

# Association of single nucleotide polymorphisms in the *USF1*, *GTF2A1L* and *OR2W3* genes with non-obstructive azoospermia in the Chinese population

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## Abstract

**Purpose** To research the association between the single nucleotide polymorphisms (SNPs) of three spermatogenesis-related genes (*USF1*, *GTF2A1L* and *OR2W3*) and non-obstruction azoospermia (NOA).

**Methods** We investigated 361 NOA cases and 368 controls from the Chinese Han population, and we used Sequenom iPLEX technology to analyze the candidate 9 SNPs from the *USF1*, *GTF2A1L* and *OR2W3* genes.

**Results** In this study, we found that the variant rs2516838 of *USF1* was associated with NOA susceptibility ( $P=0.020$ , OR=1.436), and the haplotype TCG of the variants rs1556259,

rs2516838, and rs2774276 of *USF1* conferred an increased risk of NOA ( $P=0.019$ , OR=1.436). Furthermore, we found that the rs11204546 genotype of *OR2W3* and the rs11677854 genotype of *GTF2A1L* were correlated with the FSH level in the patients ( $P=0.004$  and  $P=0.018$ , respectively).

**Conclusions** Our results provided a new insight into susceptibility of *USF1* variant with male infertility. Clinically, the SNPs (rs11204546 of *OR2W3* and rs11677854 of *GTF2A1L*) might be additional valuable molecular predictive markers for assessing the treatment of NOA patients.

**Keywords** Male infertility · *USF1* · *GTF2A1L* · *OR2W3* · Single nucleotide polymorphisms (SNPs)

**Capsule** *USF1* variant rs2516838 might confer risk to NOA.

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## Introduction

Approximately one out of six couples experiences infertility and nearly half of these cases can be ascribed to the male partner [1]. Regardless of various causative factors such as varicocele, obstruction of spermatic ducts, agglutination of sperm, impotence and hormonal imbalance, a significant proportion of male infertility attributes to idiopathic factor [2, 3]. It is demonstrated that single nucleotide polymorphisms (SNPs) in genes are associated with a variety of clinical subtypes of male infertility, including non-obstructive azoospermia (NOA), which is clinically characterized by the absence of sperm in the testis as a result of impaired spermatogenesis [4–7].

During spermatogenesis, gene expressions are regulated at the transcriptional level to ensure the differentiation of germ cells [8]. Upstream stimulatory factor (USF) is a ubiquitously expressed cellular transcription factor. Purified USF consists of 2 related polypeptides, that is USF1 and USF2. Upstream transcription factor 1 gene (*USF1*) locates in human chromosome 1q23.3. In terms of the structure of USF1 protein, USF1

contains a C-terminal DNA-binding domain that includes a helix-loop-helix motif and a leucine repeat which is required for efficient DNA binding and USF1 dimerization [9]. USF1 is one of the transcriptional factors of the spermatogenesis-related genes. There were two mechanisms elaborating how the *USF1* gene influenced the spermatogenesis-related genes. One acted through controlling the number of Sertoli cells. In the adult testis, Sertoli cells are essential to provide the physical environment for germ cells to develop mature sperms. The number of germ cells that could be supported depended on the duration of the proliferation period in Sertoli cells [10]. Therefore, timing Sertoli cell differentiation was critical for determining the final number of Sertoli cells and the upper limit of male fertility. The USFs (USF1 and USF2) regulated numerous Sertoli cell genes which were indispensable during differentiation [11, 12], and the other functioned through initiating the spermiogenesis. A previous study showed that the transcription factor USF specially bound and activated the *Miwi* promoter in mice [13]. *Miwi*, a cytoplasmic protein, was specifically expressed in the testis from pachytene spermatocytes to round spermatids and was required for initiating spermiogenesis [14, 15]. USF controlled *Miwi* expression through methylation-mediated regulation. The CpG islands in the *Miwi* promoter were unmethylated in the pachytene spermatocytes and round spermatids where *Miwi* was expressed. If the CpG island within the E2 box to which the USFs bind was methylated, the binding of the USF would be inhibited, thereby *Miwi* expression would not occur [13]. In that case, the *Miwi*-dependent initiating spermiogenesis would not take place.

In this study, we hypothesized that *USF1*, *GTF2A1L* and *OR2W3* might be potential candidates for the disruption of spermatogenesis. We assumed that there might be genetic associations between variants of the genes and NOA susceptibility. To verify this hypothesis, we performed a genotyping analysis for 9 SNPs from *USF1*, *GTF2A1L* and *OR2W3* genes in a case-control study.

## Materials and methods

### Ethic statement

All patients provided written informed consent for the collection of samples and subsequent analyses. This study was approved by the ethics committee for genome research of the Anhui Medical University and was conducted based on the Declaration of Helsinki Principles (reference no. 2008035, 10 January 2008).

### Patients of NOA and the controls

In this study, we investigated 361 NOA patients with the age ranging from 19 to 43 and 368 controls with the age ranging

from 20 to 53 from the Chinese population. Patients with NOA were collected from the Reproductive Medicine Center, the First Affiliated Hospital of Anhui Medical University, between March 2008 and July 2013. To confirm the diagnosis of azoospermia, i.e. no sperm was found after ejaculate centrifugation at 3,000 g for 10 min, we analyzed at least two semen samples. The semen analyses for sperm concentration, motility, and morphology were performed following the fifth edition of the World Health Organization manuals [16]. The NOA patients were carefully selected based on a comprehensive examination, including semen analysis, karyotype analysis, medical history, physical examination, hormone analysis and Y chromosome microdeletions screening. Patients with a history of orchitis, maldevelopment of testis, mono- or bilateral cryptorchidism, varicocele, hypogonadism, obstruction of vas deferens, chromosomal abnormalities, and Y microdeletions were excluded from this study [17, 18]. The controls were all fertility men who had fathered 1 or more healthy children. All control donors were enrolled from the same hospital where the patients were recruited. All the subjects tested were the Chinese Han population. Also, we used Electro-Chemiluminescence Immunoassay system (Roche Diagnostics, Germany) to measure the FSH level of the patients. We divided the patients into two subtypes: FSH <12.4mIU/ml and FSH ≥12.4mIU/ml, according to the normal range of FSH level (1.5–12.4mIU/ml). Furthermore, we designed 10 ml of the patient's testis volume as a threshold on the basis of the previous study [19]. All the testis volume of the controls were larger than 10 ml.

### Extraction of peripheral blood DNA

we extracted the genomic DNA from peripheral blood using QiAamp DNA Blood MiDi Kit (Qiagen Inc., Germany), according to the manufacturer's protocol. DNA purity and concentrations were determined using a NanoDrop Spectrophotometer (ND-100, wilmington, DE) of full wave length and standardized to 30 ng/μl.

### SNP selection and genotyping

We selected 9 SNPs in this study using Haploview software [20], including 3 *USF1* SNPs (rs2516838, rs1556259, rs2774276), 4 *GTF2A1L* SNPs (rs1916838, rs11677854, rs10432667, rs13024367) and 2 *OR2W3* SNPs (rs10888267, rs11204546). All the candidate SNPs were selected from the Chinese population of International HapMap Project Web site (<http://hapmap.ncbi.nlm.nih.gov/>) with MAF>0.05. Locus-specific PCR and detection primers were designed using the MassARRAY Assay Design 3.0 software (Sequenom, San Diego, California) following the manufacturer's instructions. Then we amplified the DNA samples by multiple PCR reactions, and the PCR products were used for locus-specific

**Table 1** The characteristics of the study population. (a) Continuous variables were expressed as mean±SD; *FSH* follicle stimulating hormone, *NOA* non-obstructive azoospermia; /: no data available; Bold values was statistically significant ( $P<0.05$ ). Student's *t*-test was used to analyze the data

Factors	Controls ( <i>n</i> =368) <sup>a</sup>	NOA ( <i>n</i> =361) <sup>a</sup>	<i>p</i> value
Mean age (year)	29.47±3.86	28.82±4.26d	0.070
Body mass index (kg/m <sup>2</sup> )	23.61±3.53	23.09±3.32	0.071
Right testicle volume (ml)	14.58±2.65	8.90±3.51	<b>0.000</b>
Left testicle volume (ml)	14.51±2.70	8.95±3.55	<b>0.000</b>
FSH (mIU/ml)	/	21.42±19.88	/

single-base extension reactions. The resulting products were desalted and transferred to a 384-element SpectroCHIP array. We performed a genotyping analysis of the SNPs for fast-track validation analysis using the Sequenom MassArray system (Sequenom, San Diego, CA). Subsequently, we used fifteen nanograms genomic DNA to genotype each sample. We performed allele detection using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and the mass spectrograms were analyzed by the MassARRAY Typer software (Sequenom).

Statistical analysis

We used *T*-test to compare the clinical characteristics between the controls and cases. We determined the Hardy-Weinberg equilibrium (HWE) by the controls ( $P>0.01$ ). The HWE of the patients, the differences in frequency distributions of the genotypes between the cases and the controls and the correlations between the clinical data and all the SNPs were calculated in two ways: SHEsis software (<http://analysis.bio-x.cn/myAnalysis.php>) [21] and SPSS release 17.0 statistical package. Moreover, the sample powers of each SNP were

calculated using PS (power and sample size calculations) software [22] and the power calculation of the SNPs detected reached the research need ( $>0.08$ ). In addition, for Chi-square test, Fisher exact test was used when the number of genotype≤5. In this study, of the 9 SNPs evaluated, we considered the associations with a *p* value of  $<0.05$  to be of significance. We did not adjust the data for multiple comparisons.

Results

Characteristics of the study population

We summarized clinical characteristics of the NOA cases and the controls in Table 1. The results showed that the testicle volume in NOA cases was significantly smaller than that in the controls ( $P<0.001$ , both the sides).

The polymorphisms of *USF1*, *GTF2A1L* and *OR2W3* genes and risk of NOA

In this study, we investigated 3 *USF1* SNPs (rs2516838 G/C, rs1556259 C/T, rs2774276 G/C), 4 *GTF2A1L* SNPs (rs1916838 T/C, rs11677854 C/T, rs10432667 A/G, rs13024367 C/G) and 2 *OR2W3* SNPs (rs10888267 T/C, rs11204546 C/T). All of the 9 SNPs of interest did not deviate from the HWE ( $P>0.01$ ) in the controls. We found that C allele of rs2516838 was associated with NOA ( $P=0.02$ , odds ratio [OR] =1.436, 95 % confidence interval [CI]=1.059–1.947, Table 2). Table 3 showed genetic model of the SNP rs2516838 between the controls and the cases. We found that the individuals with CG genotype of rs2516838 increased risk susceptibility to the NOA in the codominant model ( $P=0.008$ , OR=1.610, 95 %CI=1.133–2.287), and the individuals with

**Table 2** Genetic analysis of *USF1*, *GTF2A1L* and *OR2W3* polymorphisms in the risk of NOA. *NOA* non-obstructive azoospermia, *MAF* minor allele frequency, *HWE* Hardy-Weinberg equilibrium. Bold values was statistically significant ( $P<0.05$ ). Chi-square test was used for analyzing the data

Gene	SNP	Alleles	Position	HWE		MAF		<i>p</i> value	OR(95%CI)
				Controls	Cases	Controls	Cases		
USF1	rs2516838	G>C	161014370	0.291	0.687	0.114	0.156	<b>0.020</b>	1.436(1.059-1.947)
USF1	rs1556259	C>T	161014649	0.597	0.138	0.444	0.479	0.177	1.153(0.938-1.418)
USF1	rs2774276	G>C	161011716	0.448	0.447	0.114	0.117	0.894	1.022(0.741-1.410)
GTF2A1L	rs1916838	T>C	48894604	0.469	0.725	0.078	0.084	0.69	1.080(0.740-1.576)
GTF2A1L	rs11677854	C>T	48891287	1.000	0.707	0.750	0.740	0.947	0.987(0.667-1.461)
GTF2A1L	rs10432667	A>G	48873978	0.896	0.601	0.279	0.283	0.864	1.020(0.811-1.284)
GTF2A1L	rs13024367	C>G	48899516	0.728	0.725	0.083	0.084	0.987	1.003(0.691-1.455)
OR2W3	rs10888267	T>C	248059423	0.753	0.395	0.484	0.475	0.739	0.966(0.785-1.187)
OR2W3	rs11204546	C>T	248059712	0.834	0.597	0.467	0.483	0.542	1.066(0.868-1.310)

**Table 3** The genotype distributions of *USF1* rs2516838 in the cases and the controls. Codominant model: DD versus Dd and DD versus dd; Dominant model: DD versus (Dd + dd); Recessive model: (DD + Dd) versus dd; d: minor allele; D: major allele. Bold values was statistically significant ( $P < 0.05$ ). We used Chi-square test to analyze the data

SNPs	Model	Genotype	Controls N(frequency)	Cases N(frequency)	<i>p</i> value	OR(95 %CI)
rs2516838	Codominant	GG	289(0.792)	255(0.708)		1
		CG	69(0.189)	98(0.272)	<b>0.008</b>	1.610[1.133–2.287]
		CC	7(0.019)	7(0.019)	0.817	1.133[0.392–3.275]
	Dominant	GG	289(0.792)	255(0.708)		1
		CG + CC	76(0.208)	105(0.291)	<b>0.009</b>	1.566[1.115–2.199]
	Recessive	CC	7(0.019)	7(0.019)		1
		CG + GG	258(0.981)	353(0.980)	0.561	1.368[0.474–3.949]

the minor allele C of rs2516838 increased the risk susceptibility to the NOA in the dominant model ( $P=0.009$ , OR=1.566, 95 %CI=1.115–2.199).

#### Haplotypes of *USF1* gene and risk of NOA

To investigate potential combination effects of *USF1* SNPs on susceptibility of spermatogenic failure further, we did a haplotype analysis in *USF1* gene and found a significant haplotype associated with NOA. The haplotype TCG containing all the 3 SNPs of *USF1* gene (rs1556259, rs2516838, rs2774276) exhibited significantly increased susceptibility to suffering from spermatogenic failure in the Chinese patients ( $P=0.019$ , OR=1.436, 95 % CI=1.059–1.948, Table 4).

#### Association of the 9 polymorphisms with clinical data of NOA

We further performed a stratified association analysis of the 9 SNPs with the testis volume and the level of FSH in the patients. Our data indicated that there were significant correlations for rs11204546 of *OR2W3* (C>T,  $P=0.004$ ) and rs11677854 of *GTF2AIL* (C>T,  $P=0.018$ ) with the FSH level in the patients, rather than the testis volume ( $P > 0.05$ ) (Table 5).

## Discussion

In this study, we chose 9 SNPs from *USF1*, *GTF2AIL*, *OR2W3* genes to investigate the relationship between non-obstructive azoospermia and the variants. The results showed

that rs2516838 of *USF1* significantly led to an increased risk of spermatogenic failure. Due to the presence of the *USF1/USF2a* heterodimers [23], *USF1* was not yet characterized alone. Evidences showed that *USF1* played a pivotal role in spermatogenesis [11–13]. The potential mechanisms that the SNP rs2516838 involved in NOA remained unclear. It was supposed that 1) the variations might influence the *USF1* expression. Based on the analysis performed by the GoldenPath ([www.genome.ucsc.edu](http://www.genome.ucsc.edu)) [24], a database forecasting the function of SNPs in transcriptional regulation, the G to C change of rs2516838 might disturb the gene transcription. The deficient transcription of *USF1* would decrease the level of *USF1* protein. During proliferation, low level of *USF1* expression in Sertoli cells limited the *USF* recruitment to E-box motifs within the promoter regions of *Nr5a1* and *Shbg* genes which were vital for Sertoli cell differentiation [12], therefore the progress of the spermatogenesis might be repressed as a result of the aberrant differentiation of Sertoli cells, NOA would take place; 2) We found that the minor allele C of the SNP rs2284922 located in the conserved region of *USF1* gene by means of the F-SNP database [25], a web-based SNP analysis tool. Such an alteration in the gene might lead to an abnormality of gene expression or weaken the stability of gene [26]; 3) the variation of rs2516838 might relate to the initiation of spermiogenesis. There were studies revealing that the rs2516838 polymorphism of *USF1* was associated with methylation of genomic DNA [27, 28]. As *Miwi* gene was essential for initiating spermiogenesis, and a recent investigation implied that the methylation of CpG islands within the proximal promoter of *Miwi* gene revealed a marked correlation with the expression of *Miwi* [13]. We supposed that the variant rs2516838 of

**Table 4** Haplotype distributions of *USF1* in the NOA cases and the controls. <sup>a</sup>: The order of the haplotypes: rs1556259, rs2516838, rs2774276; Bold values was statistically significant ( $P < 0.05$ ). Chi-square was used for data analysis

Haplotype <sup>a</sup>	Controls N (frequency)	Cases N (frequency)	<i>p</i> value	OR(95 %CI)
TCG	83(0.114)	112(0.156)	<b>0.019</b>	1.436[1.059–1.948]
CGG	405(0.556)	374(0.521)	0.176	0.867[0.705–1.066]
TGC	84(0.115)	84(0.117)	0.924	1.016[0.736–1.401]
TGG	156(0.214)	148(0.206)	0.703	0.952[0.739–1.226]

**Table 5** Association of the 9 polymorphisms with clinical data of NOA. *FSH* follicle stimulating hormone; **(a)** the FSH level was divided into <12.4mIU/ml and ≥12.4mIU/ml according to the normal range of the FSH level (1.5–12.4mIU/ml); **(b)** the testis volume was divided into <10 ml and ≥10 ml; Bold values was statistically significant ( $P<0.05$ ). We used Chi-square for data analysis

Gene	Genotype	FSH <sup>a</sup>			Testis Volume <sup>b</sup>			
		<12.4mIU/ml	≥12.4mIU/ml	<i>p</i> value	<10 ml	≥10 ml	<i>p</i> value	
USF1	rs2516838(G>C)			0.522			0.954	
	GG	54	101		117	109		
	GC	18	45		44	38		
	CC	2	2	2	2			
	rs1556259(C>T)			0.358				0.574
	CC	19	33		39	42		
	CT	35	84		91	75		
	TT	20	30	32	32			
	rs2774276(G>C)			0.664				0.701
GG	57	118	128		111			
GC	17	29	34		37			
CC	0	1	1	1				
GTF2A1L	rs1916838(T>C)			0.424			0.698	
	TT	56	124		134	124		
	TC	16	22		28	22		
	CC	1	2	1	2			
	rs11677854(C>T)			<b>0.018</b>				0.582
	CC	56	252		136	125		
	CT	17	40		25	23		
	TT	1	0	0	1			
	rs10432667(A>G)			0.612				0.059
	AA	34	73		90	66		
	AG	35	59		56	72		
	GG	5	13	14	11			
rs13024367(C>G)			0.302			0.883		
CC	55	124		135	123			
CG	17	22		26	24			
GG	1	2	2	1				
OR2W3	rs11204546(C>T)			<b>0.004</b>			0.960	
	CC	19	70		33	29		
	CT	30	172		84	80		
	TT	25	52	44	40			
	rs10888267(T>C)			0.219				0.938
	TT	21	27		35	32		
	TC	35	83		83	79		
CC	18	36	44		38			

*USF1* might influence the methylation status of *Miwi* gene and indirectly lead to NOA; and 4) rs2516838 might be in linkage disequilibrium with other variants in *USF1* gene or other genes that could play a concurrent role in impaired spermatogenesis, as those in other complex diseases [29].

To address potential combination effects of variants of *USF1* on risk of spermatogenesis defect, we performed a haplotype analysis among the 3 SNPs rs1556259, rs2516838 and rs2774276 of *USF1* gene. We found that the frequency of the haplotype TCG of the three SNPs was significantly higher

in the NOA patients than that in the controls ( $P=0.019$ , OR=1.436, and 95 % CI=1.059–1.948). In comparison with the other 3 haplotypes (Table 4), the minor allele C of rs2516838 would probably exert a dominant force to NOA. Thus, we supposed that the Chinese individuals who carried the haplotype TCG with the minor allele C of the rs2516838 might be liable to suffering from NOA.

Furthermore, GTF2A1L, as a germ cell-specific transcription and a substitute for TF II A in male germ cells, was involved in spermatogenesis [30]. None or less expression



of *GTF2A1L* protein in the infertile patients with spermatogenic disturbances indicated that *GTF2A1L* gene might be correlated with NOA [31]. However, in this study, we failed to identify any associations between the variants of *GTF2A1L* gene and NOA. In addition, although two independent studies demonstrated an association of the SNP rs11204546 of *OR2W3* gene with azoospermia in the European descent [32, 33], in this study we found no association of the *OR2W3* rs11204546 with Chinese NOA. The reasons for the divergences among the investigations appeared unknown. It would be attributable either to the strictly controlled recruitment of our clinical cases or to the ethnic population differences. Moreover, in this study, we found that there were significant correlations for rs11204546 of *OR2W3* (C>T,  $P=0.004$ ) and rs11677854 of *GTF2A1L* (C>T,  $P=0.018$ ) with the FSH level in the patients (Table 5). Interestingly, a previous study indicated that the FSH level might be a predictive factor to obtain elongated spermatids by testicular sperm extraction for azoospermic patients [34]. Therefore, we hypothesized these two FSH-related SNPs (rs11204546 and rs11677854) might be a valuable molecular predictive markers of sperm/elongated spermatids retrieval rate for NOA patients. But yet, additional clinical studies are needed to confirm the hypothesis.

In conclusion, our study showed that the *USF1* variant rs2516838 was associated with spermatogenesis in the Chinese Han population and indicated the susceptibility of the *USF1* haplotypes TCG to NOA. Clinically, we found that rs11204546 and rs11677854 were associated with the FSH. Nevertheless, further investigations on the molecular mechanisms in the *USF1*, *OR2W3* and *GTF2A1L* genes might provide better understanding of the development of NOA.

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