Elastin Polymorphism Haplotype and Intracranial Aneurysms Are Not Associated in Central Europe

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Background and Purpose—The occurrence of intracranial aneurysms and of aneurysmal subarachnoid hemorrhage are influenced by genetic factors. Recent genomic studies in Japan have defined 3 chromosomal loci and 1 haplotype of elastin polymorphisms as important risk factors, both for affected sib pairs and sporadic patients.

Methods—We have genotyped 2 single nucleotide polymorphisms in the elastin gene and evaluated their allelic association with intracranial aneurysm in a Central European sample of 30 familial and 175 sporadic patients and 235 population controls.

Results—We found no allelic association between this elastin polymorphism haplotype and intracranial aneurysm.

Conclusion—Our data probably reflect increased genetic heterogeneity of intracranial aneurysm in Europe compared with Japan. (*Stroke*. 2003;34:1207-1211.)

Key Words: elastin ■ genetics ■ intracranial aneurysm ■ polymorphism, single nucleotide ■ subarachnoid hemorrhage

The population prevalence of intracranial aneurysms (IA) is 3% to 4% in prospective autopsy studies and 5% to 7% in prospective angiography studies.[1] As with the prevalence of IA, the annual incidence of subarachnoid hemorrhage (SAH) varies among different ethnic groups, with the highest frequencies reported for Japan and Finland.[2,3]

The reduced penetrance of IA has made it difficult to analyze inheritance patterns.[4] It is clear, however, that a congenital predisposition plays a role at least in a subgroup of patients. Thus, a 2- to 6-fold increased risk of SAH has been reported among first-degree relatives of SAH index patients.[5–10] The largest documented pedigree included 6 clinically manifest individuals and displayed an apparently autosomal dominant inheritance.[11] The underlying molecular defect is thought to affect connective tissue development, given than IA has been reported to be part of the phenotype in several human inherited connective tissue abnormalities.[12–14] Furthermore, aortic aneurysms have been explained by mutations in the fibrillin-1 gene in some patients.[15,16] Allelic associations between matrix metalloproteinases and both intracranial and abdominal aortic aneurysms have been reported in individual studies.[17,18] Analyses of the connective tissue in walls of IA documented anomalies in the type III collagen network[19–22] but found no mutations in the COL3A1 gene itself.[23,24]

Although IA families have been collected in several countries, a sufficient number of large pedigrees for classical linkage studies has not been recruited so far. Recently, 83 affected Japanese sib pairs were genotyped with microsatellite polymorphisms at 404 chromosomal loci by Onda et al.[25] They found 3 suggestive linkages for IA on chromosomes 5q22 to 31, 14q22, and—with the highest logarithm of odds score—7q11. To corroborate these loci, the analysis of additional IA families will be necessary. On chromosome 7q11, haplotypes have already been analyzed to narrow the critical region.[25] In affected sib pairs and affected sporadic individuals, the Japanese group reported a strong and reliable association ($\chi^2=27.9$; $P=3.81\times10^{-6}$) between IA and a haplotype formed with 2 polymorphisms within the elastin (ELN) gene, namely single nucleotide polymorphisms

TABLE 1. Characteristics of our Collection of 205 Patients With Intracranial Aneurysm

Sex	123 women, 82 men
Age of manifestation, y (mean±SD)	48.0±10.9
Ruptured IA, %	80.5
Multiple IA, %	27.8
Location of IA* (%)	
ICA	25
MCA	27
ACA	6
ACoA	20
PCA	1
PCoA	5
BA/VA	10
Other	6

*Includes multiple intracranial aneurysms.

ICA indicates internal carotid artery; MCA, middle cerebral artery; ACA, anterior cerebral artery; ACoA, anterior communicating artery; PCA, posterior cerebral artery; PCoA, posterior communicating artery; BA/VA, basilar or vertebral artery.

(SNPs) in intron 20 (Int20) (rs2856728 in SNP database at http://www.ncbi.nlm.nih.gov/SNP/) and in intron 23 (Int23) (base pair 24 of the intron, position 6443039 of contig NT_007758).[25] The aim of our study was to test the relevance of this association in a Central European sample including familial and sporadic IA patients.

Subjects and Methods

Patients
Two hundred five individuals with IA were recruited by the participating institutions in Innsbruck, Austria, and Lübeck, Essen, Günzburg, Giessen, Bonn, and Mainz, Germany. The IA diagnosis was always established by angiography. Thirty of the 205 patients were members of IA families with 2 or more affected individuals. Other baseline characteristics of our collection are given in Table 1. Venous blood samples were taken in EDTA and stored at −80°C. DNA was extracted using a silica-based microtiter method (QIAamp 96 DNA Blood Kit, Qiagen). Two hundred thirty-five DNAs of healthy unrelated individuals from the same geographic areas in Central Europe were analyzed as control. Written informed consent was obtained from all patients under study. The study was part of a nationwide investigation of stroke financed by the German Ministry of Health, and a central ethics committee in the capital approved the complete stroke research program. In addition, the specific aspects of the intracranial aneurysm study was approved by the ethics committee at Frankfurt University Hospital.
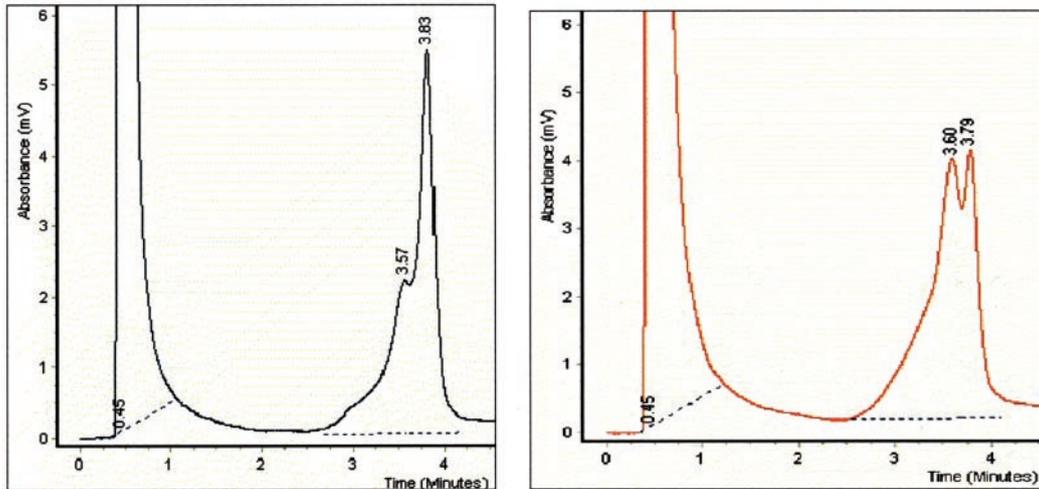
Denaturing High Performance Liquid Chromatography
Polymerase chain reaction (PCR) products including both SNPs were prepared from Int20 and Int23 of the elastin gene using primer pairs and annealing temperatures as shown in Table 2. PCR reactions were carried out in 25 μL total volume that included 40 ng DNA template in 1 μL, 10 pmol of forward and reverse primer, 0.25 mmol/L final concentration of dNTPs, 0.5 U of *Taq* DNA polymerase (AmpliTaq from PE Biosystems) with the corresponding buffer. PCR conditions were as follows: initial denaturation at 95°C for 50 seconds, cyclic denaturation at 94°C for 1 minute, annealing at the appropriate temperature for 1 minute, elongation at 72°C for 1 minute, and final extension at 72°C for 7 minutes. The PCR products were heated for 10 minutes at 95°C followed by incubation at 55°C for 10 minutes to allow heteroduplex formation. The denaturing high performance liquid chromatography (dHPLC) analysis was performed on a Wave System (Transgenomics). Homozygous and heterozygous genotypes of the known SNP 20 and SNP 23 were distinguished according to their differences in melting behavior.[26] Temperature and acetonitrile gradient were established according to sequence-specific melting profiles by predictions of the Stanford Genome Technology Center and Wave-Maker software. For Int20, the optimal conditions were 62.5°C or 63.5°C with 53% to 62% acetonitrile. For Int23, 64°C and 53% to 62% acetonitrile were optimal (Figure 1). As control samples

TABLE 2. Primers Used for Genotyping of Single Nucleotide Polymorphisms of the Elastin Gene

Primer	Annealing Temperature, °C	PCR Length, bp
For sequencing and denaturing high performance liquid chromatography		
Int20		
5′-TTTCCCGGCTTTGGTGTCGGAGTC-3′	60	265
5′-GCCCTGTAAGGCATGGATACGA-3′		
Int23		
5′-CATGGAAGGGTCCCTGGAGTTGAC-3′	59	346
5′-ATTCATCAGTGGGCCAAAAGGTCT-3′		
For mutagenically separated polymerase chain reaction		
Int20		
5′-CTTTCCCGGCTTTGGTGTCGGAGTCGG-3′		
5′-CCA<u>C</u>CCCTTCTCAACCCATGTCCCCAA**G**-3′	64	152–163
5′-ACAAGA<u>A</u>GCCCGAATCCCTTCTCAACCCATGTCCCCC**GA**-3′		
Int23		
5′-ATTACTCTCTCACCCCTTCTCTTCACACC-3′		
5′-CAG<u>A</u>AGAAAGCAGTTCTCCGTGAG<u>AC</u>A**A**-3′	57	143–158
5′-CCAGAGCAGGGC<u>A</u>CTGGAGAAAGCAGTTCTCCGTGAGCG<u>A</u>**G**-3′		

Bold letters indicate the SNP position, whereas underlined letters represent mutagenic changes from the normal sequence.

Figure 1. Denaturing high performance liquid chromatography: Retention profiles for Int23 T>C at 64°C show the homozygous and the heterozygous genotype

of both alleles we used sequence-verified German samples, and control samples donated by Prof Inoue's team in Japan. Samples that gave a homozygous profile could be defined as CC homozygotes or TT homozygotes by mixing them with DNA from CC control individuals and analyzing the formation of heteroduplexes in a second dHPLC run.

Mutagenically Separated Polymerase Chain Reaction

For each SNP, 2 allele specific reverse primers and 1 forward primer were designed to produce a length-polymorphic PCR product,[27] as shown in Table 2. PCR reactions were carried out in 30 μL total volume that included 30 ng DNA template in 1 μL, 0.05 mmol/L final dNTPs, 0.5 U of *Taq* DNA polymerase (Hot Start AmpliTaq from PE Biosystems) with the corresponding buffer. The optimal primer concentrations were as follows: primer concentration Int20: 0.13 μmol/L forward primer, 0.22 μmol/L reverse primer 1, 0.15 μmol/L reverse primer 2; Int23: 0.13 μmol/L forward primer, 0.08 μmol/L reverse primer 1, 0.24 μmol/L reverse primer 2 (Figure 2). PCR conditions for Int20 and Int23 were as follows: initial denaturation at 94°C for 15 minutes, cyclic denaturation at 94°C for 15 seconds, annealing at the appropriate temperature for 30 seconds, elongation at 72°C for 20 seconds, final extension at 72°C for 7 minutes. Again the specific detection of each allele was controlled using sequence-verified positive control samples (Figure 3).

Sequencing

For sequencing purposes, we used primers and PCR conditions as for dHPLC and used BigDye (Applied Biosystems) chemistry on an ABI 377 automated sequencer. Electropherograms were transferred to a LINUX-SUSE server and assembled and scanned for consensus/ variants with the SeqMan software of the DNAstar Lasergene

Figure 2. MS-PCR of Int20 representing the homozygous T allele, the heterozygous state, and the homozygous C allele

Navigator package. Sequencing was performed to verify selected results from dHPLC and to screen for additional polymorphisms within these 2 introns.
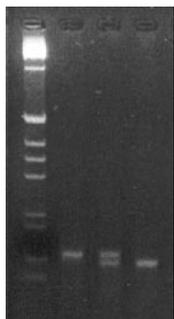
Statistical Analysis

Allelic association with IA was evaluated by traditional and Craddock-Flood chi-square test statistics for both SNPs Int20 and Int23. Haplotype frequencies of patients and controls were estimated by an expectation-maximization algorithm using the Arlequin software, version 2.000 (http://lgb.unige.ch/arlequin). Again, allelic association of patient versus control haplotypes with IA was assessed using chi-square contingency tables. A *P* value <0.05 was considered significant. Given the frequencies of 0.107 and 0.027 observed in IA patients and controls for Mm haplotype homozygotes by Onda et al,[25] our sample of 205 patients and 235 controls would have a statistical power of 0.905, as assessed by the BiAS software for binomial distributions (http://www.bias-online.de).

Results

dHPLC analysis and mutagenically separated PCR (MS-PCR) were carried out for all samples and gave completely concordant results. Automated sequencing was used to verify dHPLC and MS-PCR data in comparison with the positive controls from Japan. The distribution of alleles for Int20 and Int23 within our collection of 205 IA patients and 235 control individuals from Central Europe is shown in Table 3. For Int20, the frequency of the C allele was 18.0% (74 among 410 chromosomes) in the patient sample, compared with 15.9% (75 among 470) in the control sample. For Int23, the frequency of the C allele was 42.7% (175 among 410) in the patient sample, compared with 47.4% (223 among 470) in the control sample.

For Int20, the observed rate of homozygotes and heterozygotes corresponded to Hardy-Weinberg equilibrium predictions, whereas for Int23, the observed values differed from expectation ($\chi^2=3.607$, $P=0.0576$). Comparing the frequency of alleles in our control population with the controls reported by Onda et al,[25] a significantly lower frequency of the minor allele was observed among Germans for Int20 ($\chi^2=14.51$, $P<0.000139$) and among Japanese for Int23 ($\chi^2=26.4$, $P<0.000001$). After testing for allelic association with the disease, no significant effect or even trend could be
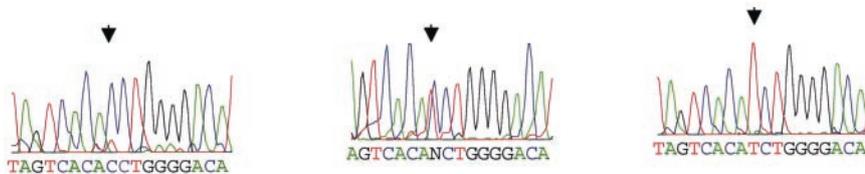
Figure 3. DNA-sequence traces of Int20 representing the homozygous C allele, the heterozygous state, and the homozygous T allele

observed between IA and Int20 ($\chi^2=0.75$, $P=0.684$) or Int23 ($\chi^2=4.33$, $P=0.11$). The estimation of haplotype frequencies of Int20/Int23 with the Arlequin software in patients were as follows: C/C, 0.066; C/T, 0.094; T/C, 0.401; T/T, 0.439; in controls, C/C, 0.047; C/T, 0.134; T/C, 0.381; T/T, 0.438. The chi-square analysis of an association between such haplotypes and IA did not generate positive evidence in our collection ($\chi^2=2.6$, $P=0.45$). The haplotype analysis in the subgroup of 30 patients from the IA multiplex families versus controls also failed to show an association ($\chi^2=6.2$, $P=0.10$), but the statistical power of this group was insufficient for conclusions ($1-\beta=0.547$).

Discussion

Apart from the genetic evidence mentioned earlier, a pathogenetic role of elastin in IA has recently become corroborated also through a global gene expression analysis approach (SAGE-Lite), in which significant overexpression of elastin was observed in tissue obtained from a 3-year-old patient.[28] Another argument for the role of elastin in IA comes from observations in the mouse mutant Blotchy, in which a mutated gene of copper metabolism leads to defective elastin and collagen synthesis and a tendency to develop IA, particularly under increased hemodynamic stress.[29] Furthermore, a well-established experimental induction of IA in rodent models is through application of elastase within arteries.[30]

In view of the good evidence arguing for the role of elastin in IA, this study analyzed polymorphisms within the elastin gene known to display a highly significant association with IA and aneurysmal SAH in Japan. Nevertheless, we were unable to confirm these previous findings by Onda et al.[25] There are possible explanations for this discrepancy: First, the Japanese study included higher proportions of patients with positive family history than ours. Testing only our subgroup of 30 familial patients also failed to reveal significant allelic association, although the statistical power of this subgroup is limited. Second, the phenotype in our collection in comparison with the Japanese study was characterized by more ruptured IAs, reduced frequency of the MCA and ICA

location, and younger manifestation. Third, if many different IA mutations at the elastin locus coexist in our sample, an association of 1 allele with IA will not be observed. Because the Japanese population has undergone fewer migrations and admixtures than those of Central Europe, such an increased genetic heterogeneity appears quite plausible.

In conclusion, limited genetic heterogeneity of IA has been shown in Japan, and additional genetic heterogeneity likely exists in Europe. Since the completion of our study, the analysis of Finnish affected sib pairs with IA has demonstrated suggestive linkage to chromosome 19q and could not substantiate the relevance of the aforementioned IA loci at 5q, 7q, and 14q in Finland.[31] Therefore, further attempts should be undertaken to collect families and affected sib pairs also from other parts of the world to advance independent genomic screens and cooperative efforts to identify the genes underlying IA. Such further elucidation of the genetic basis of IA could be a major step toward a better understanding of the pathobiology of this vessel wall disorder.

Acknowledgments

TABLE 3. Int20 and Int23 Genotype Counts in Patients With Intracranial Aneurysms Versus Controls

	n	CC	CT	TT
Int 20				
IA	205	8 (3.9%)	58 (28.3%)	139 (67.8%)
Controls	235	6 (2.6%)	63 (26.8%)	166 (70.6%)
Int 23				
IA	205	44 (21.5%)	87 (42.4%)	74 (36.1%)
Controls	235	52 (22.1%)	119 (50.6%)	64 (27.3%)

IA indicates intracranial aneurysm.

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