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Mutational landscape of *DNAH1* in Chinese patients with multiple morphological abnormalities of the sperm flagella: cohort study and literature review

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Abstract

Purpose Multiple morphological abnormalities of the sperm flagella (MMAF) are important causes of male infertility. Mutations in *DNAH1* are the main causative factors proven so far. We aim to determine the mutational landscape of *DNAH1* in Chinese patients with MMAF.

Methods Forty-one Chinese patients with MMAF were enrolled and underwent a 10-gene next-generation sequencing panel screening.

Results Only the *DNAH1* gene was found to have mutations in 12 of these unrelated individuals (29%). Combining published data from two other cohorts of Chinese men with MMAF, we suggest that p.P3909fs*33, p.R868X, p.Q1518X, p.E3284K, and p.R4096L are hotspot mutations. A polymorphism—rs12163565 (G>A)— showed linkage to p.P3909fs*33, suggesting that this involved a founder effect. Four of the 12 patients with *DNAH1* mutations were able to use intracytoplasmic sperm injection with their partners and all were successful in obtaining embryos.

Conclusions Hotspot mutations were identified for Chinese patients with MMAF. MMAF sub-phenotypes might be associated with different combinations of *DNAH1* mutations.

Keywords Morphological abnormalities · Sperm flagella · Teratospermia · Next-generation sequencing panel · Intracytoplasmic sperm injection · Male infertility

Introduction

Sperm tail abnormalities are an important cause of human male infertility. The syndrome of multiple morphological abnormalities of the sperm flagella (MMAF) is characterized by short, absent, bent, coiled, and irregular flagella. To date, nearly 20 genes have been found to be associated with this phenotype [1]. Mutation in the gene encoding the dynein axonemal heavy chain 1 (*DNAH1*) that encodes for one of the inner axonemal dynein arms (7IDAs) is the first identified cause of MMAF in humans [2]. Multiple ultrastructural defects of the sperm flagella can occur in the same individual, such as lack of IDAs, axonemal disorganization, complete absence of the central doublet microtubule pair, supernumerary dense fibers, and absence of mitochondria [2].

Genetic testing among patients with the MMAF phenotype can have clinical implications. First, any therapies to improve sperm quality will not be recommended for patients with MMAF caused by genetic variants, so they can avoid unnecessary medication. Second, limited evidence suggests that MMAF caused by variants in different genes might be associated with intracytoplasmic sperm injection (ICSI) outcome. For example, good ICSI outcomes can be expected for those patients with MMAF carrying *DNAH1* [3], *CFAP43*, and

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CFAP44 [4] mutations, while mutations to *CFAP65* [5] and *CEP135* [6] can impair ICSI outcomes. Third, genetically diagnosed patients need to be informed that any male offspring might also have MMAF and infertility if their spouse also carries a pathogenic variant of the same gene.

Clarification of the proportion of genetic causes in patients with MMAF is important for the design of cost-effective clinical diagnostic next-generation sequencing (NGS) panels. However, screening studies on such cohorts are still limited. Here we used a targeted NGS panel to screen 41 Chinese men with an MMAF phenotype and review previously reported *DNAH1* mutations to identify recurrent and novel mutations. This work expands the mutational and phenotypic spectrum of *DNAH1* and lays the foundation for the design of MMAF-specific diagnostic screening kits.

Materials and methods

Study subjects

This study was approved by the ethics committees of Renji Hospital, Shanghai Jiao Tong University, School of Medicine and Nanjing Drum Tower Hospital, Nanjing University Medical School. Forty-one patients with primary male infertility were diagnosed with severe asthenozoospermia and MMAF from November 2017 to June 2020. All patients underwent a comprehensive semen analysis according to the World Health Organization laboratory manual [7]. None was from consanguineous families. Some patients underwent light microscopy-based morphological analysis of semen samples. None of them was found to have the typical phenotype of primary ciliary dyskinesia such as chronic respiratory tract infections and abnormally positioned internal organs.

Genetic testing

Genomic DNA was extracted from blood samples collected from all enrolled patients using DNeasy Blood and Tissue Kits (Qiagen, Valencia, CA, USA). We used an NGS panel to perform genetic testing, including ten genes as follows: *CFAP44* (OMIM: 617559), *CFAP69* (OMIM: 617949), *ARMC2* (OMIM: 618424), *CFAP43* (OMIM: 617558), *AK7* (OMIM: 615364), *TTC21A* (OMIM: 611430), *WDR66* (OMIM: 618146), *FSIP2* (OMIM: 615796), *DNAH1* (OMIM: 603332), and *QRICH2* (OMIM: 618304). The other known MMAF genes are not included because they were discovered after panel design and sample screening, such as *DNAH6* [8], *DNAH17* [9], *CFAP65* [10, 11], *CFAP70* [12], *DNAH8* [13], *CFAP47* [14], and *TTC29* [15]. Coding exons and flanking introns (± 10 bp) of these genes were targeted and captured using IDT xGen Lockdown Probes (Integrated DNA Technologies, Coralville, IA, USA). DNA was quantified using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and the quality of libraries was assessed using 2100 Bioanalyzer High Sensitivity DNA assays (Agilent Technologies, Carlsbad, CA, USA). All DNA libraries were sequenced under 2 × 75-bp paired-end mode on the Illumina NextSeq 500 platform (Illumina Inc., San Diego, CA, USA).

Data analysis

Targeted NGS panel sequences in FASTQ format were filtered and aligned to the human reference sequence (hg19/GRCh37) with the use of BWA v0.7.13 [16]. Variants including single-nucleotide variants (SNVs) and insertion–deletion mutations (InDels) were genotyped from recalibrated BAM files using VarDict [17]. The ANNOVAR software [18] was used to annotate all detected variants. After a preliminary screening to remove silent mutations and polymorphisms (minor allele frequency > 1% in the gnomAD database), the remaining mutations were further evaluated and classified as pathogenic (P), likely pathogenic (LP), variant of unknown significance (VUS), likely benign (LB), or benign (B) following the American College of Medical Genetics (ACMG) guidelines [19]. Copy number variants were identified using the DNAcopy R package [20]. Candidate variants were checked manually using the Integrative Genomics Viewer [21], and then validated by Sanger sequencing to avoid false positives. All compound heterozygous mutations were found *in trans* by pedigree analyses. *DNAH1* mutations were annotated using transcript NM_015512.

Haplotype phasing

The *DNAH1* mutations c.11726_11727del (p.P3909fs*33, chr3: 52430998) and an upstream polymorphism rs12163565 (G>A, chr3: 52430526) were amplified at the same amplicon using primers F (5′-CCAGATATGGGTCT CAATGCTC-3′) and R (5′-TGTCTTTGTGGGAT GGGATG-3′). To phase the two loci, purified PCR products were cloned into a pMD-18T vector (Takara, Dalian, China) to transform *Escherichia coli* cells. Transformants were selected randomly and sequenced using universal primers. For the patients who carried c.11726_11727del (p.P3909fs*33), only two recent samples from patients A5389 and A6254 were tested and the remaining DNA specimens were not available.

Results

Genetic mutations

Forty-one Chinese patients with MMAF were enrolled in this study. Of these, 12 (29%) were found to have *DNAH1* homozygous or compound heterozygous mutations (Table 1). No

Table 1 *DNAH1* mutations identified in this study and in two previously reported cohorts

ID	cDNA change	AA change	Exon/ intron	Het/ Hom	Clinical significance	SIFT	PolyPhen	AF
A2191	c.4552C>T	p.Q1518X	Exon 27	Het	LP (PVS1+PM2)	-	-	0.0001669
	c.11787+1G>A	NA	Exon 73	Het	LP (PVS1+PM2)	-	-	0
A3010	c.11726_11727del	p.P3909fs*33	Exon 73	Het	P (PVS1+PM3+PP1+PP4)	-	-	0.001286
	c.12089+1G>A	NA	Intron 75	Het	P (PVS1+PM2+PP4)	-	-	0
A3298	c.4552C>T	p.Q1518X	Exon 27	Het	LP (PVS1+PM2+PM3)	-	-	0.0001669
	c.12287G>T	p.R4096L	Exon 76	Het	LP (PM1+PM2+PM3_supporting+PP2+ PP3+PP4)	D(0)	D(0.999)	0
A3342	c.7397G>A	p.R2466Q	Exon 48	Het	VUS (PM1+PM2+PP2)	D(0.05)	B(0.15)	0
	c.12287G>A	p.R4096H	Exon 76	Het	LP (PM1+PM2+PP2+PP3)	D(0)	D(0.999)	0
A5730	c.6004C>T	p.R2002C	Exon 38	Het	LP (PM1+PM2+PM3+PP3)	D(0)	D(0.988)	0
	c.10982C>A	p.S3661X	Exon 69	Het	LP (PVS1+PM2)	-	-	0
A6328	c.2602C>T	p.R868X	Exon 15	Het	LP (PVS1+PM2)	-	-	0
	c.12748C>T	p.R4250X	Exon 78	Het	VUS (PM2+PM3)	-	-	0.0001113
A3679	c.5573T>C	p.L1858P	Exon 35	Het	P (PM1+PM2+PM3+PP2+PP3)	D(0)	D(0.967)	0
	c.11726_11727del	p.P3909fs*33	Exon 73	Het	P (PVS1+PM3+PP1)	-	-	0.001286
A4218	c.11726_11727del	p.P3909fs*33	Exon 73	Hom	P (PVS1+PM3+PP1)	-	-	0.001286
A5389	c.11726_11727del	p.P3909fs*33	Exon 73	Het	P (PVS1+PM3+PP1)	-	-	0.001286
	c.12264_12265del	p.W4089Gfs*51	Exon 76	Het	P (PVS1+PM2+PM3)	-	-	0
A5601	c.4552C>T	p.Q1518X	Exon 27	Het	LP (PVS1+PM2+PM3)	-	-	0.0001669
	c.9685C>T	p.R3229C	Exon 61	Het	VUS (PM1+PM2+PP3)	D(0)	P(0.858)	0
A6137	c.6526-1G>T	NA	Intron 41	Het	LP (PVS1+PM2)	-	-	0
	c.9850G>A	p.E3284K	Exon 62	Het	VUS (PM2+PM3+PP3+PP4)	D(0.01)	P(0.64)	0.0003371
A6254	c.5104C>T	p.R1702X	Exon 32	Het	P (PVS1+PM2+PM3)	-	-	0.0003894
	c.11726_11727del	p.P3909fs*33	Exon 73	Het	P (PVS1+PM3+PP1)	-	-	0.001286
P1	c.4115C>T	p.T1372M	Exon 25	Het	VUS (PM1+PM2+PP4)	D(0)	D(0.966)	0
	c.11726_11727del	p.P3909fs*33	Exon 73	Het	P (PVS1+PM3+PP1+PP4)	-	-	0.001286
P2	c.6822C>G	p.D2274E	Exon 43	Het	VUS (PM1+PP4+BP4)	T(0.55)	B(0.065)	0.001836
	c.9850G>A	p.E3284K	Exon 62	Het	LP (PM2+PM3+PP3+PP4)	D(0.01)	P(0.64)	0.0003371
P3	c.6212T>G	p.L2071R	Exon 39	Het	VUS (PM3_supporting+PP4)	D(0)	P(0.839)	0
	c.12200_12202del	p.4067_4068del	Exon 76	Het	P (PVS1+PM2+PP4)	-	-	0
P4	c.3836A>G	p.K1279R	Exon 22	Het	B (PP4+BS1+BP4)	T(0.15)	B(0.003)	0.01207
	c.11726_11727del	p.P3909fs*33	Exon 73	Het	P (PVS1+PM3+PP1+PP4)	-	-	0.001286
P5	c.7377+1G>C	NA	Intron 47	Hom	P (PVS1+PM2+PM3_supporting+PP4)	-	-	0
P6	c.3108G>A	p.W1036X	Exon 19	Het	P (PVS1+PM2+PM3_supporting+PP4)	-	-	0
	c.5864G>A	p.W1955X	Exon 37	Het	P (PVS1+PM2+PM3_supporting+PP4)	-	-	0
P7	c.6253_6254del	p.R2085fs*8	Exon 39	Het	P (PVS1+PM2+PM3_supporting+PP4)	-	-	0
	c.11726_11727del	p.P3909fs*33	Exon 73	Het	P (PVS1+PM3+PP1+PP4)	-	-	0.001286
P8	c.2610G>A	p.W870X	Exon 15	Het	P (PVS1+PM2+PP4)	-	-	0
	c.12287G>T	p.R4096L	Exon 76	Het	LP (PM1+PM2+PM3_supporting+PP2+ PP3+PP4)	D(0)	D(0.999)	0
P9	c.12397C>T	p.R4133C	Exon 76	Het	LP (PM1+PM2+PM3_supporting+PP4)	D(0)	D(1)	0.00005564
	c.11726_11727del	p.P3909fs*33	Exon 73	Het	P (PVS1+PM3+PP1+PP4)	-	-	0.001286
P10/P11	c.7066C>T	p.R2356W	Exon 45	Het	LP (PM1+PM2+PM3+PP4)	D(0)	D(1)	0.00005585
	c.11726_11727del	p.P3909fs*33	Exon 73	Het	P (PVS1+PM3+PP1+PP4)	-	-	0.001286
P12	c.5766-2A>G	NA	Intron 36	Het	P (PVS1+PM2+PM3_supporting+PP4)	-	-	0.0004452
	c.10630G>T	p.E3544X	Exon 67	Het	P (PVS1+PM2+PP4)	-	-	0
P1’	c.7864C>T	p.R2622X	Exon 50	Het	P (PVS1+PM2+PM3_supporting)	-	-	0
	c.11726_11727del	p.P3909fs*33	Exon 73	Het	P (PVS1+PM3+PP1+PP4)	-	-	0.001286
P2’	c.7075C>T	p.R2359C	Exon 45	Het	LP (PM1+PM2+PM3_supporting+PP3)	D(0)	P(0.988)	0

Table 1 (continued)

ID	cDNA change	AA change	Exon/ intron	Het/ Hom	Clinical significance	SIFT	PolyPhen	AF
	c.11726_11727del	p.P3909fs*33	Exon 73	Het	P (PVS1+PM3+PP1+PP4)	-	-	0.001286
P3’	c.10060_ 10061insATCT	p.E3354fs*28	Exon 64	Het	LP (PVS1+PM2)	-	-	0
	c.11726_11727del	p.P3909fs*33	Exon 73	Het	P (PVS1+PM3+PP1+PP4)	-	-	0.001286
P4’	c.4987C>T	p.R1663C	Exon 31	Het	LB (PM1+BS1+BP6)	D(0)	B(0.121)	0.01706
	c.7779C>G	p.S2593R	Exon 49	Het	VUS (PM1+PM2)	D(0.01)	B(0.003)	-
P5’	c.11726_11727del	p.P3909fs*33	Exon 73	Hom	P (PVS1+PM3+PP1+PP4)	-	-	0.001286
P6’	c.3970G>A	p.A1324T	Exon 23	Het	VUS (PM1+PM2+PM3_supporting)	D(0.04)	B(0.048)	0
	c.4552C>T	p.Q1518X	Exon 27	Het	LP (PVS1+PM2+PM3)	-	-	0.0001669
P7’	c.2602C>T	p.R868X	Exon 15	Het	LP (PVS1+PM2)	-	-	0
	c.11726_11727del	p.P3909fs*33	Exon 73	Het	P (PVS1+PM3+PP1+PP4)	-	-	0.001286
P8’	c.11726_11727del	p.P3909fs*33	Exon 73	Hom	P (PVS1+PM3+PP1+PP4)	-	-	0.001286

P1–P12 [25] and P1′–P8′ [27] were described in previously reported cohorts. The clinical significance of mutations was classified as pathogenic (P), likely pathogenic (LP), variant of unknown significance (VUS), likely benign (LB), or benign (B) based on the evidence outlined in the following brackets. *NA* not available, *Het* heterozygous, *Hom* homozygous; SIFT annotation: *D* deleterious (0–0.05), *T* tolerated (> 0.05); PolyPhen annotation: *D* probably damaging (0.957–1), *P* possibly damaging (0.453–0.956), *B* benign (0–0.452); *AF* allele frequency of East Asians in the gnomAD database

candidate pathogenic variations were found in any other tested genes. A total of 17 different mutation types were identified in 12 patients, including 11 loss-of-function (LOF; six stop-gain, two frameshift, and three splicing sites) and six missense mutations. The *DNAH1* c.11726_11727del (p.P3909fs*33) and c.4552C>T (p.Q1518X) were identified in five and three unrelated patients, respectively, giving allele frequencies of 7% (6/82) and 4% (3/82).

Clinical profiles

Ten of these twelve patients with *DNAH1* mutations were from eastern China, aged between 24 and 41 years. Eight of them had oligospermia (sperm concentration < 5×10^6/mL in semen), of whom patient A3342 had severe oligospermia (< 1×10^6/mL). All patients showed loss of sperm motility and near 100% sperm tail abnormality (Table 2). Five patients underwent sperm morphology analysis, and patients A3679 and A6137 had several elongated sperm flagella visible by light microscopy, but spermatozoa with normal flagella were not found in patients A4218, A5601, and A5389. Instead, A4218 and A5601 had large residual cytoplasmic masses (Fig. 1). Four of the 12 patients underwent assisted reproduction with their partners using ICSI, and all were successful in generating embryos. Two of them had embryos transferred, one delivered successfully and the other experienced two implantation failure.

Haplotype block of p.P3909fs*33

For patients A5389 and A6254, the *DNAH1* mutation c.11726_11727del (p.P3909fs*33) was always located on the same allele as rs12163565 (G>A) (Supplementary Figure S1). For patient A4218 with the homozygous mutation c.11726_11727del (p.P3909fs*33), the genotype rs12163565 was also a homozygous locus (G>A) (Supplementary Table S1). This suggests that both loci are in the same haplotype block.

Discussion

Since *DNAH1* mutations were first discovered as genetic causes of MMAF [2], the number of identified genes has reached nearly 20 in recent years [1]. However, these genes can only account for about 50% of patients with MMAF [22], which means there are still unknown genetic factors. Homozygous or compound heterozygous mutations of *DNAH1* are common causative factors of MMAF, explaining 7.7% [23], 38.9% [2], 41.7% [24], or 57.1% [25] of the etiology in different cohort studies. Here, 41 patients with MMAF were enrolled and screened using a 10-gene NGS panel. Interestingly, only *DNAH1* mutations were identified, while mutations of other genes were not found. The *DNAH1* mutations explain the etiology of 12/41 (29%) patients with MMAF. The different rates between studies may be due to different inclusion criteria for patients with MMAF, as well as ethnic differences. However, this paper highlights the importance of *DNAH1* in the etiology of men with MMAF in China.

The mutational spectrum of *DNAH1* is likely to be ethnically specific. For example, c.11788-1G>A is a hotspot splicing mutation for Tunisian patients with MMAF [2], while p.P3909fs*33 is a recurrent loss-of-function (LOF) mutation

Table 2. Semen parameters and ICSI outcomes from 12 patients with MMAF associated with *DNAH1* mutations

			Semen parameters			ICSI outcomes				
ID	Birthplace (China)	Age	Sperm count ($\times 10^6$/mL)	Motility (a + b%)	Sperm tail abnormal rate (%)	Collected oocytes	Embryos	Transferred embryos	Clinical pregnancy	Delivery
A2191	Eastern	28	1.1	0 + 0	100	10	5	2	2	2
A3010	Eastern	27	3.5	0 + 1.0	100	12	4	4	0	0
A3298	Northeast	41	4.5	0 + 0	100	7	2	-	-	-
A6137	Eastern	33	29.1	2.8 + 0	98	13	2	-	-	-
A3342	Central	27	<1	-	-	-	-	-	-	-
A5730	Eastern	32	4.5	0 + 0	100	-	-	-	-	-
A6328	Eastern	28	4.5	0 + 0	89	-	-	-	-	-
A3679	Eastern	32	1.5	2.1 + 0	99	-	-	-	-	-
A4218	Eastern	25	24.7	0 + 0	95	-	-	-	-	-
A5389	Eastern	29	33.6	0 + 0	97	-	-	-	-	-
A5601	Eastern	30	3.5	0 + 0	95	-	-	-	-	-
A6254	Eastern	24	40.5	0 + 0	99	-	-	-	-	-

Semen analysis could not be performed for patient A3342 because of a low sperm concentration. The partner of A3010 experienced two implantation failure. Embryos were frozen and awaiting transfer for A3298 and A6137

ICSI intracytoplasmic sperm injection

for Chinese men [25, 26]. However, large cohort studies are still lacking for clarification of the genetic and phenotypic spectrum of Chinese patients with MMAF. We have summarized the *DNAH1* mutations in this study combined with previously reported cohorts of Chinese men with MMAF [25, 27]. The allele frequency of p.P3909fs*33 was 6/82 (7%) in this study, and 5/40 (13%) in Sha, Yang [25] (P10 and P11 were siblings in that study and counted as one sample, so the total allele number = [] × 2 = 40, see Fig. 2a). Wu, Wang [27] reported eight patients with MMAF caused by *DNAH1* mutations in their supplementary material, six (75%) of whom carried at least one copy of p.P3909fs*33 (P1′–P8′; Table 1). An additional cohort of nine Chinese patients with MMAF also showed the high prevalence of p.P3909fs [26]. The allele

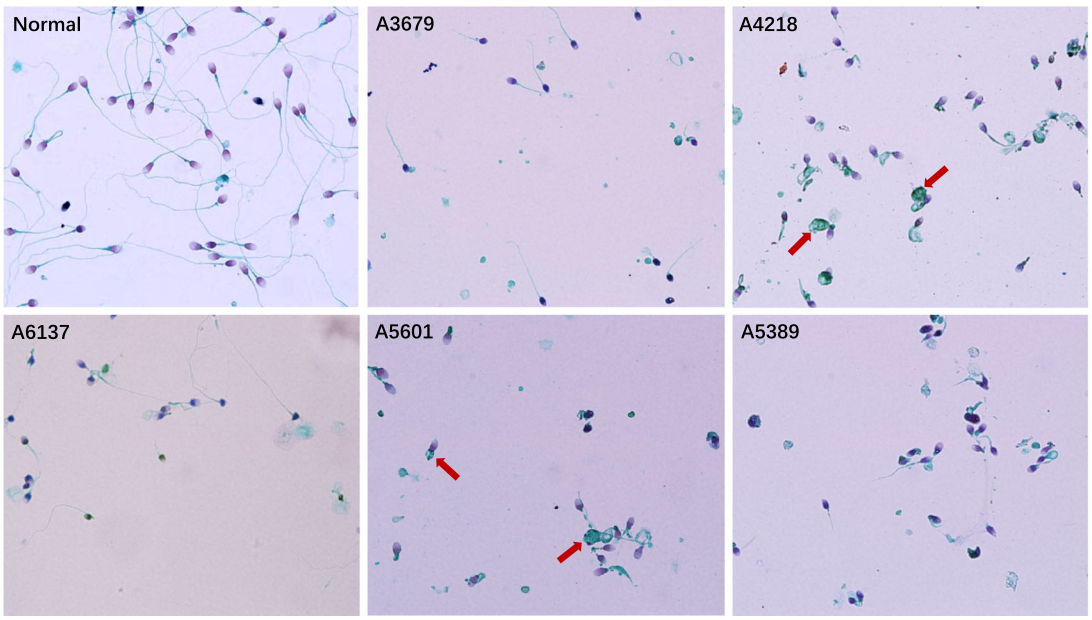

Fig. 1 Spermatozoa with normal and abnormal flagella. Short, absent, bent, coiled, and irregular sperm flagella with excess residual cytoplasm can be seen in high-power fields of view (×1000) for normal semen and five patients with MMAF. Sperms with excess residual cytoplasm are marked with arrows

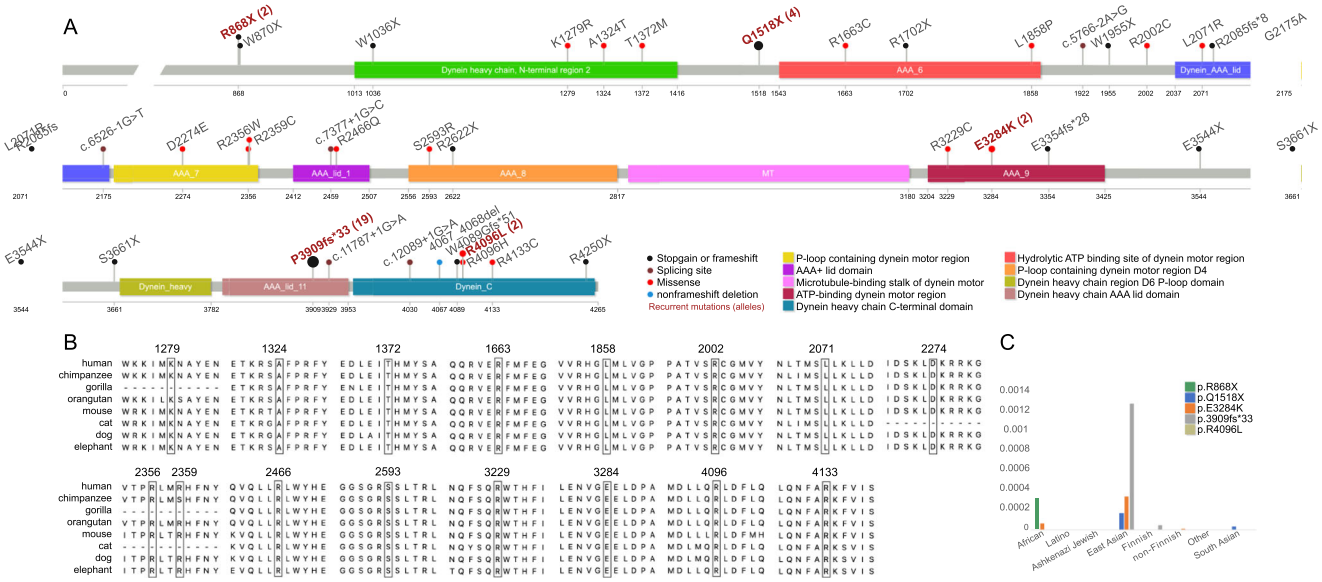

Fig. 2 Mutational landscape of patients with MMAF with *DNAH1* mutations. **a** Locations of the mutations on the *DNAH1* protein domain, mutational types, and hotspot mutations. **b** All missense mutations exhibit high sequence conservation. **c** Allele frequencies of five hotspot *DNAH1* mutations in various ethnic populations in the gnomAD database

frequency of p.P3909fs*33 is as high as 0.001286 (East Asians in the gnomAD database; https://gnomad.broadinstitute.org). We identified an upstream polymorphism, rs12163565 (G>A) that coexisted with p.P3909fs*33 in our NGS data (Supplementary Table S1) and was subsequently validated by *E. coli* cloning and sequencing (Supplementary Figure S1). This suggests that p.P3909fs*33 is in the same haplotype block as rs12163565 (G>A) and has been influenced by a founder effect in the East Asian population including Chinese men.

By combining two other previous studies [25, 27], we identified four additional candidate hotspot mutations—p.R868X, p.Q1518X, p.E3284K—and p.R4096L, which were found in two, four, two, and two unrelated individuals, respectively (Fig. 2a). Except for p.R868X and p.R4096L, which do not occur in East Asian populations according to the gnomAD database, the allele frequencies of p.Q1518X and p.E3284K in East Asians are higher than in other populations (Fig. 2c). This implies that these loci might also have been influenced by a founder effect. Although two previously reported mutations c.3836A>G (p.K1279R) and c.4987C>T (p.R1663C) showed a high allele frequency (> 1%) in East Asian populations (Table 1), they were reclassified as B and LB according to the ACMG guidelines [19], so might not be causal MMAF.

Targeted NGS panel may has important clinical implications for patients with MMAF. For all those patients with biallelic pathogenic mutations, they were informed that ICSI is currently the only feasible treatment. Four of the 12 patients with *DNAH1* mutations in our study underwent ICSI with their partners and all were successful in obtaining embryos (Table 2). Two couples underwent embryo transfer, one of whom delivered successfully and the other suffered an early miscarriage. Because patients with MMAF caused by *DNAH1* mutations may have good ICSI outcomes [3], we encourage the couples to try for another cycle of ICSI. In addition, all these female partners were recommended to be screened for *DNAH1* to avoid the possibility of the male offspring having MMAF and none of them was found to carry the pathogenic variant.

Five patients underwent sperm morphology analysis. The degree of flagellar abnormalities appeared to be more severe in patients A4218, A5601, and A5389. In contrast, more than two spermatozoa with elongated tails could be found per microscope field for patients A3679 and A6137 (Fig. 1). There are no published studies to date discussing whether combinations of different *DNAH1* mutations result in MMAF subphenotypes. Both patients A3679 and A6137 each have one allele with a missense mutation, while both alleles of patients A4218 and A5389 carry LOF mutations. This speculation seems reasonable because LOF affects the integrity of the *DNAH1* protein and thus affects the axonemal assembly process, leading to the phenotype of excess residual cytoplasm mainly comprising unassembled axonemal and periaxonemal components [28]. However, this hypothesis does not explain the severe phenotype of patient A5601. To further validate this hypothesis, more samples and further structural studies on spermatozoa are needed to establish the relationship between *DNAH1* mutation combinations and MMAF subphenotypes.

Conclusion

Forty-one patients with MMAF were enrolled and underwent 10-gene NGS panel screening. Only the *DNAH1* gene was found to have mutations in 12 unrelated individuals, giving a diagnostic rate of 29%. In these mutations, p.P3909fs*33 is a widely reported hotspot mutation and is found to be linked to polymorphism rs12163565 (G>A), suggesting that it arose as a founder effect. Moreover, p.R868X, p.Q1518X, p.E3284K, and p.R4096L were also identified as hotspot mutations by combining two previously reported cohorts. Limited evidence suggests that the MMAF sub-phenotypes may be associated with the different *DNAH1* mutation combinations, which needs further cohort studies.

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