Single-nucleotide polymorphisms in HORMAD1 may be a risk factor for azoospermia caused by meiotic arrest in Japanese patients

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ORIGINAL ARTICLE

Single-nucleotide polymorphisms in *HORMAD1* may be a risk factor for azoospermia caused by meiotic arrest in Japanese patients

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Genetic mechanisms are implicated as a cause of some male infertility, yet are poorly understood. Meiosis is unique to germ cells and essential for reproduction. The synaptonemal complex is a critical component for chromosome pairing, segregation and recombination. *Hormad1* is essential for mammalian gametogenesis as knockout male mice are infertile. *Hormad1*-deficient testes exhibit meiotic arrest in the early pachytene stage and synaptonemal complexes cannot be visualized. To analyze the hypothesis that the human *HORMAD1* gene defects are associated with human azoospermia caused by meiotic arrest, mutational analysis was performed in all coding regions by direct sequence analysis of 30 Japanese men diagnosed with azoospermia resulting from meiotic arrest. By the sequence analysis, three polymorphism sites, Single Nucleotide Polymorphism 1 (c. 163A>G), SNP2 (c. 501T>G) and SNP3 (c. 918C>T), were found in exons 3, 8 and 10. The 30 patients with azoospermia and 80 normal pregnancy-proven, fertile men were analyzed for *HORMAD1* polymorphisms. Both SNP1 and SNP2 were associated with human azoospermia caused by complete early meiotic arrest ($P<0.05$). We suggest that the *HORMAD1* has an essential meiotic function in human spermatogenesis.
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Keywords: azoospermia; *HORMAD1*; male infertility; male meiosis; SNP

INTRODUCTION

One of the most serious social problems facing Japan today is the declining birth rate. However, it is generally not well recognized that the number of infertile couples is on the rise in Japan. Although social and environmental factors—such as social progress for women and the resulting increase in the age at which women marry, pollution and global warming—are behind part of the increase in the number of patients with infertility, approximately half of all cases of infertility are generally caused by factors related to the man. To date, a variety of treatments have been developed for male infertility, and these are steadily producing results. At present, however, there is no effective treatment for patients with non-obstructive azoospermia, in which there is an absence of mature sperm in the testes. Although evidence suggests that many patients with azoospermia have a genetic predisposition to the condition, the cause has not been elucidated in the vast majority of cases.[1] The most frequent genetic cause of azoospermia is represented by Klinefelter syndrome,[2] and many cases of meiotic arrest (MA) are caused by the presence of balanced chromosomal translocations.[3] More genetic causes of azoospermia in humans include Y-chromosome microdeletions and mutations in specific genes, including *SYCP3, PRM1, SPATA16, AURKC* and *KLHL10*.[4–8] As Y-chromosome deletions account for only 9.4% of the cases of male infertility,[9] azoospermia may be caused by autosomal gene mutations. Genetic polymorphisms also increase susceptibility to some forms of male infertility, e.g., the human *SPATA17*, *PARP-2* and *UBR2* genes are linked to male infertility.[10–12] Meiosis is a fundamental process in sexually reproducing species that allows genetic exchange between maternal and paternal genomes.[13] Genetic regulation of meiosis in mammals is poorly understood when compared to that in lower eukaryotes such as yeast.

Proteins that contain a HORMA (Hop1, Rev7 and Mad2)-domain regulate interactions between homologous chromosomes during meiosis in a wide range of eukaryotes. Biochemical and cytological observations demonstrate that a mouse HORMA domain-containing protein, HORMAD1, is associated with the meiotic chromosome axis.[14] *Hormad1* is essential for mammalian gametogenesis as knockout male and female mice are infertile.[15] *Hormad1*-deficient (*Hormad1*$^{-/-}$) male germ cells arrest in the early pachytene stage, and synaptonemal complexes are not evident in electron micrographs of *Hormad1*-deficient male germ cells. HORMAD1 is a critical component of the synaptonemal complex, which affects synapsis, recombination and meiotic sex chromosome inactivation and transcriptional silencing.[15]

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Here, we analyzed possible associations between *HORMAD1* mutations and azoospermia caused by MA in humans.

MATERIALS AND METHODS

Study participants

Japanese patients with azoospermia caused by MA (n=30) were included in the study, and 80 healthy, pregnancy-proven, Japanese fertile men were examined as controls. All normal controls 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.

Azoospermia in each patient was confirmed 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, pituitary failure or other causes of possible testicular damage revealed at clinical examination were excluded from the study. Final diagnosis of azoospermia was carried out by histological examination. Samples from each patient were subjected to more than one pathologic test. All patients included in the study had a normal 46: XY karyotype based on chromosome analysis of peripheral lymphocytes. There were no patients with Klinefelter syndrome and no patients with balanced chromosome translocations. In addition, no patients had Y-chromosome microdeletions.

Mutation screening

We screened 30 Japanese patients with azoospermia secondary to MA for mutations in the *HORMAD1* gene. Full-length cDNA sequences (NM_032132) were compared to human genomic sequences (NW_923184.1) by BLAST, and all exon/intron borders were determined. The following *HORMAD1* primers were used for mutational analysis: Exon 2: E2F1 and E2R1; Exons 3: E3F1 and E3R1; Exon 4: E4F1 and E4R1; Exon 5: E5F1 and E5R1; Exon 6 and Exon 7: E6F1 and E6R1; Exon 8: E8F1 and E8R1; Exon 9: E9F1 and E9R1; Exon 10: E10F1 and E10R1; Exon 11 and Exon 12: E11F1 and E11R1; Exon 13: E13F1 and E13R1; Exons 14: E14F1 and E14R1; and Exon 15: E15F1 and E15R1. Sequences of oligonucleotide primers are listed in **Table 1**.

PCR was performed using primers for each intron region (**Table 1**): with a final volume of 25 μl, consisting of genomic DNA (50 ng), dNTPs (0.32 mmol l^{-1} each), two primers (0.2 μmol l^{-1} each), 0.2 μmol l^{-1} Taq polymerase (0.625 IU) and reaction buffer containing $MgCl_2$ 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. 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 *HORMAD1* gene of 80 healthy, fertile control men was also analyzed by direct sequencing and subsequent sequence analysis. Sequence analysis was carried out on the patients with polymorphisms four times and two times on normal controls; the patients and controls were sequenced simultaneously.

Genotyping and statistical analyses

Single-locus analysis. To investigate the role of *HORMAD1* polymorphisms in azoospermia, Fisher's exact test was used to identify meaningful differences. Hardy–Weinberg equilibrium was tested using SNPAlyze software (Windows 2000/XP/Vista/7, 32-bit version; Dynacom, Chiba, Japan). Linkage disequilibrium of all possible two-way combinations of single-nucleotide polymorphisms (SNPs) with the absolute value of the correlation coefficient (D') were tested. P values were determined by χ^2 approximation. Haplotype frequencies were estimated by the maximum likelihood method based on the expectation–maximization (E–M) algorithm under the assumption of Hardy–Weinberg equilibrium. Linkage disequilibrium and haplotype frequencies were tested using SNPAlyze software. P values were determined by χ^2 approximation; $P<0.05$ was considered to be statistically significant.

RESULTS

Mutation analysis of the *HORMAD1* gene in the 30 patients revealed three nucleotide changes: c. 163A>G in exon 3, c. 501T>G (Met 128 Arg) in exon 8, and c. 918C>T in exon 10. These changes, or SNPs, were based on comparisons with the sequences published in the NCBI dbSNP database. Only SNP3, c. 918C>T in exon 10, had been reported previously; the others were newly identified SNPs (**Table 2**). Among the three coding SNPs (cSNPs)—SNP1, 2 and 3, only SNP2 was non-synonymous. The non-synonymous cSNP, SNP2, was tested using two software tools, SIFT (free web site by University of British Columbia, Canada) and PolyPhen (free web site by Harvard Medical School, USA). The SIFT score of the Met128Arg change was 0.96 (>0.50), and the Met128Arg was designated 'benign' based on the PolyPhen analysis. Genotyping for the *HORMAD1* SNP alleles among the 30 patients and 80 controls revealed significantly different genotype distribution and allele frequencies of SNP1 and SNP2 between the two groups ($P<0.005$) (**Table 2**).

At the c. 163A>G site (SNP1), the proportion of GA heterozygote/AA homozygote was 0.33/0.67 in the patient group and 0.00/1.00 in the control group ($P<0.001$). The allele frequency of c. 163A>G was

Table 1 Sequences of oligonucleotide primers used for mutational screening of *HORMAD1*

	Forward primer	*Reverse primer*
Exon 2	E2F1: 5′-TGTATAGGGAATAAAAATAGGA-3′	E2R1: 5′-AATACTTCAGCAAATATCTTCAT-3′
Exon 3	E3F1: 5′-CTTTTGGGGGATTACTAACC3′	E3R1: 5′-ACAAGTGAACTGTCAGGTACG-3′
Exon 4	E4F1: 5′-TGTCACCGCACTCCATCCT-3′	E4R1: 5′-GGGGCACAAAATAAAGAAACA-3′
Exon 5	E5F1: 5′-CCGAAGTTTTCCTCTCCTTG-3′	E5R1: 5′-ATTCAGACTGACCTACACTC-3′
Exons 6, 7	E6F1: 5′-TTTGTTCTTTGTTGTATTTCAGC-3′	E6R1: 5′-TCCTAAAACTCAAGCCCTTTCA-3′′
Exon 8	E8F1: 5′-AGACTGAAGCCCCAAACCCAAAC	E8R1: 5′-CTAATCACCTAAGTTCTCTTTCCAC-3′
Exon 9	E9F1: 5′-GTGGAAAGAGAACTTAGGTGATT-3′	E9R1: 5′-ATTTGAGTGAGGTTATACTTCACA3′
Exon 10	E10F1: 5′-ACAGACAGTCAAGTGAAGAAA-3′	E10R1: 5′-CAATGTTGTTTGGGCTAAGTA-3′
Exons 11, 12	E11F1: 5′-CTTCCAAAGTGCTAGTAATA-3′	E11R1: 5′-TAAACAAATCCACCAGTAATAT-3′
Exon 13	E13F1: 5′-TGGTACTTTCTCAGTTCAGTGG-3′	E13R1: 5′-TTCTTTGTGCCTATGTAGCCTA-3′
Exon 14	E14F1: 5′-TTAAAAAAATTGTATATCCAGC-3′	E14R1: 5′-AATGAGACAGGAGATGTTTA-3′
Exon 15	E15F1: 5′-GAAACCCAGATATAGTTATGCT-3′	E15R1: 5′-AGTAAAAAGTGAATCCATACCA-3′

Table 2 Genotype and allele frequencies of three coding single-nucleotide polymorphisms (cSNPs) in the human *HORMAD1* gene

SNP	*Alteration*		*Genotype frequency*			*Allele frequency*		
	Nucleotide	*Amino acid*	*Genotype/total no. of samples (%)*			*Minor allele/total no. of chromosomes (%)*		
			(G) MA[a]	*Controls*	P *value*	*(A) MA*[b]	*Controls*	P *value*
SNP1	c. 163A>G	Synonymous	GA 10/30 (33.30)	0/80 (0.00)	<0.001	G 10/60 (16.70)	0/160 (0.00)	<0.001
SNP2	c. 501T>G	Met128Arg	GT 4/30 (13.30)	0/80 (0.00)	<0.05	G 4/60 (6.67)	0/160 (0.00)	<0.05
SNP3	c. 918C>T	Synonymous	TT 2/30 (6.67)	8/80 (10.00)	0.725	T 21/60 (35.00)	55/160 (34.40)	1.00

Abbreviation: MA, meiotic arrest.
[a] (G) indicates genotype.
[b] (A) indicates allele.

0.17/0.83 in the patient group and 0.00/1.00 in the control group ($P<0.001$). At the c. 501T>G (SNP2, Met128Arg) site, the proportion of GT heterozygote/TT homozygote was 0.13/0.87 in the patient group and 0.00/1.00 in the control group ($P<0.05$). The allele frequency of c. 501T>G (Met128Arg) was 0.07/0.93 in the patient group and 0.00/1.00 in the control group; the difference was significant ($P<0.05$) (**Table 2**). In the Hardy–Weinberg equilibrium test of the distribution of genotypes for each SNP of the patient group, there were no SNPs showing a significant deviation ($P>0.05$). Haplotype analysis revealed similar haplotype frequencies estimated for all three polymorphisms in the groups ($P>0.05$). Haplotype estimation and linkage disequilibrium analysis also revealed no critical differences ($P>0.05$).

DISCUSSION

We predicted that mutations or polymorphisms in the *HORMAD1* participate in azoospermia caused by MA. Based on these results, we could not state that *HORMAD1* mutations directly caused azoospermia. The number of analyzed patients is not enough to achieve a final decision. However, we identified three cSNPs in *HORMAD1*. The present association study revealed significantly different allele frequencies at SNP1 (163A>G) and SNP2 [501T>G (Met128Arg)] between patients with azoospermia caused by MA and fertile men. These findings indicated that A allele at nt 163 in exon 3 and T allele at nt 501 in exon 8 or their flanking regions may play a role in the disruption of spermatogenesis in Japanese patients. The genotype and allele frequency of SNP1 and SNP2 were much higher in the azoospermia men than in the controls, indicating that *HORMAD1* might play a key role in human spermatogenesis, although the number of patients analyzed was not large enough to allow a definitive conclusion to be drawn. Regardless, the biochemical effects of the changes at SNP1 and SNP2 are unknown. However, both SNP1 and SNP2 were never detected in 80 normal controls. Then, the detected SNP1 and SNP2 might be in linkage with other genes in the same locus. There is a possibility that some genes playing the critical roles in human spermatogenesis will be found in this region in the future. In addition, the RNA including these two SNPs may translate a different protein compared to the normal one. We believe that analysis of 30 men is far too small for an association study. However, azoospermia caused by MA is very rare and our histological diagnostic criteria are very strict; we have DNA samples from more than 5000 patients with azoospermia, but only 30 of these patients had azoospermia caused by MA.

In vitro fertilization is often an efficient way to resolve infertility associated with female factors, but it is not as effective for severe oligospermia in males. Although testicular sperm extraction–intracytoplasmic sperm injection is now performed for many patients with azoospermia, it cannot benefit patients that lack spermatozoa in their testes because of a complete failure in spermatogenesis. Therefore, treatment for infertility due to non-obstructive azoospermia is an important topic for advances in assisted reproductive technology.

In conclusion, this is the first report showing that *HORMAD1* SNP may predispose men to a defect in spermatogenesis, although the causal and potential mechanistic relationships between these *HORMAD1* SNPs and azoospermia remain unclear. Our results may provide insight into the molecular basis of MA as a cause of non-obstructive azoospermia. Additionally, whether this association exists in similar patients from other ethnic groups must be determined in future studies.

AUTHOR CONTRIBUTIONS

TM carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. AT, YM, EK and MN diagnosed the patients, collected the blood samples and purified the DNAs. In addition, they diagnosed all of the patients by histological analysis. MH performed the molecular genetic studies. YS carried out the statistical analysis and revised the manuscript. KS conceived the study, and participated in its design and coordination and helped to draft the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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