A familial study of twins with severe asthenozoospermia identified a homozygous *SPAG17* mutation by whole-exome sequencing

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/cge.13059

Tel: 86-592-2187228

Conflict of interest: The authors declare no conflict of interest.

Abstract

Asthenozoospermia (AZS) is a common cause of male infertility, characterized by abnormal reduction in the motility of ejaculated spermatozoa. Here, in a patient from a consanguineous family, we identified a homozygous mutation (c.G4343A, p.R1448Q) in *SPAG17* by whole-exome sequencing. The encoded protein, SPAG17, localizes to the axonemal central apparatus and is considered essential for flagellar waveform. *In silico* analysis revealed that R1448Q is a potential pathogenic mutation. Immunostaining and western blot assays showed that the R1448Q mutation may exert a negative effect on the steady-state of the SPAG17 protein. Therefore, *SPAG17* may be a new pathogenic gene causing AZS.

Key Words: asthenozoospermia, axoneme, flagella, SPAG17, whole-exome sequencing.

Introduction

Asthenozoospermia (AZS), also known as reduced sperm motility (Human Phenotype Ontology, HP: 0012207), is a common type of male infertility and is characterized as an abnormal reduction in the mobility of ejaculated sperm. Approximately 70% of infertile males have AZS (1). The mechanisms of AZS are complex, but the exact pathogenesis is unclear. Previous studies have suggested that AZS is of genetic origin (2, 3). To date, several genes have been reported to cause AZS, including *AKAP3* and *AKAP4* (4), *SLC26A8* (5), *DNAH1* (6), *CATSPER2* (7), *GALNTL5* (8), and *NSUN7* (9). These genetic studies suggested that an abnormal structure of the fibrous sheath (4), axoneme (6) and midpiece (9, 10), dysfunction on anion fluxes (5, 7), and reduced levels of glycolytic enzymes (8) affect the motility of the sperm flagellum, resulting in decreased sperm motility and the occurrence of AZS.

Here, we report in a consanguineous family twin brothers with severe AZS. Whole-exome sequencing and functional analysis revealed that the homozygous *SPAG17* mutation may be associated with AZS.

Materials and methods

Patients

The proband (29 years of age, II:5) was recruited from the Xiamen Maternity and Child Care Hospital. He had normal erection and ejaculation and sexual life 2-3 times per week, but his wife did not become pregnant or use contraception. The parents of the proband were in a consanguineous marriage in their close three generations and gave birth to three boys and a

girl (Fig. 1A) . The patient's brother (II:1) has a son, his sister (II:4) had two births, but his twin brother (II:7) is infertile. The patient is engaged in sales of aquatic products and has no bad chemical contact history or bad habits such as smoking and drinking. Physical examination results were as follows: height, 169 cm; weight, 65 kg; external genital development, normal; and bilateral testicular size and bilateral spermatic vein, normal. The semen examination results from our hospital were as follows: semen volume, 2.8 mL; sperm density, 27.5 million/mL; prorsad percentage motility, 1.8%; and non-prorsad percentage motility, 1.6%. Sperm morphology examined by Papanicolaou staining showed that the normal morphology sperm amounted to 4.5% and sperm acrosin level was 28 mIU/mL. Seminal plasma biochemical testing indicated that fructose level, neutral glycosidase activity, and seminal plasma zinc level were normal. On the basis of these results, the patient was diagnosed with severe AZS. The patient's wife was treated with *in vitro* fertilization (IVF). Twenty-four eggs (23 2-PN, 1 GV) were collected and formed 9 available embryos (one good-quality embryo). Two embryos were transplanted on January 2017 and the pregnancy was successful. Semen examination of his twin brother (II:7) in another hospital showed that semen volume was 2.2 mL, sperm density was 32.8 million/mL, prorsad percentage motility was 2.1%, and non-prorsad percentage motility was 2.5%. Sperm morphology examined by Papanicolaou staining showed that 5% of sperm had normal morphology. The proband's twin brother was also diagnosed with severe AZS. IVF for the twin brother's wife was also successful. These twin brothers did not have respiratory disorders or history of sinusitis.

The chromosomal karyotypes of the two patients were normal 46, XY; and no microdeletions were found in the Y chromosome. This study was approved by the Ethics

Committee of Xiamen Maternity and Child Care Hospital. Written informed consent was obtained and then 5 mL of peripheral blood was collected from each participant.

Exome sequencing and Sanger sequencing validation

Exome sequencing was carried out as previously described (11). The average depth of exome sequencing reads was more than 100×. Sanger sequencing was performed to validate the missense mutation of the *SPAG17* gene in the proband and his twin brother, sister, elder unaffected brother, mother, and father.

Immunostaining of spermatozoa and western blot analysis

Immunostaining and western blot analysis were carried out as previously described (12). The specific antibodies used in these assays are listed in Supplementary Table 2.

Results

Whole-exome sequencing analysis of the patient with AZS

The proband with severe AZS (Fig. 1A) was recruited to this study. Pedigree analysis suggested an autosomal recessive mode of inheritance of AZS, as the patients' parents were in a consanguineous marriage (Fig. 1A). Thus, we focused on homozygous mutations identified in the patient by whole-exome sequencing. After filtering out polymorphisms with allele frequencies greater than 1% in the dbSNP, 1000 Genomes, and ESP6500 databases, we compiled a list of genes harboring homozygous mutations (Supplementary Table 1). Among these genes, only *SPAG17* is closely related to flagellar function and localized to the axonemal central apparatus. By Sanger sequencing, the homozygous mutation in *SPAG17* (NM_206996:exon30:c.G4343A:p.R1448Q) was confirmed in the proband and his twin brother (Fig. 1B). The proband's unaffected brother, mother, and father all carried the

heterozygous allele. The proband's sister harbored the wild-type alleles (Fig. 1B).

In silico analysis of p.R1448Q mutation

In silico analysis predicted that the SPAG17 p.R1448Q mutation is a disease-associated mutation (Table 1). The allele frequency of c.G4343A is 0.0016 in the East Asian population according to ExAC database (Table 1). R1448Q is located in the C-terminus of the SPAG17 protein, which is necessary for flagellar motility. The basic amino acid arginine located at position 1448 in human SPAG17 is highly conserved between different species, from human to Western clawed frog (Fig. 1C), indicating the functional importance of the R1448 site. The proband's unaffected brother and father carried the heterozygous mutation, indicating that *SPAG17* was tolerant to heterozygous mutation. This was further supported by the finding that *SPAG17* had a residual variation intolerance score of 0.60 (13). Constraint metrics (intolerance to variation) reported in the ExAC Browser (http://exac.broadinstitute.org/gene/ENSG00000155761) also suggested that *SPAG17* is tolerant to both heterozygous missense (Z score = -2.39) and heterozygous loss-of-function (probability of LoF intolerance = 0.00) mutations, indicating that *SPAG17* has more heterozygous variants in the general population than expected. Thus, the R1448Q homozygous mutation may be associated with AZS.

Deceased expression of SPAG17 in patient's spermatozoa

To determine the potential effect of the R1448Q mutation on SPAG17, immunofluorescence analysis was carried out. SPAG17 was localized to the sperm flagella and caudal region of sperm head in normal controls (Fig. 2A). However, in the patient with AZS, SPAG17 showed low expression in the flagella (Fig. 2A). The low expression level was

also confirmed by western blot analysis (Fig. 2B and C). Therefore, the low expression level of SPAG17 protein in the patient suggested that the R1448Q mutation affects the steady-state level of the protein.

Discussion

SPAG17 is a central pair protein present in the axoneme of cells with a "9+2" organization of microtubules. *SPAG17* is expressed in the human and mouse testis and its encoded protein is localized to the axonemal central apparatus (14), which controls flagellar waveform and maintains the structural integrity of the axoneme. SPAG17 was found to interact with SPAG6 (14), another central apparatus protein essential for sperm motility, as *Spag6*-deficient male mice are sterile because of sperm motility defects (15). In *Spag6* mutant mice, SPAG17 was missing from the sperm (14, 16). SPAG17 protein is highly conserved throughout eukaryotes and *Chlamydomonas* homolog PF6, on which the carboxyl-terminus is necessary for flagellar motility (17). Ultrastructural analysis of *Spag17* knock-out mice revealed the loss of one central pair microtubule in cilia axonemes (18). Although SPAG17 has been found to play an important role in sperm flagellar function, the association of *SPAG17* with human male infertility remains unclear.

In this study we report a homozygous mutation, p.R1448Q, of *SPAG17* in twin brothers with severe AZS. Bioinformatics prediction and amino acid conservation analysis suggested that R1448Q is a pathogenic mutation. Immunostaining and western blot assays showed that R1448Q mutation may exert a negative effect on the steady-state level of the SPAG17 protein.

It has been reported that the cilia of *Spag17* homozygous mutant mice did not move, while those of *Spag17*$^{+/-}$ and *Spag17*$^{+/+}$ mice moved normally. This suggests that the nexus between the central pair (CP) protein (divided into C1 and C2) and radial spokes is critical for stabilizing the axoneme. The main reasons for cilia/flagella movement abnormalities in mice and *Chlamydomonas* are as follows: 1) *Spag17*-deficient mice were also missing a C1 projection. In addition, approximately 25% of the tracheal ciliary axonemes were missing one CP microtubule (18); 2) Analysis of the *Chlamydomonas* PF6 gene (SPAG17 homologous gene) showed that the PF6 protein interacts with a number of other proteins, including calmodulin, and ultimately influences the radial spokes attached to the outer microtubule doublets (19). Thus, the mutation in SPAG17 may affect the structure and function of CP microtubules and outer microtubule doublets, ultimately leading to AZS.

In conclusion, our study demonstrated that a homozygous mutation p.R1448Q of *SPAG17* may lead to severe AZS, possibly by affecting the steady-state level of the SPAG17 protein and structure and function of the microtubules. This finding will aid researchers and clinicians in better understanding the molecular etiology of AZS.

Acknowledgements

Research funding was supported by the Youth Scientific Research Project of Fujian Provincial Health and Family Planning Commission [Grant no. 2015-2-52 and 2016-2-72], the Science and Technology Project of Fujian Province [Grant No. 2015D018, 2016D10 and 2017D011], the Xiamen Science and Technology Planning Project [Grant no. 3502Z20154033], the Major/Important Disease Research Project [Grant No. 3502Z20159022], the Young/Middle-aged Talent Cultivation Project [Grant no.

2015-ZQN-JC-44], and the Open Project of Key Laboratory of Male Reproductive and Genetics, National Health and Family Planning Commission [Grant No. KF201704]. We thank the participants described in this report for their consent and support to publish this manuscript. We also thank the experts from Editage for their hard work on the redaction.

Contributions

YW.S., ZY.J., LB.M, PP.Q. P.L., and H.J. collected and provided clinical information, YW.S., XH.X, and L.L. carried out whole-exome sequencing and data analysis; XH.X. performed functional experiments; L.L., XH.X., and TL.W. designed experiments and wrote the manuscript.

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Figure Legends

Figure 1. *SPAG17* mutation in twins with AZS. (A) Patients with AZS in a pedigree. The affected family members (proband and his twin brother) are indicated as black squares. The proband indicated with an arrow. (B) Sanger sequencing confirmation of *SPAG17* mutation, c.G4343A. The proband and his twin brother carried the homozygous mutation. His father, mother, and elder brother harbored the heterozygous allele, while his sister's genotype showed wild-type alleles. The red arrow indicates the c.G4343A site. (C) Alignment of SPAG17 proteins in different species. The R1448 site of human SPAG17 was highly conserved in the aligned sequences.

Figure 2. SPAG17 mutated protein showed decreased expression. (A) Immunofluorescence for SPAG17 in spermatozoa. Immunofluorescence staining of normal male sperm (upper) and patient's sperm (lower) with SPAG17 antibody. SPAG17 was labeled with rabbit anti-SPAG17 primary antibody and DsRed-conjugated anti-rabbit secondary antibody. Nuclei were stained with DAPI. (B) Western blot of SPAG17 level in spermatozoa from patient and healthy controls. β-Tubulin was used as a positive control. (C) Quantification analysis of the western blot. Integrated intensity of SPAG17 bands were normalized against those of β-tubulin.

Table 1. *In silico* analysis of *SPAG17* mutation

Mutation	Amino acid change	Polyphen-2[a]	SIFT[b]	Mutation Taster[c]	SNPs&GO[d]	ExAC (total)[e]	ExAC (East Asian)[f]
c.G4343A	p.R1448Q	Possibly-damaging (0.874)	Tolerated (0.28)	Polymorphism (0.986)	Neutral (0.178)	2.999e-05	0.0016

[a]Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/). Prediction Scores range from 0 to 1 with high scores indicating probably or possibly damaging.

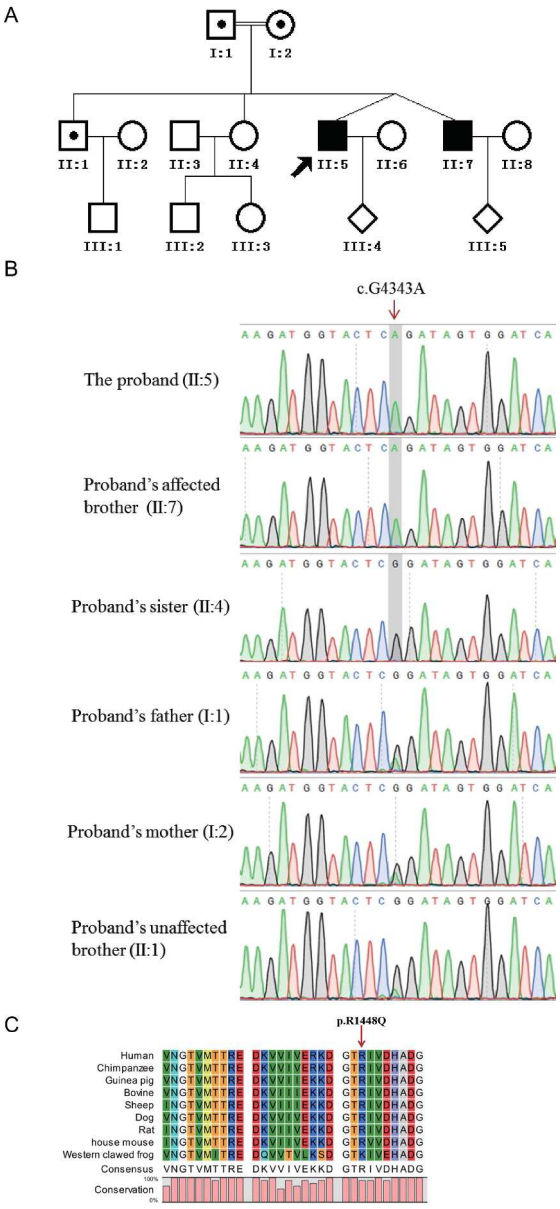
[b]SIFT, i.e., Sorting Intolerant From Tolerant (http://sift.jcvi.org/). Scores vary between 0 and 1. Variants with scores close or equal to 0 are predicted to be damaging.

[c]Mutation Taster (http://www.mutationtaster.org/). The probability value is the probability of the prediction, i.e., a value close to 1 indicates a high 'security' of the prediction.

[d]SNPs&GO (http://snps.biofold.org/snps-and-go/). Probability: Disease probability (if >0.5 mutation is predicted Disease).

[e]Frequency of variation in total of ExAC database.

[f]Frequency of variation in East Asian population of ExAC database.



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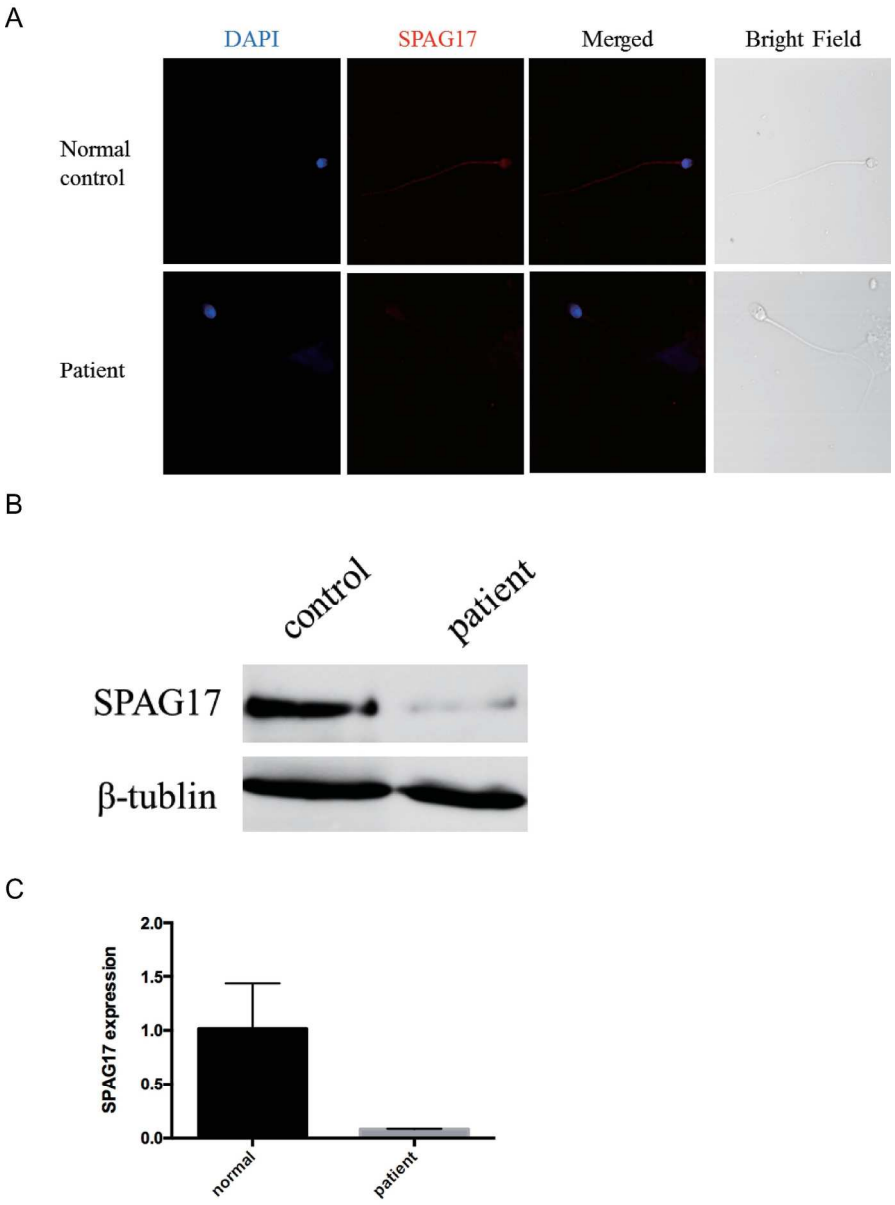

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