Genome-Wide Linkage Mapping for Valve Calcification Susceptibility Loci in Hypertensive Sibships
The Hypertension Genetic Epidemiology Network Study


Abstract—It remains unclear whether genetic factors contribute to the susceptibility to valve calcification. Accordingly, echocardiograms and genotyping were performed in 1871 hypertensive siblings who participated in the Hypertension Genetic Epidemiology Network Study. Genome-wide affected sibpair nonparametric linkage analysis was conducted using the allele-sharing method implemented in the Merlin computer program. A total of 1014 sibships from 858 families were evaluated for aortic valve sclerosis or mitral annular calcification. Of these, 78 sibships from 68 families contained ≥2 affected siblings with ≥1 type of valve calcification (142 affected siblings). All 3 of the traits showed a modest degree of familial aggregation, with sibling recurrence risk (SD) and sibling recurrence risk ratio (95% CI) being 0.25 (0.035) and 2.31 (1.72 to 3.11) for aortic valve sclerosis, 0.25 (0.035) and 1.78 (1.36 to 2.33) for mitral annular calcification, and 0.31 (0.030) and 1.52 (1.24 to 1.85) for aortic valve sclerosis and mitral annular calcification, respectively. Affected sibpair linkage analysis revealed the highest logarithm of odds score (3.14) in chromosome 16 at 105.6 cM for aortic valve sclerosis. Other chromosomal regions with logarithm of odds score ≥1.9 were found in chromosomes 19 (2.88), 16 (2.63), 1 (2.12), and 2 (2.03) for aortic valve sclerosis and chromosome 13 (2.12) for any valve calcification. There was no logarithm of odds score ≥1.9 for mitral annular calcification. Our study shows strong linkage of aortic valve sclerosis to chromosome 16q22.1-q22.3 and suggestive linkage to chromosome 19p13.11-p11 and identifies several other promising genomic regions that may contain specific susceptibility loci for valve calcification. (Hypertension. 2007;49:1-8.)

Key Words: genetics ▪ valves ▪ calcification ▪ echocardiography ▪ epidemiology

Among older adults, valve calcification, that is, aortic valve sclerosis (AVS) or mitral annular calcification (MAC), is a common finding on echocardiography.1–4 Although it has been thought previously that these are generally benign conditions, recent evidence indicates the contrary. The Cardiovascular Health Study first identified that AVS was independently associated with 1.35-fold higher all-cause and 1.52-fold higher cardiovascular (CV) death in older adults without baseline CV disease.5 Recent reports from the Losartan Intervention for Endpoint Reduction in Hypertension Study indicated that AVS was associated with a higher composite end point of CV death, fatal and nonfatal myocardial infarction, and fatal and nonfatal stroke independent of covariates that included prevalent coronary heart disease, diabetes mellitus, left ventricular geometry, and ejection fraction or albuminuria.6,7 Data from the Framingham Heart Study found that each 1-mm increase in MAC was associated with a 10% increased risk of incident CV disease, CV death, and all-cause death. The associations of valve calcification with increased CV morbidity and mortality have been attributed to atherosclerosis, because the risk factors for valve calcification and atherosclerosis are similar.2,5–11

Recent studies have provided initial evidence that calcification in the CV system is, at least in part, under genetic control. Substantial proportions of the variability in coronary artery calcification, as assessed by electron beam computed tomography, and abdominal aortic calcification, as determined by lateral lumbar radiographs, have been found to be because of additive genetic effects.12,13 More recent evidence indicates that valve calcification is also influenced by genetic factors. In a case–control study, Ortlepp et al14 reported the distribution of vitamin D receptor polymorphisms in patients...
Methods
The HyperGEN Study is 1 of 4 components of the Family Blood Pressure Program, funded by the National Heart, Lung, and Blood Institute to assess the genetic basis of hypertension in population-based samples. As described previously, HyperGEN primarily relied on a sibling pair design that recruited hypertensive members of sibships in which ≥2 siblings with onset of high blood pressure, without known cause, by age 60 were willing to enroll in the study. The institutional review board of participating institutions approved the protocol, and all of the participants gave informed consent. HyperGEN participant characteristics have been described previously, and the protocol, and all of the participants gave informed consent. HyperGEN participant characteristics have been described previously, and the procedures followed were in accordance with institutional guidelines.

Results
Clinical Characteristics
In HyperGEN, there are 1871 hypertensive siblings from 858 families (1014 sibships) who were evaluated for AVS or MAC. Of these, 396 individuals from 319 families (329 sibships) were affected with ≥1 type of valve calcification, and 1475 siblings were unaffected. More than one third (142 of 396 [36%]) of the affected individuals were from families with ≥2 affected siblings (68 families and 78 sibships). Three sample pedigrees showing affected siblings with valve calcification are shown in Figure 1. The number of families and siblings with valve calcification is summarized in Table 1. Forty-one siblings had AVS only, 57 siblings had MAC only, and 44 siblings had both AVS and MAC. Twelve (6.1%) HyperGEN participants in the 68 families had unknown valve calcification status because of inability to evaluate 1 or both valves for calcification. HyperGEN participants with unknown valve calcification status were included in the analyses because they had genotype information. All 3 of the traits showed a modest degree of familial aggregation, with sibling recurrence risk (SD) and sibling recurrence risk ratio (95% CI) being 0.25 (0.035) and 2.31 (1.72 to 3.11) for AVS, 0.25

Echocardiographic Methods
Imaging and Doppler echocardiograms were performed using protocols and methods adapted from those used in previous studies from the Cornell Medical Center Echocardiography Laboratory. Correction orientation of planes for imaging and Doppler recordings was verified using standard procedures. Valve calcification was defined as the presence of AVS or MAC. AVS was defined as a focal area of echogenicity and thickening of the aortic valve leaflets on the long- or short-axis views with or without stenosis. MAC was defined as intense echogenicity at the junction of the atrioventricular groove and mitral valve leaflets in the parasternal and apical views. In a previous study that used identical echocardiographic methods, there was 90% consistency between readers to detect valve calcification. Participants with mitral stenosis (n=3) were excluded from the analyses.

Estimating Sibling Recurrence Risk Ratio for Valve Calcification
Sibling recurrence risk ratio, which is defined as the ratio of sibling recurrence risk to the overall population prevalence, is a commonly used index to measure familial aggregation of a disease trait. The HyperGEN Study, the ascertainment of hypertensive sibships was based on hypertension, not on valve calcification, so the proband status is unknown. Therefore, we used the method proposed by Olson and Cordell for the situation of unknown proband status and complete ascertainment to calculate sibling recurrence risk. The population prevalence was estimated from the randomly ascertained sample recruited as part of the HyperGEN Study (429 blacks and 263 whites).

Genotyping Techniques and Linkage Analyses
Genotyping was carried out by the National Heart, Lung, and Blood Institute Mammalian Genotyping Service using Cooperative Human Linkage Center Screening set 8, which included 387 microsatellite markers spaced out approximately every 9 cm throughout the genome and an average marker heterozygosity of 0.76. Analyses and assignment of marker alleles were done using computerized algorithms. The quality check of marker data was completed using a group of quality check programs (PEDSYS, MAPMAKER SIBS, ASPEX, and PEDCHECK) to assure Mendelian consistency at the Data Coordinating Center.

AVS and MAC were considered individually and as a single composite phenotype because of the strong association between AVS and MAC. In HyperGEN, after adjusting for age, sex, ethnicity, body mass index, systolic BP, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) cholesterol, the presence of AVS was independently associated with MAC (odds ratio: 4.8; 95% CI, 3.7 to 6.4; P<0.001). Because the mode of inheritance of valve calcification is unknown, evidence of linkage was assessed by a nonparametric or model-free approach. Both affected and unaffected siblings were included in the calculation of identical-by-descent statistics. Multipoint nonparametric linkage analysis was performed using the allele-sharing model program under the Kong and Cox exponential allele-sharing model implemented in the computer program Merlin. The exponential model had been shown to fit the data well when there are a small number of pedigrees with extreme identical-by-descent sharing. A nonparametric logarithm of odds (LOD) score, which estimates the statistical significance of identical-by-descent shared alleles between all affected relatives, was calculated with the use of the score function. Families were weighted equally when the family scores were combined to obtain an overall score. Allele frequencies were estimated based on marker allele frequencies of the random sample in each ethnic group separately (232 blacks and 212 whites). Genome scans were conducted separately for each ethnic group with the use of ethnicity-specific allele frequency, and, then, an ethnicity-combined LOD score was derived by summing LOD scores for all pedigrees output from the race-specific analyses.

Sibships in which ≥2 siblings with onset of high blood pressure, without known cause, by age 60 were willing to enroll in the study. The present study sought to identify specific chromosomal regions contributing to the presence of valve calcification using genome-wide linkage analyses in black and white hypertensive sibships participating in the HyperGEN Genetic Epidemiology Network (HyperGEN) Study.

with and without aortic stenosis and found a higher prevalence of the B allele in those with aortic stenosis. Novaro et al showed that the apolipoprotein E4 allele was an independent predictor of aortic stenosis but not MAC, although a more recent report suggested otherwise. However, these polymorphisms and clinical risk factors did not completely identify individual susceptibility to development of valve calcification, thus, other yet unidentified genes potentially exist. The present study sought to identify specific chromosomal regions contributing to the presence of valve calcification using genome-wide linkage analyses in black and white hypertensive sibships participating in the HyperGEN Genetic Epidemiology Network (HyperGEN) Study.

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and 1.78 (1.36 to 2.33) for MAC, and 0.31 (0.030) and 1.52 (1.24 to 1.85) for AVS and/or MAC, respectively.

As may be seen in Table 2, HyperGEN siblings with valve calcification (n = 142) were older and more likely to be male and had higher BP, LDL cholesterol, and serum creatinine; lower body mass index and HDL cholesterol; and higher prevalence of diabetes mellitus, coronary heart disease, and current smoking than HyperGEN siblings without valve calcification (n = 1475). Prevalent bicuspid AV and aortic stenosis was similar in the HyperGEN siblings with or without valve calcification. Demographic and clinical characteristics of siblings with valve calcification compared with those without valve calcification parallel findings in the entire HyperGEN population sample.29,30

**Linkage Analysis**

Whole genome linkage analysis is presented in Figure 2 and shows several chromosomal regions with LOD score ≥ 1.9 for valve calcification (Table 3). We chose to highlight LOD scores ≥ 1.9 as recommended by Lander and Kruglyak.31 We found the highest LOD score of 3.14 in chromosome 16 at 105.6 cM (between markers D16S516 and D16S402) for AVS. Other chromosomal regions with LOD score ≥ 1.9 were found in chromosomes 19, 16, 1, and 2 for AVS and chromosome 13 for the composite phenotype (Table 3). There were no LOD scores ≥ 1.9 for MAC alone.

**Discussion**

To our knowledge, this is the first study to identify specific chromosomal regions that may contribute to susceptibility to valve calcification. We found strong evidence for linkage of AVS in chromosome 16q22.1-q22.3 (LOD score: 3.14). Other chromosomal regions with LOD score ≥ 1.9 for AVS were found in chromosome 19p13.11-p11 (LOD score: 2.88), chromosome 1q42 (LOD score: 2.12), chromosome 16q22.1-q22.3 (LOD score: 2.63), and chromosome 2q37 (LOD score: 2.03). No chromosomal regions were linked to the presence of MAC alone. The presence of multiple suggestive peaks in several chromosomal regions suggests pleiotropy in susceptibility genes predisposing to valve calcification, each with probably small-to-moderate effects that summate to influence risk in the general population.32 Interestingly, whereas the presence of AVS is strongly associated with the presence of MAC, we found suggestive evidence of linkage of AVS and MAC in only 1 locus, chromosome 13q32-q33 (LOD score: 2.12), suggesting that genes may have differential effects depending on the type of valve involved or variable penetrance of mutated candidate genes.

We found the highest LOD score (3.14) for AVS in chromosome 16q22.1-q22.3 (Figure 3). The HDLC3 gene maps from 99.4 to 100.4 cM within 1 LOD support interval of the peak LOD score for AVS in this region.33 Pajukanta et al35 initially reported evidence of a susceptibility locus for low HDL cholesterol in Dutch and Finnish families in chromosome 16q. They found that variation in the human winged helix/foxhead transcription factor (FOXC2) gene was associated with low HDL cholesterol in Scandinavian families with familial combined hypercholesterolemia. Among HyperGEN participants, there was no evidence of linkage in variation in total cholesterol, LDL, and HDL cholesterol levels in this chromosomal region.34 Thus, although LDL cholesterol levels were slightly but statistically significantly higher, and HDL cholesterol levels were slightly but statistically significantly lower among HyperGEN siblings with valve calcification as compared with HyperGEN siblings without valve calcification, the significant linkage that we obtained in this region is unlikely the result of high LDL cholesterol or low HDL cholesterol levels, per se, but might reflect effects of cholesterol on valve calcification.35,36 Other

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**Figure 1.** Three sample HyperGEN pedigrees showing valve calcification affected (A), unaffected (U), or indeterminate (I) status. The left side of the circle or square represents affected status for AVS, whereas the right side represents affected status for MAC.

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**Table 1.** Distribution of Valve Calcification in HyperGEN Siblings

<table>
<thead>
<tr>
<th>HyperGEN Participants</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of families (family size)</td>
<td>68 (2 to 7)</td>
</tr>
<tr>
<td>No. of sibships (sibship size)</td>
<td>78 (1 to 7)</td>
</tr>
<tr>
<td>No. of subjects</td>
<td>198</td>
</tr>
<tr>
<td>No. of affected, %</td>
<td>142 (71.7)</td>
</tr>
<tr>
<td>AVS only, %</td>
<td>41 (20.7)</td>
</tr>
<tr>
<td>MAC only, %</td>
<td>57 (28.8)</td>
</tr>
<tr>
<td>AVS and MAC, %</td>
<td>44 (22.2)</td>
</tr>
<tr>
<td>Unknown valve status, %</td>
<td>12 (6.1)</td>
</tr>
<tr>
<td>Relative pairs</td>
<td></td>
</tr>
<tr>
<td>Affected sibling pairs</td>
<td>73</td>
</tr>
<tr>
<td>Affected half-sibling pairs</td>
<td>7</td>
</tr>
<tr>
<td>Discordant sibling pairs</td>
<td>83</td>
</tr>
<tr>
<td>Discordant half-sibling pairs</td>
<td>6</td>
</tr>
</tbody>
</table>
potential candidate genes within 1 LOD score support interval of the peak include musculoaponeurotic fibrosarcoma oncogene (at 104 to 105 cM) and cadherin 13 (at 111.2 cM).37,38 Musculoaponeurotic fibrosarcoma oncogene has been shown to be an essential transcription factor in interleukin-10 expression in macrophages39; interleukin-10 is associated with the development and progression of valve lesions in rheumatic heart disease.40 Cadherins are adhesion molecules that mediate calcium-dependent cell–cell adhesion.41 Cadherin 13 or T-cadherin.41

Horne et al42 found recent evidence of a genetic component for death resulting from nonrheumatic aortic and mitral valve disease. Using a genealogical index of familiality (GIF) that measures risk beyond first-degree relatives, the authors found excess relatedness in death resulting from aortic (GIF: 3.44) and mitral (GIF: 4.44) valve disease. Furthermore, when restricting to early age at death, the GIFs for aortic and mitral valve disease–related death increased (GIF: 8.47 and 5.66, respectively), suggesting a strong heritable component for death associated with valve disease. Our study extends the above findings by showing that valve calcification is also influenced by familial factors, as indicated by the sibling recurrence risk ratio (range: 1.52 to 2.10). The finding of a susceptibility locus for AVS in chromosome 16q and suggestive loci in several other chromosomal regions confirms that genetic factors influence the development of valve calcification, particularly AVS. Indeed, a recent report from Probst et al43 identified clusters of large families affected by classic forms of aortic stenosis in Western France. That study also indicated that offspring of affected individuals had AVS, suggesting that AVS may be an early manifestation of those affected with this familial form of aortic stenosis. Further studies are needed to clarify whether genes that influence valve calcification also influence valve stenosis and death from valve disease.

Recent studies indicate that mutations in NOTCH1 cause a spectrum of developmental aortic abnormalities and severe AVS in nonsyndromic autosomal-dominant human pedigrees and in patients with bicuspid AV.44,45 NOTCH proteins are single-pass transmembrane receptors that regulate cell fate decisions during development and include 4 receptors: NOTCH1, NOTCH2, NOTCH3, and NOTCH4.46 NOTCH1 and NOTCH2 map to chromosomes 9q34.3 and 1p13-p11, respectively.47 We found no evidence of linkage for valve calcification to these chromosomal regions. NOTCH3 maps to chromosome 19p13.2-p13.1 at 42.3 cM,47 within 1 LOD support interval of our peak with a LOD score of 2.88. Further studies are needed to determine the role of NOTCH3 in valve calcification. NOTCH4 maps to chromosome 6p21.3.48 We found no evidence of linkage for valve calcification in this locus. Although other studies have shown associations among apolipoprotein E, vitamin D receptor polymorphisms, and transforming growth factor β receptor (TGFBR) mutations to valve disease,14–16,49 we found no evidence of a linkage of valve calcification to the apolipoprotein E gene in chromosome 19q13.2, vitamin D receptor gene in chromosome 12q12-q14, TGFBR1 gene in chromosome 9q33-34, or TGFBR2 gene in chromosome 3p22.50–53 in our study.

Studies have indicated that substantial proportions of the variability in coronary artery calcification and abdominal aortic calcification, both markers of subclinical atherosclerosis, are because of additive genetic effects.10,11 The Genetic

| TABLE 2. Clinical Characteristics of HyperGEN Siblings With Valve Calcification |
|---------------------|---------------------|---------------------|---------------------|
| Variable            | Siblings With Valve Calcification* (n = 142) | P                  | Siblings Without Valve Calcification† (n = 1475) |
| Age, y              | 63±9                | <0.001              | 53±11               |
| Male, %             | 46                  | 0.01                | 35                  |
| Body mass index, kg/m² | 30.7±5.3           | 0.03                | 32.1±7.3            |
| Blood pressure, mm Hg  | 136/72±23/12        | 0.04/0.02           | 132/75±21/12        |
| Total cholesterol, mg/dL | 207±42             | NS                  | 200±39              |
| HDL cholesterol, mg/dL | 49±14              | 0.02                | 52±15               |
| LDL cholesterol, mg/dL | 128±36             | 0.03                | 121±36              |
| Serum creatinine, mg/dL‡ | 0.99 (0.61 to 1.62) | 0.02                | 0.95 (0.60 to 1.49) |
| Serum calcium, mg/dL | 9.37±0.48           | 0.22                | 9.42±0.46           |
| Serum phosphate, mg/dL | 3.47±0.59          | NS                  | 3.55±0.53           |
| Diabetes mellitus, % | 24.7                | NS                  | 18.6                |
| Coronary heart disease, % | 21.1                | <0.001              | 9.6                 |
| Current smoking, %   | 22.1                | 0.01                | 12.1                |
| Bicuspid aortic valve, % | 0.70                | NS§                 | 0.14                |
| Aortic stenosis, %   | 0.70                | NS§                 | 0.41                |

NS indicates not significant.

*Affected siblings included in the linkage analysis.
†Unaffected siblings from the whole population.
‡Geometric means (95% CI), naturally log-transformed value was used.
§Fisher’s exact test was used.
Figure 2. Genome-wide nonparametric linkage analysis results for valve calcification in HyperGEN siblings. LOD scores (y axis) and their respective centimorgan (x axis) from the p-telomere (left) to the q-telomere (right) are shown. Broken black line indicates AVS; solid black line, MAC; solid gray line, AVS and/or MAC (composite).
Epidemiology Network of Arteriopathy Study has recently reported linkage of coronary artery calcification to chromosomes 6 (76.4 cM; LOD score: 2.22) and 10 (91.8 cM; LOD score: 3.24). We found a suggestive peak in a different region (127 cM; LOD score: 1.82) in chromosome 10 for MAC (Figure 2). In our study, there were no suggestive peaks influencing valve calcification in chromosome 6 at or near the region identified by the Genetic Epidemiology Network of Arteriopathy Study. Whether similar genes influence valve and vascular calcification remain to be elucidated.

The purpose of the study was to identify specific chromosomal regions contributing to presence of valve calcification in hypertensive siblings. In the HyperGEN Study, there were 7 participants with aortic stenosis. None of these participants were included in the linkage scan for AVS, because they did not belong to families that had another affected sibling with AVS. However, 1 participant with aortic stenosis had a sibling with MAC and, thus, was included in the linkage analyses for the composite phenotype. Similarly, of the 3 HyperGEN participants with bicuspid AV, none were included in the linkage scan for AVS, but 1 participant with AVS had a sibling with MAC and, thus, was included in the linkage scan for the composite phenotype. Excluding these 2 sibpairs from the linkage analyses for the composite phenotype did not significantly alter the results (data not shown). As have been shown by the NOTCH1 and TGFBR1/TGFBR2 studies, affected individuals do not necessarily share the same cardiac abnormalities. Thus, our analysis focusing on valve calcification alone may actually have underestimated the identification of affected individuals in our cohort. At the present time, the roles of aging and elevated BP on the candidate genes that may be responsible for the observed linkage results remain to be clarified. The cross-sectional nature of our study precludes us from identifying the exact onset of valve calcification or assessing the effect of aging on regulation and modulation of candidate genes influencing valve calcification in the cohort. Valve calcification in older individuals could represent late expression of the phenotype or age-related penetrance of mutated candidate genes similar to that found in some forms of hypertrophic cardiomyopathy. Longitudinal studies are needed to determine the effect of age on candidate genes influencing valve calcification and disease. Similarly, because our cohort was ascertained for hypertension, we were unable to perform separate linkage analyses in siblings with or without hypertension to determine the effect of hypertension on candidate genes influencing valve calcification. In a recent report, Simmons et al implicated the role of endothelial phenotypes in regulating and modulating valve calcification in normal porcine aortic valves suggesting that hemodynamic factors influence the development of valve calcification. However, whereas there are relations between BP and valve calcification, further studies are needed to identify correlative links between BP and other hemodynamic variables to candidate genes influencing susceptibility to valve calcification.

Perspectives
Our study shows linkage of AVS to chromosome 16q22.1-q22.3 and suggestive linkage to chromosome 19p13.11-p11; several other promising genomic regions may contain specific susceptibility loci for valve calcification. Studies are underway to identify the specific genes contained in these novel chromosomal regions that we found in this study that are responsible for the observed linkage results. Recent studies indicate that valve disease is increasing with age and associated with adverse outcomes and, thus, represents an emerging public health problem. Although the identification of genes influencing valve calcification is challenging, it offers much promise in defining novel mechanistic paradigms and developing therapeutic strategies in the prevention and treatment of valve calcification and valve disease.

Acknowledgments
We thank study coordinators, cardiac sonographers, the HyperGEN participants, and the participating communities for the extraordinary effort.
cooperation and involvement that made this study possible. We wish to thank the University of Minnesota Supercomputing Institute for use of the IBM and Netfinity Linux supercomputers.

Sources of Funding

This study was supported in part by grants RO1 HL55673 and U10-HL-54471, 54472, 54473, 54495, 54496, and 54509 from the National Heart, Lung, and Blood Institute and M10RR0047-34 (General Clinical Research Center) from the National Institutes of Health. W.T. was supported in part by National Heart, Lung, and Blood Institute training grant T32-HL07972.

Disclosures

None.

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