GENETICS

Single nucleotide polymorphism in the *UBR2* gene may be a genetic risk factor for Japanese patients with azoospermia by meiotic arrest

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Abstract
Purpose To investigate the association between the *UBR2* gene and the risk of azoospermia caused by meiotic arrest.
Methods Mutational analysis of the *UBR2* gene was performed using DNA from 30 patients with azoospermia by meiotic arrest to 80 normal controls.

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Results The genotypic and allelic frequencies of c.1,066A>T variant were significantly higher in patient than control groups ($p<0.001$).
Conclusion The c.1,066A>T variant in the *UBR2* gene is associated with increased susceptibility to azoospermia caused by meiotic arrest.

Introduction

Genetic causes of azoospermia in humans include Y-chromosome microdeletions, and specific gene mutations, for example *DAZ, RBMY, USP9Y, SYCP3, PRM1, SPATA16, AURKC* and *KLHL10* [1–9]. As Y-chromosome deletions account for only 16% of men with infertility [10], azoospermia in many infertile men may be caused by autosomal gene mutations. Genetic polymorphisms may also increase susceptibility to some forms of male infertility; e.g., the human *BCL2* and *eNOS* genes are linked to male infertility [11, 12]. 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 [13]. Genetic regulation of meiosis is poorly understood in mammals 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, Meisetz, Cdk2, Hop2*, have been identified by disruption experiments in embryonic stem cells [14–24].

Mouse UBR2, a homolog of UBR1, was identified in 2003 [25]. The substrate-binding properties of UBR2 are similar to those of UBR1 and it is the second E3 of the mammalian N-end rule pathway. While most UBR2$^{-/-}$ female mice die as embryos, UBR2$^{-/-}$ males are viable but infertile due to the postnatal degeneration of the testes. Although the gross architecture of UBR2$^{-/-}$ testes is normal and spermatogonia are intact, UBR2$^{-/-}$ spermatocytes are arrested between leptotene/zygotene and pachytene by meiotic failure [25]. UBR2 localizes to meiotic chromatin regions, including unsynapsed axial elements linked to chromatin inactivation, and mediates transcriptional silencing via the ubiquitination of histone H2A [26].

In the present study, we analyzed possible associations between UBR2 mutations and azoospermia caused by meiotic arrest (MA) in humans.

Materials and methods

Patients and controls

Azoospermia 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 was carried out by histological examination. Every patient would carry out more than one pathologic test. Chromosome analysis of peripheral lymphocytes showed a karyotype of 46 and XY in all patients. No patients had Y chromosome microdeletions. A total of 30 Japanese patients with azoospermia caused by MA were included in the study; 80 healthy, pregnancy-proven, fertile men were also examined as controls. All normal controls were Japanese men and had normal sperm inspections, in addition to all having a child by spontaneous pregnancy. However, all of them were medical doctors, indicating their average intelligence may be higher than the average of the general Japanese population. 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 UBR2 gene. Their full-length cDNA sequences (BC024217.2) were compared to human genomic sequences (NT_007592.15) by BLAST, and all exon-intron borders were determined.

The following UBR2 primers were used for mutational analysis. Exon 1: E1F1 and E1R1; Exon 2: E2F1 and E2R1; Exons 3: E3F1 and E3R1; Exon 4: E4F1 and E4R1; Exon 5: E5F1 and E5R1; Exons 6: E6F1 and E6R1; Exon 7: E7F1 and E7R1; Exon 8: E8F1 and E8R1; Exon 9: E9F1 and E9R1; Exon 10: E10F1 and E10R1; Exon 11: E11F1 and E11R1; and Exon 12: E12F1 and E12R1. Sequences of oligonucleotide primers are listed in Table 1.

PCR was performed using primers for each intron region (Table 1). PCR was performed in a final volume of 25 μl, consisting of genomic DNA (50 ng), dNTP (0.32 mM each), each primer (0.2 μM), 0.2 μM 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 direct sequencing of each product was conducted. To confirm the role of the detected polymorphisms in azoospermia, the coding region of the UBR2 gene of 80 healthy, fertile control men was also analyzed by direct sequencing 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 UBR2 polymorphisms in azoospermia, Fisher's exact test was used to determine a meaningful difference. $P<0.05$ 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 χ^2 approximation. Importance was determined with a $P<0.05$ as described above. Haplotype frequencies were estimated by the method of maximum likelihood based on the expectation-maximization (E-M) algorithm under the assumption of HWE. Linkage disequilibrium and haplotype frequency were tested using SNPAlyze software. P values were determined by χ^2 approximation; significance was determined at the $P=0.05$ level.

Results

Mutation analysis of the UBR2 gene revealed four nucleotide changes among the 30 patients: c.322C>T in

Table 1 Sequences of oligonucleotide primers used for mutational screening of *UBR2*

	Forward primer	Reverse primer
Exon 1	E1F1: 5′-TGTACCTACAGCACCCAGAA-3′	E1R1: 5′-ATTACCCCACAAGGCGAGG-3′
Exon 2	E2F1: 5′-CGGTGGTGAGTGTTGTAAAC-3′	E2R1: 5′-TTGTAATACAGATTTTTAAGCAG-3′
Exon 3	E3F1: 5′-ATTATGGAGACGCAGGTCCT3′	E3R1: 5′-TCTACACTGGCACCCTATAT-3′
Exon 4	E4F1: 5′-AACATTGAGAGGTTTGGCAT-3′	E4R1: 5′-CAACTGTGTACTATTAGCAAC-3′
Exon 5	E5F1: 5′-TGGTAATGTATAGGATCAAAT-3′	E5R1: 5′-ACAGGAAGAAATCTCCACC-3′
Exon 6	E6F1: 5′-GTAGTATCACAGAGCATAGC-3′	E6R1: 5′-GTTATACCCAATATACATTTG-3″
Exon 7	E7F1: 5′-CTTGGAAAAGTTAGCATAGC-3′	E7R1: 5′-CTATATCAATCCTACACAGC-3′
Exon 8	E8F1: 5′-GATCTGAATCAAGTAAGGCA-3′	E8R1: 5′-AAAAATATCCAGTATGACCAAA-3′
Exon 9	E9F1: 5′-GTGTGTGATGTGAGATTTGA-3′	E9R1: 5′-ATATAAAGTCCATTTAAGTTGG-3′
Exon 10	E10F1: 5′-AAAATGCTGTGTTGGCTGGG-3′	E10R1: 5′-AGGAGTCTCACGGCTAAAAT-3′
Exon 11	E11F1: 5′-TCTCCCCCTTGTTGTTTAGAA-3′	E11R1: 5′-TACCAGATGACTTTGATATTCA-3′
Exon 12	E12F1: 5′-TTTAGGAAGCACTAACATCTT-3′	E12R1: 5′-TATTCAGGAAGTAAACAAGAG-3′

exon 1, c.821C>A (His182Asn), c.829A>T in exon 5, and c.1,066A>T in exon 6. These SNPs were compared to the NCBI dbSNP database and all of them were confirmed to be novel SNPs (Table 2). Among the four cSNPs (SNP1-SNP4), only SNP2 was non-synonymous. Genotyping for the *UBR2* SNP alleles among the 30 patients and 80 controls revealed significantly different genotype distribution and allele frequency of SNP4 between the two groups (Table 2).

At the c.1,066A>T site, the proportion of TA heterozygote/AA homozygote was 0.20/0.80 in the patient group and 0.00/1.00 in the control group ($P<0.001$). The allele frequency of c.1,066A>T was 0.10/0.90 in the patient group and 0.00/1.00 in the control group; the difference was significant ($P<0.001$). Haplotype analysis revealed similar haplotype frequencies estimated for all four polymorphisms in the groups ($P>0.05$). Haplotype estimation and LD analysis also revealed no critical differences ($P>0.05$).

Discussion

In this study, we hypothesized that mutations or polymorphisms of the *UBR2* participate in azoospermia caused by MA. We could not detect any *UBR2* mutations that directly cause azoospermia in the 30 patients with MA. Instead, we identified four novel cSNPs in the gene. The present association study revealed that the genotype distribution for SNP4 (c.1,066A>T) is significantly different between Japanese azoospermic patients and healthy controls ($P<0.001$). This finding suggests that allele A at nucleotide 1,066 in exon 6, 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. In addition, as the encoded amino acids remain unchanged, the function of the SNP is unknown. We believe that a cohort of 30 men is far too small for an association study. However,

Table 2 Genotype and allele frequencies of four coding single-nucleotide polymorphisms (cSNPs) in the human *UBR2* gene of 30 azoospermic patients with meiotic arrest and 80 control individuals

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	322C>T	Synonymous	TT 6/30 (20.0)	17/80 (21.3)	0.886	T 29/60 (48.3)	78/160 (48.8)	0.956
SNP2	821C>A	His182[His, Asn]	AC 1/30 (3.33)	1/80 (1.25)	0.468	A 1/60 (1.67)	1/160 (0.625)	0.469
SNP3	829A>T	Synonymous	TA 6/30 (20.0)	16/80 (20.0)	1.000	T 6/60 (10.0)	18/160 (11.3)	0.792
SNP4	1,066A>T	Synonymous	TA 6/30 (20.0)	0/80 (0.00)	<0.001[c]	T 6/60 (10.0)	0/160 (0.00)	<0.001[c]

[a] (G) indicates genotype. [b] (A) indicates allele. [c] Statistically significant

azoospermia by MA is very rare and our histological diagnostic criteria are very strict—i.e., we have DNA samples from more than 5,000 patients with azoospermia, of which only 30 had azoospermia caused by MA.

In vitro fertilization has been proven to be an efficient way to resolve infertility due to female factors, but not as effective for severe oligospermia in the male partner. Although TESE-intracytoplasmic sperm injection is now performed for patients with azoospermia, it cannot 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 preeminent topic for assisted reproductive technology.

In conclusion, this is the first report showing that *UBR2* SNP may predispose men to a defect in spermatogenesis, although the mechanism of the SNP in azoospermia remains unclear. Our results may provide insight into the molecular basis of meiotic arrest as a cause of non-obstructive azoospermia. It remains to be confirmed whether an association exists in similar patients from other ethnic groups.

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