Mutations in the COL5A1 Coding Sequence Are Not Common in Patients With Spontaneous Cervical Artery Dissections

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Background and Purpose—The dermal connective tissue of most patients with spontaneous cervical artery dissections (sCAD) contains abnormal collagen fibers. This suggests a predisposing connective tissue defect. The ultrastructural abnormalities in the skin of patients with sCAD have similarity with the morphological alterations in patients with Ehlers-Danlos syndrome type II, a dominant hereditary disorder that has been correlated in some patients to mutations within the genes encoding type V collagen. The aim of this study was to assess the alpha 1 chain of type V collagen (COL5A1) as a candidate gene for sCAD.

Methods—We searched for mutations in the COL5A1 gene in cDNA from cultured fibroblasts of 19 patients with sCAD using single-strand conformational polymorphism analysis and nucleotide sequence analysis of polymerase chain reaction–amplified fragments of the whole COL5A1 coding sequence.

Results—We detected 1 missense mutation leading to a predicted amino acid (192D/N) substitution within the N-terminal propeptide in 2 siblings. All other patients showed regular COL5A1 sequences with some silent polymorphisms.

Conclusions—Mutations in the COL5A1 gene do not appear to be a major factor in the etiology of sCAD. (Stroke. 1999;30:1887-1890.)

Key Words: connective tissue disorders  dissection  genetics  mutation

Spontaneous cervical artery dissections (sCAD) are increasingly recognized as a cause of stroke among young and middle-aged patients. A predisposing arteriopathy in these patients has been postulated. A recent study by Brandt et al substantiated the hypothesis of a predisposing defect in the extracellular matrix. These authors found ultrastructural abnormalities in skin biopsies of a majority of patients with sCAD and suggested an association with a connective tissue disorder. Major findings included numerous enlarged and irregular collagen fibrils and pronounced elastic fiber fragmentation. The abnormalities resembled the morphological alterations in patients with Ehlers-Danlos syndrome type II or III (EDS II/III). Those changes were not observed in age-matched stroke patients with alternative etiologies of cerebral ischemic infarcts or in healthy control subjects. None of the patients with sCAD had other phenotypic manifestations of a known hereditary connective tissue disorder.

Type V collagen belongs to the family of fibrillar collagens. It is expressed at a low level in many tissues and plays an important role in type I fibrillogenesis by modulating and influencing fibril diameter. Mutations in COL5A1 and COL5A2 may lead to ultrastructural abnormalities similar to those observed in sCAD patients. Furthermore, some patients with EDS I/II carry mutations in the COL5A1 or the COL5A2 gene (for reference, see Michalickova et al). The ultrastructural similarities of dermal connective tissue aberrations of EDS II patients and sCAD patients prompted us to start this study and to analyze the full coding sequence of COL5A1 in patients with sCAD.

Subjects and Methods

We investigated 15 sCAD patients with pronounced electron microscopic alterations in the dermal connective tissue, 4 sCAD patients without ultrastructural abnormalities, and 1 healthy control subject. The mean age of the patients (16 males and 3 females) at the time of dissection was 41.4 years. One patient suffered from bilateral dissections, and 2 patients subsequently developed a second dissection. Two patients without ultrastructural abnormalities are sibs.

Skin biopsies were taken from the outer side of the upper arm by open deep knife biopsy. Part of the material was processed for electron microscopy according to the method of Hausser and Anton-Lamprecht. Fibroblasts from another part of the biopsy were
cultured in MEM supplied with 10% FCS. RNA was prepared from cultured skin fibroblasts with RNA-zol (AGS-Heidelberg) and cDNA synthesized with muLV reverse transcriptase and random hexamers (Perkin-Elmer). A mixture of deaza-dGTP and dGTP (3:1) was used for the synthesis of cDNA. DNA was isolated from 3 mL peripheral EDTA blood with DNA-zol (AGS-Heidelberg) or from the midphase of the fibroblast RNA-zol preparations by ethanol precipitation. The whole coding sequence of the \textit{COL5A1} cDNA was amplified in the presence of 7% DMSO by polymerase chain reaction (PCR) in 16 overlapping fragments. PCR primers with melting temperatures of approximately 60°C (GC rule) were designed with the aid of the PRIDE program.\textsuperscript{12} The last primer, located in the 3' untranslated region, was published by Wenstrup et al.\textsuperscript{13} Amplified DNA fragments were digested with restriction enzymes to produce fragments of 100 to 300 bp for multiplex single-stranded conformational polymorphism analysis (SSCP).\textsuperscript{14–15} Most fragments were digested twice with 2 different restriction enzymes to generate different patterns of fragments. Following denaturation and subsequent cooling on ice, SSCP fragments were loaded on at least 2 different nondenaturing gel systems (2% or 10% glycerol) and run in a cooled vertical electrophoresis chamber at 15°C overnight. Gels were stained with silver. If single-strand bands were weak or fuzzy or if complementary strands comigrated during electrophoresis, additional electrophoretic conditions were applied by modifying either the run temperature (room temperature or 4°C) or the acrylamide concentration of the gel. PCR products were prepared for dye terminator cycle sequence analysis (Perkin-Elmer) and analyzed at the 310 Abi Prism genetic analyzer following the instructions of the producer.

**Results**

SSCP indicated the presence of polymorphisms in DNA fragments 0656 to 1157 after digestion with AluI. Lane 1, non-denatured DNA probe; lane 2, molecular size marker; lanes 3 to 25, denatured DNA probes of different patients. The denatured 198-bp fragment shows a polymorphism at high frequency (upper band, arrowhead), whereas an SSCP arising out of the double-strand 139-bp fragment is observed in 1 of the siblings' DNA (lane 22, arrow). DNA of the other sibling was not loaded on this gel. B. Sequence analysis of genomic DNA of the unique SSCP in panel A. Sequencing both in forward (upper lane) and reverse (lower lane) directions revealed a (G/A) heterozygocity in exon 4 of the \textit{COL5A1} gene, corresponding to position 700 of the cDNA, according to Takahara et al.\textsuperscript{7} R=G/A heterozygocity; Y=C/T heterozygocity.
correlated with single-base heterozygocities at positions 700, 864, 1218, and 3018 of the cDNA sequence. Region 4559 to 5670 of cDNA, encoding the C-terminal propeptide and part of the triple helix, was analyzed by direct sequencing in all patients. It contained an additional, tri-allelic DNA polymorphism at position 4608. The heterozygocities at positions 864 (22xC/18xT), 1218 (1xC/39xT), 3018 (3xC/37xT), and 4608 (1xA/14xG/25xG) are silent, because substitutions do not result in changes of the predicted polypeptides. 17 of 20 patients were heterozygous for at least 1 of the polymorphisms in the COL5A1 coding sequence.

The heterozygosity at position 700 in the 2 patients who are brother and sister (Figure, panel B) results in a substitution in the predicted amino acid sequence, with aspartic acid replaced by asparagine in 1 of both alleles at position 192 of the N-terminal procollagen amino acid sequence. The TaqI restriction fragment length polymorphism (RFLP) caused by this mutation was also studied in genomic DNA. The mother of both patients carries the mutant allele. It is absent in a healthy brother and the father as well as in 25 control subjects. A comparison of the predicted 3D-reconstructed polypeptides encoded by the normal and the mutant allele suggests that the amino acid substitution does not lead to a conformational change of the protein (data not shown).

Discussion

The high incidence of ultrastructural dermal connective tissue abnormalities found in patients with sCAD suggests a causal relationship between sCAD and a connective tissue disorder. This observation stimulated us to start a search for mutations in a possible candidate gene involved in collagen fibril organization.

Two more groups searched systematically for mutations in patients with sCAD or intracranial aneurysms. Kuivaniemi et al17 analyzed parts of the coding sequence of the a (I) chain of collagen type III in 55 patients. They found no significant mutation. In a recent analysis of the whole COL5A1 coding sequence, mutations were observed neither in patients with intracranial aneurysms nor those with sCAD.18–19

We found 4 silent polymorphisms in the COL5A1 gene. The polymorphism at position 864 that results in an RFLP after digestion with PstI was earlier described as genomic RFLP.20 Seventeen of 20 patients were heterozygous in at least 1 position in the COL5A1 coding sequence. Because these heterozygocities were detected in cDNA, these patients are neither hemizygous for the COL5A1 gene nor do they carry regulatory mutations that result in the transcriptional inactivation of 1 allele (null allele), as described for other collagen genes.21–23

The only missense mutation found in this investigation was observed in 2 sibs. It is difficult to prove whether the resulting amino acid substitution has any phenotypic effect. The mother of the patients, who carries the same mutant allele, had a normal ultrasound study of the cervical arteries and did not suffer from symptomatic dissections. Therefore, the mutant condition may not increase the risk of sCAD. However, it is also possible that the mother did not develop sCAD despite an increased risk.

Greenspan et al5 compared the amino acid sequences of the alpha 1 chains of type V collagen in humans and Chinese hamsters. Position 192 of the sequence is not conserved between both species, which suggests that divergence at this position does not disturb an important function. The predicted secondary structures of the proteins encoded by the mutant and the wild-type alleles lead to a similar conclusion.

In summary, the majority of patients with sCAD do not carry mutations in COL5A1. Moreover, we cannot decide whether the only missense mutation detected has any phenotypic effect. Our data therefore suggest that COL5A1 is not a major candidate gene for sCAD.

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