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LETTER TO THE EDITOR

Male Infertility

An association study of the single-nucleotide polymorphism c190C>T (Arg64Cys) in the human testis-specific histone variant, *H3t*, of Japanese patients with Sertoli cell-only syndrome

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Dear Editor,

Approximately 20% of men with nonobstructive azoospermia (NOA) are diagnosed with infertility caused by genetic defects.[1] These include chromosomal abnormalities, Y-chromosome microdeletions, and several specific gene mutations/deletions, such as in *DAZ*, *RBMY*, *USP9Y*, *SYCP3*, *HSF2*, *PLK4*, and *TEX11*.[1,2] Several histones have been detected in mammalian testes, and testis-specific variants are specifically and highly expressed during spermatogenesis.[3] Recently, histone H3 variants of human and mouse genomes have been identified by *in silico* hybridization screening.[4] The mouse H3t histone has a human counterpart, H3T (H3.4), and shares a common chaperon recognition motif with H3.1 and H3.2.[5] Knockout mice for *H3t* were first generated in 2017; both male and female *H3t* null mice were viable and healthy, but the male mice were sterile.[6] *H3t* deficiency leads to azoospermia because of the loss of haploid germ cells.[6] The phenotype of *H3t* null male mice is identical to that of Sertoli cell-only syndrome (SCOS) in humans. Therefore, we analyzed human *H3T* in genomic DNA from Japanese patients with SCOS.

This study was approved by the Ethics Committee of Asahikawa Medical University, Japan. Written informed consent was obtained from each participant. Patients with azoospermia secondary to SCOS with no chromosomal abnormalities were recruited from three national hospitals in Japan between 2001 and 2017. Those with defective spermatogenesis caused by infections, seminal tract obstruction, pituitary gland dysfunction, and other causes of testicular disorder were excluded from the study. A total of 178 Japanese patients with SCOS, mainly from Kanazawa, Osaka, and Tokyo, were included, together with 110 fertile Japanese men as normal controls. All patients underwent testicular microdissection with sperm extraction; however, no spermatozoa were present in their testes. A final diagnosis of SCOS was performed by two pathologists. All fathers of the patients were fertile, and none of their brothers suffered from azoospermia.

Direct sequencing of the *H3T* coding region from chromosome 1 was performed on PCR-amplified fragments using peripheral leukocyte DNA and gene-specific primers: H3T-cds1-Fw (5′-CCAACAGGCATGAATATAAG-3′) and H3T-cds1-Rv (5′-ACCCTAATCAGAAGTAGGTA-3′). Fisher's exact test was used to evaluate the statistical significance of *H3T* variants in patients. Hardy–Weinberg equilibrium (HWE) was tested for the variants using SNPAlyze software (Dynacom, Chiba, Japan). Linkage disequilibrium of all possible two-way single-nucleotide polymorphism (SNP) combinations was tested by calculating absolute correlation coefficient values. Haplotype frequencies were estimated by the maximum likelihood method based on the expectation-maximization algorithm under the assumption of HWE. Linkage disequilibrium and haplotype frequency were tested using SNPAlyze. All *P* values were determined by Chi-square approximation, with significance assumed at $P < 0.05$. The potential pathogenicity of *H3T* mutations was predicted by *in silico* analysis using three different software packages: MutationTaster http://www.mutationtaster.org/), Polyphen-2 (http://genetics.bwh.harvard.edu/pph 2), and SIFT (http://sift.jcvi.org/).

The *H3T* coding region was sequenced in all 178 patients with SCOS. Seven variants were detected in this patient group (**Table 1**) – SNP1: c15G>A, Chr1:228613012, rs199672652; SNP2: c88G>A, Chr1:228612939, rs531385963; SNP3: c109A>C, Chr1:228612918, rs201151997; SNP4: c135C>T, Chr1:228612892, rs56336130; SNP5: c158G>A, Chr1:228612869, rs201904037; SNP6: c189A>C, Chr1:228612838, rs2230656; and SNP7: c190C>T, Chr1:228612837, rs201294185. All seven SNPs have been reported previously; however, we found no information about their frequencies in the Japanese population.

Allele and genotype distributions of the seven SNPs in the patients with SCOS and 110 controls are listed in **Table 1**. A significant association with SCOS was observed only for SNP7 ($P = 0.0465$: genotypes and $P = 0.0478$: alleles). We also found that the distributions of SNP7 (c190C>T [Arg64Cys]) genotypes and allele frequencies

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Table 1: Genotype and allele frequencies for seven coding single-nucleotide polymorphisms in human H3T identified in 178 patients with SCOS and azoospermia and 110 normal controls

SNP	*Alteration*		*Genotype frequency*			*Allele frequency*		
	Nucleotide	*Amino acid*	*Genotype/total patients (%)*			*Minor allele/total chromosomes (%)*		
			(G) SCOS	*Control*	*P*	*(A) SCOS*	*Control*	*P*
SNP1	c15G>A		(AG) 3/178 (1.7)	0/110 (0)	0.289	(A) 3/356 (0.84)	0/220 (0)	0.291
SNP2	c88G>A	NS	(AG) 2/178 (1.1)	1/110 (0.9)	1.000	(A) 2/356 (0.56)	1/220 (0.5)	1.000
SNP3	c109A>C	NS	(CA) 16/178 (9.0)	12/110 (10.9)	0.683	(C) 16/356 (4.49)	16/220 (7.3)	0.190
SNP4	c135C>T		(TC) 8/178 (4.5)	6/110 (5.5)	0.781	(T) 8/356 (2.24)	6/220 (2.7)	0.783
SNP5	c158G>A	NS	(TC) 1/178 (0.6)	0/110 (0)	1.000	(T) 1/356 (0.28)	0/220 (0)	1.000
SNP6	c189A>C		(CA) 62/178 (34.8) (CC) 33/178 (18.5)	47/110 (42.7) 10/110 (9.1)	0.068	(C) 128/356 (36.0)	67/220 (30.5)	0.205
SNP7	c190C>T	NS	(TC) 7/178 (3.9)	0/110 (0)	0.0465*	(T) 7/356 (0.28)	0/220 (0)	0.0478*

*P<0.05, the patient group compared with the control group. SCOS: Sertoli cell-only syndrome; NS: nonsynonymous substitution; SNP: single-nucleotide polymorphism

differed significantly between patients and controls. Seven patients carried the T allele at SNP7, but this was absent from all 110 controls. Therefore, this T allele might have been inherited from their mothers. The SNP7 change was predicted to be "deleterious" and "disease causing" in an *in silico* analysis using SIFT and MutationTaster; however, it was predicted to be "benign" by PolyPhen-2. Haplotype analysis revealed similar estimated haplotype frequencies for all seven SNPs (P = 0.2595–1.0000). Haplotype estimation and linkage disequilibrium analysis also revealed no statistically significant critical differences between groups (P > 0.05).

We hypothesized that mutations or polymorphisms in *H3T* may be associated with SCOS. An earlier study demonstrated that human nucleosome assembly protein 2 (hNap2) catalyzes the formation of H3t-containing nucleosomes *in vitro*.[7] Previous mutational analyses using recombinant H3t revealed that its Val111 residue plays an essential role in hNap2-mediated nucleosome formation.[7] However, the SNPs identified in the present study do not change the Val111 residue of H3t, indicating that they do not have an impact on nucleosome formation.

This study had a number of limitations. First, the sample size was not determined before the start. Second, the number of patients analyzed was not sufficient to allow a definitive conclusion to be drawn. However, retrospective power calculations demonstrated that this study had 80% power to detect an increased genotype prevalence of 23.4% in cases against a control of 10%, and 86% power to detect an increased genotype prevalence of 9% against a control of 1%. Third, all patients were from Kanazawa, Osaka, or Tokyo, so were not representative of all areas of Japan. The *H3t* null male mice are sterile, but the patients we identified with SNP7 are heterozygous, so clearly there are biological discrepancies between mice and humans for this genotype.

In conclusion, our results provide insights into the molecular basis of SCOS as a possible cause of NOA. It remains to be determined whether any association between this variant and azoospermia caused by SCOS exists in similar patients from other ethnic groups.

AUTHOR CONTRIBUTIONS

TM, GM, and HU performed molecular analysis. MI, TS, and HO examined and diagnosed the patients and collected DNA samples. TM, YS, and KS wrote and revised the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declared no competing interests.

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