ORIGINAL ARTICLE

Differences in allele frequencies of autosomal dominant hypercholesterolemia SNPs in the Malaysian population

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Hypercholesterolemia is caused by different interactions of lifestyle and genetic determinants. At the genetic level, it can be attributed to the interactions of multiple polymorphisms, or as in the example of familial hypercholesterolemia (FH), it can be the result of a single mutation. A large number of genetic markers, mostly single nucleotide polymorphisms (SNP) or mutations in three genes, implicated in autosomal dominant hypercholesterolemia (ADH), viz *APOB* (apolipoprotein B), *LDLR* (low density lipoprotein receptor) and *PCSK9* (proprotein convertase subtilisin/kexin type-9), have been identified and characterized. However, such studies have been insufficiently undertaken specifically in Malaysia and Southeast Asia in general. The main objective of this study was to identify ADH variants, specifically ADH-causing mutations and hypercholesterolemia-associated polymorphisms in multiethnic Malaysian population. We aimed to evaluate published SNPs in ADH causing genes, in this population and to report any unusual trends. We examined a large number of selected SNPs from previous studies of *APOB*, *LDLR*, *PCSK9* and other genes, in clinically diagnosed ADH patients ($n=141$) and healthy control subjects ($n=111$). Selection of SNPs was initiated by searching within genes reported to be associated with ADH from known databases. The important finding was 137 mono-allelic markers (44.1%) and 173 polymorphic markers (55.8%) in both subject groups. By comparing to publicly available data, out of the 137 mono-allelic markers, 23 markers showed significant differences in allele frequency among Malaysians, European Whites, Han Chinese, Yoruba and Gujarati Indians. Our data can serve as reference for others in related fields of study during the planning of their experiments.

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INTRODUCTION

Hypercholesterolemia, the presence of high level of cholesterol in the blood, is the key factor in the development and progression of atherosclerosis, and consequently is the second major cause of coronary artery disease.¹ Hypercholesterolemia is influenced by many factors including diet, heredity and other lifestyle factors.

The hereditary type of hypercholesterolemia, called FH (OMIM 143890) is clinically characterized by highly elevated level of plasma LDL cholesterol; deposition of cholesterol in peripheral tissues including xanthelasma, arcus senilis and tendon xanthomas; premature or accelerated atherosclerosis; and increased risk of premature coronary heart disease. Genetically, FH is an autosomal dominant disorder caused mostly by genetic defects found in the three genes involved in cholesterol homeostasis: *LDLR* (OMIM 606945), *APOB* (OMIM 107730) and *PCSK9* (OMIM 607786).² Cholesterol can be obtained from both diet and biosynthesis in cells. Cholesterol is hydrophobic, and thus depends on lipoproteins for its transport within the bloodstream. Cholesterol, in cholesteryl ester form, is

transported in the plasma mostly via low density lipoprotein (LDL). The homeostasis of cholesterol can be regulated by *PCSK9*.³ *PCSK9* is a serine protease of proprotein convertase family.⁴ Mature *PCSK9* is secreted from the cell into circulation.^{5–8} The circulating *PCSK9* can bind to *LDLR*, leading to the co-internalization, followed by the degradation of *LDLR*.⁹ Studies demonstrated that the overexpression of *PCSK9* results in depletion of *LDLR*, and thus a dramatic increase in plasma level of cholesterol.^{10–12} Conversely, the depletion or removal of *PCSK9* protein can substantially increase the level of *LDLR* and decrease the plasma cholesterol.^{13–16}

It is very important that haplotype signatures of prominent SNPs of these three genes are studied in all populations rather than inferring or assuming frequencies from the HAPMAP data, where only a few populations are tested. *LDLR* is a highly polymorphic gene but how relevant is the CHB (Han Chinese in Beijing) frequencies to other similar races in other locations? There may be many more causal variants waiting to be discovered in other populations; for example, most of the CHB samples from HAPMAP are northern

Hans. The southern Hans are separated from northern Hans by the Yangtze river. There has been no study so far done that has analyzed the utility of the CHB data in other Asian populations.

A large number of hypercholesterolemia-associated and ADH-causing genetic variants, mainly SNPs or mutations in the *LDLR*, *APOB* and *PCSK9* genes have been documented worldwide.^{17–19} These data are invaluable for the purpose of universal screening and management of ADH. Unfortunately, only a handful of such studies have been conducted in Malaysian and related populations, and thus the repertoire of known ADH-associated or unassociated genetic variants are limited.^{20–23} In the present study, in order to identify ADH variants, specifically ADH-causing mutations and hypercholesterolemia-associated polymorphisms in Malaysian population, we genotyped both healthy subjects and clinically diagnosed ADH patients for 310 previously documented single nucleotide mutations or SNPs located in *LDLR*, *APOB* and *PCSK9* genes. We found that 137 out of 310 markers were devoid of polymorphic allele in the Malaysian population.

MATERIALS AND METHODS

Instruments/software used: Illumina Bead Station (Illumina Inc., San Diego, CA, USA) Genome studio software (Illumina Inc.), all accessories related to hybridization work from Illumina Inc.

Study subjects

Dutch Lipid Clinic Network (DLCN) criteria for the diagnosis of heterozygous FH were employed for the selection of study subjects.²⁴ The inclusion and exclusion criteria are given in appendix-A. A total of 140 clinically diagnosed ADH and 111 control subjects were recruited between January 2007 and September 2009 from the University Malaya Medical Center (UMMC) at Kuala Lumpur. The protocol was reviewed and approved by the UMMC's ethics committee (Ref: 546.16:Detection of FH (*LDLR* AND *APOB*) mutations amongst Malaysian population).

DNA isolation

Blood was collected in various vacutainers in the hospital from the clinically diagnosed ADH and control subjects after overnight fasting of 12–14 h and the lavender cap EDTA vacutainers came to the lab for DNA extraction and the others went for biochemical profiling. The genomic DNA from all subjects was extracted from the whole blood using QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instruction. The DNA was adjusted to a final concentration of 50 ng μl^{-1} before microarray analysis.

Genetic marker selection, genotyping assay and data analysis

Following literature review, polymorphisms previously implicated in ADH with the following attributes were selected: (i) SNPs in genes encoding *LDLR*, *APOB*, *PCSK9* and a few other associated genes; (ii) SNPs that were known to have functional effects on *in vitro* assays or were non-synonymous or in regulatory regions. In *LDLR*, 75% of these SNPs were located in exons with 15% resulting in stop codons whereas 25% were in the noncoding region. Three publically available databases: British Heart Foundation (BHF) (http://www.ucl.ac.uk/ldlr/Current/search.php?select_db=LDLR&srch=all&page=11), dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP/>) and SNPedia (www.snpedia.com) were referred for the design of the custom probes.

For the design of the microarray chip, single nucleotide mutations and polymorphisms in *APOB*, *LDLR* and *PCSK9* genes (GenBank accession numbers for *APOB*, *LDLR* and *PCSK9* are AY324608, AY324609 and AY829011, respectively) were selected from two publically available databases: British Heart Foundation (BHF, http://www.ucl.ac.uk/ldlr/Current/search.php?select_db=LDLR&srch=all&page=11) and dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP/>).

Illumina ranks SNPs based on an in-built algorithm in the ADT where SNPs scoring below 0.4 have a rank of zero suggesting the probe is not designable by Illumina. A score between 0.4 and 0.6 gives a rank 0.5 whereas a score above

0.6 is ranked 1 and both these ranks technically can be successfully designed as probes (Oligo Pool All (OPA)) by Illumina. Probes were submitted to ADT in the sequence list format. In all, 231 probes had score of 0.5 and 1305 had a score of 1.0.

Genotyping was performed using Illumina GoldenGateGenotyping (GGGT) Microarray Assay. The assay has an average 30-fold redundancy for each probe. All assays were performed according to the manufacturer's protocol and were in compliance with MIAME guidelines.²⁵ The microarray data were deciphered using GenomeStudio software and statistical analysis was performed using STATA10 (Stata Corp., College Station, TX, USA). HWE is a basic principle of population genetics. Genotype frequencies at any locus remain same in the absence of migration, mutations, natural selection and assortative mating. When these assumptions are not met, it may result in a deviation from the expected. Hence, a HWE analysis of controls was carried out to exclude the non-conforming SNPs.

Natural selection and genetic drift can often cause genetic differences between populations. Human allele frequencies for various genetic variants differ by geographical locations. The genetic diversity for each SNP used in this study was measured using F-statistics. A rough estimate of genetic differentiation can be made by looking up the fixation index (F_{ST}) values. Wherever available, F_{ST} values were extracted for the SNPs studied.

Verification of genotyping

Verification for the allele calls by microarray was done by blindly regenotyping some SNPs in a number of subjects randomly selected from both case and control groups, using DNA sequencing.

RESULTS AND DISCUSSION

The characteristics of the study subjects are detailed in Table 1. Genomic DNA of 111 healthy control subjects (DLCN criteria <3 points) and 140 patients diagnosed with ADH (>3 points) were genotyped. The mean age was 39.9 ± 0.90 and 47.0 ± 0.90 years for the controls and ADH cases, respectively. The diversity of Malaysia's population was also reflected in the ethnic composition of the study subjects.

In total, 310 markers from the public databases were studied, including 130, 73 and 107 markers from *APOB*, *LDLR* and *PCSK9* genes, respectively. Among them, no minor allele was observed in 137 markers (73 *APOB*, 28 *LDLR* and 36 *PCSK9* markers) in both our control and ADH populations (Table 2 and Supplementary Table 1). The failure to find a minor allele among the ADH patients suggested that these genetic markers cannot be used as susceptibility marker for this disorder among Malaysians.

The allelic frequencies of the 137 identified mono-allelic genetic markers, in Han Chinese and European populations, if available, were

Table 1 Characteristics of the study subjects

	Control, N = 111	Study, N = 140
Age (years $\pm$ s.e.)	39.9 $\pm$ 0.90	47.0 $\pm$ 0.90
Ethnicity		
Malay, n (%)	71 (64.0)	84 (60.0)
Indian, n (%)	22 (19.8)	35 (25.0)
Chinese, n (%)	18 (16.2)	21 (15.0)
Gender		
Female, n (%)	65 (58.6)	67 (47.9)
Male, n (%)	46 (41.4)	73 (52.1)
Dutch Lipid Network		
not FH (<3), n (%)	111 (100)	0
possible (3–5), n (%)	0	93 (66.4)
probable (6–8), n (%)	0	23 (16.4)
definite (>8), n (%)	0	24 (17.1)

Table 2 Summary of genetic markers tested

Gene	Markers examined	Mono-allelic markers	% of total
<i>APOB</i>	130	73	56.2
<i>LDLR</i>	73	28	38.4
<i>PCSK9</i>	107	36	33.6
Total	310	137	—

then extracted from dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP/>) and compared with our data (Supplementary Table 1). In total, the allelic frequencies of 84 out of the 137 markers for Han Chinese or European, or both, were available (Supplementary Table 1). Based on these data, we have identified that the allelic frequencies of 23 markers among Malaysians differed from that of either Han Chinese or European, or both (Table 3). Where available, we also extracted the minor allele frequencies (MAF) of major populations listed in HAPMAP viz. YRI (Yoruba tribe of Ibadan), ASW (African ancestry in southwest USA), GIH (Gujarati Indians of Houston), LWK (Luhya in Webuye, Kenya), TSI (Tuscans of Italy) and JPT (Japanese of Tokyo area). Most of these markers have a MAF of <10%, with the exception of rs61318752 (CHB), rs127208519 (YRI, ASW and LWK), rs10422244 (YRI, ASW and LWK), rs12720828 (CEU and TSI) and rs10199768 (CEU, YRI, ASW, GIH, LWK and TSI). Interestingly, rs61318752 has a reported MAF of 11.4% among Han Chinese, but was devoid of minor allele among Malaysians even though our Malaysian subjects also included 39 individuals of ethnic Chinese origin. One possible reason for this discrepancy is that almost all Chinese Malaysians are of Southern Chinese origin; and the minor allele of rs61318752 could be absent in population which originates from Southern China. Also, it is of interest to see that rs12720851, rs10422244 and rs17111557 have a MAF in the range of 10–18% for the African ancestry populations, YRI and LWK showing a positive correlation between the two tribes. From the figures listed, it seems that they both share common antecedents. Joubert *et al.*,²⁶ in their study on African ancestry populations also show the average r^2 between two SNPs of YRI and LWK to be around 0.857. Adjacent SNP-SNP linkage disequilibrium was highly correlated across populations of African ancestry except for the Malawian Population. Geographic distance and inbreeding seem to be the two most common parameters for genetic heterogeneity. Thus, the frequencies of risk alleles of SNPs and mutations defining the susceptibility to ADH differ significantly between population to population and natural selection and genetic drift are the two commonest factors responsible.

FST is an appropriate index of allele-frequency differentiation, which captures the difference in allele frequency between two populations at any given SNP and ranges from 0 (no differentiation) to 1 (fixed difference between populations). FST values plummet if there is greater gene flow and less of inbreeding. With migrant number influx increasing into each population, it is expected that FST will decrease further but we still see such sharp genetic differences between populations. (At equilibrium between drift and gene flow, $FST = 1 / (1 + 4Nm)$, where Nm is the number of migrants moving into each sub-population.²⁷ It is most likely that it is due to selection.) SNPs follow peculiar distribution according to the geographic nature of the selection pressure.

Although FST has a theoretical range of 0–1.0, the observed maximum is usually much <1.0. Wright (1978) suggests the

following qualitative guidelines for the interpretation of FST (based on allozyme loci):

- the range 0.0–0.05 may be considered as indicating little genetic differentiation
- the range 0.05–0.15 indicates moderate genetic differentiation
- the range 0.15–0.25 indicates great genetic differentiation
- values of FST above 0.25 indicate very great genetic differentiation

The basis of such varied differentiation is still a partial mystery. The types of selection acting on SNPs in a particular population need to be understood better.

Common alleles are shared by all populations. Genetic drift or natural selection reduces heterozygosity by the random loss of alleles. Infinitely large populations will not experience drift whereas small populations will experience major drift. Drift is thus one of the major forces of evolution along with natural selection, mutation, non random mating etc.

The two markers rs41288783 and rs10422244 in *APOB* and *LDLR*, respectively, show an FST of ~0.3, which is quite pronounced and the MAF also correlates as well (Table 3). The frequency of a risk allele discovered in one population is not always a strong predictor of the disease frequency in other populations. Several disease-associated alleles or protective alleles appear to have been driven to high frequency by positive selection in certain human populations.

Out of these 23 SNPs reported in ADH cases, rs1801703, rs12720848, rs568413, rs12713844, rs41288783 lead to non-conservative amino-acid replacements of Val to Met, His to Asn, Cys to Tyr, Asp to His, Pro to Leu, respectively, but to our dismay, we found the wild-type allele present in the Malaysian population, both in cases and controls, proving that either these variations thought to contribute to ADH susceptibility as seen in other populations is not a cause of concern in this ethnicity or more robust functional assays are expected on these SNPs to ascertain an associative role.

Genetic heterogeneity in any complex disease leads us to hunt for novel genes and the risk alleles therein. Similarly genetic heterogeneity for ADH among different populations provides the possibility of uncovering other uncharacterized genes that may be involved in the pathogenesis of the disease. For example, in a Mexican population, no *PCSK9* mutations were found in one large ADH family that showed positive linkage to the 1p34.1–32 locus; this indicates genes other than *PCSK9* in the locus may be involved.²⁸ In addition, a Portuguese ADH study found only 48% of its total received cases with clinical diagnosis of ADH had genetic defects on *LDLR*, *APOB* or *PCSK9*, leaving the other 52% of ADH cases with possible undiscovered gene mutations.²⁹

In a study by Garcia *et al.*,³⁰ the researchers used gene chip (robo arrays) to resequence the coding regions of 10 key genes of lipid metabolism in 80 dyslipidemic cases. In all, 14 non-synonymous and 22 synonymous SNPs were identified, which were confirmed by conventional sequencing. Coding sequence variations thought to be associated with dyslipidemia were also seen in controls. This study is certainly a good evidence or instance for all researchers to understand that work on common variants and causal variant need to be published regardless of how significant or insignificant it may be considered.

There is a dire need for systematic sequencing of all genes involved in cholesterol metabolism in different populations to decipher which sequence variation causes moderate or high elevations of lipoproteins. Normally 10–20% of SNPs lead to a non-conservative change amino-acid replacement. SNPs are commonly used to study genetics for

Table 3 The list of mono-allelic markers that are polymorphic in various populations

Markers	Gene	Region	Nucleotide change	Amino-acid change	MAF (%)								MAF sources	F _{ST} values
					CHB	CEU	JPT	YRI	ASW	GIH	LWK	TSI		
rs1801703	APOB	Exon 29	[T/C]	Val4128Met	5	0.8	0.05	0	0	1.1	0.6	3.4	HapMap	0.004
rs12720851	APOB	Intron 27	[A/G]	N/A	0	0.4	0	13.4	14.3	N/A	10.6	1.7	HapMap	0.1385
rs12720848	APOB	Exon 26	[T/G]	His3182Asn	4.9	0.9	0	0	1	0.6	1.1	1.2	HapMap	0.0167
rs6413458	APOB	Exon 26	[A/G]	Ile2716Ile	0	1.7	0	2.5	N/A	N/A	N/A	N/A	HapMap	0.0000
rs1042013	APOB	Exon 26	[A/T]	Leu2680Gln	N/A	N/A	0.6	0	N/A	N/A	N/A	0.6	N/A	0.0040
rs12713771	APOB	Intron 25	[A/G]	N/A	0	0.8	0	0	N/A	N/A	N/A	N/A	HapMap	0.0040
rs12713844	APOB	Exon 22	[G/C]	Asp1113His	0	0.8	0	0	N/A	N/A	N/A	N/A	HapMap	0.0040
rs12713911	APOB	Intron 20	[T/C]	N/A	0	0.8	0	0	N/A	N/A	N/A	N/A	HapMap	—
rs12720828	APOB	Intron 20	[A/G]	N/A	3.5	18.8	2.3	0	2.1	12.2	N/A	14.8	HapMap	—
rs41288783	APOB	Exon 19	[A/G]	Pro994Leu	N/A	0.8	N/A	N/A	N/A	N/A	N/A	N/A	HapMap	0.3552
rs10199768	APOB	Intron 18	[A/C]	N/A	3.5	46	6	23.9	25.5	19.9	17.8	40.3	HapMap	—
rs12720779	APOB	Intron 17	[A/T]	N/A	N/A	0.8	N/A	5.9	2.1	N/A	N/A	N/A	HapMap	0.0056
rs3828293	APOB	Intron 14	[T/C]	N/A	1.2	0	0.6	0	N/A	N/A	N/A	N/A	HapMap	—
rs12720842	APOB	Intron 7	[A/G]	N/A	0	1.8	0	N/A	N/A	N/A	N/A	N/A	HapMap	—
rs1800478	APOB	Intron 5	[A/G]	N/A	0	1.3	0	12.1	5.1	N/A	7.2	1.1	HapMap	0.1835
rs12720796	APOB	Intron 4	[A/C]	N/A	0	1.7	0	0	N/A	N/A	N/A	N/A	HapMap	0.0233
rs12720789	APOB	Intron 4	[T/C]	N/A	0	0.4	0	1.8	N/A	N/A	5.6	N/A	HapMap	0.0423
rs10422244	LDLR	Intron 15	[T/C]	N/A	0	0.4	0	16.8	15.3	0.6	10.6	N/A	HapMap	0.3364
rs61318752	LDLR	Intron 6	[G/T]	N/A	11.4	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1000 Genomes Project	—
rs6658758	PCSK9	Intron 5	[T/C]	N/A	1.1	0	0	5.3	N/A	N/A	N/A	N/A	HapMap	—
rs28385711	PCSK9		[C/G]	N/A	N/A	2.5	N/A	N/A	0	N/A	N/A	N/A	Coriell cell repository (CCR)	—
rs17111555	PCSK9		[A/G]	N/A	0	0.9	4.1	8.4	5.1	N/A	13.9	N/A	HapMap	0.1285
rs17111557	PCSK9		[T/C]	N/A	0	0.8	4.1	10.6	7.1	N/A	17.4	N/A	HapMap	

Abbreviation: MAF—minor allele frequency

 F_{ST} values are from SNP at evolution

CHB—Han Chinese in Beijing, China CEU—Utah residents with Northern and Western European ancestry from the CEPH collection JPT—Japanese in Tokyo, Japan YRI—Yoruba in Ibadan, Nigeria ASW—African ancestry in Southwest USA GIH—Gujarati Indians in Houston, Texas LWK—Luhya in Webuye, Kenya TSI—Toscans in Italy

common diseases and predict pharmacological response. The selection of likely informative SNPs in association studies depends on their allele frequencies and on the linkage disequilibrium between SNPs, both of which may show inter ethnic differences.

Ng *et al.*³¹ compared the allele frequency distributions of 64 intragenic SNPs of 35 candidate genes for cardiovascular diseases in three populations consisting of 207 Chinese, 858 French and 395 Spanish individuals. In all, 28 of these SNPs from 12 genes were also examined for intragenic linkage disequilibrium. About 20% of SNPs were restricted to Europeans, being monomorphic in Chinese, among them mostly nonsynonymous coding SNPs and noncoding SNPs. Only 1.6% of SNPs were specific in Chinese, commensurate with the detection of these SNPs almost exclusively in Caucasians. Similarly, these SNPs were more often rare (<0.1 minor allele frequency) in Chinese (44.3%) than in Europeans (31.1%). The variant allele frequencies and intermarker linkage disequilibria in terms of D' and Δ^2 were highly correlated between French and Spanish populations ($r=0.98$ – 0.99 , $P<0.001$). However, only moderate correlations of allele frequencies and D' were found between the Chinese and the European populations ($r=0.7$ and 0.3 , respectively) despite a high correlation of Δ^2 values ($r=0.8$).³¹

Hence, it is very suggestive and ethical that we pay utmost care in interpreting meaning of variations in the clinical setting in various ethnicities. The 310 genetic markers studied represent only a small subset of all genetic alterations and diversities in *LDLR*, *APOB* and *PCSK9* genes that may potentially affect cholesterol metabolism,

homeostasis and ADH documented thus far. It is known that genetic variants associated with complex diseases vary from population to population.³² The different repertoires of genetic markers associated with hypercholesterolemia or causing ADH are population specific,¹⁸ more studies are needed to characterize the full range of genetic alterations among Malaysians. By disclosing the identities of these mono-allelic loci, we hope we may contribute to the better understanding of genetic markers associated with cholesterol metabolism and disorders in Malaysian population and provide useful insights.

CONFLICT OF INTEREST

The authors declare no conflict of interest. The views and interpretations expressed in this paper are those of the authors based on past studies and do not reflect the views of the management sponsoring the project.

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