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Genetic association of *UBE2B* variants with susceptibility to male infertility in a Northeast Chinese population

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ABSTRACT. The ubiquitin-conjugating enzyme 2B gene (*UBE2B*) is involved in the regular and symmetric organization of the fibrous sheath of sperm flagella. This study aimed to examine the relationship between single nucleotide polymorphisms (SNPs) in *UBE2B* and infertility in Northeast Chinese men. We carried out a polymerase chain reaction-restriction fragment length polymorphism analysis for SNPs in 312 fertile males and 388 infertile males in Northeast China. Taking advantage of the high degree of linkage disequilibrium among SNPs surrounding *UBE2B* ($r^2 > 0.90$), we selected 2 haplotype-tagging SNPs with a minor allele frequency of 5% or greater (rs17167484: g.-293T>G and rs3777373: g.20016A>G) that captured the majority of the genetic variations in a 40-kbp region of this gene. No significant differences

between cases and controls were found in the allelic and genotype distribution of the 2 SNPs. However, the haplotype analysis for the 2 SNPs showed that the GA haplotype was significantly associated with a greater than 3-fold decreased risk of male infertility (P = 0.003). Because the frequency of the GA haplotype (1.1%) is relatively low in Chinese men, such a significant finding may occur by chance, but the results are still significant after multiple comparison adjustments (P = 0.012 after Bonferroni's correction). We conclude that the *UBE2B* polymorphisms g.-293T>G, g.20016A>G and g.9157A>G are not associated with male infertility, and the GA haplotype is likely a protective factor for male fertility in Northeast Chinese men.

Key words: *UBE2B*; Single nucleotide polymorphism; Haplotype; Male infertility

INTRODUCTION

Approximately 15% of couples who attempt to conceive are unsuccessful within a 2-year period. The causal frequency of male and female infertility is relatively equal. More than half of male infertility cases have uncertain causes and are thus idiopathic. In male germ cells, many dramatic morphological changes occur during spermatogenesis, particularly in haploid spermatids after meiotic division. In human men, the complicated processes of spermatogonial stem cell proliferation and differentiation, meiosis, generation of haploid germ cells, and morphogenesis of developing sperm in the seminiferous tubules take 2 months to complete (Nishimune and Tanaka, 2006). During the differentiation process, some sperm-specific proteins involved in flagellum formation, signal transduction, and energy metabolism play important roles in maintaining the shape and function of sperm.

In mice, ubiquitin-conjugating enzyme (UBE2B) is involved in the regular and symmetric organization of the fibrous sheath of sperm flagella. Baarends et al. (2003) have reported that the rate of apoptosis of primary spermatocytes in *Ube2b* knockout mice increases during meiotic prophase, and the structure and telomeric localization of synaptonemal complexes are altered within the nuclei of pachytene and diplotene spermatocytes. In these infertile mice, the shape of the sperm head and diameter of the flagella are abnormal, which leads to a major impairment of spermatogenesis (Roest et al., 1996; Grootegoed et al., 2000). The flagellar phenotype of *Ube2b*-null mice is very similar to the known human phenotype of "aberrant distribution of periaxonemal structures" (Escalier and Serres, 1985), which leads to severe defects in sperm motility, with abnormal flagellar curvature or impaired progressivity (Serres et al., 1986). Therefore, *UBE2B* may be associated with male infertility caused by spermatogenetic impairment or testicular dysfunction (Pengo et al., 2006).

In mammals, the highest rate of ubiquitination is found in the testes, and it is especially increased in testes that contain haploid spermatids (Rajapurohitam et al., 1999, 2002). Genetic engineering has demonstrated that the ubiquitin pathway plays a major role in spermatogenesis (Roest et al., 1996). UBE2B, a key protein in the ubiquitin pathway, transfers activated ubiquitin to the substrate once it is bound to E3 ubiquitin ligase (Ciechanover, 1996). *UBE2B*, which is approximately 21 kb long, is located on 5q3.11 and comprises 6 exons.

Ube2b$^{-/-}$ mice exhibit male infertility and abnormalities in sperm head shape (Escalier et al., 2003). In addition, the diameters of the sperm flagella are irregular, sperm motility is impaired, germ cells show extensive apoptosis, and the testis is depleted (Roest et al., 1996; Baarends et al., 2003). These findings suggest that UBE2B may be associated with dependent ubiquitination in chromatin remodeling to play a critical functional role during both meiosis and spermiogenesis. Most previous studies have focused on infertility mutations on the Y chromosome, such as *AZF* (azoospermia factor gene). Recent studies have indicated that genetic variants of autosomal genes may also be associated with infertility through effects on gene expression and function. Suryavathi et al. (2008) have reported that *UBE2B* genetic variants are associated with male infertility in Indians, and they found 2 novel substitutions that were observed only in infertile men. In the present study, we selected 3 *UBE2B* polymorphisms (g.-293T>G in the 5'-untranslated region, g.20016A>G in the 3'-untranslated region, and g.9157A>G in exon 4) that are significantly associated with infertility in Indian men. We carried out a genetic analysis using these variants in a case-control sample cohort from Northeast China to verify the relationship between *UBE2B* genetic variants and male infertility.

MATERIAL AND METHODS

Subjects

A total of 388 infertile men with non-obstructive azoospermia were recruited from the Infertility Clinic of Jinghua Hospital, Shenyang, China, between October 2007 and October 2009. The inclusion criteria were as follows: a minimum of 1-2 years of infertility and a sperm count below 20 x 10^6/mL determined by at least 2 semen analyses. Patients were excluded if they had 1) hypogonadotropic hypogonadism, 2) obstructive azoospermia, 3) genetic defects that caused azoospermia (Klinefelter syndrome or deletions of the Y chromosome), or 4) undergone treatment with chemotherapeutic agents or radiotherapy. A control group consisting of 312 fertile men who had at least one child with no history of requiring assisted reproduction technology was obtained randomly from a natural Northeast Chinese population receiving medical checkups between October 2007 and October 2009. The study was approved by the Jinghua Hospital Ethics Committee, and informed consent was obtained from all participating individuals.

Semen analysis was performed according to World Health Organization criteria (World Health Organization, 1999). Patients with non-obstructive infertility were categorized into 3 subtypes based on semen parameters: an oligozoospermia and asthenozoospermia (OA) group (sperm count of <20 x 10^6/mL and progressive sperm motility of <50%), a severe OA group (sperm count of <5 x 10^6/mL and progressive motility of <10%), and an azoospermia group (no spermatozoa). The diagnosis of azoospermia (no spermatozoa in the ejaculate) was based on double-check results from 2 independent semen samples.

Polymorphism selection

Genetic variations around *UBE2B* are well characterized, and 22 single nucleotide

polymorphisms (SNPs) have a minor allele frequency exceeding 1% in Chinese population. Taking advantage of the high degree of linkage disequilibrium among these SNPs (r^2 > 0.90), we selected haplotype-tagging SNPs using the Tagger software implemented in Haploview 5.0 (Broad Institute, USA). Two haplotype-tagging SNPs with a minor allele frequency of 5% or greater (rs17167484: g.-293T>G and rs3777373: g.20016A>G) capture the majority of the genetic variations in a 40-kbp region surrounding *UBE2B* and are significantly associated with infertility in Indian males (Suryavathi et al., 2008). Because the SNP g.9157A>G (no National Center for Biotechnology Information cluster ID number) was only observed in infertile males (Suryavathi et al., 2008), it was also selected into our case-control association study.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis

Genomic DNA was extracted from peripheral blood using a proteinase K digestion followed by standard phenol-chloroform extraction. For all subjects, 3 SNPs of the *UBE2B* (g.-293T>G, g.20016A>G, and g.9157A>G) were analyzed using PCR-RFLP. The 3 primer pairs were designed based on the genomic sequences flanking these SNPs (Table 1). PCRs for g.-293T>G were cycled in a 5-min 94°C predenaturation followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 45 s, and extension at 72°C for 45 s. The PCRs for the other SNPs were similar, except that annealing temperatures of 62° and 56°C were used for g.9157A>G and g.20016A>G, respectively. The PCR products were digested with the appropriate restriction enzyme, separated using 8% neutral polyacrylamide gel electrophoresis, and visualized with routine silver staining. The sequences of the primers, the restriction enzymes, and the size of the digested fragments are shown in Table 1.

Table 1. Primer sequences, restriction enzymes and PCR and digested conditions.

SNP site	Prime sequence	Annealing temperature (°C)	Genotype	Fragment size (bp)	Restriction enzyme	Digested temperature (°C)
g.-293T>G	F: 5'-GGATAGTGTTTCTGTTTCGTGGTCT-3'	60	TT	240	*Taq*I	65
	R: 5'-ACTGACAAACAACCCTGCAATGAC-3'		TG	170+70		
			GG	240+170+70		
g.20016A>G	F: 5'-AGAAAGCTAATACAAAACTATCCTA-3'	56	AA	161+45	*HpyCH4*III	37
	R: 5'-TGTTAAACGTTGCATAATGAAT-3'		AG	206		
			GG	206+161+45		
g.9157A>G	F: 5'-TGGGAGGGTCCT TTGCC-3'	62	AG	525	*Eco*RV	37
	R: 5'-AACAACTCTTTTCCCCACTATCATC-3'		AA	420+105		
			GG	525+420+105		

Statistical analysis

Cases and normal controls were examined for significant differences in genotype (allele) distributions in each of the *UBE2B* polymorphisms at the population level. The Hardy-Weinberg equilibrium (HWE) assumption was checked for each polymorphism in the cases

and controls separately. After checking the validity of HWE, we performed chi-square tests for each polymorphism by comparing the distribution of the genotypes (alleles) of the cases to that of the normal controls. Using the counts of one of the genotypes (allele) as a reference, we computed the odds ratios (ORs) for the remaining genotype (allele) variants through a logistic regression analysis with SPSS 16.0 (SPSS Inc., USA). The associated 95% confidence intervals (95%CIs) for the ORs were obtained from the logistic regression models. Haplotype frequency was analyzed online with the SHEsis software (http://analysis.bio-x.cn) (Shi and He, 2005; Li et al., 2009). Haplotype analysis was evaluated with the Fisher exact test, and each haplotype was compared with the others in the case groups or subgroups against the control group. The ORs and 95%CIs for each haplotype were determined using a logistic regression model. Bonferroni's correction for multiple tests was applied to judge the significance of the resulting test statistics in the haplotype analysis. All P values (two-sided) below 0.05 were considered to be statistically significant.

RESULTS

The average age (mean ± SD) was 35.8 ± 6.3 years for the controls and 31.8 ± 5.1 years for the patients. A significant difference in the average age was found between patients and controls ($P < 0.001$). The case subgroups contained 147 OA patients, 72 severe OA patients, and 169 azoospermia patients. Their average ages were 32.6 ± 5.5 years, 31.2 ± 4.6 years, and 31.5 ± 5.0 years, respectively.

As shown in Table 2, the genotype distributions of the polymorphisms in the cases and controls did not deviate from HWE ($P > 0.05$). No significant differences in the allelic frequencies between the cases and controls were found for SNPs g.-293T>G ($P = 0.150$) and g.20016A>G ($P = 0.373$). The genotype frequencies and distributions of *UBE2B* SNPs g.-293T>G and g.20016A>G in the control and case groups are listed in Table 2. For the g.9157A>G substitution, all participants were homozygous for the AA genotype. No significant differences in the genotype frequencies between the cases and controls were found for SNPs g.-293T>G ($P = 0.207$) and g.20016A>G ($P = 0.373$). Despite analyzing the association under dominant, recessive, and additive models, we observed no significant results (data not shown). In the 3 case subgroups (OA, severe OA, and azoospermia), no significant differences were found in either the allelic or the genotype distribution compared with those of the control group (Table 2).

Haplotype analysis for the SNPs g.-293T>G and g.20016A>G revealed 4 haplotypes: TA, TG, GA, and GG (Table 3). The distributions of the TA, TG, and GG haplotypes in the cases and the controls were not significantly different (see Table 3), but the frequency of the GA haplotype in the case group was significantly lower than that of the control group (OR = 0.32; 95%CI = 0.14-0.71; $P = 0.003$), which remained significant after multiple comparison adjustments ($P = 0.012$ after Bonferroni's correction). When the 3 subgroups were compared with the control group, no differences were observed with respect to the haplotype distribution of TA, TG, and GG, but the frequency of the GA haplotype was significantly lower in the azoospermia and OA subgroups (Table 3). However, after correction for multiple comparisons, the results for the 2 subgroups failed to show a statistically significant result ($P > 0.05$ after Bonferroni's correction; Table 3).

Table 2. *UBE2B* genotype frequencies and odds ratio (OR) against controls.

Genotype	Controls	Cases	OR (95%CI)	Azoospermia	OR (95%CI)	Severe OA	OR (95%CI)	OA	OR (95%CI)
	N (%)	N (%)		N (%)		N (%)		N (%)	
g.-293T>G									
TT	275 (88.1)	356 (91.8)	1.00	153 (90.5)	1.00	66 (91.7)	1.00	137 (93.2)	1.00
GT	36 (11.5)	30 (7.7)	0.64 (0.37-1.12)	14 (8.3)	0.70 (0.35-1.41)	6 (8.3)	0.67 (0.26-1.69)	10 (6.8)	0.46 (0.20-1.08)
GG	1 (0.3)	2 (0.5)	1.01 (0.09-11.5)	2 (1.2)	2.20 (0.19-25.1)	0 (0.0)	-	0 (0.0)	-
g.20016A>G									
AA	267 (85.6)	324 (83.5)	1.00	137 (81.1)	1.00	60 (83.3)	1.00	127 (86.4)	1.00
AG	45 (14.4)	63 (16.2)	1.25 (0.80-1.95)	31 (18.3)	1.46 (0.85-2.49)	12 (16.7)	1.14 (0.54-2.41)	20 (13.6)	0.93 (0.50-1.75)
GG	0 (0.0)	1 (0.3)	-	1 (0.6)	-	0 (0.0)	-	0 (0.0)	-

The ORs were adjusted by age. 95%CI = 95% confidence interval; OA = oligozoospermia and asthenozoospermia.

Table 3. *UBE2B* haplotype frequencies and odds ratio (OR) against controls.

Group	Haplotype		%	OR (95%CI)	P[a]	P[b]
	g.-293T>G	g.20016A>G				
Control	T	A	89.4	1.00 (reference)		
	T	G	4.5	1.00 (reference)		
	G	A	3.4	1.00 (reference)		
	G	G	2.7	1.00 (reference)		
Case	T	A	90.4	1.12 (0.79-1.58)	0.541	
	T	G	5.2	1.17 (0.71-1.92)	0.534	
	G	A	1.1	0.32 (0.14-0.71)	0.003	0.012
	G	G	3.3	1.22 (0.65-2.27)	0.536	
Azoospermia	T	A	89.3	0.99 (0.64-1.52)	0.961	
	T	G	5.4	1.20 (0.66-2.21)	0.548	
	G	A	0.6	0.18 (0.04-0.75)	0.008	0.095
	G	G	4.7	1.76 (0.88-3.54)	0.107	
Severe OA	T	A	89.4	1.00 (0.51-1.80)	1.00	
	T	G	6.4	1.46 (0.68-3.14)	0.332	
	G	A	2.2	0.66 (0.20-2.16)	0.488	
	G	G	1.9	0.70 (0.19-2.52)	0.583	
OA	T	A	92.1	1.38 (0.84-2.27)	0.197	
	T	G	4.5	1.00 (0.51-1.95)	0.994	
	G	A	1.1	0.31 (0.10-1.03)	0.043	0.516
	G	G	2.3	0.85 (0.35-2.09)	0.724	

[a]P was analyzed for statistical significance by the Fisher exact test. [b]After Bonferroni's correction. 95%CI = 95% confidence interval. OA = oligozoospermia and asthenozoospermia.

DISCUSSION

Suryavathi et al. (2008) have reported that *UBE2B* is associated with male infertility in Indian men. They identified 2 novel substitutions (g.5197T>G in intron 3 and g.9157A>G in exon 4) that were found only in infertile men. Analyzing the potential function of these substitutions with various bioinformatics tools, the authors predicted that g.9157A>G might result in the loss of a binding site for the RNA binding protein TRA2Beta. The authors also performed genotype analysis of the g.-293T>G and g.20016A>G transversions and found that the frequency distributions of these transversions were significantly different in infertile and fertile men. Their results also suggested that haplotype TG conferred a significantly increased risk of male infertility. Therefore, they proposed that UBE2B might be involved in sperm production in humans. However, Huang et al. (2008) have demonstrated that the allelic frequency of g.-293G is 5.2% in infertile males of northern European descent and reported no statistically significant differences from the reference frequency (4.4%) published by the International HapMap Project (http://hapmap.ncbi.nlm.nih.gov/), although it is lower than the previously reported frequency of 20% from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/).

We selected 3 *UBE2B* SNPs for our study that were significantly associated with infertility in Indian males. According to the International HapMap Project data on Han Chinese in Beijing, the frequency of the G allele is 6.5% for both g.-293T>G and g.20016A>G, whereas the frequency of the TG+GG and AG+GG genotypes is 13.1%. Our fertile control group data was 11.8 and 14.4%, respectively, which were not significantly different from the frequencies given in the International HapMap Project reference data (P = 0.7). However, the frequencies of the genotypes TG+GG and AG+GG for g.-293T>G and g.20016A>G (11.8 and

14.4%, respectively), in our control group were significantly higher than those of the Indian control group (2 and 5.3%, respectively) (Suryavathi et al., 2008).

In the present study, no significant differences were found between infertile and fertile men in distributions of alleles and genotypes for g.-293T>G and g.20016A>G before or after adjustment for age. Similarly, no significant differences were observed among these 3 subgroups of infertile men or between these subgroups and the control group (see Table 2). The g.9157A>G substitution of *UBE2B* was absent in both the case group and the control group. Haplotype analysis for the SNPs g.-293T>G and g.20016A>G showed that the GA haplotype was significantly associated with a greater than 3-fold decreased risk of male infertility (see Table 3). These results should be interpreted with caution because the frequency of the GA haplotype (1.1%) is relatively low in Chinese men, leaving the possibility that this finding occurred through chance. However, the finding remained significant after multiple comparisons adjustment (P = 0.012 after Bonferroni's correction).

In conclusion, our genetic analysis suggests that the *UBE2B* polymorphisms g.-293T>G, g.20016A>G, and g.9157A>G are not associated with male infertility, and the GA haplotype is likely to be a protective factor for male fertility in Northeast Chinese men. Considering that the significant variant may not function because nearby SNPs have a higher linkage disequilibrium with one another, we must identify additional causative variants through genomic resequencing. In addition, the discordant results between Indian and Chinese men may originate in ethnic and geographical differences. Therefore, further genetic and functional studies are needed to validate these findings, and these results require confirmation by multiethnic cohort studies with larger sample sizes.

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