Genomewide Linkage in a Large Dutch Family With Intracranial Aneurysms

Replication of 2 Loci for Intracranial Aneurysms to Chromosome 1p36.11-p36.13 and Xp22.2-p22.32

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Background and Purpose—Approximately 2% of the general population harbor intracranial aneurysms. The prognosis after rupture of an intracranial aneurysm is poor; 50% of the patients die as a result of the rupture. Familial occurrence of intracranial aneurysms suggests there are genetic factors involved in the development of such aneurysms.

Methods—A large, consanguineous pedigree with 7 of 20 siblings affected by intracranial aneurysms was compiled and a genomewide linkage analysis on this family was performed using Illumina’s single nucleotide polymorphism-based linkage panel IV, which includes 5861 single nucleotide polymorphisms. A nonparametric linkage affecteds-only approach with GENEHUNTER was used.

Results—Two loci with suggestive linkage (nonparametric linkage = 3.18) on chromosome regions 1p36 and Xp22 were identified. Additional microsatellite markers were genotyped in the 2 candidate loci and showed suggestive linkage to the locus on chromosome 1 with a nonparametric linkage of 3.18 at 1p36.11-p36.13 and significant linkage to the locus on chromosome X with a nonparametric linkage of 4.54 at Xp22.2-p22.32.

Conclusions—The 2 potential loci for intracranial aneurysms, which we identified in this large Dutch family, overlap with loci that have already been identified in previous linkage studies from different populations. Identification of genes from these loci will be important for a better understanding of the disease pathogenesis. (Stroke. 2008;39:1096-1102.)

Key Words: aneurysm ■ genetics ■ subarachnoid hemorrhage

Genetic factors are likely to be involved in the development of intracranial aneurysms (Mendelian Inheritance in Men 105800) because familial predisposition is the strongest risk factor for intracranial aneurysms and aneurysmal subarachnoid hemorrhage (SAH). Familial clustering of SAH is found in approximately 10% of patients with SAH, and first-degree relatives of patients with SAH have a 2.5 to 7 times greater risk of developing SAH than the general population. However, the segregation of SAH and intracranial aneurysms does not follow a strict Mendelian inheritance pattern in families, so the complex inheritance pattern seen is assumed to be caused by the interaction of several genes and environmental factors.

In complex diseases, the identification of susceptibility genes is hampered by their multigenic origin and genetic heterogeneity. Variants of susceptibility genes in complex diseases are expected to be common. Not all carriers of these common variants will become affected by the disease because they may not carry all the remaining disease-associated alleles of the susceptibility genes necessary to develop the disease. In addition, different combinations of genes may lead to the same phenotype in different populations. To overcome these difficulties, large single families can be studied in which the disease is expected to be genetically homogeneous and the disease-associated genes will lead to the same phenotype. Such large single families may represent highly penetrant and rare alleles.

We previously reported on a genomewide linkage study in a large, Dutch consanguineous family. Using a recessive mode of inheritance, positive evidence of linkage on chromosome 2p13 was found with a maximum logarithm of odds (LOD) score of 3.55. Because aneurysms develop over time, relatives with familial intracranial aneurysms are often screened at intervals; this yields a considerable benefit because new aneurysms are detected in approximately 10% of the relatives who previously had a negative screening.

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Indeed, since the completion of our genomewide linkage study in 2004, repeated screening has revealed newly developed aneurysms in 2 siblings of our family 5 years after their last negative screening. Because these newly affected siblings did not carry the risk haplotype of chromosome 2p13, our previous results of positive linkage, assuming an autosomal-recessive mode of inheritance, were no longer significant. A follow-up genomewide linkage study in this family was therefore decided on. A model-free, nonparametric linkage, affecteds-only approach was used to overcome the possible problem of complex etiology.

Subjects and Methods

Family

The family pedigree has already been described in our previous linkage study. Details of the family members are shown in Table 1 and Figures 1 and 2. The parents are first cousins and only their children are affected. DNA samples are available for the mother (I-2) and for 16 of her 20 children (see Table 1). The father (I-1) and the mother (I-2) of the children both died, but not from aneurysmal SAH. The father died at 62 years of age and the mother at 89 years of age. The children with aneurysmal SAH (II-6, II-9, and II-12) were defined by symptoms suggesting SAH combined with subarachnoid blood on a CT scan and a proven aneurysm on CT angiography or conventional angiography. Individual II-2 had an episode suggestive of aneurysmal SAH at the age of 36, but she died before a CT scan or angiography could be performed. Because there is no DNA sample available from this patient, she was not included in the present analysis. The children, II-11, II-15, II-17, and II-19, were diagnosed with an unruptured intracranial aneurysm identified by magnetic resonance angiography (MRA). The intracranial aneurysms in children II-11 and II-17 were identified after the end of our previous linkage study, 5 years after their last negative MRA. In our previous linkage study, individual II-13 was designated as having an unruptured intracranial aneurysm identified by MRA. However, at follow-up (MRAs done 1 and 6 years after the initial positive MRA), an aneurysm could no longer be visualized. This possible false-positive finding can be explained by the improved image quality of recent MRAs performed with increasing magnetic field strengths and leading to an improved sensitivity and, more importantly, to an improved specificity in detecting intracranial aneurysms. In our present analysis, individual II-13 was therefore designated as being unaffected. In conclusion, 7 of the 16 children participating in this analysis are affected and have been diagnosed with either ruptured or unruptured intracranial aneurysms.

Genotyping

A 2-stage design was used for the linkage analysis. First, a whole-genome screen was performed in all the available individuals (n = 17) using Illumina’s single nucleotide polymorphism (SNP)-based linkage panel IV. Although we use an affecteds-only approach, it is not sufficient to only genotype the affecteds. Nonaffected family members are also genotyped to determine the phase of the affecteds. Genomic regions of potential interest were then followed up using microsatellite markers.

Genotyping of Single Nucleotide Polymorphism Markers

Illumina’s SNP-based linkage panel IV includes 5861 informative SNP markers distributed evenly across the human genome with an average...
distance of 0.64 cM.\textsuperscript{17} The information content of this linkage panel is comparable to standard 5 cM short-tandem-repeat marker maps. The SNPs were genotyped using the BeadArray technology on an Illumina BeadStation following the manufacturer’s protocol (www.illumina.com). All SNPs were examined for their resulting quality and those that had a low signal or were poorly clustered were excluded (n=109).

Genotyping of Microsatellite Markers

In potentially interesting regions (see “Linkage Analysis” section), additional microsatellite markers were genotyped (n=24; see supplemental Table I, online only). The markers were selected from the Marshfield Center for Medical Genetics marker set. Genotyping for the microsatellite analysis was performed by polymerase chain reaction with detection of fluorescent polymerase chain reaction products on a 3700 DNA sequencer (Applied Biosystems) and analyzed with GeneScan and Genotyper software (Applied Biosystems). For details of genotyping with microsatellite markers, see van Belzen et al.\textsuperscript{18} A Mendelian inheritance check was performed for both the SNP and microsatellite markers; markers with Mendelian errors were excluded (for SNPs n=41; for microsatellite markers n=0) from the linkage analysis.

Linkage Analysis

Although the consanguinity of the parents in our family suggests an autosomal-recessive mode of inheritance, other modes of inheritance cannot be ruled out. Therefore, a model-free, nonparametric linkage (NPL) analysis was performed with the linkage program GENEHUNTER (version 2.1_r2 beta)\textsuperscript{19} using an affecteds-only approach. Using the NPL statistics, allele sharing in all possible pairs of affected individuals was determined and compared with the expected values for allele sharing. All the available 17 individuals who were genotyped were included in the analysis, and the disease status of the 7 patients with proven ruptured or unruptured intracranial aneurysms was set as affected. The other 10 were set as unknown. Because we had no DNA sample of individual II-2, who had an episode suggestive of aneurysmal SAH, she was not included in this analysis. Due to limitations of the GENEHUNTER program, we were only able to analyze microsatellite markers on the autosomal chromosomes with this program. For the analysis of the microsatellite markers on the X-chromosome, the nonparametric analysis of the MAPMAKER/SIBS program of GENEHUNTER (version 2.1_r2 beta)\textsuperscript{20} was used. Because the SNP marker sets per

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Chromosome 1p36.11-36.13 haplotypes of microsatellite markers. Ambiguous recombination are indicated in bold.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Chromosome Xp22.2-p22.32 haplotypes of microsatellite markers. Ambiguous recombination are indicated in bold.}
\end{figure}
chromosome were too large for the GENEHUNTER program, they were divided into smaller sets of 100 SNPs with a sliding window of 20 SNPs. The family studied was also too large for analysis with either the GENEHUNTER and the MAPMAKER/SIBS programs and was therefore divided into 2 smaller families (family 1: IDs I-2, II-3, II-4, II-5, II-6, II-8, II-9, II-11, II-12; family 2: IDs I-2, II-13, II-14, II-15, II-16, II-17, II-18, II-19, II-20). After scanning these 2 families separately, the NPL scores were combined.

Probability values corresponding to the obtained NPL scores were determined according to Kruglyak et al. The threshold levels for linkage were determined according to the genomewide significance levels proposed by Kruglyak et al with suggestive linkage as an NPL score $>3.18 \ (P=7.4 \times 10^{-4})$, significant linkage as an NPL score $>4.08 \ (P=2.2 \times 10^{-5})$, and highly significant linkage as an NPL score $>4.99 \ (P=3.0 \times 10^{-7})$. Chromosomal regions with NPL scores $>3.18$ were considered potentially interesting.

**Results**

**Analysis of Single Nucleotide Polymorphism Markers**

Of the 5861 genotyped SNPs from Illumina’s SNP-based linkage panel IV, 109 random SNPs were excluded because...
Table 2. NPL Scores Using Additional Microsatellite Markers at the Chromosome 4 and 21 Regions

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position</th>
<th>NPL Score</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.00</td>
<td>0.58</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>2.84</td>
<td>0.64</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>5.68</td>
<td>0.70</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>8.51</td>
<td>0.76</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>11.35</td>
<td>0.83</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>14.19</td>
<td>0.91</td>
<td>0.16</td>
</tr>
<tr>
<td>21</td>
<td>0.00</td>
<td>-0.75104</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>0.96</td>
<td>-0.71658</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>1.91</td>
<td>-0.68387</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>2.87</td>
<td>-0.65282</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>3.82</td>
<td>-0.62333</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>4.78</td>
<td>-0.59531</td>
<td>0.57</td>
</tr>
</tbody>
</table>

of poor quality and 41 random SNPs because of Mendelian errors during the inheritance check, leaving a total of 5711 SNPs for analysis (97.4%). The maximum NPL scores per chromosome are shown in supplemental Figure IA (available online at http://stroke.ahajournals.org). The analysis of the SNPs identified 4 intervals of potential interest with maximum NPL scores of 3.18 ($P=7.4 \times 10^{-5}$): 1p36.13-36.21, 4p14-15.1, 21q22.3, and Xp22.2-22.3. We have also analyzed our data assuming an autosomal-recessive and autosomal-dominant mode using different disease gene frequencies (frequency of 0.1, which is comparable with a disease prevalence of 2% and frequency of 0.01). Under these assumptions, the results remained essentially the same. The NPL scores in the previously identified locus on chromosome 4p13 vary between $-0.75$ ($P=0.77$) and 0.64 ($P=0.26$; supplemental Figure IB, online only).

Analysis of Microsatellite Markers

Analysis with 2 additional microsatellite markers (Table 2) reduced the evidence of linkage to 4p14-15.1 with NPL scores varying between 0.58 ($P=0.28$) and 0.90 ($P=0.18$), and to 21q22.3, with NPL scores between $-0.59$ ($P=0.72$) and $-0.75$ ($P=0.77$).

Analysis of 10 additional microsatellite markers at the 1p36.13-36.21 locus showed a maximum NPL score of 3.18 ($P=7.4 \times 10^{-5}$; Figure 3A). The maximum NPL score peak occurred at markers D1S199, D1S552, and D1S2702 and the NPL-1 interval was flanked by markers D1S2826 and D1S234, which define an 18.05-cM interval. This corresponds to a 6-Mb segment from 18.6 million bp to 24.9 million bp on chromosome Xp22.2-22.32. Six of the 7 patients share a 5-marker haplotype ranging from DXS6807 to DXS1224 inherited from the mother (Figure 2). However, patient II-15 who has an unruptured intracranial aneurysm did not inherit the risk haplotype from the mother, whereas sibling II-18, who is as-yet unaffected, did inherit this haplotype.

The presence of high linkage disequilibrium between SNP markers can cause inflation of multipoint linkage statistics, which leads to false-positive results. Therefore, the 2 intervals of potential interest identified by analysis of the SNP markers on chromosome 1 and X were confirmed by analyzing additional microsatellite markers in these regions. In view of the replication of linkage with the microsatellite markers, the NPL scores of the chromosome 1 and X regions are not likely to be inflated due to the presence of linkage disequilibrium between the SNP markers analyzed.

Discussion

A follow-up linkage analysis in a large Dutch family with intracranial aneurysms is reported. In this family, the disease is expected to be genetically homogeneous in a single family, but due to the complex etiology of intracranial aneurysms, it is still possible that the disease in this family has a multigenic origin. Thus, using a model-based, parametric linkage analysis may lead to misclassifying the model. Therefore, a model-free, nonparametric linkage analysis was performed and significant linkage to 2 different loci, at 1p36.11-36.13 and Xp22.2-p22.32, was identified. In the previous linkage analysis in this family, a model-based recessive mode of inheritance was used because of consanguinity of the parents of the affected children. However, this consanguinity may be a coincidence and may not lead to a recessive mode of inheritance in this family. Transmission of locus 1p36.11-36.13 does seem to follow a recessive mode of inheritance, but that of locus Xp22.2-p22.32 seems to be inherited in a dominant mode. Not all affecteds share the risk alleles at both the loci, but each affected has at least one of the risk alleles. The 2 loci may therefore compensate each other, which would indeed suggest a multigenic origin for the disease in
this family. Multigenic origin for diseases in single families has been described previously, for example, in a family with celiac disease. However, as long as the disease-associated mutation(s) in our family has not been identified, we cannot rule out that one of the associated loci might be spurious and that in fact in this large family, only a single disease-associated mutation will be found.

The locus at 1p36.11-36.13 overlaps with the previously reported locus on chromosome 1p34.3-p36.13 (ANIB3; HUGO Nomenclature Committee) identified in a single North American family. This family was analyzed under a dominant model and a maximum limit of detection score of 4.2 was found at marker D1S234, which appeared to be located adjacent to the marker generating the highest NPL score in our family (D1S2702). An excellent candidate gene in the overlapping region between the 2 studies is the perlecan gene, which codes for a heparan sulfate proteoglycan involved in the maintenance of the extracellular matrix of the arterial wall. It is a major component of basement membranes interacting with other basement membrane components such as laminin, collagen type IV, and other extracellular matrix molecules such as fibronectin. Perlecan is believed to be involved in stabilizing macromolecules and in cell adhesion. In a comprehensive case–control study with tag SNPs, we recently demonstrated SNPs in the perlecan gene to be associated with intracranial aneurysms in 3 independent populations (combined \( P = 0.0005^{24} \)), further emphasizing the possible involvement of this gene in the pathogenesis of intracranial aneurysms. However, by sequencing of all the 96 exons and exon–intron boundaries of the perlecan gene, initially in 2 affected individuals of our consanguineous family and in 2 control subjects, no mutations were detected (data not shown).

The second locus on Xp22.2-p22.32 has also been previously reported in the literature. A nonparametric linkage analysis in 29 Japanese families, with 3 or more family members with intracranial aneurysms, identified 3 chromosomal regions, including the Xp22 locus with a maximum NPL score of 2.16. Because the authors defined the boundaries of the locus by NPL scores with nominal probability values <0.05, it is not clear if this locus overlaps with our 90% support interval defined by the maximum NPL score −1. A Finnish linkage study analyzing affected sibpairs with intracranial aneurysms also found evidence for linkage to Xp22 with a maximum limit of detection score of 2.08, but after genotyping additional markers, their evidence for linkage was reduced in this region and it was not explored further. A susceptibility locus for intracranial aneurysms on the X chromosome may explain the preponderance of affected women with intracranial aneurysms because many genes on chromosome Xp escape X inactivation.

Because intracranial aneurysms are found in 2% of the population and intracranial aneurysm is a complex disease, phenocopies may occur in our family. Phenocopies may influence linkage studies by lowering or by obscuring true linkage signals. Consequently, in theory, we may have left other loci than the 1p36.11-p36.13 and Xp22.2-p22.32 loci undetected in our family.

In our previous linkage study, one of the siblings was assigned as being affected because MRA had revealed an unruptured intracranial aneurysm. However, in follow-up MRAs, this aneurysm could no longer be identified. The misspecification of phenotype in our previous study is especially a problem in parametric analyses and the problem can even be ameliorated in parametric analyses by providing age-based penetrance functions. The misspecification once again emphasizes that the definition of the phenotype should meet high standards of reproducibility and validity. For our study, an improved specificity and sensitivity in detecting intracranial aneurysms is obtained by the improved image quality of MRAs performed with increasing magnetic field strengths. To guarantee reproducibility of the intracranial aneurysm diagnosis, conventional catheter angiography was performed for confirmation of the MRA findings in case an aneurysm was detected on MRA.

The 9 loci list detected in a variety of linkage studies (loci on chromosomes 1p34.3-p36.13, 2p15.2-14.3, 7q22-31, 7q11, 11q24-25, 14q22, 17cen, 19q13.3, and Xp22.2-p22.32) confirm the genetic heterogeneity of intracranial aneurysms. Even in our single family, in which 2 loci on chromosome 1p36.11-36.13 and Xp22.2-p22.32 were identified, the disease appears to be genetically heterogeneous and multigenic. Because the locus on chromosome 1 had already been identified in another single, large family with intracranial aneurysms from North America, and evidence of linkage to the locus on chromosome Xp22 was obtained in affected sibpairs and multiple families with intracranial aneurysms in Japanese and Finnish populations, the 2 loci may thus be general risk factors for intracranial aneurysms in different populations.

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Disclosures

None.

References

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