Genetic Polymorphisms in LDLR, APOB, PCSK9 and Other Lipid Related Genes Associated with Familial Hypercholesterolemia in Malaysia

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Abstract

Familial hypercholesterolemia (FH) is an autosomal dominant disorder characterized by elevations in total cholesterol (TC) and low density lipoprotein cholesterol (LDLc). Development of FH can result in the increase of risk for premature cardiovascular diseases (CVD). FH is primarily caused by genetic variations in Low Density Lipoprotein Receptor (LDLR), Apolipoprotein B (APOB) or Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9) genes. Although FH has been extensively studied in the Caucasian population, there are limited reports of FH mutations in the Asian population. We investigated the association of previously reported genetic variants that are involved in lipid regulation in our study cohort. A total of 1536 polymorphisms previously implicated in FH were evaluated in 141 consecutive patients with clinical FH (defined by the Dutch Lipid Clinic Network criteria) and 111 unrelated control subjects without FH using high throughput microarray genotyping platform. Fourteen Single Nucleotide Polymorphisms (SNPs) were found to be significantly associated with FH, eleven with increased FH risk and three with decreased FH risk. Of the eleven SNPs associated with an increased risk of FH, only one SNP was found in the LDLR gene, seven in the APOB gene and three in the PCSK9 gene. SNP rs12720762 in APOB gene is associated with the highest risk of FH (odds ratio 14.78, p<0.001). Amongst the FH cases, 108 out of 141 (76.60%) have had at least one significant risk-associated SNP. Our study adds new information and knowledge on the genetic polymorphisms amongst Asians with FH, which may serve as potential markers in risk prediction and disease management.

Introduction

Familial hypercholesterolemia (FH) (ICD-10 code E78.0) was the first genetic disease of lipid metabolism to be clinically and molecularly characterized [1]. It is an inherited disorder of lipoprotein metabolism, transmitted in an autosomal dominant manner [2]. FH is characterized by elevated levels of low density lipoprotein cholesterol (LDLc) and total cholesterol (TC) in the circulation, deposits of cholesterol in peripheral tissues, presence of tendon xanthomas and accelerated atherosclerosis, leading to premature cardiovascular events [1,3,4,5,6].

Heterozygous FH is one of the most frequent Mendelian disorders with a frequency of 1 in 500 but has a much higher incidence in certain populations, such as the Afrikaners, Christian Lebanese, Finns, and French-Canadians [7]. The frequency of homozygous FH is 1 in a million, with symptoms appearing in childhood [8].

FH can result primarily from mutations in either Low Density Lipoprotein-Receptor gene (LDLR), Apolipoprotein B-100 gene (APOB), or Proprotein Convertase Subtilisin/Kexin type 9 gene (PCSK9), singly or in combination [1]. Genetic variations in the LDLR gene are commonly loss-of-function mutations, which result in increased plasma LDLc levels [9]. Genetic variations in the APOB gene, and the PCSK9 gene give rise to the same lipid homeostasis functional defects [1]. To date, genetic variants in an excess of 1000 have been identified in the LDLR, APOB & PCSK9 genes as reported by British Heart Foundation (BHF) and other public databases. In addition, other genes associated with lipid control and regulatory regions such as Upstream Transcription Factor 1 gene (USF1), Apolipoprotein E gene (APOE), Lipoprotein Lipase gene (LPL), Fibrinogen Beta Chain gene (FGB), and Hepatic Lipase gene (LIPC) can manifest as hypercholesterolemia and have been shown to predispose to premature cardiovascular diseases [10,11,12,13,14]. Results from SNPs study should be interpreted with caution, as SNPs may exert their effects individually, or multiple SNPs can act synergistically to cause a functional difference between haplotypes. Interaction among multiple SNPs may jointly affect a disease’s risk. Assessing the independent individual SNP without considering the SNP-SNP interactions forms (even on SNPs that show very weak associations with
estimated odds ratios) will fail to discover weak associations [15,16].

Clinical management of FH focuses on early detection and control of hypercholesterolemia to decrease the risk of atherosclerosis and to prevent premature cardiovascular disease [17]. Establishing an accurate diagnosis of FH is often difficult. In spite of its prevalence, and considerable benefit associated with its early detection and treatment, FH is often under-diagnosed in many countries [18,19]. Systematic genetic screening for mutations in those at risk of FH has been found to be cost effective and will help in better prognosis [20]. However, most genetic studies in FH were conducted in non-Asian populations and the allelic variants prevailing here in South East Asia are not known. In South East Asia, specifically in Malaysia, only few studies have been carried out on these genes [21,22,23,24,25,26]. Thus, the aim of this study is to determine the genetic variants in the LDLR, APOB, PCSK9 and other lipid related genes in a study cohort with clinical FH.

**Materials and Methods**

**Subject Recruitment**

Consecutive 141 patients with high LDLc levels above 4 mmol/L were recruited between January 2007 and September 2009 from the medical out-patient clinics at the University Malaya Medical Centre (UMMC), Kuala Lumpur. The FH-Dutch Lipid Clinic Network (DLCN) criteria [27,28] was adopted as the diagnostic scoring method to clinically diagnose/screen for FH, excluding molecular diagnosis criterion, and stratify subjects into possible FH, probable FH or definite FH. One hundred and one control subjects consisted of those who were genetically unrelated, with normal LDLc levels, absence of family history of FH, hyper/hypothyroidism, chronic kidney disease, diabetes, chronic liver disease and characterized as not FH by DLCN criteria (Table S1). The protocol was approved by the UMMC’s Medical Ethics Committee (Ref: 546.16) and written informed consents were obtained from all patients.

**Questionnaire and Data Collection**

The data included socio-demographic characteristics (age, sex, and occupation), personal and family history of hypertension, hypercholesterolemia, CVD and other lifestyle habits, such as smoking status, and physical activity. Body mass index (BMI), waist circumferences (WC) and blood pressure were also measured.

**DNA Isolation**

Genomic DNA from all subjects was isolated from whole blood using QIAamp DNA Mini Kit (QIAGEN, USA) in 200 μl of total volume according to user protocol. Qualitative and quantitative estimations were carried out on the DNA samples. All DNA samples were normalized to concentration of 50 ng/μl for genotyping.

**Selection of Genes/SNPs and Microarray Probes Synthesis**

Genetic variations implicated in FH from three publically available databases, BHF (www.ucl.ac.uk) [29], dbSNP (ncbi.nlm.nih.gov/SNP/) [30] and SNPedia (www.snpedia.com), were selected based on the following attributes: i) conventional SNPs known to cause FH in genes encoding LDLR, APOB and PCSK9 ii) SNPs in USF1, APOE, LPL, FGB and LIPC that were known to have functional effects by in vitro assays or were non-synonymous in lipid regulatory regions. Though our initial research and mining led us to 1850 SNPs, which were sent to Illumina for designing the probes, only 1536 could be of designable standards as per Illumina criteria.

A tool called Assay Design Tool (ADT) of Illumina ranks SNPs based on an in-built algorithm where SNPs scoring below 0.4 have a rank of zero suggesting the probe is not designable by Illumina. SNPs scoring between 0.4 and 0.6 get a rank 0.5 whereas a score above 0.6 is ranked 1. SNPs scoring 0.5 and 1 are technically ranked as SNPs that can be successfully designed as probes by Illumina. The assay has an average 30-fold redundancy for each probe thus making the quality control robust. In all, 231 probes had score of 0.5 and 1305 had a score of 1.0. Only 1536 SNPs were chosen because that was the maximum plexity Illumina platform could accommodate.

Most of the reported studies till date were from Caucasian population, and hence we were keenly interested to re-look at the reported SNPs using a population based approach, and check if the interpretations are extraplatable to Asian population.

Designability scores were graded and qualified probes were selected and synthesized for the custom GoldenGate™ genotyping assay (GGGT) (www.illumina.com). Of the 1850 SNPs mined, 1536 SNPs were synthesized as probes, comprising of 811 in LDLR, 245 in APOB, 284 in PCSK9, and another 196 lipid-regulatory related SNPs.

**Genotyping**

Genotyping was performed on Universal BeadChips (Illumina, USA) according to the manufacturer’s protocol and was carried out in compliance with MIAME (Minimum Information about a Microarray Experiment) guidelines [31]. All the raw data from our GGGT microarray assays were imported into the GenomeStudio™ software (Illumina, USA) for allelic analysis and deviation from Hardy-Weinberg equilibrium. Average call rate of 70–80% was observed, which is expected of a custom GGGT assay.

**Statistical Analysis**

Statistical analysis was performed using the SPSS software v16.0 (SPSS Inc., Chicago, Illinois). The test of normality (Kolmogorov-Smirnov) was employed to determine the normality of the variables. Descriptive analysis and statistical significance of the association were assessed by independent t-test. Logistic regression was applied to obtain the Odds Ratio (OR) and the p-values for the tested SNPs (p-values ≤0.05 were considered to be significant). An OR>1.0 was used as the cut-off for the baseline of risk-associated SNPs, and the baseline risk-lowering SNPs as OR<1.0. An OR equal to 1 was a neutral value and deemed as normal. Analysis of variance (ANOVA) test was conducted for comparison of means between clinical profiles and three genotype groups. Minor allele frequency (MAF) for this study was calculated. MAF for the most closely related ethnic group to our study were also extracted from public database (NCBI dbSNP Build 137). A Chi-Square Test was performed to determine whether there was a significant difference between our study’s MAF and public databases’ MAF. Bonferroni correction for multiple comparisons of SNPs on the same gene was performed. Unless otherwise specified, all data were presented as means and standard deviations.

**Additional Validation of Genotype Calls by Sequencing**

Microarray calls were validated by blindly re-genotyping some SNPs in a number of subjects randomly selected from cases using DNA sequencing. Primers were synthesized for regions encompassing a few significant SNPs and the PCR products amplified from the genomic DNA of FH cases were sent for sequencing (First BASE Laboratories, www.base-asia.com).
Results

Subjects Demographics and Clinical Profiles

Of the 141 FH subjects and 111 control subjects included in the study, 24 were classified as definite FH, 25 as probable FH and 92 as possible FH from cases based on DLCN criteria. There was no significant gender bias observed between FH subjects (73 males; 68 females) and control subjects (46 males; 65 females) that were recruited into the study (p = 0.104). The mean age of the FH subjects was 46.84 (SD ± 11.2), while control subjects were 44.00 (SD ± 9.9) with significant difference (p < 0.001). The BMI (p = 0.239), waist circumference (p = 0.356) and HDL cholesterol (HDLc) level (p = 0.420) were similar between the two study groups. FH subjects were found to have significantly higher levels of triglycerides (TG) (p = 0.001), TC and LDLc (p < 0.001) as compared to controls (Table 1). 

FH Associated SNPs

A total of 14 SNPs were found to be significantly associated with FH. Eleven out of 14 were associated with high risk of FH (OR >1), while the remaining three were protective against FH (OR <1). Of the 11 associated SNPs, one (rs2569556) was found in the LDLR gene, seven in the APOB gene (rs1720762, rs13306187, rs13306194, rs12714238, rs12720772, rs57825321 and rs41291161) and three (rs12084215, rs565436 and rs28362269) in the PCSK9 gene. The APOB rs12720762 is associated with the highest risk of FH with OR of 14.78. Amongst the FH subjects, 108 (76.60%) subjects have had at least one significant risk-associated SNP. 17 out of 24 definite FH subjects (70.83%), 19 out of 25 probable FH subjects (76%) and 72 out of 92 possible FH subjects (78.26%) had at least one significant risk-associated SNP (Table 2). 

The APOB rs57825321 and USF1 rs3737787 and rs2516839 were found to have a protective effect against FH in this case-control association study (Table 2). No significant associations were found with the other 1522 SNPs (99.09%) genotyped (Statistical data not shown but available upon request).

SNPs Association with Clinical and Demographic Profile

We also investigated the association of significant SNPs with the clinical and demographic profile of FH patients. There was no significant association between most of the SNPs with the clinical and demographic profile. However, the APOB rs13306194 and rs57825321 were significantly associated with HDLc level (p < 0.001). APOB rs12720772 was associated with TC (p = 0.0275) and BMI (p = 0.0337) while the PCSK9 rs12084215 was associated with HDLc level (p = 0.0090) and BMI (p = 0.0228) (Table 3, 4, 5, and 6). Clinical and demographic profile of control subjects was also examined similarly. No significant association was seen between most of the SNPs with the clinical and demographic profile. However, rs12084215 (PCSK9) was significantly associated with TG level (p = 0.0052) and waist circumference measurement (p = 0.0214) (Table 7).

Table 1. Demographics and clinical profiles of the subjects.

| Characteristic         | Over all FH cases | Definite FH | Probable FH | Possible FH | Controls | p-value Over all FH cases vs. Controls |
|------------------------|-------------------|-------------|-------------|-------------|----------|--------------------------------------|
| Males : Females        | 73:68             | 11:13       | 15:10       | 47:45       | 46:65    | 0.104                                |
| Age (years)            | 46.84 (±11.2)     | 42.37 (±17.4)| 45.60 (±18.6)| 48.34 (±8.5)| 40.00 (±9.3)| <0.001| |
| BMI (kg/m²)            | 26.42 (±5.4)      | 22.79 (±5.1)| 26.49 (±5.2)| 27.32 (±5.2)| 25.62 (±4.9)| 0.239| |
| WC (cm)                | 86.21 (±17.5)     | 76.64 (±19.1)| 85.37 (±18.6)| 88.90 (±16.0)| 83.05 (±12.2)| 0.356| |
| TG (mmol/L)            | 1.79 (±1.0)       | 1.99 (±1.8)| 2.00 (±1.0)| 1.69 (±0.7)| 1.23 (±0.7)| 0.001| |
| TC (mmol/L)            | 8.86 (±5.1)       | 13.23 (±10.7)| 9.51 (±2.4)| 7.47 (±1.2)| 5.18 (±0.9)| <0.001| |
| HDLc (mmol/L)          | 1.25 (±0.7)       | 1.17 (±0.4)| 1.53 (±1.5)| 1.20 (±0.3)| 1.34 (±0.3)| 0.420| |
| LDLc (mmol/L)          | 6.37 (±2.3)       | 9.23 (±3.4)| 6.96 (±2.3)| 5.49 (±1.0)| 3.28 (±0.7)| <0.001| |

BMI, Body Mass Index. 
WC, Waist circumference. 
TG, Triglyceride. 
TC, Total Cholesterol. 
HDLc, High Density Lipoprotein Cholesterol. 
LDLc, Low Density Lipoprotein Cholesterol. 
The data are expressed as mean (±SD). 
p-values were obtained by comparing the phenotypes between the two groups using Student’s t-test. 
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Table 2. FH associated SNPs, (p<0.05).

| Gene | rs number | Nucleotide change | p-value | OR (CI) |
|------|-----------|------------------|---------|---------|
| LDLR | rs2569556 | [G>A]            | 0.0140  | 1.77 (1.12–2.78) |
| APOB | rs13306187| [G>A]            | <0.0001 | 6.76 (3.28–13.90) |
| APOB | rs13306194| [G>A]            | 0.0154  | 2.25 (1.17–4.34) |
| APOB | rs12714238| [G>A]            | <0.001  | 8.04 (3.20–20.20) |
| APOB | rs12720772| [G>A]            | 0.0130  | 2.00 (1.16–3.46) |
| APOB | rs12720762| [G>C]            | <0.001  | 14.78 (5.03–43.44) |
| APOB | rs41291161| [T>A]            | <0.0001 | 11.51 (4.32–30.69) |
| APOB | rs57825321| [A>T]            | 0.0304  | 2.02 (1.07–3.83) |
| APOB | rs12714254| [T>G]            | <0.001  | 0.22 (0.11–0.50) |
| PCSK9| rs12084215| [C>A]            | 0.0064  | 3.87 (1.46–10.23) |
| PCSK9| rs565436  | [A>G]            | 0.0020  | 5.00 (1.80–13.89) |
| PCSK9| rs28362269| [G>A]            | <0.001  | 5.43 (2.76–10.65) |
| USF1 | rs3737787 | [A>G]            | 0.0174  | 0.55 (0.33–0.90) |
| USF1 | rs2516839 | [A>G]            | 0.0317  | 0.67 (0.46–0.97) |

rs number, NCBI Reference SNP (rs) Number, an identification tag assigned by NCBI to SNPs [30]. 
CI, Confidence interval. Odds ratio (OR) between groups was determined by logistic regression. 
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Polymorphisms of Familial Hypercholesterolemia

BMSX Polymorphisms of Familial Hypercholesterolemia

The data are expressed as mean (±SD). 
p-values were obtained by comparing the phenotypes between the two groups using Student’s t-test. 
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Additional Validation of Genotype Calls by Sequencing

Bonferroni Correction

Five out of the eight significant SNPs of \textit{APOB} gene (rs13306187, rs12720772, rs12714238, rs12720762, rs41291161, rs12714254) and all three \textit{PCSK9} gene SNPs (rs12084215, rs565436 and rs28362269) survived significance after a conservative Bonferroni correction for multiple testing, (0.05/9, p<0.0056) and (0.05/4, p<0.0125) respectively, none of the SNPs of \textit{USF1} gene is significant following Bonferroni correction. These 8 SNPs are thus of sufficient interest to warrant further investigation.

Additional Validation of Genotype Calls by Sequencing

A few samples were sent for sequencing to rule out any genotyping errors and results were concordant with the genotype calls generated from the microarray data (Figure S1).

Discussion

Fourteen out of 1536 SNPs evaluated in this study were significantly associated with FH, with 11 SNPs associated with increasing risk for FH, while the remaining three SNPs associated with decreasing risk for FH. Among the risk-increasing SNPs, allele A of rs2569556 in \textit{LDLR} gene was identified among 55 out of 141 FH cases (39.0%), with 48 heterozygous and seven in homozygous genotypes. SNP rs13306187 in \textit{APOB} gene also demonstrated risk association on allele A among 11 FH cases. Ten of the FH cases were heterozygous and only one of the FH case was observed in homozygous genotype. Other risk associated SNPs on \textit{APOB} gene were only observed to occur in heterozygous genotype and these included 64 cases for rs12720772; six cases for rs12714238, 121 cases for rs57825321, 17 cases for rs13306194, six cases for rs12714238, and four cases for rs12720762. Three \textit{PCSK9} SNPs were observed to be associated with increased risk in a heterozygous manner (six cases for rs65436, six cases for rs12084215, and 15 cases for rs28362269). Only two risk-elevating SNPs (rs2569556 in \textit{LDLR} gene and rs13306187 in \textit{APOB} gene) were observed to be in the homozygous state; while other 9 SNPs presented in a heterozygous manner, which may demonstrate a milder phenotypic effect of FH. In general, 17 definite FH subjects (70.83%), 19 probable FH subjects (76%) and 72 possible FH subjects (78.26%) had at least one SNP out of the risk-increasing SNPs. The remaining 33 FH subjects (23.40%) did not have any risk-increasing SNPs indicating that there are other genetic or environmental factors causing hypercholesterolemia that were undetected by our study and which has the potential for future investigation. Besides the risk-increasing SNPs, there were 3 other SNPs with OR<1 (rs12714254 in \textit{APOB} gene, and rs2516839 and rs3737787 in the \textit{USF1} gene) which confer lower risk against FH. \textit{USF1} gene was studied because \textit{USF1} protein regulates the transcriptional activation of a variety of genes involved in glucose, lipid and apolipoproteins (\textit{APOCIII}, \textit{APOAI} and \textit{APOE}) metabolism in the development of atherosclerosis [32,33,34,35]. Results for SNPs in other candidate genes such as \textit{APOE}, \textit{LPL}, \textit{FGB} and \textit{LIPC} were analysed and found not to be significant in our study (p>0.05).

Clinical parameters were compared between the significant genotypes among FH patients (Table 3, 4, 5, and 6). Allele A of \textit{APOB} rs12720772 in heterozygous GA patients is associated with significantly higher level of plasma TC compared to the G allele (p = 0.0275), while allele A in \textit{APOB} rs12720772 (p = 0.0337) and \textit{PCSK9} rs12084215 (p = 0.0228) were associated with higher BMI.

| Table 3. Comparison of clinical profiles between rs13306194 genotypes among FH patients. |
| --- |
| Clinical Profiles | GG (% = 88) | GA (% = 12) | AA (% = 0) | p-value |
| Age | 47.11±(10.9) | 46.18±(12.3) | – | 0.7463 |
| TG | 1.83±(1.1) | 1.54±(0.7) | – | 0.2905 |
| TC | 8.89±(5.4) | 8.86±(2.7) | – | 0.9848 |
| HDLc | 1.15±(0.3) | 1.94±(1.8) | – | <0.001 |
| LDLc | 6.42±(2.4) | 6.16±(2.4) | – | 0.6622 |
| BMI | 26.44±(5.5) | 26.29±(5.5) | – | 0.9173 |
| WC | 86.57±(17.3) | 82.38±(19.6) | – | 0.3720 |

Data are presented in mean ± SD.
p-values were obtained by comparing the phenotypes among the genotypes using Analysis of Variance (ANOVA).
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| Table 4. Comparison of clinical profiles between rs57825321 genotypes among FH patients. |
| --- |
| Clinical Profiles | AA (% = 86) | AT (% = 14) | TT (% = 0) | p-value |
| Age | 47.02±(11.2) | 45.95±(12.0) | – | 0.6965 |
| TG | 1.83±(1.1) | 1.53±(0.7) | – | 0.2456 |
| TC | 8.89±(5.5) | 8.76±(2.5) | – | 0.9169 |
| HDLc | 1.15±(0.3) | 1.88±(1.6) | – | <0.001 |
| LDLc | 6.43±(2.4) | 6.13±(2.2) | – | 0.5995 |
| BMI | 26.51±(5.5) | 25.97±(5.1) | – | 0.6817 |
| WC | 86.79±(17.4) | 82.44±(18.6) | – | 0.3310 |

Data are presented in mean ± SD.
p-values were obtained by comparing the phenotypes among the genotypes using Analysis of Variance (ANOVA).
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| Table 5. Comparison of clinical profiles between rs12720772 genotypes among FH patients. |
| --- |
| Clinical Profiles | GG (% = 48) | GA (% = 52) | AA (% = 0) | p-value |
| Age | 46.69±(9.0) | 47.39±(12.0) | – | 0.7175 |
| TG | 1.63±(0.8) | 1.80±(0.8) | – | 0.2683 |
| TC | 7.89±(1.9) | 8.88±(2.8) | – | 0.0275 |
| HDLc | 1.23±(0.5) | 1.30±(0.9) | – | 0.6228 |
| LDLc | 5.89±(1.6) | 6.68±(2.8) | – | 0.0614 |
| BMI | 25.18±(3.5) | 27.10±(5.9) | – | 0.0337 |
| WC | 85.04±(13.5) | 86.11±(18.8) | – | 0.7290 |

Data are presented in mean ± SD.
p-values were obtained by comparing the phenotypes among the genotypes using Analysis of Variance (ANOVA).
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Interestingly, we observed that 3 risk alleles have the effect of significantly higher level of plasma HDLc in their heterozygous form in 3 SNPs (GA, rs13306194; AT, rs57825321 of APOB; CA, rs12084215 of PCSK9). For control subjects, similar approach for clinical and demographic profile was also performed. Only allele A in SNP rs12084215 of PCSK9 was associated with higher level of TG (p = 0.0052) and waist circumference measurement (p = 0.0214) with heterozygous AC (Table 7). It is noteworthy that all the parameter-associated SNPs presented with homozygous wild type (+/-+) and heterozygous (+/--) but extremely low or no homozygous mutant (-/-). This finding is commonly observed among the heterozygous FH associated SNPs [36] (Table 3, 4, 5, 6, and 7).

MAF from the studied population were calculated and compared with the MAF information on public database. Han Chinese subjects, where available, were selected as the targeted group for comparison as this Asian ethnicity were believed to closely resemble the ethnic groups of our study population. The results demonstrated that six of our SNPs differed in the frequency of MAF in public database [37]. Disparity in frequency could be due to founder-effects, natural selection or multi-ethnic groups in the study population [38] (Table 8).

Out of the 1536 SNPs that were studied, 1522 SNPs (99.09%) did not show any significant result or association. This is because besides the SNPs being mono-allelic (non-polymorphic) [37], we also observed an almost equal number of the predicted risk alleles present in both case subjects and control subjects across our 252 samples. Thus analyses of these polymorphisms were not statistically significant and therefore, regarded as having non-pathogenic phenotype. These findings suggest that many SNPs published in public databases might just be non-pathogenic polymorphisms in Malaysia. This will require further validation in a larger population of Asian descent.

For the SNPs association study, we included all significant SNPs, inclusive of SNPs with relatively low odds ratio (LDLR rs2569556, OR 1.77), as SNPs usually work with other functionally relevant SNPs additively or synergistically, to manifest a disease condition in certain population [16]. Therefore, including these SNPs with relatively low ORs might aid future research on SNP-SNP interaction and polygenic effect of FH. Furthermore, 12 SNPs that were reported as significant were in the intronic and untranslated region (UTR) of genes. These SNPs could be in linkage disequilibrium with other functional SNPs involved in potential regulatory regions or splice site variants that may be associated with lipid related disorders. Exons 2 to 6 fall in the untranslated region (UTR) of genes. These SNPs could be in linkage disequilibrium with other functional SNPs involved in potential regulatory regions or splice site variants that may be associated with lipid related disorders. Exons 2 to 6 fall in the

### Table 6. Comparison of clinical profiles between rs12084215 genotypes among FH patients.

| Clinical Profiles | CC (% = 91) | CA (% = 9) | AA (% = 0) | p-value |
|-------------------|------------|-----------|-----------|---------|
| Age               | 44.39 ± (11.3) | 53.50 ± (2.6) | -         | 0.0558  |
| TG                | 1.72 ± (0.8)    | 1.68 ± (0.9)    | -         | 0.9069  |
| TC                | 8.35 ± (3.1)    | 7.65 ± (1.4)    | -         | 0.5927  |
| HDLc             | 1.18 ± (0.5)    | 2.32 ± (2.8)    | -         | 0.0090  |
| LDLc             | 6.32 ±(3.0)    | 4.56 ± (1.8)    | -         | 0.1602  |
| BMI               | 25.80 ± (5.7)   | 31.57 ± (5.9)   | -         | 0.0228  |
| WC                | 84.33 ± (17.0)  | 94.58 ± (6.4)   | -         | 0.1518  |

Data are presented in mean ± SD. p-values were obtained by comparing the phenotypes among the genotypes using Analysis of Variance (ANOVA).

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### Table 7. Comparison of clinical profiles between rs12084215 genotypes among Control subjects.

| Clinical Profiles | CC (n = 71%) | AC (n = 26%) | AA (n = 3%) | p-value |
|-------------------|------------|-----------|-----------|---------|
| Age               | 38.05 ± (9.11) | 37.67 ± (5.92) | 41.00 ± (4.24) | 0.8686  |
| TG                | 1.07 ± (0.57)    | 2.90 ± (n/a)    | -         | 0.0052  |
| TC                | 5.11 ± (0.93)    | 6.20 ± (n/a)    | -         | 0.2659  |
| HDLc             | 1.42 ± (0.35)    | 0.98 ± (n/a)    | -         | 0.2368  |
| LDLc             | 3.20 ± (0.78)    | 3.90 ± (n/a)    | -         | 0.3934  |
| BMI               | 25.55 ± (4.91)   | 24.98 ± (5.08)   | 25.14 ± (2.27) | 0.9313  |
| WC                | 78.64 ± (8.96)   | 98.50 ± (14.85)  | -         | 0.0214  |

Data are presented in mean ± SD. p-values were obtained by comparing the phenotypes among the genotypes using Analysis of Variance (ANOVA).

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Table 8. Minor allele frequency of significant SNPs.

| Gene | rs no.       | Region | Chr | Nucleotide change | MAF (Total) | MAF (Cases) | MAF (Controls) | MAF (PD) | p-value (Total vs. PD) | MAF source                                                                 |
|------|--------------|--------|-----|-------------------|-------------|-------------|----------------|-----------|------------------------|----------------------------------------------------------------------------|
| LDLR | rs2569556    | Intron 6 | 19  | G>A               | 0.263       | 0.316       | 0.223          | 0.209     | 0.3853                 | HapMap-HCB                                                                  |
| APOB | rs13306187   | Exon 25 | 2   | G>A               | 0.111       | 0.197       | 0.043          | 0.035     | 0.0314                 | HapMap-HCB                                                                  |
| APOB | rs13306194   | Exon 12 | 2   | G>A               | 0.091       | 0.128       | 0.061          | 0.133     | 0.2784                 | Pilot 1 CHB+JPT low coverage panel                                       |
| APOB | rs12714238   | Intron 5 | 2   | G>A               | 0.072       | 0.136       | 0.022          | 0.011     | 0.0311                 | Pharmacogenetics Network for Cardiovascular Risk Therapy                 |
| APOB | rs12720722   | Intron 18 | 2   | G>A               | 0.301       | 0.352       | 0.260          | 0.000     | <0.0001                | HapMap-CHB                                                                  |
| APOB | rs12720762   | Intron 1 | 2   | G>C               | 0.076       | 0.159       | 0.014          | 0.021     | 0.0741                 | Pharmacogenetics Network for Cardiovascular Risk Therapy                 |
| APOB | rs41291161   | Intron 14 | 2   | T>A               | 0.077       | 0.149       | 0.021          | 0.001     | <0.0001                | ABECASIS CLINICAL PANEL                                                   |
| APOB | rs57825321   | Intron 16 | 2   | A>T               | 0.403       | 0.429       | 0.369          | 0.158     | <0.0001                | Pilot 1 CHB+JPT low coverage panel                                       |
| APOB | rs12714254   | Intron 3 | 2   | T>G               | 0.405       | 0.342       | 0.454          | 0.100     | <0.0001                | Pilot 1 CHB+JPT low coverage panel                                       |
| PCSK9| rs12084215   | Intron 3 | 1   | C>A               | 0.102       | 0.164       | 0.046          | NA        | NA                     | Pilot 1 CHB+JPT low coverage panel                                       |
| PCSK9| rs565436     | Intron 9 | 1   | A>G               | 0.079       | 0.145       | 0.036          | 0.100     | 0.6627                 | Pilot 1 CHB+JPT low coverage panel                                       |
| PCSK9| rs28362269   | Intron 9 | 1   | G>A               | 0.112       | 0.188       | 0.053          | 0.059     | 0.1289                 | Pilot 1 YRI low coverage panel                                           |
| USF1 | rs2516839    | 5′ UTR  | 1   | G>A               | 0.408       | 0.360       | 0.447          | 0.366     | 0.5197                 | HapMap-CHB                                                                  |
| USF1 | rs3737787    | 3′ UTR  | 1   | G>A               | 0.163       | 0.117       | 0.199          | 0.250     | 0.1032                 | HapMap-HCB                                                                  |

rs no, NCBI Reference SNP (rs) Number, an identification tag assigned by NCBI to SNPs.
Chr, Chromosome.
p-value obtained by comparing frequencies using Chi-Square Test.
MAF (Total), minor allele frequency obtained from total sum of case and control subjects in this study.
MAF (PD), minor allele frequency information from public database, NCBI dbSNP Build 137.
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homeostasis by either modulating the expression of hepatic \textit{LDLR} [45] or controlling the activity of cholesterol \textit{タルハ-ヒドロキシラーゼ} (CYP7A1), a rate-limiting enzyme in the synthesis of bile acid from cholesterol [46]. Presence of FH causal SNPs along with increasing age accelerates the hypercholesterolemia. This bias was beyond our control as our recruitment of subjects was based on volunteered patients who visit UMMC.

Another limitation of our study was that the microarray genotyping platform used in this study only allowed us to study published SNPs. It is possible that rare risk-associated SNPs may be discovered in future using other existing technologies such as sequencing. The authors also recognise that the genotype scores are not true reflection of the biological characteristics of FH as all alleles were given the same statistical weight. Therefore, a model of association of SNPs should be created to calculate the overall genetic risk. These 14 SNPs identified could provide more insights in future studies on screening markers for FH management and warrants further investigation.

**Supporting Information**

**Figure S1 Validation by sequencing.**

**Table S1 The Dutch lipid network criteria.**

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