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Molecular Insights into Proprotein Convertase Subtilisin/Kexin Type 9 Mutations in Vietnamese Hypercholesterolemia Cases

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Abstract

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a crucial enzyme involved in the regulation of circulating low-density lipoprotein cholesterol (LDL-C). Variations in the PCSK9 gene can have significant effects on LDL receptor degradation—gain-of-function mutations accelerate receptor breakdown and contribute to familial hypercholesterolemia (FH), while loss-of-function mutations increase receptor availability, lower LDL-C levels, and reduce the risk of coronary heart disease. The genetic variants of PCSK9 in the Vietnamese population remain unknown. Therefore, this study aimed to investigate the molecular characteristics of the PCSK9 gene in Vietnamese patients diagnosed with hypercholesterolemia. Peripheral blood samples were collected from 26 patients at a local hospital, and the PCSK9 gene was analyzed using PCR-sequencing. The identified variants were compared to the reference sequence (NG_009061) for screening. A total of 60 variants were identified in 14 out of 26 patients (53.85%). Among them, 50 variants (83.33%) were novel, including 24 predicted to be damaging, 11 classified as benign, and 15 classified as variants of uncertain significance. The functional impact of the novel variants was assessed using PolyPhen-2 and FSPLICE. This study provides insight into the spectrum of PCSK9 variants in Vietnamese hypercholesterolemia patients and emphasizes the relevance of genetic diagnosis for the management of FH.

Keywords: Familial Hypercholesterolemia, PCSK9 gene, Mutation, Vietnamese

Introduction

Familial hypercholesterolemia (FH, OMIM: #143890), also called familial hypercholesterolemia type 2 or Fredrickson class 2A hyperlipidemia, is an inherited metabolic disorder transmitted in an autosomal dominant manner [1]. The heterozygous form (HeFH) is estimated to affect approximately 1 in 244 individuals, whereas the rarer homozygous form (HoFH) is found in about 1 in 160,000 to 300,000 people [2]. This condition is marked by significantly increased levels of total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C), often accompanied by tendon xanthomas and early-onset atherosclerosis, increasing the likelihood of premature cardiovascular complications [1, 3-6].

Genetic mutations contributing to FH have been identified in several key genes, including proprotein convertase subtilisin/kexin type 9 (PCSK9), apolipoprotein B (APOB), and LDL receptor (LDLR), either individually or in combination [4, 5, 7, 8]. More than 10,000 variations in these genes have been documented in genetic databases such as ClinVar and LOVD. Among diagnosed cases, mutations in LDLR account for 85–90%, APOB for 1–12%, and PCSK9 gain-of-function (GOF) mutations for 2–4% [9, 10].

The PCSK9 gene, positioned on chromosome 1 (p32.3), is also referred to as Neural Apoptosis-Regulated Convertase-1 (NARC1) and encodes a 692-amino-acid protein that belongs to the subtilase family. This protein is predominantly produced in the liver, kidney, cerebellum, and small intestine [11]. Functionally, PCSK9 is integral to LDL metabolism, as it regulates the

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degradation of LDL receptors. By binding to LDL receptors, PCSK9 facilitates their lysosomal breakdown, which ultimately leads to higher LDL-C levels in circulation [12].

Mutations in PCSK9 are generally categorized into two types: gain-of-function (GOF) and loss-of-function (LOF) [11]. GOF mutations—such as R496W, R469W (C-terminal), R128S, F216L, R215H (catalytic domain), and D129N, S127R (prodomain)—reduce the number of LDL receptors on the cell surface, thereby exacerbating hypercholesterolemia and raising the risk of coronary heart disease (CHD). Conversely, LOF mutations—including C679X, A443T (C-terminal), L253F (catalytic domain), and Y142X, G106R, and R46L (prodomain)—lead to lower cholesterol levels by preserving LDL receptors, reducing LDL-C concentration, and lowering CHD risk [12].

Advancements in genetic screening have facilitated early detection of FH, but such studies have primarily been conducted in developed countries, including the United Kingdom, France, Germany, Taiwan, China, and Japan [10]. In contrast, Vietnam has seen limited research in this field, leaving FH largely undiagnosed and untreated, which poses a significant public health concern. To date, there has been no reported genetic screening of PCSK9 in the Vietnamese population. Therefore, this study aimed to investigate the genetic variants of PCSK9 in Vietnamese patients with hypercholesterolemia.

Materials and Methods

Ethical Approval and Sample Collection

This study received ethical clearance from the Institutional Ethics Board at Medic Medical Center, Ho Chi Minh City, Vietnam (Ethics code: 02/06/2020/HĐĐĐ/YTHH). Before participation, all individuals provided written informed consent, permitting the use of their biological samples for genetic experiments.

Twenty-six blood samples were obtained from patients diagnosed with hypercholesterolemia at Medic Medical Center in Ho Chi Minh City, Vietnam. The inclusion criteria for patient selection were based on elevated total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels, specifically LDL-C ≥ 4.13 mmol/L and TC ≥ 6.20 mmol/L.

DNA Extraction, PCR Amplification, and Genetic Analysis of PCSK9

Genomic DNA was extracted using the TopPURE® Blood DNA Extraction Kit (HI-132). Target regions of the PCSK9 gene were amplified through PCR sequencing, with the primer sequences listed in **Table 1**. Each PCR reaction was conducted in a 50 µL reaction volume containing 100 ng of template DNA. The thermal cycling conditions consisted of an initial denaturation step at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for half a minute, annealing at 56 °C for 30 seconds, and extension at 72 °C for 30 seconds, with a final extension step at 72 °C for ten minutes.

PCR products were resolved on a 2% agarose gel, stained with GelRed, and visualized under UV light. The amplified products were subsequently sequenced to detect genetic variations in exon 1 and exon 2 of the PCSK9 gene. These sequences were analyzed by comparison with reference sequences NG_009060 and NM_000527 to determine the presence of mutations.

Table 1. Primers used in the current study

Primer	Sequence	Reference
FO-E1F	AAAACGACGGCCAGTTGAACTTCAGCTCCTGCACAG	Ohta *et al*., 2016
FO-E1R	ACTCCACTTCCTCTCTTACAT	
FO_E2F	AAAACGACGGCCAGTTGGTCCGCATTTGGTAACTTC	
FO-E2R	CTCAATACATACTTGCTGTCC	

Note: E1: exon 1; E2: exon 2.

The placement of the novel variants within the encoded PCSK9 protein was determined by aligning them with the reference sequence NP_777596. To assess their pathogenicity, each part was classified as either pathogenic or non-pathogenic using data from ClinVar and LOVD. For variants that had not been previously documented, their potential functional impact was predicted using PolyPhen-2 and FSPLICE.

Results and Discussion

Clinical Characteristics

This study analyzed 26 blood samples from individuals who met the clinical criteria for definite hypercholesterolemia, as defined by the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III). The diagnostic thresholds included total cholesterol (TC) levels of ≥ 6.2 mmol/L, low-density lipoprotein cholesterol (LDL-C) levels of ≥ 4.1 mmol/L, and high-density lipoprotein cholesterol (HDL-C) levels below 1.03 mmol/L. A summary of the patient's clinical profiles is provided in **Table 2**.

Table 2. Clinical profile of index patients

Characteristic	Index level
Age (years)	49 ± 5.64
Gender (Male/Female)	57.69%/42.31%
TC (mmol/L)	6.97 ± 0.43
LDL-C (mmol/L)	4.32 ± 0.46
HDL-C (mmol/L)	1.38 ± 0.30
TG (mmol/L)	3.63 ± 1.28

Note: TC (total cholesterol), TG (triglyceride), LDL-C (low-density lipoprotein cholesterol), and HDL-C (high-density lipoprotein cholesterol). Data are presented as mean ± standard deviation (SD).

Variant Annotation in Hypercholesterolemia Patients

Genetic analysis revealed a total of sixty variants across 14 out of 26 patients, representing 53.85% of the study group. Of these, 10 variants (16.67%) had been previously reported in existing literature **(Table 3)**, while the remaining 50 variants (83.33%) were classified as novel **(Table 4, Figure 1)**.

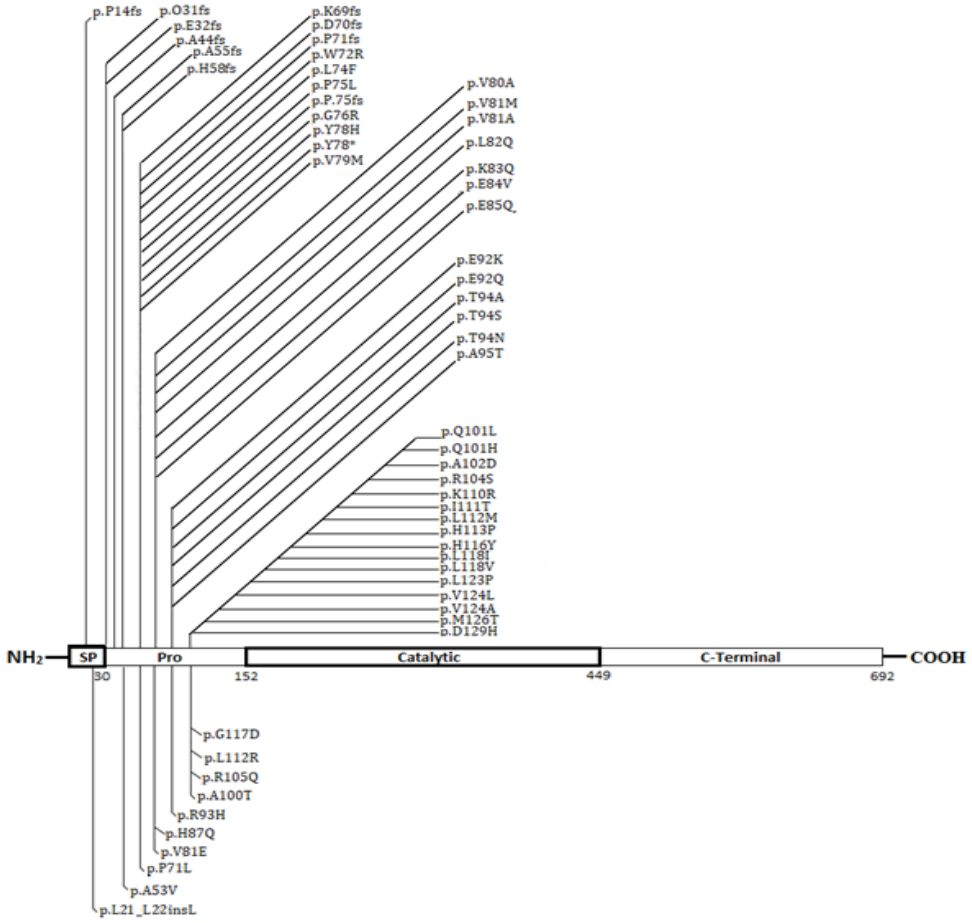

Figure 1. Variants located in the signal peptide domain and N-terminal prodomain of PCSK9

Characterization of Identified Variants

Among the 10 identified variants, one frameshift insertion was classified as benign about FH pathogenesis. The remaining 9 were missense variants, with one categorized as potentially pathogenic, four considered benign or likely benign, and five classified as variants of

uncertain significance. Notably, the c.63_64insCTG (p.L21_L22insL) variant was positioned within the signal peptide domain (residues 1–30), while all missense variants were found within the N-terminal prodomain (residues 31–69).

Table 3. 10 known variants of *PCSK9* identified in the study subjects

Type of variants	Nucleotide change	Amino acid change	rs no.	Pathogenic*
Frame insertion	c.63_64insCTG	p.L21_L22insL	rs371488778	B
Missense	c.158C > T	p.A53V	rs11583680	B/LB
	c.212C > T	p.P71L	rs569379713	LP
	c.242T > A	p.V81E	N/A	VUS
	c.260A > T	p.H87Q	N/A	VUS
	c.278G > A	p.R93H	rs763855534	LB
	c.298G > A	p.A100T	rs564681731	VUS
	c.314G > A	p.R105Q	rs754143671	VUS
	c.335T > G	p.L112R	rs1021372164	VUS
	c.350G > A	p.G117D	rs761417131	LB

Note: *Data was classified based on the database of Clinvar and/or LOVD; N/A: Not assignment; B: benign; LB: Likely benign; LP: Likely pathogenic; VUS: Variant of uncertain significance.

Novel Variant Identification and Functional Assessment

In total, fifty novel variants were found in this study. These included 11 frame insertions: seven in exon 1, three in exon 2, and one in the splicing region. Additionally, there were 37 missense variants, with 35 in exon 2 and 2 in the splicing region, as well as two nonsense variants, both located in exon 2.

To predict the potential effects of these novel variants, PolyPhen-2 and FSPLICE were utilized. Among the missense variants, 11 were assessed as likely damaging, 11 as benign, and two as variants of uncertain significance (VUS). All frame insertions and nonsense variants were classified as VUS, indicating unclear functional consequences.

Table 4. The novel variants of *PCSK9* were identified in hypercholesterolemia patients

Type of variants	Nucleotide change	Amino acid change	Location	Pathogenic^{+}	Frequency
Frameshift	c.41_42insT	p.P14fs	Exon 1	VUS	1 of 14 (7.14%)
	c.91_92insT/G	p.Q31fs	Exon 1	VUS	1 of 14 (7.14%)
	c.93_94insA	p.E32fs	Exon 1	VUS	1 of 14 (7.14%)
	c.131_132insT	p.A44fs	Exon 1	VUS	1 of 14 (7.14%)
	c.164_165insT	p.A55fs	Exon 1	VUS	1 of 14 (7.14%)
	c.172_173insT	p.H58fs	Exon 1	VUS	1 of 14 (7.14%)
	c.204_205insT	p.K69fs	Exon 1	VUS	1 of 14 (7.14%)
	c.208_209insC	p.D70fs	Exon 2	VUS	1 of 14 (7.14%)
	c.212_213insA	p.P71fs	Exon 2	VUS	1 of 14 (7.14%)
	c.225_226insA	p.P75fs	Exon 2	VUS	1 of 14 (7.14%)
	c.208-1_208-2insC	N/A	Splicing Region	VUS	1 of 14 (7.14%)
Nonsense	c.234C > A	p.Y78*	Exon 2	VUS	1 of 14 (7.14%)
	c.266C > A	p.S89*	Exon 2	VUS	2 of 14 (14.28%)
Missense	c.214T > C	p.W72R	Exon 2	P. damaging	2 of 14 (14.28%)

	c.222G > C	p.L74F	Exon 2	P. damaging	1 of 14 (7.14%)
	c.224C > T	p.P75L	Exon 2	P. damaging	2 of 14 (14.28%)
	c.226G > C	p.G76R	Exon 2	P. damaging	1 of 14 (7.14%)
	c.232T > C	p.Y78H	Exon 2	P. damaging	3 of 14 (21.43%)
	c.235G > A	p.V79M	Exon 2	Benign	3 of 14 (21.43%)
	c.239T > C	p.V80A	Exon 2	P. damaging	2 of 14 (14.28%)
	c.241G > A	p.V81M	Exon 2	P. damaging	1 of 14 (7.14%)
	c.242T > C	p.V81A	Exon 2	P. damaging	3 of 14 (21.43%)
	c.245T > A	p.L82Q	Exon 2	P. damaging	7 of 14 (50.00%)
	c.247A > C	p.K83Q	Exon 2	P. damaging	4 of 14 (28.57%)
	c.251A > T	p.E84V	Exon 2	P. damaging	3 of 14 (21.43%)
	c.253G > C	p.E85Q	Exon 2	Benign	2 of 14 (14.28%)
	c.274G > A	p.E92K	Exon 2	Benign	1 of 14 (7.14%)
	c.274G > C	p.E92Q	Exon 2	Benign	1 of 14 (7.14%)
	c.280A > G	p.T94A	Exon 2	P. damaging	3 of 14 (21.43%)
	c.280A > T	p.T94S	Exon 2	P. damaging	2 of 14 (14.28%)
	c.281C > A	p.T94N	Exon 2	P. damaging	1 of 14 (7.14%)
	c.283G > A	p.A95T	Exon 2	Benign	4 of 14 (28.57%)
	c.302A > T	p.Q101L	Exon 2	Benign	1 of 14 (7.14%)
	c.303G > T	p.Q101H	Exon 2	Benign	5 of 14 (35.71%)
	c.305C > A	p.A102D	Exon 2	P. damaging	2 of 14 (14.28%)
	c.310C > A	p.R104S	Exon 2	P. damaging	2 of 14 (14.28%)
	c.329A > G	p.K110R	Exon 2	P. damaging	1 of 14 (7.14%)
	c.332T > C	p.I111T	Exon 2	Benign	1 of 14 (7.14%)
	c.334C > A	p.L112M	Exon 2	P. damaging	2 of 14 (14.28%)
	c.338A > C	p.H113P	Exon 2	P. damaging	1 of 14 (7.14%)
	c.346C > T	p.H116Y	Exon 2	Benign	3 of 14 (21.43%)
	c.352C > A	p.L118I	Exon 2	Benign	2 of 14 (14.28%)
	c.355C > G	p.L118V	Exon 2	Benign	3 of 14 (21.43%)
	c.368T > C	p.L123P	Exon 2	P. damaging	1 of 14 (7.14%)
	c.370G > T	p.V124L	Exon 2	P. damaging	2 of 14 (14.28%)
	c.371T > C	p.V124A	Exon 2	P. damaging	1 of 14 (7.14%)
	c.377T > C	p.M126T	Exon 2	P. damaging	1 of 14 (7.14%)
	c.385G > C	p.D129H	Exon 2	P. damaging	3 of 14 (21.43%)
	c.399 + 1G > T	N/A	Splicing Region	VUS	1 of 14 (7.14%)
	c.399 + 2T > A	N/A	Splicing Region	VUS	1 of 14 (7.14%)

Note: *Codon stop; [+]data was predicted by PolyPhen-2 and FSPLICE. P. damaging: probably damaging; VUS: Variant of uncertain significance.

Traditionally, the diagnosis of FH relied on clinical criteria, such as elevated LDL-C levels, physical examination findings, and a history of hypercholesterolemia or cardiovascular diseases within the family [13-16]. In past years, advances in molecular diagnostic methods, such as polymerase chain reaction (PCR) and genome sequencing, have significantly improved genetic testing capabilities [17]. In the study, PCR sequencing was used to investigate the genetic

variants of PCSK9 in Vietnamese hypercholesterolemia patients.

Among the 26 patients with hypercholesterolemia, 53.85% were found to carry 60 PCSK9 variants. Of these, 10 variants were previously documented in databases like ClinVar and LOVD, while 50 variants were novel and identified for the first time in the study. Of the 10 identified variants, four were classified as benign/likely benign, one as likely damaging, and five as variants of uncertain significance (VUS). For the 50 novel variants, functional predictions were made using PolyPhen-2 and FSPLICE, with 24 variants predicted to be probably damaging, 11 classified as benign, and 15 marked as VUS.

Interestingly, among the 14 patients carrying these variants, the individual with the highest levels of hypercholesterolemia was found to have the largest number of novel variants (18 variants), all of which were located in exon 2 of the PCSK9 gene. Further in vivo functional studies are necessary to confirm whether these variants contribute to the pathogenesis of hypercholesterolemia.

A limitation of this study was the absence of data on the familial history of the patients. Future research, incorporating family studies, is essential to explore whether these novel variants could indeed be causative for hypercholesterolemia.

Conclusion

This study identified and characterized 50 novel PCSK9 variants in patients with hypercholesterolemia. Functional prediction models suggested that 48% (24 out of 50) of these variants were likely to be damaging. To validate the pathogenicity of these variants in familial hypercholesterolemia, further in vivo functional analyses and family studies are needed.

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