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GENETICS

Association of polymorphisms in *tektin-t* gene with idiopathic asthenozoospermia in Sichuan, China

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Abstract
Purpose The purpose of this research was to study the association between the single nucleotide polymorphisms (SNPs) of the *tektin-t* gene and idiopathic asthenozoospermia.
Methods We conducted sequence analyses of the *tektin-t* gene in 104 idiopathic asthenozoospermia and 102 fertile men with normospermic parameters in Sichuan, China.
Results In this study, we found that allele 136 T (odds ratio [OR] 1.745, 95 % confidence interval [CI] 1.146–2.655, P=0.009) was significantly increased in idiopathic asthenozoospermic patients compared with fertile men. This mutation substitutes a highly conserved arginine at position 46 to cysteine. Moreover, PolyPhen-2 analysis predicted that this variant was "probably damaging". In addition, a novel heterozygous mutation, R207H (c.620G >A), was detected in five asthenozoospermic patients, while there was no detection of this genotype among the fertile candidates, indicating that the mutation was located within a conserved domain predicted by PolyPhen-2 analysis as "probably damaging" to the protein.
Conclusions These results suggested that tektin-t variants (Arg/Cys+Cys/Cys) were probably one of the high risk genetic factors for idiopathic asthenozoospermia among males in Sichuan, China, while the R207H polymorphism may be associated with idiopathic asthenozoospermia risk.

Keywords Genetic polymorphisms · *Tektin-t* · Asthenozoospermia · Sperm motility

Capsule These results suggested that tektin-t variants (Arg/Cys+Cys/Cys) were probably one of the high risk genetic factors for idiopathic asthenozoospermia among males in Sichuan, China, while the R207H polymorphism may be associated with idiopathic asthenozoospermia risk.

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Introduction

Infertility is a worldwide problem affecting about 15 % of couples trying to conceive, and male factor may partially or fully account for approximately 50 % of all infertility cases [1]. Genetic factors may account for a high percentage of male infertility. Four major semen anomalies (azoospermia, oligozoospermia, asthenozoospermia (AZS), and teratospermia) are present in almost 50 % of infertile couples and in approximately 90 % of infertile males [2]. In these cases, AZS is one of the major causes of male infertility second only to oligospermia [3]. AZS may exist as an isolated disorder, in association with other sperm anomalies or as parts of syndromic association. As an isolated disorder, AZS was found in as many as 18.71 % of patients presenting for male infertility and might be a significant factor in another 63.13 % of patients with combined oligo- and/or teratozoo-spermia [4]. Thus, AZS may be one of the main seminal pathologic infertilities among males.

Tektins have been proven to be indispensable for spermatozoa motility. Tektins were first isolated from sea urchin sperm flagellar microtubules [5] and named as tektin A, B, and C [6]. As the sequences of sea urchin tektin A, B, and C have been obtained [7–9], the facts were found that the

tektin A and B were closely related, and the models of dimers and polymers molecules were devised [10]. Several functional studies have demonstrated tektins including tektin-2, tektin-3, tektin-4, and tektin-5 are critical for sperm motility [11–16]. These studies imply tektins play important roles in sperm function. Tanaka et al. [16] generated *tektin-t*-deficient mice and proved that females were fully fertile but males were infertile caused by debilitating sperm motility and impairing motility of both flagella and cilia. Moreover, Bhilawadikar R et al. [17] found that tektin-t levels were positively associated with sperm motility parameters, fertilization rate, embryo quality, and pregnancy rate. The human *tektin-t* (or *h-tekB1* or *Tektin-2*) gene has been cloned [18–20], showing a specific expression in testis, and precisely localized in flagella of mature sperm. The localization of this protein and its association with sperm motility make it a good candidate gene for research into the causes of AZS. Therefore, we screened 104 idiopathic AZS patients as a case group and 102 fertile men as a control group to study the association of polymorphisms of the *tektin-t* gene with idiopathic AZS infertile patients in Sichuan, China.

Patients and methods

Patients

We recruited 104 male partners from successively enrolled couples who had their first infertility consultation in the Affiliate Reproductive Hospital Genitalia Hygiene Research Center (Sichuan, China) between May 2014 and March 2015. The asthenozoospermic infertility patients were aged between 23 and 44 years old and were not related. To screen the idiopathic AZS, patients with known diseases such as cryptorchidism, orchitis, epididymitis, varicocele, obstruction of the vas deferens, endocrine hypogonadism, karyotype anomalies, and Y chromosome microdeletions were excluded from the study. Patients with drug, alcohol, substance abuse, and tobacco use were also excluded. The control group consisted of 102 fertile and healthy males of a comparable age who had fathered at least one child. All subjects were given informed consent for molecular analysis of their semen samples and blood samples.

Semen analyses were performed at least twice for all subjects according to the World Health Organization (WHO) recommendations [21]. At least one sperm sample per individual collected after 3–5 days of abstinence through masturbation was analyzed. These isolated asthenozoospermic infertility patients had concentration of spermatozoa $>20\times10^6$/ml and rapid forward progressive motile sperm (grade A <25 %) and total progressive motile sperm (grades A+B <50 %) in fresh ejaculation according to the WHO (2010) criteria [21].

Extraction of genomic DNA

Total DNA of human spermatozoa was extracted using the EasyPure Blood Genomic DNA Kit (Transgen, Beijing, China). Briefly, the spermatozoa pellet was resuspended in sterile water and mixed with lysis solution containing 20-mg/ml proteinase K and Binding Buffer 3. Lysis was incubated at room temperature for 10 min. Lysates were added to centrifugal column to bind the DNA. Bound DNA was washed and then eluted from the centrifugal column. Quantification of the extracted genomic DNA was conducted by the spectrophotometry analysis. All of the DNA samples were stored at −20 °C until examination.

Polymerase chain reaction amplification and genotyping

PCR amplification of all exons of *Tektin-t* gene and the parts of flanking intronic sequence were performed using the primer sets as described in Table 1. PCR was performed in A200 Gradient Thermal cycler (Long Gene) in a total volume of 25-μL buffered solution containing 1.5 mM Mg^{2+} (Fermentas International Inc., Burlington, Ontario, Canada), 0.25 mM dNTPs (TransGen, Beijing, China), 0.2 μM of each primer (Sangon Biotech, Shanghai, China), 2 U of DNA polymerase (Fermentas International Inc., Burlington, Ontario, Canada), approximately 200 ng genomic DNA. The PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation (94 °C, 1 min), annealing (56 °C, 1 min), extension (72 °C, 1 min), and a final extension at 72 °C for 5 min.

All PCR products were analyzed by electrophoresis using 2 % agarose gel prepared in 1×TAE buffer containing DuRed nucleic acid gel stain (Abgent Biotechnology Co., Ltd., Suzhou, China) run at 120 V for 35 min at room temperature. Subsequently, the PCR products were sequenced with the ABI3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using the BigDye fluorescence labeling Terminator Cycle Sequencing kit (Sangon Biotech, China). The sequence alignment of the *Tektin-t* gene was performed using DNAMAN. Sequences from the species were obtained from NCBI.

Statistical analysis and the pathogenicity prediction

Hardy-Weinberg equilibrium and the comparison of genotype frequencies between patients and control groups were performed using the chi-squared (χ^2) test. Using the unconditional logistic regression analysis to calculate odds ratio (OR) and 95 % confidence interval (95% CI) were to measure the risk associated with variant genotypes. $P<0.05$ was considered to be statistically significant. All data were analyzed using Statistical Package for Social Sciences software version 20.0 (SPSS Inc., Chicago, IL, USA).

Table 1 Primers used for the amplification of the 10 exons of the human Tektin-t gene

Exon	Primer	Primer sequence	Size (bp)	PCR product size (bp)
1	tektin-t 1 F	5′ AGTGAAAGCGCTGCCCTGAT 3′	20	384
	tektin-t 1R	5′ GGAGAGCCCCGTGGGAAGAG 3′	20	
2-3	tektin-t 2 F	5′ CAGAGCAAAATGGACAGGAA 3′	20	661
	tektin-t 3R	5′ TAGCGCTTGTCCCCACTTTA 3′	20	
4	tektin-t 4 F	5′ GCCATCACTCCCAGCTAA 3′	18	488
	tektin-t 4R	5′ GCCAAAATGTAGCACGAGAG 3′	20	
5-7	tektin-t 5 F	5′ CCTGTGGTGTCCCTTTAGTA 3′	20	894
	tektin-t 7 F	5′ TCAGGCTCAGGAGCTTTGTG 3′	20	
8-10	tektin-t 8 F	5′ ACTTCCTGCTGTGGGATGAG 3′	20	936
	tektin-t 10R	5′ CCTCTGCATTTATTCCATTC 3′	20	

F forward primer, *R* reverse primer

The prediction of the damaging effect of missense mutation to protein structure and function was performed using PolyPhen-2 bioinformatic program (http://genetics.bwh.harvard.edu/pph2/) [22]. For a false positive rate of 20 %, PolyPhen-2 achieved true positive prediction rates of 92 % and 73 % on HumDiv and HumVar datasets, respectively [22, 23]. We also used the ExPASy-ProtScale (http://web.expasy.org/protscale/) to analyze the changes in hydrophobicity of the tektin-t protein due to the mutation.

Results

A descriptive comparison of our study population is presented in Table 2. After sequencing the 10 exons of *tektin-t* and the flanking intronic sequence of 104 AZS patients, we screened five nucleotide substitutions: R46C (c.136C >T) in exon3, S174S (c.522C >T) in exon 5, R207H (c.620G >A) in exon 5, K267N (c.801G >T) in exon 7, and T393T (c.1179A >G) in exon 10. The five nucleotide changes were all detected in the heterozygotes excepted for a part of c.136C >T. We confirmed that c.522C >T, c.801G >T, and c.1179A >C were common mutations after screening the 102 controls and analyzing online database; however the genotype and allele frequencies of the missense mutation c.136C >T changing the arginine at position 46 to Cysteine were different. The amino acid 46R (Fig. 1a–c) is located in a highly conserved domain from invertebrate to higher species (Fig. 1d). This mutation in both asthenozoospermic patients and controls were in Hardy-Weinberg equilibrium (χ^2=1.5622, 0.9418; P=0.4579, 0.6244, respectively). The frequencies of genotype CT (OR=1.867, 95 % CI=1.047–3.328, P=0.034) and TT (OR=3.503, 95 % CI=1.138–10.778, P=0.023) were significantly increased compared with controls. The frequencies of allele 136 T (OR=1.745, 95 % CI=1.146–2.655, P=0.009) showed a significant increase in asthenozoospermic patients. Overall, R46C showed a significant difference of distribution between the idiopathic asthenozoospermia and fertile controls (Table 3). Moreover, PolyPhen-2 analysis predicted that this mutation is "probably damaging" with the score of 0.986 on HumVar model (Fig. 2). In addition, a hydropathy plot of the R46C mutant polypeptide generated with the Kyte-Doolittle algorithm by using an online tool ExPASy-ProtScale demonstrated a further imbalance in its hydrophobicity caused by the a.136C >T mutation and may result in modification of the protein structure (Fig. 3).

A novel heterozygous missense mutation (c.620G>A) was dectected in five asthenospermic patients (4.8 %), but this mutation was absent in controls, suggesting that it may be associated with asthenospermia. PolyPhen-2 analysis predicted that this mutation is "probably damaging" with the score of 0.999 on HumVar model.

Table 2 Comparison of age and semen parameters between asthenozoospermic group and controls

Clinical parameters	Asthenozoospermic group (n=104)[a]	Control group (n=102)[a]	P[b]
age (years)	31.2±6.2	34.9±3.5	0.496
Sperm concentration (×10^6/mL)	38.0±17.7	62.8±16.3	0.018
Rapid progressive motility (%)	9.9±5.9	37.9±8.1	<0.001
Total progressive motility (%)	21.2±10.3	63.53±12.8	0.006

[a] Data are presented as mean±SD

[b] The comparison between groups was done with the Student's t test. P<0.05 was considered statistically significant

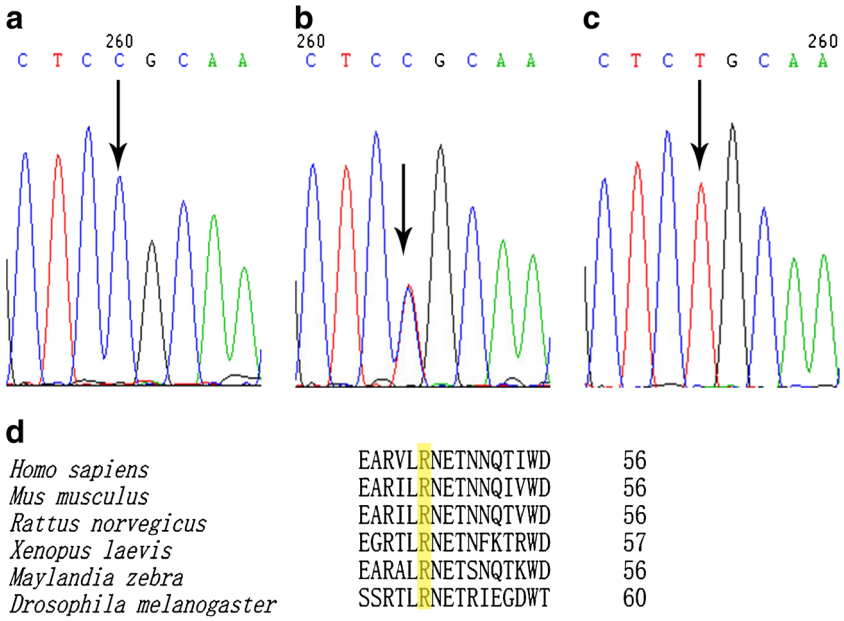

Fig. 1 Sequencing analysis of the 136 C >T of exon 3 in the *tektin-t* gene with different allele expressions. **a** Wild-type homozygous (CC) genotype. **b** Heterozygous (CT) genotype. **c** Mutant homozygous (TT) genotype. **d** Sequence alignment of the *tektin-t* gene in six different species. The position of our reported mutated amino acid (R46C) is framed

Discussion

Sperm with low motility has been identified as one of the important factors of male infertility [24]. AZS is a phenotype often present in primary ciliary dyskinesia (PCD) that is a rare autosomal recessive genetic disorder which could cause a defect in the action of the cilia lining of the respiratory tract and the flagella of sperm cells [25–27]. About 50 % of all affected PCD patients have laterality defects such as situs inversus (heterotaxy), this syndrome is referred as Kartagener Syndrome (KS) [28]. Approximately, 90 % of PCD/KS male patients are affected by AZS and most of them showing dynein genes mutation [16]. It has been demonstrated that tektins participated in the assembly of axonemal microtubules and offered stability to axonemal microtubules [29]. Tektins are a highly conserved family of flagellar and ciliary proteins in many species from invertebrate to higher species, such as *Chlamydomonas*, sea urchins, zebrafish, hedgehog, rodents, and humans [18, 19, 28, 30, 31]. In mammals, tektins exist in the testis, brain, retina, and other tissues containing ciliated cells [32, 33]. Tektins are insoluble α-helical proteins and are related to intermediate filament (IF) proteins and nuclear lamins. Thus, tektins may play a fundamental role in ciliary movement [10]. The human *tektin-t* gene has been first cloned in 2002, and tektin-t protein is localized to the tail of mature sperm [19]. Its highly conserved sequence suggested that tektin-t has an important role in the formation of ciliary and flagellar [17, 34].

In our study, we found a significant difference in frequency of the missense mutation (c.136C >T) of the *tektin-t* gene between idiopathic asthenozoospermic patients and the control groups. The incidences of the missense *tektin-t* gene mutation (R46C) (heterozygote [CT] and homozygote [TT]) were 64.42 % in asthenozoospermic patients and 47.06 % in the controls, showing a significant increase between the patients and the controls (OR=2.037, 95 % CI=1.165–3.562,

Table 3 Genotype and allele frequencies for *tektin t* C136T among idiopathic asthenozoospermia and controls

	Controls (N=102)	Asthenozoospemia (N=104)	Odds ratio (95 % CI)	P
136CC	(52.94 %)54	(35.58 %)37		
136CT	(42.16 %)43	(52.88 %)55	1.867 (1.047–3.328)	0.034*
136TT	(4.90 %)5	(11.54 %)12	3.503 (1.138–10.778)	0.023*
136 (CT+TT)	(47.06 %)48	(64.42 %)67	2.037 (1.165–3.562)	0.012*
136C	(74.02 %)151	(62.02 %)129		
136 T	(25.98 %)53	(37.98 %)79	1.745 (1.146–2.655)	0.009**

CI confidence interval

* $P<0.05$

** $P<0.01$

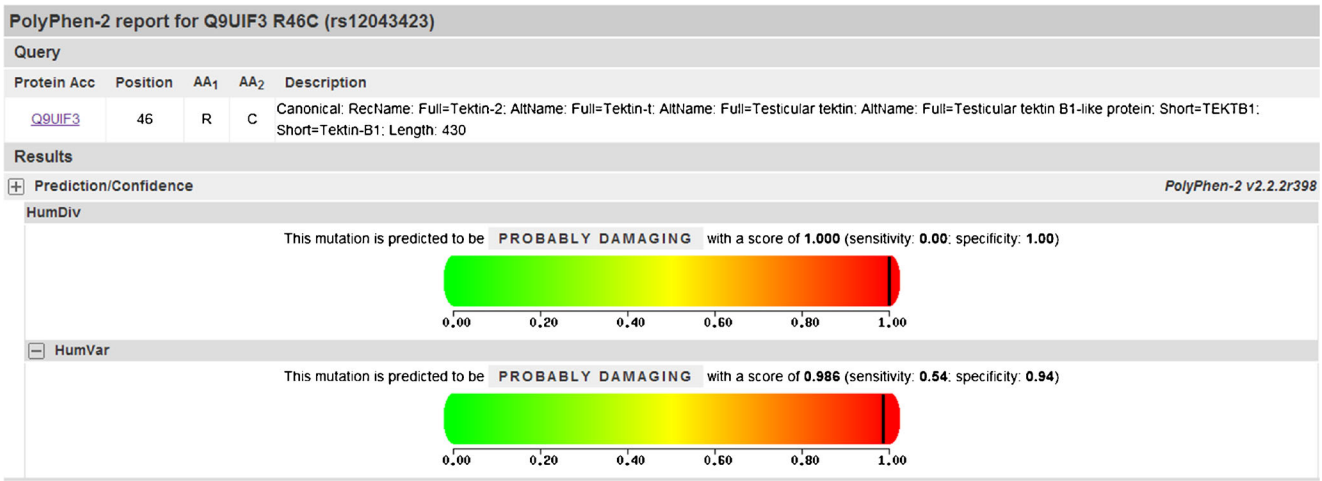

Fig. 2 PolyPhen-2 analysis predicting the pathogenicity, the p.R46C substitution on the tektin-t protein

P=0.012) which indicated that this mutation could be a possible risk (OR=1.745, 95 % CI=1.146–2.655, *P*=0.009) for AZS. These results were consistent with the facts that the mutated amino acid is located in a highly conserved position in the tektin-t protein (Fig. 1d), and this variant was analyzed using PolyPhen-2 with a result of "probably damaging" (Fig. 2). Additionally, the hydrophobicity of the arginine at the position 46 of the tektin-t protein was significantly different from the wild type, and the substitutions may result in modification of the protein structure (Fig. 3). Moreover, a novel mutation R207H was found in our study in five patients and predicted that this change is "probably damaging" with the score of 0.999 on HumVar model by PolyPhen-2 analysis. In addition, the frequency of R207H is more in Asian population (0.005) than non-Asian population (0.00003) which supports our finding.

Zuccarello et al. [35], analyzed 90 isolated non-syndromic AZS patients and found a heterozygous mutation A229V in one patient and predicted that a.a 229 holds some important roles in the tektins' functions. However, there was no detection of this mutation in our study. Furthermore, the R46C mutation was found to be associated with asthenozoospermia by statistical analysis and predicted to have a possible damaging effect to the protein structure and function, while this change was also found in previous study [35], but was regarded as a common SNP. In addition, the frequency of R46C is higher in Asian population (0.24) than non-Asian population (0.006), which is inconsistent with our result, which could be explained by the difference in geographical location. Moreover, a novel mutation R207H was found in our study in five patients, this mutation was never found in the previous study. The differences between our study and the

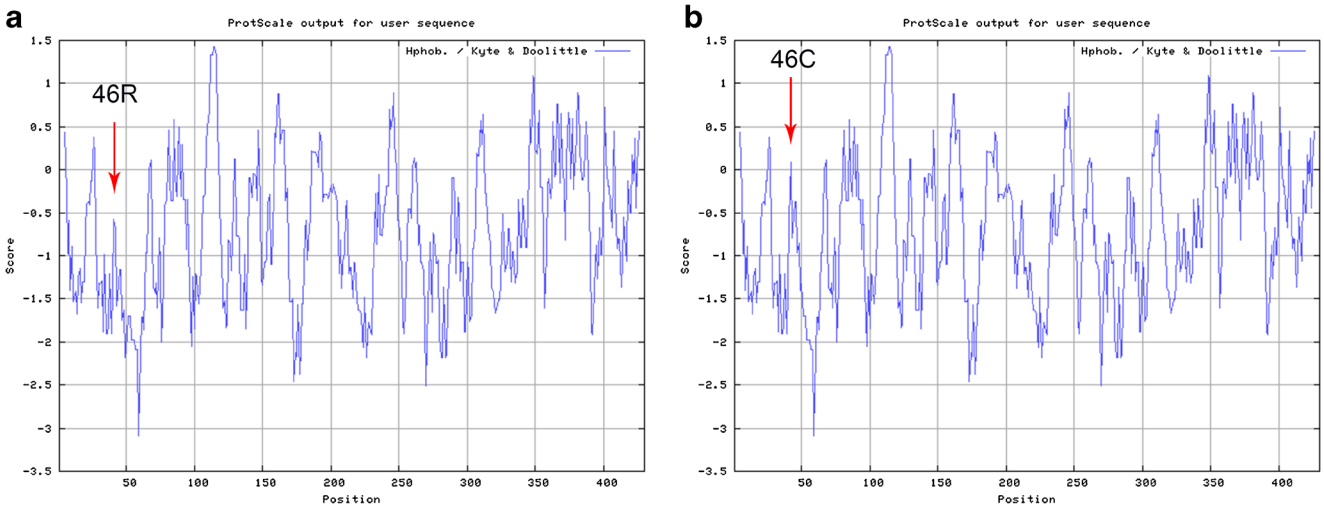

Fig. 3 Hydropathy plot for the tektin-t protein prepared in the Expasy ProtScale Website according to the Kyte and Doolittle algorithm. The hydrophobicity of the wild-type tektin-t protein a. is compared to the mutant form, including the novel p.R46C mutation b. The hydrophobicity scores of p.R46C of the tektin-t protein are higher than the wild type. The mutated site is indicated with an *arrow*

previous study could be explained by many factors as follows: the difference in geographical location and genetic background of the study populations, different screening standards of samples, and study sample size.

Conclusion

In conclusion, this is the first Chinese study on the association between SNPs of the *tektin-t* gene and idiopathic asthenozoospermia. Our results suggest that the tektin-t variant (R46C) is probably associated with asthenozoospermia. We also found a novel missense (R207H) variant in five asthenozoospermic patients but absent in fertile men, suggesting that this mutation might be associated with asthenozoospermia. Further studies are needed to confirm our results with larger groups of participants. Analyzing the structure of the tektin-t protein and functional studies should be taken to demonstrate these variants affect tektin-t protein activity, protein synthesis, and flagellar impairment of mature sperm. Furthermore, future studies including subjects of different ethnic and geographic origins should be conducted for *tektin-t* gene combined with other genes associated with AZS.

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