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GENETICS

Single nucleotide polymorphisms in the *SEPTIN12* gene may be associated with azoospermia by meiotic arrest in Japanese men

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Abstract
Purpose To investigate the association between *SEPTIN12* gene variants and the risk of azoospermia caused by meiotic arrest.
Methods Mutational analysis of the *SEPTIN12* gene was performed using DNA from 30 Japanese patients with azoospermia by meiotic arrest and 140 fertile male controls.
Results The frequencies of the c.204G>C (Gln38His) allele and the CC genotype were significantly higher in patients than in fertile controls ($p<0.05$).
Conclusion The c.204G>C (Gln38His) variant in the *SEPTIN12* gene was associated with increased susceptibility to azoospermia caused by meiotic arrest.

Keywords Male infertility · Meiosis · Septin · SNP

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Capsule c.204G>C (Gln38His) variant in the *SEPTIN12* gene was associated with increased susceptibility to azoospermia by meiotic arrest in Japanese men.

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Introduction

Genetic causes of azoospermia in humans include chromosomal abnormalities, e.g., Y-chromosome microdeletions and mutations in specific genes; e.g., *DAZ, SYCP3, PRM1, SPATA16, AURKC* and *KLHL10* [1–7]. As Y-chromosome deletions account for only 16% of men with infertility [8], azoospermia in many infertile men may be caused by mutations in autosomal genes. Genetic polymorphisms may also increase susceptibility to some forms of male infertility; e.g., mutations in the human *BCL2* and *eNOS* genes are linked to male infertility [9, 10]. Defective meiosis during spermatogenesis causes azoospermia; however, the mechanisms leading to defective meiosis remain unknown. Meiosis is a fundamental process in sexually reproducing species that allows genetic exchange between maternal and paternal genomes [11]. Our understanding of the genetic regulation of meiosis in mammals is poor relative to that in lower eukaryotes, such as yeast.

Several critical genes expressed in mouse meiosis, such as *Dmc1, Fkbp6, Scp3 (Sycp3), Spo11, Msh4 and Msh5, Meisetz, Cdk2*, and *Hop2*, have been identified by disruption experiments in embryonic stem cells [12–22]. In 2006, Lin et

al. identified 10 genes involved in human spermatogenesis by subtractive hybridization microarray analysis of mRNA expression in testicular tissue [23]. Their analysis of normal and Sertoli cell-only syndrome (SCOS) testicular tissues identified the human *SEPTIN12* gene (NM_144605.3). This gene encodes SEPT12 (NP_653206.2), which belongs to the septin family of polymerizing GTP-binding proteins that are required for many cellular functions; e.g., membrane compartmentalization, vesicular trafficking, mitosis, and cytoskeletal remodeling [24]. SEPT12 can interact with SEPT6, independently of the coiled-coil domain of SEPT6 [25]. The testicular tissues of men with either hypospermatogenesis or maturation arrest have lower levels of the *SEPTIN12* transcripts than those of fertile men [26]. Moreover, most chimeric mice derived from *Septin12* knockout embryonic stem cells generated using gene targeting were infertile and had a decreased sperm count, decreased sperm motility, and spermatozoa with defects involving all subcellular compartments [26]. Oocytes fertilized with spermatozoa obtained from Septin12$^{+/-}$ chimeric mice failed to develop beyond the morula stage after in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) because of significant DNA defects in the spermatozoa [27]. Previously, we investigated whether *SEPTIN12* gene variants are associated with azoospermia caused by SCOS [28]. The coding regions of *SEPTIN12* from 100 infertile Japanese male patients and 140 fertile control males were directly sequenced, and the data were subjected to statistical analysis. No mutations were found in *SEPTIN12*; however, eight coding single nucleotide polymorphisms (SNP1-SNP8) could be detected in the patients with SCOS. The frequencies of particular SNP3, SNP4, and SNP6 variants and the frequencies of some genotypes were notably higher in the SCOS group than in the control group ($P<0.001$). These results suggest that *SEPTIN12* might play a critical role in human spermatogenesis [28].

In the present study, we analyzed possible associations between *SEPTIN12* mutations and azoospermia caused by meiotic arrest (MA) in humans.

Materials and methods

Patients and controls

Azoospermia was confirmed in patients by two consecutive semen analyses obtained after 5–7 days of sexual abstinence and by examination of a centrifuged semen pellet. Patients with defective spermatogenesis following infection or due to obstruction of the seminal tract, to pituitary failure, or to other causes of possible testicular damage revealed upon clinical examination were excluded from the study. Final diagnosis was carried out by histological examination; more than 20 testicular specimens from each of the 30 patients included in the study were examined by a pathologist and urologists at Osaka University. Briefly, it was determined based on the most advanced pattern of spermatogenesis. MA is defined as spermatocyte stage arrest without spermatozoa throughout the seminiferous tubules in the specimen, so-called “uniform MA” [29]. We carefully distinguished MA from hypospermatogenesis, which is characterized with reduction in the degree of normal spermatogenesis and focal spermatogenesis with spermatid stage arrest [30]. Each of the 30 patients had a normal 46, XY karyotype based on analysis of chromosomes in peripheral lymphocytes. No patients had Y chromosome microdeletions. Ultimately, 30 Japanese patients with azoospermia secondary to MA were included in the study; 140 healthy, pregnancy-proven, fertile men were also examined as controls [28]. All normal controls were Japanese men and had normal sperm inspections, in addition to all having a child by spontaneous pregnancy. All subjects were Japanese and provided written informed consent for molecular blood analysis. This study was approved by the local ethics committee.

Mutation screening

We screened 30 Japanese patients diagnosed with azoospermia secondary to MA for mutations in the *SEPTIN12* gene. Full-length cDNA sequences were compared to human genomic sequences (NT_037887.4) from the NCBI reference database by BLAST, and all exon-intron borders were determined. The following *SEPTIN12* primers were used for mutational analysis. Exon 2: E2F1, E2F2, E2R1 and E2R2; Exons 3, 4: E3F1, E3F2, E3R1 and E3R2; Exon 5: E5F1, E5F2, E5R1 and E5R2; Exons 6, 7: E6F1, E6F2, E6R1 and E6R2; Exon 8: E8F1, E8F2, E8R1 and E8R2; and Exon 9: E9F1, E9F2, E9R1 and E9R2 [28]. Nested PCR was performed using primers for each intronic region and 10-fold diluted first PCR products as templates. The initial PCR was performed in a final volume of 25 μl, consisting of genomic DNA (10 ng), dNTP (0.32 mM

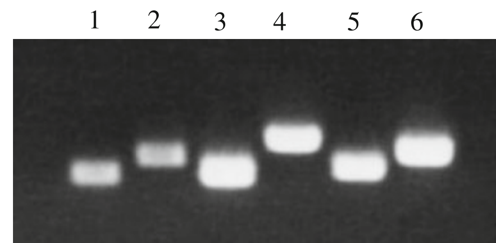

Fig. 1 Six PCR products include the exons and adjacent introns. Lane1: the region including exon2 (E2), 336 bp; Lane2: E3&E4, 473 bp; Lane3: E5, 342 bp; Lane4: E6&E7, 637 bp; Lane5: E8, 395 bp; Lane6: E9, 533 bp

Table 1 Genotype and allele frequencies of three coding single-nucleotide polymorphisms (cSNPs) in the human *SEPTIN12* gene of 30 azoospermic patients with MA and 140 fertile male controls

SNP	Alteration		Genotype frequency		Allele frequency	
	Nucleotide	Amino acid	Genotype/Total no. of samples (%)		Minor allele/Total no. of chromosomes (%)	
			(G) MA	Controls *p* value	(A) MA	Controls *p* value
SNP1	204G>C	Gln38His	CC 2/30 (6.67)	0/140 (0.00) 0.030*	C 9/60 (15.0)	0/280 (0.00) <0.001*
SNP2	422C>A	Thr111[Thr, Lys]	AC 1/30 (3.33)	1/140 (0.714) 0.323	A 1/60 (1.67)	1/280 (0.357) 0.322
SNP3	564G>A	Synonymous	AA 1/30 (3.33)	15/140 (10.7) 0.310	A 12/60 (20.0)	92/280 (32.9) 0.063

(G) and (A) indicate genotype and allele, respectively.

each), each primer (0.2 μM), 0.2 μM Taq polymerase (0.625 IU) and reaction buffer containing $MgCl_2$. First PCR was performed 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 PCR was carried out for 20 cycles under the same conditions as above, except that 2 μl 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 direct sequencing of each product was conducted (Fig. 1). To confirm the role of the detected polymorphisms in azoospermia, the coding region of the *SEPTIN12* gene of 140 healthy, fertile control men was also analyzed by direct sequencing analysis previously [28].

Genotyping and statistical analyses

Single-locus analysis

To investigate the role of *SEPTIN12* polymorphisms in azoospermia, Fisher's exact test was used to determine a meaningful difference in allele and genotype frequencies between the patient and control groups. A $P<0.05$ was considered to be statistically critical. Hardy-Weinberg equilibrium (HWE) was tested using SNPAlyze software (Dynacom; Chiba, Japan). Linkage disequilibrium (LD) of all possible two-way combinations of single nucleotide polymorphisms (SNPs) with the absolute value of the correlation coefficient (D') was tested; *P* values were determined by a χ^2 approximation, and again a $P<0.05$ was consider significant. Haplotype frequencies were estimated by the method of maximum likelihood based on the expectation-maximization (E-M) algorithm under the assumption of HWE. LD and haplotype frequency were tested using SNPAlyze software. *P* values were determined by χ^2 approximation, and a $P=0.05$ level was considered significant.

Table 2 Pairwise linkage disequilibrium (D'-value above right triangle) and statistical significance (*P*-value below left triangle) for the three SNPs. Significant at $P=0.05$ level by χ^2 tests

	SNP1	SNP2	SNP3
Case group			
SNP1		1	1
SNP2	0.5808		−0.1158
SNP3	0.5808	0.2595	
Control group			
SNP1		0.1002	1
SNP2	0.1049		1
SNP3	0.1049	0.1406	

Results

Sequence analysis of the *SEPTIN12* gene revealed three nucleotide changes in the 30 patients: 204G>C (Gln38His) in exon 2, 422C>A (Thr111Lys) in exon 4, and 564G>A in exon 5. All three SNPs were identified in the previous study (Table 1) [28]. SNP1 and SNP2 were non-synonymous, and SNP3 was synonymous. Genotyping for the *SEPTIN12* SNP alleles among the 30 patients and 140 controls revealed significantly different genotype distributions and allele frequencies of SNP1 between the two groups (Table 1).

Table 3 Haplotype frequency estimation in the patient group and control group

SNP1-SNP2-SNP3	Case	Control	*P*-value
G - C - G	0.6944	0.7857	0.2719
G - C - A	0.2500	0.1735	0.3209

However, there were no meaningful differences on SNP2 and SNP3 between the two groups ($p>0.05$) (Table 1).

At the c.204G>C (Gln38His) site, the proportion of CC homozygote/GG homozygote was 0.067/0.933 in the patient group and 0.00/1.00 in the control group ($P<0.05$). The allele frequency of c.204G>C (Gln38His) was 0.15/0.85 in the patient group and 0.00/1.00 in the control group; again, the difference was significant ($P<0.001$). Haplotype analysis revealed no significant difference in the haplotype frequency estimates for the three polymorphisms in the patient and control groups ($P>0.05$). Haplotype estimation and LD analysis also revealed no significant differences between the patient and control groups ($P>0.05$) (Tables 2 and 3).

Discussion

In this study, we hypothesized that mutations or polymorphisms of the *SEPTIN12* may be associated with azoospermia caused by MA. We could not state that we could find *SEPTIN12* mutations that directly cause azoospermia by these results. The number of analyzed patients is not enough to achieve a final decision. However, we identified three cSNPs in the gene at least. The present association study revealed that the genotype distribution for SNP1, 204G>C (Gln38His), is significantly different between Japanese azoospermic patients and fertile controls ($P<0.05$). This finding indicated that the G allele at SNP 204 in exon 2 and the resulting Gln amino acid, or their flanking regions, may play a role in the disruption of spermatogenesis in Japanese patients. However, the number of patients analyzed was not large enough to allow a definitive conclusion to be drawn. Moreover, the function of the SNP at position 204 is unknown. Nevertheless, azoospermia by MA is very rare, and our histological diagnostic criteria were very strict—i.e., we have DNA samples from more than 5,000 patients with azoospermia, and only 30 of these patients had azoospermia secondary to MA.

In conclusion, we suggest that the C variant of the SNP at position 204 might be associated with azoospermia secondary to MA in humans, but the relationship between the C allele and the mechanistic cause of azoospermia was not investigated. Our results may provide insight into the molecular basis of meiotic arrest as a cause of non-obstructive azoospermia. It remains to be determined whether an association between this variant and azoospermia secondary to MA exists in similar patients from other ethnic groups.

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