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MOLECULAR CHARACTERIZATION OF PROPROTEIN CONVERTASE SUBTILISIN/KEXIN TYPE 9 GENE MUTATIONS IN VIETNAMESE PATIENTS WITH HYPERCHOLESTEROLEMIA

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ABSTRACT

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is an enzyme that plays a key role in regulating the circulating low density lipoprotein cholesterol (LDL-C). Among the *PCSK9* variants, while gain-of-function mutations aggravate the degradation of LDL receptors, leading to familial hypercholesterolemia (FH), whereas loss-of-function mutations increase LDL receptor levels, lower LDL levels, and prevent coronary heart disease. The variants of *PCSK9* have not been clarified in the Vietnamese population yet. Hence, the present study aimed to present the molecular characteristics of *PCSK9* gene in Vietnamese hypercholesterolemia patients. A total of 26 peripheral blood samples were collected from the patients who were diagnosed with Hypercholesterolemia in a local hospital. The PCR-sequencing method was applied to amplify and sequencing *PCSK9*. Then, variant screening was performed by comparing with the reference sequence (NG_009061).
60 variants were identified in 14 of 26 patients (accounting for 53.85%): 50 (83.33%) variants were identified as novel variants among which, 24 were probably damaging, 11 were benign, and 15 were variants of uncertain significance. Functional effects of novel variants were predicted by using PolyPhen-2 and FSPLICE. This study analyzed the variants spectrum in Vietnamese Hypercholesterolemia patients and expresses the importance of genetic diagnosis in FH patients.
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Introduction

Familial Hypercholesterolemia (FH, OMIM: #143890), also known as Familial Hypercholesterolemia type 2 or Fredrickson Class 2A Hyperlipidemia, is a prevalent autosomal dominant hereditary genetic metabolic disease [1]. The prevalence of the heterozygous form of FH (HeFH) is about 1/244 individual, while that of the homozygous form (HoFH) is about 1/160,000-300,000 [2]. FH is characterized by extremely raised levels of total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) in the circulation, the presence of tendon xanthomas and accelerated atherosclerosis, resulting in an increased risk of premature cardiovascular events [1, 3-6].

Accumulating evidence of molecular pathogenesis of FH, FH can be caused by mutations in either *Proprotein convertase subtilisin kexin 9* (*PCSK9*), *Apolipoprotein B* (*APOB*), or *LDL receptor* (*LDLR*), and others singly or combinations [4, 5, 7, 8]. Up to date, more than 10000 genetic variants of those genes have been identified and reported in several public databases, such as Clinvar, LOVD, etc. From all mutations described, approximately 85-90%, 1-12% of patients with FH have been associated with *LDLR* mutations, and *APOB* mutations, respectively. Additionally, 2-4% gain-of-function *PCSK9* mutations were identified in the patients of FH [9, 10].

Located on the p-arm of chromosome 1 (p32.3), *PCSK9* gene, also known as *Neural Apoptosis Regulated Convertase1* (*NARC1*), encodes for the 692 amino acids proprotein convertase subtilisin/kexin type 9, a proprotein convertase of subtilase family, and predominantly expressed in the kidney, liver, cerebellum, and small intestine [11]. The PCSK9 protein plays an important role in the LDL metabolism, in detail, PCSK9 functions as a chaperone to mediate the degradation of LDL receptors by interacting with the extracellular domain leading to the degradation of lysosome, which is directly related to high LDL-C level in plasma [12].

Two types of mutations: Gain-of-function (GOF) and Loss-of-function (LOF) have been identified in the variants of *the PCSK9* gene [11]. Many GOF mutations in *PCSK9*, such as R496W, R469W (in C-terminal); R128S, F216L, R215H (in the catalytic domain); D129N, S127R (in prodomain); etc., have been reported. Its function is to decrease the number of LDLRs at the cell surface, leading to hypercholesterolemia and increase the risk of coronary heart disease (CHD). While the LOF mutations, including C679X, A443T (in C-terminal); L253F (in the catalytic domain); Y142X, G106R, R46L (in

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prodomain); etc., are associated with lower cholesterol levels. As a result, it leads to the reduction of LDL-C in the bloodstream and also CHD risk [12]. The studies related to the genetic screening of genes involves in cholesterol metabolism will light out the era of early diagnosis of FH. Despite the prevalence and significant benefits associated with FH, most of those molecular screenings have been conducted in developed countries, including United Kingdom, France, Germany, Taiwan, China, Japan, etc. Especially, in Vietnam, it is markedly under-recognized, under-diagnosed, and under-treated, and is a public health problem that requires attention [10]. Molecular screening of *PCSK9* has not been previously described. Therefore, this study aimed to evaluate the genotype variants of *PCSK9* in Vietnamese Hypercholesterolemia patients.

Materials and Methods

Ethics statement, sample collection

The Institutional Ethics Broad approval was obtained from Medic Medical Center, Ho Chi Minh City, Vietnam (Ethics code: 02/06/2020/HĐĐĐ/YTHH). All patients enrolled in the study signed the consent forms to approve the usage of the samples for laboratory work and analysis.

A total of 26 blood samples from Hypercholesterolemia patients were collected at Medic Medical Center, Ho Chi Minh, Vietnam. The patients were diagnosed with increased the concentration of TC and LDL-C (LDL-C ≥4.13 mmol/L, TC ≥6.20 mmol/L).

DNA isolation, amplification, and analysis of PCSK9 gene

Genomic DNA was isolated from whole blood samples using TopPURE ® Blood DNA Extraction Kit (HI-132). The amplification of targets was performed by the PCR-sequencing method. The used primers are shown in **Table 1**. The PCR assay was done in a total volume of 50µl, containing 100 ng template. PCR reaction was subjected to initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 56°C for 30 s, 72°C for 30 s, and finally 72°C for 10 minutes. PCR products were directly loaded onto a two percent agarose gel, stained with GelRed, and visualized directly under UV illumination. The PCR products were sequenced to identify whether there were mutations in exon 1 and 2 of *PCSK9* gene in comparison to the references of *PCSK9* gene (NG_009060; NM_000527).

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 position of the novel variants in its encoded protein was identified based on the comparison with the reference sequence NP_777596. The functional effect of each variant was classified as pathogenic or non-pathogenic based on the database of ClinVar and LOVD. For those variants, which have not been previously recorded, the PolyPhen-2 and FSPLICE were used for predicting the functional effects.

Results and Discussion

Clinical profile

Of all 26 blood samples included in this study, which were clinical diagnosis as definite hypercholesterolemia based on the criteria of National Cholesterol Education Program Adult Treatment Panel III - NCEP ATP III (Total cholesterol ≥ 6.2 mmol/L; LDL cholesterol ≥ 4.1 mmol/L; HDL cholesterol < 1.03 mmol/L). **Table 2** summarized the clinical characteristics of patients.

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; HDL-C: High Density Lipoprotein Cholesterol. The data were expressed as mean±SD.

Annotation of variants in hypercholesterolemia subject

A total of 60 variants were identified in 14 of 26 patients (accounting for 53.85%). Among variants, 10 were found to correspond to previously published variants (accounting for 16.67%) **(Table 3)** and 50 were identified as novel variants (accounting for 83.33%) **(Table 4; Figure 1)**.

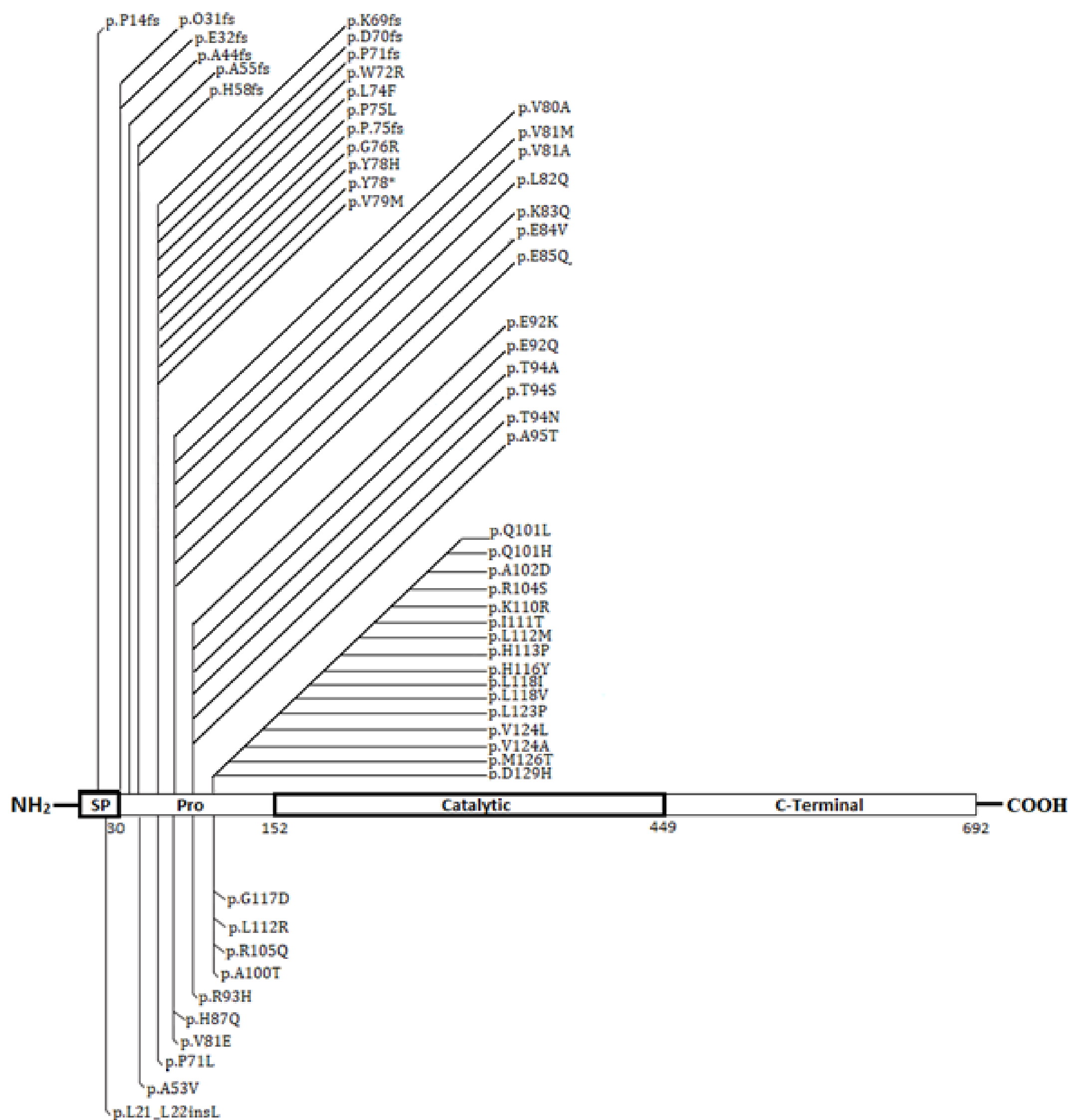

Figure 1. Variants located in the signal peptide domain and N-terminal prodomain of PCSK9

In the total of 10 variants, one frame insertion was identified as benign in FH pathogenesis, and nine were missense variants, among which one was probably pathogenic, four were benign/likely benign, and 5 variants had uncertain significance. Among them, the variant of c.63_64insCTG (p.L21_L22insL) was located at the domain of the signal peptide (residue 1 to 30). All missense variants were located at the N-terminal prodomain (residue 31 to 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

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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.

A total of 50 novel variants were identified of which 11 were frame insertion (seven located in exon 1, three located in exon 2, and one located in the splicing region); 37 were missense variants, (35 located in exon 2, and 2 located in the splicing region); and 2 were nonsense variants (located in exon 2). The functional prediction of those novel variants was performed by PolyPhen-2 and FSPLICE. The results showed that among 37 missense variants, 11 were probably damaging, 11 were benign, and 2 were VUS. All frameshift variants and nonsense variants were identified as VUS.

Table 4. The novel variants of *PCSK9* 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.

Historically, the diagnosis of FH was based on clinical characteristics, including the high LDL-C levels, physical examination findings, and clinical/familial history of hypercholesterolemia and/or cardiovascular diseases [13-16]. In recent years, numerous molecular diagnostic techniques have been developed, including polymerase chain reaction and genome sequencing for genetic testing [17]. In this study, the PCR-sequencing was applied to investigate the genotype variants of *PCSK9* in Vietnamese hypercholesterolemia patients. In 26 hypercholesterolemia patients, 53.85% carried a total of 60 *PCSK9* variants Among which 10 were in Clinvar and LOVD and 50 were newly identified in this study. Of 10 known variants, 4, 1, and 5 were identified as benign/Likely benign, Likely damaging, and VUS, respectively. The functional prediction of these 50 novel variants was performed by PolyPhen-2 and FSPLICE. As the results, 24, 11, and 15 of the novel variants were identified as probably damaging, benign, and VUS, respectively. Notably, among 14 patients within the identified variants, the patient, who was diagnosed with the highest levels of hypercholesterolemia, contained the highest number of novel variants (18 variants), and all of them were located in the exon 2 of *PCSK9*. It was necessary to perform an *in vivo* functional analysis to determine those variants as whether or not pathogenic in hypercholesterolemia.

This study had a limitation. The familial history of patients was not recorded. Therefore, to further study whether these novel variants could be considered the cause of hypocholesterolemia, family studies should be conducted.

Conclusion

In conclusion, this study annotated 50 novel variants of *PCSK9* on the clinical phenotype of patients with hypocholesterolemia. By *in vivo* functional analysis, 24 of 50 novel variants (48.00%) were predicted as probably damaging. To further study, *in vivo* functional analysis, as well as family studies, will be performed to determine those variants as whether or not pathogenic in familial hypercholesterolemia.

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Conflict of interest: None

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Ethics statement: This study was approved by the Institutional Review Board (IRB) of Medic Medical Center and the protocols used in the study were approved by the Committee of Human Subjects Protection of the Medic Medical Center, Ho Chi Minh City, Vietnam (Ethics code: 02/06/2020/HĐĐĐ/YTHH).

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