Genetic Analysis of Familial Connective Tissue Alterations Associated With Cervical Artery Dissections Suggests Locus Heterogeneity

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Background and Purpose—Cervical artery dissections (CAD) can be associated with connective tissue aberrations in skin biopsies. The analysis of healthy relatives of patients suggested that the connective tissue phenotype is familial with an autosomal dominant inheritance.

Methods—We performed genetic linkage studies in 3 families of patients with CAD. Connective tissue phenotypes for the patients and all family members were assessed by electron microscopic study of skin biopsies. A genome-wide linkage analysis of 1 family (1 patient with 8 healthy relatives) indicated 2 candidate loci. Three genes were subsequently studied by sequence analysis. Part of the genome was also studied by linkage analysis in 2 further families.

Results—The genome-wide scan in a single family suggested linkage between the hypothetical mutation causing the connective tissue phenotype and informative genetic markers on chromosome 15q24 (logarithm of the odds score: \( Z = 2.1 \)). A second possible candidate locus (\( Z = 1.9 \)) was found on chromosome 10q26. Sequence analysis of 3 candidate genes in the suggestive locus (chondroitin sulfate proteoglycan4 [CSPG4], lysyl oxidase-like1 [LOXL1] and fibroblast growth factor receptor2 [FGFR2]) did not lead to the identification of a mutation responsible for connective tissue alterations. In 2 additional smaller families the loci on chromosome 15q24 and 10q26 were excluded by linkage analysis.

Conclusions—Linkage analysis of a large family with CAD-associated connective tissue alterations suggested the presence of a candidate locus on chromosome 15q2 or on chromosome 10q26. Sequence analysis did not lead to the identification of a mutated candidate gene in 1 of these loci. The study of 2 additional pedigrees indicated locus heterogeneity for the connective tissue phenotype of CAD patients. (Stroke. 2006;37:1697-1702.)

Key Words: cerebrovascular disorder ▪ cervical artery dissection ▪ genetic linkage analysis

Cervical artery dissections (CAD) are an important cause of stroke in young and middle-aged patients. In few patients the development of the dissection is thought to be triggered or caused by mechanical injury, but the pathogenesis of the disease remains unclear in most cases. Several risk factors were associated with CAD, as for instance hereditary connective tissue disorders, in particular the vascular type of Ehlers-Danlos syndrome (EDS IV), recent infection, mild hyperhomocysteinemia, α1-antitrypsin deficiency, migraine or fibromuscular dysplasia. However, the possible role of these risk factors in the development of dissections is not self-evident. The hypothesis of a structural defect of the arterial wall and the association with recent infections lead to the study of several candidate genes in CAD patients by DNA sequence analysis or by genetic association analysis. Most genes studied so far were directly involved in the biosynthesis of extracellular matrix components. However, despite many efforts the candidate gene approach did not yet lead to the identification of genes that are involved in the pathogenesis of CAD. We therefore aimed at a genome-wide scan by linkage analysis to search for a candidate locus. However, familial CAD is rare. The CAD disease phenotype might have a low penetrance, CAD might occur asymptomatically or unrecognized as such, and healthy persons without CAD might develop the disease in the future. We therefore preferred to analyze a subclinical marker associated with CAD and not the CAD phenotype itself.
Skin biopsies from most patients with CAD revealed morphological alterations of the dermal connective tissue, which suggested the presence of a further unknown risk marker. Different patterns of connective tissue aberrations were found in patients with CAD. The most common type of connective tissue aberrations with composite collagen fibrils and fragmentation of elastic fibers was studied previously in a small pedigree and found to be familial with an autosomal dominant pattern of inheritance. In this study we present 2 additional and larger families with inherited connective tissue aberrations of the same pattern. We performed a genome-wide genetic linkage analysis to identify a susceptibility locus, responsible for the connective tissue phenotype and increasing the risk for CAD.

Materials and Methods

Materials

The index patient of family A had a stroke at the age of 70. Bilateral CAD resulting in high-grade stenosis of both extracranial internal carotid arteries was revealed whereas signs of atherosclerotic stenocclusive disease of the carotid arteries were absent. The index patient of family B developed a dissection of the right internal carotid artery at the age of 26. His medical history was presented in more detail elsewhere. Family C was analyzed in a previous exclusion mapping study. Deep-knife skin biopsies were sampled from all 3 index patients and prepared for histology, electron microscopy and for culturing of fibroblasts as described. All patients showed clear and reproducible connective tissue alterations of the same ("Ehlers Danlos syndrome-III like") type. Relatives were informed by the index patients and asked to participate in the study. The performance of skin biopsies and the sampling of blood were approved by the local ethical committee (University of Heidelberg) and required informed consent from each patient. Eight relatives of patient A, 5 relatives of patient B and 4 relatives of index patient C were phenotyped by an electron microscopic inspection of a skin biopsy.

EDTA-blood was sampled from all phenotyped family members and also from their partners. DNA was isolated after SDS-proteinase K digestion, phenol-chloroform extraction and ethanol precipitation following standard procedures.

Linkage Analysis

Three hundred and eighty-three autosomal microsatellite markers of the Applied Biosystems MD10 linkage mapping set (mean distance of the genetic markers 10 cM) were used for an initial genome scan of family A. Fluorescent amplification products were visualized on the ABI-310 automated analyzer and analyzed with the GeneScan software. Logarithm of the odds (LOD) profiles were calculated in multipoint analyses for whole chromosomes with the Genehunter Software (version 2.1). The position of the microsatellite markers were calculated as the mean of male and female cM localization as indicated in the cedar genetics data bank (http://www.cedar.genetics.soton.ac.uk/summaryml.html). For the estimation of unknown allele frequencies of microsatellite markers we followed the CEPH database (http://www.cephb.fr/test/cephdb/). Additional microsatellite markers were selected and analyzed in a second phase of linkage analysis for chromosome regions with inconclusive results (uninformative genetic markers, LOD-scores > -2). In total the segregation of 453 informative microsatellite markers was analyzed in family A.

Moreover, 2 members of the family (a brother of the index patient as well as his grandson, both displaying a connective tissue phenotype) were analyzed with the aid of Affymetrix 10K single-nucleotide polymorphism (SNP) arrays. Among about ten thousand genotyped markers we found 531 informative SNPs that were suitable for additional exclusion mapping. Ninety-one of those informative SNPs were located in regions with low density of informative microsatellite markers and were included in the LOD score calculation.

Family B was selected by linkage analysis for those genomic regions where we found LOD-scores above -2 in family A. Segregation of 113 microsatellite markers for these genomic regions was analyzed in these kindred. Family C had previously been analyzed with genetic markers flanking 32 candidate loci. We now analyzed in this family the segregation of 28 additional CA-repeat markers.

Polymerase Chain Reaction and Sequencing Analysis

The LOXL1 gene (lysyl oxidase-like 1; GC15P072005; 7 exons) is located on position 72,005,852 to 72,031,529 of chromosome 15. No additional degenerate copies were detected by BLAST searching with single LOXL1 exons as search sequences.

The CSPG4 gene (chondroitin sulfate proteoglycan 4; GC15M073754; 10 exons) is located on position 73,754,019 to 73,792,151 of chromosome 15. Multiple sequence copies with high similarity to Exons 2 to 8 of the CSPG4 gene were found during BLAST search. Homologous sequences are detected on the Y chromosome (AC006328) and in multiple loci of 15q21 (AC019294; AC104758; AC136698; AC135995; AC135735; AC127482; AC136704; AC048660; AC126605; AC110291; AC005630; AC010725; AC11295; AC10724; AC012064). In order to design specific primers for single exons all these different pseudogene sequences were added to the sequence for primer search. Specific primer sequences were particularly difficult to find for exon 3. For this exon only few specific primers were found, and nested polymerase chain reactions were required to generate overlapping fragments not longer than about 600 bp for direct sequencing analysis.

The FGFR2 (fibroblast growth factor receptor 2 precursor) gene is located on chromosome 10 (122,473,377 to 123,347,936). The coding region of FGFR2 was amplified in 4 overlapping fragments after RT-PCR of RNA from dermal fibroblasts and analyzed by sequence analysis. The sequence of exons 2 to 5 of the cDNA could not be analyzed properly in cDNA, because of alternative splicing. Therefore, exons 2, 3, 4 and 5 were analyzed in amplified genomic DNA sequences.

Each sequence region was amplified from family members with connective tissue alterations as well as relatives without connective tissue alterations and subsequently studied by sequencing analysis.

Results

Connective Tissue Morphology

Pedigrees of the analyzed families are shown in Figure 1. The connective tissue morphology of skin biopsies from all family members was studied by electron microscopy. Mild but significant connective tissue alterations with “flower-like” composite fibrils in some collagen bundles and with fragmentation of the elastic fibers (“moth eaten aspect”) were found in the index patients and in some of their relatives. In all 3 families we found the same EDS-III like morphology, which is the most common pattern of morphological aberrations in CAD. The connective tissue alterations of members of family C were presented in detail before. The segregation pattern of the connective tissue phenotype is in agreement with the assumption of autosomal dominant inheritance. X-chromosomal markers were not studied, because the observed pattern of inheritance excluded sex-linked transmission.

Genetic Linkage Analysis

DNA from all members of family A was analyzed with 453 informative microsatellite markers and 91 SNPs. The mean distance between (CA)-repeat markers was 7.6 cM, with a maximum of 23 cM (between D20S173 and the telomere of chromosome 20q) and of 26 cM (between D8S505 and D8S285). For the analysis of the genetic linkage data we assumed a model of autosomal dominant inheritance with full penetrance of the connective tissue phenotype, absence of
phenocopies, and a mutation prevalence of 0.001 in the normal population.

LOD scores under –2, virtually excluding the presence of a hypothetical mutation, were found in 80.7% of the (autosomal) genome of family A (Figure 2). Inconclusive LOD scores (above –2) were found for the remaining autosomal region. The maximum LOD score ($Z=2.1$) was detected only in 1 locus in this family. We initially found perfect cosegregation of 1 allele of marker D15S131 and the connective tissue phenotype. In a subsequent analysis of 11 other microsatellite markers in close vicinity of D15S131 the candidate locus with maximum LOD score of 2.1 appeared to include 6 markers in a genetic region of about 5 cM between D15S131 and D15S1023 (Figure 3). One further very short region with $Z=1.9$ was detected at chromosome 10 between D10S1757 and D10S1483. No further suggestive loci were detected in the genescan of this family.

All regions with LOD scores $>−2.0$ in family A were subsequently analyzed in family B. The segregation of 113 genetic markers was studied and the results for both pedigrees were used for the calculation of LOD scores. Both suggestive loci in family A on chromosomes 10 and 15 were excluded in family B and very few loci were not excluded (LOD score $<−2.0$) in 2 family analyses. Three loci with slightly positive LOD score were found in the combined analysis of family A and B (data not shown). However, all these 3 loci are excluded by genetic linkage analysis in Family C.

**Sequence Analysis of Selected Candidate Genes**

Genetic analysis of family A suggested linkage with an assumed autosomal dominant mutation between genetic markers D15S131 and D15S1023. Within this region of chromosome 15 we analyzed 2 candidate genes in more detail (**LOXL1** and **CSPG4**). Because exons 2 to 8 of **CSPG4** were found to be present in many highly homologous copies throughout the genome, the analysis of these exons turned out to be extraordinary hard. We produced specific sequences of about 75% of the coding sequence of **CSPG4**, but we were unable to generate specific amplification products from parts of exon 3. The genomic sequences of **CSPG4** and **LOXL1** in the index patient were compared with the sequences from his healthy brother without the connective tissue phenotype and with published consensus sequences. In **CSPG4** we found a heterozygous G/A substitution at position G5525 in both the index patient and his brother. The index patient was homozygous and his brother heterozygous for a known SNP in the **LOXL1** gene (Table). The specific amplification of sequences from **ADAMTS7**, a third candidate gene within this region,
turned out to be too difficult in our hands and we gave up the initial idea to analyze the coding sequence of this gene completely. In the **FGFR2** gene which is located on chromosome 10q26 we found a single silent variant (a G to A substitution at position 1288). Hence, no sequence variants were found in any of these genes that are likely to explain the connective tissue phenotype.

**Discussion**

Patients with a family history of CAD are rare, and families with >3 affected members were not described. Hence, a genetic analysis of CAD on the basis of large families with affected and unaffected subjects is not possible. However, the finding of inherited connective tissue alterations in patients as well as in their healthy relatives enables the genetic analysis.
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Genetic Variation in Candidate Genes. Sequence Variation Was Detected in Individuals From Family A With Connective Tissue Alterations

<table>
<thead>
<tr>
<th>Gene</th>
<th>Exon Position</th>
<th>Nucleotide Variation</th>
<th>Nucleotide Position</th>
<th>Amino Acid Variation</th>
<th>Amino Acid</th>
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<tr>
<td>LOXL1</td>
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<td>141</td>
<td>R→L</td>
<td>rs 1048661</td>
<td></td>
</tr>
<tr>
<td>CSPG4*</td>
<td>10</td>
<td>G→A</td>
<td>1842</td>
<td>R→Q</td>
<td>Not described</td>
<td></td>
</tr>
</tbody>
</table>

Because the LOXL1 G748T and the CSPG4 G5525A SNPs were also found in a sibling with wild-type (normal) connective tissue phenotype, these nucleotide substitutions are considered as polymorphisms without effect on the pathogenesis of CAD.

*The sequence of part of exon 3 of the CSPG4 gene was not analyzed because we could not generate specific polymerase chain reaction products for this region of the gene.

of large families. Not the disease itself is considered as the mutation phenotype, but the presence of ultrastructural connective tissue alterations. In this genome-wide linkage analysis study we initiated a candidate locus search for the connective tissue phenotype.

In family A we identified a suggestive candidate locus responsible for the connective tissue phenotype. The locus between D15S131 and D15S1023 on chromosome 15q2 was the only locus with maximum LOD score (Zmax = 2.1), attributable to perfect cosegregation of a putative mutation with highly informative genetic markers. However, this LOD score of 2.1 is below the required standard for the demonstration of linkage, because the analyzed pedigree does not contain enough informative meioses. In a second family with the same connective tissue phenotype, the region between D15S131 and D15S1023 was excluded as candidate locus for a disease-causing mutation. All genome regions with inconclusive results (Z > -2) in family A were studied by genetic linkage analysis in Family B. Linkage was excluded for the majority of the genome in this combined genetic analysis of 2 pedigrees. Additional linkage studies in family C finally resulted in exclusion of the whole genome, which indicated locus heterogeneity of the connective tissue phenotype.

Locus heterogeneity was also found in linkage studies of families with familial aorta dissections (with loci on chromosome regions 3p24–25, 5q13–15 and 11q23–24) and with intracranial aneurysms (with proposed candidate loci on chromosomes 1p34, 2p13, 7q11, 7q22, 17cen, 19q13 and Xp22). The connective tissue phenotype in our families has similarities with the findings in EDS-III, a connective tissue disorder characterized by joint hypermobility. Chromosome region 15q2 was reported to harbor a candidate gene involved in a psychiatric disorder associated with joint hypermobility.29,30 Chromosome region 15q2 has a complex sequence structure with many degenerate long repeats (which, moreover, makes this chromosome region particularly difficult to study). The original finding of an additional large disease causing duplication (DUP25)29 in patients with joint hypermobility and a psychiatric disorder was not confirmed by other researchers.31 The hypothetical unknown gene in this region, involved in joint hypermobility, is not yet identified.

The suggestive loci on 15q24 and 10q26 that we found in our linkage studies contains several promising candidate genes. The CSPG4 gene shows sequence homology with CSPG2, the versican encoding gene. CSPG2 was repeatedly discussed as a candidate gene involved in aneurysm formation15,24 and versican is considered to be a key molecule in vascular disease.24,25 The LOXL1 gene is highly expressed in large arteries and involved in elastic fiber deposition.26 Inactivation of LOX leads in mice to the development of aortic aneurysm and perinatal death.27 Recently a dramatic down regulation of LOX gene expression was reported in a patient with spontaneous coronary artery dissection.28 FGFR2 shares sequence homology with TGFBR2, a gene that was recently shown to carry mutations in some families with aortic dissections and aneurysms.32

Possible criticism of the present study might include the size of family A, which is too small to demonstrate linkage with certainty and solely enables the detection of suggestive linkage. However, the occurrence of familial CAD is extremely rare, and the study of the connective tissue phenotype as a subclinical marker for CAD enabled for the first time a genome-wide linkage for this disease. A further criticism might be that we only analyzed the coding sequences of 3 candidate genes. We prefer to perform additional linkage studies of further large families first. The identification of a locus with LOD score >3 should have priority over further sequence analysis of candidate genes.

The identification of the connective tissue phenotype enabled a genome-wide linkage analysis for the investigation of a genetic predisposition to cervical artery dissections. We found 2 loci with suggestive linkage for CAD. The analysis of 3 families with a similar connective tissue phenotype demonstrated locus heterogeneity.

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Disclosures
None.

References


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