Madison, WI, USA). Polymerase chain reaction (PCR) primers for *VASA* and β-actin were synthesized by Shanghai Bioengineering Inc. (Shanghai, China). The primers were, for *VASA*: 5'-TCTTCACA-AGCTCCCAATCC-3' (sense) and 5'-TGAGAATA-CAAGG ACAGGAGCT-3' (antisense), for β-actin: 5'-CCTGTGGCATCCACGAAACTA-3' (sense) and 5'-TGTCAAGAAAGGGTGTAACGCAA-3' (antisense). The PCR reaction was initiated by hot start at 95°C for 5 min, followed by 30 cycles at 95°C for 30 s, 55–58°C for 30 s, and 72°C for 30 s, then 72°C for 5 min extension. The PCR products were analyzed using a Rapid Agarose Gel Electrophoresis System (Wealtec CORP, Sparks, NV, USA) in 2.0% agarose gels in 0.5 × Tris-Borate-EDTA (TBE) buffer. The size of PCR products for VASA and β-actin was 164 bp and 356 bp, respectively.

2.3 SYBR green quantitative PCR

SYBR green quantitative reverse transcription PCR (QRT-PCR) was carried out using Perkin-Elmer's ABI Prism 7000 Sequence Detector System (Applied Biosystems, Foster City, USA). Platinum SYBR Green qPCR SuperMix Uracil-DNA glycosylase (UDG) (Invitrogen, California, USA) kit was used. The initial step was 50°C for 2 min for the activation of UDG, then inactivated by a high temperature of 95°C for 2 min during normal PCR cycling, followed by 50 cycles of 15 s of denaturation at 95°C and 40 s of annealing and elongation at 58°C. ROX Reference Dye was included as a separate component to normalize the fluorescent signal between reactions. The data from QRT-PCR were analyzed with ΔΔCt method. The ΔCt value was determined by subtracting β-actin Ct value from the studying group Ct value. The ΔΔCt was calculated by subtracting the ΔCt value of the normal group from the ΔCt value of each group. $2^{-\Delta\Delta Ct}$ represented the average relative amount of mRNA for each group.

2.4 Immunofluorescence

For immunoflurescence analysis, the purified spermatozoa was coated on slides, fixed with 4% paraformaldehyde in PBS at room temperature for 20 min, then blocked and permeabilized with 0.1% Triton X-100, 1% BSA and 10% normal goat serum in PBS at room temperature for 45 min. Spermatozoa smears were incubated with diluted primary antibodies VASA (1 : 100; R&D System, Minneapolis, USA) overnight at 4°C. FITC-conjugated rabbit anti-goat IgG (CHEMICON, California, USA) was directed against primary antibodies. Slides were mounted in antifade medium and viewed on a microscope equipped with a fluorescent attachment (OLYMPUS, Shinjuku-ku, Tokyo, Japan).

2.5 Western blot

Protein from spermatozoa was extracted by lysis buffer (0.15 mol/L NaCl, 5 mmol/L EDTA pH 8.0, 1% Triton X-100, 10 mmol/L Tris-HCl pH 7.4, 5 mol/L DTT, 100 mmol/L PMSF, 5 mol/L aminocaproic acid), and was measured by the BCA method (BCA Protein Assay#23225, PIERCE, Rockford, USA). For Western blotting, 30 μg protein from each sample was separated by denaturing polyacrylamide gel electrophoresis (PAGE), then transferred to PVDF membrane. The membrane was blocked for 1 h with Tris-buffered saline (TBS) solution containing 5% non-fat milk and 0.2% Tween-20 at room temperature. Specific antibody goat anti-human VASA (1:1 000, R&D system, USA) was incubated overnight at 4°C. The membranes were washed, then incubated for 1 h with peroxidase-conjugated anti-goat antibodies (1:1 000) and internal control HRP-GAPDH (1:5 000, Kangchen, China), followed chemiluminescence detection by SuperSignal® West HisProbe™ kit (Pierce, Rockford , USA).

2.6 Data analysis

The data were expressed as mean ± SD. Differences of VASA expression between normozoospermia and oligozoospermia men were examined using paired *t*-test and $P < 0.05$ was considered as statistically significant.

3 Results

With RT-PCR, the expression of *VASA* mRNA was detected in human ejaculated spermatozoa (Figure 1). To compare the difference of *VASA* mRNA expression in normozoospermic and oligozoospermic men, QRT-PCR was used. The data from QRT-PCR were analyzed with ΔΔCt method (Table 1). The ΔCt values from normozoospermic men and patients with oligozoospermia were 6.15 ± 0.64 and 8.58 ± 0.91, respectively. With paired *t*-test, a significant difference was detected between the two groups. The values of $2^{-\Delta\Delta Ct}$ from normozoospermic men and the patients were 1 and 0.19, respectively. The data showed that the expression of *VASA* mRNA in the spermatozoa from the patients with oligozoospermia was more than 5-fold lower than that in normozoospermic men.

To further confirm the results of RT-PCR, immuno-

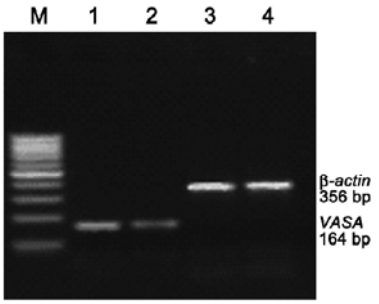

Figure 1. Expression of *VASA* mRNA in ejaculated spermatozoa from normozoospermic men and patients with oligozoospermia. M: DNA marker; Lane 1, 3: normal spermatozoa; Lane 2, 4: spermatozoa from the patients with oligozoospermia.

Table 1. Expression of *VASA* mRNA in ejaculated spermatozoa from normozoospermic men and oligozoospermic patients by quantitative reverse transcription polymerase chain reaction (QRT-PCR) (mean ± SD, $n = 15$). *The ΔCt value was determined by subtracting β-actin Ct value from *VASA* Ct value. Differences of *VASA* mRNA expression between normozoospermic and oligozoospermic men were examined using paired *t*-test. There was a significant difference between the two groups ($^cP < 0.01$). **The ΔΔCt was calculated by subtracting the ΔCt of the normal group from the ΔCt value of each group. This is the subtraction of an arbitrary constant, so the standard deviation of ΔΔCt is the same as the standard deviation of ΔCt. $2^{-\Delta\Delta Ct}$ represented the average relative amount of *VASA* mRNA for each group.

Group	*VASA* Ct	β-actin Ct	ΔCt*	ΔΔCt **	$2^{-\Delta\Delta Ct}$
Normozoospermia	32.13 ± 0.74	25.98 ± 0.63	6.15 ± 0.64	0 ± 0.64	1 (0.64–1.55)
Oligozoospermia	34.40 ± 1.07	25.82 ± 0.89	8.58 ± 0.91^c	2.43 ± 0.91	0.19 (0.10–0.34)

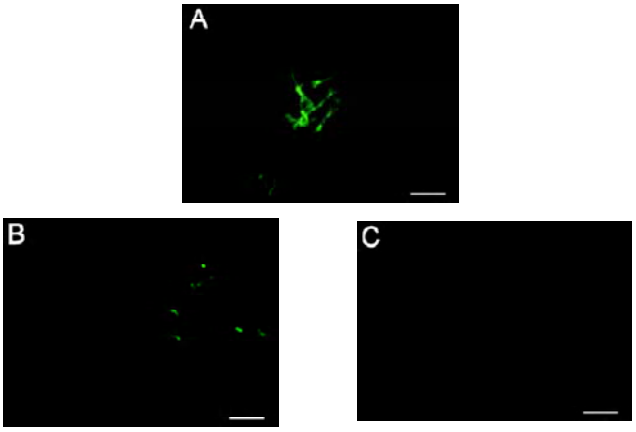

Figure 2. Immunofluorescence analysis of VASA protein in ejaculated spermatozoa from normozoospermic men and the patients with oligozoospermia (× 1 000). VASA protein was located on the plasma membrane of the heads and tails of spermatozoa. Compared with normozoospermic men (A), the expression of VASA was significantly decreased in the spermatozoa from the patients with oligozoospermia (B). The slide omitted anti-VASA primary antibody was used as negative control (C). Scale bar = 50 μm.

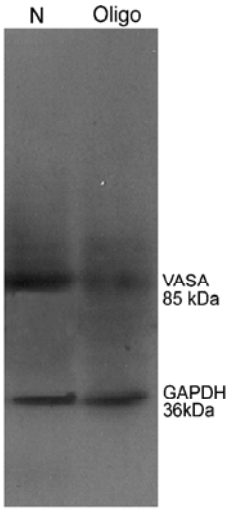

Figure 3. Western blot analysis of VASA expression in ejaculated spermatozoa from normozoospermic men and patients with oligozoospermia. Compared with normozoospermic men (N), the expression of VASA was significantly decreased in the spermatozoa from the patients with oligozoospermia (Oligo).

cytochemistry was used to detect VASA protein expression in the same samples. VASA protein was specifically located in the membrane and cytoplasm of the spermatozoa (Figure 2). Notably, the positive signals of the neck segment, middle piece and principal piece of tails on spermatozoa were powerful, and the end piece of the tails was weak. Compared with normozoospermic men (Figure 2A), the expression of VASA was significantly decreased in the spermatozoa from the patients with oligozoospermia (Figure 2B). The slide omitted anti-VASA primary antibody was used as negative control (Figure 2C).

The protein expression of VASA in ejaculated spermatozoa was also confirmed by Western blot and a band at approximately 65 kDa was detected (Figure 3). Again, the expression of VASA was significantly lower in oligozoospermic men than that in normozoospermic men, which was consistent with the results from both RT–PCR and immunocytochemistry.

4 Discussion

Mature spermatozoa are usually considered to be tools only to transfer genetic messages. However, findings from several studies have shown that mature ejaculated spermatozoa contain a complex repertoire of mRNAs, which play a key role in sperm motility, capacitation and acrosomal reaction [13–16]. Ostermeier *et al.* [15] identified at least 2 686 transcripts in ejaculated spermatozoa of normal fertile men with microarray techniques, and Wang *et al.* [16] identified 149 genes which were expressed at higher levels in both testis and ejaculated spermatozoa. A recent study showed that messenger RNAs of ejaculated spermatozoa were also delivered to the egg at fertilization, which suggested that these transcripts could be important in the early development of the human embryo [17]. The origin of mRNAs in human sperm is not very clear. Some studies have shown that transcript remnants of human mature spermatozoa were leftovers from spermatogenesis, and reflected the conditions of spermatogenesis, thus, the ejaculated spermatozoa could be used as a noninvasive proxy for investigations of testis-specific infertility [15, 16, 18–20].

The *VASA* gene is conserved in invertebrate and vertebrate species, and plays a very important role in the process of spermatogenesis [7–11]. Recently, it has been reported that the *VASA* gene and its homologues have become the genetic selection markers of male germ cell lineage derived from embryonic stem cells [21, 22]. Previous study showed that *VASA* was expressed in human spermatocytes but not spermatozoa [7]. In the present study, we have verified that *VASA* mRNA is expressed in

the ejaculated spermatozoa. QRT–PCR analysis showed that the *VASA* mRNA level was approximately 5-fold higher in normozoospermic men than in oligozoospermic men. With immunofluorescence and Western blotting, the present study showed that VASA protein was located on the cytoplasmic membrane of the heads and tails of spermatozoa, which is significantly downregulated in oligozoospermic men. These data suggested that *VASA* could be used as a molecular marker for the diagnosis of male infertility.

How does theVASA gene regulate spermatogenesis? Two previous studies [9, 23] reported that Vasa protein in *Drosophila* was distributed uniformly in the cytoplasm of cells, which acted as RNA chaperones and associated with chromatoid body (CB). The findings of the present study substantiate these views that the *VASA* served as CB and transcribed mRNA remaining in spermatozoa when the genome becomes dormant. *VASA* is not only required for spermatogenesis, but also for the embryonic stem cells differentiating into primordial germ cells and spermatogonium stem cells [21, 22, 24]. From these results, we speculate that lower *VASA* expression during spermatogenesis might be associated with the abnormal differentiation of primordial germ cells or spermatogonia cells, which leads to the decreasing of production of spermatogenic cells and decreased sperm production.

In summary, the present study showed that the *VASA* gene was expressed in human ejaculated sperm, and its expression was significantly decreased in the infertile men with oligozoospermia. Anomalies in the expression of this gene are associated with spermatogenic dysfunction and involved in the pathogenesis of some cases of male infertility. Sperm mRNA analysis might thus be a useful tool in evaluating the testis function of infertile men.

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