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Single-nucleotide polymorphisms in the human *RAD21L* gene may be a genetic risk factor for Japanese patients with azoospermia caused by meiotic arrest and Sertoli cell-only syndrome

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Abstract

Genetic mechanisms are implicated in some cases of male infertility. Recently, it was demonstrated that male mice lacking RAD21L exhibited azoospermia caused by meiotic arrest. Mouse RAD21L is a functionally relevant meiotic α-kleisin that is essential for male fertility. Therefore, we hypothesized that *RAD21L* mutations or polymorphisms may be associated with male infertility, especially azoospermia secondary to meiotic arrest. To determine if *RAD21L* defects are associated with azoospermia in groups of patients with meiotic arrest, we performed direct sequencing of the *RAD21L* coding regions in 38 Japanese patients with meiotic arrest and in 200 normal controls. Three coding single-nucleotide polymorphisms (SNP1–SNP3) were detected in the meiotic arrest patient group. Sertoli cell-only syndrome is considered a common cause of nonobstructive azoospermia. For comparison, the *RAD21L* coding regions in which SNP1–SNP3 were detected were sequenced in 140 patients with Sertoli cell-only syndrome. Statistical analyses were used to compare the two groups of patients with the control group. Genotype and allele frequencies of SNP2 and SNP3 were notably higher in the two patient groups compared with the control group (Bonferroni adjusted p-value <0.016). These results suggest a critical role for *RAD21L* in human spermatogenesis.

Keywords: azoospermia, meiotic arrest, *RAD21L*, SCOS, single-nucleotide polymorphism

Introduction

Infertility affects approximately 15% of couples trying to conceive, and approximately half of these cases involve male factors (Krausz *et al.*, 2015). Genetic causes of azoospermia include chromosomal abnormalities, Y chromosome microdeletions, and specific mutations/deletions of several Y chromosomal genes (Miyamoto *et al.*, 2015). Approximately 10% of all male infertility cases (<1% of all men) are associated with nonobstructive azoospermia (Jarow *et al.*, 1989; Anniballo *et al.*, 2000; Vloeberghs *et al.*, 2015). This is a histopathological diagnosis based on testicular biopsy findings including hypospermatogenesis, maturation arrest (also called meiotic arrest (MA)), or Sertoli cell-only syndrome (SCOS). 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 (Nasmyth, 2002). Mutations in the RNA binding motif protein gene *RBMY* (Yq11.223) and the synaptonemal complex protein gene *SYCP3* (12q23) are known to cause azoospermia secondary to MA (Elliott *et al.*, 1997; Matzuk & Lamb, 2008; Miyamoto *et al.*, 2003). Genetic polymorphisms may also increase susceptibility to some forms of male infertility including azoospermia via MA (Barda *et al.*, 2014; Eggers *et al.*, 2015; Lu *et al.*, 2013).

The cohesion subunit RAD21 is a member of the α-kleisin family of proteins, and is present from fish to mammals (Gutiérrez-Caballero *et al.*, 2011; Ishiguro *et al.*, 2011; Lee & Hirano, 2011). A RAD21 paralogue (RAD21L) is abundantly transcribed in the testis, and is suggested to be a canonical cohesion subunit. RAD21L interacts with the structural maintenance of chromosomes proteins SMC3 and SMC1α/β, and with the stromal antigen STAG3 (Gutiérrez-Caballero *et al.*, 2011; Ishiguro *et al.*, 2011; Lee & Hirano, 2011).

Moreover, RAD21L localizes along the axial elements of the synaptonemal complex of mouse meiocytes (Herrán *et al.*, 2011). Male mice lacking RAD21L are defective in the full synapsis of homologous chromosomes at the zygotene stage, which leads to total azoospermia and consequently infertility (Herrán *et al.*, 2011).

To determine if RAD21L plays a critical role in human spermatogenesis, we performed mutational analysis of the human *RAD21L* gene using genomic DNA from Japanese patients with azoospermia caused by MA and SCOS.

Materials and methods

Patients and controls

This study was approved by the Ethics Committee of Asahikawa Medical University, Japan. Written informed consent for this study was obtained from each participant. Patients with azoospermia secondary to MA without any chromosomal abnormalities were selected and histopathologically examined. A total of 38 Japanese patients with azoospermia due to MA and 140 patients with azoospermia caused by SCOS were included in the study. Two hundred fertile men who had produced at least one child and lacked any history of infertility treatment were included as normal controls. All participants were Japanese.

Mutation screening

Peripheral blood leukocytes were obtained from 38 and 140 patients with MA and SCOS, respectively, and 200 normal controls. Genomic DNA was extracted using a Wizard™ Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The *RAD21L* cDNA sequence (HQ603827.1) was compared

with the human genomic sequence (NC_018931) by BLAST, and all exon-intron borders were determined. The PCR primer sets used to amplify the coding regions are listed in Table 1. Nested PCRs were performed using the DNAs from the MA patients and the normal controls as templates. The PCR products were purified using a QIAquick PCR Purification kit (Qiagen, Tokyo, Japan). Direct sequencing of each product was performed. The DNAs from the SCOS patients were sequenced using the same procedure, but only in the three regions in which SNPs were detected.

[Table 1 near here]

Genotyping and statistical analysis

Fisher's exact test was used to evaluate the statistical significance of *RAD21L* variants in the azoospermia patients. Bonferroni *post hoc* corrections were applied to correct for comparisons among the three *RAD21L* variants discovered (adjusted $P < 0.016$).Hardy-Weinberg equilibrium (HWE) was tested for the variants using SNPAlyze software (Dynacom, Chiba, Japan). Linkage disequilibrium of all possible two-way 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 software. All p-values were determined by χ^2 approximation, with significance considered at p-values <0.05.

Results

The *RAD21L* coding region was sequenced in 38 MA patients. No apparent mutations were found, but three variants were detected in the patient group compared with the control group (Table 2). The detected variants were: c.454C>A (SNP1) (chr20: 1234085) in exon 4;

c1268A>C (SNP2) (chr20: 1243196) in exon 10; and c1610G>A (SNP 3) (chr20: 1254311) in exon 14. Only SNP1 has been reported previously (rs755285899); however we were unable to find information on its frequency in the Japanese population. The allele and genotype distributions of the three SNPs in both MA patients and controls are shown in Table 2. Allele and genotype distributions adhered to HWE ($p > 0.05$). Association was observed for SNP2 ($p < 0.016$) and SNP3 ($p < 0.016$), but not for SNP1 (Table 2). For comparison, 140 patients with azoospermia caused by SCOS were also analysed but only in the coding regions containing SNP1–SNP3. The frequencies of SNP2 and SNP3 were also higher in the SCOS patients compared with the control group ($p < 0.016$).

[Table 2 near here]

Haplotype analysis revealed similar estimated haplotype frequencies for all three SNPs ($p > 0.05$). Haplotype estimation and linkage disequilibrium analysis also revealed no critical differences ($p > 0.05$). In addition, SNP2 and SNP3 were detected in different individuals in both patient groups.

Discussion

In this study, we hypothesized that mutations or polymorphisms in *RAD21L* may be associated with azoospermia caused by MA and SCOS. No *RAD21L* mutations that directly cause azoospermia were detected; however, the small numbers of patients analysed, especially those with MA, are not enough to reach an absolute conclusion. Nevertheless, we did identify three SNPs within the *RAD21L* coding regions. Moreover, we found that the distribution of the SNP2 (1268A>C (His423Pro)) and SNP3 (1610G>A (Ser537Asn))

146 genotypes was significantly different between Japanese azoospermia patients with MA or
147 SCOS, and fertile controls ($p < 0.016$). This indicates that the C allele of SNP2 (in exon 10)
148 and the resulting Pro amino acid substitution, and the A allele of SNP3 (in exon 14) and the
149 resulting Asn amino acid substitution, or their flanking regions, may play a role in the
150 disruption of spermatogenesis in Japanese patients. Again, the numbers of patients analysed
151 were not large enough to allow a definitive conclusion to be drawn. The function of the
152 SNPs at positions 1268 and 1610 in the gene sequence is unknown; however, the SNP2 and
153 SNP3 changes were predicted "benign" in an *in silico* analysis using PolyPhen2 and
154 MutationTaster. Azoospermia with MA and SCOS is very rare, and our histological
155 diagnostic criteria were very strict. Indeed, we have DNA samples from more than 5000
156 patients with azoospermia, and only 38 and 140 of them had azoospermia caused by MA
157 and SCOS. Among the others, 15 and 1286 cases were hypospermatogenesis and
158 obstructive azoospermia, respectively. For the remaining patients, we could not determine if
159 they had obstructive or nonobstructive azoospermia because most of them were too old
160 when we began to carry out the microdissection testicular sperm extraction (MD-TESE)
161 procedure. Further, some patients did not agree to have MD-TESE or histological biopsy.
162 In conclusion, we suggest that the C and A variants of the SNPs at positions 1268
163 and 1610 might be associated with azoospermia secondary to MA and SCOS in human, but
164 the relationship between the identified SNPs and the mechanistic cause of azoospermia has
165 not yet been determined. Our results provide insight into the molecular basis of MA and
166 SCOS as possible causes of nonobstructive azoospermia. It remains to be determined
167 whether an association between these variants and azoospermia secondary to MA and
168 SCOS exists in similar patients from other ethnic groups.
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Declaration of interest statement

The authors report no conflicts of interest.

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Table 2. Genotype and allele frequencies for three coding SNPs in the human *RAD21L* gene identified in 38 azoospermia patients with meiotic arrest, 140 with Sertoli cell-only syndrome, and 200 normal controls

SNP	Alteration		Genotype frequency			Allele frequency		
	Nucleotide	Amino acid	Genotype/Total no. of samples (%)			Minor allele/Total no. of chromosomes (%)		
			(G) MA[a]	SCOS[b] p-value[c]	Control	(A) MA[a]	SCOS[b] p-value[c]	Control
SNP1[d]	454C>A	NS[e]	(CA) 12/38 (31.6)	46/140 (32.9)	53/200 (26.5)	(A) 58/76 (76.3)	222/280 (79.3)	297/400 (74.3)
						0.7749	0.1427	
			(AA) 23/38 (60.5)	88/140 (62.9)	122/200 (61.0)			
			0.7289	0.0226				
SNP2	1268A>C	NS	(AC) 4/38 (10.5)	10/140 (7.14)	2/200 (1.00)	(A) 4/76 (5.26)	10/280 (3.57)	2/400 (0.50)
			0.007*	0.005*		0.007*	0.005*	
SNP3	1610G>A	NS	(GA) 3/38 (7.89)	6/140 (4.29)	0/200 (0.00)	(A) 3/76 (3.95)	6/280 (2.14)	0/400 (0.00)
			0.004*	0.005*		0.004*	0.005*	

[a]Meiotic arrest. [b]Sertoli cell-only syndrome. [c]The p-values are for the patient group compared with the control group. [d]SNP1 is known SNP rs755285899. [e]NS, nonsynonymous substitution. * p-value $< p<0.016$.