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THE ASSOCIATION OF *CFAP65* WITH MALE INFERTILITY IN VIETNAMESE INDIVIDUALS

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SUMMARY

Cilia- and flagella-associated protein (CFAP) is a well-known protein family that plays a vital role in the spermatogenic process. Recently, the gene *CFAP65*, which encodes the cilia- and flagella-associated protein 65, has been focused on as a new candidate for male infertility. Mutations in this gene are frequently detected in patients with primary infertility, especially among cases with combined phenotypes of acrosome abnormalities and multiple morphological abnormalities of the flagella (MMAF). In addition, mice carrying both a complete knock-out of the *CFAP65* gene and a *CFAP65* homozygous frameshift mutation exhibited sterility with the typical phenotypes of MMAF. However, no case-control study has been performed on the relationship between polymorphisms in *CFAP65* and male infertility in any population. Hence, our study aimed to investigate the correlation between the polymorphism *CFAP65* rs117885048 and male infertility in a Vietnamese population comprising 207 infertile men and 217 healthy controls. As a result, the studied population followed Hardy-Weinberg equilibrium (HWE) ($p > 0.05$) and the frequencies of genotypes CC/CT/TT were 0.875, 0.12, and 0.003, respectively. The Chi-square test revealed no association between the polymorphism *CFAP65* rs117885048 and the disease in this population ($p > 0.05$). To further interpret the correlation between single nucleotide polymorphisms in the *CFAP65* gene and male infertility, a more comprehensive study with other polymorphisms needs to be considered.

Keywords: male infertility, *CFAP65***,** rs117885048, Vietnam, PCR-RFLP

INTRODUCTION

Male infertility is an urgent, global problem since its age-standardized prevalence rate has risen by 8.224% from 1990 to 2017, increasing at a rate of 0.291% per year (Sun *et al.*, 2019). Moreover, among multiple likely causes of male infertility, genetics accounts for at least 15% (Krausz *et al.*, 2015). However, identifying the precise genetic cause of male infertility is challenging since thousands of genes participate in the spermatogenic process (Krausz *et al.*, 2018), with about 30% of the cases remaining unknown (Fainberg *et al.*, 2019). Therefore, advanced technologies such as whole-exome sequencing (WES), whole-genome sequencing (WGS), and next-generation sequencing (NGS) have been applied to uncover the potential genes associated with different types of male infertility (Krausz *et al.*, 2018; Xavier *et al.*, 2021; Robay *et al.*, 2018), including cilia- and flagella-associated protein genes (*CFAP*) *CFAP43*, *CFAP44*, *CFAP65*, *DNAH1*, *TEX11*, *MEIOB*, *SYCE1*, etc. Among them, the *CFAP65* gene has

recently been investigated.

The *CFAP65* gene, located on chromosome 2q35, contains 35 exons and encodes a protein belonging to the cilia- and flagella-associated protein family. Previous studies have suggested that *CFAP65* is involved in sperm flagellar development or sperm motility. It is expressed in the acrosome area and flagella midpiece, specifically localized in the cytoplasm of round spermatids and elongating spermatids (Wang *et al.*, 2019). In addition, recessive pathogenic variants, including nonsense, missense, splice site, and deletion in *CFAP65*, are frequently found in patients with asthenozoospermia or defects in sperm motility (Zhang *et al.*, 2019; Wang *et al.*, 2019; Li *et al.*, 2020). Furthermore, mutations with similar inherited forms in other CFAP-encoding genes, including *CFAP43*, *CFAP44*, *CFAP69*, and *CFAP70*, are also reported in patients with sperm motility defects in infertility (Tang *et al.*, 2017; Sha *et al.*, 2019; Dong *et al.*, 2018; Beurois *et al.*, 2019). Therefore, it could be inferred that *CFAP65* is required for sperm flagella development and is crucial for the spermatogenic process. Although *CFAP65* is one potential gene responsible for male infertility, no study has been conducted on the association of this gene and the development of male infertility in any population in the world.

Hence, to understand the effect of *CFAP65* polymorphisms on male infertility at the population scale, a first-time case-control study of *CFAP65* polymorphism rs117885048 was conducted in a Vietnamese population.

MATERIALS AND METHODS

Study participants

The study consists of 424 subjects (207 infertile male patients and 217 controls). The selection criteria of patients include (1) experiencing childless status after at least 12 months of regular unprotected sexual intercourse and never having children before; (2) having a normal karyotype, and no AZF microdeletion; and (3) having no medical history of fertility-affecting diseases including mumps, sexually transmitted diseases, and drug addiction. Controls were selected among men who had fathered at least one child without seeking assisted reproductive technologies (ARTs). Subjects that fulfilled the requirement above gave informed consent for blood donation. The study was approved by the Institutional Review Board of the Institute of Genome Research, Vietnam Academy of Science and Technology.

SNP genotyping

Genomic DNA was extracted from blood samples by GeneJET Whole Blood Genomic DNA Purification Kit (Thermo Fisher Scientific). They were then diluted to the appropriate concentration (about 10 ng/µL) and used as the template for the PCR reaction, amplifying the region containing the polymorphism rs117885048. The total volume of the PCR reaction was 10 µL, including 1 µL of Dream Taq buffer (10X); 0.6 µL of dNTPs (2.5 mM); 0.05 µL Taq polymerase (5 U/µL); 0.2 µL of specific primer forward and reverse (10 pmol); 2 µL of DNA template and nuclease-free water (H_2O). The PCR conditions started with an initial denaturation of 95^{o}C for 5 minutes, followed by 35 cycles of denaturation at 95^{o}C for 30 seconds, annealing at 57^{o}C for 30 seconds, extension at 72^{o}C for 30 seconds, and final extension at 72^{o}C for 5 minutes. The PCR products were digested with the restriction enzyme *Hha*I (Thermo Fisher) based on the polymerase chain reaction-restriction fragment length polymorphism (PCR_RFLP) method. The digested reaction was carried out in a volume of 5 µL, containing: 3 µL of PCR products; 0.3 µL buffer Tango (10X); 0.1 µL of *Hha*I (10 U/µL); and nuclease-free water (H_2O). Reaction tubes were incubated at 37^{o}C for approximately 3 hours. The digested products were run on an agarose gel at 1.5% to observe the genotypes of samples based on the number and size of DNA bands (Table 1).

Statistical analysis

Data obtained from the above methods were analyzed using Microsoft Excel (Microsoft Corp., Washington, DC, USA) and R version 4.0 (R Core Team, 2020). The Chi-square test ($\chi 2$)

of the package "Hardy Weinberg" was used to calculate the Hardy-Weinberg equilibrium (HWE) of the population (Graffelman, 2015). In addition, the association between the polymorphism and male infertility was tested in 3 test models: additive, dominant, and recessive, using the package "epitools" (Aragon, 2020). Finally, an odds ratio with a confidence interval of 95% was used to assess the association. All the statistical tests were two-sided. The result was considered to be statistically significant if the p-value < 0.05.

Table 1. Genotypes of *CFAP65* rs117885048 based on PCR-RFLP methods

Genotypes	Number of DNA bands	Size of DNA bands (bp)
CC	2	100; 195
CT	3	295; 195; and 100
TT	1	295

Note: PCR-RFLP: Polymerase Chain Reaction - Restriction Fragment Length Polymorphism; bp: base pair; Homozygous form CC (2 bands of 195 bp and 100 bp); Heterozygous form CT (3 bands of 295 bp, 195 bp, and 100 bp); Homozygous form TT (1 band of 295 bp).

RESULTS

Genetic analysis of *CFAP65* rs117885048

The targeted region containing *CFAP65* rs117885048 was amplified successfully with a specific, sharp, and bright DNA band (Fig. 1). The PCR products obtained from 424 samples (207 cases and 217 controls) were then digested with *Hha*I (Thermo Fisher) to observe the genotypes. Seven representative samples (1–7) of *CFAP65* rs117885048 indicated that the genotypes of samples 1, 3, 4, 5, and 6 were homozygous genotype CC, sample 2 was heterozygous genotype CT, and sample 7 was homozygous TT (Fig. 1).

The minor allele frequencies of the polymorphism in cases, controls, and the whole population were 0.065 (Table 2). No significant discrepancy was detected in the frequency between the case and control groups. However, using Chi-square to test the Hardy-Weinberg equilibrium (HWE) showed that the studied population followed HWE (p> 0.05).

Association of *CFAP65* rs117885048 with male infertility

To understand more about the correlation of the polymorphism with male infertility, statistical analyses were performed on three test models: additive, dominant, and recessive (Table 3). The result showed no association in any tested model (p > 0.05). Thus, the *CFAP65* rs117885048 was not associated with male infertility in this Vietnamese population.

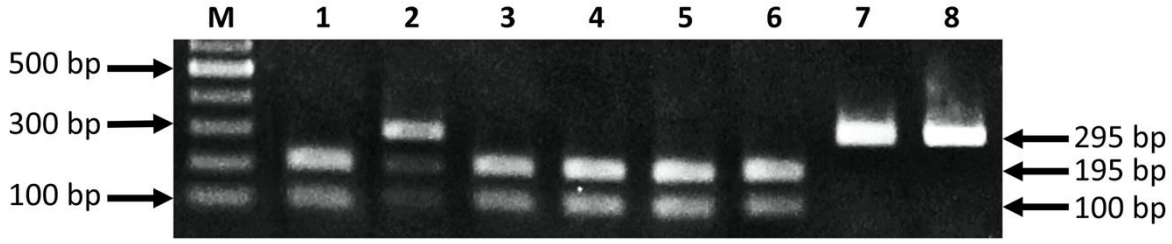

Figure 1. Analysis of 7 *Hha*I-digested PCR products on agarose gel 1.5%. M: Marker 100 bp; 1, 3-6: Homozygous samples CC (2 bands of 195 bp and 100 bp); 2: Heterozygous sample CT (3 bands of 295 bp, 195 bp, and 100 bp); 7: Homozygous sample TT (1 band of 295 bp); 8: Uncut PCR product.

Table 2. *CFAP65* rs117885048 genotypes of 424 samples.

	Genotypes			Allele frequencies		HWE *p*-value
	CC	**CT**	**TT**	**C**	**T**	
Case (n = 207)	181	25	1	0.935	0.065	0.892
Control (n = 217)	190	26	1	0.935	0.065	0.913
Total (n= 424)	371	51	2	0.935	0.065	0.862

Note: HWE: Hardy-Weinberg equilibrium; n: number.

Table 3. Correlation of *CFAP65* rs117885048 with male infertility.

Test model	Controls (n = 217)	Cases (n = 207)	OR	95% CI	p-value
		Additive			
CC	190 (87.56%)	181 (87.44%)	1.000		
CT	26 (11.98%)	25 (12.08%)	0.916	0.507 – 1.654	0.769
TT	1 (0.46%)	1 (0.48%)	0.953	0.024 – 37.348	0.973
		Dominant			
CC	190 (87.56%)	181 (87.44%)	1.000		
CT+TT	27 (12.44)	26 (12.56%)	1.011	0.565 – 1.806	0.971
		Recessive			
CC+CT	216 (99.54%)	205 (99.52%)	1.000		
TT	1 (0.46%)	1 (0.48%)	1.054	0.027 – 41.273	0.971
		Allele			
C	406 (93.55%)	387 (93.48%)	1.000		
T	28 (6.45)	27 (6.52%)	1.012	0.582 – 1.756	0.967

Note: n: number; OR: odds ratio; 95% CI: 95% confident intervals; p-value was measured using Chi-square test.

DISCUSSION

Previous studies reported that genetic variants in *CFAP* were frequently reported in infertile men with primary infertility. The first nonsense mutation was identified in a Chinese patient with spermatogenic failure due to MMAF (Tang *et al.*, 2017). Using WES, biallelic null mutations in *CFAP65* were detected in six Chinese probands with primary infertility from unrelated families. Those six accounted for 6.8% (6/88) (Li *et al.*, 2020). Likely pathogenic variants in *CFAP65* were identified in two additional MMAF-affected men of Iranian and North African origin, suggesting that the alterations in *CFAP65* might affect male fertility in other populations. Another study found mutations on the *CFAP65* gene with the same prevalence of 6.7% (3/45) in Chinese families, including a missense variant NM_194302: c.C3021A; p.N1007K (Wang *et al.*, 2019). No deleterious variants were found in the control cohort of 637 men. The results also indicated that patients carrying mutations in *CFAP65* had a poor result of intracytoplasmic sperm injection (ICSI), suggesting that screening of *CFAP65* mutations could be necessary before conducting ICSI.

The *CFAP65* gene is evolutionarily conserved in several species, but its function has not been identified (Pazour *et al.*, 2005). It is suggested to be a transmembrane protein containing two putative domains (ASH and MSP). The ASH domain of abnormal spindle-like microcephaly-associated protein has been found in proteins associated with cilia, flagella, the centrosome, and

the Golgi complex (Shima *et al.*, 2004). As a consequence, its vital role in sperm motility is indicated. The MSP domain, which is mainly involved in a protein-protein interaction module, performs other functions related to forming the dynamic MSP cytoskeleton and serves as a signaling molecule (Tarr *et al.*, 2005). *CFAP65*, in particular, has been proposed to participate in calcium-mediated activities and to mediate the anchoring of mitochondria and acrosomes to the outer membrane forming cytoskeleton in spermatozoa (de Boer *et al*., 2015; Amaral *et al*., 2013), explaining why infertile patients with *CFAP65* mutations frequently have acrosome abnormalities. Furthermore, a recent study demonstrated that CFAP65 interacted with CFAP47 in human spermatozoa and mouse testis using coimmunoprecipitation analysis (Liu *et al.*, 2021). Immunofluorescence analysis revealed abnormal localization of CFAP65 in spermatozoa from men with *CFAP47* mutations and loss of CFAP47 staining in sperm cells from men with *CFAP65* mutations, indicating that the interaction or regulation of these genes is indispensable for male infertility. Additionally, both mouse models with a *CFAP65* homozygous frameshift mutation and a complete knock-out of the *CFAP65* gene showed sterility with typical phenotypes of MMAF (Li *et al.*, 2020; Wang *et al.*, 2021).

Although all the previous findings support that CFAP65 is required for sperm flagellar development, no association was obtained between the polymorphism of *CFAP65* and male infertility in the current studied population. However, given the great potential of the gene, a more comprehensive study with other polymorphisms is needed to confirm the relationship between *CFAP65* and male infertility.

CONCLUSION

This study analyzed the correlation of the polymorphism *CFAP65* rs117885048 with infertility in a Vietnamese population. Results revealed the genotypes of this polymorphism followed Hardy-Weinberg Equilibrium. However, the statistical analysis showed no association between rs117885048 and male infertility. This study could be considered as a pilot study in the assessment of the correlation between the *CFAP65* gene and male infertility in Vietnam.

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