

## CORRELATION BETWEEN AK7 rs2275554 AND MALE INFERTILITY IN 421 VIETNAMESE INDIVIDUALS

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### ABSTRACT

Male infertility is a global health problem caused by many factors, including primary ciliary dyskinesia (PCD), multiple morphologic abnormalities of the flagella (MMAF), and genetic factors, in which PCD and MMAF have been reported to be associated with variants in the AK7 gene. So, this study aimed to evaluate the association of polymorphisms AK7 rs2275554 with infertile men in the Vietnamese population. Total DNAs were isolated from 421 samples, including 199 males diagnosed with infertility and 222 healthy individuals having at least one child. All 421 samples were applied for genotyping using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Using statistical methods, we showed that the distribution of their genotypes conformed with Hardy-Weinberg equilibrium ( $p$ -values  $> 0.05$ ). There was no association between polymorphism rs2275554 and male infertility in the Vietnamese population ( $p$ -values  $> 0.05$ ). These findings in this study will contribute to the knowledge base about the underlying genetics of male infertility in the Vietnamese population.

**Keywords:** Male infertility, AK7, Vietnam, PCR-RFLP.

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## INTRODUCTION

Male infertility is the inability of a man to conceive with a healthy partner within one year of unprotected sex, a problem affecting the psychological health and marital discord of couples (Kumar & Singh, 2015). Many factors can lead to male infertility, such as an unhealthy lifestyle, environment, certain medical conditions, hormonal disorders, or genetic disorders including multiple morphological abnormalities of the sperm flagella (MMAF) and primary ciliary dyskinesia (PCD) (Durairajanayagam, 2018; Leigh et al., 2019; Leslie et al., 2021). MMAF is a disorder characterized by asthenoteratozoospermia with irregular morphology of the sperm flagellum, which helps the sperms move through the female reproductive tract and meet an egg (Wang et al., 2020). First named in the 2014 study by Ben Khelifa and colleagues, this syndrome has been described with features like “dysplasia of the fibrous sheath”, “short tails”, or “stump tails” (Ben Khelifa et al., 2014). The flagellum consists of the structures necessary for the movement and penetration of sperm through the fluids upon fertilization with the egg. PCD is a genetic disorder of motile cilia dysfunction characterized by various clinical symptoms such as upper and lower respiratory tract infections, lateral malformations, and male infertility (Leigh et al., 2019). Motile cilia are tiny, whip-like organelles that generate directional fluid flow by beating (Ringers et al., 2020). Studies have shown that motile cilia are present in the efferent ductules and these cilia stir up the seminal fluid to prevent blockage in the vas deferens, one of the causes of low sperm count (Sironen et al., 2020). The cilia and the flagellum have a similar cytoskeletal framework of microtubules that form the cores of both organelles known as axoneme, which is highly preserved throughout evolution. The axoneme consists of a pair of central microtubules, surrounded by nine pairs of peripheral microtubules (“9+2” structure), bound to many proteins involved in the structure and beating coordination (Lores et

al., 2018). The fringe doublets have inter-doublet connections by the nexin-dynein administrative complex and multiprotein T-molded structures, called the radial spokes (RSs), interfacing each fringe doublet to the central pair. RSs are considered essential to control the movement of the axoneme. Outer (ODAs) and inner (IDAs) dynein arms are multiprotein ATPases complexes attached to the doublet microtubules. These complexes simultaneously promote the sliding of the fringe doublet and drive the beating and motility of cilia and flagella (Lores et al., 2019). Therefore, mutations in axoneme proteins can lead to loss of ODAs, IDAs, or RSs, thereby affecting the structure and motility of flagella. Many genes have also been strongly associated with MMAF, one of which is the *AK7* gene.

The *AK7* gene (Adenylate kinase 7) is located on chromosome 14, at position 14q32.2, contains 18 exons, and encodes for a protein of 723 amino acids. This peptide product is a member of the human Adenylate kinase (AK) family (Panayiotou et al., 2011). AKs are important phosphotransferases that catalyze the mutual conversion of adenosine phosphates, facilitating the reversible reaction of  $2\text{ADP} \leftrightarrow \text{ATP} + \text{AMP}$ . This catalytic process is crucial to the cellular energy homeostasis as a continuous and comprehensive supply of adenosine nucleotides is necessary to generate ATP, the primary energy source for the assembly and beating of the cilia and flagella (Lores et al., 2018). In addition, ATP hydrolysis plays an important role in transporting axonemal components required for the elongation and maintenance of flagella (Fernandez-Gonzalez et al., 2009). *AK7* is the only adenylate kinase containing the C-terminal DPY30 domain among AK family members. This domain is known to help specify AK activity near axonal components and is involved in interactions with A-kinase anchor proteins (AKAP) (Nsota Mbango et al., 2019). AKAP proteins are abundant in testes and essential for sperm function as they regulate motility,

sperm storage capacity, and acrosome response (Carnegie et al., 2009).

So far, there were only a few studies on the AK7 gene in male infertility patients with MMAF and PCD that showed a strong association between AK7 and male infertility patients. The polymorphism AK7 rs2275554, a missense variant (NM\_152327.5:c.305G>A,p.R102Q), was analyzed *in silico* using the CADD (Combined Annotation Dependent Depletion) software (Rentzsch et al., 2021). This software can predict the deleteriousness of single nucleotide variants in the human genome by integrating multiple annotations, including conserved and functional information into one index. The obtained score of 22.5 showed that AK7 rs2275554 was a promising candidate for an association study. Therefore, to investigate the potential correlation between AK7 SNPs and male infertility, we conducted a case-control study of AK7 rs2275554 in the Vietnamese population.

## MATERIALS AND METHODS

### Study subjects

This study collected blood samples from 199 Vietnamese male subjects in the case group and 222 subjects in the control group. 199 men from the case group were diagnosed with male infertility based on these criteria: having regular sex with their partner for more than 1 year without any contraception method but not seeing pregnancy. Their sperm analysis results showed that they either had azoospermia or oligozoospermia. All subjects, who had karyotype abnormalities, chromosomal disorders, deletion, or suffered from diseases affecting fertility (inflammation of the epididymis or testicles, sexually transmitted infections, including gonorrhea or HIV,...) were disqualified for the study. All subjects in the control group (n = 222) were fertile men with normozoospermic and had given birth to at least one child by spontaneous conception. The conduct of human sampling and research was approved by the Ethical Council in Biomedical

Research of the Institute of Genome Research, Vietnam Academy of Science and Technology. All subjects signed consent forms for blood samples and medical records in the research process.

### Methods

#### *Extracting genomic DNA from whole blood samples*

The blood samples were used to extract and purify total DNA with Thermo kit “GeneJET Whole Blood Genomic DNA Purification” following the manufacturer’s instructions. Electrophoresis 2 µL of the subject’s DNA samples in 1% agarose gel and using the spectroscopic method to check the quantity and quality of the extracted DNA. All DNA samples were stored at -20 °C.

#### *PCR and RFLP technique*

The total DNA of 421 samples was used to amplify the AK7 gene fragment containing the polymorphism AK7 rs2275554 by PCR reaction with specific primers (Table 1). Evaluation of PCR products quality was assessed by electrophoresis on 1.2% agarose gel. The PCR products were then digested with restriction enzyme *Xho*I (Thermo Fisher). The digestion reaction mixture was incubated at 37 °C in a water bath for 4–6 hours. The DNA fragments produced by the digestion were then separated by length on 2% agarose gel. Based on the size and number of DNA bands appearing on the gel, the genotypes of AK7 rs2275554 were determined.

#### *Sanger sequencing*

To confirm the results of the PCR-RFLP method, 5% out of 421 samples were randomly selected for purification by Gene JET PCR Purification Kit (Thermo Fisher) and Sanger sequencing using the ABI Big Dye Terminator v3.1 sequencing kit (Applied Biosystems, CA) on an ABI sequencer 3500 (Applied Biosystems). Sequencing results were analyzed using SnapGene Viewer bioinformatics software.

Table 1. Primers for PCR, size, and the number of DNA bands corresponding with three genotypes of AK7 rs2275554

Primer sequence	PCR product (bp)	PCR-RFLP		
		Genotype	Number of DNA bands	Fragment size (bp)
F: 5'CTCCTGCAAGTGTTCCTGTG3' R: 5'AGCCCTACTTTGTCTAGTGTTC3'	332	GG	2	136; 196
		GA	3	136; 196; 332
		AA	1	332

**Statistical analysis**

Data obtained from the PCR-RFLP method were analyzed using R version 4.0.3 (R Core Team, 2020) and Microsoft Excel (Microsoft Corp., Washington, DC, USA). Using the Chi-square test ( $\chi^2$ ) of the “Hardy-Weinberg” package (Graffelman, 2015) to check Hardy-Weinberg equilibrium (HWE) of a population and to evaluate the correlation between genotypes and alleles of the polymorphism with the probability of male infertility. The “epitools” package (Tomas J. Aragon et al., 2020) was used to evaluate the correlation between polymorphism and male infertility in 3 test models: additive, dominant and recessive. Association was estimated by calculating odds ratio (OR) with 95%

confidence intervals. The estimation was considered to be statistically significant if  $p$ -value  $< 0.05$ .

**RESULTS**

**Genotype identification of polymorphism AK7 rs2275554**

The DNA region containing the polymorphism AK7 rs2275554 was amplified by PCR reaction with specific primers (Table 1), and agarose gel electrophoresis showed a specific, sharp, and bright DNA band with the correct molecular weight (around 300 bp). Then, PCR products were digested with *XhoI* restriction enzyme, and the results of electrophoresis of 6 samples are shown in Figure 1.

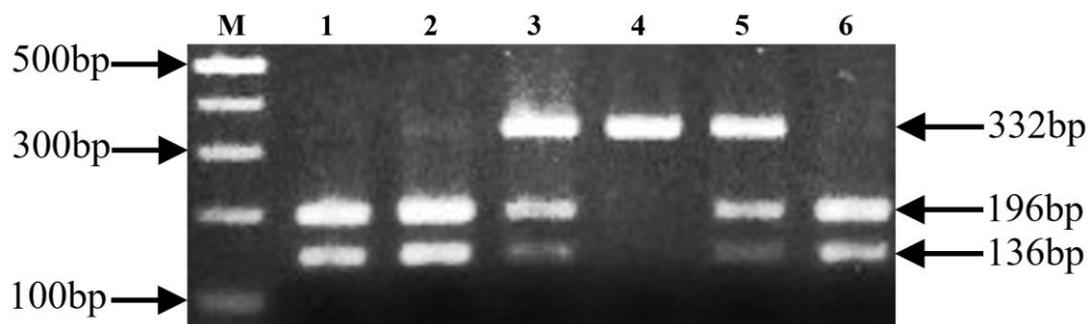


Figure 1. Restriction enzyme-digested PCR products of 6 samples on 2% agarose gel.

M: Marker 100 bp; 1, 2, and 6: Wildtype GG (2 bands of 196 bp, and 136 bp);  
3 and 5: Heterozygous GA (3 bands of 332 bp, 196 bp, and 136 bp);  
4: Homozygous AA (1 band of 332 bp)

Six representative *XhoI*-digested products from 421 samples in the study clearly displayed the genotype of each sample: samples 1, 2 and 6 were wildtype (GG), samples number 3 and 5 were heterozygous

(GA) and sample 4 was homozygous (AA). The genotypes of all 421 samples were determined and shown in Table 2. The frequency of allele A (minor allele) in the cases, controls, and whole studied population

were 0.183, 0.212, and 0.192 respectively (Table 2). Besides, the distribution of 3 genotypes of the AK7 rs2275554 polymorphism with  $p$ -values were 0.08 in the case group, 0.984 in the control group, and 0.275 in the population ( $p$ -values  $>0.5$ ), showing that all these values followed the Hardy-Weinberg equilibrium (HWE).

Table 2. General information on the polymorphism AK7 rs2275554

	Genotype			Allele		HWE $p$ -value
	GG	GA	AA	G	A	
Case (n = 199)	129 (64.82%)	67 (33.67%)	3 (1.51%)	0.817	0.183	0.080
Control (n = 222)	138 (62.16%)	74 (33.34%)	10 (4.5%)	0.788	0.212	0.984
Total	267 (63.4%)	141 (33.5%)	13 (3.1%)	0.802	0.192	0.275

Note: n: Number of participants; HWE: Hardy-Weinberg Equilibrium.

Sanger sequencing was used to confirm the genotypes of about 5% of the samples (20 samples), validating the findings by the PCR-RFLP method (Fig. 2).

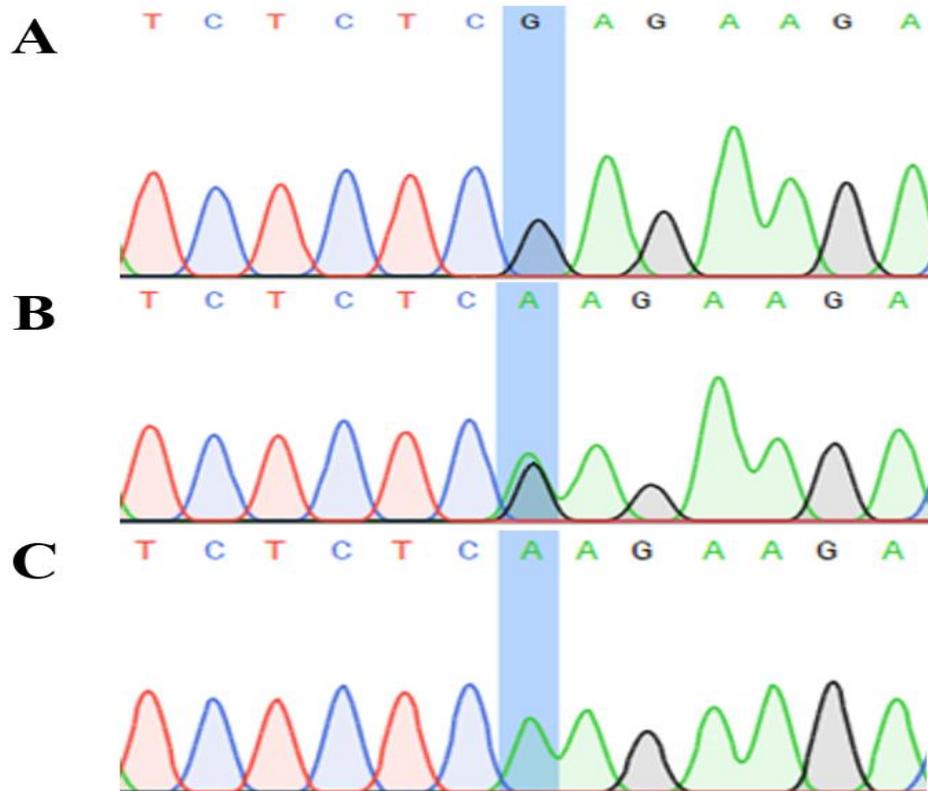


Figure 2. Genotyping AK7 rs2275554 using Sanger sequencing. A: Wildtype form GG; B: Heterozygous form GA; C: Homozygous form AA

#### Association analysis between AK7 rs2275554 and male infertility

To analyze the correlation between the polymorphism AK7 rs2275554 and male

infertility, 3 models were tested for the minor allele (additive, dominant, and recessive). The  $p$ -values were greater than 0.05, indicating no statistically significant association between genotypes and male infertility (Table 3).

Table 3. The association between rs2275554 and male infertility

Test model	Cases (n = 199)	Controls (n = 222)	OR	95% CI	p-Value
Additive					
GG	64.82%	62.16%	1.000		
GA	33.67%	33.33%	1.032	0.685–1.555	0.878
AA	1.51%	4.51%	2.999	0.877–14.289	0.075
Dominant					
GG	64.82%	62.16%	1.000		
GA + AA	35.18%	37.84%	1.121	0.753–1.672	0.571
Recessive					
GG + GA	98.49%	95.49%	1.000		
AA	1.51%	4.51%	2.969	0.877–14.057	0.075

Note: n: Number of participants; OR: odds ratio; 95% CI: 95% confident intervals; p-values measured by Chi-square test.

## DISCUSSION

Infertility is a global medical problem, affecting an estimated 15% of couples worldwide, with male infertility being the sole cause for 20–30% of infertility cases and 50% of cases in general (Agarwal et al., 2015). A decrease or slow movement of sperm in the ejaculate (asthenozoospermia), often accompanied by oligospermia or teratozoospermia, occurs in about 19% of infertile men (Curi et al., 2003). The multiple morphological abnormalities of the sperm flagella (MMAF) is a specific form of asthenozoospermia, characterized by abnormal flagellate phenotypes and severely impaired sperm motility (Wang et al., 2020). In addition, another similar form of asthenozoospermia is primary ciliary dyskinesia (PCD), an inherited disorder caused by motor defects in the cilia and flagella, which can also lead to male infertility. The axoneme is an extremely conserved microtubule-based cytoskeleton, present in both cilia and sperm flagella, that helps maintain the functioning of these organelles (Lores et al., 2018). Abnormalities in the structure and function of the axoneme are known to cause primary ciliary dyskinesia (PCD). There have been studies describing the relationship between the AK7 gene and PCD. AK7 deficiency in animals causes severe PCD phenotypes, including possibly impaired sperm motility and defects in sperm

maturation (Fernandez-Gonzalez et al., 2009), and this protein is a component of axonemal and peri-axonemal structures in mouse sperm flagella (Vadnais et al., 2014). In another study in 2012, 2 variants rs2369679 and c.1214insT in the AK7 gene were identified from PCD patients. The c.1214insT mutation may be related to PCD, and AK7 could be associated with the development of PCD (Mata et al., 2012). Besides, in a 2018 study, Lores et al studied two siblings with MMAF without any features of PCD and identified the c.2018T>G (p.Leu673Pro) mutation in AK7 that results in the loss of AK7 protein only in sperms but not in respiratory cilia, although both cell types carry the mutated transcript (Lores et al., 2018). The study also showed that the p.Leu673Pro mutant did not reduce protein levels but was able to affect the structure of the coil-coiled domain previous to the DPY30 domain, forming the interaction and oligomerization domain (Lores et al., 2018). These results suggest that the absence of AK7 in sperm cells is not caused by faulty gene expression or protein production. Still, it may be due to the structural defects that affect the protein's interaction with axonemal partners and anchoring within the axoneme in the sperm cells (Lores et al., 2018). This literature indicated an association between the gene AK7 and male infertility factors like MMAF and PCD in European-descendant/Caucasian groups. Our study focused on studying the effect of AK7

rs2275554 on male infertility in the Vietnamese population. The polymorphism rs2275554 is a missense variant, changing the amino acid at position 102 (NM\_152327.5:c.305G>A,p.R102Q).

However, the survey results of AK7 rs2275554 in Vietnamese male infertility patients and controls did not reveal any association of this variant with male infertility in the Vietnamese population.

## CONCLUSION

In this study, we performed the PCR-RFLP method to determine the distribution of AK7 rs2275554 in the Vietnamese population and analyzed the relationship of this polymorphism with male infertility. Genotypic ratios GG, GA, AA in our sample were 63.4%, 33.5%, and 3.1%, respectively, and all followed Hardy-Weinberg equilibrium. However, genotype and allele frequency analysis of AK7 rs2275554 polymorphism revealed no relationship between this polymorphism and male infertility. This study expanded the understanding of the genetics of male infertility in Vietnam.

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