Novel biallelic loss-of-function mutations in CFAP43 cause multiple morphological abnormalities of the sperm flagellum in Pakistani families

Ihsan Khan*, Basit Shah*, Sobia Dil, Nadeem Ullah, Jian-Teng Zhou, Da-Ren Zhao, Yuan-Wei Zhang, Xiao-Hua Jiang, Ranjha Khan, Asad Khan, Haider Ali, Muhammad Zubair, Wasim Shah, Huan Zhang, Qing-Hua Shi

Multiple morphological abnormalities of the sperm flagellum (MMAF) is a specific type of asthenoteratozoospermia, presenting with multiple morphological anomalies in spermatozoa, such as absent, bent, coiled, short, or irregular caliber flagella. Previous genetic studies revealed pathogenic mutations in genes encoding cilia and flagella-associated proteins (CFAPs; e.g., CFAP43, CFAP44, CFAP65, CFAP69, CFAP70, and CFAP251) responsible for the MMAF phenotype in infertile men from different ethnic groups. However, none of them have been identified in infertile Pakistani males with MMAF. In the current study, two Pakistani families with MMAF patients were recruited. Whole-exome sequencing (WES) of patients and their parents was performed. WES analysis reflected novel biallelic loss-of-function mutations in CFAP43 in both families (Family 1: ENST00000357060.3, p.Arg300Lysfs*22 and p.Thr526Serfs*43 in a compound heterozygous state; Family 2: ENST00000357060.3, p.Thr526Serfs*43 in a homozygous state). Sanger sequencing further confirmed that these mutations were segregated recessively in the families with the MMAF phenotype. Semiquantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was carried out to detect the effect of the mutation on mRNA of the affected gene. Previous research demonstrated that biallelic loss-of-function mutations in CFAP43 accounted for the majority of all CFAP43-mutant MMAF patients. To the best of our knowledge, this is the first study to report CFAP43 biallelic loss-of-function mutations in a Pakistani population with the MMAF phenotype. This study will help researchers and clinicians to understand the genetic etiology of MMAF better.

Asian Journal of Andrology (2021) 23, 627–632; doi: 10.4103/aja.aja_26_21; published online: 28 May 2021

Keywords: cilia and flagella-associated proteins; male infertility; multiple morphological abnormalities of the sperm flagella; whole-exome sequencing

INTRODUCTION

Multiple morphological abnormalities of the sperm flagellum (MMAF) is one of the more severe forms of sperm defect, characterized by bent, coiled, irregular, short, or absent sperm flagella. The sperm flagellum in MMAF patients often shows ultrastructural abnormalities associated with the “9 + 0” arrangement of dynein microtubules, such as lacking the central pair of microtubules, disorganized axoneme, and mitochondrial sheath, which in turn affects sperm motility and leads to male infertility.

In the past few years, the development of next-generation sequencing technology has led to identification of a genetic cause in MMAF patients. Various pathogenic mutations have been found in genes encoding cilia and flagella-associated proteins (CFAPs), such as CFAP43, CFAP44, CFAP65, CFAP69, CFAP70, and CFAP251. It has been noted that all these CFAP-associated genes have diverse functions and location. For example, CFAP43, CFAP44, and CFAP65 are associated with the inner dynein arm (IDA) complex tether/tether head (T/TH); CFAP69 is associated with intraflagellar transport (IFT); CFAP70 is related to the outer dynein arm (ODA)-associated complex; and CFAP251 is identified in the calmodulin and spoke-associated complex (CSC). In 2017, Tang et al. identified biallelic loss-of-function mutations of CFAP43 in Chinese MMAF patients and further confirmed the pathogenicity in knockout mouse models of the Cfap43 ortholog gene. Later, in 2018, Coutton et al. also identified CFAP43 biallelic mutations in MMAF patients from different ethnic groups. Biallelic mutations of CFAP43 and CFAP44 have been reported to be associated with MMAF patients. However, the genetic causes of MMAF among Pakistani patients remain unexplored. Given the existence of a traditional and close-knit society in Pakistan, approximately 65% of the population have consanguineous marriages. A high proportion of consanguineous marriage increases the risk of autosomal recessive disorders in offspring. Such kinds of...
as per the manufacturer's instructions. For WES, AllExome Enrichment Kit V1 (GeneTech, Beijing, China)-captured libraries were constructed for family members of Family 1 (I:1, I:2, II:1, and II:2) and Family 2 (III:1, III:2, IV:3, and IV:4) as instructed by the manufacturer. Sequencing was carried out on a Hiseq2000 platform (Illumina, San Diego, CA, USA). Clean reads were mapped to the human reference genome (hg19) by Burrows–Wheelier Alignment tool. Variants were discovered and annotated with the Genome Analysis Toolkit (GATK)\(^2\) and ANNOVAR.\(^3\) After that, specific filtration pipelines for each family are described in Supplementary Figure 1 and detailed in Supplementary Table 2 and Supplementary Table 3. Sanger sequencing was performed to verify the selected variants in all the available family members. The primers for PCR are listed in Supplementary Table 1.

Transmission electron microscopic (TEM) analysis of spermatozoa TEM analysis was performed according to Zhang \(\text{et al.}\)\(^{1,1}\) in 2019. Spermatozoa from the patient and a fertile control individual were taken and fixed in 0.1 mol l\(^{-1}\) phosphate buffer (PB; pH 7.4), comprising 0.2% picric acid, 8% glutaraldehyde, and 4% paraformaldehyde and stored at 4°C overnight. Samples were washed with 0.1 mol l\(^{-1}\) PB, postfixed with 1% osmium tetroxide. Spermatozoa cells were dehydrated through graded alcohol (30%, 60%, 90%, 100%, 100%, and 100%; 10 min for each bath) followed by infiltration of an epon resin and acetone mixture. Ultrathin (70 nm) sections were cut from the samples followed by staining with lead citrate and uranyl acetate. Tecnai 10 or 12 Microscopes (Philips CM10, Philips Electronics, Eindhoven, The Netherlands) at 120 kV or 100 kV were used to capture TEM analysis. The samples were dehydrated through graded alcohol (30%, 60%, 90%, 100%, 100%, and 100%; 10 min for each bath) followed by infiltration of a resin mixture. Ultrathin sections (70 nm) were cut from the samples followed by staining with lead citrate and uranyl acetate. Tecnai 10 or 12 Microscopes (Philips CM10, Philips Electronics, Eindhoven, The Netherlands) at 120 kV or 100 kV were used to capture TEM analysis.

RNA extraction and semiquantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) Total sperm RNA from patient (Family 2-IV:3) and a fertile male was extracted with RNAiso Plus (TAKARA, Beijing, China) and reverse-transcribed into cDNA by PrimeScript RT Reagent Kit (TAKARA) as per the manufacturer’s instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward: 5'-GTCAAGGCTGAGAAGGGA-3'; reverse: 5'-AAATGAGCCCCAGCCTTCTC-3') was used as an internal control and CFAP43 (Ensembl transcript ID: ENST00000357060.3) primers used were as follows: forward: 5'-AGCACGTCGTTTATGATCAG-3'; reverse: 5'-AGCACGTCGTTTATGATCAG-3'.

RESULTS
Clinical features of patients
This study was performed on two Pakistani families with three infertile men. Family 1-II:1 (57 years), Family 1-II:2 (55 years), and Family 2-IV:3 (39 years) had been married for 31 years, 26 years, and 14 years, respectively, but all were infertile. Detailed information was collected from each patient to exclude the possibility of associated infertility-related disease. All the individuals were healthy, with no previous history of any testicular injury or obstruction, no symptoms of Primary Ciliary Dyskinesia (PCD; disease ID: #MIM 244400). Detailed pedigree charts were constructed on the basis of information provided by their parents (Figure 1). All the physical characteristics and semen parameter values of the patients are presented in Table 1. The semen volumes, pH, and viscosity fell within the normal ranges according to the World Health Organization guidelines (2010).\(^{27}\) However, sperm concentrations were lower than the normal range (Table 1). Sperm morphological analysis reflected severe abnormalities of flagella including bent, short, coiled, irregular, and absent that are typical characteristics of MMAF (Figure 2a).
Novel biallelic loss-of-function mutations in CFAP43 are candidate pathogenic variants in the families

To identify the genetic cause of MMAF, WES was performed for all available family members as shown in Figure 1. WES data were filtered according to the detailed pipeline in Supplementary Figure 1. As stated in a previous study, MMAF is an autosomal recessive inheritance,17 so as from the family history of Family 1, and the parents in Family 2 were in a consanguineous marriage, we focused on homozygous/compound heterozygous mutations shared by patients. Finally, the filtration pipeline identified novel biallelic loss-of-function mutations in CFAP43 in both families (Family 1: ENST00000357060.3, c.899_900del and c.1577_1578del in a compound heterozygous state; Family 2: ENST00000357060.3, c.1577_1578del in a homozygous state). It is noteworthy that the frameshift mutation (c.1577_1578del) was identified in both families.

CFAP43 mutation induced severe axonemal disorganization

TEM was performed to observe the ultrastructure defects of patient II:1’s spermatozoa of Family 1, as well as normal sperm ultrastructure from a fertile control individual. For TEM, a typical microtubule structure was presented in the spermatozoa of the fertile control that contains a "9 + 2" axonemal arrangement of nine doublets of microtubules (DMTs) and two central pairs (CP), surrounded by a fibrous sheath (FS) and outer dense fibers (ODF) as shown in Figure 2b. In contrast to the fertile male spermatozoa, CFAP43-deficient sperm cross-sections showed axonemal and periaxonemal defects and approximately 82% of the cross-sections were abnormal (Figure 2b). The main defect was severe disorganization of the FS, ODF, and axonemal disassembly, and in some cross-sections the absence of central pair complex (CPC) (9 + 0 conformation).

CFAP43 mutations cosegregated with MMAF phenotype in the families and induced CFAP43 mRNA decay

Sanger sequencing confirmed that the WES-identified CFAP43 mutations cosegregated with MMAF phenotype in both families (Figure 3a and 3b). To determine the effects of the frameshift mutation (c.1577_1578del) on CFAP43 expression, we measured CFAP43 mRNA in spermatozoa of the patient from Family 2, using the sperm sample from a fertile male as control. As shown in Figure 3c, CFAP43 mRNA was detected in the control sample, but not in the patient IV:3. Owing to the unavailability of Family 1 patients’ fresh semen samples for mutant CFAP43 protein/mRNA detection, we compared the mutation c.899_900del with reported CFAP43 mutations that had been confirmed in mRNA or protein level. Our mutation, c.899_900del (predicted truncate protein, p.Arg300Lysfs*22), was close to p.Asn380Lysfs*3, which was previously identified by Wu et al.14 and has been confirmed to cause mRNA decay by quantitative polymerase chain reaction (qPCR), as well as the lack of CFAP43 protein by immunofluorescent staining in patients’ semen samples.

DISCUSSION

In the current study, we recruited two Pakistani families with MMAF patients. After WES of all available family members, novel biallelic loss-of-function mutations in CFAP43 were identified in both families (Family 1: ENST00000357060.3, c.899_900del and c.1577_1578del in a compound heterozygous state; Family 2: ENST00000357060.3, c.1577_1578del in a homozygous state), as shown in Figure 4. Sanger sequencing further confirmed that these mutations were segregated recessively in the families with MMAF phenotype. Furthermore, the mutation c.1577_1578del has been confirmed to cause mRNA degradation in patient’s spermatozoa from the Family 2. TEM results of the patient II:1’s spermatozoa of Family 1 showed severe disorganization of the axoneme. This is the first report of novel biallelic loss-of-function mutations in CFAP43 causing MMAF in the Pakistani population.

Of all identified CFAP43 mutations, 80% are loss-of-function mutations, which include frameshift, nonsense, and splice-site mutations (Figure 5 and Supplementary Table 4 Ref 2,16,11,32). These loss-of-function mutations (frameshift and nonsense) might cause...
Mutations in *CFAP43* cause MMAF

I Khan et al

mRNA degradation or produce truncated protein. Detailed sperm analyses indicated an increased number of immotile spermatozoa (98%–100%), and all patients’ spermatozoa had typical MMAF characteristics. Furthermore, no significant differences were observed among the semen parameters of the patients harboring *CFAP43* mutations in the current study compared with the previously reported patients with other *CFAP43* mutations (Supplementary Table 4). Wu et al.\(^1\) first examined two *CFAP43* mutations’ effects (p.Asn380Lysfs*3 and p.Gln492Arg) on mRNA and protein level in patients’ spermatozoa and found that both mutations cause *CFAP43* mRNA degradation. In the current study, we could not obtain fresh semen samples from patients of Family 1 to verify the *CFAP43* mutation effects on mRNA and protein level. However, since the mutation (p.Arg300Lysfs*22) is close to the mutations verified by Wu et al.\(^1\) (p.Asn380Lysfs*3 and p.Gln492Arg), we speculate that *CFAP43* mutations identified in our study have a similar effect on *CFAP43* expression, resulting in complete loss of *CFAP43* (Figure 4).

*CFAP43* and *CFAP44* mutations account for 7.5%–30.8% of MMAF patients from a different study cohort, specified in a recent review.\(^1\) Tang et al.\(^2\) identified patients harboring *CFAP43* or *CFAP44* mutations, explaining 7.5% (4/30) of all patients with MMAF. However, Yan et al.\(^3\) identified 22.2% of 27 patients carrying *CFAP44* or *CFAP43* mutations. The most recent study by Wu et al.\(^1\) reported 30.8% of all patients.

---

**Figure 3:** Sanger sequencing results of *CFAP43* mutations in DNA and mRNA levels. Chromatograms of the *CFAP43* mutations from (a) Family 1 and (b) Family 2. Red/Blue arrows show the genomic position of *CFAP43* mutations. (c) SqRT-PCR analysis of *CFAP43* mRNA levels in male control and Family 2-IV.3 sperm samples. SqRT-PCR: semiquantitative reverse-transcriptase polymerase chain reaction; *CFAP*: cilia and flagella-associated protein; bp: base pair; Ref: reference; Het: heterozygous; chr10: chromosome 10; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase; del: deletion.

**Figure 4:** The identified mutations in *CFAP43* gene and predicted mutant proteins. *CFAP43* gene structure (Ensembl transcript ID: ENST00000357060) is shown with mutations identified in both families. Vertical bars indicate exons and slashed lines represent introns. *CFAP43* (1655 AA) comprises two domains: WD (tryptophan-aspartic acid (W-D)) repeat domain and SMC_N coil domain. *CFAP*: cilia and flagella-associated protein; AA: amino acid; SMC_N: N-terminus of structural maintenance of chromosome; del: deletion.

---

According to previous information, good intracytoplasmic sperm injection (ICSI) outcomes are reported for MMAF patients with *CFAP43* and *CFAP44* mutations. The recorded rates of transferable embryo, implantation, and clinical pregnancy were 80%, 50%, and 100%, respectively, in *CFAP43*.\(^5\) Hence, it is worth mentioning that it would be more interesting for researchers and clinicians to apply ICSI for *CFAP43*-mutant MMAF patients and
Table 1: Characteristics and sperm morphology in the patients

| Characteristic | Reference value | Family 1–II:1 | Family 1–II:2 | Family 2–IV:3 |
|---------------|----------------|--------------|--------------|--------------|
| Genotype      | –              | c.899_900del/ | c.899_900del/ | c.1577_1578del/ |
|               | –              | c.1577_1578del | c.1577_1578del | c.1577_1578del |
| Age (year)²   | –              | 57           | 55           | 39           |
| Years of marriage³ | –     | 31           | 26           | 14           |
| BMI (kg m⁻²)  | –              | 37.1         | 31.3         | 23.5         |
| Semen parameters |              |              |              |              |
| Semen volume (ml) | >1.5         | 2.0          | 3.0          | 3.3          |
| Semen pH       | Alkaline       | Alkaline     | Alkaline     | Alkaline     |
| Sperm concentration (x 10⁶ ml⁻¹) | >15          | 9            | 6            | 7            |
| Motility (%)   | >40            | 0            | 0            | 0            |
| Progressively motility (%) | >32         | 0            | 0            | 0            |
| Sperm morphology |              |              |              |              |
| Normal flagella (%) | >4.0         | 3.2          | –            | 0.8          |
| Abnormal flagella (%) | –            | 96.7         | –            | 99.1         |
| Short flagella (%) | –            | 70.9         | –            | 44.5         |
| Absent flagella (%) | –            | 17.2         | –            | 18.3         |
| Bent flagella (%) | –            | 5.4          | –            | 14.9         |
| Coiled flagella (%) | –            | 5.0          | –            | 12.6         |
| Irregular/caliber (%) | –            | 0            | –            | 8.8          |
| Head defects |              |              |              |              |
| Normal head (%) | –            | 6.8          | –            | 4.9          |
| Abnormal head (%) | –            | 93.1         | –            | 95.2         |
| Tapered head (%) | –            | 45.9         | –            | 71.1         |
| Pyriform head (%) | –            | 25.0         | –            | 14.7         |
| Double head (%) | –            | 1.4          | –            | 0.9          |
| Large/amorphous head (%) | –        | 0            | –            | 0.9          |
| Round head (%) | –            | 10.9         | –            | 5.5          |
| Small head (%) | –            | 6.5          | –            | 0.9          |
| Absent head (%) | –            | 3.4          | –            | 1.2          |

²Reference values were published in WHO (2010). ³The current years of marriage. –: not available; BMI: body mass index; WHO: World Health Organization; del: deletion

In conclusion, our study identified novel loss-of-function mutations in CFAP43 in Pakistani MMAF patients. These findings highlight the significance for genetic counseling and diagnosis for MMAF patients in the Pakistani population, while CFAP43 could be routinely genetic diagnosed. Further studies are needed to identify improvements in the prediction of ICSI outcomes for MMAF patients in Pakistan. However, it is very important to know the genetic screening of the wives of male patients carrying CFAP43 mutation before the couple asks for ICSI, to reduce the chances of genetic diseases in the offspring.
other pathogenic genes to characterize better MMAF in the Pakistani population.

AUTHOR CONTRIBUTIONS
IK and BS wrote the manuscript and performed semen analysis; SD, NU, AK, HA, XHJ, WS, MZ, and RK collected patients’ samples. JTZ, DRZ, and YWZ performed the WES sequencing and WES data analysis. QHS and HZ conceived and supervised the study, designed and analyzed data, and wrote the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS
All authors declared no competing interests.

ACKNOWLEDGMENTS
This work was supported by the National Natural Science Foundation of China (No. 32070850), the National Natural Science Foundation of China (No. 31630050, 31890780, and 32061143006), the National Key Research and Development Program of China (2018YFC1003900, 2019YFA0802600, and 2016YFC1000600), the Strategic Priority Research Program of the Chinese Academy of Sciences (No. XDB19000000), and the Fundamental Research Funds for the Central Universities (No. YD2070002006).

Supplementary Information is linked to the online version of the paper on the Asian Journal of Andrology website.

REFERENCES
1. Touré A, Martínez G, Kheirafi ZE, Cazin C, Beurois J, et al. The genetic architecture of morphological abnormalities of the sperm tail. J Hum Genet 2021; 140: 21–42.
2. Tang S, Wang X, Li W, Yang X, Li Z, et al. Biallelic mutations in CFAP43 and CFAP44 cause male infertility with multiple morphological abnormalities of the sperm flagella. Am J Hum Genet 2017; 100: 854–64.
3. Chemes HE, Brugo S, Zanchetti F, Carrere C, Lavieri JC. Dysplasia of the fibrous sheath: an ultrastructural defect of human spermatozoa associated with sperm immotility and primary sterility. Fertil Steril 1987; 48: 664–9.
4. Rawe V, Galaverna G, Acosta A, Olmedo SB, Chemes H. Incidence of tail structure distortions associated with dysplasia of the fibrous sheath in human spermatozoa. Hum Reprod 2001; 16: 879–86.
5. Sha YW, Wang X, Su ZY, Mei LB, Ji ZY, et al. Patients with multiple morphological abnormalities of the sperm flagella harbouring CFAP44 or CFAP43 mutations have a good pregnancy outcome following intracytoplasmic sperm injection. Andrologia 2019; 51: 121–31.
6. Barthélémy C, Trasanne M, Lebas C, Lecomte P, Lanskaj T. Tail stump spermatozoa: morphogenesis of the defect. An ultrastructural study of sperm and testicular biopsy. Andrologia 1990; 22: 417–25.
7. Khelifa MB, Coutton C, Zouari R, Karouzène T, Rendu J, et al. Mutations in DNAH1, which encodes an inner arm heavy chain dynein, lead to male infertility from multiple morphological abnormalities of the sperm flagella. Am J Hum Genet 2014; 94: 95–104.
8. Yang SM, Li HB, Wang JX, Shi YC, Cheng HB, et al. Morphological characteristics and initial genetic study of multiple morphological anomalies of the flagella in China. Asian J Androl 2015; 17: 513.
9. Chemes HE, Rawe VY. Sperm pathology: a step beyond descriptive morphology. Origin, characterization and fertility potential of abnormal sperm phenotypes in infertile men. Hum Reprod Update 2003; 9: 405–28.
10. Coutton C, Vargas AS, Amir-Yekta A, Kheirafi ZE, Mustapha SF, et al. Mutations in CFAP43 and CFAP44 cause male infertility and flagellum defects in Trypanosoma and human. Nat Commun 2018; 9: 1–18.
11. Wu H, Li W, He X, Liu C, Fang Y, et al. Novel CFAP43 and CFAP44 mutations cause male infertility with multiple morphological abnormalities of the sperm flagella (MMAF). Reprod Biomed Online 2018; 38: 769–78.
12. Wang WL, Tu CF, Nie HC, Meng LL, Li Y, et al. Biallelic mutations in CFAP65 lead to severe asthenoteratospermia due to acrosome hypoplasia and flagellum malformations. J Med Genet 2019; 56: 750–7.
13. Liu CY, Liu MR, He XJ, Zhu Y, Amir-Yekta A, et al. Homozygous mutations in SPEF2 induce multiple morphological abnormalities of the sperm flagella and male infertility. J Med Genet 2020; 57: 31–7.
14. Liu CY, He XJ, Liu WJ, Yang SM, Wang LB, et al. Bi-allelic mutations in TTC29 cause male subfertility with asthenoteratospermia in humans and mice. Am J Hum Genet 2019; 105: 1168–81.
15. Kheirafi ZE, Amir-Yekta A, Dacheux D, Karouzène T, Coutton C, et al. A homoygous ancestral SVA-insertion-mediated deletion in WDR66 induces multiple morphological abnormalities of the sperm flagellum and male infertility. Am J Hum Genet 2018; 103: 400–12.
16. Auguste Y, Delagve V, Des virtues JP, Longepied G, Grisac A, et al. Loss of calmodulin- and radial-spoke-associated complex protein CPAF251 leads to immotile spermatozoa lacking mitochondria and infertility in men. Am J Hum Genet 2018; 103: 413–20.
17. He XJ, Li WY, Wu H, Lv MR, Liu WJ, et al. Novel homozygous CFAP69 mutations in humans and mice cause severe asthenoteratospermia with multiple morphological abnormalities of the sperm flagella. J Med Genet 2019; 56: 96–103.
18. Dong FN, Amir-Yekta A, Martinez G, Saut A, Tek J, et al. Absence of CFAP69 causes male infertility due to multiple morphological abnormalities of the flagella in humans and mouse. Am J Hum Genet 2018; 102: 636–48.
19. Beurois J, Martinez G, Cazin C, Kheirafi ZE, Amir-Yekta A, et al. CFAP70 mutations lead to male infertility due to severe asthenoteratospermia. A case report. Hum Reprod 2019; 34: 2071–9.
20. Manzoor R, Imran M, Ayes A, Syed T. Consanguineous marriages: effects on pregnancy outcomes in Pakistan. J Dev Policy Pract 2018; 2: 78–105.
21. Ansari M, Ebstein F, Özköç H, Paracha SA, Iwaszkiewicz J, et al. Biallelic variants in PSMB1 encoding the proteasome subunit 6 cause impairment of proteasome function, microcephaly, intellectual disability, developmental delay and short stature. Hum Mol Genet 2020; 29: 1132–43.
22. Santos-Cortez RL, Faridi R, Rehman AU, Lee K, Ansar M, et al. Autosomal-recessive hearing impairment due to rare missense variants within S1PR1. Am J Hum Genet 2016; 98: 331–8.
23. Khan MI, Kersten FF, Azam M, Collin RW, Hussain A, et al. CLRN2 mutations cause nonsyndromic retinotopic pigmentosa. Ophthalmology 2011; 118: 1444–8.
24. Zhang B, Ma H, Khan T, Ma A, Li T, et al. DNAH17 missense variant causes flagella destabilization and asthenospermia. J Exp Med 2020; 217: e20182365.
25. Yin H, Ma H, Hussain S, Zhang H, Xie X, et al. A homozygous FANCM frameshift pathogenic variant causes male infertility. Genet Med 2018; 21: 62–70.
26. Yu Y, Wang J, Zhou L, Li H, Zheng B, et al. CFAP43-mediated intra-manchette transport is required for sperm head shaping and flagella formation. Zygote 2021; 29: 75–81.
27. World Health Organization. Laboratory Manual for the Examination and Processing of Human Semen. 5th ed. Geneva: WHO Press; 2010.
28. Li H, Durbin R. Fast and accurate short read alignment with Burrows–Wheeler transform. Bioinformatics 2009; 25: 754–60.
29. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 2010; 20: 1297–303.
30. Wang K, Li MY, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res 2010; 38: e164.
31. Zhang B, Khan I, Liu C, Ma A, Khan A, et al. Novel loss-of-function variants in DNAH17 cause multiple morphological abnormalities of the sperm flagella in humans and mice. Clin Genet 2020; 99: 176–86.
32. Sha YW, Wang X, Xu X, Su ZY, Cui Y, et al. Novel mutations in CFAP44 and CFAP43 cause multiple morphological abnormalities of the sperm flagella (MMAF). Reprod Sci 2019; 26: 26–34.
33. Martinez G, Kheirafi ZE, Zouari R, Mustapha SF, Saut A, et al. Whole-exome sequencing identifies mutations in FSIP2 as a recurrent cause of multiple morphological abnormalities of the sperm flagella. Hum Reprod 2018; 33: 1973–84.
34. Lorès P, Coutton C, El Khouri E, Stouvenel L, Givelet M, et al. Homozygous missense mutation L1573P in adenylate kinase 7 (AK7) leads to primary male infertility and multiple morphological anomalies of the flagella but not to primary ciliary dyskinesia. Hum Mol Genet 2018; 27: 1196–211.
35. Coutton C, Martinez G, Kheirafi ZE, Amir-Yekta A, Bouguenet M, et al. Bi-allelic mutations in ARMC2 lead to severe asthenoteratospermia due to sperm flagellum malformations in humans and mice. Am J Hum Genet 2019; 104: 331–40.

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

©The Author(s)(2021)
Supplementary Table 1: Primers for polymerase chain reaction and Sanger sequencing of CFAP43 variants

| CFAP43 variants  | Product size (bp) | Forward primer          | Reverse primer          |
|------------------|-------------------|-------------------------|-------------------------|
| c.1577_1578del, p.Thr526Serfs*43 | 442               | ATCAGGAGAATCCCTCATCC    | TTACCTCTTCACATGCCAAG    |
| c.899_900del, p.Arg300LysfsTer22    | 395               | GCTCCTCTCTATAATCAAG     | ATGTGACAGATCTGACCATCC  |

Supplementary Figure 1: Whole-exome sequencing (WES) analysis pipeline for (a) Family 1 and (b) Family 2.

Supplementary Table 1: Whole-exome sequencing (WES) analysis pipeline for (a) Family 1 and (b) Family 2.
Supplementary Table 2: Details of filtered variants from whole-exome sequencing analysis pipeline for family 1

| Gene name | Mutation type          | cDNA change | Phenotypes of mutant mice from MGI or literature, or expression in testes                                                                 |
|-----------|------------------------|-------------|------------------------------------------------------------------------------------------------------------------------------------------|
| ANKRD36C  | Nonsynonymous SNV      | C98T        | Mutant mice have a mottled retina with photoreceptor degeneration and male infertility associated with oligozoosperma and asthenozoosperma |
| ANKRD36C  | Frameshift substitution| 1577_1579G  | The same as above                                                                                                                         |
| CELA3B    | Nonsynonymous SNV      | G358A       | The expression of this gene is not detectable in human testis                                                                           |
| CELA3B    | Frameshift substitution| 2752_2753T  | The same as above                                                                                                                         |
| CFAP43    | Frameshift substitution| 1577_1578G  | Mice homozygous for a knock-out allele exhibit complete male sterility, asthenozoosperma, and teratozoosperma characterized by short, thick, and coiled flagella and sperm axonemal defects |
| CFAP43    | Frameshift substitution| 899_901A   | The same as above                                                                                                                         |
| NBPF1     | Nonsynonymous SNV      | G171A       | Mice homozygous for a null allele exhibit partial (in utero or perinatal) lethality, hyperactivity, and increased vertical activity         |
| NBPF1     | Nonsynonymous SNV      | T35G        | Mice homozygous for a knock-out allele display delayed mammary tumor progression, impaired intestinal absorption of cholesterol, decreased gastric mucus accumulation, reduced secretion and accumulation of gallbladder mucin, and decreased susceptibility to cholesterol gallstone formation |
| PABPC3    | Frameshift substitution| 232_236T    | Homozygotes for a null allele show high brain AEA levels, reduced pain sensation, altered behavioral responses to AEA, and sex-specific changes in ethanol intake and sensitivity. Homozygotes for the C385A variant show enhanced cued fear extinction and reduced anxiety-like behavior |
| PABPC3    | Nonframeshift substitution|            | The same as above                                                                                                                         |
| PABPC3    | Frameshift substitution| 301_309G    | Homozygotes for a null allele show high brain AEA levels, reduced pain sensation, altered behavioral responses to AEA, and sex-specific changes in ethanol intake and sensitivity. Homozygotes for the C385A variant show enhanced cued fear extinction and reduced anxiety-like behavior |
| PABPC3    | Nonsynonymous SNV      | C17T        | The same as above                                                                                                                         |
| PIK3C2G   | Frameshift substitution| 595_596G    | Homozygous null mice display hypoplasia of gut-associated lymph tissue due to defects in lymphocyte migration                               |
| PIK3C2G   | Frameshift substitution| 24_25T      | The same as above                                                                                                                         |
| PRIM2     | Frameshift substitution| 899_901A    | The same as above                                                                                                                         |
| PRIM2     | Frameshift substitution| 497_498A    | The same as above                                                                                                                         |
| RRP12     | Splicing               | 1657+3A>C   | Homozygotes for targeted null mutations exhibit a 1 h shorter circadian period under constant darkness and reduced expression of another circadian gene in the suprachiasmatic nucleus in response to acute light exposure |
| RRP12     | Nonsynonymous SNV      | A1178T      | The same as above                                                                                                                         |
| SPTA1     | Splicing               | 565-3C>T    | Mice homozygous or heterozygous for alleles of this gene exhibit varying degrees of hematopoietic defects                                    |
| SPTA1     | Frameshift substitution| 51_52A      | The same as above                                                                                                                         |

MGI: mouse genome informatic; AEA: anandamide; SNV: single-nucleotide variant

Supplementary Table 3: Details of filtered variants from whole-exome sequencing analysis pipeline for family 2

| Gene name | Mutation type | cDNA change | Phenotypes of mutant mice from MGI or literature                                                                 |
|-----------|---------------|-------------|------------------------------------------------------------------------------------------------------------------|
| CFAP43    | Frameshift    | 1577_1578G  | Mice homozygous for a knock-out allele exhibit complete male sterility, asthenozoosperma, and teratozoosperma characterized by short, thick, and coiled flagella and sperm axonemal defects |
| MYO15A    | Nonsynonymous SNV | C10393T | Mutations in this gene result in profound deafness and neurological behavior                                      |
| KRTAP9-9  | Nonsynonymous SNV | G422A | In the hair cortex, hair keratin intermediate filaments are embedded in an interfilamentous matrix, consisting of hair KRTAP, which are essential for the formation of a rigid and resistant hair shaft through their extensive disulfide bond cross-linking with abundant cysteine residues of hair keratins. The matrix proteins include the high-sulfur and high-glycine-tyrosine keratins |
| HTT       | Nonsynonymous SNV | A107C | Null mutants gastrulate abnormally and die in utero. Conditional mutants are small with progressive neurodegeneration. Knock-ins of 20–150 CAG repeat units variably mimic Huntington’s with late-onset motor defects, reactive gliosis, and neuronal inclusions |
| KRT25     | Nonsynonymous SNV | A716C | Mutations in this gene have a defect in hair formation resulting in a wavy coat and curly vibrissae |
| DONSON    | Nonsynonymous SNV | A752G | Homozygous knockout is early embryonic lethal. Heterozygous knockout causes no observable phenotype |

MGI: mouse genome informatic; SNV: single-nucleotide variant; KRTAP: keratin-associated protein
Supplementary Table 4: Semen characteristics in the subjects carrying CFAP43 mutations

| Patient identified in the study | cDNA change | Effect on protein, or protein alteration | Semen volume (ml) | Sperm count (10⁶ ml) | Motility (%) | Immotile (%) | MMAF phenotype | Reference |
|---------------------------------|-------------|----------------------------------------|------------------|---------------------|-------------|-------------|----------------|-----------|
| P003                            | c.2802T>A   | p.Cys934*                              | 2.2–3.8          | 16.1–39.4           | 0           | 100         | Yes            | 2         |
| P028                            | c.253C>T    | p.Arg85Trp                             | 1.5–2.5          | 16.1–39.4           | 2           | 98          | Yes            |           |
| P029                            | c.386C>A    | p.Ser129Tyr                            | 2.5–4.0          | 12.2–18.9           | 1           | 99          | Yes            |           |
| P6                              | c.3661-2A>  | NA                                     | 3.0              | 15.8                | 0           | 100         | Yes            | 32        |
| P1                              | c.1140_1143del | p.Asn380Lysfs'3                      | 2.3              | 7.6                 | 0           | 100         | Yes            | 11        |
| P8                              | c.739A>T    | p.Lys247*                              | 2.4              | 25.8                | 0           | 100         | Yes            |           |
| P9                              | c.1474G>C   | p.Gln492Arg                            | 3.5              | 32.1                | 0           | 100         | Yes            |           |
| P10                             | c.4600C>G   | p.Leu1534Val                           | 4.1              | 19.2                | 0           | 100         | Yes            |           |
| P5                              | c.4963C>T   | p.Arg1655*                             | 2.9              | 20.1                | 0           | 100         | Yes            |           |
| P=10                            | c.3541–2A>C | p.Ser1181Lysfs'4                       | 3.5±1.4          | 27.2±23.4           | 0±0 (n=9)  | 100         | Yes            | 10        |
| c.1240_1241delGT                | c.1577_1578del | p.Val141LeufsTer46                  |                  |                     |             |             |                 |           |
| c.2658G>A                      | c.1577_1578del | p.Val886Ter                           |                  |                     |             |             |                 |           |
| c.2680C>T                      | c.1577_1578del | p.Arg944Ter                           |                  |                     |             |             |                 |           |
| c.3882delIA                    | c.1577_1578del | p.Glu294AsfsTer47                    |                  |                     |             |             |                 |           |
| c.3352C>T                      | c.1577_1578del | p.Arg1118Ter                          |                  |                     |             |             |                 |           |
| c.3882delIA                    | c.1577_1578del | p.Leu435SerfsTer26                   |                  |                     |             |             |                 |           |
| c.1040T>C                      | c.1577_1578del | p.Val347Ala                           |                  |                     |             |             |                 |           |
| c.2141+5G>A                    | c.1577_1578del | p.Lys141Val11                        |                  |                     |             |             |                 |           |
| P=2                            | c.899_900del | p.Arg300Lysfs'22                      | 3.3              | 07                  | 0           | 100         | Yes            | Current study |
| c.1577_1578del                 | c.1577_1578del | p.Thr526Serfs'43                     | 2–3              | 6–9                 | 0           | 100         | Yes            | Current study |

Supplementary Table 5: Percentages of involvement of the different sperm flagellum reported genes in the different cohorts

| Gene               | Protein features                          | Percentage of involvement (%) | Reference |
|--------------------|------------------------------------------|------------------------------|-----------|
| DNAH1              | Dynein heavy chain                       | 28                           | 7         |
| CFAP65             | Coiled-coil domain-containing protein     | 6.8                          | 12        |
| CFAP43 and CFAP44  | WD repeat domains                        | 7.5                          | 2         |
| FSIP2              | AKAP4 interacting domain                 | 5.1                          | 33        |
| AK7                | ADK domain, coiled coil domain, DPY30 domain | 1.2                          | 34        |
| WDR66 (CFAP251)    | calcium regulating EF-hand domain        | 9                            | 15        |
| CFAP69             | Armadillo-type α-helical repeats         | 2.6                          | 18        |
| ARM2               | Armadillo repeat-containing protein 2     | 2.4                          | 35        |