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GENETICS

Association of a *TDRD1* variant with spermatogenic failure susceptibility in the Han Chinese

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Abstract
Purpose Piwi-interacting RNAs (piRNAs) are a broad group of noncoding small RNAs that have important biological functions in germline cells and can maintain genome integrity via silencing of retrotransposons. In this study, we aimed to explore the associations between genetic variants of important genes involved in piRNA biogenesis and male infertility with spermatogenic impairment.
Methods To this end, five single-nucleotide polymorphisms (SNPs) in the *ASZ1*, *PIWIL1*, *TDRD1*, and *TDRD9* genes were genotyped by TaqMan allelic discrimination assays in 342 cases of nonobstructive azoospermia (NOA) and 493 controls.
Results The SNP rs77559927 in *TDRD1* was associated with a reduced risk of spermatogenic impairment. The genotypes TC and TC+CC showed odds ratios and 95 % confidence intervals of 0.73 (0.55–0.98, $P=0.034$) and 0.73 (0.56–0.97, $P=0.030$), respectively, in patients with NOA compared with those in the controls.
Conclusion Thus, our results provided the first epidemiological evidence supporting the involvement of *TDRD1* genetic polymorphisms in piRNA processing genes in determining the risk of spermatogenic impairment in a Han Chinese population.

Keywords *TDRD1* · Piwi-interacting RNA · Polymorphism · Spermatogenesis impairment · Nonobstructive azoospermia

Capsule Thus, our results provided the first epidemiological evidence supporting the involvement of *TDRD1* genetic polymorphisms in piRNA processing genes in determining the risk of spermatogenic impairment in a Han Chinese population.

Xiao-Bin Zhu and Jian-Qi Lu contributed equally to this work.

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Introduction

Infertility has been reported to affect 10–15 % of couples; approximately half of these cases are related to male infertility problems [1, 2]. Spermatogenic impairment is the most common form of male infertility, and many genetic factors have been shown to contribute to this disorder [3–6]. Spermatogenesis is a unique and complex developmental process regulated by many genes [7]. Thus, mutations or other alterations may cause spermatogenic impairment and male infertility [8].

Piwi-interacting RNAs (piRNAs) are a broad group of noncoding small RNAs with a length of 26–31 nucleotides. These RNAs are abundantly expressed in animal gonads [9] and exhibit repeat-derived sequences. piRNAs were first discovered in *Drosophila* and mapped to serve as an endogenous defense mechanism to suppress retrotransposon activity by silencing gene expression [10]. To date, however, the molecular mechanisms that mediate piRNA-induced DNA methylation remain unclear. Previous reviews [11, 12] have indicated that piRNAs play an essential role in maintaining DNA integrity in germline cells.

Numerous genes are involved in the generation of piRNAs. In particular, proteins encoded by *Piwi* genes are essential for

the biogenesis and function of piRNAs [13, 14]. In addition, genes such as *piwi* and *tdrd* have been implicated in the biogenesis of piRNAs [15–18]. Tudor domain-containing proteins or Tudor domain-related proteins (TDRDs) have been extensively studied in the context of Piwi proteins [19], playing a key role in piRNA biogenesis. TDRD9 is expressed in mitotic spermatogonia, meiotic spermatocytes, and haploid spermatids in the mouse testis [20]. In mice, the Piwi clade consists of *Miwi2*, *Mili*, and *Miwi*. Miwi has been shown to be expressed in meiotic pachytene germ cells, Miwi2 has been reported to be localized only in embryonic stages, and Mili has been found at all stages but is enriched in spermatocytes [15, 21].

Piwi genes and piRNA biogenesis-associated genes have been shown to be expressed at various stages of germ cell development in the testis [22]. Studies in animal models with deletions of these genes have consistently shown impairment of gametogenesis. Aravin and Bourc'his reported that mutations in the *Mili* and *Miwi2* genes lead to elimination of DNA methylation around transposable elements and sterility in male mice [23]. Consistent with studies in flies, mutations in the *Miwi2* gene have been shown to result in the accumulation of DNA damage [24]. Houwing et al. studied the Piwi pathway proteins Ziwi and Zili in zebrafish. In their study, eggs from female zebrafish with mutations in the *zili* gene were shown to exhibit defects in the meiotic process as both Piwi-piRNA complexes and piRNA biogenesis-associated proteins are localized in the nucleus in *Drosophila* [25]. These results reflect the potential role of piRNA proteins in regulating DNA transcription and cell cycle progression.

Considering the essential role of piRNA biogenesis pathway genes in spermatogenesis, we hypothesized that genetic variants in these genes would potentially affect spermatogenesis. Therefore, to test our hypothesis, we performed genotyping analyses for five SNPs in these piRNA pathway genes and carried out a case-control study on the association between these SNPs and susceptibility to spermatogenic impairment.

Materials and methods

Patient recruitment and sample collection

Three hundred forty-two (342) patients with nonobstructive azoospermia (NOA) were strictly screened by clinicians as cases from two hospitals in China. In order to confirm either azoospermia, all patients underwent at least two semen analyses, and no sperms were found after centrifugation of the ejaculate at 3000×*g* for 10 min, according to World Health Organization (WHO) guidelines [26]. For a diagnosis of NOA, all patients underwent standard clinical examinations. Patients with a history of Y chromosome microdeletions, karyotype anomalies, genetic abnormalities, cryptorchidism, and orchitis were excluded [27]. Finally, only NOA patients without known pathogenicity factors were included in this study. Additionally, patients having unique occupational exposure that was thought to affect semen quality were excluded. Each patient donated 5 mL blood for genomic DNA extraction. Four hundred ninety-three (493) controls with normozoospermia were randomly recruited from the Shanghai Human Sperm Bank (Shanghai Province, China). The criteria for the controls included in this study were as follows: (1) sperm concentration more than 15×10^6/mL, (2) progressive motility over 32 %, and (3) round cells $<1 \times 10^6$/mL, according to the WHO criteria. Both the control and case groups were from Han Chinese populations. This study was conducted following the approval of the Ethics Committee of Renji Hospital, Shanghai Jiao Tong University School of Medicine, and all patients and controls provided written informed consent for participation in this genetic study.

SNP selection and genotype analyses

We first selected some genes associated with the piRNA biogenesis pathway as target genes to prepare RNA probes and performed target capture sequencing for 59 NOA patients. Some SNPs were found with higher frequencies in patients with NOA than in Han Chinese in Beijing (CHB) and Han Chinese South (CHS) populations in China. Based on the 1000 Human Genomes Project, SNPs in Han Chinese with higher frequencies compared with those in other populations were selected as the target sites. Finally, a total of five potential functional SNPs in four genes were selected randomly, including rs1029396 in *ASZ1*, rs1106042 in *PIWIL1*, rs77559927 in *TDRD1*, and rs45550534 and rs3783312 in *TDRD9* (Fig. 1, Table 1, Supplementary Tables 1 and 2). In the case of multiple SNPs in the same haplotype block (linkage coefficient $r^2 < 0.8$), only one was selected. The allele frequencies of these SNPs in CHB and CHS were significantly higher than the 1000G global frequency, particularly for rs77559927 in *TDRD1*. Therefore, we speculated that these SNPs might have special functions in the Chinese population.

Genomic DNA from the patients was isolated from peripheral blood leukocytes. The SNP genotyping work was performed using an improved multiplex ligation detection reaction (iMLDR) technique developed by GENESKY Biotechnologies Inc. (Shanghai, China). Two types of negative controls were used for each plate, i.e., (1) double-distilled water as a template and (2) DNA sample without primers. Duplicate tests were designed, and the results were consistent. A random sample accounting for ~5 % ($n = 20$) of all DNA samples was directly sequenced using Big Dye Terminator version 3.1 and an ABI3730XL automated sequencer (Applied Biosystems, Foster City, CA, USA) to confirm the results of iMLDR.

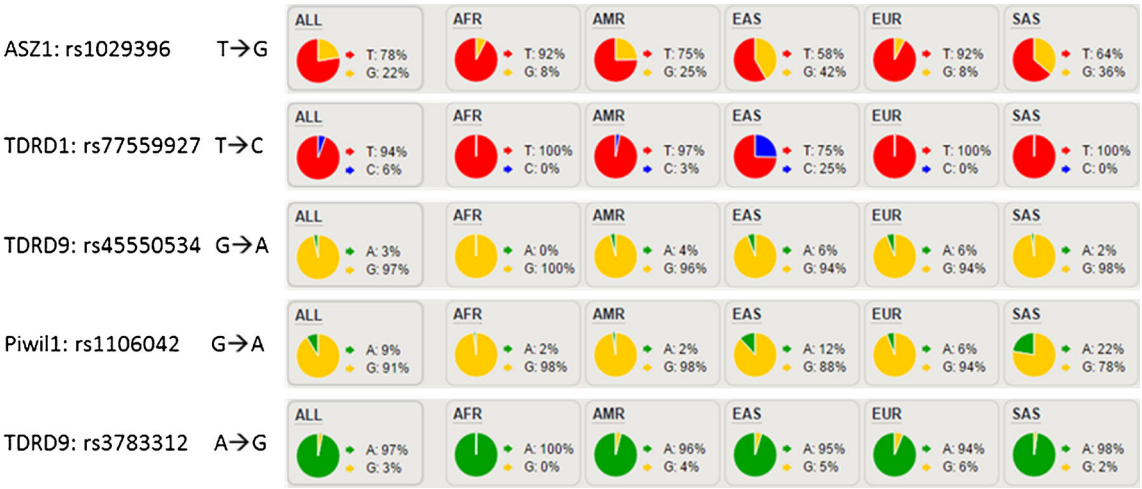

Fig. 1 Five potential functional SNPs in four genes selected randomly

Statistical analyses

Statistical analyses were performed with the Statistical Analysis System (version 9.1.3; SAS Institute, Cary, NC, USA). The Hardy-Weinberg equilibrium was tested using Hardy-Weinberg equilibrium calculator [28]. The risk of spermatogenic failure was estimated as the odds ratios (ORs) and 95 % confidence intervals (95 % CIs) using unconditional univariate logistic regression. All P values were two-sided, with $P = 0.05$ considered the threshold of significance.

Results

Characteristics of the study population

The clinical characteristics of the 342 cases and 493 controls are shown in Table 2. We found that the testis volume in the cases was significantly smaller than that in the controls ($P < 0.01$). No significant differences in age or body mass index (BMI) were detected between the two groups ($P > 0.05$).

Table 1 piRNA biogenesis-associated genes and polymorphisms evaluated in this study

SNP	Chr	Gene	Alleles	SNP property	Sense change
rs45550534	14	*TDRD9*	A/G	3′UTR_exon35	ND
rs77559927	10	*TDRD1*	T/C	5′UTR_exon1	ND
rs1029396	7	*ASZ1*	T/G	nonsynon_exon6	p.Lys216Thr
rs1106042	12	*PIWIL1*	G/A	nonsynon_exon13	p.Arg527Lys
rs3783312	14	*TDRD9*	A/G	3′UTR_exon35	ND

ND not determined

Association of spermatogenic failure with the TDRD1 gene

All SNP frequencies were consistent with Hardy-Weinberg equilibrium (HWE) in controls, which had a p value of less than 0.0001 for deviation from HWE. The associations between SNPs in piRNA biogenesis-related genes and the risks of male infertility are shown in Table 3 and Supplementary Table 3. The genotype frequency of rs77559927 in the *TDRD1* gene was significantly different between patients with NOA and controls for the χ^2 trend test ($P = 0.0467$). Significantly decreased risk of spermatogenic impairment was found for carriers of the rs77559927 TC genotype of *TDRD1* when compared with homozygous carriers of the T allele in patients with NOA (OR 0.73, 95 % CI 0.55–0.98; $P = 0.034$). The homozygous carriers of the C allele may also have a lower risk of spermatogenic impairment (OR 0.75, 95 % CI 0.41–1.39). However, because the number of individuals with this genotype was too small, our study did not have sufficient statistical power to identify any differences. Instead, when we combined the rs77559927 TC and CC genotypes, assuming a dominant model effect, the combined rs77559927 TC + CC variant genotypes were associated with a 26.6 % reduced risk of spermatogenic impairment (OR 0.73, 95 % CI 0.56–0.97). This OR value suggested that the variant rs77559927T > C may be a protective factor against the risk of spermatogenic impairment. The SNP rs77559927 is a T to C change in the 5′ untranslated region (UTR) of exon 1 of the *TDRD1* gene, which may lead to increased or decreased expression of certain transcript isoforms. The altered expression of *TDRD1* may affect the roles of piRNAs and Piwi proteins during spermatogenesis, resulting in alterations in spermatogenesis. With regard to the other SNPs, no significant differences in distribution frequencies were identified among patients with NOA and controls (Supplementary Table 4).

Table 2 Patient and control characteristics

Characteristic	Controls ($n=493$)	Patients with NOA ($n=342$)	All participants ($n=845$)
Age (years)	26.95 ± 3.40	27.30 ± 4.05*	27.13 ± 4.42
Body mass index	22.51 ± 2.27	23.28 ± 3.24*	22.85 ± 2.81
Right testicle volume (mL)	15.48 ± 1.76	5.81 ± 1.66**	11.53 ± 5.06
Left testicle volume (mL)	15.43 ± 1.87	5.87 ± 1.77**	11.531 ± 5.04

*$P>0.05$, **$P<0.01$

We also assessed the discriminative accuracy of the prediction model with and without addition of the identified SNP to the significant demographic and clinical features (age, BMI) by comparing the area under the receiver operating characteristic curve (AUC; Fig. 2). When adding the dominant model of rs77559927 in the prediction model of oligospermia, the AUC increased to 0.76 (95 % CI 0.73–0.79) compared with the model that only included age and BMI (AUC 0.758; 95 % CI 0.726–0.790; $P=0.336$).

Discussion

Spermatogenesis defects may have various causes; for example, some conditions, such as cryptorchidism and orchitis, can cause NOA, and other known genetic factors, such as Y chromosome microdeletions and Klinefelter's syndrome, are directly associated with spermatogenesis failure [29–31]. In this study, we aimed to identify unknown genetic factors that may be associated with spermatogenesis defects in patients with unexplained NOA. Therefore, we enrolled patients according to strict inclusion and exclusion criteria.

piRNAs have a wide range of functionalities, which are not always easy to identify. Numerous studies have found associations between piRNA biogenesis-related genes and fertility [11, 12]. However, the exact functional mechanism of Piwi/piRNA complexes remains unclear. Based on the findings of all reviewed studies, piRNA biogenesis-associated genes have been shown to be vital for spermatogenesis [32].

Studies on spermatogenesis impairment have focused on genetic variants or polymorphisms in candidate genes [33, 34]. However, available information regarding how genetic variants influence gene expression levels in spermatogenesis is limited. If a genetic polymorphism is located in the 3′UTR, it may influence messenger RNA (mRNA) stability and change the ability of mRNAs to bind microRNAs; this may result in decreased gene expression owing to mRNA cleavage or translational repression [35]. Alternatively, if a gene polymorphism is located in one of the binding motifs for splicing, pre-RNA splicing activity may be decreased, resulting in downregulation of genes involved in spermatogenesis [36, 37].

As mentioned previously, some studies in humans have also reported an association between piRNA biogenesis-associated gene polymorphisms and infertility [38]. Thus, variations in piRNA pathway genes may play a role in spermatogenic impairment. Accordingly, in this study, we evaluated the possible associations between common SNPs in piRNA pathway genes and male infertility with spermatogenesis impairment in Chinese patients with NOA and fertile controls to test whether polymorphisms in piRNA pathway genes were involved in human spermatogenic impairment. Because of phenotypic multiplicity in male infertility, only patients with NOA were recruited to avoid interference from other etiological factors in our study. However, we found no significant differences in the frequencies of SNP genotypes in the *TDRD9*, *PIWIL1*, and *ASZ1* genes between patients with NOA and controls. Because our sample size was limited, the results indicated that these SNPs had relatively weak or no associations with spermatogenic impairment.

Table 3 Association of rs77559927 with NOA risk

SNP	Genotype	Controls ($n=493$)		Patients ($n=342$)		OR (95 % CI)	$P^{corrected}$	*P* value
rs77559927		*N*	%	*N*	%			
	TT	255	52.0	203	59.4	1.00		
	TC	208	42.0	121	35.4	*0.73 (0.55–0.98)*	*0.17*	*0.034*
	CC	30	6.1	18	5.3	0.75 (0.41–1.39)	1.00	0.366
	Dominant model	TT vs. TC + CC				*0.73 (0.56–0.97)*	*0.15*	*0.030*
	Recessive model	CC vs. TT + TC				0.86 (0.47–1.56)	1.00	0.616
P^{trend}	*0.047*							
P for HWE	0.144							

Italic values indicate significant findings ($P<0.05$)

OR odds ratio, *CI* confidence interval, P^{trend} *P* value for X^2 trend test, $P^{corrected}$ *P* value after correction

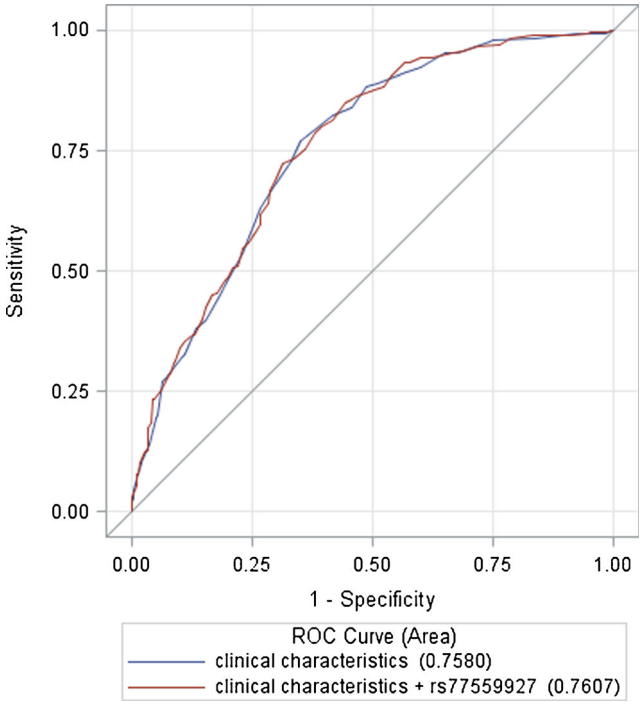

Fig. 2 Area under the receiver operating characteristic curve

Notably, we detected significant differences in the frequencies of genotypes between patients with NOA and controls in the SNP rs77559927. The frequencies of genotypes TC and CC were significantly lower in patients with NOA than those in controls, suggesting that the variant rs77559927 T > C was associated with a lower risk of spermatogenic impairment (OR = 0.73), indicating that the genotypes TC and CC may have some protective effects on spermatogenic impairment. However, the mechanisms through which this SNP affects spermatogenic impairment are not yet clear. The SNP rs77559927 is a T to C change at the 5′-UTR of exon 1 of the *TDRD1* gene, which may lead to increased or decreased expression of certain transcript isoforms. Altered expression of *TDRD1* may affect the roles of piRNAs and Piwi proteins during spermatogenesis, subsequently altering spermatogenesis; thus, this SNP may alter the susceptibility to male infertility with oligospermia. Further studies are required to examine this hypothesis.

In conclusion, in this study, we investigated the relationships between polymorphisms in piRNA pathway genes and male infertility with spermatogenesis impairment in humans. The results of this study revealed that the SNP rs77559927 in *TDRD1* was negatively associated with male infertility with oligospermia and may have some protective effects against spermatogenic impairment in the Chinese population. Furthermore, additional independent validation studies and functional experiments are needed to determine the specific role of the SNP rs77559927 in the *TDRD1* gene. Because the sample size in this study was limited and restricted to the Chinese population, the findings of this study need to be validated in further studies with larger samples and other ethnic populations.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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