·Original Article·

Association of *USP26* haplotypes in men in Taiwan, China with severe spermatogenic defect

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Abstract

Aim: To complete comprehensive haplotype analysis of *USP26* for both fertile and infertile men. **Methods:** Two hundred infertile men with severe oligospermia or non-obstructive azoospermia were subjected to sequence analysis for the entire coding sequences of the *USP26* gene. Two hundred men with proven fertility were genotyped by primer extension methods. Allele/genotype frequencies, linkage disequilibrium (LD) characteristics and haplotypes of fertile men were compared with infertile men. **Results:** The allele frequencies of five single nucleotide polymorphisms (370-371insACA, 494T>C, 576G>A, ss6202791C>T, 1737G>A) were significantly higher in infertile patients than control subjects. The major haplotypes in infertile men were TACCGA (28% of the population), TGCCGA (15%), TACCAA (8%), TGCCAA (6%), TATCAA (5%) and CATCAA (5%). The major haplotypes for the control subjects were TACCGA (58% of the population), CACCGA (7%), CATCGA (6%) and TGCCGA (5%). Haplotypes TGCCGA, TATCAA, CATCAA, CATCGC, TACCAA and TGCCAA were over-transmitted in patients with spermatogenic defect, whereas haplotypes TACCGA, CACCGA, and CATCGA were under-transmitted in these patients. **Conclusion:** Some *USP26* alleles and haplotypes are associated with spermatogenic defect in the Han nationality in Taiwan, China. *(Asian J Androl 2008 Nov; 10: 896–904)*

Keywords: single nucleotide polymorphism; *USP26* gene; spermatogenic defect; linkage disequilibrium

1 Introduction

Infertility affects one in 10 couples worldwide. In roughly half of these couples, the male factor is implicated. Although there are many factors that contribute to male infertility, the cause cannot be identified for the majority of infertile men [1]. In recent years, there

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has been an intensive search for genetic causes of male infertility. Screening with markers on the long arm of the human Y chromosome has detected Yq microdeletions in 5%–15% of males with spermatogenic defect. It is believed that many genes located on the long (q) arm of the Y chromosome are required for spermatogenesis. This region, banding in q11.23 of the Y chromosome, includes the azoospermia factor (AZF) locus, which contains a gene or genes that are required for normal spermatogenesis [2, 3]. Other genetic factors that have been reported to be involved in spermatogenic defect include mutations at the mitochondrial DNA polymerase locus, a polymorphism of the cytochrome P4501A1 gene, mutations of the follicle-stimulating hormone receptor gene (*FSH*), the deleted in azoospermia-like gene (*DAZL*), the synaptonemal complex protein 3 gene (*SYCP3*), human *BOULE*, casein kinase 2 alpha genes, dystrophia myotonica protein kinase gene (*DMPK*), DNA polymerase gamma (*POLG*), methylenetetrahydrofolate reductase (*MTHFR*) and follicle-stimulating hormone receptor (*FSHR*) [4–16].

USP26 appears to be an attractive candidate for a sterile gene considering its unique expression pattern. *USP26* comprises a single exon and is located on Xq26. 2. Its messenger RNA (mRNA) sequence is 2 794 bp long and its protein consists of 913 amino acids (Genbank: NM_031907.1). Its mouse homolog was found to be only expressed in spermatogonia [17]. *USP26* belongs to a large family of deubiquitinating enzymes (DUB) [18]. Deubiquitination of macromolecules by DUB, including ubiquitin proteases, can rescue macromolecules from degradation through substrate-specific, N-terminal-dependent, enzymatic reaction [19, 20]. Previous studies have addressed the importance of the ubiquitin pathway during mammalian fertilization, including acrosomal function and spermatozoa–zona pellucida (ZP) penetration [21,22]. During spermatogenesis, deubiquitination enzymes have been shown to be involved in the regulation of protein turnover (e.g., replacement of histones by protamine, germ cell apoptosis, mitotic proliferation and differentiation of spermatogonial stem cells) [23–28]. Several papers have been published on the role of *USP26* in male infertility. Stouffs *et al.* [29] analyzed patients with various histological patterns of spermatogenic defect (Sertoli cell-only syndrome and maturation arrest) for the presence of mutations in *USP26.* They found some Sertoli cell-only syndrome (SCOS) patients had the genotype of 370-371insACA (rs3840975), 494T>C and 1423C>T (rs41299088) [29]. A study by Paduch also provided evidence linking *USP26* mutation to male infertility [30]. Although these two studies suggested that a specific genetic cluster (370-371insACA, 494T>C and 1423C>T) might be associated with testicular dysfunction, two other studies showed that this genetic cluster was not restricted to men with testicular dysfunction [31, 32]. In a recent publication on *USP26*, the authors identified several novel mutations in the *USP26* gene that might cause spermatogenesis impairment in a small group of infertile men (n = 41) [33]. However, meta-analysis of previous case-control studies (in total, 544 patients and 1 705 controls) revealed no significant association of the 370-371insACA, 494T>C and 1423C>T genotype with male infertility [14, 29–32]. Further studies based on a large group of patients with diversified ethnic backgrounds will be valuable to bring more insight into the role of *USP26* in male infertility.

In the present study, we set out to analyze genetic variants and haplotypes of *USP26* for both fertile and infertile men by sequence analysis for the entire coding region of *USP26*. We found some *USP26* genetic variants and haplotypes were overt-transmitted and some were under-transmitted in patients with severe spermatogenic defect in the Han nationality in Taiwan, China. We also identified some novel genetic variants or mutations in patients. Our result supports important roles of *USP26* in human spermatogenesis.

2 Materials and methods

2.1 Subjects

The study was approved by the Institutional Review Board of National Cheng Kung University Medical Centre (Taiwan, China). From January 2001 to December 2005, 200 infertile men presenting with severe oligozoospermia (spermatozoa count < 5×10^6/mL) or non-obstructive azoospermia and 200 men with proven fertility (control group) were enrolled. All of the control subjects had fathered at least two children within 5 years without assisted reproductive technologies. The paternal relationship between control subjects and their offspring was confirmed using the AmpF/STR-Profiler-Plus zygosity determination system (Applied Biosystems, Foster City, CA, USA). All patients and control subjects belonged to the Han nationality in Taiwan, the major ethnic group in China (making up more than 95% of this province's population). The patients were evaluated according to the protocol described in our previous studies [6, 34]. In

brief, all patients underwent comprehensive characterization, including a detailed history, physical examination, at least two semen analyses, hormone assays (luinizing hormone [LH], fouicle stimulating hormone [FSH], prolactin [PRL] and testosterone [T]), karyotyping and a molecular test for Y-chromosomal deletions. Patients suspected to have non-obstructive azoospermia were advised to undergo bilateral testicular biopsies. Serum levels of FSH, LH, PRL and T were measured using commercial radioimmunoassay kits: Coat-A-Count FSH IRMA, Coat-A-Count LH IRMA, Coat-A-Count PRL IRMA and IMMULITE Total Testosterone (Diagnostic Products, Los Angeles, CA, USA). Chromosome analysis was performed using the GTG method (G-banding by Trypsin-Giemsa technique). Molecular analysis of Y-chromosomal deletions included a combination of 16 gene-based primers, as described previously [34]. Patients with any identifiable cause of male infertility, including congenital bilateral absence of vas deference (CBAVD), cryotorchidism, varicocele, diabetes mellitus or hypertension, or with history that may affect spermatogenesis (e.g., orchitis, trauma, malignancies, *etc.*) were excluded from the study group. No significant difference was found between age, serum LH, estradiol and T concentrations of infertile men and control subjects. Sperm count and FSH concentrations were significantly higher in patients compared with the control group ($P < 0.005$). Of 200 study subjects, 137 had severe oligospermia and 63 had azoospermia.

2.2 Sequence analysis of patients

Genomic DNA samples of patients were subjected to sequence analysis for the entire coding sequences of the *USP26* gene. Genomic DNA was extracted from peripheral blood samples using a Puregene DNA isolation kit (Gentra, Minneapolis, MN, USA). *USP26* genomic sequence (AF285593) was obtained from the NCBI web site (http://www.ncbi.nlm.nih.gov). The entire gene (2 794 bp) was divided into five overlapping fragments ranging from 409 to 600 bp in length. To amplify partial fragments of the *USP26* exon, polymerase chain reaction (PCR) reactions were performed in 20 µL volumes containing 200 ng of genomic DNA, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 0.1% Triton X-100, 200 µmol/L deoxyribonucleotide triphosphates (dNTP), 100 pmol of each primer and 1 U *Taq* DNA polymerase (Promega Corp., Madison, WI, USA). The primer sets for *USP26* are described in (Table 1). PCR amplification was performed in an automated thermal cycler (OmniGene Thermal Cycler; Hybaid Ltd., Ashford Middlesex, UK). PCR products were sequenced to identify mutations or polymorphisms. Sequence analysis was performed with an automatic sequencer (ABI 377, Applied Biosystems/PE).

2.3 Genotyping for fertile controls

Genomic DNA samples of the control subjects were subjected to genotyping by primer extension methods. The amplicons were amplified in a multiplex fashion and each 20 µL reaction consisted of 50 ng of genomic DNA, 10 pmol of primers, 4.0 mmol/L $MgCl_2$, 0.2 mmol/L dNTP and 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems). The cycling conditions were: 95ºC for 5 min, followed by 35 cycles at 95ºC for 1 min, 55ºC for 1 min and 72ºC for 1 min, with a final extension for

Table 1. Primer sets used for PCR and DNA sequencing of the *USP26* gene. [a]The nucleotide sequences is according to AF285593. [b]The lengths of polymerase chain reaction product size.

Fragment	Primer pairs	Primer sequence[a]	Product size (bp)[b]
1	Usp26-1F	CTTCAAGATTATCAATAATCGG	680
	Usp26-1R	AATCTGCCTTGGATTTCTTG	
2	Usp26-2F	GCTCTCATCTAGCTCAGAGA	687
	Usp26-2R	CCAAATTCACTTTTAGG CTTCC	
3	Usp26-3F	GCTCATGAGTTTTTAGCTCACT	682
	Usp26-3R	GTTCAGACTCCTTATCTGATC	
4	Usp26-4F	CATCAGTGTATCATGGCCTG	562
	Usp26-4R	TTGTGTGCCCCTGGGTGCC	
5	Usp26-5F	CGGTATGAGAATCTGTGAAC	511
	Usp26-5R	TTCCTTCTGAAGGGTCTCCTC	

10 min at 72ºC. Each allele was measured by primer extension and SNaPshot chemistry (Applied Biosystems). Multiplex PCR products (3 µL) were treated with 2.5 U of shrimp alkaline phosphatase (SAP; Amersham Pharmacia Biotech, Uppsala, Sweden) and 2 U of exonuclease I (BM Biochemica, Mannheim, Germany) in a 10 mL reaction volume for 1 h at 37ºC. SNaPshot multiplex PCR was performed in a 10 µL reaction volume containing 1.25 µL of SNaPshot Multiplex Ready Reaction Mix (Applied Biosystems), 4 µL of SAP/ exonuclease I-treated PCR products, and 1 to 3 pmol of each SNaPshot primer. Thermal cycling for SNaPshot reactions consisted of 25 cycles at 96ºC for 10 s, 50ºC for 5 s and 60ºC for 30 s. The SNaPshot multiplex reaction was then treated by SAP in an 11 µL reaction volume containing 10 µL of SNaPshot multiplex PCR product and 1 U of SAP. The samples were analyzed using an ABI 3100 genetic analyzer (Applied Biosystems) and the allele determination was carried out with the Genotyper 3.7 program (Applied Biosystems).

2.4 Statistical analysis

Tests for association with single markers and haplotypes in control samples were performed using the χ^2 test. $P < 0.05$ was considered statistically significant. The relative risk of spermatogenic defect was estimated from logistic odds ratios (OR) and 95% confidence intervals (CI) in multivariate analysis. Tests for haplotype association with spermatogenic defect were performed using EHPLUS software [35], and statistical significance was estimated using the permutation test PMPLUS [36]. Associations between haplotypes and male infertility were analyzed using linear regression models. The linkage disequilibrium (LD) coefficient (D') between each pair of single nucleotide polymorphisms (SNPs) was calculated using the ldmax program within the GOLD software package [37].

3 Results

3.1 Polymorphisms in men in Taiwan, China

The coding sequences of the *USP26* gene were examined by sequencing analysis for the presence of mutations of sequence variants in infertile men. The sequence from GenBank (AF285593) was used as the reference sequence and the A of ATG of the start codon was numbered + 1. In total, seven genetic variants were observed, including one insertion variant 370-371insACA (rs3840975), causing a threonine insertion in amino acid position 121, and six SNPs, 494T>C, 576G>A (rs41304540), ss6202791C>T, 1423C>T (rs41299088), 1737G>A and 2202A>C. All of these genetic variants were available in the web site (http://www.ncbi.nlm.nih.gov/) and peer-review articles [29–32]. One substitution, 576G>A (E192), does not alter the amino acid sequence: a glutamic acid at position 192. The other five SNP were predicted to create amino acid alteration: 494T>C changes a leucine into a serine (L165S); ss6202791C>T changes a serine into a phenylalanine (S600F); 1423C>T substitutes a histidine for a tyrosine (H475Y); 1737G>A substitutes a methionine for a isoleucine (M579I); and 2202A>C substitutes a lysine for a asparagine (K734N). The following substitutions occurred only once in the patients group but not in the fertile controls group: 393A>T (S131S); 468T>G (Q156H); 1976C>T (T659M); 2144A>T (N715I); 2182A>T (I128F); 2195T>C (F732S); 2204T>G (V735G); 2239A>T (I747F); 2247A>C (E749D); 2250A>G (Q750Q); and 2271T>C (P757P). Except for 1976C>T (T659M), these substitutions have not been reported [30]. The substitution of 520T>G and 565T>G is predicted to cause premature stop codon at codon 175 and 189, respectively. Haplotype analyses ruled out that patients carrying these variant sites occurred only once in the infertility group.

3.2 Allelic and genotypic frequencies in fertile and infertile men

Allelic frequencies and odds ratios for each sequence variant of *USP26* are shown in Table 2. Of the seven variants, 370-371insACA, 494T>C, 576G>A, ss6202791C>T and 1737G>A are significantly associated with spermatogenic defect. The *P*-value for these five variants was less than 0.05 and the odds ratios were larger than 1. For example, the *P*-values for 370-371insACA and 576G>A are less than 0.0001 and the OR are 35.86 and 4.095 (Table 2).

3.3 Reproductive effect of the 70-371insACA, 494T>C, 1423C>T genetic cluster and its derivation

The ancestral *USP26* cluster [370-371insACA, 494T>C, 1423C>T] and its first derivation only occur in the group of infertile men. In contrast, the second derivation only occurs in the group of fertile men (Table 3). The frequency of the [370-371insACA, 494T>C, 1423C>T] cluster is 3% in the infertile group but none in fertile men.

Table 2. Allelic frequencies of seven *USP26* sequence variants in fertile and infertile men. Allele frequencies were compared between infertile men and control subjects. SNPs, single nucleotide polymorphisms; OR, odds ratio; CI, confidence interval.

SNPs	Allele	Allele frequency: Control (n = 200) n (%)	Spermatogenic defect (n = 200) n (%)	P-value (χ^2)	OR	95% CI
370-371insACA	No ins	200 (100)	184 (92)	< 0.0001	35.860	2.135–602.400
	insACA	0 (0)	16 (8)			
494T>C	T	164 (82)	146 (73)	0.0311	1.685	1.046–2.715
	C	36 (18)	54 (27)			
576G>A	A	172 (86)	120 (60)	< 0.0001	4.095	2.510–6.682
	G	28 (14)	80 (40)			
ss6202791C>T	C	162 (81)	144 (72)	0.0338	1.658	1.037–2.651
	T	38 (19)	56 (28)			
1423C>T	C	192 (96)	186 (93)	0.1882	1.806	0.740–4.408
	T	8 (4)	14 (7)			
1737G>A	G	172 (86)	120 (60)	< 0.0001	4.095	2.510–6.682
	A	28 (14)	80 (40)			
2202A>C	A	196 (98)	194 (97)	0.5218	1.515	0.421–5.456
	C	4 (2)	6 (3)			

Table 3. The genotype frequency of the 370-371insACA, 494T>C and 1423C>T cluster, its first and second derivative in fertile and infertile men. [a]Analyzed for statistical significance by Fisher's exact test. [b]According to [37]. OR, odds ratio.

	370-371ins ACA	494T>C	1423C>T	Spermatogenic defect n (%)	Controls n (%)	P-value[a]	OR
Ancestral[b]	Yes	C	C	8 (4%)	0 (0%)	0.0073	17.71
1st derivation[b]	Yes	C	T	6 (3%)	0 (0%)	0.0301	13.40
2nd derivation[b]	No	T	T	0 (0%)	4 (2%)	0.1231	0.1089

3.4 Linkage disequilibrium (LD) between different SNPs

(Table 4) shows the pairwise LD (D') between different alleles. For patients with spermatogenic defect, 2202A>C was found to be in complete LD with 370-371insACA, 494T>C, 576G>A and 1423C>T (D' = 1). 494T>C had strong LD with 370-371insACA and 1423C>T (D' = 0.829 and 0. 804 respectively). For control subjects, 370-371insACA was found to be in weak LD with the other six variants (D' = 0). Except 2202A>C, no strong linkage disequilibrium (D' > 0.8) was observed for these seven variants in the control group. Therefore, the LD characteristic of infertile men was different from that of control subjects.

3.5 Haplotype analysis

The major haplotypes (defined as haplotype frequency in either group ≥ 5%) were TACCGA (28% of the population), TGCCGA (15%), TACCAA (8%), TGCCAA (6%), TATCAA (5%) and CATCAA (5%) for patients. The capital letters represented 494T>C, 576G>A, ss6202791C>T, 1423C>T, 1737G>A and 2202A>C from left to right, respectively. The major haplotypes for the control subjects were TACCGA (58% of the population), CACCGA (7%), CATCGA (6%) and TGCCGA (5%). Six of the nine major haplotypes showed significant differences in frequency between infertile men and control subjects (Table 5). Haplotypes TGCCGA, TATCAA, CATCAA, CATCGC, TACCAA and TGCCAA were over-transmitted in patients with severe spermatogenic defect, whereas haplotype TACCGA, CACCGA and CATCGA were under-transmitted in these patients. Of 134 patients with major haplotypes, only nine azoospermic men

Table 4. Pairwise linkage disequilibrium (D') in fertile and infertile men. Estimates in the upper right are for infertile men and in the lower left are for control subjects.

	370-371insACA	494T>C	576G>A	ss6202791C>T	1423C>T	1737G>A
370-371insACA	—	0.829	0.375	0.107	0.534	0.583
494T>C	0	—	0.136	0.331	0.804	0.259
576G>A	0	0.206	—	0.107	0.762	0.042
ss6202791C>T	0	0.451	0.206	—	0.008	0.464
1423C>T	0	0.390	0.128	0.691	—	0.286
1737G>A	0	0.206	0.336	0.206	0.128	—
2202A>C	0	1	1	0.383	1	1

Table 5. Major haplotypes of fertile and infertile men. [a]Analyzed for statistical significance by Fisher's exact test and $P < 0.05$ was considered statistically significant. OR, odds ratio; CI, confidence interval.

	Haplotype							Spermatogenic defect *vs.* controls				
Patterns	370-371 insACA	494T >C	576G >A	ss6202791C >T	1423C >T	1737G >A	2202A >C	Spermatogenic defect	Controls	*P*-value[a]	OR	95% CI
1	No	T	A	C	C	G	A	56 (28%)	116 (58%)	< 0.0001	0.282	0.186–0.428
2	No	T	G	C	C	G	A	30 (15%)	10 (5%)	0.0013	3.353	1.591–7.064
3	No	C	A	C	C	G	A	2 (1%)	14 (7%)	0.0035	0.134	0.030–0.599
4	No	T	A	T	C	A	A	10 (5%)	2 (1%)	0.0358	5.211	1.127–24.100
5	No	C	A	T	C	A	A	10 (5%)	2 (1%)	0.0358	5.211	1.127–24.100
6	No	C	A	T	C	G	C	8 (4%)	0 (0%)	0.0073	17.710	1 .014–309.100
7	No	T	A	C	C	A	A	16 (8%)	8 (4%)	0.1391	2.087	0.872–4.995
8	No	T	G	C	C	A	A	12 (6%)	8 (4%)	0.4923	1.532	0.612–3.833
9	No	C	A	T	C	G	A	8 (4%)	12 (6%)	0.4923	0.653	0.261–1.633

Table 6. Clinical features of patients with major haplotypes. HS, Hypospermatogenesis; MA, maturation arrest; SCOS, Sertoli cell-only syndrome.

Haplotype	Number of patients	Severe oligospermia (No.)	Azoospermia (No.)	Testicular histology (Biopsy: No.)
1	56	48	8	HS: 1, MA: 1
2	30	26	4	MA: 2
4	10	4	6	SCOS: 1
5	10	6	4	MA: 1
7	16	8	8	HS: 1, SCOS: 1
8	12	10	2	HS: 1
Total number of patients	134	102	32	9

had testicular histology available. We found their testicular histological features were highly variable, ranging from hypospermatogenesis, MA (maturation arrest at apermatocyte or spermatid) to SCOS (Table 6).

4 Discussion

In the present study, we found allele frequencies of five SNPs (370-371insACA, 494T>C, 576G>A,

ss6202791C>T and 1737G>A) were significantly higher in infertile patients than those in control subjects (Table 2). As compared with previous studies, our data represents comprehensive coverage of *USP26* genotypes by sequence analysis for the entire coding region. By sequence analysis for the entire coding region, we also discovered some novel variants (e.g., 393A>T, 468T>G, 520T>G, 565T>G, 2144A>T, 2182A>T, 2195T>C, 2204T>G, 2239A>T, 2247A>C, 2250A>G and 2271T>C). These substitutions occurred only once in the patients group but not in the fertile men. Genotyping for more subjects will be required to determine if these substitutions are rare SNP or true mutation.

Although our study is in accordance with two previous studies that described association of genetic cluster (370-371insACA, 494T>C, 1423C>T) with male infertility [29, 30, 32], the role of 370-371insACA, 494T>C and 1423C>T genotypes remains uncertain. Meta-analysis of four studies (a total of 544 patients and 1705 controls) revealed no significant association of the 370-371insACA, 494T>C and 1423C>T genotypes with male infertility (14, 29–32). After incorporating our data (a total of five studies with 744 patients and 1905 controls), meta-analysis still revealed negative association (OR: 0.778; 95% CI: 0.484–1.252). We speculate that the uncertainly could be accounted for by genetic backgrounds of the enrollees in different studies. The frequencies of the 370-371insACA, 494T>C and 1423C>T genotypes seem highly varied demographically even in the Chinese population. We could hardly detect the 370-371insACA, 494T>C and 1423C>T genotypes in fertile men of the Han nationality in Taiwan, China (Table 3). The frequency of ancestral cluster in our study is 4% in infertile men and 0% in fertile controls. The overall frequency in fertile men is 2% for the Han nationality in Taiwan, China, much lower than that reported in the Chinese Han (8%) [31]. The genes involved in reproduction have been shown to be under strong positive selection [38]. The Han immigrated from Mainland China to Taiwan Provice relatively recently – within the past several hundred years [39]. The low prevalence of 370-371insACA, 494T>C and 1423C>T genotypes in the Han nationality in Taiwan, China could be interpreted as a result of migration followed by genetic drift.

Recently, LD has been used to track down candidate genes in association studies, in which the disease variants are detected through the presence of linkage signal at nearby sites [40]. Patterns of LD are also important for unraveling the evolutionary history of humans, including identification of demographic effect and the detection of natural selection [41]. We found different LD characteristics of *USP26* for fertile and infertile men. This finding may imply distinct genetic backgrounds for fertile and infertile men. In infertile men, 2202A>C was found to be in complete LD with 370-371insACA, 494T>C, 576G>A and 1423C>T (D' = 1). The 494T>C allele had strong LD with 370-371insACA and 1423C>T (D' = 0.829 and 0.804, respectively) (Table 4). The major haplotypes for patients with spermatogenic defect were TACCGA (28% of the population), TGCCGA (15%), TACCAA (8%), TGCCAA (6%), TATCAA (5%) and CATCAA (5%). These six haplotypes accounted for 67% of the patient population. The major haplotypes for control subjects were TACCGA (58% of the population), CACCGA (7%), CATCGA (6%) and TGCCGA (5%), which altogether constitute 76% of the control population. It appeared that haplotypes TGCCGA, TATCAA, CATCAA, CATCGC, TACCAA and TGCCAA conferred susceptibility to spermatogenic defect, whereas haplotype TACCGA, CACCGA and CATCGA were protective against spermatogenic defect (Table 5). It is noteworthy that, of the nine deleterious haplotypes, five belonged to minor haplotype (< 5%) of the fertile men, reflecting negative selection in the population. It is interesting to note occurrence of various testicular phenotypes in men with deleterious *USP26* haplotypes. This observation is in accordance with a complex disease model for spermatogenic defect [42]. Male infertility represents a classical example of a complex disease with substantial genetic contribution. *USP26* genotype is among one of the many factors that may contribute to spermatogenic defect in humans.

Taken together, we showed association of specific genetic variants with severe spermatogenic defect in the Taiwanese Han. We identified haplotypes of *USP26* and found association of specific haplotypes with impaired sperm production for the first time. Our result seems to support important roles of *USP26* in human spermatogenesis. Considering contradictory data about the clinical implication of specific *USP26* genotypes, *USP26* may be just among one of the many factors contributing to spermatogenic defect. Other factors that are involved in diverse regulatory pathways of human spermatogenesis may jointly modify the effect of *USP26*.

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