



Evaluation of The 1499T>C Variant in The *AKAP3* Gene of Infertile Men with Multiple Morphological Abnormalities of The Sperm Flagella Phenotype: A Case-Control Study

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Abstract

Background: Infertile men with multiple morphological abnormalities of the sperm flagella (MMAF) phenotype exhibit mosaic sperm flagella abnormalities such as short, bent, coiled, and irregular flagella or absent flagella. Sperm flagellum has an ultrastructurally axonemal structure that contains a large number of proteins. A-Kinase Anchoring Protein 3 (AKAP3) is expressed in spermatozoa. It may function as a regulator of motility and the acrosome reaction. This study aimed to compare genetic changes in infertile men suffering MMAF phenotype with the control group.

Materials and Methods: In this case-control study, genetic variants of the *AKAP3* gene were evaluated in 60 infertile men with MMAF phenotype and 40 fertile men, as control. As exon five of the *AKAP3* gene encodes the functional domain of this protein, its genetic variants were studied. Therefore, polymerase chain reaction (PCR)-sequencing was undertaken on the DNA extracted from control and patients' blood samples.

Results: Sixty infertile men with MMAF phenotype and 40 normozoospermic men, as control, were enrolled in this study. Four haplotype variants 1378T>C (rs10774251), 1391C>G (rs11063266), 1437T>C (rs11063265), and 1573G>A (rs1990312) were detected in all patients and controls. On the other hand, a missense mutation 1499T>C (rs12366671) was observed in four patients with the homozygous form while seven patients carried the heterozygous form. No mutation was identified in the controls ($P=0.04$). The difference between the variation allele frequencies was assessed in the patient and control groups by the Fisher Exact Test.

Conclusion: In the homozygous form, this mutation changed Isoleucine to Threonine. This alternation occurred inside the AKAP4 binding domain of the AKAP3 protein. The observed variants caused no significant deviation in the secondary structure of AKAP3 protein and probably its function in spermatozoa flagella. So, these variants cannot be considered as the causes of MMAF phenotype in the studied patients.

Keywords: AKAP3 Gene, Male Infertility, Sperm Flagella

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Introduction

Male factors are presented in roughly 40-50% of all infertility cases (1). Male infertility diagnosis is merely descriptive, as etiology of the sperm abnormalities, remains idiopathic in about 40% of infertile cases. One of the reasons leading to the lack of basic understanding is heterogeneity of the contributing factors (2). Although male infertility is a multifactorial defect with strong genetic rudiments, to date, only a few genes have been

properly elucidated to be correlated with spermatozoa defects in humans (3). Multiple morphological abnormalities of the sperm flagella (MMAF) phenotype was mostly diagnosed by asthenozoospermia, due to the existence of different types of sperm flagella anomalies, such as bent, short, coiled, irregular, and absent flagella with a variable percentage among MMAF patients; however, all of these defects could be observed in healthy men but with extremely lower levels (4). Until now, 24

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MMAF-associated genes have been identified which are responsible for manifestation of the primary infertility without occurrence of any primary ciliary dyskinesia symptom, like left-right laterality disturbances and recurrent respiratory infections (5).

A healthy sperm flagellum has three main parts: the midpiece, principal piece, and end piece. The sperm flagellum contains an axonemal structure, along with accessory structures, such as fibrous sheath (FS), the outer dense fibers (ODFs), and mitochondrial sheath (MS) (6). In the midpiece part, a MS surrounds the axoneme structure (7, 8). Different components of sperm flagellum represents various ultra-structural and morphological defects in MMAF phenotype (4).

There are numerous proteins in different parts of the sperm flagellum, accountable for unique structural properties of spermatozoa. A-kinase anchoring protein 3 (AKAP3) and A-kinase anchoring protein 4 (AKAP4) are two essential constituents of the tail FS (9). AKAPs are a group of signal organizing scaffold proteins. Collaboration of AKAPs with kinase A-dependent CAMP causes phosphorylation of a fundamental group of proteins in flagella (10). AKAP3 is implicated to organize the fibrous sheath. This protein exists in the acrosome of the sperm head, so it is considered as a regulator of acrosome reaction and sperm motility (11).

AKAP3 protein comprises of two main domains. An RII binding domain exists at the N-terminal of the protein (12). This contains an amphipathic peptide structure and provides a binding site for the protein kinase A regulatory subunit (13). In mammals, impaired interaction between the protein kinase A regulatory subunit and AKAP3 protein could result in immotile spermatozoa (14). The C-terminal domain of AKAP3 protein constructs a binding site for AKAP4. Relationship of these two proteins can play an important role in the structure of sperm flagella's FS (9).

In this research, the aim of study is correlation of the genetic variants of the exon 5 of AKAP3 gene and male infertility, as a result of the MMAF phenotype in humans, was determined by DNA sequencing.

Materials and Methods

Participants

The current study is a case and control study which was approved by the Ethics Committee of Royan Institute (Tehran, Iran, EC/93/1045). Sixty infertile men with MMAF phenotype (P1-P60) and 40 normozoospermic men (C1-C40) as controls were enrolled and all the participants confirmed their consents. As this phenomenon is a rare sperm characteristic, it took near four years to collect the cases. Inclusion criteria for controls were according to the standard values for semen analysis according to World Health Organization (WHO) 2010 guideline.

Papanicolaou staining

Papanicolaou (PAP) staining was performed to provide valid staining of spermatozoa. This kind of staining is routinely used in andrology laboratories, as one of the most popular methods for identifying human sperm morphology (15).

DNA extraction, polymerase chain reaction and sequencing

DNA was extracted from peripheral blood leucocytes by using the salting-out method. Three primer pairs (*AKAP3-1*, *AKAP3-2*, and *AKAP3-3*) were used to amplify the exon 5 of the *AKAP3* gene. The exon 5 was studied in three coding regions: RII binding domain (*AKAP3-1*), the downstream RII binding domain till AKAP4 binding region (*AKAP3-2*) and AKAP4 binding region (*AKAP3-3*, Table 1). Polymerase chain reaction (PCR) amplification was performed in 50 µl mixture volume, containing 1.5 µM MgCl₂, 1 µM dNTP, 5 µl PCR buffer (1X), 0.8 unit/µl Taq DNA polymerase enzyme (all from Cinagen, Iran), 100 ng of the genomic DNA, and 1.5 µl for each 10 pmol/ul primer. The PCR cycle included an initial DNA denaturation at 95°C for 4 minutes, followed by 30 cycles of DNA denaturation at 94°C for 45 seconds, annealing at melting temperature (TM) set for 45 seconds, extension at 72°C for 45 seconds and ultimately one cycle of the final extension at 72°C for 7 minutes.

Table 1: The primers used for PCR and sequencing			
Primers name	Sequence (5'-3')	Product size	TM (°C)
<i>AKAP3-1</i>	F: AGACTATTACAACACCACCA	372	53
	R: TGTGATGATCCCGAGAC	372	53
<i>AKAP3-2</i>	F: ATCTCCACAGCGTCACAG	682	55
	R: GCTACAGGAGGTTTCATTG	682	55
<i>AKAP3-3</i>	F: AGGAGGAGACTTGTGCGA	755	56
	R: CAACGAAGCATCACAGGA	755	56

PCR; Polymerase chain reaction and TM; Melting temperature.

PCR products were sequenced and the results were analyzed by FinchTV software version 1.4.0 and they were compared to the reference sequences from Ensembl release 103 databases.

Statistical analysis

The difference between the variations' allele frequencies in the patient and control groups was assessed by the Fisher Exact Test. GraphPad Prism version 7.04 (USA, GraphPad by Dotmatics) was used for statistical analyses. P<0.05 was considered statistically significant.

Results

MMAF phenotype was identified in the patients using PAP staining. Short, coil, absent and bent flagellum defects were observed (Fig.1).



Fig.1: Papanicolaou staining shows morphological defects of flagella in spermatozoa of MMAF patients (scale bar: 10 μ M). MMAF; Multiple morphological abnormalities of the sperm flagella.

Four haplotype variants were detected in all patients and controls. These variants were detected in all samples as homozygous genotype. The haplotype variants included ENSG00000111254.8, chromosome 12: 1378 T>C (rs10774251), 1391 C>G (rs11063266), 1437 T>C (rs11063265), and 1573 G>A (rs1990312, Fig.2). The other variant was ENSG00000111254.8, chromosome 12: 1499T>C (rs12366671) which was observed in four patients in the homozygous genotype and seven patients in the heterozygous form. Notably, this variant was not detected in the controls (Fig.3). In the homozygous form, Isoleucine was altered to Threonine.

With regard to 1499T>C variant, frequency of the genotype ($P=0.04$) and C alleles ($P=0.0005$) were significantly higher in the patients compared to the controls (Table 2).

Another variant was ENSG00000111254.8, chromosome 12: 1982T>C (rs953905145) which was only observed in three patients as heterozygote form. However, this variant was not significantly different between the patients and controls (Table 3).

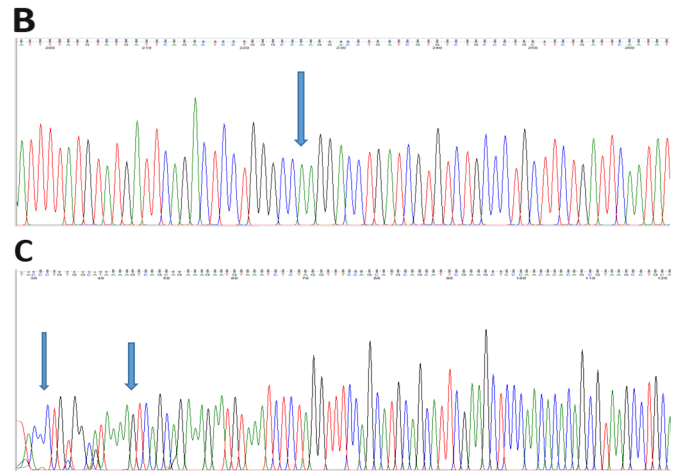
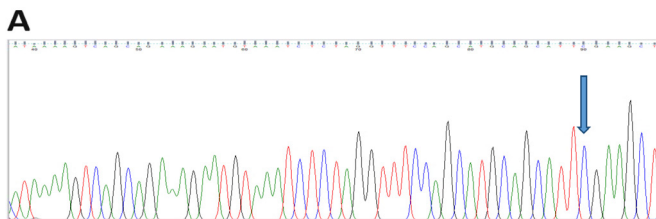


Fig.2: The sequencing results showing four different haplotype polymorphisms of the AKAP3 gene. **A.** 1437T>C, **B.** 1573G>A, and **C.** 1378T>C and 1391C>G polymorphisms observed in different patients and controls.

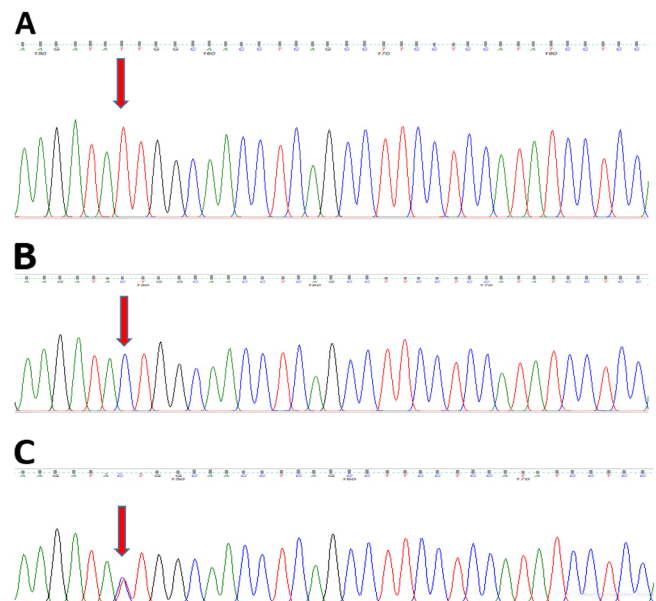


Fig.3: The different sequencing forms of 1499T>C (rs12366671) variant of the AKAP3 gene. **A.** Wildtype (Homozygote (TT)), **B.** Mutant homozygote (CC), and **C.** Heterozygote forms (C/T).

Table 2: Genotype distribution and proportion of the genetic variants observed in the MMAF patients and controls

Variations	Case (total) 100%			Case (total) 100%			Significant P value
	WT	HETERO	HOMO	WT	HETERO	HOMO	
rs10774251 T>C	0	0	100	0	0	100	-
rs11063266 C>G	0	0	100	0	0	100	-
rs11063265 T>C	0	0	100	0	0	100	-
rs1990312 G>A	0	0	100	0	0	100	-
rs12366671 T>C	49	7	4	100	0	0	0.041*
rs953905145 T>C	57	3	0	100	0	0	0.053*

MMAF; Multiple morphological abnormalities of the sperm flagella and *; The significance difference is $P \leq 0.05$.

Table 3: Distributions of the (rs12366671 T>C) polymorphism allele frequencies in the studied groups

rs12366671 T>C Allels	Case alleles (total=120) 100%			Control alleles (total=80) 100%			Significant P value
	WT	HETERO	HOMO	WT	HETERO	HOMO	
C	49	7	4	100	0	0	0.041*
C	57	3	0	100	0	0	0.052*

*; The significance difference is P≤0.05.

Discussion

Male infertility is becoming an expanding multifactorial defect. Considerably, a large number of genes and genetic factors are correlated with spermatozoa defects including low sperm concentration, abnormal sperm morphology, and reduced motility (15).

In spite of many attempts to identify the responsible factors in the spermatogenesis, genetic factors involved in male infertility has not been completely discovered yet (16).

In our study 60 infertile men with MMAF phenotype were recruited and the *AKAP3* gene was evaluated as a gene with a causative role in creating MMAF phenotype. This phenomenon is a mosaic morphological sperm disorder, mainly affecting the sperm flagella. This commonly results in asthenozoospermia and male reduced fecundity. Consequently, four haplotype variants were detected in all samples of both patients and control groups, including 1378T>C (rs10774251), 1391C>G (rs11063266), 1437T>C (rs11063265), and 1573G>A (rs1990312). The previous reports performing in the East Asia population, indicated frequencies of 92% C allele and 8% G allele in 1391C>G variant.

Meanwhile, G allele was observed in 100% of present study cases. The difference observed in the mutant allele frequency could be due to ethnicity differences of the Asian population from our population.

1499T>C (rs12366671) variant was observed in four patients with homozygous genotype and seven patients in heterozygous form, whereas this variant was not identified in the control individuals. This genetic alternation changed Isoleucine (a hydrophobic amino acid) to Threonine (a polar amino acid) which occurs inside the AKAP4 binding domain of AKAP3 protein.

Baccetti et al. (17) reported some deletions in the coding regions of the AKAP3 and AKAP4 genes, however, these deletions were only characterized in one patient and more data is needed to confirm their role in male infertility.

Our new published experiment (18), which was based on structural modeling and in silico analysis of single nucleotide polymorphisms (SNPs), revealed that 1499C>T variant (and the other variants identified in the present study) caused no critical deviation in the secondary structure of AKAP3 protein and probably its function in sperm flagella. Therefore, according to our new findings, 1499C>T cannot be the cause of MMAF phenotype and male infertility. Additionally, our results are in line with

the findings of the previous study performed in 2001. In this study, Turner et al. did not observe any evidence to hold up the hypothesis that *AKAP3* and *AKAP4* genes mutations lead to dysplasia of the fibrous sheath (DFS) in humans (9). The patients with 1499T>C variants were followed up, but unfortunately none of them requested for assisted reproductive technology (ART).

Conclusion

As no significant difference was observed in the four haplotype variants of the *AKAP3* gene [1378T>C (rs10774251), 1391C>G (rs11063266), 1437T>C (rs11063265), and 1573G>A (rs1990312)], therefore they cannot be considered as the causes of MMAF phenotype in the population of Iranian patients studied.

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Authors' Contributions

E.P.T.; Performed the experiments and collected some of the samples and drafted the manuscript. S.-H.H.; Drafted the manuscript and collected some of the samples and edited the article. H.G.; Provided some advice and guide during the project. M.S., A.M.M.; Developed the concept, supervised and interpreted the findings of the study. All authors read and approved the final manuscript.

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