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Analysis of the COL3A1 gene in patients with spontaneous cervical artery dissections

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Abstract The etiology of spontaneous cervical artery dissection (sCAD) is unknown. An underlying connective tissue disorder has been suggested. As a collagen disease is conceivable several genes encoding fibrillar collagens have been condsidered as candidate genes for sCAD.

We analysed the COL3A1 gene in patients with spontaneous cervical artery dissection (sCAD) and in healthy controls, using three different genetic methods. 1) The promoter region, the 5' and 3' untranscribed regions and the N- and C-peptide encoding regions were studied by direct sequencing analysis of DNA from 12 patients. 2) A possible association of sCAD and the COL3A1 gene was tested for with 5 different DNA polymorphisms in 45 patients and 50 healthy control subjects. 3) DNA samples from a father and his two daughters, all suffering from spontaneous dissections of a cervical artery, were analysed with CA-repeat markers that flank the COL3A1 locus.

No disease-causing mutations were found in an extended sequence analysis of the COL3A1 gene in patients with sCAD. However, we identified a single nucleotid polymorphism (SNP) in the promotor region in 2 patients and a 2 bp deletion in the 3' UTR in 7 patients. These sequence variants were also found among 50 healthy subjects. An analysis of multiple DNA polymorphisms of the COL3A1 locus in patients and healthy control persons did not indicate a significant association of sCAD with COL3A1. A deletion polymorphism in the 3' UTR was, however, found more often amongst patients with sCAD. The possible linkage of a hypothetical disease mutation with the COL3A1 locus was tested in a small family with three affected patients. As the affected daughters did not inherit the same COL3A1 allele from their affected father (LOD < – 2.3) COL3A1 was excluded as a disease gene in this family.

This study confirms and extends earlier work which suggests that COL3A1 mutations are not a major cause for isolated sCAD.

Key words spontaneous dissections · COL3A1 · mutation analysis · linkage analysis

Introduction

Spontaneous cervical artery dissection (sCAD) is an important cause of stroke among young and middle-aged patients [3, 15]. Ultrastructural abnormalities have been observed in skin biopsy specimens of about 60 % of patients with sCAD [4, 5]. Major findings included enlarged and irregular collagen fibrils and pronounced elastic fiber fragmentation. This suggested that patients are predisposed to sCAD because of a defect in the extracellular matrix. However, patients with sCAD rarely show signs of known hereditary connective tissue disorders [5, 16]. Defects in the biosynthesis of type III collagen have been searched for repeatedly in patients with sCAD, because such defects are typically found in patients with the vascular type of Ehlers Danlos syndrome (EDS IV), a connective tissue dissease which predisposes to arterial dissection [16] and the development and rupture of arterial aneurysms [14]. Moreover, the synthesis of type III collagen by cultured fibroblasts from some sCAD patients was found to be reduced [18]. However, DNA sequence analysis of the coding region of the COL3A1 gene from patients with sCAD did not result in the detection of mutations [12, 18]. Nor have mutations been found in other candidate genes [8, 9]. To date disease-causing mutations have only been identified in the minority of sCAD patients with a known hereditary connective tissue disorder. The molecular defect in the majority of patients with only cerebrovascular symptoms is unknown.

In this study of sCAD patients, we analysed sequences from the COL3A1 locus that have not been studied before in patients with cerebrovascular diseases, notably the promoter and untranslated regions. Neither transcribed nor untranscribed flanking sequences were found to carry disease-causing mutations. Moreover, an analysis of 5 polymorphic DNA markers did not reveal a clear association between COL3A1 and sCAD. We introduced linkage analysis as a new approach in the study of sCAD and demonstrated that the disease-phenotype did not co-segregate with COL3A1 flanking DNA markers in a small family.

Materials and methods

From two series of patients with sCAD (n = 90, described by Brandt et al. [4, 5]) we selected 12 patients with pronounced morphological alterations in their dermal connective tissue for cDNA sequence analysis. A further consecutive series of 45 sCAD patients, that were only partially investigated by electron microscopy, was selected for association analysis. None of the patients included in this study showed signs of a known connective tissue disorder. Fifty genomic DNA samples from healthy students and staff-members of the department were used as controls. The performance of skin biopsies and the sampling of blood was approved by the local ethical committee (University of Heidelberg) and required informed consent of each patient.

DNA was isolated from EDTA blood samples after SDS-proteinase-K digestion and phenol-chlorophorm extraction following standard procedures. A DNA fragment containing both intron 24 and 25 of COL3A1 was amplified by PCR. The variations in a CA repeat in intron 24 and in a VNTR in intron 25 [13, 17] were analysed with the GeneScanning software on an ABI-Prism 310 genetic analyser. For the analysis of a biallelic polymorphism in exon 31 [20] amplified DNA fragments were digested with Alu I and analysed on a 3 % agarose gel. A deletion polymorphism in the 3' UTR of the COL3A1 transcript [10] was studied by GeneScanning of specific PCR-products. The Ava-II RFLP described by Dalgleish et al. [7] was found to be a single nucleotid polymorphism (SNP) located about 4 kb downstream of the COL3A1 coding sequence. It was analysed on 2 % agarose gels after Ava-II digestion of PCR-amplified DNA. Table 1 lists the sequences of the oligonucleotides used as primers. The CA repeat markers D2S118 and D2S364, flanking a 4.8 cM genetic interval surrounding the COL3A1 gene, were supplied by PE-Biosystems and were tested and analysed following the instructions of the supplier.

RNA was isolated from cultured dermal fibroblasts and reverse transcribed into cDNA as described previously [8]. cDNA was used for the sequence analysis of the regions encoding the N- and C-terminal propeptides and the 3'UTR (X14420) [1]. Genomic DNA was used for the analyses of promotor sequences M26939) [2]. Amplified DNA fragments were purified on Qiagen columns, processed for Big Dye terminator cycle sequencing (ABI-Prism) following the supplied instructions and analysed on a ABI-Prism 310 automatic sequencer. All sequences were determined in both directions.

For the statistical comparison of the allelic distributions of the various DNA sequence polymorphisms we used the chi square test. The allelic distribution of the control group was taken as the "expected distribution". We tested the hypothesis (H_0) that the group of patients is a sample taken from the population of the healthy control subjects.

LOD scores were calculated with the Genehunter Complete Linkage Analysis Program (version 2.0).

Table 1 PCR primers used in this study

Region of COL3A1	sequence of primer (forward/reverse)
Intron 24/25	AGTGATGGGAAACCAGGGCCTCCC-FAM/GGTGCTCCTGGTAAGAATGGAGAA
Exon 31	CTGGTGAACGTGGACCTCCTGGATTGG/ATAAATGATCAGAAGGAAATCA
3'UTR, (Ä)	ACAGTTTAGGTTTAAGATGACCAATGA/AAGCTACCTGCCAGGAAGAAGAATT-FAM
3'-flanking	ACTGAACTCAGGCCTCAGAGGTT/CTGGGAATTGAAGAAAGTCTTGAGG
5'-flanking	ACTAGATAAATGGGCATCAAGCAGT/GAAAAGCAGTTCAAAGTAGCACCAT
N-propeptide	TGATGGTGCTACTTTGAACTG/GCTACTCCAGACTTGACATC
N-propeptide	CCTACTGGTCCTCAGAACTA/TGACCATCACTGCCTCGAGCACCGTCA
C-propeptide	TCAGCAGGGTGCAATCGGCAGTCCA/AGTTCAGGATTGCCGTAGC
C-propeptide	GGACAGATTCTAGTGCTGAG/ACAAGATTAGAACAAGAGGAAC
3'UTR	TGTGTTCCTCTTGTTCTAATCT/TCTGACCAGTTGAGGTAGTT
3'UTR	GAAATAGTCAAATACGAAATTAGA/AGATCAACAACCA

Results

Twelve patients with sCAD with pronounced morphological alterations in the dermal connective tissue [5] were selected for sequencing analysis (11 male and 1 female patient, 1 patient with a recurrent dissection and 2 patients with a familial dissection, mean age 42.5 years). A series of 45 further patients (29 male and 16 female patients, mean age 44.8 years, 2 patients with recurrent dissections, 3 patients with multiple dissections, 1 patient with FMD) for the association analysis.

Sequence analysis of the COL3A1 gene (the full length cDNA apart from the central part that codes for the triple-helical region of the propeptide) and about 400 bp of upstream sequences did not reveal disease mutations. We identified a SNP in the 5' flanking sequence (position 1333 in sequence M26939) in two patients. This SNP was confirmed by Ppu10I digestion of the amplified DNA and subsequently detected as a Ppu10I RFLP in one out of 50 control persons (data not shown). A 2 bp deletion in the 3' UTR of the gene was initially found in 7 out of 12 patients. We detected this deletion in control subjects as well.

The analysis of allelic distributions of four different DNA polymorphisms in the COL3A1 gene did not indicate the presence of a significant association between sCAD and the COL3A1 locus (Table 2). However, the 2 bp deletion in the 3' UTR of the gene was found more frequently among patients.

A father and two daughters suffered from sCAD. None of the five DNA polymorphisms of the COL3A1 locus were informative in this family. We therefore studied the segregation of the paternal COL3A1 alleles with D2S118 and D2S364, two microsatellite markers that flank the COL3A1 gene. Between the microsatellite markers LOD values are under –2.3, which excludes COL3A1 as a possible candidate gene in this family.

Discussion

No mutations were found in coding or flanking sequences of COL3A1 from 12 sCAD patients. The region of the gene coding for the (alpha)1-chain of type III collagen was analysed in detail by Kuivaniemi et al. [12] and the full length coding region by van der Berg et al. [18]. Neither study resulted in the identification of a disease mutation. Here we extended this mutation search and analysed also the UTRs of the COL3A1 transcript and about 400 bp of upstream sequences in 12 patients. Again we did not identify any putative disease-causing mutations. The alanine-threonine variant in the (alpha)1(III)-chain [20] was found to occur as frequently in patients as in controls.

We also could not convincingly demonstrate an association between sCAD and a particular COL3A1 haplo-

Table 2 The allelic distribution of COL3A1 DNA polymorphisms in patients and healthy subject

polymorphism	alleles	control subjects (n = 50)	patients (n = 45)
intron 24 (CA repeat)	243bp	1	1
	259bp	13	8
	261bp	53	47
	263bp	5	3
	265bp	1	1
	267bp	27	30
intron 25 (VNTR)	4 repeats	47	49
	5 repeats	27	22
	6 repeats	26	19
exon 31 Alu I – RFLP	G-allele	63	61
	A-allele	37	29
3' UTR 2 bp deletion	deletion	16	22
	wildtype	84	68*
3' flanking Ava II – RFLP	A-allele	23	25
	G-allele	77	65

The allelic distributions of 5 DNA polymorphisms in COL3A1 in patients and controls. Six different alleles were found for the CA repeat in intron 24 (PCR-products of 243, 259, 261, 263, 265 or 267 bp length). Three different alleles were found for the VNTR in intron 25 (4, 5 or 6 copies of a tandemly arranged 16 bp repeat). The alanine-threonine polymorphism in exon 31 was determined by Alu I restriction digestion (agaggtggaA/Gctggtcccct). The Ava-II polymorphism in the 3' flanking sequences of the COL3A1 gene was visualized after digestion of a PCR amplified fragment of 580 bp. The A-allele (ctcaaggtggA/Gccagtacggtt) contains an AvaII site, the G allele cannot be digested by Ava II.
* The frequency of the deletion differs between patients and control persons. Chi-square tested with $p < 0.03$.

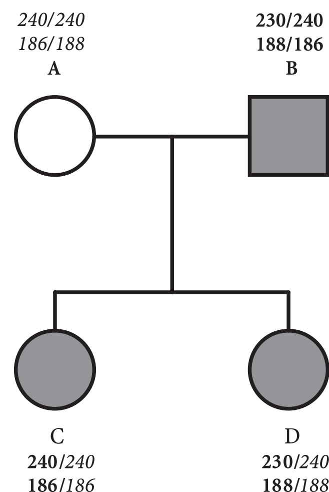

Figure Segregation of two microsatellite markers in a family with three affected sCAD patients. D2S364 (= AFM303ya9/$(AC)_n$) flanks the COL3A1/COL5A2 locus in the direction of the centromere ($\theta = 0.080$ according to http://www.cephb.fr), D2S118 (= AFM066xc1/$(AC)_n$) in the direction of 2qtel ($\theta = 0.030$). The distance between the markers is about 4.8 cM.
▢ patient with sCAD, ○ healthy subject. We observed two different alleles of D2S364 (with PCR fragment lengthes of 230 and 240 bp) and two alleles of D2S118 (186 and 188 bp). The segregation patterns of the microsatellite markers exluded the COL3A1/COL5A2 locus as a possible disease locus (LOD < –2.3) in this family.

type. Analysis of a deletion polymorphism suggested the presence of an association of sCAD and COL3A1 [10], but the allelic distribution of four other DNA-polymorphisms did not reveal any difference between patients (n = 45) and healthy controls (n = 50). The association of sCAD with the 2bp deletion polymorphism might be a statistical artifact, or it might be a weak but real association, due to a slight effect of the 2bp deletion on the expression of COL3A1. However, the 2 bp deletion did not seem to have any effect on the stability of the RNA, as the same amounts of both alleles are observed in cDNA of heterozygous carriers (data not shown). The possibility of an impact on translation efficiency of the 2bp deletion was not studied.

We introduced linkage analysis as a new approach in the study of dissections. Familial occurrence of cervical artery dissections is rare [11, 15]. Families must have at least three affected members, in order to be suitable for linkage analysis, since non-affected members cannot be phenotyped for certain. Carriers might be phenotypically normal, might develop a dissection in the future, an asymptomatical dissection or even no dissection at all. We studied the co-segregation of two informative flanking markers (D2S118 and D2S364) with the disease-phenotype in a three point linkage analysis and found a LOD < -2.3 for the interval between the markers. The hypothesis of a disease-causing mutation between these markers is therefore excluded. Since COL3A1 and COL5A2 map very close to each other [6, 19] both genes are excluded as candidate genes in this family.

Three different genetic methods, mutation search, association analysis and linkage analysis were used in this study. Mutations in or around the COL3A1 gene were not found. An association between the COL3A1 locus and sCAD could not be demonstrated. The evidence from these methods is negative and as long as no mutations are found, the research might be extended to other sequences or to further DNA polymorphisms. The exclusion of the COL3A1 locus by genetic linkage analysis, on the other hand, is definite: Further searching for a disease-causing mutation in this genetic interval will – at least in this family – never lead to the identification of a disease-causing mutation.

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