Genetic Linkage of Serum Homocysteine in Dominican Families

The Family Study of Stroke Risk and Carotid Atherosclerosis

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Background and Purpose—Homocysteine levels are determined by genetic and environmental factors. Several studies have found high plasma levels of total homocysteine to be associated with premature onset of cardiovascular disease, stroke, and many other conditions. However, the exact mechanisms of documented and novel total homocysteine quantitative trait loci to that risk is unknown.

Methods—We have performed linkage analysis in 100 high-risk Dominican families with 1362 members. Proband were selected from the population-based Northern Manhattan Study. A set of 405 microsatellite markers was used to screen the whole genome. Variance components analysis was used to detect evidence for linkage after adjusting for stroke risk factors. Ordered-subset analysis based on Dominican Republic enrollment was conducted.

Results—Total homocysteine levels had a heritability of 0.44 (P<0.0001). The most significant evidence for linkage was found at chromosome 17q24 (maximum logarithm of odds [MLOD]=2.66, P=0.0005) with a peak at D17S2193 and was significantly increased in a subset of families with a high proportion of Dominican Republic enrollment (MLOD=3.92, P=0.0022). Additionally, modest evidence for linkage was found at chromosome 2p21 (MLOD=1.77, P=0.0033) with a peak at D2S1356 and was significantly increased in a subset of families with a low proportion of Dominican Republic enrollment (MLOD=2.82, P=0.0097).

Conclusions—We found a strong evidence for novel quantitative trait loci on chromosomes 2 and 17 for total homocysteine plasma levels in Dominican families. Our family study provides essential data for a better understanding of the genetic mechanisms associated with elevated total homocysteine levels leading to cardiovascular disease after accounting for environmental risk factors. (Stroke. 2010;41:1356-1362.)

Key Words: cardiovascular disease ■ Dominican families ■ genetic linkage ■ homocysteine

Cardiovascular disease and stroke are the most common causes of death in Western countries.1 Several studies have demonstrated that increased plasma levels of total homocysteine (tHcy)2 are associated with premature onset of cardiovascular disease3 and stroke.4 Homocysteine (Hcy)2 is formed from methionine as a result of cellular methylation reactions.5 The exact mechanisms by which Hcy promotes cardiovascular disease are not yet fully understood, although it has been proposed that Hcy may play a role in endothelial injury, high-density lipoprotein inhibition, thrombogenesis, and autoimmune response.6 However, clinical trials using vitamin B12 and folic acid to decrease the levels of tHcy failed to demonstrate a clinical benefit in secondary prevention against stroke6 or myocardial infarction.7 In contrast, other trials have shown benefits from vitamin B supplementation in high-risk patients with stroke8 but not in patients with myocardial infarction,9 suggesting that tHcy may play a pivotal role in stroke.

For these reasons, a great effort has been made to identify the genetic determinants of plasma tHcy. Polymorphisms in genes encoding for methylenetetrahydrofolate reductase have been associated with variations in plasma levels of tHcy. Specifically, the methylenetetrahydrofolate reductase 677 C→T polymorphism was the most important known genetic determinant of folate and tHcy status.10 We have previously reported that vascular risk associated with elevated tHcy levels is greatest among whites and Hispanics compared with blacks.9 Few studies have documented differences in heritability for tHcy by race-ethnicity,11,12 but the data are still limited. The aim of the present study was to detect novel quantitative trait loci, a region on a chromosome that influences the trait, for tHcy among high-risk Dominican families.
Table 1. Demographic and Clinical Characteristics by the Country of Enrollment

<table>
<thead>
<tr>
<th></th>
<th>Not DR Enrolled</th>
<th>DR Enrolled</th>
<th>Total</th>
<th>Testing DR Versus non-DR Enrolled Wilcoxon-Rank Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(N=844)</td>
<td>(N=402)</td>
<td>(N=1246)</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>45.5 ± 17.1</td>
<td>47.0 ± 17.0</td>
<td>46.0 ± 17.1</td>
<td>0.1269</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>29.3 ± 5.8</td>
<td>27.5 ± 5.7</td>
<td>28.7 ± 5.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Waist circumference, inches</td>
<td>36.8 ± 5.5</td>
<td>35.7 ± 5.6</td>
<td>36.4 ± 5.6</td>
<td>0.0016</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>185.5 ± 40.0</td>
<td>183.8 ± 42.5</td>
<td>185.0 ± 40.8</td>
<td>0.6545</td>
</tr>
<tr>
<td>Low-density lipoprotein, mg/dL</td>
<td>111.7 ± 35.4</td>
<td>106.4 ± 33.1</td>
<td>110.0 ± 34.8</td>
<td>0.0209</td>
</tr>
<tr>
<td>High-density lipoprotein, mg/dL</td>
<td>48.8 ± 14.0</td>
<td>53.0 ± 11.8</td>
<td>50.2 ± 13.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>126.7 ± 84.0</td>
<td>122.6 ± 81.7</td>
<td>125.3 ± 83.2</td>
<td>0.5463</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>120.2 ± 19.1</td>
<td>124.9 ± 21.5</td>
<td>121.8 ± 20.0</td>
<td>0.0005</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>76.0 ± 9.9</td>
<td>79.4 ± 12.2</td>
<td>77.1 ± 10.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total folate, mg/dL</td>
<td>530.5 ± 287.4</td>
<td>579.7 ± 308.9</td>
<td>546.4 ± 295.3</td>
<td>0.0054</td>
</tr>
<tr>
<td>Total B6</td>
<td>2.6 ± 1.7</td>
<td>3.0 ± 2.0</td>
<td>2.8 ± 1.8</td>
<td>0.0013</td>
</tr>
<tr>
<td>Total B12</td>
<td>5.9 ± 5.3</td>
<td>6.1 ± 5.1</td>
<td>6.0 ± 5.2</td>
<td>0.2926</td>
</tr>
<tr>
<td>Creatinine, mg/dL</td>
<td>0.9 ± 0.3</td>
<td>0.9 ± 0.6</td>
<td>0.9 ± 0.4</td>
<td>0.2472</td>
</tr>
<tr>
<td>Pack-years smoked, packs/day*years</td>
<td>3.7 ± 9.9</td>
<td>4.4 ± 11.4</td>
<td>3.9 ± 10.4</td>
<td>0.3519</td>
</tr>
<tr>
<td>Homocysteine, µmol/L</td>
<td>7.9 ± 3.1</td>
<td>10.9 ± 4.3</td>
<td>8.9 ± 3.8</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Materials and Methods

Subjects

Details of the Family Study of Stroke Risk and Carotid Atherosclerosis have been described in full elsewhere. Briefly, high-risk probands were selected from the population-based Northern Manhattan Study (NOMAS) according to the following criteria: (1) report of a sibling with a history of myocardial infarction or stroke; or (2) having 2 of 3 quantitative risk phenotypes (maximal carotid plaque thickness, left ventricular mass, or tHcy level above the 75th percentile in the NOMAS cohort). Most probands (80%) were recruited based on the first criterion. Families were enrolled if the proband was able to provide a family history, obtain consent from family members, and had at least 3 first-degree relatives able to participate. No probands were excluded by disabling or fatal vascular events prohibiting consent of family members. Although probands were identified in Northern Manhattan, we enrolled family members in New York (Columbia University) and in the Dominican Republic (DR; Clinicas Corazones Unidos, Santo Domingo). All subjects provided informed consent and the study was approved by the Institutional Review Boards of Columbia University, University of Miami, the National Bioethics Committee, and the Independent Ethics Committee of Instituto Oncologico Regional del Cibao in the DR.

Overall, 1362 individuals from 100 Dominican families with complete phenotype and genotype data were analyzed. Thirty percent of subjects were enrolled in the DR. Because sequential oligogenic linkage analysis routines analyzes relative pairs in an extended family framework, these 1362 individuals were part of a larger family structure of 2184 individuals and resulted in 1460 sib pairs, 452 half-sib pairs, and 2273 avuncular pairs. Mean family size was 22±11 (median, 20; range, 4 to 87).

Data Collection

Demographic, socioeconomic, and risk factor data were collected through interviews based on The Family Study of Stroke Risk and Carotid Atherosclerosis instruments. Questionnaires regarding diet, vitamin use, hypertension, diabetes, smoking, alcohol use, and physical activity were administered. Vitamin intake was assessed using the Block Food Frequency Questionnaire. Dietary folate, B12, and B6 intake were calculated from questionnaire responses using Block DIETSYS Version 3.0 software. This questionnaire was found reliable and valid in multiple epidemiological studies. Measurements of height, weight, hip and waist circumference, and skin-fold thickness were also obtained as were serial blood pressures.

Fasting blood samples were drawn into serum tubes and spun within 1 hour at 3000 g at 4°C for 20 minutes and frozen at −70°C. The blood samples were processed for lipids (total cholesterol, low-density lipoprotein, triglyceride, high-density lipoprotein), glucose levels, creatinine, methymalonic acid as well as tHcy. Fasting serum tHcy and methyamalonic acid were measured by licensed methods for commercial use.
Table 2. Sequential Oligogenic Linkage Analysis Routine Multipoint LOD Scores >1 and Ordered-Subset Analysis Results Ranking Families by Proportion of DR Enrollment

<table>
<thead>
<tr>
<th>Location</th>
<th>CM</th>
<th>Marker</th>
<th>LOD</th>
<th>P</th>
<th>h2q</th>
<th>Order</th>
<th>P (no. Family)</th>
<th>Maximum LOD</th>
<th>Order</th>
<th>P (no. Family)</th>
<th>Maximum LOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1p37</td>
<td>18</td>
<td>D1S508</td>
<td>1.34</td>
<td>0.0099</td>
<td>0.17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2p21</td>
<td>61</td>
<td>D2S1356</td>
<td>1.17</td>
<td>0.0033</td>
<td>0.21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3q25</td>
<td>26</td>
<td>D3S4545</td>
<td>1.59</td>
<td>0.0053</td>
<td>0.22</td>
<td>1.60 (0.79, 56)</td>
<td></td>
<td></td>
<td>2.82 (0.0097, 81)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3q27.3</td>
<td>200</td>
<td>D3S1262</td>
<td>1.21</td>
<td>0.0127</td>
<td>0.17</td>
<td>1.21 (1, 100)</td>
<td>1.55 (0.30, 92)</td>
<td></td>
<td>1.60 (0.79, 99)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4q15</td>
<td>78</td>
<td>D4S2367</td>
<td>1.13</td>
<td>0.0106</td>
<td>0.16</td>
<td>1.45 (0.23, 42)</td>
<td>1.25 (0.58, 98)</td>
<td></td>
<td>1.55 (0.30, 92)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9q34.3</td>
<td>164</td>
<td>D9S1838</td>
<td>1.14</td>
<td>0.0153</td>
<td>0.15</td>
<td>1.14 (1, 100)</td>
<td>1.52 (0.34, 64)</td>
<td></td>
<td>1.55 (0.30, 92)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15q11.2</td>
<td>1</td>
<td>D15S128</td>
<td>1.44</td>
<td>0.0082</td>
<td>0.22</td>
<td>1.71 (0.44, 36)</td>
<td>1.44 (1, 100)</td>
<td></td>
<td>1.55 (0.30, 92)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17q11.2</td>
<td>56</td>
<td>D17S1880</td>
<td>2.45</td>
<td>0.0006</td>
<td>0.26</td>
<td>3.23 (0.06, 58)</td>
<td>2.55 (0.60, 92)</td>
<td></td>
<td>1.55 (0.30, 92)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17q21.3</td>
<td>75</td>
<td>D17S2180</td>
<td>2.47</td>
<td>0.0006</td>
<td>0.30</td>
<td>3.92 (0.0022, 58)</td>
<td>2.48 (0.74, 92)</td>
<td></td>
<td>1.55 (0.30, 92)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17q24.2</td>
<td>91</td>
<td>D17S2193</td>
<td>2.66</td>
<td>0.0005</td>
<td>0.27</td>
<td>2.74 (0.57, 92)</td>
<td></td>
<td></td>
<td>1.55 (0.30, 92)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22q13.3</td>
<td>55</td>
<td>D22S1169</td>
<td>1.36</td>
<td>0.0097</td>
<td>0.20</td>
<td>1.59 (0.45, 98)</td>
<td></td>
<td></td>
<td>1.55 (0.30, 92)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**h2q** indicates locus-specific heritability; **P**, empirical *P*-based on 10 000 replicates; **H to L**, ranking families from high to low percent DR enrollment; **L to H**, ranking families from low to high percent DR enrollment.

Note: 37 of 100 families had no members living in the DR and, therefore, proportion of DR enrollment was 0. If Ordered-Subset Analysis results are blank, the maximum LOD occurred somewhere within the group of families with 0 proportion of DR enrollment (essentially randomly ordered since they are all 0), and so results are not valid for inference.

Genotyping and Quality Control

Extraction of DNA was done by the Columbia University Genome Center. DNA was sent to the Center for Inherited Disease Research for genotyping at Johns Hopkins University. A set of 405 microsatellite markers at an average interval of 10 cM across the genome was genotyped. Family structure was verified and adjusted using Relpair and PREST.19,20 Mendelian error checking was performed using Pedcheck.21

Statistical Analyses

**Heritability**

To minimize ascertainment bias, the sequential oligogenic linkage analysis routine ascertainment correction was used in all analyses. Heritability was estimated using a pedigree-based maximum-likelihood method implemented in sequential oligogenic linkage analysis routine.14 This heritability represents the genetic proportion of total phenotypic variance after the effect of all covariates has been removed (per sequential oligogenic linkage analysis routine parameterization). Thus, the residuals of tHcy are used for analysis and checked for normality (kurtosis <0.8) before proceeding. tHcy was natural-log transformed, multiplied by 10, and observations beyond 3 SD from the mean were dropped. An initial polygenic model was used to estimate significant covariates (P<0.10) that were used in final analyses.

Covariates that were tested included age, sex, age*sex, age², B12 deficiency, B6, B12, folate, vitamin use (self-report), creatinine, pack-years smoked, body mass index, alcohol use, and country of enrollment. Vitamin B12 deficiency was defined by methylmalonic acid level >0.8 before proceeding. tHcy was natural-log transformed, multiplied by 10, and observations beyond 3 SD from the mean were dropped. An initial polygenic model was used to estimate significant covariates (P<0.10) that were used in final analyses.

**Candidate Genes**

Genes located in the 1 LOD support interval surrounding each linkage peak (LOD >1) were identified using the University of California, Santa Cruz human genome annotation database (www.genome.ucsc.edu). Genes were considered as likely candidates if they belonged to the canonical methionine metabolism pathway and were in the SAM-dependent methyltransferase family of genes.11 In addition, genes related to Hcy in the gene database for the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/gene) were considered (Supplemental Table I; available at http://stroke.ahajournals.org).

**Ordered Subset Linkage Analysis**

Ordered subset linkage analysis22 was performed using proportion of family members living in the DR as the ranking phenotype. Family-specific LOD scores were output for quantitative trait loci with LOD >1 in the multipoint linkage analysis. For each peak, family-specific LOD scores were added in trait rank order (decreasing and increasing) until a maximum LOD (MLOD) score was obtained. A permutation procedure was implemented to test the hypothesis that ordering by family phenotype gave stronger linkage than random ordering. Specifically, 10 000 random family orderings were permuted and empirical probability values derived.

**Results**

Of the 1362 Dominican individuals, a total of 1246 were included in the final analysis after outliers and individuals with missing data for significant covariates were removed. The mean tHcy level was 8.9 μmol/L and was significantly higher in individuals living in the DR (Table 1). The covariate screening identified age, sex, age*sex, age², B12 deficiency, B6, B12, folate, creatinine, vitamin intake, pack-years of smoking, alcohol, and DR enrollment as significant covariates, explaining 50% of the total variance of tHcy. The heritability estimate of tHcy was 0.44 (P<0.0001). The proportion of the total variance of tHcy explained by genes was 22%.

To identify the genetic loci underlying variation in tHcy, we performed quantitative trait loci linkage analysis. This
identified 11 regions with a multipoint LOD > 1 on chromosomes 1, 2, 3, 4, 9, 15, 17, and 22 (Table 2; Figure 1). This included 2 distinct peaks on chromosomes 3 and 3 distinct peaks on chromosome 17. There were a total of 19 candidate genes in the 1-LOD support regions for all peaks (Table 3). The region on chromosome 17q24 was suggestive for linkage with a peak at D17S2193 (MLOD = 2.66, empirical probability value = 0.0005). The 1-LOD supportive interval across all 3 peaks extends approximately from 16.2 to 71.2 Mb on chromosome 17 encompassing 788 protein coding genes.

Among the 58 families with the highest proportion of DR enrollment, the LOD score significantly increased from 2.47 to 3.92 (P = 0.0022) on chromosome 17q21 at D17S2180 (Table 2; Figure 2B). The LOD score on chromosome 2p21 increased from 1.77 to 2.82 (P = 0.0097) among the 81 families with the lowest proportion of DR enrollment (Table 2; Figure 2A). In addition, this ranking strategy also reduced the 1-LOD supporting interval size on 17q from 55 Mb to 37 Mb. This narrowed critical linkage region harbors 565 protein-coding genes. The critical region on 2p extends from approximately 32.7 to 60.8 Mb and harbors 111 protein-coding genes.

Discussion

Several studies have shown that moderate and high tHcy plasma levels may play a pivotal role in increasing the risk for cardiovascular disease. We have shown that vascular risk associated with elevated tHcy levels differed by race–ethnicity. In the current study using quantitative trait loci mapping in extended DR families, we found 11 regions with suggestive linkage (multipoint LOD > 1) on 8 different chromosomes after controlling for significant covariates. The highest LOD scores were found on chromosomes 2 and 17. The heritability estimate of tHcy in our study was 0.44, which was similar to those reported in European populations.

The metabolism of Hcy is a complex system involving several enzymes and cofactors. Genetic analysis may help us to understand the mechanisms leading to higher levels of tHcy and increased risk for cardiovascular disease. The most widely studied variants have been in methylenetetrahydrofolate reductase, especially the methylenetetrahydrofolate reductase 677 C →T polymorphism, and 5-methyltetrahydrofolate homocysteine methyltransferase. Other polymorphisms that may affect plasma tHcy include methyltransferase 2756A →G, MTRR 66A →G, cSHMT 1420C →T, TC 67A →G, TC 776C →G, and GCPL1 1561C →T. In the current study, we report novel linkage for tHcy to chromosome 2p21 and to chromosome 17q21 in a Caribbean population. Different study populations and differences in the environmental factors may explain the lack of replication between studies.
Among the 565 protein coding genes in the 1-LOD supportive interval for chromosome 17, there are 2 genes related to Hcy metabolism: phenylethanolamine N-methyltransferase (PNMT), which binds the S-adenosyl-L-homocysteine and inhibits its synthesis; pyridoxamine 5'-phosphate oxidase (PNPO), which catalyzes conversion of pyridoxine 5'-phosphate to pyridoxal 5'-phosphate (PLP), the metabolically active form of vitamin B6 that is required as a coenzyme for Hcy metabolism; and methyltransferase like 2A (METTL2A) involved in the metabolism of the methionine cycle and therefore in the Hcy metabolism.

The improvement in LOD scores when accounting for the geographical location impacts tHcy levels with \( P=1.49e-20 \) for the effect of enrollment location in the polygenic model screen. Interestingly, folate levels are higher among those living in the DR than in the United States (Table 1). This seems counterintuitive because the United States has fortified certain foods with folate for over a decade, whereas the DR is just starting to fortify its foods with folate. Additional analysis (not shown) reveals that this higher folate level is actually driven by higher folate levels among younger (range, 18 to 40 years) residents in the DR compared with their US counterparts. Perhaps, despite folate supplementation in the United States, the younger immigrant US population may consume less folate-supplemented food. Further investigation may be needed to determine whether the younger US Caribbean Hispanic population characterized by low socioeconomic status levels and recent immigration lacks access to or chooses a low-folate diet.

We also found significant differences in B12 deficiency between the DR- and US-enrolled individuals (Table 1). We hypothesize that there is an additional unidentified environmental factor that causes those living in the DR to metabolize B12 poorly, although they are receiving adequate amounts in their diet and through supplements. Observations such as ours have been previously reported from the National Health and Nutrition Examination Survey (NHANES 1999 to 2004). In addition, a study from NHANES 1999 to 2002 showed that in
people with B12 deficiency, higher serum folate is associated with increased tHcy levels as seen in our subjects enrolled in the DR (Table 1). We have also found significantly higher waist circumference and body mass index in the Dominican participants enrolled in the United States than in the DR (Table 1). A suggestive linkage with dietary macronutrient (total calories, total proteins, total fat, saturated fat, monounsaturated fat, and polyunsaturated fat) intake and adiposity phenotypes within chromosome region 2p22 near marker D2S1346 was previously reported in extensive Mexican American families. This chromosome region neighbors our strongest linkage for tHcy (D2S1356). Further investigation between nutritional and environmental factors and variation in tHcy levels in various populations is warranted.

Strengths of the present study include the large Dominican family study with a comprehensive baseline assessment combined with rigorous phenotype measurement. By focusing on 1 ethnic group, we have minimized the effects of heterogeneity; however, this could explain why we did not replicate the results from other related studies. Approaches to mapping quantitative phenotypes also offer efficient statistical advantages over discrete traits. We believe some of the unknown determinants of tHcy may be related to diet or other environmental information, which has not yet been analyzed. One methodological limitation is that dietary intake was estimated using a single food frequency questionnaire that asked about food consumption over the prior 12 months, resulting in possible dietary misclassification.

With the unbiased genomewide approach, we identified several likely quantitative trait loci controlling tHcy plasma levels among Dominican families. We found novel evidence for linkage between regions in chromosomes 2 and 17 and tHcy levels in plasma. Our Family Study of Stroke Risk and
Carotid Atherosclerosis provides essential genetic and environmental data among Dominican families not available from other studies and may help to better understand the genetic mechanisms of increased tHcy levels leading to high risk of stroke and other vascular diseases.

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Disclosures
None.

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