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Genetic Study of *Low-Density Lipoprotein Receptor* Gene and Apolipoprotein B-100 Gene among Malaysian Patients with Familial Hypercholesterolaemia

ORIGINAL

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Abstract

Background: Autosomal dominant hypercholesterolaemia (ADH) is a genetic disorder that is mainly resulted from defects in the *low-density lipoprotein receptor* (*LDLR*) and *apolipoprotein B-100* (*APOB-100*) genes. Few studies of familial hypercholesterolaemia (FH) have been conducted in Malaysia, that makes the underlying main defect remains not well understood.

Objectives: This study was aimed to describe the molecular aspects of FH among Malaysian subjects.

Methods and Findings: We studied a group of 74 familial hypercholesterolaemic patients and 77 healthy control subjects. The promoter region and the 18 exons of the low-density lipoprotein receptor gene were screened by denaturing high-performance liquid chromatography (DHPLC) to detect small deletions, insertions and nucleotide substitutions, while DNA sequencing was applied to look for gene variants in amplicons of exon 26 and 29 in the *APOB-100* gene. A total of five gene sequence variants in the *LDLR* gene were reported in 54.1% of the studied patients. *P.Arg471Arg* variant has the highest frequency of 20.3% among the study subjects. One intronic mutation (*c.313+1G>A*) and one missense mutation (*p.Arg 385Try*) were found to be pathogenic, while the other three variants were reported to be non-pathogenic by the *in silico* analyses. Nine variants were reported in the *APOB-100* gene among familial hypercholesterolaemic patients with a non-significant difference in their frequency from the control subjects.

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Conclusions: Five *LDLR* gene sequence variants were reported. However, nine polymorphisms were stated in the *APOB-100* gene. Those polymorphisms were not associated with FH phenotype among this cohort. These findings suggested that *LDLR* gene mutation is the major genetic cause for FH among Malaysians. The results offer information on the genetic spectrum of FH among Malaysian cohort which can serve as a platform for further genetic studies.

Keywords
Familial Hypercholesterolaemia, *LDLR* Gene, *APOB-100* Gene, DHPLC, Malaysians.

Introduction

Familial hypercholesterolaemia (FH), (OMIM143890) is the first genetic disorder of lipid metabolism that is characterized both clinically and molecularly [1]. It is transmitted in an autosomal dominant manner [1]. Familial hypercholesterolaemia is characterized by elevated serum level of low density lipoprotein cholesterol (LDL-C) and total cholesterol (TC), tendon xanthomas (TX) and early atherosclerosis, leading to premature atherosclerotic coronary artery disease (CAD) [2, 3]. Heterozygous FH is the commonest monogenic disorder, that is affecting 1 in 200–250, which is double as high as thought previously [4], with a penetrance rate of more than 90 % [5]. The frequency can be higher in certain populations, such as the Afrikaners, French-Canadians and Christian Lebanese [6] due to the founder effect [1]. Data from a large community study in Denmark suggested that the prevalence of FH may reach 1:137 [7]. Homozygous FH has a severe phenotype with an early symptoms during childhood [8].

Monogenic FH is mostly attributed to defects in *low-density lipoprotein receptor* (*LDLR*) gene. The *LDLR* gene was first discovered by Goldstein et al., it encodes for LDL receptor [9]. The *LDLR* gene composed of 18 exons and is located at chromosome 19 [10]. The *LDLR* gene defect was reported as the most common genetic cause of FH. Recently, the LOVD FH variant database describes 1741 mutations [11].

A similar phenotypic presentation to FH can be observed in patients with defective apolipoprotein B function (the main protein components of the LDL) [12]. Mutations in the *apolipoprotein B-100 gene* (*APOB-100*), can cause a clinical phenotype that is named as Familial Defective Apolipoprotein B-100 (FDB). Mutations that can cause FDB are mostly reported at the LDL binding domain of *APOB-100* gene, i.e at exons 26 and 29 of this gene [13].

Autosomal dominant hypercholesterolaemia (ADH) can also be a result of mutation in *Proprotein Convertase Subtilisin/Kexin type 9* (*PCSK9*) gene that was identified on chromosome 1p32 [14].

Clinical management of FH should focus on its early diagnosis, coronary risk assessment, plus managing CAD risk factors as hypertension and glucose intolerance, additionally reducing cholesterol level by the use of lipid lowering therapy in order to decrease atherosclerosis risk and subsequently prevents CAD [15]. It is often difficult to establish an accurate diagnosis for FH despite having various available international diagnostic criteria such as Simon Broome's Registry, Dutch Lipid Clinic Criteria and US MedPed [1]. Phenotypic criteria that requires details of the family history and detection of subtle physical signs such as arcus cornealis or xantomata that are frequently detected and are age dependent, making them insensitive indicators for the clinical detection of FH.

It is believed that there are about 34 million FH cases globally [16]. In spite of the high prevalence of

FH and the considerable advantage of its early detection and treatment, only around 1% of FH cases are diagnosed worldwide [16] with few exceptions where 71% in the Netherlands and 43% in Norway with FH were diagnosed [16].

Systematic genetic screening programs for mutation detection in those who are clinically determined as FH have become less costly and can facilitate a better prognosis of the disease [17]. It was estimated that in the Asia-Pacific region alone, approximately 3.6 million people are suffering from FH [18]. The International FH Foundation freshly announced guidelines for FH diagnosis [19], and in Asia, the Japanese guidelines for FH have been recently published [20]. However, most of the genetic researches about FH were conducted in non-Asian populations with very few researches that were performed in South East Asia, specifically in Malaysia to determine the genetic mutations of FH [21-27].

Therefore, the current study was aimed to elucidate the molecular spectrum of FH among Malaysian subjects through the screening for mutations in the 18 exons and promoter region of *LDLR* gene, in addition to the exons 26 and 29 of *APOB-100* gene.

Materials and Methods

Study population

Seventy four FH subjects from the Specialist Lipid and Coronary Risk Prevention Clinic of a teaching institution were recruited for this study. Familial hypercholesterolaemia was diagnosed based on the Simon Broome Familial Hypercholesterolaemia Register diagnostic criteria [28]. Patients with secondary hyperlipidaemia such as those with diabetes mellitus, hypothyroidism and nephrotic syndrome, were excluded from the study [29].

Seventy seven normolipaemic controls were also taken on to detect any nucleotide substitutions that could be regarded as single nucleotide polymorphism (SNP). The control subjects were randomly chosen as healthy volunteers with TC level of < 6.5 mmol/L and/or LDL-C< 3.8 mmol/L with no previous history or family history of hyperlipidaemia or premature CAD, no history of secondary causes of hyperlipidaemia nor clinical signs of hyperlipidaemia.

Demographic data, medical history, smoking habits, history of personal CAD and family history of premature CAD were documented. Physical examination for stigmata of hyperlipidaemia: presence of TX, xanthelasma, and arcus cornealis were recorded. Blood pressure (BP) was measured with the subject in a seated position and after resting for 5-10 minutes, BP was measured by an automated BP reader (cuff size 12 x 33cm, Colin press-mate, Japan). The systolic (SBP) and diastolic blood pressures (DBP) were measured to the nearest 1 mmHg. Height and weight were measured to obtain BMI by using the formula: BMI=weight (kg)/height^2 (m^2). Presence of CAD was confirmed depending on the clinical history, previous medical records and exercise tolerance test reports.

The study protocol was approved by the institutional research and ethics committees. Written informed consent was obtained prior to the commencement of this study.

Sample collection

Overnight fasting venous blood samples (4 ml) were collected from patients and controls into tubes containing potassium ethylene diamine tetra acetic acid (EDTA). Genomic DNA was extracted by Maxwell® 16 Blood DNA Purification kit on Maxwell® 16 Automated DNA Extraction System (Promega, USA). The samples were then stored at -20°C until further analysis. A further 6 ml of blood was collected into plain tubes and serum was separated within two hours of collection by centrifugation at 4,000 rpm for 7 minutes for biochemistry testing.

Routine biochemical analyses were performed for all subjects (both cases and controls) which consisted of fasting serum lipid (FSL) that included TC,

triglyceride (TG), high density lipoprotein cholesterol (HDL-C) and LDL-C. Fasting plasma glucose, liver function tests, renal profile and thyroid function test (composed of thyroid stimulating hormone, free thyroxine and tri-iodothyronine) were also measured to exclude secondary causes of hyperlipidaemia. TC, TG and HDL-C were performed on an automated analyser (Cobas Integra 400 plus, Roche Diagnostics, Germany). LDL-C was derived using the Friedewald calculation [30]. All these tests have been accredited by an international accredited body MS ISO 15189:2007 (SAMM no. 688).

Molecular analysis

Both patients and control subjects were screened for mutations in the *LDLR* and *APOB-100* genes. All coding regions including intron-exon junctions of *LDLR* gene were screened based on the *LDLR* gene reference sequence that was obtained from the database of the GenBank (accession no. NT_011295), the primers for *LDLR* gene were adapted from Bodameret al., [31] (supplementary).

For variants within the *APOB-100* gene, the reference gene sequence was obtained from Genbank (accession no. NM_000384). **Table 1** shows three sets of primers that were designed to amplify the previously reported variants associated with FDB in the *APOB-100* gene as follows: one amplicon in exon 29 at nucleotides 12452-13113, that is related to codons 4151-4372; two amplicons in exon 26: one at nucleotides 10352-11632 that is related to codons 3451-3878, and the other at nucleotides 7328-7818 that is related to codons 2443-2606.

PCR program for *APOB-100* gene is illustrated in **Table 1**.

For the *LDLR* and *APOB-100* genes, samples were analysed by PCR standardized using genomic DNA and primer pairs to amplify the target regions. One hundred nanograms of genomic DNA was mixed with 10X PCR buffer, 2 mM $MgCl_2$, 200 µM deoxynucleoside triphosphates, 2.5U of Taq polymerase and 0.2 µM of forward and reverse primers, respectively. The amplification was performed in a final volume of 50 µl and carried out with the use of the Mastercycler Gradient (Eppendorf, Germany). Cycling conditions for the *LDLR* gene were 95 ^{O}C for 5 minutes, followed by 35 cycles at 95 ^{O}C for 1 minute, 57 ^{O}C for 1 minute (except for exon 16, which was run at 65 ^{O}C),72 ^{O}C for 1 minute and final extension for 7 minutes at 72 ^{O}C.

The PCR reaction mixture was run in 2% agarose gel electrophoresis with a 100-bp ladder for comparison.

DHPLC and direct sequencing

Using DHPLC, all patients and controls samples were investigated for point mutations, short deletions and duplications in the *LDLR* gene. Mutation screening for *LDLR* gene was performed using partial denaturation mode of DHPLC on Wave Nucleic Acid Fragment Analysis System (Transgenomic, USA). The melting temperature for each DNA fragment was predicted using http://insertion.stanford.edu./melt.html software. The PCR products were denatured at 95°C for 5 min and then cooled to 65°C at a rate of 1°C/min. After slow re-annealing,

Table 1. Primer sequences, PCR protocols and the expected amplicon size of *APOB- 100* gene.

Exon	Primer name	Forward primer (5' – 3')	PCR protocol	Product size (bps)
26	Primer A	F: GGAAACCAAGGCCACAGTTG R: GCGATACCTGCTTCGTTTGC	94^{O}C, 3 min, 35 cycles (94^{O}C, 60 s, 59.8^{O}C, 60 s, 72^{O}C, 60 s), 72^{O}C 10 min	311
	Primer B	F: ACCGCTAAAGGAGCAGTTGA R:TCCTACCAATGCTGGTGGTGGTT	94^{O}C, 3 min, 35 cycles (94^{O}C, 60 s, 59.8^{O}C, 60 s, 72^{O}C, 60 s), 72^{O}C 10 min	897
29		F:GAACTCTTAAGTTCCACATTGC R: CTGGGACACATAGTCTCTGC	94^{O}C, 5 min, 35 cycles (94^{O}C, 60 s, 64^{O}C, 30 s, 72^{O}C, 30 s), 72^{O}C 10 min	713

it was essential to generate the heteroduplexes by mixing equal ammounts of subject samples and a wild type sample at the optimum temperature identified by mapping, the elution profile of the sample was compared to the elution profile of the control. Under partial denaturation temprature, the single peak pattern of the sample indicated the absence of mismatch i.e gene variant. In contrast, samples that showed different peak patterns from the controls were considered to be heteroduplex gene variants, and DNA sequencing of the purified PCR products of these samples was performed to confirm the results of DHPLC. For *APOB-100* gene, PCR samples were preceded directly to sequencing to look for the variants that are located in LDL binding domain of the *APOB-100* gene rather than screening the whole gene. PCR fragments were sequenced using the ABIPRISM Big Dye terminator cycle model ABI 3730xl DNA Analyser (Applied Biosystem, USA) according to the manufacturer's recommendations.

In silico analyses of variant effects

Online computer programs were used to investigate the effects of the gene variants. All the variants were subjected to *in silico* analyses using Alamut Visual Version 2.7.1 that screens for splicing abnormalities as well as protein changes. Variants that were located within exons in which Alamut Visual could not predict any pathogenicity were subjected to analysis by Polymorphism Phenotyping (Poly Phen) software [32], which is an automatic tool for predicting the possible effect of an amino acid substitution on the structure and function of a protein. Poly Phen software classifies amino acid substitutions as benign, probably damaging, or possibly damaging. Nucleotide numbers were chosen using the LDLR gene sequence from http://www.ucl.ac.uk/fh database, with cDNA numbering that begins with A of ATG = 1. Mutations were named following the Human Genome Variation Society http://www.hgvf.org. Mutation was defined as sequence change which is clearly defined as FH causing, such as frameshift mutation also variants that are predicted to be pathogenic by *in silico* programs.

Statistical analyses

The distribution of quantitative variables was tested for normality. An initial descriptive analysis was carried out using number of subjects and percentages for qualitative variables. Mean (SD) was used for quantitative variables. Student's t-test was used to compare two groups. Categorical data and proportions were analysed using Chi-square test. A p-value <0.05 was considered statistically significant. The statistical analysis was performed on the Statistical Package for Social Sciences (SPSS version 16.0) software.

Results

Study Subjects

A total of 74 FH patients (50 Malays and 24 Chinese) were collected.

Sixty-one (82.4 %) patients were clinically diagnosed as definite FH and 13 (17.6 %) as possible FH according to the Simon Broome Criteria [28].

Their clinical characteristics are presented in **Table 2**. Out of the 74 patients, 28 (37.8 %) were males while 46 (62.2 %) were females. Coronary artery disease was present in 27.4% of the patients.

Table 2. Background, clinical characteristics and lipid profiles of FH and control subjects.

Baseline Characteristics	FH group n=74	Control group n=77	P value
Age (years)*[a]	45.9 + 12	44.7 + 10.3	NS
Gender(%)[b]			
Males	37.8	33.8	NS
Females	62.2	66.2	
Current smoker (%)[b]	14.5	17.4	NS
Systolic BP (mmHg)*[a]	136.9 + 22.3	133.2 + 16.4	NS
Diastolic BP (mmHg)*[a]	77.8 ± 11.1	76.7 ± 10.8	NS

Baseline Characteristics	FH group n=74	Control group n=77	P value
Hypertension (%)[b]	20.4	9.0	<0.05
Tendon Xanthomas(%)	85.1	-	
Corneal Arcus (%)	95.9	-	
Xanthelasma (%)	20	-	
Glucose (mmol/L)*[a]	5.2 + 0.5	4.8 + 011	<0.01
BMI (kg/m2)*[a]	24.3 ± 4.6	23.0 ± 4.6	NS
WHR*[a]	0.84 ± 0.07	0.81 ± 0.06	<0.05
Waist circumference (cm)*[a]	84.2 + 12.0	78.2 + 11.2	<0.001
Central Obesity (%)[b]	45.9	27.0	<0.005
CAD (%)[b]	27.4	0	<0.001
TC (mmol/L)*[a]	8.6 + 0.2	5.4 + 0.1	<0.001
TG (mmol/L)*[a]	2.0+ 0.2	1.3 + 0.6	<0.001
LDL-C (mmol.L)*[a]	6.4 + 0.2	3.4 + 0.1	<0.001
Death and Dyeing	70.05	70.05	81.25
HDL-C (mmol/L)*[a]	1.3 ± 0.4	1.4 ± 0.3	<0.001

*: Data expressed as mean ± SD, NS: not significant. BMI: Body mass index, BP: Blood pressure, CAD: coronary artery disease. [a]: independent T test was used, [b]: Chi square test was used.

Mutations Screening by DHPLC

LDLR gene variants were identified by analysing the promoter region and the exon-intron boundaries of the 18 exons of the *LDLR* gene using DHPLC, the heteroduplex peaks were further analysed by DNA sequencing to confirm the presence of the gene variants.

Screening of all clinically diagnosed cases with FH revealed five *LDLR* gene variants among 40 out of the 74 clinically diagnosed FH patients (54.1%). None of the variants could be detected in the control group. The most frequent variant was the silent variant *p.Asn591Asn* that was reported among 19 patients (25.6%). It resulted from the substitution of T>C at nucleotide 1773 in exon 12 (*c.1773T>C*). Patients who carried this variant had a high mean value for serum TC and a very high mean value for serum LDL-C according to the NCEP classification [29] **(Table 3)**.

The second most common *LDLR* gene variant was c.1413G>A substitution in exon 10, resulting in the silent variant *p.Arg450Arg*, which was detected at a frequency of 20.3%.

Table 3. Different mutation categories of *LDLR* gene and lipid profile parameters among FH patients (n=74).

Mutation categories in *LDLR* gene (%)[A]	Lipid profile parameters		
	TC (mmol/l)	LDL-C (mmol/l)	HDL-C (mmol/l)
p.Asn591Asn (25.6%)[B]	8.1 ± 1.3	5.9 ± 1.2	1.3 ± 0.3
p. Arg450Arg (20.3%)[B]	8.0 ± 0.8	5.8 ± 0.9	1.3 ± 0.3
p.Cys255Ser (5.4%)[B]	9.9 ± 3.1	8.1 ± 3.1	1.4 ± 0.3
c.313+1G>A (1.3%)	11.9	9.8	1.2
p.Arg385Try (1.3%).	8.9	6.6	1.4

[A]: Percentage within the study group of 74 subjects. [B]: Groups are presented as mean (SD).

Also the carries of this variant had a high mean value for serum TC and a very high mean value for serum LDL-C according to the NCEP classification [29] **(Table 3)**.

One splice site mutation (*c.313+1G>A*) in intron 3, was reported in a Chinese FH subject (1.3%). The patient had sever hypercholesterolaemia with serum TC and LDL-C values of 11.9 mmol/L and 9.8 mmol/L, respectively **(Table 3)**.

In exon 5, *p.Cys255Ser* variant was reported among four FH patients from one family of Malay ethnicity (5.4%). They were found to carry this variant of which one member was homozygous while the other three were heterozygous. The four FH patients presented with a mean of high TC and very high LDL-C values (9.9 ± 3.1 mmol/L) and (8.1 ± 3.1 mmol/L), respectively [29], **Table 3**. The homozygous FH patient for this variant presented with prominent xanthelasma, corneal arcus and xanthomata with severely elevated TC, TG and LDL-C levels of 15.3 mmol/L, 1.4 mmol/L and 13.5 mmol/L, respectively and HDL level of 1.2 mmol/L, whereas, the heterozygotes FH patients presented with corneal arcus and xanthomata only.

Another variant reported in this study was found in exon 9 which is *p.Arg385Try*. This variant was identified in a Chinese male. The identified patient

Table 4. *LDLR* gene variants detected among FH subjects

Location	Variant name*	n(%)	Reference	In-silico analysis
Intron 3	c.313+1G>A	1(1.3%)	LDLR_00442**	Pathogenic Disrupt normal splicing No SIFT or polyphen analysis
Exon 5	c.763T>A; *p.Cys255Ser*	4(5.4%)	Azian et al., Alicezah et al., Alyaa et al.	SIFT: deleterious. Polyphen: probably damaging
Exon 9	c.1216C>T; *p.Arg 385Try*	1(1.3%)	LDLR_00451**	Pathogenic SIFT: Deleterious PolyPhen: probably damaging
Exon 10	c.1413 G > A; *p.Arg471Arg*	15(20.3%)	Rs5930***	Synonymous Unlikely to cause pathogenicity
Exon 12	c.1773T>C; *p.Asn591Asn*	19(25.6%)	LDLR_01195** Rs 688***	Synonymous Unlikely to cause pathogenicity

*: Naming of the variants was done according to Nomenclature of the Human Genome Variation Society (HGVS). **: www.ucl.ac.uk/ldlr/LOVDv.1.1.0/. ***: Rs reference sequence from NCBI

with heterozygous p.Arg385Try mutation presented with high TC and very high LDL-C levels of 8.9 mmol/L and 6.6 mmol/L, respectively [29] **(Table 3)**.

In silico analyses

Alamut Visual software identified the two synonymous variants, *p.Asn591Asn* and *p.Arg471Arg* as non-pathogenic because there were no evidence of splicing aberrations or changes in protein structure, although it may stall translation by requiring the use of low abundance tRNAs. The selected SNP ID was obtained from the NCBI database, **(Table 4)**.The c.313+1G>A variant was classified as pathogenic splice site mutation as it can disrupt the normal splicing process, **(Table 4)**.

For the missense mutation (*p.Arg 385Try*), it was classified as pathogenic by Alamut Visual software, while PolyPhen predicted it as probably damaging. The *p.Cys255Ser* variant was predicted to be Deleterious by SIFT and probably damaging by Polyphen software, **(Table 4)**.

Based on the *in silico* analyses results, the two synonymous variants were reported as non pathogenic, while the other three mutations were discovered to be pathogenic, these results bring together the mutation detection rate for this research of about 8.1%(*c.313+1G>A, p.Arg 385Try* and *p.Cys255 Ser* with frequencies of 1.3%, 1.3% and 5.4%, respectively).

Table 5. *APOB-100* gene defect in FH patients and control subject

APOB-100 gene variant [A]	FH group çn=74		Control group n=77		p-value*
	No	%	No	%	
Exon 26					
p.Thr2515Thr	10	13.5	11	14.3	0.8
p.Gly2540Val	1	1.4	2	2.6	0.1
p.Ile2716Ile	2	2.7	1	1.3	0.1
p.Pro2739Leu	41	55.4	35	45.5	0.2
p.Thr3567Thr	0	0	1	1.3	0.3
Exon 26					
p.Glu4181Lys	6	8.1	6	7.7	0.9
p.Arg4270Thr	3	4.1	5	6.5	0.5
p.Arg4297His	1	1.4	1	1.3	0.3
p.Ser4338Asn	35	47.3	36	46.7	0.9

A: Variant sequence name according to Nomenclature of the Human Genome Variation Society (HGVS). *: Chi- square test was used

APOB-100 Gene Sequencing Analysis

Concerning the *APOB-100* gene, Nine variants were identified in the study subjects **(Table 5)**.

The frequency of the gene variants among FH patients and the control group was shown in **Table 5**.

No significant difference could be reported for all the variants between FH and the control groups.

All the variants were exposed to *in silico* analyses. *p.Gly2540Val* gene variant was reported to be

possibly damaging while *p.Pro2739Leu* was found to be probably damaging, the rest of the variants were established to be benign by Polyphen software, **Table 6**.

Table 6. Gene variants in the *APOB-100* gene among the study subjects.

Exon	Codon[A]	*Variant sequence name*[B]	Reference ID[C]	*In silico* analysis[D]
26	2515	*p.Thr2515Thr*	Rs693	Benign
	2540	*p.Gly2540Val*	Rs571626569	Possibly damaging
	2716	*p.Ile2716Ile*	Rs 6413458	Benign
	2739	*p.Pro2739Leu*	Rs 676210	Probably damaging
	3567	*p.Thr3567Thr*	Rs12713558	Benign
29	4181	*p.Glu4181Lys*	Rs10142031	Benign
	4270	*p.Arg4270Thr*	Rs1801702	Benign
	4297	*p.Arg4297His*	Rs375701380	Benign
		p.Ser4338Asn	Rs1042034	Benign

[A]: Numbering was done according to the accession no NP_000375.2 from GenBank. [B]: Variant sequence name according to Nomenclature of the Human Genome Variation Society (HGVS). [C]: Reference ID from NCBI, [D]: In-silico analysis according to Polyphen

Discussion

At present, genetic diagnosis is the most precise method for diagnosing familial hypercholesteolemic patients. Although plentiful mutations were reported in the *LDLR* gene among FH patients, genetic data for the Malaysian population remain scarce [21-25]. The current study screened and investigated for both *LDLR* and *APOB-100* genes variants from a cohort of clinically diagnosed definite and possible Malaysian FH patients.

The present study cohort of 74 patients with clinical features of FH was relatively young (mean±SD age:45.9±12 years), and have a low prevalence of CVD (27.4%) compared to a previous study (32.6%) [33]. Together with an average LDL-C level of 6.4 ± 0.2 mmol/L which is also lower than the average LDL-C level for another FH population [33]. Such variation can be explained by the variation in the cardiovascular risk factors, the underlying causative mutation and the difference in the lifestyle among the respective population.

The *LDLR* gene mutations were identified in 8.1% of clinically diagnosed FH patients. This result is lower than that reported among other Malaysian FH (42.2 %) [22] and among those who were collected by screening program for FH in the Netherlands (32.0%) [34], and Filipino FH (20%) patients [35]. This can be explained by the different inclusion criteria that were used (Simon Broome). Additionally, because of the different screening methods which were used among the different studies.

Large rearrangements that may be present in the *LDLR* gene can increase the mutation detection rate if they were screened in this cohort. Furthermore, the presence of mutations or polymorphism in other candidate genes that are concerned to the LDL-C metabolisms, the co-existed environmental factors that may give to a similar phenotypic presentation as FH and finally the existence of *LDLR* gene mutation in the introns of the gene, all these factors can increase the mutation detection rate in the study subjects.

Five previously reported *LDLR* gene sequence variations could be discovered in this research. Two pathogenic missense mutations and one intronic mutation (*c.313+1G>A*) could be reported.

The commonest variant that was reported in this study is *p.Asn591Asn* in exon 12, which was found in 25.6% of the patients. The frequent detection of a mutation may be the result of consanguinity, founder effect, frequent introduction of the mutation into the cohort or can be due to recurrent mutational events, [36]. *P.Asn591Asn* variant was previously reported among the same ethnic group by Alyaa et al in a frequency of 4.3 % [22]. Also this synonymous variant was recently reported among possible FH cases of Azorean background in Portugal [37] and among Russian FH population [38].

The intronic mutation, *c.313+1G>A* has been previously described by Khoo and his colleagues of the same ethnic group [25]. This is the second report for this mutation among Malaysian cohort. This mutation, affecting the 5' splice site domain(splice donor is deleted), was predicted to be a pathogenic by the in-silico analysis. According to Saphiro and Senapathy (1987) [39], it occurred within a conserved 5'splice-donor site. Cameron et al reported that this mutation could result in skipping of exon 3 and inclusion of intron 3 [40].

In exon 5, *p.Cys255Ser* mutation has also been previously described in Malaysian FH population, [22, 27, 41]. It resulted in substitution of a Cystein by a Serine that may disrupt the ligand binding site of the LDLR gene so it may affect gene binding's affinity to the LDL-C.

While *p.Arg 385Try* mutation, at exon 9, was previously described among both Malaysian Azian et al [27] and Ashkenazi Jews populations [42]. It was reported as 'probably damaging' by *in silico* analysis.

p.Arg471Arg polymorphic variants was reported for the first time among Malaysian patients. How ever, it was previously reported among Russian FH population [43].

Both *p.Asn591Asn* in exon 12 and *p.Arg471Arg* in exon 10 variants are viewed as "silent," and were found to be non-pathogenic by *in sililco* analysis. Which may indicate that they are not disrupting the normal splicing, codon usage, mRNA stability and folding, all these can adversely alter the normal peptide synthesis [44].

Familial defective apoB is not able to be clinically distinguished from FH without genetic investigation. A variety of *APOB-100* gene mutations have been identified in FDB patients [45, 46], while other FH research among Malaysian population were not able to detect mutations in the *APOB-100* gene [24, 25, 41]. The present study was performed to screen for known and novel *APOB-100* gene mutations among subjects who are clinically diagnosed with FH. DNA sequencing was operated as a mutation screening technique for the regions that are known to be crucial for the structural conformation of *APOB-100* protein and its LDLR binding function [47, 48].

In the present study, nine variants were identified in the *APOB-100* gene among the patients with no significant difference from the healthy controls, suggesting that these are more likely polymorphisms rather than mutations, and all are previously reported in the NCBI database as polymorphisms.

In the present study, *p.Arg3500Glu* mutation (the most common *APOB-100* mutations) could not be identified, suggesting that this mutation is not a common variant in the Malaysian FDB cohort. This finding is similar to another FH study that was conducted among Malaysians [46,41].

From this report, five polymorphisms (*p.Thr2515Thr, p.Thr3567Thr, p.Arg4270Thr, p.Arg4297His* and *p.Ser4338Asn*) were previously reported among Malaysians [46], while the rest, to the best of our knowledge, are reported for the first time among Malaysian FDB patients.

p.Gly2540Val variant was predicted to be Possibly damaging while *p.Pro2739Leu* variant was predicted to be Probably damaging by *in silico* analysis. However, family and in vitro studies are needed to confirm the pathogenesis of this variant.

In conclusion, among the 74 studied FH patients, we found five variants in the *LDLR* gene (one reported for the first time among Malaysian FH) and nine polymorphisms in the *APOB-100* gene (four reported for the first time among Malaysians) which indicate that *LDLR* gene variants appear to be a more leading cause to the FH phenotype rather than the *APO B- 100* gene variants among Malaysian FH patients.

Future studies involving larger sample size of FH cases that can achieve a better representation of the general population plus genetic studies on other genes mutations that can be responsible for the ADH phenotype such as *PCSK9* and *LDLR-AP1* (Low Density Lipoprotein Receptor Adaptor Protein

1), may further contribute to the development of the genetic information about FH among Malaysians.

We recommend the use of microarray analysis that can be designed to detect the novel and the known variants in *LDLR* and *APOB-100* genes among Malaysian FH subjects. A combination of such highly sensitive and specific technique together with the detection of large rearrangements by Multiplex Ligation dependent Probe Amplification (MLPA) plus screening of other genes that may lead to FH phenotype will considerably increase the mutation detection rate among the study subjects. Additionally, those discovered mutations need for further confirmatory studies among families of index cases to highlight if they are associated with hypercholesterolaemia among the family members or no. In heterogeneous population such as in Malaysia, the presence of mutations in other candidate genes such as *PCSK9* may possibly cause hypercholesterolaemia in the clinically-diagnosed FH patients. Mutations identification in these genes may contribute to gain a better understanding of the diversity of disease-causing genes in FH.

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Conflict of interest

The authors declare no conflicts of interest.

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