Polymorphisms at newly identified lipid-associated loci are associated with blood lipids and cardiovascular disease in an Asian Malay population

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Abstract We conducted a cross-sectional study of Malay participants aged 40–80 years (n = 2,932) to examine the associations between polymorphisms at newly identified, lipid-associated loci with blood lipid levels and prevalent cardiovascular disease (CVD) in a Malay population in Asia. A polymorphism adjacent to the TRIB1 locus (rs17321515) was associated with elevated total cholesterol and LDL-cholesterol (LDL-C) after adjustment for age and sex (both P values <0.007) and with increased risk of coronary heart disease and CVD (odds ratio (OR) 1.23, 95% confidence interval (95% CI) 1.03–1.46; and OR 1.2, 95% CI 1.02–1.42, respectively) under an additive model of inheritance. In addition, using recessive models of inheritance, polymorphisms on chromosome 19 adjacent to the CILP2 and PBX4 loci (rs16996148) and on chromosome 1 at the GALNT2 locus (rs4846914) were associated with elevated HDL-C (P = 0.005) and lower LDL-C (P = 0.048), respectively. Although novel, the former is consistent with the association between this polymorphism and lower blood triglycerides observed in the initial studies conducted in populations of European ancestry. Neither showed statistically significant association with CVD. These observations should form the basis of further investigation to identify the causative polymorphisms at this locus, and also to understand the mechanistic roles that this protein may play in lipidprotein metabolism in Asians and other populations.

Blood lipids are key modifiable risk factors for coronary heart disease (CHD) and other cardiovascular diseases (CVDs) (1). They show significant heritability (2). Recently, through genome-wide association studies, we and others have identified single-nucleotide polymorphisms (SNPs) at six novel loci associated with blood lipid concentrations (3–6) and CHD (6). These observations were made primarily in individuals of European ancestry. Our initial study did include a multiethnic population derived from the 1998 National Health Survey in Singapore (3). This was a randomly selected sample of the population in Singapore. The study population comprised primarily Chinese. Despite oversampling of the minority ethnic groups in Singapore (Malays and Asian Indians), the numbers of these ethnic groups were small (781 Malays and 587 Asian Indians). More importantly, no data on the presence of CVD were available in that population that would...
allow us to assess the association between the presence of these polymorphisms and the risk of CVD.

The importance of studying these other ethnic groups becomes apparent when we examine the epidemiology of CVDs in the world today (7). Socioeconomic development, accompanied by rapid urbanization, has resulted in an epidemiologic transition in the burden of diseases from those associated with infection and malnutrition to those associated with noncommunicable chronic diseases. CVDs, ischemic heart disease in particular, represent some of the major causes of morbidity and mortality in developed countries today. In developing countries, this transition is still in progress, and many populations in Asia can be expected to experience a doubling of the burden of CVD over the next several decades. As a consequence, there has been emerging interest in the risk factors driving this increase and in potential preventive measures that can be taken in these countries. Most studies in this region have focused on Japanese, Chinese, and South Asians (8). Asia also comprises a large number of islands that have been jointly referred to as “other Asian islands.” The populations of these islands are ethnically distinct from those of China, Japan, and South Asia and can be expected to experience a greater increase in the burden of CVD than either India or China.

Malays and other related ethnic groups represent 300–400 million persons inhabiting these other Asian islands. This ethnic group represents a large understudied population in relation to CVD. Differences in genetic architecture between these and other ethnic groups could mean that the genetic variants identified that show associations with blood lipids and CVD in populations of European ancestry may have different effects in Malays compared with populations of Chinese or European ancestry. Furthermore, exposure to different lifestyles and environments in these populations resident in Asia may further modify the effect of genetic variation on blood lipids and CVD risk. To fill this knowledge gap, we examined a large cross-sectional population of Malays that was recruited independently from the population included in our original study. We assessed the association between polymorphisms at these novel loci and 1) blood lipid levels and 2) prevalent CHD and CVD.

MATERIALS AND METHODS

Study population

The Singapore Malay Eye Study is a population-based, cross-sectional epidemiological study of 3,280 adults residing in Singapore, aged 40 to 79 years. All participants were of Malay ethnicity. Although no data are available from participants from this particular study, recent genome-wide studies carried out in Chinese, Malays, and Asian Indians in Singapore suggest that all three ethnic groups are genetically homogeneous and distinct from each other, as well as from HapMap populations of European (Utah residents with Northern and Western European ancestry from the Centre Etude Polymorphism Humain collection) and West African (Yoruba in Ibadan, Nigeria) ancestry (unpublished data available to editors and reviewers on request). The study was approved by the Singapore Eye Research Institute Institutional Review Board. Informed consent was obtained from all participants. Details of the study design, sampling plan, and methodology have been reported elsewhere (9–11). Essentially, in April 2004, the Ministry of Home Affairs in Singapore provided an initial computer-generated list of 16,069 Malay names, derived from a simple random sampling of all Malay adults aged 40–79 years residing in 15 residential districts in the southwestern part of Singapore. The residential districts selected for this study were classified according to postal sectors and were chosen because according to the 2000 Singapore Census (12, 13), the residents were a fair representation of the Singapore population in terms of age distribution, housing type, and socioeconomic status. Therefore, we believe that our findings from this study population are broadly generalizable to the Malay population in Singapore. From this list of 16,069 names, we derived a final sampling frame of 5,600 names using an age-stratified random sampling strategy, selecting 1,400 from each decade from age 40 years onward (40–49, 50–59, 60–69, and 70–79 years) across the 15 residential districts. Of these, 4,168 individuals (74.4%) were determined to be eligible to participate. A person was considered ineligible if he or she had moved from the residential address, had not lived there in the past 6 months, was deceased, or was terminally ill (e.g., terminal cancer). Of the 4,168 eligible individuals, 3,280 participants (78.3%) took part in the study. Of the nonparticipants, 831 (95.6%) declined to participate and 57 (6.4%) were not contactable.

Of the 3,280 participants, 3,114 gave consent for DNA collection. Of these, 112 participants had insufficient DNA for genetic analysis. A total of 3,002 participants were genotyped; 2,932 with complete genotypes for all five SNPs were utilized in this analysis. These details are summarized in Fig. 1.

Information on CVD and risk factors

Although the study was primarily designed to assess the prevalence and risk factors for eye disease in Malays, one of the aims of the study was to examine the association between retinal vascular diameter and CVD. The levels of CVD risk factors as well as the presence of CVD were carefully ascertained as part of the protocol. Trained interviewers administered a standardized questionnaire. Current smoking was defined as smoking at least one cigarette per day. Individuals were classified into those who did or did not drink alcohol. Educational level was categorized into 1) less than elementary level (<6 years), 2) elementary (6 years), 3) high school (10 years), or 4) college or university (>11 years). A history of angina pectoris, myocardial infarction, or stroke, or use of cholesterol-lowering medication was obtained. CHD included either angina pectoris or myocardial infarction, and CVD included CHD or stroke.

Information on genetic variants and blood lipids

Genotyping was performed with Sequenom’s iPLEX Gold system, according to the manufacturer’s protocol (Sequenom, San Diego, CA). All assays gave a call rate above 98%. Five of the six polymorphisms identified in our previous genome-wide association study (3) were genotyped in this study population. A sixth polymorphism (rs12130333) was not included in this study; when it was first identified as a lipid-associated locus, it only showed an association with fasting triglyceride concentration, which was not measured in this study.

Total cholesterol, LDL-cholesterol (LDL-C), HDL-C, creatinine, and glucose were measured from nonfasting venous samples using enzymatic methods implemented in the Advia 2400 Chemistry System (Siemens Medical Solutions Diagnostics, Deerfield, IL). LDL-C and HDL-C were measured using direct assays.
Glycated hemoglobin A1C (HbA1C) was measured using high-pressure liquid chromatography on a Bio-Rad Variant II analyzer (Bio-Rad Laboratories, Hercules, CA), an assay accredited by the National Glycoprotein Standardization Program, with controls traceable to the Diabetes Control and Complications Trial. Diabetes mellitus was diagnosed if the participant reported a history of diabetes mellitus, or if the random (nonfasting) plasma glucose level was $\geq 11.1$ mmol/l. Estimated glomerular filtration rate was calculated using using the four-variable Modification of Diet in Renal Disease Study equation (14).

Statistical analysis

Statistical analyses were carried out using STATA version 9.1 for Windows (STATA Corp., College Station, TX). Associations between the SNPs were assessed by ANCOVA in participants with genotypic information for all five polymorphisms ($n = 2,932$). Preliminary analyses stratified by sex showed associations in the same directions for males and females. Hence, men and women were combined in subsequent analyses, which were adjusted for age and sex. Additional adjustment for current smoking, education level, alcohol consumption, body mass index, and diabetes mellitus were carried out. The associations with prevalent CHD or CVD were assessed using logistic regression, adjusted for age and sex. In line with recommendations for replication genotype-phenotype associations from the National Cancer Institute-National Human Genome Research Institute working group on Replication in Association Studies (15), statistical significance was first obtained using the genetic model used in the initial study (3), which was an additive model. Subsequently, additional analyses using a general effects, dominant and recessive models were carried out. $P$-values were derived from log-likelihood ratio tests on models with or without genetic information. Associations with blood lipid concentrations were assessed after excluding individuals ($n = 465$) who gave a history of taking cholesterol-lowering medication.

RESULTS

Table 1 shows the characteristics of the study population. Of the participants, 11.2% had a history of CVD; among these, 32.2% had a history of angina pectoris, 57.7% had a history of myocardial infarction, and 22.3% had a history of stroke. Some individuals reported more than one of these conditions. Participants with CVD were significantly older than those without. They were also more likely to be male, to be current smokers, to have diabetes mellitus, and to have lower HDL-C. Participants with CVD were also more likely to report taking lipid-lowering medication and had lower total cholesterol and LDL-C than those without CVD.
Table 2 lists the polymorphisms studied, along with their allele frequencies in the Malay population. One polymorphism (rs646776) showed significant deviation from Hardy-Weinberg equilibrium and was not included in subsequent analyses. The call rates for the other SNPs were generally high. We compared the lipid levels and the prevalence of CHD/CVD in those with complete genotype data against those with missing genotypes (see supplementary Table I). No significant differences were noted between those included in this analysis and those in whom genotyping failed.

Table 3 shows the associations between the individual polymorphisms and plasma lipids as well as CHD and CVD. Under an additive model of inheritance, only one of the polymorphisms (rs17321515) showed a statistically significant association with total cholesterol and LDL-C concentrations. The same polymorphism also showed a significant association with the presence of CHD/CVD. These associations remained statistically significant after additional adjustment for body mass index, smoking, alcohol ingestion, and the presence of diabetes mellitus. Adjustment for HbA1C (in place of diabetes mellitus), and estimated glomerular filtration rate also did not alter the associations observed (data not shown). An additional test of association between rs17321515 and CHD was carried out after excluding prevalent stroke cases from the controls. The odds ratio (OR) per allele was only slightly increased from 1.23 [95% confidence interval (95% CI) 1.03–1.46, \( P = 0.023 \) to 1.24 (95% CI 1.04–1.49), \( P = 0.017 \)).

We also carried out analyses under dominant and recessive models of inheritance (see supplementary Table II). In these analyses, rs16996148 adjacent to the CILP2/PBX4 loci, which showed a borderline association with HDL-C under an additive model (\( P = 0.065 \)), showed a statistically significant association with HDL-C under a recessive model (\( P = 0.005 \)), but no association with CHD or CVD. In addition, rs4846914 on chromosome 1 adjacent to the locus for GALNT2 also showed a borderline association with LDL-C (\( P = 0.048 \)), but not with CHD or CVD.

DISCUSSION

We describe genotype-phenotype associations, in a Malay population living in Singapore, for four out of six loci recently identified through genome-wide association studies of blood lipid levels. In our original study, the polymorphism adjacent to TRIB1, rs17321515, was associated with variation in LDL-C, HDL-C, and triglyceride levels.
Jun-kinase pathway (19). The latter study also reported smooth-muscle cell proliferation and chemotaxis via the proteins that act as secondary messengers in MAPK-related pathways. TRIB1 encodes tribbles-1, one of a family of pro-inflammatory proteins that may have a direct, non-LDL-C-mediated effect on the risk of cardiovascular disease (CVD). TRIB1 expression was elevated in human atherosclerotic arteries when compared with nonatherosclerotic controls, suggesting that this protein may have a direct role in the pathogenesis of CVD.

In our original report, a polymorphism on chromosome 19p13 in an intergenic region between CILP2 and PBX4 (rs16996148) was identified in association with lower LDL-C, but also showed an association with lower fasting triglyceride (3). In the current study, the association with LDL-C was in the same direction and of the same magnitude as that observed in populations of European ancestry, but did not reach statistical significance. However, we did not examine the association with triglyceride in the previous study, given the known negative correlation between triglyceride and HDL-C and the lower levels of triglycerides associated with the T allele in the original study.

In the Malmo Diet and Cancer Study, in which data under a general effects model were presented, the effect of the T allele on triglyceride (3) was similar to and in the same direction as that previously observed in populations of European ancestry, but did not reach statistical significance. Although we note a statistically significant association of the T allele with higher HDL-C under a recessive model of inheritance, this represents a novel observation. Although we did not examine the association with triglyceride in the current study, the elevated HDL-C is consistent with the previously observed association in populations of European ancestry, but did not reach statistical significance. However, we did not examine the association with triglyceride in the previous study, given the known negative correlation between triglyceride and HDL-C and the lower levels of triglycerides associated with the T allele in the original study. Indeed, in the Malmo Diet and Cancer Study, in which data under a general effects model were presented, the effect on triglyceride also appeared to be recessive. The roles that CILP2 or PBX4 might play in lipoprotein metabolism are unclear at this time. However, it should be noted that in vivo, TRIB1 expression was elevated in human atherosclerotic arteries when compared with nonatherosclerotic controls, suggesting that this protein may have a direct role in the pathogenesis of CVD.

### TABLE 3. Association of SNPs at newly identified lipid-associated loci with lipid levels and risk of heart disease

<table>
<thead>
<tr>
<th>SNP</th>
<th>11*</th>
<th>12*</th>
<th>22*</th>
<th>(P^a)</th>
<th>(P^b)</th>
</tr>
</thead>
</table>
| Total cholesterol [adjusted mean (SD), mmol/l] (n = 2,467)
| rs4846914    | 5.62 (1.45) | 5.6 (1.25) | 5.58 (0.98) | 0.54 | 0.493 |
| rs16996148   | 5.63 (1.61) | 5.57 (1.2)  | 5.52 (0.76) | 0.172 | 0.17  |
| rs17145738   | 5.61 (1.66) | 5.6 (1.26)  | 5.59 (0.56) | 0.849 | 0.706  |
| rs17321515   | 5.61 (1.25) | 5.61 (1.25) | 5.72 (1.22) | 0.0008 | 0.001  |
| LDL-cholesterol [adjusted mean (SD), mmol/l] (n = 2,467)
| rs4846914    | 3.63 (1.27) | 3.58 (1.09) | 3.54 (0.86) | 0.124 | 0.12  |
| rs16996148   | 3.62 (1.41) | 3.57 (1.05) | 3.53 (0.67) | 0.219 | 0.201  |
| rs17145738   | 3.6 (1.46)  | 3.6 (1.1)   | 3.6 (0.49)  | 0.981 | 0.982  |
| rs17321515   | 3.55 (1.09) | 3.61 (1.1)  | 3.68 (1.07) | 0.007 | 0.008  |
| HDL-cholesterol [adjusted mean (SD), mmol/l] (n = 2,467)
| rs4846914    | 1.23 (0.4)  | 1.24 (0.35) | 1.25 (0.27) | 0.137 | 0.085  |
| rs16996148   | 1.23 (0.45) | 1.23 (0.33) | 1.26 (0.21) | 0.113 | 0.065  |
| rs17145738   | 1.23 (0.46) | 1.24 (0.35) | 1.24 (0.15) | 0.954 | 0.554  |
| rs17321515   | 1.23 (0.35) | 1.23 (0.35) | 1.24 (0.34) | 0.84  | 0.962  |

**Coronary heart disease [OR (95% CI)] (n = 2,932)**

<table>
<thead>
<tr>
<th>SNP</th>
<th>1*</th>
<th>12*</th>
<th>22*</th>
<th>(P^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs4846914</td>
<td>1.05 (0.87–1.27)</td>
<td>0.066</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs16996148</td>
<td>0.98 (0.78–1.23)</td>
<td>0.865</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs17145738</td>
<td>0.92 (0.7–1.23)</td>
<td>0.584</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs17321515</td>
<td>1.23 (1.03–1.46)</td>
<td>0.023</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Cardiovascular disease [OR (95% CI)] (n = 2,932)**

<table>
<thead>
<tr>
<th>SNP</th>
<th>1*</th>
<th>12*</th>
<th>22*</th>
<th>(P^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs4846914</td>
<td>1.02 (0.86–1.22)</td>
<td>0.965</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs16996148</td>
<td>0.98 (0.8–1.21)</td>
<td>0.857</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs17145738</td>
<td>0.98 (0.75–1.26)</td>
<td>0.656</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs17321515</td>
<td>1.2 (1.02–1.42)</td>
<td>0.001</td>
<td></td>
<td></td>
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</tbody>
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\(P^a\) Adjusted for age, sex.
\(P^b\) Adjusted for age, body mass index, sex, education level, diabetes mellitus, current smoking, and alcohol ingestion.

Participants who gave a history of taking lipid-lowering agents (n = 465) were excluded from these analyses.

in individuals of European ancestry, but not in Asians. In the current study, with a larger sample size of Malays, the A allele was associated with increased levels of total cholesterol and LDL-C (as in those of European ancestry), but not HDL-C. The effect size for rs17321515 in LDL-C was similar to and in the same direction as that previously observed in populations of European ancestry. The presence of the polymorphism was also associated with an increased risk for prevalent CHD and CVD. In fact, the association between the polymorphism and CHD was unexpectedly large (OR 1.23 for each copy of the A allele), given the relatively modest effect on total cholesterol and LDL-C (0.1 mmol/l and 0.07 mmol/l, respectively). This finding is consistent with the large effect of mutations at the PCSK9 locus on CHD risk despite a relatively minor effect on blood lipids. In the study by Cohen et al. (16), a 1 mmol/l lower LDL-C associated with PCSK9 variants was associated with an 89% reduction in the risk of CHD in blacks, whereas a 0.5 mmol/l lower LDL-C was associated with a 47% reduction in the risk of CHD in Caucasians. This is possibly attributable to lifelong exposure to hypercholesterolemia in the presence of the genetic variant (17). Alternatively, this polymorphism may have a direct, non-LDL-C-mediated effect on the risk of CVD. TRIB1 encodes tribbles-1, one of a family of proteins that act as secondary messengers in MAPK-related signaling cascades (18) that is known to regulate vascular smooth-muscle cell proliferation and chemotaxis via the Jun-kinase pathway (19). The latter study also reported that in vivo, TRIB1 expression was elevated in human atherosclerotic arteries when compared with nonatherosclerotic controls, suggesting that this protein may have a direct role in the pathogenesis of CVD.
encoding neurocan (CSPG3). Although the literature on this latter molecule relates almost exclusively to the development and repair of neurological tissue (20), a search of the Unigene databases does suggest that it is expressed in vascular tissue. Furthermore, cell surface proteoglycans, although not specifically neurocan, have been demonstrated to influence macrophage sterol efflux through pathways that include the binding of macrophage-secreted apolipoprotein E (21, 22), suggesting a potential role in reverse cholesterol transport. Finally, polymorphism rs4846914 on chromosome 1 adjacent to the GALNT2 locus showed a borderline association with LDL-C under a recessive model of inheritance. This association was not observed in either of the original studies describing these novel loci.

The remaining SNPs did not show any association with lipid-related traits in this population, despite allele frequencies similar to those observed in individuals of European ancestry. Several possible reasons for this lack of replication exist. These SNPs have not been shown definitively to be functional variants. Differences in linkage disequilibrium between these SNPs and the functional variants in Malays compared with individuals of European ancestry at these loci could result in negative findings in our study population. Additionally, although the sampling scheme utilized in the current study was very similar to that for the Asian population in our previous study, the participants in this study were considerably older (aged 40–80) than those in our previous study (aged 18–69). Recent findings related to the association between ROBO1 variants and obesity have emphasized the importance of age-gene interactions that may result in nonreplication (23). We also cannot exclude the possibility that random sampling variation or the fact that the original findings may have been inflated (due to maximization in genome-wide association studies) could have resulted in nonreplication in the present sample.

The ability to study a relatively large sample of Malays, an ethnic group that represents a large proportion of individuals at risk of developing CVD in the coming decades, is one of the strengths of our study. We recognize the limited power to detect association with CVD for the less-common polymorphisms. Nevertheless, for rs17321515, we were able to replicate the association between this polymorphism and CVD, which has only been observed previously in individuals of European ancestry at these loci could result in negative findings in our study population. Additionally, although the sampling scheme utilized in the current study was very similar to that for the Asian population in our previous study, the participants in this study were considerably older (aged 40–80) than those in our previous study (aged 18–69). Recent findings related to the association between ROBO1 variants and obesity have emphasized the importance of age-gene interactions that may result in nonreplication (23). We also cannot exclude the possibility that random sampling variation or the fact that the original findings may have been inflated (due to maximization in genome-wide association studies) could have resulted in nonreplication in the present sample.

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Finally, owing to the cross-sectional nature of the study, we were not able to test the hypothesis that the association between rs17321515 and CHD/CVD was mediated by its effects on blood lipid levels. Because 41.5% and 40.4% of participants with CHD or CVD had a history of taking lipid-lowering medication, we felt that the lipid levels in the cases were likely to be a consequence of treatment given for the CHD/CVD and that any attempts to adjust for lipid levels would introduce confounding into the model. This is evident in the lower levels of total cholesterol and LDL-C in those with CVD compared with those without CVD. In addition, we cannot exclude the possibility that the observed association between rs17321515, LDL-C, and CHD/CVD, while biologically plausible, is due to a survival advantage among cases related to the presence of this SNP. To test both of these hypotheses would require prospective data from a cohort study in which lipid levels were measured prior to the occurrence of CHD/CVD. Such studies would represent an important next step in the investigation of the genotype-phenotype association related to these polymorphisms.

In summary, we have replicated associations between total cholesterol and LDL-C and a polymorphism adjacent to the TRIB1 locus in a Malay population. The polymorphism is also associated with CHD and CVD. In addition, we also observed an association between a polymorphism on chromosome 19 adjacent to the CILP2 and PBX4 loci, and elevated HDL-C, which, although novel, is consistent

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with the association between this polymorphism and lower blood triglycerides observed in the initial studies conducted in populations of European ancestry. However, this was observed under a recessive model of inheritance, a different model from that used in the initial studies through which the association between this polymorphism and lipid traits was first identified. This observation therefore requires replication in other studies. Finally, a polymorphism at the GALNT2 locus on chromosome 1 showed a borderline association with lower LDL-C under a recessive model of inheritance, an association that was not observed in the initial studies. These observations should form a basis for further investigation to identify the causative polymorphisms at this locus and to understand the mechanistic roles that this protein may play in lipoprotein metabolism in Asians and other populations.

REFERENCES


