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INTRACRANIAL ANEURYSM AS A PARADIGM FOR THE GENETIC ANALYSIS OF COMPLEX HUMAN TRAITS

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

by

Ali Kemal Ozturk

Yale University School of Medicine, 2006

INTRACRANIAL ANEURYSM AS A PARADIGM FOR THE GENETIC ANALYSIS OF COMPLEX HUMAN TRAITS

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The genetic analysis of complex human traits is hampered by its multifactorial nature, since potentially many genes are imparting a relatively small effect on the disease trait. This makes it considerably more difficult to identify these genes as compared to single-gene diseases, whereby the disease trait is attributable to only one locus, due mostly to the difficulties of carrying out robust linkage analyses.

Several strategies have been employed in order to overcome the obstacles of complex human traits, including the candidate gene, non-parametric, and parametric linkage approaches. Of these, the latter two enjoy the benefit of being genome-wide, while carrying the potential of missing genes that impart slight effect on the disease trait, a weakness that the candidate gene approach may overcome to some degree.

Our approach to unravel the complex genetics of Intracranial Aneurysms (IA) has been a blend of the parametric linkage approach, followed by a smaller-scale candidate gene approach, where genes within a linked interval are sequentially analyzed based on relevance. We are able to conduct the parametric linkage approach by identifying rare, outlier families whereby the disease trait is ostensibly being passed on in an identifiable, Mendelian fashion, enabling us to set the parameters required to perform parametric linkage analysis.

Using this method on four of our largest families, we have achieved linkage to chromosomes 1p34-36, 11q24-25, and 14q23-31 exceeding the statistical threshold of significance. Importantly, the latter two loci have also been identified in a non-parametric linkage study in Japanese sib-pairs. Anaylsis of genes that lie in these regions are ongoing.

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Table of Contents

Introduction 6

Statement of Purpose, Specific Hypothesis and Aims 17

Materials and Methods 18
- Collection of blood samples and Isolation of Genomic DNA 18
- Phenotype assignment of family members 18
- SNP Genotyping 18
- Confirmation of Linkage Using STR Markers 19
- Mutational Analysis 20

Results 23
- IA 20 23
- IA 100 30
- IA 101 36
- IA 42 41

Discussion 44
- Genetics of Complex Human Traits 44
- Genetics of Intracranial Aneurysm 45

References 48

INTRODUCTION

The genetic analysis of complex human traits is hampered by locus heterogeneity, whereby multiple genes may be contributing to a disease trait via small or large effects. This makes the identification of susceptibility genes considerably more difficult than single-gene diseases, due to the difficulties of performing robust linkage analyses. The aim of this work is to consider various approaches to identifying genes leading to complex traits using intracranial aneurysm (IA) as a paradigm.

Intracranial Aneurysms (IA): Background and Epidemiology

In the United States, nearly 700,000 people suffer from stroke every year (AHA Stroke Statistics, 2005 Update, http://www.americanheart.org). Subarachnoid hemorrhage (SAH), one form of hemorrhagic stroke, carries the highest mortality with an approximately 50% mortality rate one month after the initial bleed (1, 2, 3). Furthermore, only 25% of those who live past the first month will recover completely, leaving the majority of survivors requiring a life-time of care (4-6). In the absence of trauma, intracranial aneurysm (IA) is the primary cause of SAH, accounting for approximately 85% of cases (7). Despite recent advances in diagnostic and therapeutic modalities, the morbidity and mortality from SAH remains disturbingly high.

IA is surprisingly prevalent; various series have estimated the incidence to be between 0.2 and 9.9% with a mean of 5% (8). Fortunately, only a fraction of these will rupture with the majority remaining asymptomatic and discovered only incidentally or during autopsy. Thus, SAH from IA rupture comprises only 3% of all strokes (5), and the incidence of SAH remains 6 in 100,000 with 28,000 IA ruptures per year (7). The

ruptures are, however, devastating when they do occur, and SAH accounts for nearly 25% of all life-years lost due to stroke (9).

While the majority of IA occurs sporadically, familial forms also exist, and they are being increasingly recognized with recent studies attributing approximately 10% of all IAs to a genetic predisposition (10). These patients often have two or more first-degree relative members with IA.

It was once believed that all IAs occurred sporadically. The observation that IA is associated with known genetic disorders such as Autosomal Dominant Polycystic Kidney Disease (ADPKD) (11) and Ehlers-Danlos syndrome type IV etc (12), however, introduced doubt into this observation. Furthermore, these syndromes fall far from explaining the majority of familial IA cases, defined as two or more relatives with IA. Familial clustering of IAs has been documented since the 1950's (13), and the importance of heritable factors has been increasingly recognized. It is now known that first-degree relatives of patients with SAH from aneurysm rupture are three to five times more likely to harbor an IA (14). The relative risk may be even greater in siblings, who have been reported to have a six-fold higher risk compared to the general population (10).

Familial IA's behave somewhat differently than sporadic cases. They are prone to rupture at younger ages, they are more likely to be multiple, and finally, they have a strong predilection for the middle cerebral artery (MCA) (15-19). With increasing recognition of familial IA's as a separate entity, our group, as well as others, has begun searching for a mutation in a specific gene that specifically causes the formation and rupture of non-syndromic, inherited IA.

GENETIC ANALYSIS of COMPLEX TRAITS

Both association (candidate gene) and linkage (parametric and non-parametric) studies can be used for the genetic analysis of complex traits. In a linkage study, one investigates the co-segregation of two loci (one of which may be the disease locus) in families, whereas an association study looks for the co-existence of a disease trait and genetic polymorphisms. Each has their advantages and disadvantages, and should not be considered mutually exclusive.

1) <u>Association Studies (Candidate Gene Approach):</u>

In design, association studies are similar to epidemiologic case-control studies, such that a given trait is compared across allelic variants of a particular DNA marker. Thus, the frequencies of different allelic variants may be compared in subjects with particular phenotypes in an attempt to identify a positive association.

Whereas a linkage study is genome-wide, association studies are limited to candidate genes or regions (20). This is because screening the entire genome would require too many markers, making it impractical and inefficient. Thus, investigators will frequently confine themselves to particular genes or regions that they feel have potential to impart an effect (major or minor) on the disease phenotype. A potential weakness in this strategy is that it assumes that the disease causing mechanism is one that is already known, and thus, novel pathways that may be contributing to disease pathophysiology may be overlooked (20).

Furthermore, while linkage analysis is confined to families or sib-pairs, the candidate gene approach can be undertaken at a population level, allowing for numerous

unrelated individuals with the same disease to be analyzed at the same time. While potentially powerful to detect genes of modest effect contributing to a phenotype (21), candidate gene approaches are limited by the fact that only a small number of genes can be studied at one time.

Most candidate genes that consistently have been shown to impart significant effect on a disease trait has been demonstrated to be functional, that is, they influence the concentration of a protein, its functionality or efficiency, or its expression patterns (20). Several strategies have been employed in the past to identify potential candidate genes. First, if there are genes known to cause a specific disease in animals related to the disease trait being studied in humans, then the human analogues of those genes may be studied. Next, genes that take part in certain physiologic mechanisms that are known to be involved in the disease being studied may be investigated. This approach, as mentioned earlier, can not identify genes that are part of novel, unstudied pathways. Another potential weakness emerges here, whereby an overwhelming number of genes may be identified using these candidate gene finding strategies.

Perhaps the most important and most widely criticized weakness of the candidate gene approach is the potential for false positive results. In a comprehensive review of this method, Hirschhorn et al compiled 166 positive association studies that were analyzed three or more times between single nucleotide polymorphisms and complex genetic traits (22). Only 6 of the 166 were robust and consistently replicated (22). This demonstrates an important weakness in the candidate gene approach whereby the more genes that are analyzed using this strategy, the higher the likelihood of false positive results. While approaches driven by hypotheses regarding disease mechanisms are

intuitively attractive, experience has shown repeatedly that genome-wide linkage analysis is a more fruitful method to isolate causative genes of a complex disorder whose pathophysiology and molecular biology is not fully established.

2) Linkage Studies:

As previously mentioned, linkage analysis has the benefit of being genome-wide when compared with association studies. Thus, research is not confined to candidate genes or regions, and the power to detect proteins that are involved in novel pathways is much greater using this strategy. Linkage analysis can be carried out in two different modes: one where the mode of inheritance needs to be specified *a priori*, called "parametric" or model-based linkage analysis, and another where the mode of inheritance, along with other genetic parameters do not need to be specified, so called "non-parametric linkage analysis." Traditionally, it has been believed that parametric linkage analysis is the ideal method for single-gene diseases, in other words diseases where one gene imparts a major effect on the phenotype. Non-parametric linkage analysis, on the other hand, has been preferred for the analysis of complex traits with locus heterogeneity, where several or many genes may be imparting minor effects on the disease trait.

A) Non-parametric Linkage Analysis:

Non-parametric, or model-free methods were first developed for sibling pairs. More recently, this method has been extended to general pedigrees (20). As stated previously, in this method of linkage, no explicit model needs to be specified ahead of

time for the genome-wide analysis. In non-parametric linkage analysis, many DNA markers (single nucleotide polymorphisms-SNP's, or short tandem repeats-STR's) dispersed throughout the genome are obtained from siblings, and where possible, from their parents. Ultimately, allele sharing between affected sibling pairs is investigated and compared to the expected allele sharing under Mendelian principles. This method is called the "affected sib-pair" method, since more information lies in the relationship between two affected siblings than two unaffected siblings (23).

Since no genetic parameters need to be specified with this analysis, non-parametric linkage has been considered the most powerful method for studying complex traits where identifying a single mode of inheritance (along with other genetic parameters such as penetrance, phenocopy rate, etc.) may often prove to be difficult. Thus, in theory, non-parametric methods have the capability to detect multiple loci of minor effect in polygenic diseases.

This method, however, is not without its weaknesses. First, for the analysis to be powerful, significant numbers of sib-pairs must be identified and genotyped. Furthermore, this number that is required to achieve statistical significance will vary depending on the heritability of the trait. Thus, where 200 sib-pairs may be a sufficient number for a highly heritable trait, it may prove to be inadequate when searching for loci with more modest effects. This example illustrates that with non-parametric analyses, it is still difficult to achieve significance when attempting to detect genes of small effect, or genes that are affecting only a small proportion of the families being analyzed (20). Finally, while non-parametric linkage may identify many loci that may be linked to the

disease trait with varying degrees of significance, the researcher is still left to speculate as to which of the loci are disease-causing in each individual family.

B) Parametric Linkage Analysis:

Parametric linkage approaches are also referred to as model-based linkage, since an explicit genetic model (along with all of the genetic parameters) needs to be specified before the experiments are started. This model is the preferred method when a disease with a Mendelian mode of inheritance is being studied, and it is hypothesized that a single gene with a major effect is responsible for causing the phenotype. Typically, in parametric linkage analysis, one extended family pedigree (as opposed to multiple sib-pairs used in non-parametric linkage) will be analyzed. A number of DNA markers (in the form of SNP's or STR's) of known location dispersed throughout the entire human genome will subsequently be used to investigate the inheritance of portions of the DNA, statistically computing the recombination frequencies. For each marker, evidence for linkage is sought using the co-segregation of the trait (and presumably the disease causing gene) and a particular variant of the marker being analyzed. This is possible since the closer the two loci (disease locus and marker locus) are located on a chromosome, the less likely it is that a recombination event will occur between the two, and the more likely that the specific marker variant will be present in all individuals who are affected with the disease. Thus the genetic distance between the marker locus and the disease causing gene can be estimated in centimorgan (cM) units, with 1 cM corresponding to a recombination fraction of 1% (20).

Statistically, the LOD score function has added much to parametric linkage analysis (24). LOD score stands for the logarithm of the odds and is a robust measure of the strength of the linkage. It is the logarithm of the odds that the DNA marker locus is linked to the disease trait, and is calculated according to the following formula:

$$\text{LOD score} = \log_{10} \frac{\text{probability that two loci are linked}}{\text{probability that two loci are not linked}}$$

Thus, a LOD score of 3.0 would mean that the probability that two loci are linked is 1000 times more likely than them being unlinked (on separate chromosomes). However, any two given loci are approximately 50 times more likely to be unlinked than linked. Correcting for this factor would mean that the two loci with a LOD score of 3.0 are 1000/50=20 times more likely to be linked than unlinked. This means that there is a 1 in 20 probability that the linkage is due to chance findings, corresponding roughly to a *p* value of 0.05. Thus, a LOD score of 3.0 is considered to be the threshold of significance.

Using this approach, the area(s) of the genome with the highest LOD score is considered to be the most likely to contain the disease causing gene(s). Two methods may follow the initial genome-wide linkage. First, if the linked region is sufficiently small one may directly proceed to sequence that portion of the DNA looking for allelic variants that may directly explain the disease. If the area in question is comparatively large, one may use STR's in the region to fine map and look for additional recombinations that may further refine and shorten the linked interval. This is referred to as a two-stage design in linkage analysis (25). It is possible, that at the end of both the initial genome-wide scan and fine mapping using STR's, the interval is still several million base pairs long, rendering it unpractical to sequence in its entirety. At this point,

the researcher may only sequence candidate genes, i.e. those genes in the region that seem relevant to the disease based on its function, if known, and expression pattern. This final step may re-introduce the aforementioned risks and potential weaknesses of the candidate gene approach.

While quite robust in diseases in which a specific inheritance pattern can be identified, the parametric linkage analysis loses favor for the analysis of complex traits, due mostly to difficulties in discerning the genetic parameters, i.e. the inheritance mode, penetrance, phenocopy rate, etc.

Our Approach:

Our approach to unravel the complex genetics of IA formation is a blend of the previously mentioned approaches, relying most heavily on parametric linkage analysis. In order to benefit from the robustness of parametric linkage analysis in complex human traits, one needs to be able to reconcile the need to accurately determine the genetic parameters, which can prove to be difficult for complex, non-Mendelian inheritance forms. This can be done with the identification and use of rare, extended kindreds that are ostensibly passing the trait on from generation to generation in a predictable, Mendelian manner. Though difficult to find, once identified, these families enable the use of robust parametric linkage analysis for gene identification while avoiding the majority of the pitfalls that accompany non-parametric and candidate gene approaches (26). By restricting our analysis to these rare, "outlier" families, we are, in essence, converting the complex trait of intracranial aneurysm into a simple, single-gene disease.

In an attempt to recruit such large, multi-generational IA families, the Gunel lab has, over the past 10 years, screened over 3200 IA patients and identified 168 IA kindreds, with are a total of more than 450 affected patients. Critically, four of these families are sufficiently large to support genome-wide linkage independently (IA 20, 42, 100, and 101).

Having identified and recruited these families, our approach is that of a two-stage design in linkage analysis. First, we use Affymetrix gene chips that utilize 10,000 SNPs dispersed relatively evenly throughout the human genome on all of the affected members of the kindred. This initial, affected-only analysis, is the most dependable and accurate, demonstrating the areas of the genome that are shared by all individuals with IA. In this phase, the pre-defined penetrance rate is not an issue, since unaffected members that may be inheriting the high-risk portion of the genome that may not be phenotypically affected are excluded. Following the initial genome-wide scan of affected members, we genotype all candidate loci that give a theoretical maximum LOD score using STR's that span the candidate loci. At the end of this second phase, our goal is to be left with one candidate interval that is demonstrating maximum LOD score with all individuals included, both affected and unaffected. This second stage of genetic analysis using STR markers also has the added benefit of potentially narrowing and refining the locus at hand, presumably minimizing the amount of DNA needing to be directly sequenced while looking for allelic variations (26).

As mentioned previously, if, after this two-stage approach to linkage analysis we are still left with a region spanning millions of base pairs precluding direct sequencing of the entire region, we proceed to take a pseudo-candidate gene approach, identifying the

genes that lie within the region and sequencing directly those which we feel may have a role in aneurysm pathogenesis, based on function and expression pattern. Identification of a frameshift mutations or a stop codon resulting in a truncated protein is considered strong evidence in favor of disease-causing potential. If, on the other hand, a point mutation leading to an amino acid change is noted, then this change needs to be analyzed more aggressively with the use of additional patients with IA, and control patients.

STATEMENT OF PURPOSE

We hypothesize that a single-gene defect may be identified in rare, outlier families with IA which leads directly to the disease phenotype. While identification of such families is difficult, they enable the use of parametric linkage approaches, since the mode of inheritance can be identified. We believe that parametric linkage approaches, in such outlier families, are more robust than the non-parametric or candidate gene approaches.

Identification of a gene that directly leads to IA phenotype in the aforementioned families can subsequently be analyzed in other forms of familial IA, as well as sporadic ones, to investigate whether the protein product of the identified gene plays any role in rare, and more common forms of the disease.

Finally, the protein product of the identified gene will help elucidate the pathophysiological mechanisms leading to IA, and may even result in a screening test that could detect IA before they rupture.

All presented work was performed by Ali K. Ozturk, with the exception of the linkage analysis of IA 20, which was performed by Brian V. Nahed.

METHODS

Collection of blood samples and Isolation of Genomic DNA

After HIPAA 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 K - SDS lysis and subsequent phenol and chloroform extractions (26).

Phenotype assignment of family members

All phenotypes were assigned prospectively. Affected status was assigned after confirmation of the presence of an IA based on MRA, CTA, or conventional angiogram. In cases where 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 are <30 years of age were classified as phenotype unknown, as were members with aneurysms of the aorta or other extracranial vessels (26). All other members were designated unaffected phenotype.

SNP genotyping:

We used the GeneChip Mapping 10K Xba Array containing 10,044 SNP markers (Affymetrix: Santa Clara, CA) for genome wide linkage analysis. SNP genotypes were obtained by following the Affymetrix protocol for the GeneChip Mapping 10K Xba

Array. Briefly, genomic DNA was digested with *Xba*I; adapters were ligated to the product and an adapter-specific primer was used to amplify the product by PCR. The products were purified, fragmented and labeled with biotin-ddnTP. Biotin-labeled DNA fragments were hybridized to the mapping 10K array chip. After hybridization, arrays were washed, stained, and scanned. Affymetrix MicroArray Suite 5.0 software was used to obtain raw microarray feature intensities which were processed using the Affymetrix Genotyping Tools software package to derive SNP genotypes.

An average of 9468 genotypes was scored per subject (SNP call rate range: 91% – 97%). To analyze the Genechip data for linkage we created a UNIX based program (Chunky) that parses the data sheet into individual files per chromosome in linkage format. Information captured includes chromosome number, SNP markers, map distances, genotype calls, and allele frequencies.

We performed multipoint analysis of linkage, specifying the disease locus as autosomal dominant with penetrance varying from 70 to 99%, a mutant disease gene frequency of 0.001, and a phenocopy rate of 0.001. SNP allele frequency data for the Caucasian population, as supplied by Affymetrix, was used for the analysis of linkage which was performed using the Allegro program (DeCode Genetics, Iceland).

Confirmation of Linkage Using Microsatellite Short Tandem Repeat (STR) Markers:

Suggestive genomic regions were identified using the above approach. Regions with lod scores close to the theoretical maximum were identified and microsatellite short tandem repeat (STR) markers were then found within these regions by using the physical map data from the UCSC Genome Browser (May 2004, http://genome.ucsc.edu). All

members of the family, both affected and unaffected, were genotyped. This strategy is often referred to as a two-stage design in linkage analysis. 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, Norwalk, CT). The results were analyzed using the SimWalk2 program (http://www.genetics.ucla.edu/software/simwalk2) with penetrances specified between 70 and 99%.

Mutational Analysis:

As a first step, we have already determined the transcripts located in each of the intervals. Even though we mainly rely on the Genome Browser (May 2004 version) of the University of California, Santa Cruz (UCSC) website (http://genome.ucsc.edu), we use a number of other databases to search for additional transcripts in the linked intervals. These databases include the Mapview of the NCBI (http://www.ncbi.nlm.nih.gov/mapview/) and the Ensembl website (http://www.ensembl.org).

Among the genes located in a linked interval, we prioritize transcripts for further study based on known function and/or expression patterns. These data are obtained through both the Affymetrix expression data available at the UCSC genome browser as well as via data-mining of the PubMed database. For example, genes that are expressed in vascular structures, especially arteries, such as those expressed by vascular smooth muscle cells, endothelium, or those that are abundant in the brain along with extracellular matrix (ECM) genes are viewed as likely candidates.

The genomic sequence of these genes along with the exon/intron boundaries that are determined using the above programs are downloaded to the Sequencher program. The primers for PCR amplification of the exons are designed using PRIMER3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and the coding regions of the gene of interest are amplified and sent for direct sequencing. Given the relatively small number of samples to evaluate, we prefer direct sequencing to other mutation screening technologies as it is the most sensitive and reliable method.

For large families that show exclusive linkage to a locus, we will sequence the index case and another affected member to determine if an identified polymorphism segregates among the affected members. If a polymorphism is found to be present in both of these two patients, we then sequence additional affected and unaffected members to test whether the observed polymorphism segregates with the IA phenotype. We also check the available databases to see if this polymorphism is a previously reported SNP. A polymorphism segregating on the affected chromosome that leads to a stop codon or a frameshift mutation is considered highly likely to be the disease causing mutation.

Any other polymorphism on the affected chromosome that is not reported in the SNP databases (http://www.ncbi.nlm.nih.gov/projects/SNP/) is investigated in control chromosomes. The identification of a rare, segregating mutation in an affected family but not in several thousand controls, in conjunction with the relevant expression data, will strongly implicate mutations in a particular gene in the pathogenesis of IA.

The same gene is then tested for mutations in additional IA families that link to the respective IA locus and familial IA cases without any linkage information, along with 250 sporadic IA cases, in an effort to identify additional mutations in the same gene. For

this stage, we rely on a two-stage strategy of temperature gradient column electrophoresis with a Spectrumedix REVEAL system followed by direct sequencing on both strands of putative heteroduplexes to identify any additional mutation. Identification of additional mutations will strongly support the hypothesis that the disease causing gene is identified.

RESULTS

Parametric Linkage Analysis in IA:

As mentioned previously, four of our 168 IA families were large enough to support linkage independently, and we have finished the majority of the analysis. The following are the results from the genome-wide linkage studies performed on these families.

1) **IA 20:**

Epidemiology:

This family is originally from West Virginia. In 1980, Fox and Ko (27) reported this family as being the largest reported IA kindred with six affected members, at the time, all of whom were in generation II. Since then, we have recruited this family for our study, and extended the pedigree to include ten documented IAs, one subject with distinctive multiple intracranial vessel occlusions and extensive collateral vessel formation of unknown etiology (subject III-3), and one subject with abdominal aortic aneurysm (AAA) at a young age (age 32; individual II-5); this latter trait is sometimes associated with IA (Figure 1A).

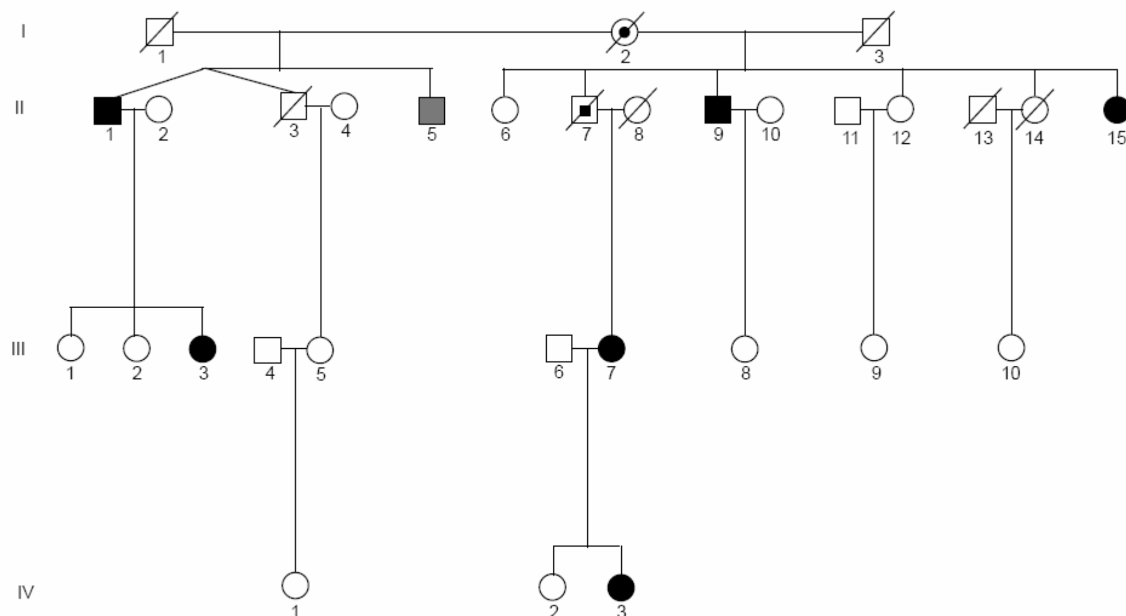


Figure 1A. IA 20 kindred. Affected and unaffected individuals are shown as filled and unfilled symbols, respectively. Obligate carriers are shown as partially filled symbols. Individual II-5 was assigned affection status unknown prior to linkage analysis and is shown as a grey symbol.

During our phenotypic assignment, we prospectively designated the family member with vessel occlusions as being affected, and the member with an AAA as being phenotype unknown. All subjects that were asymptomatic, over the age of 30, and had negative screening studies were designated as unaffected (individuals II-3, II-6, II-11, II-14, III-1, III-2, III-8, IV-2), while those under the age of 30 were assigned an unknown phenotype (individual IV-1). Finally, those subjects without screening studies who were over age 30 and asymptomatic were also designated unaffected (individuals III-4, III-9, III-10). The aneurysm characteristics and age at diagnosis are shown in Table 1A.

Table 1A: Clinical features of affected members of kindred IA 20

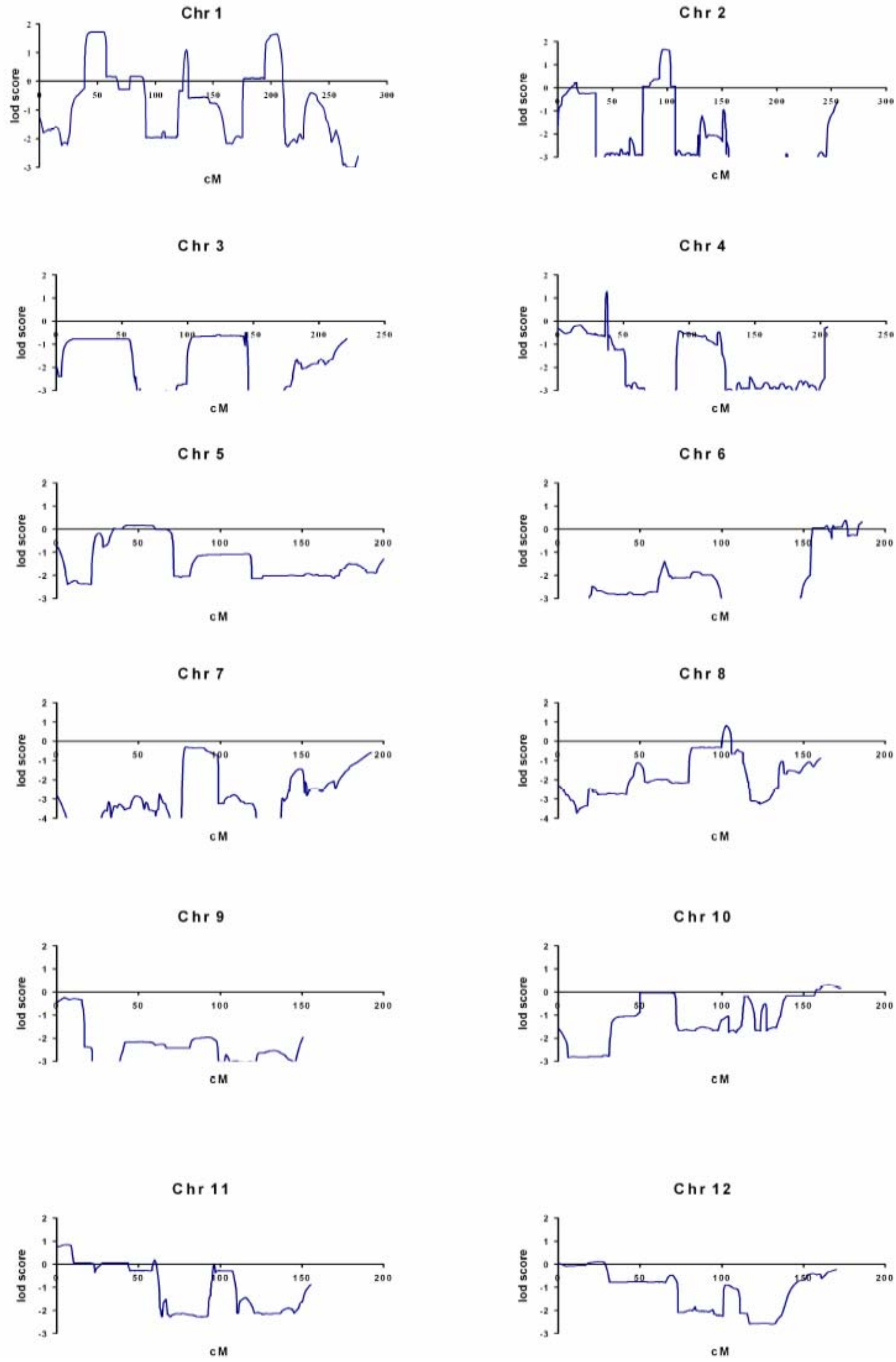
ID	Aneurysm Location	Age of diagnosis
II-1	AcoA	38
II-7	ACA, Lt MCA	53
II-9	AcoA	40
II-15	Lt MCA	29
III-3	Bilateral MCA occlusion	30
III-7	Lt MCA	36
IV-3	Basilar, Rt MCA X 2	21

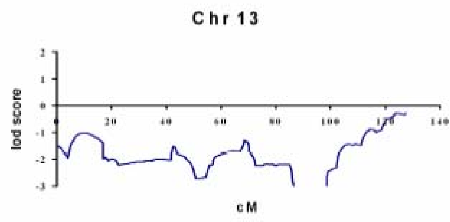
* ACA: anterior cerebral, ACoA: anterior communicating, MCA: middle cerebral artery.

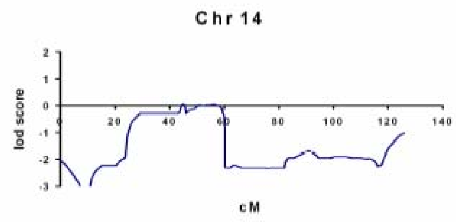
Linkage Analysis:

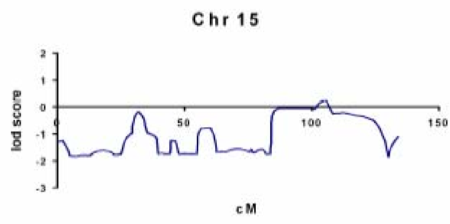
As a first step, we performed an affected only analysis using the six affected members of this family with Affymetrix genechips and the Allegro program as previously described (see Methods section). This analysis led to three peaks throughout the human genome with a LOD score at or near the theoretical maximum expected for this family. These candidate regions were located at 1p34 – 1p36, 1q31 – 1q41, and 2p11 – 2p14, all of which resulted in a LOD score of approximately 1.5 (Figure 1B). Following this, we genotyped all members of the family, both affected, unaffected, and unknown with STR markers spanning the three candidate loci to see which of the three loci were most likely to contain the disease-causing gene. Following this analysis, the locus on chromosome 1p34-36 demonstrated a LOD score of 4.2 specifying a penetrance of 99% (Figure 1C),

Figure 1B. Analysis of linkage in IA 20 from GeneChip data of affected individuals. Linkage graphs for all chromosomes are shown; x-axis corresponds to genetic distance (cM) and y-axis shows lod scores.




Chr 13
lod score
cM


Chr 14
lod score
cM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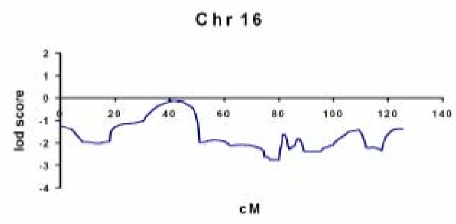
cM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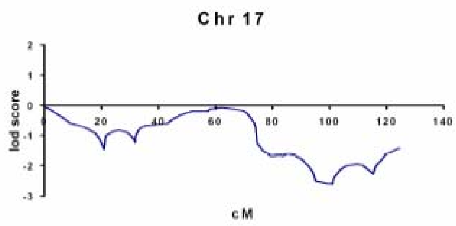
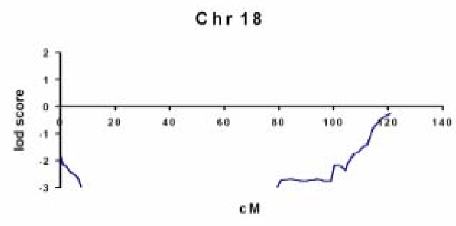
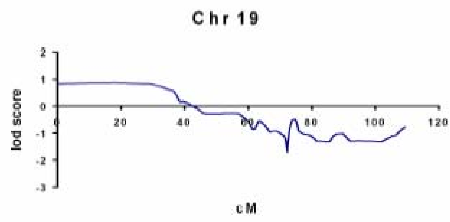
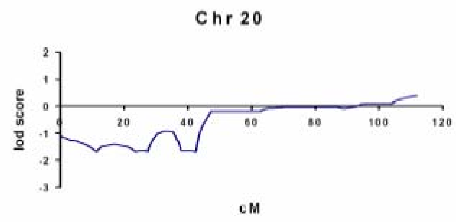
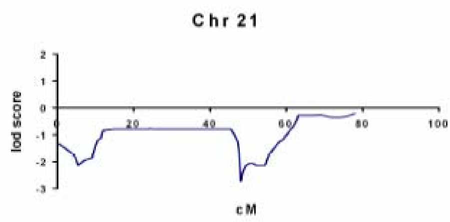
lod score
cM

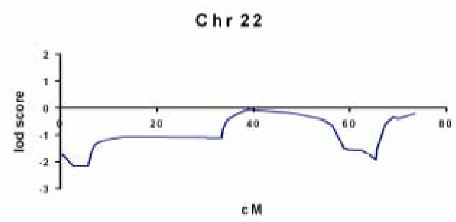

Chr 17
lod score
cM


Chr 18
lod score
cM


Chr 19
lod score
cM


Chr 20
lod score
cM


Chr 21
lod score
cM


Chr 22
lod score
cM

while the LOD scores on the remaining two loci diminished significantly (Table 1B). Of note, if family member III-3, with bilateral MCA artery occlusions is designated as phenotype unknown, the maximum LOD score at 1p34-36 diminishes to 3.9 with no significant effect on chromosomal location (26).

Figure 1C. Analysis of linkage with STR markers on 1p34-36 localizes an IA gene to a 12.5 cM region between markers D1S199 and UT5144 with a maximum lod score of 4.2. Multipoint analysis of linkage comparing segregation of IA and marker loci was performed. The location of marker loci used is indicated at the top of the diagram. The horizontal bar indicates the extent of the lod-1 interval.

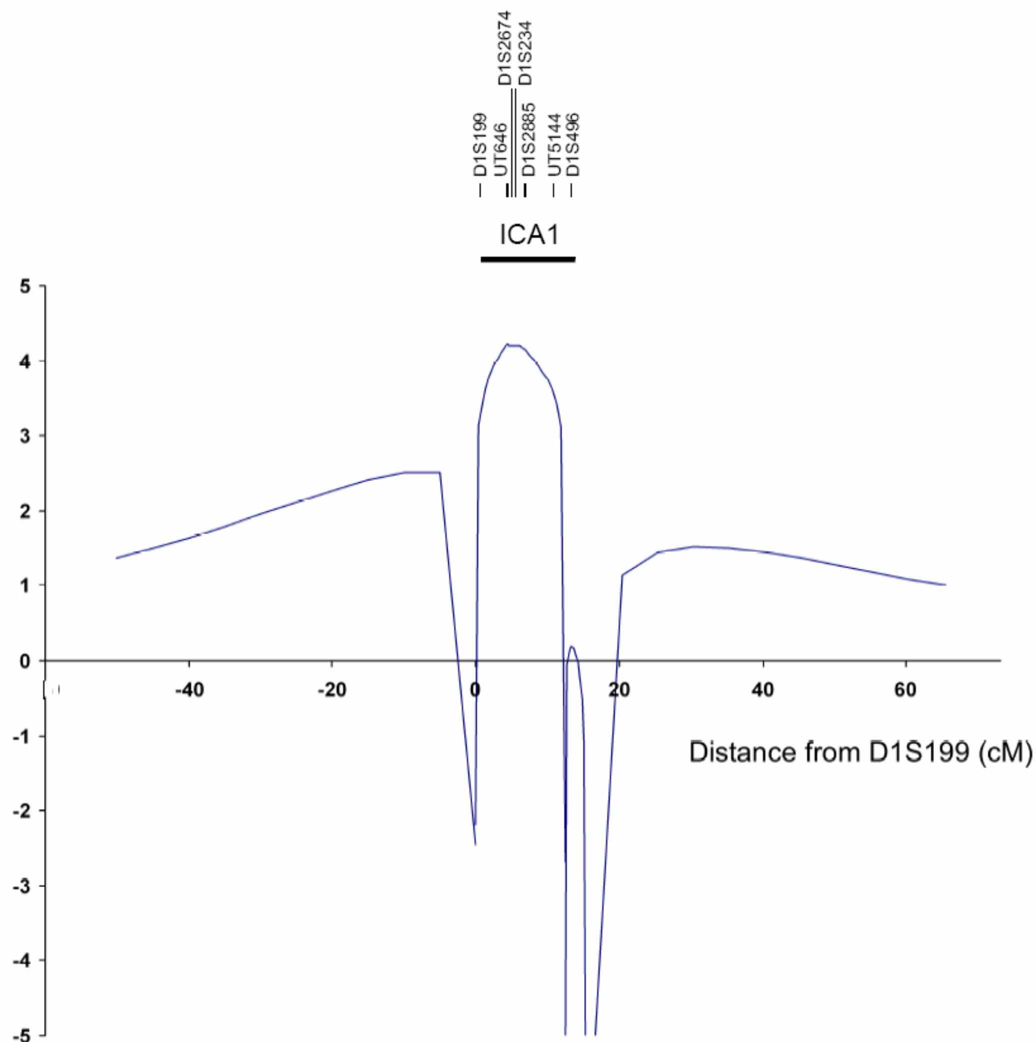


Table 1B: Maximum lod scores for linkage of STRs and IA

Interval	Penetrance		
	70%	90%	99%
1p34 – 1p36	3.4	3.9	4.2
1q31 – 1q41	1.3	-0.1	-5.6
2p11 – 2p14	-0.3	-2.3	-6.6

Maximum lod scores are reported for 1p34 – 1p36, 1q31 – 1q41, and 2p11 – 2p14 using STR markers in all family members with varying estimates of penetrance (Table 1B). We thus concluded that a gene located on 1p34-36 was the cause of IA in this family.

There are approximately 240 genes located within this interval spanning nearly 14 million base pairs (bp). As stated previously, this is too many to allow for direct sequencing of the entire region. We have thus taken a candidate gene approach within the linked interval, specifying genes that were more likely to be associated with IA based on function and expression pattern, and sequencing them first. We only sequence coding exons, introducing room for error in the event of a genetic abnormality located within non-coding exons, or intronic sequenes. However, to sequence whole genes is, with current technology, impractical and unfeasible.

Using this approach, we have sequenced nearly 60 genes located within this interval, including *Brain-Specific Angiogenesis Inhibitor (BAI)*, *Collagen Type XVI, alpha1 (COL16A1)*, and *Heparan Sulfate Proteoglycan 2*. While we have identified numerous polymorphisms in these genes, none of them co-segregated with the trait, and

we have identified no allelic variation that could potentially cause IA in this family. Sequencing of additional genes is ongoing.

2) IA 100

Epidemiology:

This family is originally from Columbia, but several members, including the index case, reside in the United States. The index case (III-7, Fig. 2A), works frequently with the PI, and upon mentioning her extensive family history, was screened and found to have an unruptured 8mm 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 three other affected members of her family (III-9, III-16, and III-17) (Fig. 2A). 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 (grey symbols in Fig. 2A). Furthermore, two subjects, II-2 and III-4, have documented AAAs (Fig. 2A). The characteristics of the family are presented in Table 2.

Figure 2A. IA 100. Affected and unaffected individuals are shown as filled and unfilled symbols, respectively. Individuals II-2 and III-4 were assigned affection status unknown prior to linkage analysis and are shown as grey symbols.

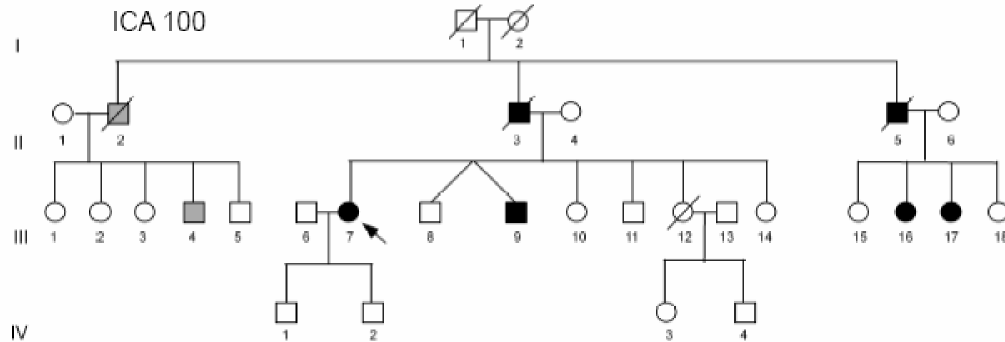


Table 2

Clinical Features of Affected Members in ICA 100

ID	Aneurysm Location and Size	SAH	Age at Diagnosis (years)
III-7	AComA, 8mm	-	40
III-9	ACA, A2/3 junction, 4mm	-	56
III-16	MCA	-	57
III-17	Right MCA bifurcation, 3-4 mm	-	54

[a] AComA, anterior communicating artery; ACA, anterior cerebral artery; MCA; middle cerebral artery.

<u>Linkage Analysis:</u>

Affected only analysis on the four family members with documented IAs demonstrated theoretical maximum linkage on multiple chromosomes throughout the

genome (Figure 2B). We performed linkage under two 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.

Due to limitations in the genechip software, we were only able analyze seven family members at one time. Using this method, we first added unaffected family members III-5, 10, and 15 to the four affected individuals in all of the chromosomes which yielded a theoretical maximum lod score in the affected only linkage analysis. Only the loci on chromosomes 3, 6, 11, and 17 (Figure 2C) continued to demonstrate linkage. Subsequently, we added three different unaffected individuals (III-2, 8, and 12) to our four affecteds in these four chromosomes, and found that only the locus on chromosome 11 remained while the other three loci showed significantly diminished evidence of linkage (Figure 2D). Finally, the locus on chromosome 11 is also the only one to be inherited by individuals II-2 and III-4 under the model that mutations in the same gene are causing their AAAs. Further genotyping of all unaffected individuals revealed a maximum lod score of 4.3 on chromosome 11q specifying a penetrance of 99% if individuals with AAAs (II-2 and III-4) are considered as affected. Assigning an affection status unknown phenotype to these two individuals gives a lod score of 3.6 without any effect on the chromosomal localization. The lod-1 interval for the IA 100 locus lies between 125.6 to 131.4 million base pairs (mbp) on chromosome 11q24-25, between SNP markers rs618176 and rs1940033. Interestingly, this is one of the 14 regions linking to IA in a Japanese sib pair study, demonstrating a statistically significant linkage (p=0.023) between marker D11S910, located at 131.2 mbp on 11q, and the IA phenotype.

Figure 2B. Analysis of linkage in IA 100 from GeneChip data of affected individuals. Linkage graphs for all chromosomes are shown; x-axis corresponds to genetic distance (cM) and y-axis shows lod scores.

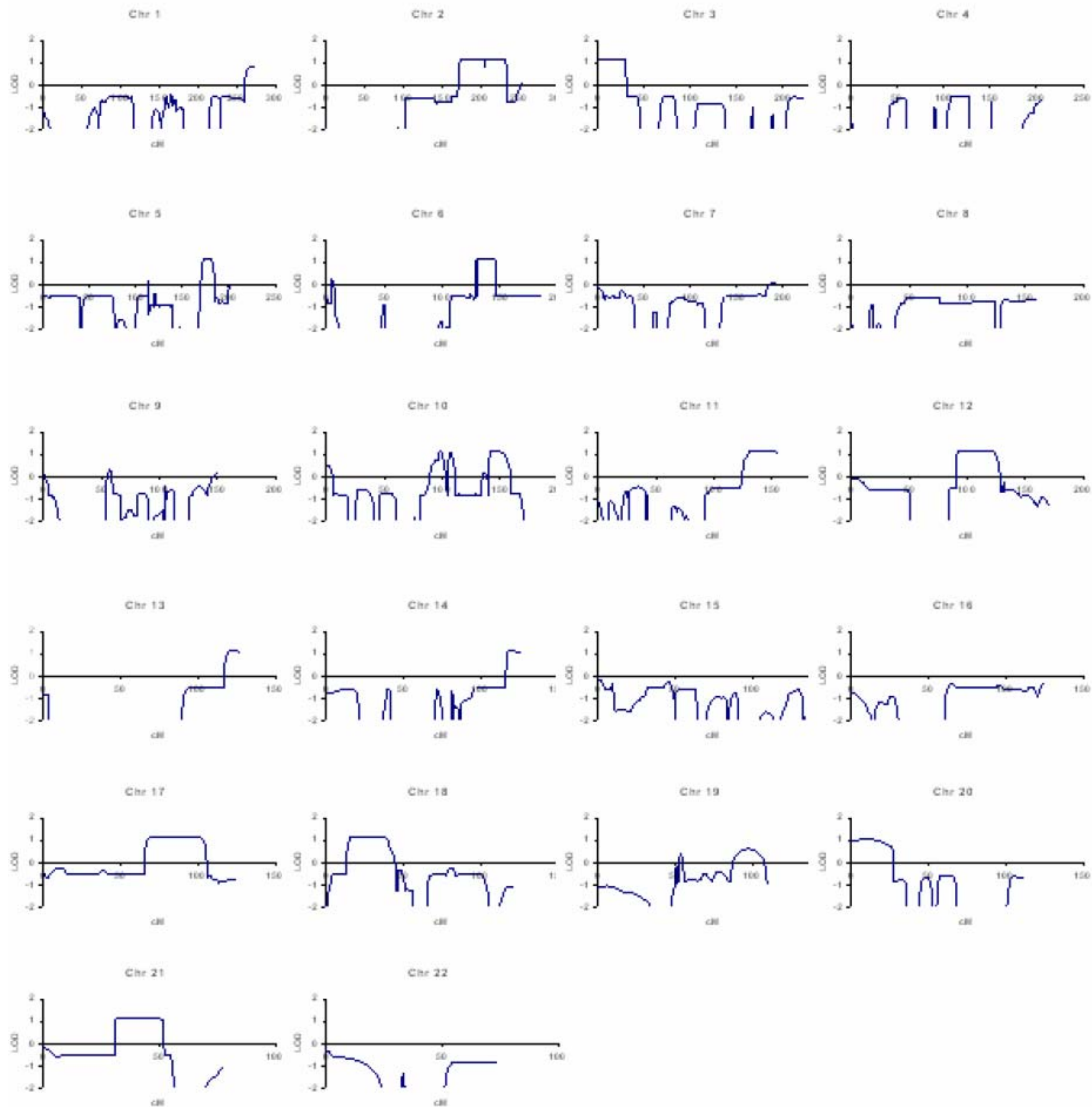
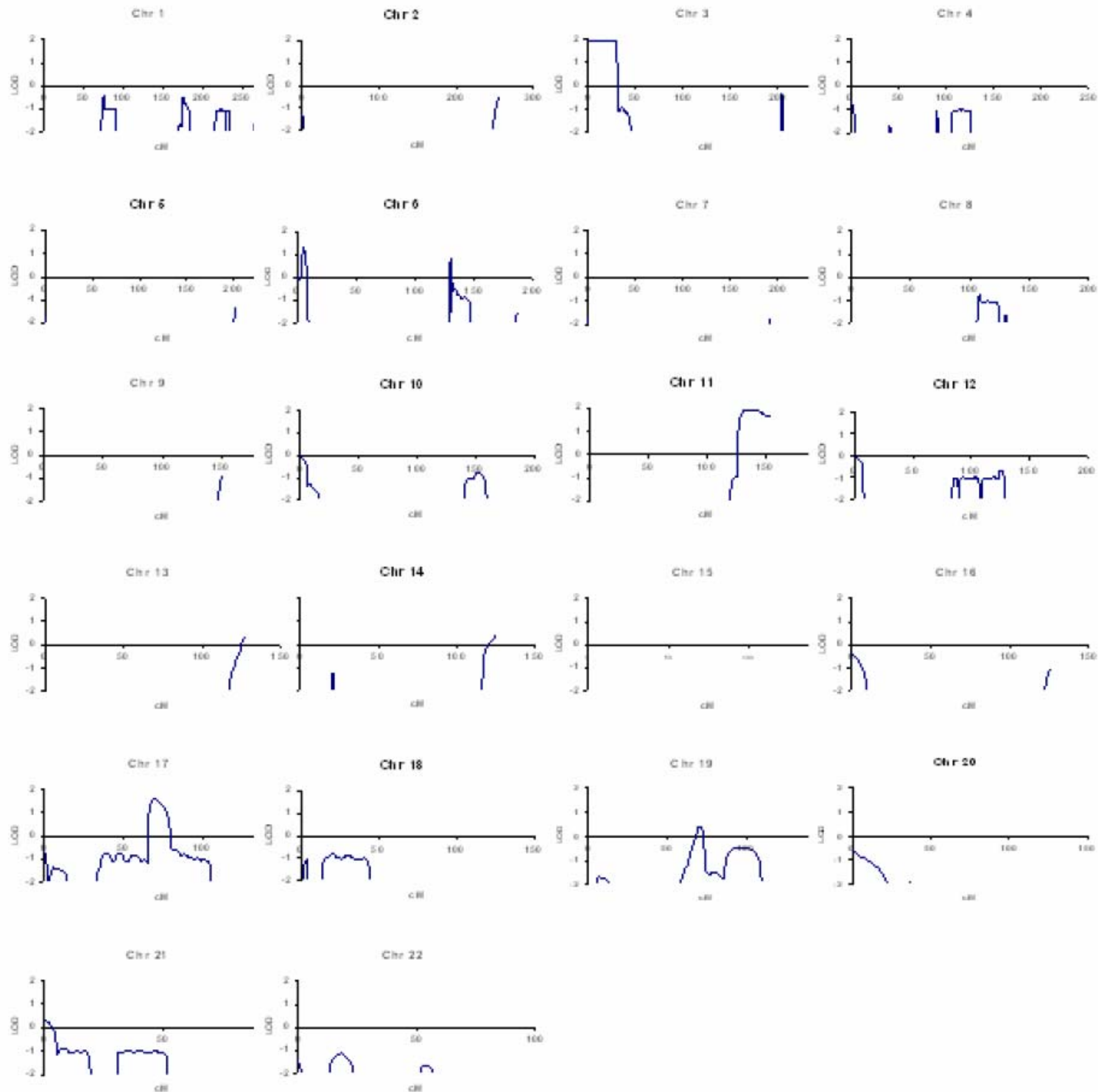
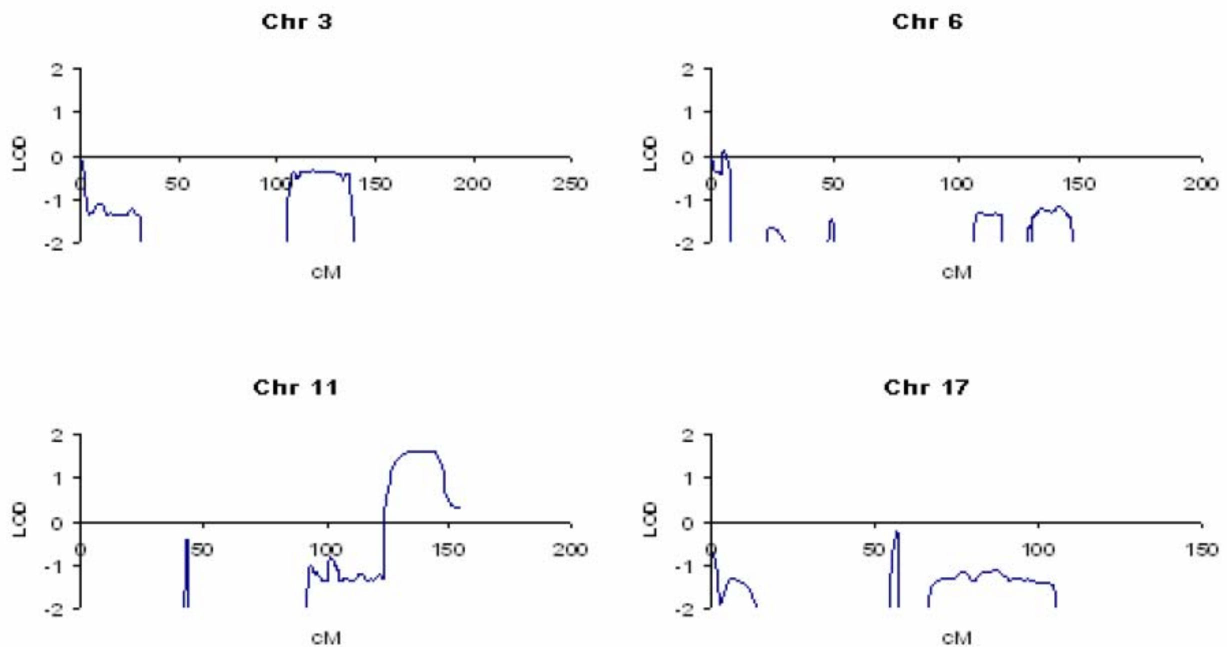


Figure 2C. Analysis of linkage in IA 100 from GeneChip data of affected and unaffected individuals. Linkage graphs for all chromosomes are shown; x-axis corresponds to genetic distance (cM) and y-axis shows lod scores.



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 mbps, respectively. This suggests that the longest interval that can contain an IA susceptibility gene on 11q is between 125.8 to 133.7 mbp. Thus, the IA 100 locus that lies between 125.6 to 131.4 mbp is almost fully contained within the region defined by the Japanese study.

Figure 2D. Analysis of linkage in IA 100 from GeneChip data of affected and unaffected individuals. Linkage graphs for all chromosomes are shown; x-axis corresponds to genetic distance (cM) and y-axis shows lod scores.



Sequencing of the genes located within this interval is ongoing. Thus far, we were able to identify no conclusive polymorphism that appears to be directly leading to IA in this family (Ozturk, 2006).

3) IA 101

Epidemiology:

This kindred was identified in Los Angeles. There are a total of 9 members with documented IAs, 4 of whom are deceased and one of whom refused to provide a blood sample (IV-5) (Figure 3A). We thus collected samples from four affected individuals. In addition, samples from 24 unaffected members were collected. The aneurysm characteristics in this family are presented in Table 3.

Figure 3A. IA 101. Affected and unaffected individuals are shown as filled and unfilled symbols, respectively. Individual V-2 was assigned affection status unknown prior to linkage analysis and is shown as grey symbol.

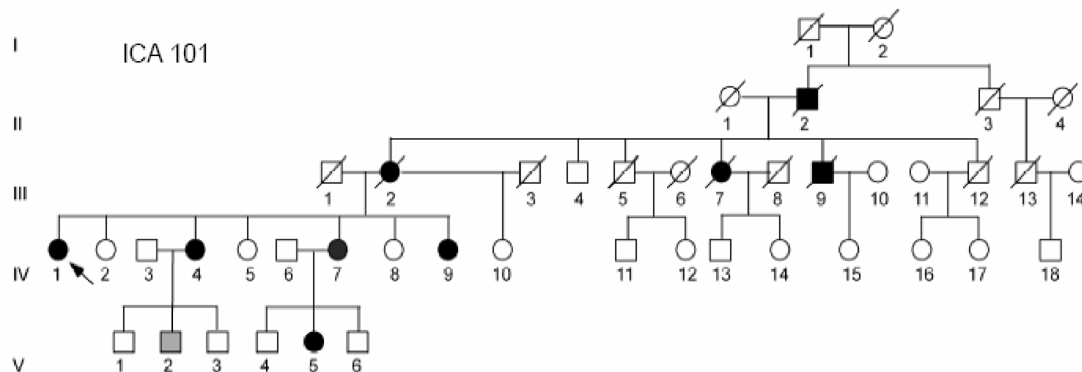


Table 3

Clinical Features of Affected Members in ICA 101

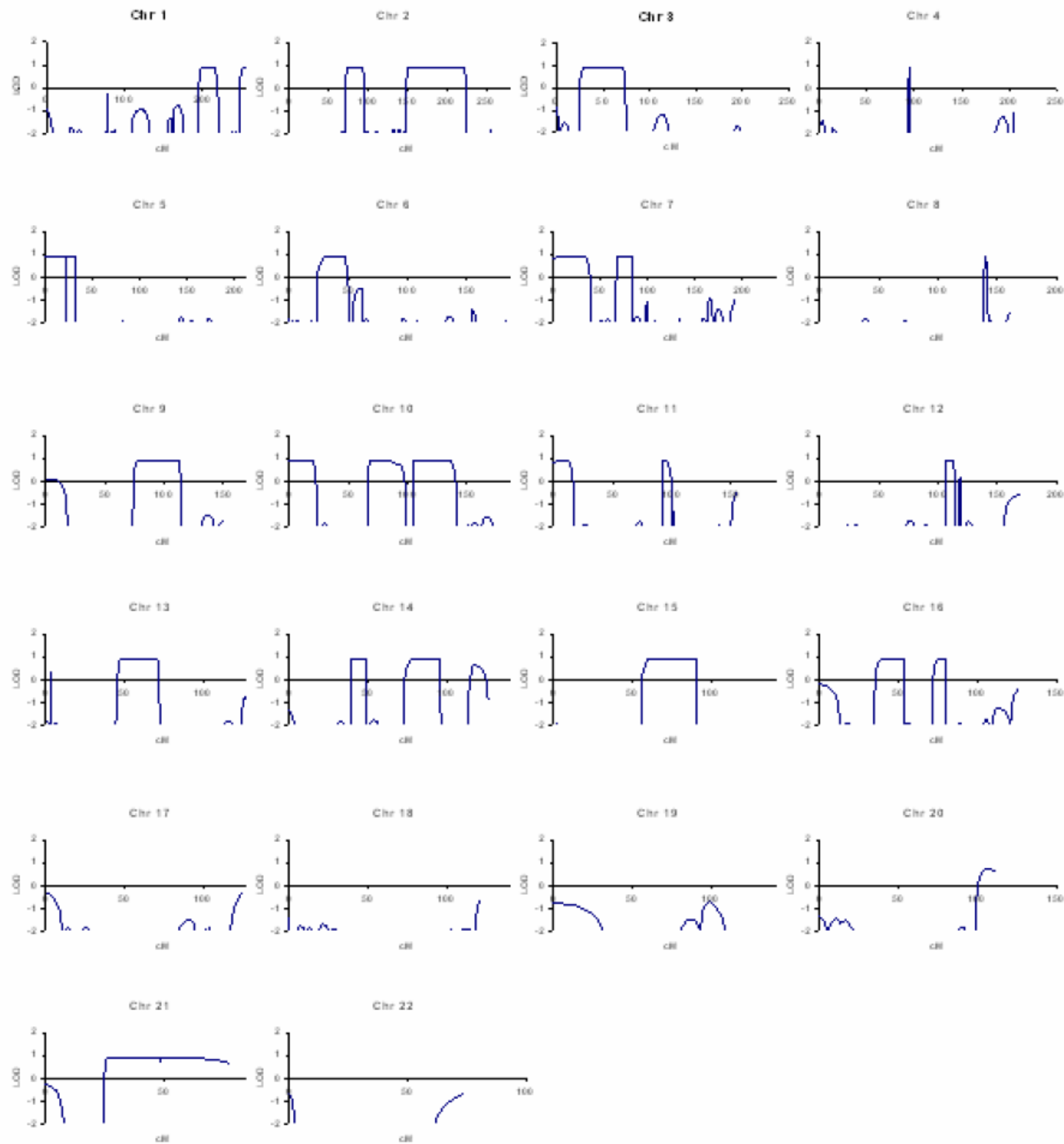
ID	Aneurysm Location and Size	SAH	Age at Diagnosis (years)
IV-1	AComA	-	41
IV-4	MCA	-	42
IV-7	AComA	-	57
IV-9	ACA	-	59

[a] AComA, anterior communicating artery; ACA, anterior cerebral artery; MCA; middle cerebral artery.

<u>Linkage Analysis:</u>

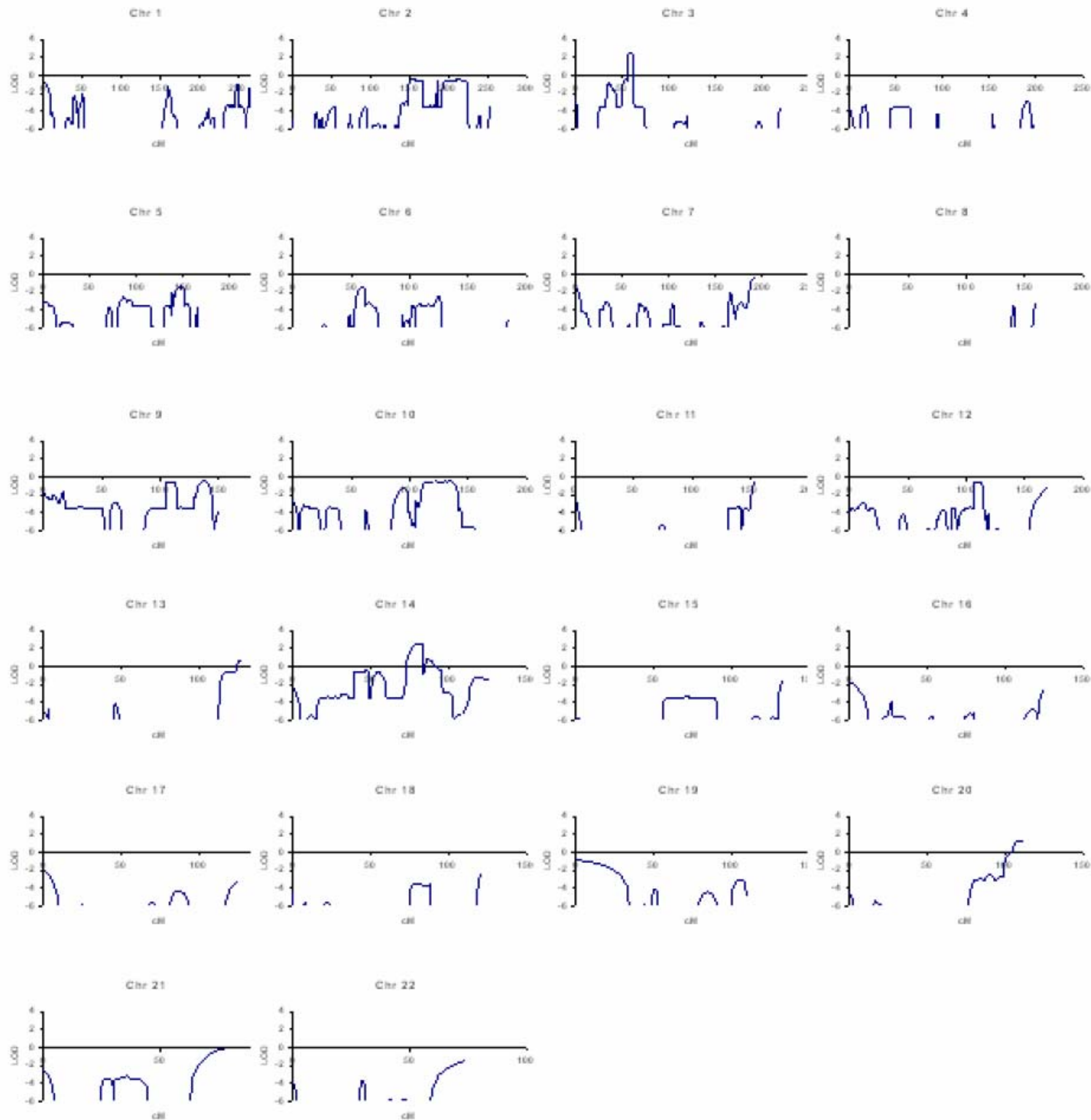
Affected-only genome wide linkage analysis revealed suggestive linkage to various regions (Figure 3B). Additional GeneChip analysis of 7 unaffected members (individuals III-4, IV-2, IV-5, IV-8, IV-10, V-1, and V-3), all with negative imaging studies, revealed linkage to chromosomes 3 and 14, both with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

Figure 3B. Analysis of linkage in IA 101 from GeneChip data of affected individuals. Linkage graphs for all chromosomes are shown; x-axis corresponds to genetic distance (cM) and y-axis shows lod scores.



The lod-1 interval is located between SNP markers rs2359991and rs2373098 which are at 75.5 and 85.6 mbp, respectively, on 14q23-31. Similar to the IA 100 locus, two markers located within the IA 101 locus were found to have statistically significant linkage with the IA phenotype in the Japanese study. 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 to 87.6 mbp based on the location of the two surrounding markers, D14S63 and D14S68, with no significant linkage to IA ($p>0.05$). Thus the overlap between the suggestive intervals in the two studies is between 75.5 to 85.6 mbp on chromosome 14q (Ozturk, 2006).

Figure 3C. Analysis of linkage in IA 101 from GeneChip data of affected and unaffected individuals. Linkage graphs for all chromosomes are shown; x-axis corresponds to genetic distance (cM) and y-axis shows lod scores.



4) IA 42

Epidemiology:

This family was recently identified in Pennsylvania. Currently, there are a total of five living affected members, four of whom are siblings; the fifth is their cousin (Figure 4A). There are also three deceased members with reported IAs. Furthermore, we have collected blood samples from 25 unaffected individuals in the family. Aneurysm characteristics of this family are shown in Table 4.

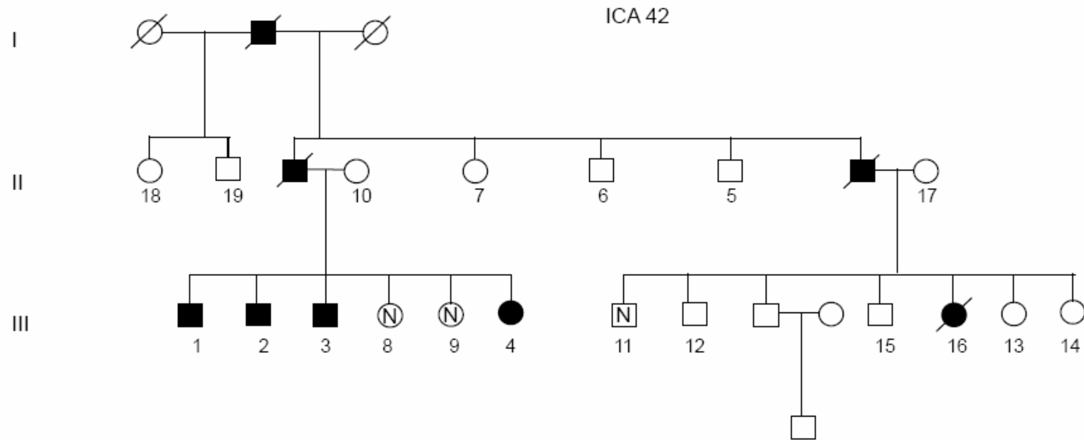


Figure 4A. IA 42. Affected and unaffected individuals are shown as filled and unfilled symbols, respectively. Family members with negative imaging studies are designated with an "N".

Table 4

Clinical Features of Affected Members in ICA 42

ID	Aneurysm Location and Size	SAH	Age at Diagnosis (years)
III-1	AComA	+	40
III-2	MCA	+	56
III-3	MCA	+	57
III-4	MCA, ACA	-	38
III-16	AComA	+	54

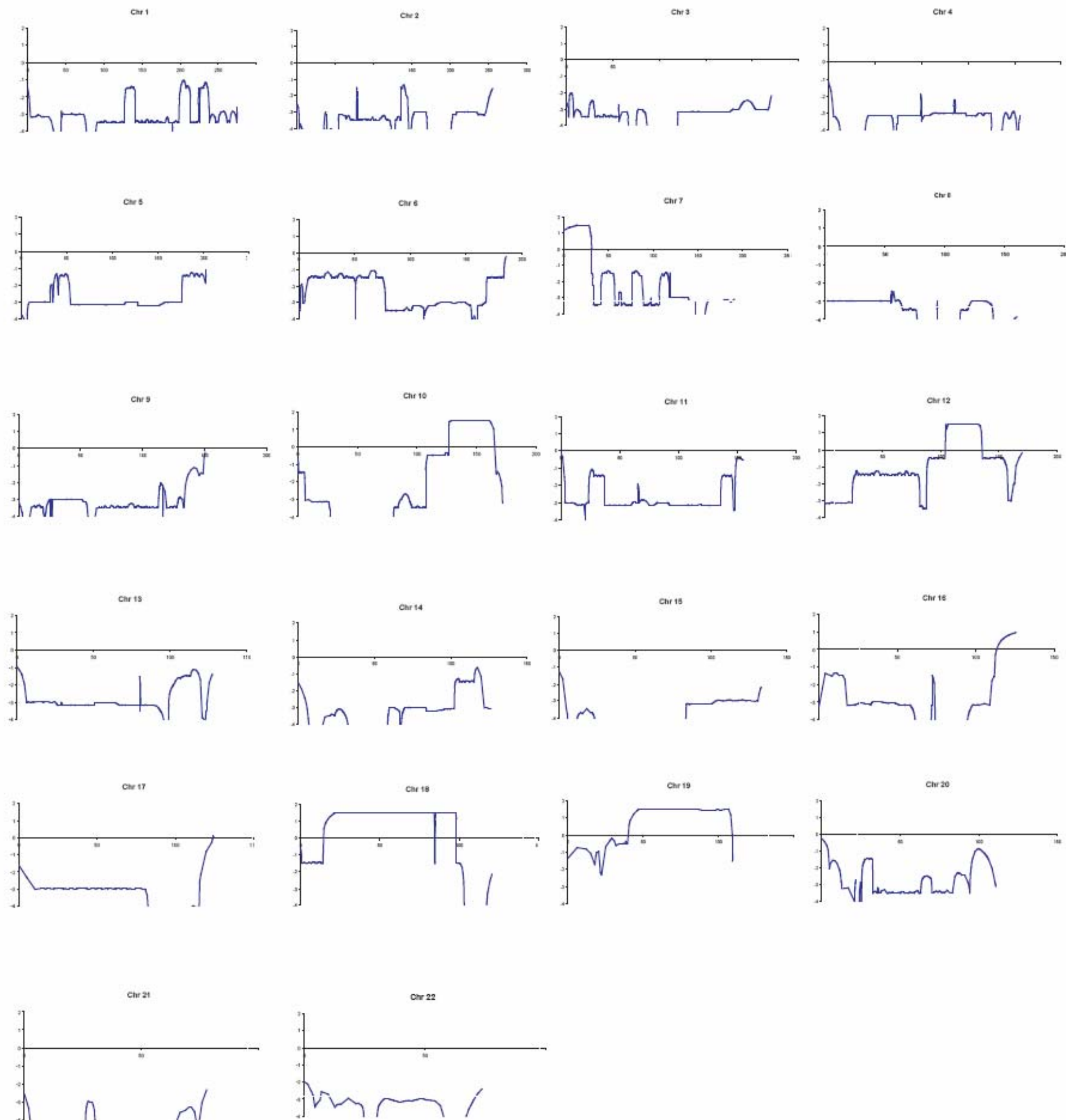
[a] **AComA, anterior communicating artery; ACA, anterior cerebral artery; MCA; middle cerebral artery.**

Linkage Analysis:

While genetic linkage analysis of this family is not complete, we have performed an affected only analysis of the five members with IA. Initial linkage studies demonstrated robust linkage to chromosomes 7, 10, 12, 16, 18, and 19 (Figure 4B). In order to narrow down these candidates to only one interval, we will use unaffected members of the family, as described previously, but also will rely on the MRA results of the five family members who are currently being evaluated for the presence of an IA. The addition of one or more affected members to this family will also narrow the candidate intervals, while avoiding the complications of incomplete penetrance. Seemingly unaffected individuals, on the other hand, who inherit the high-risk portion of the genome, make the analysis more difficult to interpret by lowering the LOD score in

the disease gene-containing region. It is for this reason that when possible, we prefer the affected only analysis as the most reliable and robust in linkage analysis.

Figure 4B. Analysis of linkage in IA 42 from GeneChip data of affected individuals. Linkage graphs for all chromosomes are shown; x-axis corresponds to genetic distance (cM) and y-axis shows lod scores.



DISCUSSION

Linkage and association studies are not without their inherent strengths and weaknesses. While linkage is more thorough, inasmuch as it takes into account the entire genome, it may fail to detect genes imparting small effects on the disease trait. Association studies, on the other hand, rely on pathways of disease that have already been identified, and are not good tools to decipher novel pathways leading to disease. Given these strengths and shortcomings of association and linkage studies (both parametric and non-parametric), research needs to focus on employing them as best as possible to benefit from their strengths while avoiding the pitfalls inherent in all of the mentioned approaches. These methods, thus, should not be thought of mutually exclusive, but rather accepted as being fully compatible with one another, and used in a complementary way. Thus, association studies may be performed *after* an initial genome-wide scan, limiting the room for error introduced with this method, and also, presumably, limiting the number of genes needing to be directly sequenced.

The challenge, then, is accomplishing a robust linkage analysis at or above statistical significance. In the setting of substantial locus heterogeneity, as is the case with all complex human traits including IA, this is no small feat. The identification of rare families, who are ostensibly inheriting the disease trait in a simple Mendelian fashion, circumvents this obstacle. While difficult to find, such rare, extended families reduce the complex trait into one that is caused by a single gene imparting a large effect on the disease trait, and by so doing, enable the use of parametric linkage approaches. The gene that is causing the disease in the outlier family, if identified, may subsequently

be analyzed in other, more common, forms of the disease to determine any, if not more subtle, roles the gene may have in these seemingly sporadic cases.

Genetics of Intracranial Aneurysm:

Multiple lines of evidence suggest that genetic factors are involved in IA formation. The existence of families with multiple members with IA has been known for some time. In the 1960's Ulrich and Sugar reported four families with two or more siblings affected by IAs. In the 1970's Brisman et al reported families with an autosomal dominant inheritance of intracranial aneurysms (28). In 1983, Fox and Ko reported the largest kindred at the time in which 6 of 13 siblings were found to harbor IAs (27). It is now known that first degree relatives of affected individuals have a three to five-fold increase in risk compared to the general population (14).

In light of these developments, several groups have begun to search for genes that may directly lead to IA phenotype in select families. Various groups have used all of the above approaches to identify an IA gene with only limited success.

Several candidate genes have been implicated in the pathogenesis of IA but have yet to display a causal relationship. These genes range from those associated with vascular wall formation to those that are mutated in connective tissue disorders and include Elastin, Collagen III, Fibrillin, Polycystin, and Endoglin (12, 29-40). Despite several loose associations identified with a few of these genes and the IA trait, none of these associations have been consistently reproducible, leaving many in doubt in terms of the effectiveness of the candidate gene approach with regards to IA. While approaches driven by hypotheses regarding disease mechanisms, such as candidate gene mutational

analysis are intuitively attractive, experience has shown time and time again that positional cloning is the most productive method for isolating causative genes when the pathophysiology and molecular biology of a disorder is not well elaborated.

Overall, non-parametric linkage analyses have identified multiple loci that may be contributing to IA on several chromosomes including 5q22-31(41-43), 17cen (44), 19q (44, 45) and Xp (44). 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 non-parametric approaches are attractive with respect to IA as 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 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 due to genetic heterogeneity or environmental factors. This approach has been successful in identifying rare mutations imparting large effects on blood pressure, lipid metabolism, insulin resistance, and obesity, 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. While there have been fewer studies utilizing 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 (46).

By using an outlier approach that relies on parametric linkage studies in large families, our group has already reported an IA susceptibility locus on 1p34-36 with a maximum lod score of 4.2. More recently, we have used the same approach on two other large families and have identified two 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. Since 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 younger than 30 years of age in our linkage analysis. Furthermore, the intervals identified in these two families are among the 14 regions reported to show significant linkage to IA in another study by Onda et al. that reported genome-wide linkage of 104 Japanese affected sib-pairs using 404 polymorphic markers throughout the human genome (41). The confirmation of these loci by another group in a separate population using a different approach is strong evidence in favor of the veracity of the results.

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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