A Novel Molecular Diagnostic Marker for Familial and Early-Onset Coronary Artery Disease and Myocardial Infarction in the LRP8 Gene

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Background—Many single-nucleotide polymorphisms have been associated with coronary artery disease (CAD)/myocardial infarction (MI) by genome-wide association studies, but the diagnostic value of these variants is limited. Functional single-nucleotide polymorphism R952Q in LRP8 is associated with familial and early-onset CAD/MI. The objective of this study is to test whether fine mapping and haplotype analysis for single-nucleotide polymorphisms flanking R952Q may identify a haplotype that may serve as a molecular diagnostic marker for familial and early-onset CAD/MI.

Methods and Results—Five single-nucleotide polymorphisms (rs7546246, rs2297660, rs3737983, R952Q, and rs5177) were genotyped and analyzed in GeneQuest (381 patients with familial, early-onset CAD and 183 patients with MI versus 560 controls) and the Italian population (248 patients with familial MI versus 308 controls). One novel risk haplotype, TACGC, was found only in patients with CAD and MI but not in controls. It was significantly associated with CAD ($P = 7.4 \times 10^{-5}$) and MI ($P = 2.2 \times 10^{-5}$) in GeneQuest. The finding was replicated in the Italian cohort ($P = 0.041$). Sib-transmission disequilibrium test analysis showed a significant association between haplotype TACGC and CAD in GeneQuest II ($P = 0.039$). Haplotype TACGC was not present in a South Korean population of 611 patients with CAD and 294 normal controls. TACGC/TACGC homozygotes tended to develop CAD/MI earlier and showed higher low-density lipoprotein cholesterol levels than heterozygotes ($P < 0.05$).

Conclusions—The rare haplotype TACGC in LRP8 confers a significant risk of familial, early-onset CAD/MI. Because the risk haplotype exists only in patients with familial and early-onset CAD/MI, we propose that it may be a molecular diagnostic marker for diagnosis of familial, early-onset CAD/MI in some white populations. (Circ Cardiovasc Genet. 2014;7:514-520.)

Key Words: cardiovascular diseases ▪ myocardial infarction ▪ polymorphism, single nucleotide

Coronary artery disease (CAD) and myocardial infarction (MI) are complex traits and the leading causes of death in the United States and other developed countries.1 Prior studies have documented the heritability of CAD and its most acute manifestation, MI.2 Development of CAD/MI is determined by multiple genes.3 A family history of early-onset CAD/MI is an independent risk factor for the development of CAD and MI.4 Many genetic factors have been identified to be involved in the pathogenesis of CAD and MI.5

Clinical Perspective on p 520

Previously, we described a genome-wide linkage analysis in 1613 individuals from 428 multiplex families with early-onset CAD and MI. We identified a genetic susceptibility locus for premature MI on chromosome 1p34-36 in an American white population.6 After analyzing candidate genes within the 1p34-36 MI locus, we found that single-nucleotide polymorphism (SNP) R952Q located in the low-density lipoprotein (LDL) receptor–related protein 8 (LRP8) gene was significantly associated with familial and early-onset CAD and MI using both population-based and family-based design.7 We also showed that SNP R952Q was associated with platelet activation. Functional studies showed that SNP R952Q was a gain-of-function variant that resulted in the increased phosphorylation of p38 mitogen-activated protein kinase.7

The LRP8 gene contains 5 linkage disequilibrium (LD or haplotype) blocks. Recently, we analyzed the association between LRP8 and CAD/MI by incorporating haplotype analysis of R952Q and its 4 neighboring SNPs in the same LD, the 5th LD at the 3’ terminus of the LRP8 gene. We found that a novel haplotype TCCGC in LD5 of the LRP8 gene confers a highly protective role in the development of familial and early-onset CAD and MI.8 In this study, we further analyzed the haplotype data of the 5 SNPs in LD5 of LRP8. We found a novel, rare risk
haplotype in the LRP8 gene, TACGC, which was detected only in 2 independent populations of patients with CAD and MI but not in controls. Haplotype TACGC was significantly associated with familial and early-onset CAD and MI.

Methods

Study Populations

The study involved 3 independent European-ancestry cohorts: GeneQuest, GeneQuest II, and the Italian Verona Heart Study and 1 Asian cohort from South Korea. The GeneQuest and GeneQuest II were American white families recruited at Center for Cardiovascular Genetics of Cleveland Clinic. The GeneQuest population consisted of 428 families with premature, early-onset CAD and MI, including 381 CAD probands and 183 MI probands. GeneQuest II consisted of 22 families with 441 family members and 140 CAD-affected individuals. For case–control studies, we selected probands from GeneQuest as cases and 560 controls without detectable stenosis by angiography. The Italian population was a case–control cohort enrolled in University of Verona, Italy, and consisted of 248 MI cases and 308 controls. Only white subjects were selected for the present study to avoid confounding ethnic factors. The South Korean cohort consisted of 611 sporadic patients with CAD and 294 normal controls ascertained at Samsung Medical Center, Seoul, South Korea. The demographic and clinical characteristics of the 4 study populations are described in detail in Table 1. The 4 populations were also used in previous studies.10,11 This study was approved by local Institutional Review Boards on Human Subject Research, and written informed consent was obtained from all participants.

Genotyping of SNPs

Whole blood samples were drawn from each participant, and genomic DNA was isolated from the blood using standard protocols.

The criteria for SNP selection and genotyping methods as well as the genotyping data for 5 SNPs, including rs7546246, rs2297660, rs3737983, R95Q, and rs5177 within the last LRP8 LD block (LD5), were reported previously.7,8 Briefly, TaqMan SNP genotyping assays were purchased from ABI (Applied Biosystems, Foster City, CA). SNP genotyping was performed as previously described.11 High-throughput SNP genotyping was performed on an ABI PRISM 7900HT Sequence Detection System. The PCR automatic allele calling was performed by ABI PRISM 7900HT data collection and analysis software version 2.1.

To ensure the quality of SNP genotyping by TaqMan assays, direct DNA sequence analysis was used to genotype SNP rs2297660 in the entire GeneQuest cohort and other 4 SNPs in randomly selected 32 samples. The results from TaqMan assays completely matched the sequencing data. Direct DNA sequence analysis was performed using the BigDye Direct Cycle Sequencing kit and with an ABI PRISM 3100 Genetic Analyzer (ABI, Foster City, CA).

Statistical Analysis

We performed both population-based case–control association studies and family-based association studies. Haplotypes were estimated using PHASE software (version 2.1.1). The frequencies of haplotypes were also estimated using PHASE version 2.1.1.

For population-based case–control association studies, the association of an SNP haplotype with a disease trait was assessed using the Fisher exact test implemented in SAS version 9.00. The P values from Fisher exact tests were referred to as $P_{\text{obs}}$. Logistic regression analysis was used to adjust for covariates of CAD and MI, and the adjustment P value was referred to as $P_{\text{adj}}$.

Empirical P values were calculated by performing 10000 Monte Carlo simulations using the CLUMP program.12 The P values from simulation tests were referred to as $P_{\text{sim}}$. Family-based association studies were analyzed using Sib-TDT analysis, which was performed using the TDT/S-TDT program 1.11

Statistical analysis of quantitative traits for the 19 study subjects with the risk haplotype were performed using the Wilcoxon rank-sum test as implemented in the R×64 3.0.1 statistical package. A value of $P$≤0.05 was considered to be statistically significant.

Results

Identification of a Risk Haplotype TACGC in LRP8 Associated With Familial and Early-Onset CAD and MI

A significant association was identified between each SNP within LRP8 LD5, including rs7546246, rs2297660,
rs3737983, R952Q, and rs5177, and CAD or MI as reported by us previously.\(^7\)\(^8\) Haplotype analysis was performed for genotyping data of LRP8 SNPs rs7546246, rs2297660, rs3737983, R952Q, and rs5177. We identified 1 haplotype in the GeneQuest population, TACGC, which was present in 3.1% of patients with CAD and 4.2% of patients with MI but did not exist in 560 controls. This haplotype conferred a significant risk of CAD \((P_{\text{obs}}=2.5\times10^{-12}; P_{\text{adj}}=7.4\times10^{-7}; P_{\text{emp}}<1.0\times10^{-7})\) and MI \((P_{\text{obs}}=1.2\times10^{-12}; P_{\text{adj}}=2.2\times10^{-6}; P_{\text{emp}}<1.0\times10^{-7})\;\text{odds ratio} = \infty\) because of the absence of this risk haplotype in the control population (Table 2).

For association studies, the positive finding in the discovery population needs to be replicated in another independent population. Thus, we also used a 2-stage association strategy in our experiment design. As in the GeneQuest population (discovery population), the TACGC haplotype was identified in patients with MI only in the Italian population but not in the normal Italian population (replication population). The TACGC risk haplotype was also significantly associated with familial MI in the Italian cohort \((P_{\text{obs}}=0.042; P_{\text{adj}}=0.041; P_{\text{emp}}=0.027;\) Table 2). We also performed haplotype analysis in a South Korean population consisting of 611 patients with CAD and 294 normal controls from Samsung Medical Center, South Korea. The TACGC risk haplotype did not exist in the South Korean population.

The association between TACGC risk haplotype and CAD/MI was also confirmed in the combined GeneQuest and Italian populations \((P_{\text{obs}}=2.4\times10^{-8}; P_{\text{adj}}=1.7\times10^{-6}; P_{\text{emp}}<1.0\times10^{-7};\) Table 2). Together, these data suggest that a rare LRP8 risk haplotype TACGC confers a significant risk of familial and early-onset CAD and MI in the 2 independent white populations.

In the GeneQuest cohort, one other risk haplotype, CCTAG, showed strong association with CAD \((P_{\text{obs}}=1.0\times10^{-4}; \text{odds ratio}=18.2)\) and MI \((P_{\text{obs}}=2\times10^{-4}; \text{odds ratio}=22.2)\). In the Italian cohort, another risk haplotype, CATAG, was significantly associated with familial MI \((P_{\text{emp}}=0.012)\). However, these positive results could not be cross-replicated between the GeneQuest and Italian populations.

### Structural Characterization of the TACGC Risk Haplotype by Fine Mapping and Sequencing Analysis

To further characterize the TACGC risk haplotype, we genotyped 19 individuals carrying the TACGC haplotype with 6 SNPs upstream of the TACGC risk haplotype and 18 SNPs downstream of it. We then analyzed the recombination events between the genotyped SNPs. As shown in the Figure, individuals 3, 7, and 17 showed a recombination event between SNP rs1288516 and rs7546246, which defines SNP rs1288516 as the upstream flanking SNP of the risk haplotype TACGC. Multiple individuals, including 6, 13, 15, and 19, showed a recombination event between SNP rs17785388 and rs6677126, which defines SNP rs6677126 as the downstream flanking SNP of the risk haplotype TACGC (Figure). Therefore, the TACGC risk haplotype is defined between SNP rs1288516 and rs6677126, a region spanning intron 4 of LRP8 to 3’-untranslated region.

We then hypothesized that there may be a rare mutation within the TACGC risk haplotype that may be causative to CAD/MI. Thus, DNA sequence analysis was then performed in the coding regions and exon-intron boundaries within the entire TACGC risk haplotype of LRP8 in the 19 patients. We identified 4 previously known SNPs (rs2297660 in exon 9, rs5173 in exon 17, rs3737983 in exon 17, and rs5174 in exon 19). Although SNP rs5173 was also on the haplotype, it was not selected for genotyping in the initial experimental design because it was only 75 bp from SNP rs3737983. Two new SNPs, c.89C>G in intron 2 and c.70T>C in intron 11, were identified. We did not identify any nonsynonymous variant in exons.

### Homozygous CAD Patients With Genotype TACGC/TACGC Showed an Earlier Age of Onset and a Higher Level of LDL Cholesterol Than heterozygotes With TACGC/TCCGC

We analyzed risk factors of CAD and MI, including age, body mass index, total cholesterol, high-density lipoprotein cholesterol, LDL cholesterol, and triglyceride levels, in 19 individuals carrying the TACGC haplotype. Interestingly, homozygotes (TACGC/TACGC) seemed to present with younger age of onset \((38.67±1.2\text{ years})\) than heterozygotes with the risk haplotype TACGC and a protective haplotype TCCGC defined in a previous study\(^9\) (TACGC/TCCGC, 44.0±3.5 years; \(P=0.032\); Table 4). Homozygous patients (TACGC/TACGC) had a significantly higher LDL cholesterol level than heterozygous patients with genotype TACGC/TCCGC \((P=0.036;\) Table 4).

No significant difference was found for systolic blood pressure, diastolic blood pressure, and hemoglobin A1c between homozygotes and heterozygotes (Table 4). The levels of high-density lipoprotein cholesterol levels, total cholesterol, very-LDL cholesterol, triglyceride, and body mass index showed a trend to be higher in homozygotes than in heterozygotes, whereas the levels of fasting glucose seemed to be lower in homozygotes than in heterozygotes, although these differences did not reach a significant level, probably because of a small sample size (Table 4).

### Discussion

Genome-wide association studies have identified \(\approx 50\) susceptibility SNPs or loci for CAD and MI.\(^1\)\(^4\)\(^13\)\(^15\) Many other CAD-MI-associated SNPs were identified by genome-wide linkage analysis followed by association studies for candidate genes in the linked region and by general candidate
LRP8 Haplotype as a Diagnostic Marker for CAD/MI

Table 2. Association Analysis of Haplotype TACGC of LRP8 With CAD and MI in GeneQuest and Italian Populations

<table>
<thead>
<tr>
<th>Population</th>
<th>Haplotype</th>
<th>Frequency</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Case (%)</td>
<td>Control (%)</td>
</tr>
<tr>
<td>GQ CAD</td>
<td>TACGC</td>
<td>3.1</td>
<td>0.0</td>
</tr>
<tr>
<td>GQ MI</td>
<td>TACGC</td>
<td>4.2</td>
<td>0.0</td>
</tr>
<tr>
<td>Italian MI</td>
<td>TACGC</td>
<td>0.7</td>
<td>0.0</td>
</tr>
<tr>
<td>Combined</td>
<td>TACGC</td>
<td>2.4</td>
<td>0.0</td>
</tr>
</tbody>
</table>

CAD indicates coronary artery disease; Combined, a combination population with CAD patients in GeneQuest (381 cases vs 560 controls) and Italian (248 cases vs 308 controls) together (629 cases vs 868 controls); GQ, GeneQuest; GQ MI, GQ myocardial infarction (183 cases vs 560 controls); P_{adj}, P value obtained after adjustment for age and sex with logistic regression analysis; P_{perm} permutation P value calculated using 1000 to 1000000 simulations using the CLUMP program; and P_{perm} uncorrected P value for association analysis using Fisher exact tests.

Note: Because the frequency of the risk haplotype in the control population is 0, an odds ratio (OR) cannot be generated because the denominator is 0 and the OR will be +∞.

The risk haplotype TACGC was not found in a South Korean population consisting of 611 patients with CAD and 294 normal controls. Thus, the diagnostic value of risk haplotype TACGC for CAD and MI in the South Korean population may be limited. Together, it seems that the TACGC risk haplotype had a high frequency in patients with familial and early-onset CAD/MI (3.1% in GeneQuest CAD and 4.2% in GeneQuest MI) and a low frequency in patients with familial MI (0.7% in our Italian cohort) and was absent in the general CAD population (0% in our South Korean cohort). The data suggest that the TACGC risk haplotype is present mostly in patients with familial, early-onset CAD/MI but absent or rare in the general CAD population. This may explain why the risk haplotype is not present in the control group of either the GeneQuest or the Italian cohort because controls were from the general population and not from families at risk. However, we cannot exclude the possibility that the absence of the TACGC risk haplotype in the South Korean population may be because of ethnic specificity.

It is interesting to note that homozygous CAD patients with 2 copies of the risk haplotype (TACGC/TACGC) tend to develop CAD/MI earlier than heterozygotes with only 1 copy of the risk haplotype (P=0.032; Table 3). Furthermore, homozygous patients (TACGC/TACGC) had a significantly higher LDL population, 4.2% in the GeneQuest MI population, and 0.7% in the Italian MI population, but 0% in both GeneQuest and Italian controls. Haplotype TACGC conferred a high risk of CAD/MI with a P_{adj} value of 7.4×10^{−2} in the GeneQuest CAD population and 2.2×10^{−9} in the GeneQuest MI population. The finding was replicated in an Italian population. In meta-analysis of combined GeneQuest and Italian populations, the risk haplotype remained significantly associated with CAD and MI (P_{adj}=1.7×10^{−6}). Because the risk haplotype TACGC was identified in patients with CAD/MI only, it may be of relevant diagnostic value for detecting individuals with a potential to develop CAD and MI.

Table 3. Sib-TDT Analysis for Association Between Coronary Artery Disease and Main Risk Haplotype in GeneQuest II

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>No. of Families</th>
<th>Z Score</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TACGC</td>
<td>7</td>
<td>2.081</td>
<td>0.039</td>
</tr>
</tbody>
</table>
cholesterol level than heterozygotes with 1 risk haplotype and 1 protective haplotype (TACGC/TCCGC; P = 0.036; Table 4).

These results are consistent with the fact that an increased LDL cholesterol level is a risk factor for CAD and MI. However, deep sequencing did not identify a nonsynonymous mutation in \textit{LRP8} that may be responsible for the increased LDL cholesterol.

### Figure

Fine mapping to define the boundaries of the TACGC haplotype. Nineteen patients with coronary artery disease/myocardial infarction (1 to 19) carrying the TACGC risk haplotype of \textit{LRP8} were genotyped with single-nucleotide polymorphisms (SNPs) upstream and downstream of the haplotype. The results define SNP rs1288516 as the upstream flanking SNP for the risk haplotype because individuals 3, 7, and 17 did not show recombination. SNP rs6677126 is the downstream flanking SNP because multiple individuals (6, 13, 15, and 19) showed recombination at this position.
level. Because intronic sequences are known to contain functional elements that may regulate gene expression through novel mechanisms, one possibility is that the risk haplotype may be associated with a functional intronic variant that can affect the expression level of LRP8 and further affect the LDL cholesterol level. But future studies are needed to test the hypothesis.

We also performed extensive fine mapping that defined the TACGC risk haplotype between SNP rs1288516 and rs6677126, a region spanning intron 4 of LRP8’-untranslated region. Sequencing analysis in the region, however, did not identify a potential causative variant for CAD and MI. Despite the fact that a causative variant was not found, we were able to identify a rare TACGC risk haplotype that seemed to be as good as a causative variant with regard to the diagnosis of CAD and MI.

Previously, we reported a common haplotype TCCGC of LRP8 that conferred protection against CAD/MI in GeneQuest and the Italian population. Compared with the risk TACGC haplotype shown here, the only difference between the 2 haplotypes is related to an A allele versus C allele of the second SNP, rs2297660. SNP rs2297660 is located in exon 9 but does not affect coding of LRP8 (synonymous SNP, Gly419Gly). Therefore, SNP rs2297660 is unlikely to affect the structure and function of LRP8. Furthermore, we searched existing expression quantitative trait loci databases (eg, Genevar or GENE Expression VARIation) and found that SNP rs2297660 was not associated with the expression level of LRP8. Therefore, it is likely that SNP rs2297660 is merely a marker for familial, early-onset CAD, and the mechanism responsible for the effect of the risk haplotype remains to be identified in the future.

It is important to note that this study has a few limitations. First, the sample size is small compared with that of contemporary studies related to genome-wide association studies of CAD and MI. The combined GeneQuest and Italian populations had only 629 cases and 868 normal controls available for use in our study. Second, although the TACGC haplotype confers a risk of CAD and MI in 2 independent populations (ie, Cleveland GeneQuest with familial, early-onset CAD/MI and the Italian Verona population with familial CAD/MI), more replication studies with large and multiple ethnic cohorts of familial and early-onset CAD/MI are needed to determine whether it is a high-risk haplotype in other populations. Third, recent large-scale genome-wide association studies did not identify an association between LRP8 and CAD/MI. We and others reported negative association between LRP8 and sporadic CAD/MI. Lieb et al reported negative association between LRP8 and familial CAD/MI in some European populations; thus, the association between LRP8 and CAD/MI may be limited to familial and early-onset CAD/MI in some specific geographic areas. Fourth, for sequencing analysis of 19 patients with the rare TACGC risk haplotype, we sequenced only exons and exon-intron boundaries within this haplotype block. We did not sequence introns because it is difficult to define intronic variants as functional variants. For example, we identified 2 new SNPs, c.89C>G in intron 2 and c.70T>C in intron 1, but it is difficult to define these 2 variants as causative variants for CAD and MI.

In conclusion, we have identified a rare, novel TACGC risk haplotype in the LRP8 gene that is present in patients with CAD and MI but not in normal controls. The TACGC haplotype is significantly associated with risk of CAD and MI in the GeneQuest population and an Italian population, as well as in the GeneQuest II population. The TACGC haplotype may serve as a novel molecular marker that may be potentially used as a screening test to identify patients at high risk for premature CAD in some white populations. At the time of online
publication of this study, Waltmann et al\textsuperscript{21} reported online that homozygous knockout mice of \textit{Lrp8} (\textit{Lrp8}\textsuperscript{-/-}) showed a phenotype of accelerated atherosclerosis at a \textit{Ldlr}\textsuperscript{-/-} background, supporting our finding that \textit{LRP8} is a susceptibility gene for CAD and MI.

### Acknowledgments

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### Disclosures

None.

### References


### CLINICAL PERSPECTIVE

Coronary artery disease (CAD) and myocardial infarction (MI) are the leading cause of death and disability. Many years of genetic and genomic research in CAD/MI have identified >50 genomic variants that either increase or decrease the risk of CAD and MI by genome-wide association studies, but the effects are too small to have a diagnostic value. We previously reported that a genetic variant, R952Q, in the \textit{LRP8} gene encoding low-density lipoprotein receptor–related protein 8 or apolipoprotein E receptor increased the risk of familial and early-onset CAD/MI. In this study, we investigated the structure of haplotypes derived from variant R952Q and 4 additional variants flanking R952Q in \textit{LRP8} and identified 1 novel haplotype, TACGC, that is present in patients with CAD/MI but not present in non-CAD controls. Statistical analyses showed that the TACGC haplotype increases risk of familial and early-onset CAD/MI in an Ohio-based GeneQuest CAD/MI population, an Italian-based population with familial and early-onset CAD/MI, and the second Ohio-based CAD/MI population consisting of large CAD/MI families (GeneQuest II). Our data show that the \textit{LRP8} TACGC haplotype may serve as a novel molecular diagnostic marker that may identify high-risk individuals for developing CAD in some ethnicities. Existing lifestyle and other measures can be taken by the high-risk individuals to prevent CAD/MI cardiac death.
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