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Single-nucleotide polymorphisms in ETV5: a risk factor for Sertoli cell-only syndrome in Japanese men?

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Summary

Purpose of investigation: Approximately 15% of couples are infertile, and male and female factors are believed to be equally important. Genetic mechanisms are implicated in aspects of male infertility and male mice lacking the gene *Etv5* exhibited azoospermia with loss of germ cells: a phenotype identical to human Sertoli cell-only syndrome (SCOS). The *ETV5* gene family encodes transcription factors sharing the evolutionarily conserved DNA-binding ETS domain. Therefore, the authors hypothesized that *ETV5* variants might be associated with azoospermia caused by SCOS. *Materials and Methods: ETV5* was sequenced in 140 Japanese men with SCOS and in 116 healthy controls using the Sanger method. *Results:* Four single-nucleotide polymorphism variants (SNPs 1-4) were detected in the patient group. An association study was performed for patients and controls. Two *ETV5* variants, SNP2 and SNP3, were nominally associated with susceptibility to SCOS ($p = 0.002$ and $p < 0.001$, respectively). *Conclusion:* These results indicate a potential role for *ETV5* in human spermatogenesis.

Key words: Azoospermia; *ETV5*; Sertoli cell-only syndrome; Single-nucleotide polymorphism.

Introduction

Infertility affects about 15% of couples wishing to have children, and approximately half of these cases involve male factors [1]. Genetic causes account for 10–15% of severe forms of male infertility, including chromosomal aberrations, Y chromosome microdeletions, and gene mutations and polymorphisms [2].

Approximately 10% of all cases of male infertility (< 1% of all men) involve nonobstructive azoospermia (NOA) [3, 4]. This is a histopathological diagnosis based on testicular biopsy findings including hypospermatogenesis, maturation arrest, or Sertoli cell-only syndrome (SCOS): first described by Del Castillo *et al*. in 1947 [5]. Germ cells are completely absent in men with SCOS, which therefore represents the most severe type of NOA. Although the pathogenesis of SCOS remains unknown, it is postulated to occur before or during the premeiotic proliferation phase in spermatogonia [6]. However, spermatogonial stem cells (SSCs) are known to be essential for maintaining human male fertility by providing the foundation for spermatogenesis [7]. Spermatogenesis is a complex process by which SSCs self-renew and differentiate into spermatozoa under the coordination of the testicular microenvironment: its SSC niche [8]. Sertoli cells surrounding the male germ cells are the most critical component of this niche [9]. Because they regulate the division and differentiation of SSCs so precisely, any abnormalities including their numbers, maturation, and/or function can result in aberrant spermatogenesis, which eventually leads to azoospermia [10].

The E-twenty-six variant gene 5 (*ETV5;* NM_004454.2) encodes the transcription factor Etv5 that is required for the fertility of male [11-13] and female [14] mice. Homozygous deletion of exons 2–6 of the mouse *Etv5* gene resulted in progressive loss of male germ cells following the first wave of spermatogenesis and led to a complete loss of all the germ cells and sterility in adulthood [11-13]. This phenotype is the same as SCOS in humans.

An association study on the human *ETV5* gene was performed on patients with SCOS and NOA and in fertile controls [15]. This analyzed Australian men (65 with SCOS, 53 with NOA and 242 controls). It was demonstrated that differences in the allele frequency between patients (SCOS and NOA) and controls were based on only one single nucleotide polymorphism (SNP) in intron 11, 51 nucleotides downstream from exon 11.

To determine whether the human *ETV5* gene might be associated with Japanese patients with SCOS and azoospermia, the *ETV5* gene was sequenced using genomic DNA. An association study was performed in 140 patients with SCOS and 116 healthy male controls.

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Table 1. — *Summary of association analysis for four SNPs detected in patients with SCOS and controls.*

SNP	Chr	Position	Allele (1/2)	SCOS 11	SCOS 12	SCOS 22	Control 11	Control 12	Control 22	RAF Case	RAF Control	p^a	OR (95%CI)[b]	p^c	OR (95%CI)[d]
SNP1	3	185.823.712	G/A	0	140	0	15	101	0	0.50	0.44	0.182	1.28 (0.90-1.81)	<0.001	0.02 (1.11-1.42)
SNP2	3	185.823.674	G/A	64	76	0	80	36	0	0.27	0.16	0.0020	2.03 (1.30-3.16)	<0.001	2.64 (1.58-4.42)
SNP3	3	185.797.835	C/A	110	30	0	116	0	0	0.11	0.00	<0.001	56.6 (3.44-931)	<0.001	64.3 (3.89-1065)
SNP4	3	185.774.813	G/T	38	73	29	33	64	19	0.47	0.44	0.478	1.14 (0.80-1.61)	0.423	1.33 (0.70-2.53)

RAF = risk allele frequency. OR or *p* values are statistically significant after Bonferroni correction.
[a]: *p* value of the Fisher's exact test with an allele frequency model.
[b]: Odds ratios (OR) and confidence intervals (CI) were calculated using the non-susceptible allele as reference.
[c]: *p* values for Fisher's exact test with a dominant-effect model.
[d]: Odds ratios (OR) and confidence intervals (CI) were calculated using the non-susceptible genotype as reference.

Materials and Methods

The Ethics Committee of Asahikawa Medical University, Japan approved this study. Written informed consent for this study was obtained from each participant. Patients with azoospermia secondary to SCOS without any chromosomal abnormalities were selected and examined histopathologically. A total of 140 patients with azoospermia caused by SCOS were included in the study. The control group included 116 men who had fathered at least one child and lacked any history of infertility treatment. All the participants were Japanese.

Peripheral blood leukocytes were obtained from the patients and controls. Genomic DNA was extracted using genomic DNA purification kits according to the manufacturer's instructions. The primer sets for polymerase chain reaction (PCR) amplification were constructed according to a previous report [15]. PCR was carried out and the products were purified using PCR purification kits and sequenced directly.

Fisher's exact test was used to evaluate any statistical significance for differences in *ETV5* variants among the patient group. Bonferroni *post hoc* corrections were applied to correct for comparisons among the four *ETV5* variants discovered (adjusted $p < 0.0125$). Hardy–Weinberg equilibrium (HWE) was tested for variants using SNPAlyze software. Linkage disequilibrium (LD) of all possible two-way combinations of SNPs with the absolute value of the correlation coefficient (D¢) was tested. *P* values were determined by c^2 approximation and $p < 0.05$ was considered as a nominal association. Multiple tests for allele evaluations were subjected to Bonferroni's correction. Haplotype frequencies were estimated by the maximum likelihood method based on the expectation–maximization (E–M) algorithm under an assumption of HWE. Linkage disequilibrium and haplotype frequencies were also tested using SNPAlyze software. The *p* values were determined by c^2 approximation; significance was determined at $p < 0.05$.

Results

The *ETV5* coding regions were sequenced in the 140 patients with SCOS, revealing no apparent mutations, but four variants were found (Table 1). The variants included c.-55G>A (SNP1) (chr3: 185823712) and c.-17G>A (SNP2) (chr3: 185823674) at the 5¢-untranslated region (5¢-UTR) of exon 2; c.421C>A (p.Leu141Ile) (SNP3) (chr3: 185797835) at exon 7; and c.1209+51G>T (SNP4) (chr3: 185774813) in intron 11. Only SNP4 had been reported previously (rs59852126) (Table 1). The four SNPs were also genotyped in the 116 controls. Allele and genotype distributions of the four SNPs in both patients and controls are shown in Table 1. Allele and genotype distributions were consistent with HWE. Significant associations with SCOS were observed for SNP2 ($p = 0.002$) and SNP3 ($p < 0.001$) even after applying the Bonferroni correction, but not for SNP1 or SNP4 (Table 1). Haplotype analysis revealed similar nonsignificant haplotype frequency estimates for all the four variants ($p > 0.05$). Haplotype estimation and LD analysis also revealed no significant differences between the patient and control groups ($p > 0.05$).

Discussion

Recent advances in molecular biology and mouse models have facilitated the identification of many fertility-related genes and their biological pathways. More than 400 genes have been shown to be critical for male and/or female fertility [16]. However, only a few genes have been proven to be associated with human male infertility. In 2012, O'Bryan *et al*. reported 12 *ETV5* SNPs—five in exons and seven in introns—in Australian and American men [15]. Differences in allele frequency between the Australian patients (SCOS+NOA) and the Australian control group were only found for SNP4. However, the present authors could not replicate this association in our Japanese cases of SCOS and there was also no association between SNP4 and infertility observed in the American cases [15]. Thus, there might be ethnic differences regarding the influence of SNP4 on male infertility.

The present authors found three hitherto unreported *ETV5* SNPs in these Japanese patients with SCOS: SNP1, SNP2, and SNP3. SNP3 is a coding region (p.Leu141Ile) that was significantly associated with SCOS ($p < 0.001$) but was not observed in the controls. Therefore, it might be not be a mere harmless polymorphism. Functional analysis of SNP3 will be needed to elucidate its association with SCOS.

In conclusion, a significant association of the *ETV5* SNP3 with infertility was found in these Japanese men with SCOS. It remains to be elucidated whether *ETV5* variants

cause abnormal spermatogenesis or show any association with infertility in other ethnic groups.

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