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GENETICS

Two single nucleotide polymorphisms in *PRDM9* (*MEISETZ*) gene may be a genetic risk factor for Japanese patients with azoospermia by meiotic arrest

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Abstract
Purpose To investigate whether defects in human *PRDM9*, *CDK2* and *PSMC3IP* are associated with azoospermia Mutational analysis was performed in Japanese patients with azoospermia caused by meiotic arrest.
Methods Mutational screening of the coding regions of human *PRDM9*, *CDK2* and *PSMC3IP* was done by direct sequencing using genomic DNA from 18 Japanese patients. Statistical analysis of the detected coding single nucleotide polymorphisms (cSNPs) in patients and normal control men was then carried out.
Results One cSNP was detected in *CDK2* and *PSMC3IP*. There were no significant differences in genotype distribution and allele frequencies between the patient and control groups in these two genes. However, three novel cSNPs were detected in the *PRDM9*. The genotype and allele frequencies of heterozygotes in SNP2 and SNP3 of *PRDM9* were significantly higher in the patient group than in the control group.
Conclusion We found a possible association between *PRDM9* and azoospermia by meiotic arrest.

Keywords Azoospermia · *CDK2* · *PRDM9* · *PSMC3IP* · SNP

Capsule The human *PRDM9 (MEISETZ)* gene might play a critical role in human spermatogenesis.

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Introduction

Genetic causes of azoospermia in humans include chromosomal abnormalities such as Y-chromosome microdeletions, and specific gene mutations such as *DAZ, RBMY, USP9Y,* and *SYCP3* [1–5]. As Y-chromosome deletions account for only 21% of men with infertility [6], azoospermia in many infertile men may be caused by autosomal gene mutations. Genetic polymorphisms may also cause susceptibility to some forms of male infertility, e.g., the human *MEI1* gene is linked to male infertility [7].

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 allows genetic exchange between maternal and paternal genomes [8]. Genetic regulation of meiosis in mammals is poorly understood, as compared 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,* have been identified by disruption experiments in embryonic stem (ES) cells [9–16]. In addition, homo-mutants with regard to three additional genes have been identified in mice with azoospermia caused by meiotic failure [17–19].

Male mice with mutant *Meisetz* (meiosis-induced factor containing a PR/SET domain and zinc-finger motif) gene

have small testes and no round spermatids, elongated spermatids or spermatozoa in the testes [17]. When the locus encoding cyclin-dependent kinase 2 (CDK2) is targeted by homologous recombination in mouse embryonic stem (ES) cells, severely atrophic testes develop, resulting in azoospermia [18]. In addition, male *Hop2 (Psmc3ip)* knockout mice show profound meiotic defects and their spermatocytes arrest at the stage of pachytene-like chromosome condensation [19].

In the present study, we analyzed possible associations between *PRDM9* (PR domain containing 9, *MEISETZ*), *CDK2* and *PSMC3IP* (*HOP2*) mutations, and azoospermia caused by meiotic arrest in humans.

Materials and methods

Subjects

Subjects were 18 infertile Japanese men with azoospermia caused by meiotic arrest and who had undergone microdissection TESE (*testicular sperm extraction*). Patients with defective spermatogenesis following infection, or due to obstruction of the seminal tract, pituitary failure or testicular damage revealed at clinical examination were excluded from the study. Final diagnoses were carried out by histological examination. The remaining subjects were 350 healthy and pregnancy-proven fertile *male* controls. All subjects gave written informed consent for molecular analysis of blood and the study was approved by a local ethics committee.

Genomic DNA extraction

Genomic DNA was extracted by a salting-out-procedure after treatment of peripheral blood leukocytes with proteinase K, as described by Tsujimura et al. [20].

Mutation screening

Full-length cDNA sequences of human *PRDM9*, *CDK2* and *PSMC3IP* were compared to human genomic sequences (NT_006576.15, NT-029419.10 and NT-010755.15) and all exon-intron borders were determined. The amounts of the genomic DNAs from the patients were limited. Therefore, nested PCR was carried out to save the DNAs. Nested PCR was performed using primers homologous with the intronic regions adjacent to each exon in the coding regions, as well as human DNA samples.

The following primer sets were used for PCR of the *PRDM9* gene:

Exon 2: MEISE2F1, MEISE2F2, MEISE2R1 and MEISE2R2;
Exon 3: MEISE3F1, MEISE3F2, MEISE3R1and MEISE3R2;
Exon 4: MEISE4F1, MEISE4F2, MEISE4R1and MEISE4R2;
Exon 5: MEISE5F1, MEISE5F2, MEISE5R1and MEISE5R2;
Exon 6: MEISE6F1, MEISE6F2, MEISE6R1and MEISE6R2;
Exon 7: MEISE7F1, MEISE7F2, MEISE7R1and MEISE7R2;
Exon 8: MEISE8F1, MEISE8F2, MEISE8R1and MEISE8R2;
Exon 9: MEISE9F1, MEISE9F2, MEISE9R1 and MEISE9R2;
Exon 10: MEISE10F1, MEISE10F2, MEISE10R1 and MEISE10R2;
Exon 11: MEISE11(F1–F12) and MEISE11(R1–11).

The following primer pairs were used for PCR for the *CDK2* gene:

Exon 1: CDKE1F1, CDKE1F2, CDKE1R1 and CDKE1R2;
Exon 2: CDKE2F1, CDKE2F2, CDKE2R1 and CDKE2R2;
Exon 3: CDKE2F1, CDKE3F3, CDKE3R1and CDKE3R2;
Exon 4: CDKE4F1, CDKE4F2, CDKE4R1and CDKE4R2;
Exon 5: CDKE5F1, CDKE5F2, CDKE5R1and CDKE5R2;
Exon 6: CDKE6F1, CDKE6F2, CDKE6R1and CDKE6R2;
Exon 7: CDKE7F1, CDKE7F2, CDKE7R1and CDKE7R2;

The following primers were used for PCR for the *PSMC3IP* gene:

Exon 1: HOPE1F1, HOPE1F2, HOPE2R1 and HOPE2R2;
Exon 2: HOPE2F1, HOPE2F2, HOPE2R1 and HOPE2R2;

Table 1 Genotype and allele frequencies of coding-nucleotide-polymorphisms (cSNPs) in human *PRDM9* in patient and control groups

Sequence change	Classification of change	Alteration in amino acid sequence	Frequency of SNP in patients		Frequency of SNP in controls		Fisher's exact test	
			Allele (%)	Genotype (%)	Allele (%)	Genotype (%)	AlleleP	GenotypeP
Exon2 C(224)T	SNP1	Silent	1/35 (2.86%)	T/C1/17(5.88%)	9/691 (1.3%)	T/C9/341 (2.6%)	0.396	0.398
Exon6 C(614)T	SNP2	Silent	1/35 (2.86%)	T/C1/17(5.88%)	0/700 (0.0%)	T/C 0/350 (0.0%)	0.049[a]	0.049[a]
Exon9T(1086)C	SNP3	Missense Tyr→His	2/34 (5.88%)	C/T 2/16(12.5%)	1/699 (0.14%)	C/T1/349 (0.29%)	0.0069[a]	0.0066[a]

[a] Statistically significant

Table 2 Genotype and allele frequencies of coding-nucleotide-polymorphism (cSNP) in human *CDK2* in patient and control groups

Sequence change	Classification of change	Alteration in amino acid sequence	Frequency of SNP in patients		Frequency of SNP in controls		Fisher's exact test	
			Allele (%)	Genotype (%)	Allele (%)	Genotype (%)	AlleleP	GenotypeP
Exon7 C (1038)G	SNP1	Missense Thr→Ser	1/35 (2.8%)	C/G 1/18 (5.5%)	1/199 (0.50%)	C/G 1/99 (1.01%)	0.282	0.282

Exon 3: HOPE3F1, HOPE3F2, HOPE3R1and HOPE3R2;
Exon 4: HOPE4F1, HOPE4F2, HOPE4R1and HOPE4R2;
Exon 5: HOPE5F1, HOPE5F2, HOPE6R1and HOPE6R2;
Exon 6: HOPE5F1, HOPE6F3, HOPE6R1and HOPE6R2;
Exon 7: HOPE7F1, HOPE7F2, HOPE7R1and HOPE7R2;
Exon 8: HOPE8F1, HOPE8F2, HOPE8R1and HOPE8R2.

The sequences of the primers are available on request. All PCR procedures 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. An aliquot of each PCR product was subsequently subjected to 20 further cycles under the same conditions. The resultant PCR products were then subjected to direct sequence analysis in both orientations.

Statistical analysis

In order to confirm the role of polymorphisms in azoospermia, the 18 patients, as well as 100 or 350 control *men*, were genotyped for polymorphic alleles, and genotypes and allele frequencies were compared among the groups. Fisher's exact test was used to determine the significance of differences. A *P* value of less than 0.05 was considered to be statistically significant.

In vitro methylation analysis

To elucidate the physiological role of the SNP3 in the *PRDM9*, methylation assay was carried out. To generate the fragments of coding region of *MEISETZ*, PCR was performed with human testis cDNA library as a template. The oligonucleotides of the Forward and Reverse primers used to amplify normal cDNA were: 5'-CGGGATCCATGGGAGACTGGGA GAAAACTC-3' (Forward) and 5'-GGGGCGGCCGCTTGA GATGAGGTTTTATTACTAATG-3' (Reverse). The amplified product (product 1) was digested with *Not*I/*Bam*HI and cloned into pET21b vector (Novagen). To generate PRDM9-SNP3, DNA fragment containing the SNP3 (1086C), semi-nested PCRs were done with product 1 as a template. The used primers were Forward and Reverse 2 and the oligonucleotides of the Reverse 2 were 5'-CATGGCAGTTTCTCCCCTTGGT GATCAGCC-3'. The product (product 2) was digested with *Bam*HI/*Bc*II. Then, semi-nested PCR was performed again with the primers, Reverse and Forward 2. The oligonucleotides of the Forward 2 were 5'-ACTCCTGGCTGATCAC CAAGG -3'. The product (product 3) was digested with *Not*I/*Bc*II. The product 2 and product 3 were ligated and cloned into pET21b vector. Both recombinant plasmids were transferred to the *E. coli* expression strain BL21 (DE3). Both protein, normal PRDM9 and PRDM9-SNP3, were purified and western blot analysis was carried out with anti-His antibody as a primary antibody. Methylation analysis was performed, as described by Hayashi et al. [17].

Results

Mutational analysis of *PRDM9* revealed three nucleotide changes (cSNPs) among the 18 Japanese patients, i.e., C224T (SNP1) in exon 2, C614T (SNP2) in exon 6 and T1086C (SNP3) in exon 9. The numbers revealed the positions from transcriptional activation point. As all of these were hitherto unreported or had not been registered in the NCBI dbSNP database, they were considered novel cSNPs (Table 1). Genotyping for *PRDM9* SNP alleles among the 18 Japanese patients and control individuals

Table 3 Genotype and allele frequencies of coding-nucleotide-polymorphism (cSNP) in human *PSMC3IP* in patient and control groups

Sequence change	Classification of change	Alteration in amino acid sequence	Frequency of SNP in patients		Frequency of SNP in controls		Fisher's exact test	
			Allele (%)	Genotype (%)	Allele (%)	Genotype (%)	AlleleP	GenotypeP
Exon6 T (530)A	SNP1	Missense Tyr→Asn	3/33 (9.1%)	A/T 3/15 (20.0%)	21/179 (11%)	A/T 21/79 (26.6%)	1.00	1.00

revealed that the genotype distribution and allele frequency of SNP2 and SNP3 were significantly different between the two groups (Table 1). At the 614T/C site (SNP2), the proportions of TT homozygotes/TC heterozygotes/CC homozygotes in the patient and control groups were 0.000/0.0588/0.9412 and 0.000/0.000/1.000, respectively (p=0.049). The allele frequencies for 614T/C in the two groups were 0.0286/0.9714 and 0.000/1.000, respectively, and were significantly different (P=0.049). Similarly, at the 1086C/T site (SNP3), the proportions of respective zygosity in the two groups were 0.000/0.125/0.875 and 0.000/0.0029/0.9971 (P=0.0066), and the allele frequencies were 0.0588/0.9412 and 0.0014/0.9986 (P=0.0069). The most common genotypes for SNP2 and SNP3 were 614C/C and 1086T/T, respectively. However, CT heterozygotes were markedly more common at the SNP2 and SNP3 sites in the patient group. There were no statistical differences in SNP1 between the two groups (p>0.05). SNP3 in the *PRDM9* is a missense mutation giving rise to a Tyr-to-His amino acid substitution. To identify the functional role of SNP3 in human spermatogenesis, normal and point-variant of PRDM9 were tested for methylation activity in vitro. However, no difference could be detected between normal and variant products.

We identified one cSNP in human *CDK2* among the 18 Japanese patients by direct sequence analysis: C1038G (SNP1) in exon 7. A search of the NCBI dbSNP database revealed that SNP1 has been already reported. Genotyping for *CDK2* SNP alleles among the 18 patients and 100 control individuals revealed no significant differences in genotype distribution and allele frequency (P>0.05) (Table 2).

With regard to the human *PSMC3IP* gene, one cSNP (SNP1) was detected among the 18 Japanese patients by direct sequence analysis; T530A (SNP1) in exon 6. According to the NCBI dbSNP database, this has also been reported previously. Genotyping for *PSMC3IP* SNP alleles among the 18 patients and 100 control individuals revealed no significant differences in genotype distribution and allele frequency (P>0.05) (Table 3).

Discussion

We identified three novel cSNPs in the *PRDM9* gene. The present association study revealed that the genotype distributions for SNP2 (T/C) and SNP3 (C/T) are significantly different between Japanese azoospermic patients and healthy controls: 0.0588/0.9412 vs. 0.000/1.000 for TC heterozygotes/CC homozygotes at the SNP2 site; and 0.125/0.875 vs. 0.0029/0.9971 for CT heterozygotes/TT homozygotes at the SNP3 site, respectively (P<0.05). Likewise, the frequencies of alleles T/C at SNP2 were 0.0286/0.9714 and 0.000/1.000, and those of alleles C/T at SNP3 were 0.0588/0.9412 vs. 0.0014/0.9986 in patients and controls, respectively (P<0.05). These findings suggest that allele C at nucleotide 614 in exon 6 and allele T at nucleotide 1086 in exon 9, or their flanking regions, may play a role in the disruption of spermatogenesis in Japanese patients, although the number of patients analyzed was not large enough to allow a definitive conclusion to be drawn. We believe that the patient group of 18 men is far too small for an association study. However, azoospermia by meiotic arrest is very rare and our histological diagnostic criteria are very strict. We have the DNA samples from more than 5,000 patients with azoospermia; however, only 18 patients had azoospermia secondary to meiotic arrest. But no such association was found in the *CDK2* and *PSMC3IP* genes. Methylation analysis using SNP3 in PRDM9 was carried out. However, no differences could be found between normal and variant products. Therefore, the functional role of the cSNPs in the *PRDM9* is unknown in human spermatogenesis

Testicular sperm extraction (TESE)-intracytoplasmic sperm injection (ICSI) is now available for patients with azoospermia; however, the procedure does not benefit patients lacking spermatozoa in their testes due to a complete failure in spermatogenesis. Therefore, treatment for infertility due to non-obstructive azoospermia is a critical immediate goal in assisted reproductive technology (ART).

In summary, this is the first report showing that *PRDM9* cSNPs may predispose men to defects in spermatogenesis; however, the mechanisms by which these cSNPs result in azoospermia remain uncertain. It remains to be confirmed whether the association is seen in larger number samples and in similar patients from other ethnic groups, although the patients with azoospermia caused by meiotic arrest are very rare.

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