

Identification of *Spata-19* New Variant with Expression beyond Meiotic Phase of Mouse Testis Development

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Abstract

Background: The study of specific genes expressed in the testis is important to understanding testis development and function. Spermatogenesis is an attractive model for the study of gene expression during germ cell differentiation. Spermatogenesis associated-19 (*Spata-19*) is a recently-identified important spermatogenesis-related gene specifically expressed in testis. Its protein product is involved in sperm cell development and reproduction. In this report we examined the expression of *Spata-19* mRNA in mouse testis, fetus, and cell lines.

Methods: Reverse transcription-polymerase chain reaction (RT-PCR), nested PCR, and PCR-restriction fragment length polymorphism (PCR-RFLP) were used to analyze *Spata-19* mRNA expression in different stages of mouse testis development, mouse fetus, mouse embryonic fibroblasts (MEF), mouse embryonic stem cells (mESC), Sertoli cells, and NIH/3T3 cells.

Results: We identified a novel splice variant of *Spata-19* in the mouse genome that it is expressed in the fetus and after the meiotic phase of spermatogenesis, and over-expressed in the post-meiotic stage of mouse spermatogenesis. This novel splice variant was absent in five days old mice testis, mESC, MEF, Sertoli, and NIH/3T3 cell lines.

Conclusion: The *Spata-19* has a large novel splice variant in mouse testis that is expressed beyond meiotic phase of testis development. We suggest that this new *Spata-19* mRNA variant might be involved in mitochondrial maintenance in sperm cells, and might be correlated with androgen secretion and male fertility.

Keywords: Fertility, *Spata-19* new variant, Spermatogenesis, Testis

Introduction

Approximately 15% of couples are infertile (1), and the cause of contributed in 15–30% of these cases are thought to be male genetic abnormalities (2).

Selectively-expressed genes in a certain organ may play physiological or functional roles in that particular tissue (3). The study and understanding of the mechanism of sperm formation, and identification and characterization of the genes specifically

expressed in the fetal or different stages of testis development may

aid in the discovery of previously uncharacterized genes related to testis function and spermatogenesis (4, 5), and understanding of the physiologic and pathogenic mechanisms of male infertility (6).

Mammalian spermatogenesis is an attractive model for the study of gene expression during male germ cell differentiation (3), and this process encompasses several rounds of mitotic division of

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spermatogonia, meiotic division, and maturation of the haploid spermatid during spermatogenesis (7). Because meiosis in testicular germ cells differs from mitosis, gene expression differs in different stages of testis development (4, 8).

Some genes that control spermatogenesis in testis may not express in other organs (9). Some of these genes have been identified as spermatogenesis-associated genes. Abnormalities in the expression of these genes may cause male infertility (3, 10-12). One such gene is spermatogenesis associated-19 (*Spata-19*), also known as Spergene-1. *Spata-19* is an important reproduction-related gene specifically expressed in testis and placenta (13). The *Spata-19* protein product is located in the sperm mitochondrion outer membrane, is involved in sperm cell and multicellular organismal development and cell differentiation (14). Previous studies have revealed that *Spata-19* has association with cancerous cell proliferation (15) and prostate cancer (16).

The purpose of this study was to analyze *Spata-19* mRNA expression in pre-meiotic, meiotic, and post-meiotic stages of mouse testis development, and also in mouse adult testes, fetus, embryonic stem cell (mESC), embryonic fibroblast (MEF), Sertoli, and NIH/3T3 cell lines to understand of its role in cellular proliferation and development.

Materials and Methods

Animals

Male Balb/c mice, 5, 15, and 25 days old, representing pre-meiotic, meiotic, and post-meiotic stages of mouse testis development, respectively (17), and adult mice were purchased from the Pasteur Institute of Iran (Tehran, Iran). Their testes were surgically removed and stored at -80°C .

Cell Line and Stem Cell Culture

Mouse embryonic fibroblast, Sertoli, and NIH/3T3 cell lines were purchased from the Pasteur Institute of Iran, and cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2 . After two days, cells were harvested and about 2×10^6 cells were separated for RNA extraction, cDNA synthesis, and reverse-transcriptase polymerase chain reaction (RT-PCR).

The mouse embryonic stem cell line C57BL/6J with normal male (XY) karyotype (Invitrogen) was

cultured on 0.1% gelatin-coated plates in Knockout Dulbecco's Modified Eagle's Medium (GIBCO-BRL), supplemented with 12.5% (v/v) ES qualified FBS (GIBCO-BRL), 2 mmol L-Glutamine (GIBCO-BRL), 1X nonessential amino acids (NEAA; GIBCO-BRL), 50 $\mu\text{g}/\text{ml}$ penicillin and streptomycin, 50 μmol β -mercaptoethanol and 10^3 U/ml Leukemia Inhibitory Factor (LIF) (Millipore). The cells were grown for 2–3 weeks. Adherent cells were detached by trypsin/EDTA for RNA extraction.

Extraction of RNA and cDNA Synthesis

Total RNA was extracted from the cell lines and testis tissues using Tri-Pure isolation reagent (Molecular Bioscience) according to the manufacturer's instructions. RNA was dissolved in DEPC-treated water and its concentration was determined with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). One μg of total RNA from each sample was used for cDNA synthesis with an M-MLV reverse transcription kit (Fermentas) and random hexamer and oligo (dT) primers (Pharmacia, Sweden).

Reverse Transcription-PCR

The housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) was used as a positive control to analyze the quality of the cDNAs. We designed a specific primer pair to amplify a 496 base pair (bp) fragment of *Spata-19* mRNA. The forward1 primer is 5'gagccaaggagtcctctgtga3' and the reverse1 primer is 5'cttagcattctgagcaagaagtc3'. The PCR amplification of *Spata-19* was performed with an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 59°C for 30 sec, extension at 72°C for 35 sec, and an additional extension at 72°C for 7 min. Reaction volumes were 25 μl containing 1 μl of cDNA sample, PCR set, and Smart Taq polymerase.

PCR-RFLP and Agarose Gel Electrophoresis

PCR amplification of full-length *Spata-19* from total testicular cDNA using specific 5' and 3' end primers generated two amplicons. The amplified PCR products from the testes of 15 and 25 days old mice were digested with the restriction enzyme BamH1 (Fermentas, Germany). The enzyme digestion mixtures contained 7 μl of PCR product, 4 units of enzyme, 2 μl of the enzyme 10X buffer and H_2O to a

final volume of 15 μ l. The samples were digested for 16 h at 37 °C. The digested and PCR products were subjected to electrophoresis in a 1.5% agarose gel and visualized under ultraviolet light after DNA staining with ethidium bromide.

cDNA Sequence Analysis and Bioinformatics tools

Bands were cut from the gel and DNAs were isolated using a Bioneer gel purification kit and sequenced on an ABI Prism 3100 sequencer (Applied Biosystems, USA). The sequence was then blasted in GenBank (<http://www.ncbi.nlm.nih.gov>) by the software BLAST to determine the homology among various species and locations in the chromosome. The nucleic and deduced amino acid sequences were also analyzed using NCBI and Ensemble databases. ClustalW2 - Multiple Sequence Alignment was used to align sequences similar to *Spata-19* in different species to study the phylogenetic relationships.

Cellular Distribution of *Spata-19* Precursor mRNA

The screening experiments identified a novel variant of *Spata-19* precursor mRNA in mouse testis. The cellular distribution of the novel *Spata-19* precursor mRNA was determined as follows: a primer pair specific to 5' end of *Spata-19* mRNA (forward primer 5'ctgaggatgatcattacaac3') and intron one (reverse primer 5'accactaagaccaatgtcac3') were designed to amplify a 198 bp fragment of the novel *Spata-19* precursor mRNA from cDNAs of mouse fetus, mESC, MEF, NIH/3T3 and Sertoli cell lines. The PCR amplification of the novel *Spata-19* precursor mRNA was performed with 35 cycles of denaturation at 94 °C for 30s, annealing at 55 °C for 25s, extension at 72 °C for 30s, and a 7 min final extension at 72 °C.

Results

Spata-19 in the genome of *Mus musculus* is located on chromosome 9 (Acc: MGI: 1922719), is about 4890 bp in length, and contains seven exons. The full-length mouse *Spata-19* mRNA is 697 nucleotides in length and contains a 465 bp open reading frame (ORF) that encodes a 154 amino acid protein.

In the present study, we used RT-PCR to analyze *Spata-19* gene expression during different stages of mouse testis development and in mouse cell lines. A product of about 496 bp was amplified using

forward 5' and reverse 3' *Spata-19* gene-specific primers.

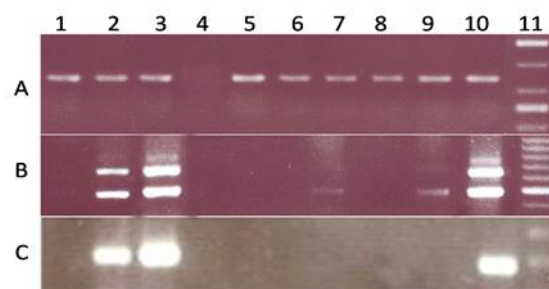


Fig. 1. RT-PCR and nested PCR result of mouse testis samples and cell lines. (A) All samples were controlled for the presence of cDNA using the housekeeping gene HPRT1. (B) *Spata-19* gene expression in mouse testis samples, mESC, Sertoli, MEF, Fetus, and NIH3T3 cell lines. (C) Nested PCR was performed to expression analyze of *Spata-19* new variant gene in mouse testis samples, mESC, Sertoli, Fetus, MEF, and NIH3T3 cell lines. Lane 1-3 is cDNA from 5, 15 and 25 days old testis, respectively. Lane 5-9 is cDNA from mESC, Sertoli, Fetus, MEF, and NIH/3T3 cell lines, respectively. Lane 4 is the negative control (water). Lane 10 is an adult testis as positive control. These experiments were repeated three independent times.

Control PCRs with HPRT primers amplified 148 bp fragments from all samples and verified the integrity of our cDNAs (Fig. 1A, Lanes 1-3 and 5-10). A negative control using water, as the template produced no bands, demonstrating that our reagents were not contaminated with genomic DNA (Fig. 1A, Lane 4).

A 496 bp *Spata-19* RT-PCR product was amplified from 5, 15, 25 days old testes, fetus and adult mice testes (Fig. 1B, lanes 1-3, 7, and 10) and the amount of product increased with time, indicating an increase in the amount of *Spata-19* mRNA (Fig. 1B, Lanes 1-3). *Spata-19* mRNA was strongly expressed in 15 and 25 days old mouse testis, as meiotic and post-meiotic stages of spermatogenesis respectively (Fig. 1B, Lanes 2 and 3). This *Spata-19* transcript was detected in NIH/3T3 (Fig. 1B, Lane 9) but not in mESC, Sertoli, or MEF cell lines (Fig. 1B, Lanes 5, 6 and 8). A 669 bp *Spata-19* RT-PCR product was amplified from 15 and 25 days old and adult mice testes (Fig. 1B, Lanes 2, 3, and 10).

Two *Spata-19* PCR products of 669 and 496 bp were amplified from the cDNAs of the testes from the 15 and 25 days old and adult mice (Fig. 1B). To compare the products from the 15 and 25 days old mice, PCR-RFLP analysis was performed. Digestion

of the PCR products from the 15 (not shown) and 25 days old mice testes with BamH1 restriction endonuclease resulted in identical restriction fragments of 502, 329, and 195 bps (Fig. 2).

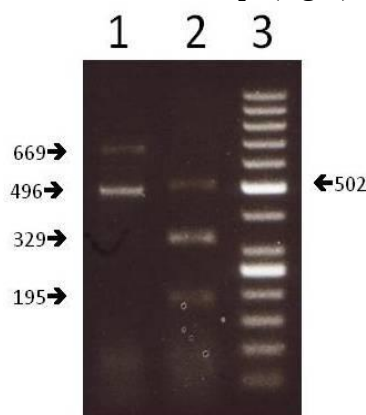


Fig. 2. Restriction fragment analysis of *Spata-19* RT-PCR products from 25 days old mouse testis by agarose gel electrophoresis. Lane 1: Undigested *Spata-19* PCR products from testis of 25 days old mouse. Lane 2: BamH1-digested products of Lane 1. These experiments were performed three independent times.

For further insight, we extracted the 669 bp fragment from the gel with a gel purification kit (Bioneer) and determined its DNA sequence. After BLASTing mouse genome sequence databases at the NCBI website, the sequence was localized to chromosome 9. The fragment was determined to be a novel precursor variant of *Spata-19* mRNA that included intron one, which comprised 173 bps of the fragment. The nucleotide sequence of the novel *Spata-19* mRNA 669 bp variant is available from the GenBank under the accession number JX089334.

Protein sequence analysis with ExPASy translate tools (<http://www.expasy.org/tools/>) identified a stop codon at residue 77; therefore, the truncated *Spata-19* mRNA variant encodes a protein of 76 amino acids (data not shown).

To further investigate the novel *Spata-19* mRNA isoform, its expression was determined in all samples by nested PCR using primers to amplify a 198 bp fragment. We found that it was expressed in the 15 and 25 days old mice testis, representing the meiotic and post-meiotic stages of testis development and its expression was higher in the post-meiotic than the meiotic phase (Fig 1C, Lanes 2 and 3). The novel *Spata-19* mRNA isoform was also expressed in adult testes (Fig 1C, Lane 10), but was not expressed in the

five day old testis, fetus and cell lines (Fig. 1C, Lanes 5-9).

Discussion

In eukaryotes, alternative splicing of precursor mRNA is the main mechanism for increasing the information content from genome to transcriptome (18). Our results indicate that *Spata-19* precursor mRNA is expressed after meiotic phase of testis development, and it is increased during mouse testis development by the time. However, its expression pattern is different in cell lines in comparison with *Spata-19*, because it has not any expression in fetus and NIH/3T3 cancer cell lines. These findings are compatible with database

(<http://www.ncbi.nlm.nih.gov/UniGene/ESTProfile>) and literature review that show the *Spata-19* expression is restricted to testis. The analysis of gene expression in Sertoli cells after treatment by follicle-stimulating hormone (FSH) revealed that *Spata-19* has no any expression in Sertoli cells (19). Also the expression analysis of genome-wide in the mouse embryonic stem cells revealed a significant inactivation of spermatogenesis related genes such as *Spata-19* (20).

The NCBI Blast searches showed that the arrangement of exons and introns in *Spata-19* are similar in mouse, rat, and human, and the 10 N-terminal amino acid residues in the *Spata-19* protein are conserved (5). The function of the *Spata-19* protein may be related to that of an adhesive molecule associated with mitochondria because of a mitochondria targeting signal sequence near the N-terminus (21, 22). Comparison and analysis of the amino acid sequence of *Spata-19* with new *Spata-19* precursor mRNA variant showed that the N-terminal region in exon one is identical between two cDNAs. According to our results that indicate the *Spata-19* precursor mRNA expression is restricted to beyond the meiotic phase of germ cell development in testis, we suggest that *Spata-19* precursor mRNA contributes to mitochondrial maintenance in sperm cells, and might be correlated with androgen secretion and male fertility.

According to a Suzuki-Toyota et al. (14) study, the *Spata-19* protein may function in developmental and differentiation processes. Previous studies revealed that *Spata-19* might be associated with cancerous cell proliferation (15) and prostate cancer disease (16). Regarding to the elevation of *Spata-19* precursor mRNA during mouse testis development, and its absence in mESC and NIH3T3 cancerous cell line, these findings lead us to suggest that *Spata-19* new variant may play roles in male germ cell development but it probably has not any roles in proliferation of cancerous cell.

In conclusion, our findings suggest that specific expression of *Spata-19* new variant after meiotic phase of testis development might be correlated with spermatogenesis and male fertility, and might have some roles in the regulatory process of male gamete

development. But exact function of this variant remain unclear. We expect that these findings will be helpful to better understand the existing variants of *Spata-19* during spermatogenesis.

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