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Original Article

A single nucleotide polymorphism in *SPATA17* may be a genetic risk factor for Japanese patients with meiotic arrest

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Abstract

Genetic mechanisms have been implicated as a cause of some cases of male infertility. Recently, 10 novel genes involved in human spermatogenesis were identified by microarray analysis of human testicular tissue. One of these is spermatogenesis-associated 17 (*SPATA17*). To investigate whether defects in the *SPATA17* gene are associated with azoospermia due to meiotic arrest, a mutational analysis was conducted, in which the *SPATA17* coding regions of 18 Japanese patients with this condition were sequenced. A statistical analysis was carried out that included 18 patients with meiotic arrest, 20 patients with Sertoli-cell-only syndrome (SCOS) and 96 healthy control men. No mutations were found in *SPATA17*. However, three coding single nucleotide polymorphisms (cSNPs: SNP1–SNP3) were detected in the patients with meiotic arrest. No significant differences in the genotype or allele frequencies of SNP1 and SNP2 were found between patients with meiotic arrest and the others. However, the frequency of the SNP3 allele was significantly elevated in the meiotic arrest group ($P < 0.05$). This study suggests that *SPATA17* may play a critical role in human spermatogenesis, especially in meiosis.

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1 Introduction

Genetic causes of azoospermia include chromosomal abnormalities, Y-chromosome microdeletions and several specific gene mutations/deletions affecting genes, such as *DAZ*, *RBM1*, *USP9Y* and *SYCP3* [1–5]. As

Y-chromosome deletions account for only 16.0% of men with infertility [6], azoospermia in many infertile men may be caused by autosomal gene mutations. Genetic polymorphisms may also increase susceptibility to some forms of male infertility. For example, the human *MEI1* and *USP26* genes are linked to male infertility [7, 8]. Further analysis, including promoter studies, have been conducted on human and mouse spermatogenesis. For example, polymorphisms in the human *UBE2B* gene are associated with the human azoospermic/oligozoospermic population [9, 10]. It is suspected that more genes play critical roles in human spermatogenesis.

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Defective meiosis during spermatogenesis is a known cause of azoospermia; however, the mechanisms leading to defective meiosis remain unknown. Meiosis is a fundamental process in sexually reproducing species that permits genetic exchange between the maternal and the paternal genomes [11]. The genetic regulation of meiosis in mammals is poorly understood when compared with that in lower eukaryotes such as yeast.

Several genes critical for mouse meiosis, such as *Fkbp6*, *Scp3* (*Sycp3*), *Spo11*, *Msh4* and *Msh5*, have been identified by disruption experiments in embryonic stem cells [12–16]. However, the number of human genes identified as causing azoospermia is quite small. The primary reason for this low number is that a reverse genetic approach is required to identify human disease-causing genes. Another reason is that biological discrepancies exist between mice and humans. To work around this latter reason, microarray analyses have been carried out with normal and abnormal human tissue. In 2006, 10 novel genes involved in human spermatogenesis were identified by microarray analysis of testicular tissue [17]. Microarray analysis was carried out using normal, meiotic arrest and SCOS (Sertoli-cell-only syndrome) testis tissues. One gene that was identified in the comparison between the testis tissues of normal and meiotic arrest azoospermia patients was spermatogenesis-associated 17 (*SPATA17*). *SPATA17* is also called *MSRG-11*. The mouse *Spata17* (*Msrg-11*) cDNA was isolated in 2005 and recognized as a member of the calmodulin (CaM)-binding protein family [18]. It is strongly expressed in adult mouse testes, but weakly expressed in the spleen and thymus. This protein contains three short CaM-binding motifs containing conserved Ile and Gln residues (IQ motifs). It has been suggested that the mouse *Spata17* gene might play an important role in the development of mouse testes, and it is a candidate gene for testes-specific apoptosis [18, 19]. However, the literature does not contain any reports on the role of the human *SPATA17* gene in spermatogenesis. In this study, we carried out a mutational analysis of human *SPATA17* using the genomic DNA of Japanese patients with azoospermia due to meiotic arrest.

2 Materials and methods

2.1 Patients and controls

Azoospermia was confirmed by two consecutive

semen analyses obtained after 5–7 days of sexual abstinence and examination of a centrifuged semen pellet. Patients with defective spermatogenesis after an infection, or because of obstruction of the seminal tract, pituitary failure or other causes of possible testicular damage revealed at clinical examination, were excluded from the study. Final diagnoses were established by histological examination. Histological diagnosis was conducted on more than 20 testicular specimens. Histological diagnosis was carried out by two pathologists and two urologists at Osaka University. Primary spermatocytes were the most mature cell types present. No postmeiotic cells (round haploid spermatids, elongating spermatids or mature spermatozoa) were present. The average hormone levels in the patients with meiotic arrest were as follows: leutinizing hormone: 5.28 mIU mL⁻¹, follicle stimulating hormone (FSH): 15.6 mIU mL⁻¹, testosterone: 2.92 ng mL⁻¹ and inhibin B: 49.0 pg mL⁻¹. Chromosome analysis of peripheral lymphocytes showed a karyotype of 46, XY in all patients. In addition, no patients analysed had Y chromosome microdeletions. A total of 18 Japanese patients with azoospermia due to meiotic failure were included in this study. A total of 96 healthy and pregnancy-proven fertile controls and 20 patients with azoospermia due to SCOS were also examined. All subjects were Japanese and gave informed consent for molecular blood analysis. This study was approved by a local ethics committee.

2.2 Mutation screening

We screened 18 Japanese patients diagnosed as having azoospermia due to meiotic arrest for mutations in the *SPATA17* gene. The full-length cDNA sequences were compared with human genomic sequences (NW_926794.1) by BLAST, and all exon–intron borders were determined. The following primers were used for mutational analysis. The sequences of the oligonucleotide primers are listed in Table 1.

Primers of *SPATA17*:

Exon 1: E1F1, E1F2, E1R1 and E1R2;

Exon 2: E2F1, E2F2, E2R1 and E2R2;

Exon 3: E3F1, E3F2, E3R1 and E3R2;

Exon 4: E4F1, E4F2, E4R1 and E4R2;

Exon 5: E5F1, E5F2, E5R1 and E5R2;

Exon 6: E6F1, E6F2, E6R1 and E6R2;

Exon 7: E7F1, E7F2, E7R1 and E7R2;

Exon 8: E8F1, E8F2, E8R1 and E8R2;

Exon 9: E9F1, E9F2, E9R1 and E9R2;

Exon 10: E10F1, E10F2, E10R1 and E10R2

Nested PCRs were carried out using primers for each intronic region (Table 1) and 10-fold diluted first PCR products as templates. The first PCR was carried out in a final volume of 25 μ L, consisting of genomic DNA (10 ng), dNTPs (0.32 m mol L⁻¹ each), primers (0.2 μ m mol L⁻¹ each), Taq polymerase (0.2 μ m mol L⁻¹, 0.625 IU) and reaction buffer containing MgCl₂. The first PCRs were carried out as follows: initial denaturation at 95 °C for 150 s, followed by 32 cycles of denaturation at 95 °C for 30 s, annealing at (primers' T_m - 5 °C) for 90 s and extension at 72 °C for 90 s. Nested PCRs were carried out for 20 cycles under the same conditions as above; however, 2 μ L of 10-fold diluted first PCR products were used as templates. Nested PCR products were purified using a QIAquick PCR Purification kit (Qiagen; Tokyo, Japan), and each product was sequenced directly. To confirm the role of

the detected polymorphisms in azoospermia, the coding region of the *SPATA17* gene of 96 healthy and fertile control individuals and the 20 patients with SCOS were also analysed by direct sequencing.

2.3 Genotyping and statistical analyses

2.3.1 Single-locus analysis

To investigate the role of *SPATA17* polymorphisms in azoospermia, Fisher's exact test was used to determine meaningful differences. $P < 0.05$ was considered to be statistically significant. Hardy-Weinberg equilibrium (HWE) was tested using the SNPalyze software (DYNACOM; Chiba, Japan). All possible two-way combinations of SNPs were tested for linkage disequilibrium (LD) with the absolute value of the correlation coefficient (D'). The P values were determined by χ^2 approximation. Significance was established at $P < 0.05$, as described above. Haplotype frequencies were estimated by the method of maximum

Table 1. Sequences of oligonucleotide primers used for mutational screening of *SPATA17*.

	Forward primer	Reverse primer
Exon 1	E1F1:5'-GTTTACTGCTTTCCCTTCC-3'	E1R1:5'-GATTCTCTCGCTCAGCCTC-3'
	E1F2:5'-CTCGCAGTAGAGACAGAGAC-3'	E1R2:5'-GAAACTCCCCGTGAGGAACA-3'
Exon 2	E2F1:5'-GGTGCCTTCCGTTCATTATC-3'	E2R1:5'-TCACTGCAAACCTTCGCCTTC-3'
	E2F2:5'-CTCACATCTGGATCACTGAC-3'	E2R2:5'-GCTGATCTCAAACCTCCTGAC-3'
Exon 3	E3F1:5'-CTCCCTGGTTCAAGTGATTC-3'	E3R1:5'-GTTCTGGGCAAGGCATTAGC-3'
	E3F2:5'-CTCAGCCTCCAGAATAGCCA-3'	E3R2:5'-CCACTCTCAAAAAGCTCACC-3'
Exon 4	E4F1:5'-GGATCAGACCAGGCAATTCA-3'	E4R1:5'-GTTACACCCAGCACTTTTCAG-3'
	E4F2:5'-CCATCACTGCATCCATATCC-3'	E4R2:5'-CAGAAACCGAGTTCAAGCTC-3'
Exon 5	E5F1:5'-CTCTCTGAAGCCCTAACTTG-3'	E5R1:5'-TGGTGAAACCCCGTCTCTAC-3'
	E5F2:5'-CAGGAAGTGGAATGGTAGG-3'	E5R2:5'-GAGGAGGAGATTGCAGTGAG-3'
Exon 6	E6F1:5'-CTCTGCTTTCCTCAGATACC-3'	E6R1:5'-CTCTATGAAGCCGCTGTCAC-3'
	E6F2:5'-GGATATGGATGATGGTTGCC-3'	E6R2:5'-TGTCCTTTCAGCCTGCTAG-3'
Exon 7	E7F1:5'-GCTGTGTGACAGATAGGAAC-3'	E7R1:5'-CTCTACCCCTTAGCCTCAGG-3'
	E7F2:5'-GTCCACCTCTGGTTAAGAAC-3'	E7R2:5'-CACACAAATGGCCAATAGGC-3'
Exon 8	E8F1:5'-CCCACAGTCTTCACTCAGTG-3'	E8R1:5'-CTTGGCTAAGTCTGAGAAGC-3'
	E8F2:5'-AGGGCTGTTGGAAGCTATG-3'	E8R2:5'-GCCTGAGAGAAAGATGTCTG-3'
Exon 9	E9F1:5'-CTGTACTGGCTGTCCTTTTG-3'	E9R1:5'-CTGGTCCCTTGGGATTAGTG-3'
	E9F2:5'-AGTGGCAGAGGTTGAAATGG-3'	E9R2:5'-GTGCATATGACACTCAGGAG-3'
Exon 10	E10F1:5'-GTGGATATGCCTGAGGTTTG-3'	E10R1:5'-AGCCTAGTGCTTTCTCCTTG-3'
	E10F2:5'-GACTTGCTTGACGGTGACAC-3'	E10R2:5'-GGAGAATCACTTGAACCTGG-3'

Abbreviation: *SPATA17*, spermatogenesis-associated 17 gene.

likelihood based on the expectation–maximization algorithm under the assumption of HWE. The LD and haplotype frequencies were tested using the SNPalyze software. *P* values were determined by χ^2 approximation; Significance was determined at the $P < 0.05$.

3 Results

Mutation analysis of the *SPATA17* gene revealed three nucleotide changes among the 18 patients: c.340A/G in exon 5, c.429C/T in exon 6 and c.608A/G in exon 7. However, all of these changes were also observed in the 96 control individuals, indicating that they are coding single nucleotide polymorphisms (cSNPs). Comparison of our results with the NCBI dbSNP database revealed that, of the three SNPs, only c.429C/T was novel (Table 2). Among SNP1 (c.340A/G), SNP2 (c.429C/T) and SNP3 (c.608A/G), only SNP1 is non-synonymous (tyrosine to cysteine).

Genotyping of the *SPATA17* SNP alleles in the 18 patients and 96 controls revealed that the genotype distribution and the allelic frequency of SNP3 were significantly different between the two groups (Table 2). At the c.608G/A site, the proportions of GG homozygotes/GA heterozygotes/AA homozygotes in the patient group were 0.333/0.333/0.333 and those in the control group were 0.031/0.323/0.646 ($P < 0.05$). The allelic frequencies of c.608G/A in the two groups

were 0.50/0.50 in the patient group and 0.193/0.807 in the control group, and this difference was significant ($P < 0.05$). The number of GG homozygotes at the SNP3 site was markedly higher in the patient group. In addition, at the c.608G/A site, the proportions of GG homozygotes/GA heterozygotes/AA homozygotes in the SCOS group were 0.00/0.15/0.85. The allelic frequencies of c.608G/A were 0.075/0.925 in the SCOS group. The allele frequencies were different between the patient group with meiotic arrest and the patient group with SCOS ($P < 0.05$). In the HWE test of the distribution of genotypes for each SNP of the patient group, SNP2 showed a meaningful deviation ($P < 0.05$). Haplotype analysis revealed that the haplotype frequencies estimated for all three polymorphisms in the groups were similar ($P > 0.05$). Haplotype estimation and LD analysis also revealed no critical differences ($P > 0.05$). In addition, average hormone levels were compared between the patients in each of the three SNP3 genotype groups (Table 3). This statistical analysis revealed no meaningful differences between the groups ($P > 0.05$), with the exception that the level of FSH in GG homozygotes was higher relative to the normal range in Japanese men.

4 Discussion

Recently, several novel genes that are downregulated

Table 2. Genotype and allele frequencies of three coding single nucleotide polymorphisms (cSNPs) in the *SPATA17* gene in 18 azoospermic patients with meiotic arrest, control individuals and in 20 azoospermic patients with SCOS.

SNPs	Alterations		Genotype frequency				Allele frequency			
	Nucleotide	Amino acid	Genotype/total no of samples (%)				Minor allele/total no of the chromosomes (%)			
			(G)	Meiotic arrest	Controls	SCOS	(A)	Meiotic arrest	Controls	SCOS
SNP1	340G/A	Tyr/Cys	GA	4/18 (22.22)	7/96 (7.29)		G	4/36 (11.11)	7/192 (3.65)	
					0.071				0.0762	
SNP2	429T/C	Synonymous	TC	2/18 (11.11)	24/96 (25.00)					
					0.235					
			TT	0/18 (0.00)	2/96 (2.08)		T	2/36 (5.56)	28/192 (14.58)	
					1.00				0.183	
SNP3	608G/A	Synonymous	GA	6/18 (33.33)	31/96 (32.29)	3/20 (15.0)				
					0.337	0.461				
			GG	6/18 (33.33)	3/96 (3.13)	17/20 (85.0)	G	18/36 (50.00)	37/192 (19.27)	3/40 (7.50)
					0.00026*	0.014			0.00022*	0.0024*

Abbreviations: SCOS, Sertoli-cell-only syndrome; *SPATA17*, spermatogenesis-associated 17; SNP, single nucleotide polymorphism. (G) and (A) reveal genotype and allele, respectively. *Statistically significant.

Table 3. Genotype of SNP3 and average hormone levels.

Genotype	FSH (mIU mL ⁻¹)	LH (mIU mL ⁻¹)	Testosterone (ng mL ⁻¹)	Inhibin B (pg mL ⁻¹)
AA (<i>n</i> = 6)	15.3	6.05	3.12	82.4
GA (<i>n</i> = 6)	10.8	3.39	2.80	41.1
GG (<i>n</i> = 6)	27.4	7.43	3.82	37.3

Abbreviations: FSH, follicle stimulating hormone; LH, leutinizing hormone; SNP, single nucleotide polymorphism.

in the testicular tissues of infertile men were identified by microarray analysis [17]. One of these genes is *SPATA17*, which is downregulated in the testes of patients with azoospermia due to meiotic arrest. The mouse *Spata17* gene is a member of the CaM-binding protein family, and it has been suggested that it might play a critical role in spermatogenic cell apoptosis in mice [18,19].

We could not detect any *SPATA17* mutations that were directly causative of azoospermia or that were limited to the 18 patients with meiotic arrest. Instead, we identified three cSNPs in the gene. This association study revealed that the allele frequencies of SNP3 (c.608A/G) were significantly different between the patients with meiotic arrest, and both the controls and the patients with SCOS. These findings suggest that the A allele at nucleotide 608 in exon 7 or its flanking region may play a role in the disruption of spermatogenesis in Japanese patients. However, the encoded amino acid is not affected. Thus, the function of SNP3 is unknown. We believe that the patient group of 18 men is far too small for an association study. However, azoospermia due to meiotic arrest is very rare, and our histological diagnostic criteria were very strict. We have DNA samples from more than 5000 patients with azoospermia; however, only 18 patients had azoospermia due to meiotic arrest. We carried out TESE (testicular sperm extraction) on > 5000 men, and > 70% of them have sperm in their testis. However, we could not find sperm in any of the patients in this study, even in the testes.

Recently, we showed that the human *PRDM9* (*MEISETZ*) gene might play an important role in human spermatogenesis [20]. The patient group in that study was also used in this study. We found SNP2 in one of the patients and SNP3 in two of the patients displaying *PRDM9* SNPs. However, these three patients revealed no variation in SNP3 of *SPATA17*. Therefore, the relationship between the *PRDM9* SNPs and *SPATA17* SNP3 remains unknown.

In vitro fertilization has been proven to be an efficient means of resolving infertility due to female factors [21]; however, it is less effective for severe oligospermia in the male partner [22]. Although TESE–intracytoplasmic sperm injection is now carried out in patients with azoospermia, it cannot benefit patients in whom the absence of spermatozoa in their testes is because of a complete failure in spermatogenesis. Therefore, a treatment for infertility due to non-obstructive azoospermia is a pre-eminent topic for assisted reproductive technology.

In conclusion, this is the first report showing that *SPATA17* SNPs may predispose men to a defect in spermatogenesis, although the mechanism of involvement of the SNPs in azoospermia remains unclear. Our results may also promote a better understanding of the molecular basis of early meiotic arrest as a cause of non-obstructive azoospermia. It remains to be confirmed whether this association is seen in a large number of samples and in similar patients from other ethnic groups, although men with azoospermia due to meiotic arrest are very rare in any ethnic group.

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