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Association of *CFAP65* rs56411706 with male infertility in 393 Vietnamese individuals

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Abstract:

Approximately two thousand genes have been found to be involved in spermatogenesis and their mutations have been reportedly associated with male infertility. Recent studies have shown that *CFAP65* was crucial for spermatogenesis, and several mutations in this gene could result in male infertility. However, the association of polymorphisms in *CFAP65* with male infertility remains unknown. In this study, the relationship between *CFAP65* rs56411706 and male infertility was assessed in a Vietnamese population by 171 male infertility patients who had been diagnosed with non-obstructive azoospermia (NOA), oligozoospermia, or asthenozoospermia while 222 healthy controls were genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Statistical analysis demonstrated that the allele frequencies of *CFAP65* rs56411706 followed Hardy-Weinberg equilibrium (HWE) ($p>0.05$). The Chi-square test revealed no correlation between the polymorphism and male infertility in this study ($p>0.05$). This is the first study on the association between a single nucleotide polymorphism in the *CFAP65* gene and male infertility in a Vietnamese population. The results of this study would help enrich the knowledge about the effects of *CFAP65* polymorphisms on male infertility in the Vietnamese population.

Keywords: ***CFAP65*, male infertility, PCR-RFLP, rs5611706, spermatogenic qualitative defects.**

Classification number: **3.2**

Introduction

Qualitative spermatogenesis defects are one of four major etiological categories of male infertility [1]. It is characterised by sperm motility, morphology, and functional parameters such as DNA and chromatin integrity [2]. Various clinical classifications for qualitative defects of spermatogenesis include "teratozoospermia" (reduced percentage of sperm with normal morphology) [3], "oligozoospermia" (reduced sperm count) [4], and "asthenozoospermia" (reduced sperm motility) [2]. However, to describe more than one abnormality within the semen parameters, other terms such as oligoteratozoospermia, oligoasthenozoospermia, and asthenoteratozoospermia are also used [2].

Asthenozoospermia, a type of asthenoteratozoospermia, is characterised by the presence of sperm flagellar anomalies that occur at the end of spermatogenesis and concurrently with head compaction and reshaping [5]. Over 80% of male infertility cases exhibit some alterations of sperm motility. The multiple morphological abnormalities of asthenozoospermia phenotypes include (1) morphological abnormalities such as short-coiled, absent, bent, and/or irregular-calibre flagella associated with dramatically declined motility or (2) ultrastructural flagellar defects, e.g., absent central pair (CP), disorganised double microtubules (DMT), dysplasia of fibrous sheath (FS), or absence of dynein arms [2]. Several genes have so far been identified as having an association with multiple morphological abnormalities of sperm flagella phenotypes [2, 6] leading to an absence of inner dynein arm (IDA), disorganized 9+2, absent CP, disorganised FS, or DMT [2]. Abnormalities of *CFAP* genes, which have been found to be involved in spermatogenesis, could cause fertility impairment in males [7-11]. Notably, *CFAP65* has been recognised

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as a new gene candidate for fertility impairment due to its functions regarding sperm flagellar morphology and paraflagellar rod synthesis [12, 13].

The *CFAP65* encodes cilia- and flagella-associated protein 65, which is highly expressed in testis during spermatogenesis. Several mutations on *CFAP65* have been reported to cause male infertility [8, 12, 14]. Consequently, the association between single nucleotide polymorphisms on this gene and the risk of male fertility is expected to be a good consideration. The polymorphism of rs56411706 occurring in the coding region of the *CFAP65* gene was analysed *in silico* using SIFT. This software predicts the effect of an amino acid substitution on protein function based on sequence homology among different species and physical properties. The obtained score of 0.09 suggested that rs56411706 is a promising candidate for an association study. Therefore, in this study, we conducted the PCR-RFLP experiment to investigate the relationship between *CFAP65* rs56411706 and the risk of male infertility in a Vietnamese population.

Materials and methods

Subjects

Our research subjects consisted of 171 men diagnosed with NOA caused by either oligo/azoospermia or asthenozoospermia and 222 healthy men with normal semen who had conceived at least one child. Unfertilised men with normal male reproductive organs and hormone balances were screened for semen and checked for microdeletion of the AZF region or other major abnormalities in the Y chromosome. The study was approved by the Ethical Review Committee of the Institute of Genomics Research (No: 9-2019/NCHG-HĐĐĐ). Prior to the commencement of the study, all participating individuals gave written informed consent.

Genotype determination

The total DNA of 393 individuals was extracted from peripheral blood samples using the GeneJet Whole Blood Genomic DNA Purification (Thermo Fisher). Quantity of genomic DNA was analysed by electrophoresis on 1% agarose, and DNA was quantified using NanoDropTM One spectrometer, Thermo Fisher. The gene fragment containing polymorphism *CFAP65* rs56411706 was amplified by PCR with a specific pair of primers sequenced F: 5'-CATTCTGCAAGGCGGTGATT-3' and R: 5'-AGGCTAAATTTTCCC TGGGGC-3'. The 10-µl PCR reaction mixture contained 1 µl of 10X PCR buffer; 100 µM dNTPs; 0.15 µM of forward and reverse PCR primer each; 0.1 U *Taq* DNA polymerase (Thermo Fisher); 10 ng genomic DNA; and H_2O. The PCR protocol consisted of denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and the final extension for 7 min at 72°C. Electrophoresis on a 1% agarose gel was performed using 3 µl of the PCR product for each sample. Products of PCR reaction were subjected to 5 h digestion by *Hha*I at 37°C. Digested products were separated by electrophoresis on 2% agarose gel. The number and size of DNA fragments used to determine the genotype of *CFAP65* rs56411706 are presented in Table 1.

Table 1. The number and size of DNA fragments of *CFAP65* rs56411706 genotypes.

Genotype	DNA fragments	DNA length (bp)
CC	2	215, 112
CA	3	215, 112, 327
AA	1	327

Statistical analysis

Statistical analyses were conducted with the statistical software R version 3.6.1 [15]. HWE equilibrium was tested using HWE exact [16, 17]. Logistic regression was used to estimate a 95% (CI) and odds ratio (OR) for binary variables, with a p-value less than 0.05 considered significant. A total of three genetic patterns were investigated: dominant, recessive, and additive. The allele composition, genotype of polymorphism *CFAP65* rs56411706, and the association between single nucleotide polymorphism and infertility risk were examined. SNPassoc [18] was used to perform association analyses.

Results

Genotyping CFAP65 rs56411706

The specific PCR products were processed with the restricted enzyme *Hha*I. The genotypes of *CFAP65* rs56411706 were determined based on the size and number of DNA bands of the restricted product (Table 1, Fig. 1).

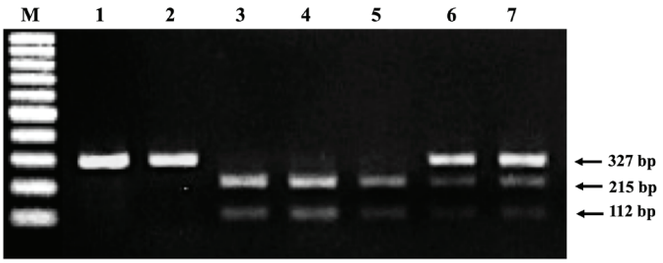

Fig. 1. Restriction enzyme-digested PCR products on agarose gel 2%.

Note: M: marker 100 bp; 1: uncut PCR product; 2: homozygous AA (1 band of 327 bp); 3-5: wildtype with the homozygous CC (2 bands of 112 and 215 bp); 6, 7: heterozygous CA (3 bands of 112, 215, and 327 bp).

Statistical analyses showed no differences in genotype composition and allele frequency between the case and control groups (Table 2). The distribution of this polymorphism was examined across two groups and as a whole by using the Chi-square test. The population followed HWE equilibrium (p>0.05) with CC/CA/AA genotype compositions of 0.72, 0.24, and 0.04, respectively (Table 2).

Table 2. Genotype composition and allele frequency of polymorphism *CFAP65* rs56411706.

Group	Genotype			Allele frequency (%)		p	HWE
	CC	*CA*	*AA*	*C*	*A*		
Control (n=222)	123 (0.72)	42 (0.24)	6 (0.04)	0.84	0.16	0.99	+
Case (n=171)	160 (0.72)	55 (0.24)	7 (0.04)	0.84	0.16	0.70	+
Total (n=393)	283 (0.72)	97 (0.24)	13 (0.04)	0.84	0.16	0.99	+

Note: HWE: Hardy-Weinberg equilibrium; +: follow the Hardy-Weinberg equilibrium.

Table 3. Association between *CFAP65* rs56411706 and male infertility.

Model	Control (n=222)	Case (n=171)	OR	95% CI	p
Additive					0.296
CC	160(72.07%)	123 (71.93%)	1.00		
CA	55 (24.77%)	42 (24.56%)	1.006	0.631-1.609	0.977
AA	7 (3.16%)	6 (3.51%)	0.894	0.283-2.909	0.848
Dominant					
CA+CC	215 (96.84%)	165 (96.49%)	1.00		
AA	7 (3.16%)	6 (3.51%)	0.89	0.28-2.86	0.84
Recessive					
AA+AC	160 (72.07%)	123 (71.93%)	1.00		
CC	62 (27.93%)	48 (28.07%)	0.99	0.636-1.553	0.975

Note: n: number of individuals; OR: odds ratio; 95% CI: 95% confidence interval; p: measured using Chi-square test.

Association analysis of CFAP65 rs56411706 with risk of male infertility

Chi-square testing on all three dominant, recessive, and additive models showed no association between this polymorphism and the risk of male infertility with a p-value greater than the significance level of 0.05 (Table 3).

Discussion

The gene *CFAP65* is evolutionarily conserved in several species [8]. The putative homolog of *CFAP65* is abundantly expressed in adult male mice, according to the murine testicular transcriptome analysis [8, 19]. Similarly, in Chlamydomonas, *CFAP65* is highly activated during flagellar regeneration [20]. The disruption of sperm motility caused by *CFAP65* was first discovered in Rose comb chickens [21]. A 2 bp frameshift mutation later confirmed the pathogenicity of *CFAP65* mutation in male mice [8]. Remarkably, phenotypes of mutated mice with *CFAP65*-frameshift-caring mutation were consistent with human asthenozoospermia [8].

In humans, *CFAP65*, comprising 13 transcripts, is found on 2q35. The cilia and flagella-associated protein 65, which is encoded by this gene, is highly and preferentially expressed in the testis according to the ENCODE [22], FANTOM [23], and GTEx databases [24]. Furthermore, proteomic analyses have found *CFAP65* in the centrioles of human spermatozoa [19]. In a study by W. Wang, et al. (2019) [14], *CFAP65* was identified as the causative gene for completely immotile spermatozoa. According to the proteomic analysis, in the absence of *CFAP65* during manchette organization, both mitochondrial sheath (MS) assembly and acrosome formation were unable to function properly [8]. Importantly, endogenous immunoprecipitation and immunostaining experiments revealed that *CFAP65* might form a cytoplasmic protein network with *MNS1, ZPBP1, RSPH1, TPPP2*, and *SPACA1* [13] in which the perturbations to the *CFAP65*-centered proteostasis network caused a series of defects in sperm head and flagella leading to severe asthenoteratospermia [13]. A homozygous nonsense mutation (p.Glu1781*) in *CFAP65* was identified in a consanguineous Chinese family with an abnormal flagella patient [19], and a biallelic mutation in *CFAP65* was also identified to cause

asthenozoospermia.

In *CFAP65* rs56411706 polymorphism, a nucleotide was substituted at NM_194302.4:c.1024G>T. This substitution altered alanine to serine at position 342. Although the *CFAP65* rs56411706 polymorphism did not show any association with male infertility in the Vietnamese population, our findings provide insights into *CFAP65* single nucleotide polymorphisms of male infertility.

Conclusions

In this study, the *CFAP65* rs56411706 polymorphism was analysed among 393 studied subjects. Results revealed the frequencies of genotypes CC/CA/CC to be 0.72/0.24/0.04, respectively. Their distribution all followed HWE. However, no relationship was established between *CFAP65* rs56411706 and male infertility ($p>0.05$). To gain more insight into this association, examinations of other polymorphisms in the *CFAP65* need to be considered.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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