Molecular Genetic Analysis of Two Large Kindreds With Intracranial Aneurysms Demonstrates Linkage to 11q24-25 and 14q23-31

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Background and Purpose—Both environmental and genetic factors contribute to the formation, growth, and rupture of intracranial aneurysms (IAs). To search for IA susceptibility genes, we took an outlier approach, using parametric genome-wide linkage analysis in extended IA kindreds in which IA is inherited as a simple Mendelian trait. We hereby present the molecular genetic analysis of 2 such families.

Methods—For genome-wide linkage analysis, we used a 2-stage approach. First, using gene chips in affected-only analysis, we identified genomic regions that provide maximum theoretical logarithm of odds (lod) scores. Next, to confirm or exclude these candidate loci, we genotyped all available family members, both affected and unaffected, using polymorphic microsatellite markers located within these regions.

Results—We obtained significant lod scores of 4.3 and 3.00 for linkage to chromosomes 11q24-25 and 14q23-31, respectively.

Conclusions—Molecular genetic analysis of 2 large IA kindreds confirms linkage to chromosome 11q and 14q, which were suggested to contain IA susceptibility genes in a previous study of Japanese sib pairs. Independent identification of these 2 loci strongly suggests that IA susceptibility genes lie within these regions. While demonstrating the genetic heterogeneity of IA, these results are also an important step toward cloning IA genes and ultimately understanding its pathophysiology. **(*Stroke*. 2006;37:1021-1027.)**

Key Words: aneurysm ■ genetics

Despite clinical advances, morbidity and mortality attributable to subarachnoid hemorrhage from a ruptured intracranial aneurysm (IA) remain a major public health problem. The basic biological mechanisms underlying IA are poorly understood, and little is known about the pathophysiology of IA except for certain risk factors defined by epidemiological studies. IA is more common among females, with a male to female ratio between 1:2 and 1:3.[1] Environmental risk factors such as hypertension, atherosclerosis, and diabetes are known to contribute to the formation and possible rupture of IAs.[2,3] Social factors such as smoking, alcohol, and diet have also been implicated.[4] However, these risk factors fall short of explaining the complete pathogenesis of this disease, and more recent efforts have been directed toward a possible genetic predisposition. Given the large number of familial cases, a genetic basis of IAs has long been appreciated.

Different strategies have been used to identify IA susceptibility genes, including candidate gene and nonparametric approaches with conflicting results. To dissect the genetics of this complex disorder, we took an outlier approach with the use of large families in which IA is inherited as a simple Mendelian trait. Although difficult to find, such families enable the use of parametric approaches to identify IA susceptibility genes with major effect and have the capacity to identify pathways that might play a key role in the pathogenesis in rare as well as common forms of the disease. Recently, we and others applied this methodology to the study of rare families with respect to IA, identifying susceptibility loci on 1p34-36 and 2p13.[5,6]

In this article, we report the analysis of 2 other multigenerational families, identifying 2 additional IA susceptibility loci on chromosomes 11q and 14q. Interestingly, these 2 intervals have been reported to contain IA susceptibility loci

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previously in a nonparametric linkage analysis using Japanese sib pairs.[7] Independent confirmation of these 2 intervals strongly suggests that they contain IA susceptibility genes. Further studies are now under way to clone these IA genes.

Materials and Methods

Collection of Blood Samples and Isolation of Genomic DNA

After HIPAA (Health Insurance Portability and Accountability Act of 1996) and HIC (Yale HIC 7680) consents were obtained, blood samples were collected from affected subjects and, where possible, from all members of the family regardless of affection status. Total genomic DNA was prepared by isolation of nuclei followed by proteinase sodium dodecyl sulfate lysis and subsequent phenol and chloroform extractions as described previously.[5]

Phenotype Assignment of Family Members

All phenotypes were assigned prospectively. Affected status was assigned after confirmation of the presence of an IA based on magnetic resonance angiography (MRA), computed tomography angiography (CTA), or conventional angiogram. In cases in which the diagnostic imaging studies were performed at outside institutions, we obtained original images whenever possible; these images were blindly read by a senior interventional radiologist at Yale who then assigned the phenotype status. Otherwise, medical records including the official dictation of the diagnostic studies were obtained. At-risk individuals with no symptoms who were <30 years of age were classified as phenotype unknown, as were members with aneurysms of the aorta or other extracranial vessels.[5] All other members were designated unaffected phenotype.

Single Nucleotide Polymorphism Genotyping

We used the GeneChip Mapping 10K Xba Array containing 10 044 single nucleotide polymorphism (SNP) markers (Affymetrix) for genome-wide linkage analysis. SNP genotypes were obtained by following the Affymetrix protocol for the GeneChip Mapping 10K Xba Array as described previously.[5]

Genechip Data Analysis

Using the Genome Analysis programs provided by Affymetrix, we performed basic analysis and manipulation of the Genechip data. We created a UNIX-based program (Chunky)[5] followed by the use of the Allegro software (DeCode Genetics, Inc) to perform multipoint linkage analysis. We assumed autosomal dominant inheritance and assigned a 70%, 90%, or 99% penetrance and phenocopy rate varying from 0.001 to 0.01. Allele frequencies for the GeneChips SNPs were obtained from Affymetrix.

Confirmation of Linkage Using Microsatellite Short Tandem Repeat Markers

Suggestive genomic regions were identified using the above approach. Regions with logarithm of odds (lod) scores close to the theoretical maximum were identified and microsatellite short tandem repeat markers were then found within these regions by using the physical map data from the University of California at Santa Cruz (UCSC) Genome Browser (May 2004). All members of the family, both affected and unaffected, were genotyped. This strategy is often referred to as a 2-stage design in linkage analysis.[8] All genotyping for microsatellite analysis was performed by polymerase chain reaction, with detection of fluorescent products on an ABI 3700 sequencer from Applied Biosystems equipped with Genescan and Genotyper software (ABI). The results were analyzed using the SimWalk2 program with penetrances specified between 70% and 99%.

Results

Family Collection

Over the past 10 years, we screened >3200 IA patients and identified 168 IA kindreds, with a total of >450 affected patients. Five of these families are large enough to support genome-wide linkage independently. We previously used 1 of these families to report a novel locus on 1p34-36.[5]

Phenotypic Information

Kindred IA100

The index case (III-6; Figure 1A), an employee of Yale–New Haven Hospital, occasionally works with the principal investigator (PI) in the outpatient clinic. After an interaction with a patient who experienced subarachnoid hemorrhage because of an IA rupture, she mentioned her extensive family history to the PI, was screened, and found to have an unruptured 8-mm anterior communicating artery IA. She later underwent successful clipping of her aneurysm by the PI and became an advocate for genetic studies. The rest of her family lives in Colombia, and, with her help, screening MRA or CTA studies were performed and samples were collected. Results of the imaging studies identified 3 other affected members of her family (III-8, III-14, and III-15; Figure 1A). Members III-3 and III-9 were both found to have mild a fusiform dilation of the cavernous segment of the internal carotid artery based on MRA and were therefore prospectively designated as phenotype unknown (Figure 1A, gray symbols). Furthermore, 2 subjects, II-2 and III-4, have documented abdominal aortic aneurysms (AAAs; Figure 1A). Screening results and phenotypic assignments for this family were presented (Goksu et al, unpublished data, 2006).

Kindred IA 101

This kindred was identified in Los Angeles, Calif. There are a total of 9 members with documented IAs, 4 of whom are deceased and 1 of whom refused to provide a blood sample (IV-5; Figure 1B). We thus collected samples from 4 affected individuals. In addition, samples from 24 unaffected members were collected.

Linkage Analyses

Kindred IA 100

Genome-wide linkage analysis using only affected members revealed linkage to various loci throughout the genome (supplemental Figure A, available online at http://stroke.ahajournals.org). Next, we genotyped all available affected plus unaffected individuals with negative screening MRAs or CTAs (n=17) using GeneChips to confirm or exclude linkage to these loci. For this analysis, samples from all the individuals in generation III were used. Individuals in the IVth generation were <30 years of age and did not have imaging studies; as a result they were not genotyped. We performed linkage under 2 models: (1) we considered individuals II-2 and III-4, who are known to harbor AAAs as affection status unknown; (2) we considered them as affected.

Because of limitations in the Allegro software, we were only able to analyze 7 family members at 1 time. Using this method, we first analyzed the genotyping results of 4 affected individuals plus unaffected family members III-5, 10, and 16 for all of the chromosomes that yielded a theoretical maximum lod score in the affected-only linkage analysis. Only the loci on chromosomes 3, 6, 11, and 17 continued to demonstrate near maximum lod scores (Figure 2A). Subsequent

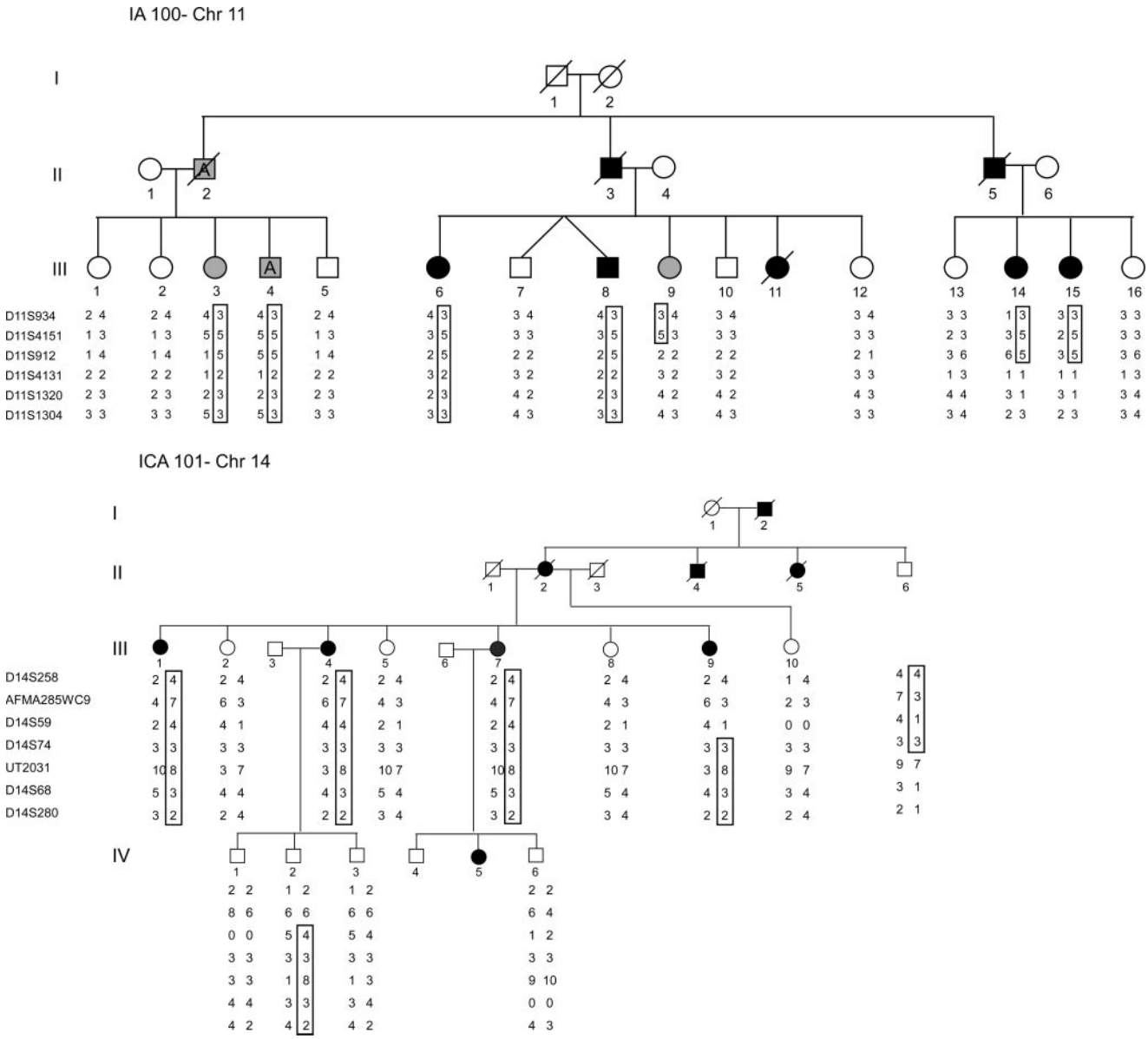

Figure 1. Family pedigrees. Unaffected and affected members are shown as unfilled and filled symbols, respectively. Subjects with unknown phenotype status are shown as gray symbols, whereas patients harboring aortic aneurysms are designated with an "A." Genotyping of short tandem repeat marker loci that span the intervals are shown with segments linking to disease phenotype enclosed in a box. A, Kindred IA 100. Genotyping of all kindred members with 6 short tandem repeat markers that span 11q24-25. B, Kindred IA 101. Genotyping of all kindred members with 7 short tandem repeat markers that span 14q23-31.

analysis with 3 different unaffected individuals (III-2, 7, and 12) plus affected patients significantly diminished evidence of linkage to all of these 4 chromosomes except the locus on chromosome 11 (Figure 2B). Interestingly, individuals II-2 and III-4, who are known to harbor AAAs, inherit the affected haplotype on chromosome 11.

To confirm or exclude this locus on chromosome 11, we next genotyped all available individuals. Under our prospectively determined model of considering AAA patients (II-2 and III-4) as phenotype unknown and specifying a penetrance of 99%, this analysis revealed a maximum lod score of 3.6. If these individuals are considered affected, the lod score is 4.3 without any effect on the chromosomal localization (Figures 3 and 4A).

Subject III-3, who we designated phenotype unknown because of a mild fusiform dilation of the left cavernous internal carotid, inherits the affected haplotype based on our linkage analysis (Figure 1A). Because her father and brother were found to have AAA, she also underwent screening with abdominal ultrasound, which revealed enlargement of her aorta to 27 mm (accepted criteria for AAA >30 mm[9]). Of note, this was larger than any of her remaining unaffected siblings. Subject III-9 was also found to inherit a portion of the affected haplotype. Like subject III-3, she was also previously found to have a dilatation of the cavernous portion of the left internal carotid and was therefore prospectively designated phenotype unknown before genotyping.

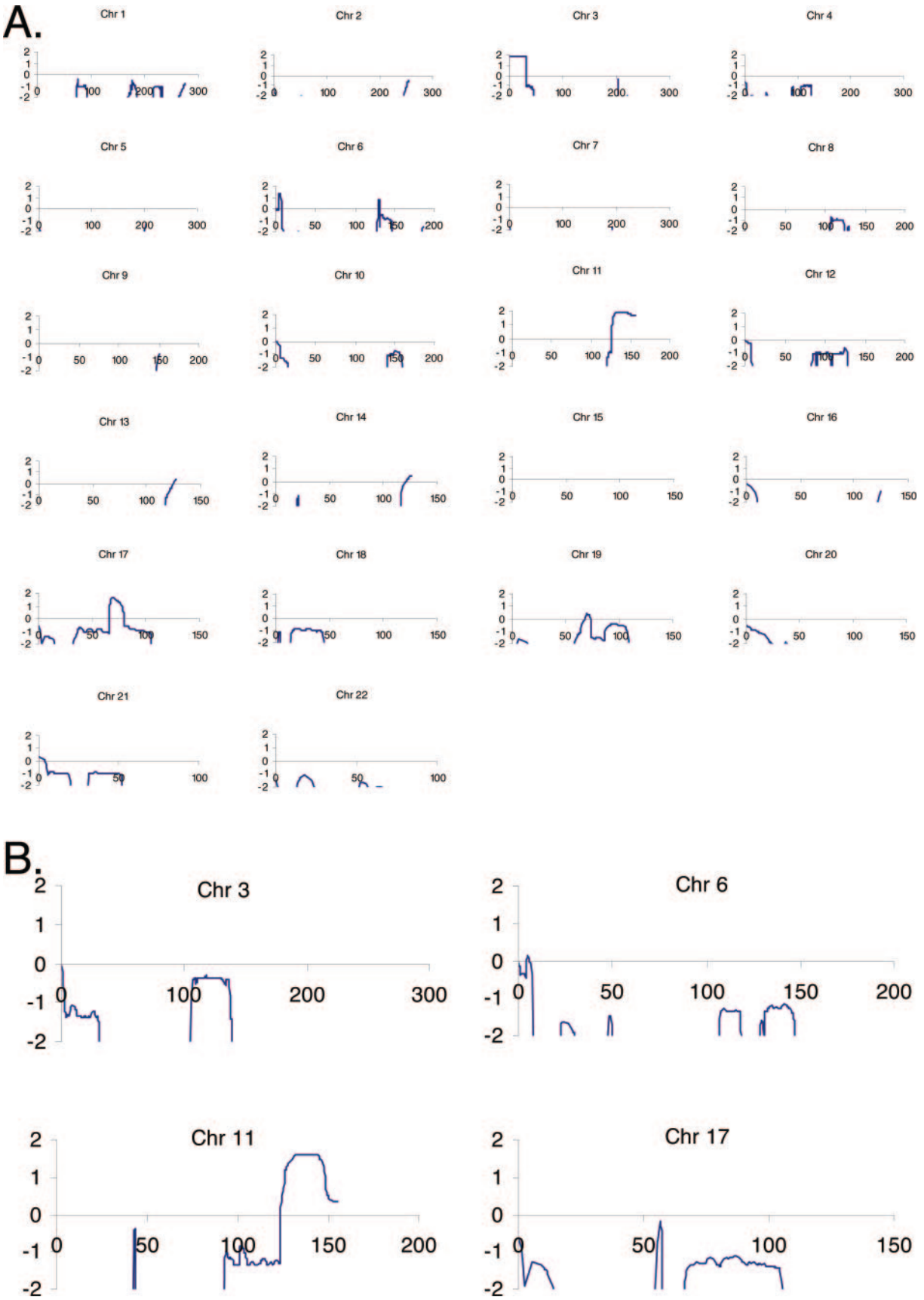

Figure 2. Genome-wide linkage analysis of IA 100 with affected and unaffected members. The *y* axis corresponds to lod score and the *x* axis shows genetic distance (in cM). A, Genotyping of 4 affected and 3 unaffected members reveals theoretical maximum lod scores on chromosomes 3, 6, 11, and 17. B, Subsequent genotyping of 3 different unaffected individuals diminished evidence of linkage to all loci except the locus on chromosome 11.

The lod-1 interval for the IA 100 locus lies between 125.6 and 131.4 million base pairs (mbp) on chromosome 11q24-25, between SNP markers rs618176 and rs1940033. Interestingly, this is 1 of the 14 regions linking to IA in a Japanese sib pair study,[7] demonstrating a statistically significant linkage (P=0.023) between marker D11S910, located at 131.2 mbp on 11q, and the IA phenotype. Furthermore, the markers immediately centromeric and telomeric to D11S910 that did not show significant linkage with IA (P>0.05) are D11S4151 and D11S4125, located at 125.8 and 133.7 mbp, respectively. This suggests that the longest interval that can contain an IA susceptibility gene on 11q is between 125.8 and 133.7 mbp. Thus, the IA 100 locus that lies between 125.6 and 131.4 mbp is almost fully contained within the region defined by the Japanese study.

Kindred IA 101

Affected-only genome-wide linkage analysis revealed suggestive linkage to various regions (supplemental Figure B). Additional GeneChip analysis of 7 unaffected members (individuals III-4, IV-2, IV-5, IV-8, IV-10, V-1, and V-3), all with negative imaging studies, revealed linkage to chromosomes 3 and 14, both with a maximum lod score of 3.0 (Figure 3) at 99% penetrance. We then identified highly polymorphic microsatellite markers in both of these regions and genotyped all of the kindred members (Figure 1B). This analysis showed that the locus on chromosome 14 is 100× more likely to harbor an IA susceptibility gene than the locus on chromosome 3.

The lod-1 interval is located between SNP markers rs2359991 and rs2373098, which are at 75.5 and 85.6 mbp, respectively, on 14q23-31. Similar to the IA 100 locus, 2 markers located within the IA 101 locus were found to have

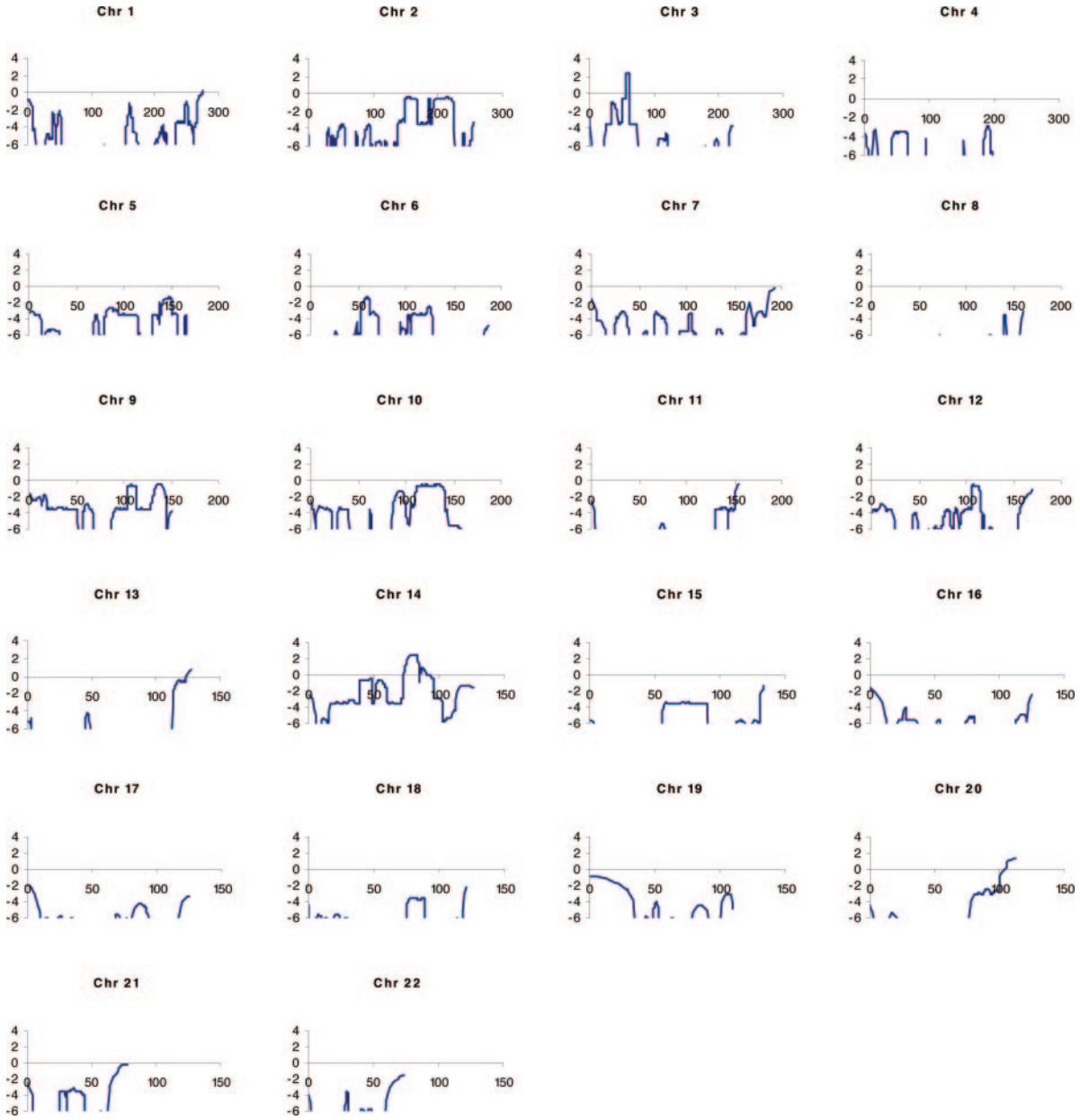

Figure 3. Genome-wide linkage analysis on IA 101 using 4 affected and 7 unaffected members reveals a theoretical maximum lod score of 3.0 on chromosomes 3 and 14. The *y* axis corresponds to lod score, and the *x* axis shows genetic distance (in cM).

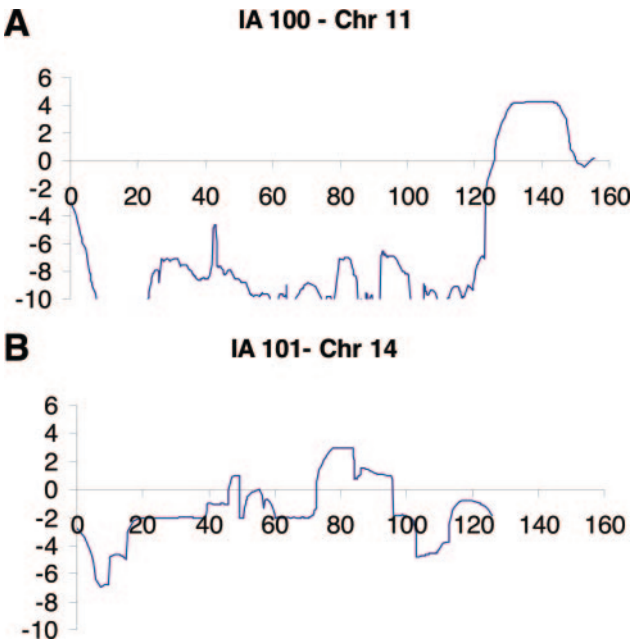

Figure 4. Genome-wide linkage analysis of kindreds IA 100 and IA 101. The *y* axis corresponds to lod score, and the *x* axis shows genetic distance (in cM). A, Analysis of all family members in IA 100 reveals a maximum lod score of 4.3 at 11q24-25 specifying 99% penetrance where members II-2 and III-4, known to harbor AAAs, are designated as affected. If they are assigned unknown affection status, the lod score is 3.6 with no effect on chromosomal location. B, Analysis of 4 affected plus 7 unaffected family members in IA 101 gives a maximum lod score of 3.0 at 14q23-31.

statistically significant linkage with the IA phenotype in the Japanese study.[7] Markers D14S74 and D14S258, located at 77.7 and 77.8 mbp, showed highly significant linkage to IA ($P=0.003$ and 0.034, respectively). This study suggested an IA susceptibility locus between 63.6 and 87.6 mbp[7] based on the location of the 2 surrounding markers, D14S63 and D14S68, with no significant linkage to IA ($P>0.05$). Thus, the overlap between the suggestive intervals in the 2 studies is between 75.5 and 85.6 mbp on chromosome 14q.

Discussion

Multiple lines of evidence suggest that genetic factors are involved in IA formation. The existence of families with multiple members with IA has been suggested as early as the 1960s, when Ulrich and Sugar reported 4 families with ≥2 siblings affected by IAs.[10] In the 1970s, Brisman et al reported families that showed autosomal dominant inheritance of IAs.[11] In 1983, Fox and Ko reported a large kindred in which 6 of 13 siblings were found to harbor IAs.[12] It is now known that first-degree relatives of affected individuals have a 3- to 5-fold increase in risk compared with the general population.[13]

In light of these developments, a number of groups have used different approaches to map genes that may be contributing to IA pathophysiology, including candidate gene, nonparametric, and parametric linkage analyses. The candidate gene approach, although promising at first, gave negative results with no association identified between IA and various genes including elastin, collagen III, fibrillin, polycystin, or endoglin.[14] Overall, nonparametric linkage analyses have identified multiple loci that may be contributing to IA on several chromosomes including 5q22-31,[7,15] 7q11,[7,15,16] 17cen,[17] 19q,[17–19] and Xp.[17] The strongest evidence to date implicates regions on chromosomes 7q and 19q, both of which have been suggested to contain an IA locus in independent studies.

Although nonparametric approaches are attractive with respect to IA because they are robust in the face of misspecification of inheritance and do not rely on recruitment of multigenerational families, an alternative strategy to gene discovery in complex genetic disorders, including IA, involves the use of traditional parametric linkage analysis in unusual, extended families. By confining analyses to a single or a few large families that appear to demonstrate simple Mendelian inheritance, one minimizes the chance of obscuring linkage because of genetic heterogeneity or environmental factors. This approach has been successful in identifying rare mutations imparting large effects on blood pressure,[20] insulin resistance,[21,22] and obesity,[23] leading to a better understanding of the molecular pathophysiology of these traits. In these and other conditions, the study of outlier families affected with Mendelian forms of the disease have had a major scientific impact by providing a launching point for investigations aimed at elucidating relevant pathophysiological mechanisms. Although there have been fewer studies using this approach in IA, the preliminary results have been quite promising. A recent report by Roos et al identified an IA locus on 2p13 by studying a consanguineous Dutch family with a maximum lod score of 3.55 linking IA to a 7-cM region containing 150 genes.[6]

By using an outlier approach that relies on parametric linkage studies in large families, our group previously reported an IA susceptibility locus on 1p34-36[5] with a maximum lod score of 4.2. In this article, we used the same approach on 2 other large families and identified 2 more loci on 11q and 14q. In all these studies, during the second stage of linkage analysis, we genotyped all individuals, affected and unaffected, to confirm or exclude candidate loci identified during the first stage of linkage. Because affected plus unaffected analysis is not as reliable as affected-only analysis, we took several measures to ensure accuracy. We screened all the at-risk individuals with imaging studies and accounted for age-dependent penetrance by not including any individual <30 years of age in our linkage analysis. Furthermore, the intervals identified in this study are among the 14 regions reported to show significant linkage to IA in an initial genome-wide linkage analysis of 83 Japanese affected sib pairs.[7] We believe that the confirmation of these loci by our group in a separate population using a different approach is strong evidence in favor of the veracity of the results. The multitude of IA susceptibility loci identified by our group, as well as others, alludes to the substantial locus heterogeneity of this disease.

Previous studies suggested an association between IA and AAA.[5,24] Two members in IA 100 (subjects III-4 and II-1) have documented AAAs. Although a previous genetic study found significant associations between AAA and polymorphisms in the tissue inhibitor of metalloproteinase 1 (*TIMP1*), *TIMP3*, matrix metalloproteinase 10 (*MMP10*), and elastin

(*ELN*) genes,[25] none of these genes lie within our lod-3 interval and were therefore not sequenced. Further, a locus on chromosome 19q13.3, which was found to be linked to AAA phenotype in 3 Dutch families,[26] was formally excluded in our linkage analysis of this family.

Identification of IA susceptibility loci is the first step in the positional cloning of IA genes. This will be followed by mutational analysis of candidate genes in these intervals, which eventually will lead to the cloning of IA genes. The identification of responsible proteins that cause aneurysms is an important first step in the development of new therapeutic approaches to this devastating disease.

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