A Novel Mutation in the Notch3 Gene in an Italian Family With Cerebral Autosomal Dominant Arteriopathy With Subcortical Infarcts and Leukoencephalopathy

Genetic and Magnetic Resonance Spectroscopic Findings

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Background: Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is an adult-onset hereditary syndrome characterized by recurrent transient ischemic attacks and strokes.1 Mutations within the Notch3 gene (chromosome 19p13.1) have been identified as the underlying genetic defect associated with CADASIL.2,3 Most of these mutations have been localized to exons 3 and 4.4 Herein we report a novel mutation within exon 6 of the Notch3 gene, identified in a kindred from southern Italy with CADASIL. Clinical and neuroimaging data are also presented for affected individuals.

Results: Sequence analysis of the Notch3 gene showed a new missense mutation CGC→TGC in codon 332 of exon 6, resulting in the replacement of an arginine residue with a cysteine. This mutation was never observed in the 7 unaffected members of the family and the 200 control chromosomes examined. Proton magnetic resonance spectroscopy showed a diffuse decrease in cerebral N-acetylaspartate, indicating the presence of widespread axonal damage.

Conclusions: Our findings emphasize the role of direct DNA sequence analysis for the diagnosis of CADASIL. Moreover, the results of proton magnetic resonance spectroscopy suggest that widespread axonal damage may be an early finding of the disease.

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SUBJECTS AND METHODS

FAMILY

The simplified pedigree of the family is shown in Figure 1. Data on the medical histories of 8 previously deceased subjects were ascertained through medical records and detailed accounts from the older members of the family at the time of the study. Family members younger than 18 years were not included in the study. Two living affected members (III:16 and III:17) were studied in detail. The phenotype of CADASIL in all other deceased subjects with the disease was quite uniform and characterized by migraine with and without aura and recurrent strokes developing from the third to fourth decades of life, progressing to a pseudobulbar palsy, spastic quadriplegia, and dementia. Death occurred in the fifth to sixth decades.

Subject III:16

The proband was a 42-year-old, right-handed man. At age 37 years, he experienced a sudden onset of right hemiparesis, which resolved partially in 1 month. One year later, he manifested diplopia, which disappeared in 10 days. At age 41 years, he experienced another acute attack characterized by right hemiparesis and dysarthria, followed by partial recovery. A few months later, he noted a worsening of both dysarthria and hemiparesis and reported to our department for hospitalization.

Neurologic examination showed right hemiparesis, dysarthria, diffuse brisk deep tendon reflexes, right-sided Babinski sign, and bilateral dysmetria. Results of routine blood examination and cerebrospinal fluid analysis were unremarkable, and cerebrospinal fluid examination was negative. Magnetic resonance imaging (MRI) revealed a right-sided thalamic lesion. Results of routine blood examination were normal, as were multimodality evoked potentials. Neuropsychological testing indicated diffuse cognitive impairment, particularly evident for memory and executive functions.

Subject III:17

A 32-year-old, right-handed man, the brother of the proband, experienced an acute episode characterized by left hemiparesis, together with bladder and emotional incontinence, at 30 years of age. These symptoms partially resolved. Neurologic examination showed mild left hemiparesis, diffuse brisk deep tendon reflexes, left-sided Babinski sign, and pseudobulbar palsy. Results of routine blood examination were unremarkable, and cerebrospinal fluid analysis gave normal results. Very-long-chain fatty acids, vitamin E, and arylsulfatase A levels were within normal limits. Mutation screening for the mitochondrion myopathy, encephalopathy, lactacidosis, and stroke syndrome was negative, and no mutations were found in the factor V Leiden or prothrombin genes. Results of electromyography and electroencephalography were normal, as were multimodality evoked potentials. Neuropsychological testing indicated diffuse cognitive impairment, particularly evident for memory and executive functions.

GENETIC ANALYSIS

Mutation analysis of the Notch3 gene of these 2 affected subjects was undertaken. Genetic analysis was also performed in 7 clinically unaffected members of the family, including members II:9, III:14, and III:15. Genomic DNA was extracted from peripheral blood after informed consent had been obtained. Polymerase chain reaction (PCR) was performed with primers specific for exons of the Notch3 gene. Because most previously described mutations occur in exons 3 and 4, these regions were screened first. When no mutations were detected in these exons, we designed primer pairs comprising the intron-exon boundaries and sequenced the remaining exons. The PCR products were sequenced by means of forward primers (ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit; Perkin Elmer, Foster City, Calif). To confirm the mutation, PCR products were digested with MsrI restriction enzyme. To control for polymorphisms at this site, the Notch3 genes of 100 unrelated healthy white subjects originating from the same geographic area as the affected patients were examined.

IMAGING

Magnetic resonance (MR) imaging and MR spectroscopy were used to estimate metabolite resonance intensities in the 2 affected subjects. Combined brain proton (1H)-MR imaging and MR spectroscopy was performed with a scanner (Philips Gyroscan NT; Philips Medical Systems, Best, the Netherlands) operating at 1.5 T. Conventional multislice spin-echo and fluid-attenuated inversion recovery images were obtained in transverse planes parallel to the anterior commissure-posterior commissure line. The fluid-attenuated inversion recovery MR images were then used to position an intracranial volume of interest (VOI) for spectroscopy parallel to the anterior commissure-posterior commissure line and centered craniocaudally on the corpus callosum. The VOI measured approximately 100 mm anteroposteriorly×20 mm craniocaudally×90 mm left to right, thus including a large proportion of the brain white matter. Two-dimensional spectroscopic images were obtained with a point-resolved spectroscopy sequence for volume selection (repetition time, 2000 milliseconds; echo time, 272 milliseconds; 250×250-mm field of view; 32×32 phase encoding steps; 1 signal average per step), as previously described. Raw data were then postprocessed as previously described. Metabolite resonance intensities of N-acetyl groups (mainly N-acetylaspartate [NAA], a marker of axonal integrity), choline (mainly from choline-containing phospholipids [Cho], reflecting myelin integrity), and creatine (Cr) were determined automatically from peak areas relative to a spline-corrected baseline. As Cr is evenly distributed throughout the brain and relatively refractory to change, metabolite resonance intensity values were normalized to Cr resonance intensity. Metabolite ratios were considered abnormal if they were more than 2 SDs outside the mean values derived from 20 normal adult control subjects. In this study, metabolite ratios were expressed as a global value, which was obtained by averaging the metabolite/Cr for all the voxels in the spectroscopic VOI for each subject.
The fluid-attenuated inversion recovery and T2-weighted MR images showed diffuse hyperintense lesions in white matter of both subjects. The $^1$H-MR spectroscopy showed diffuse decreases in brain NAA/Cr (NAA/Cr = 2.58 and 2.68 in subjects III:1 and III:7, respectively, and mean NAA/Cr = 3.05±0.19 in normal controls), whereas values of Cho/Cr were within normal limits and lactate signals were not found (Figure 3).

**COMMENT**

Herein we describe a novel pathogenetic mutation occurring in the exon 6 of Notch3 gene, a location not previously recognized in patients with CADASIL. Previously identified mutations in the Notch3 gene occur mainly in exons 3 and 4, although mutations have been reported in other exons.7,8 Similar to previously reported mutational modifications in other exons of Notch3,4,7 this mutation is located at the 5’ end of the gene and is characterized by the gain of a cysteine residue. It has been proposed that these modifications to the primary sequence affect the folding of Notch3 or induce inappropriate disulfide bonding between Notch3 and other cysteine-containing proteins.4

Our finding further supports the notion that mutational analysis is a valuable tool for this complex disease, both in confirming the diagnosis of CADASIL and, as in our case, in finding novel mutations.9 In the past few years, there has been increasing evidence that the clinical picture of CADASIL is highly heterogeneous, ranging from severe dementia to migraine with aura, associated with white matter abnormalities.4,8,10 Moreover, it has recently emerged that sporadic cases also exist, as mutation in the Notch3 gene may occur de novo.9 In addition, although electron microscopy of skin biopsy specimens is considered a useful screening method for the diagnosis of CADASIL,12 with the finding of the characteristic granular, electron-dense, osmiophilic material attached to vascular smooth muscle cells, there is now evidence that skin biopsy specimens may occasionally be negative.12 All of these findings account for the difficulty in identifying patients with CADASIL. Thus, a mutational screening for the Notch3 gene should be performed in patients who present with the aforementioned clinical features, regardless of the family history. A final molecular diagnosis of CADASIL, in fact, has important implications for the patient and for the family. The Notch3 gene, however, contains 33 coding exons and, therefore, sequencing the whole gene is time-consuming and expensive. Thus, as also suggested by other authors,9 a rational testing strategy is the following: the first step consists of direct sequencing analysis of exons 3 and 4, where mutations are located in most cases. If no mutation is found, screening of other exons that contain more rare
mutations should be pursued. If no mutation is found by the second step, direct sequencing of the remaining exons may be performed as the last step.

Single-voxel $^1$H-MR spectroscopy and MR spectroscopic imaging results have been reported by Desmond et al\textsuperscript{13} in 4 patients with CADASIL with variable disease duration. Elevated lactate levels in the right frontal lobe of 1 patient and normal findings in 3 patients were observed in that study.\textsuperscript{13} It is difficult to compare the results of the previous study with those reported here, as Desmond and coworkers used different spectroscopic techniques (single-voxel and multivoxels) and placed their VOI for spectroscopy in different brain regions for each patient. In the present study, with the use of a VOI always localized to include most of the cerebral white matter (Figure 3), $^1$H-MR spectroscopy of our patients showed a diffuse decrease in cerebral NAA/Cr. This suggests that marked axonal damage can occur in the deep white matter of patients with CADASIL, even relatively early in the course of the disease. In contrast, we did not find changes in Cho/Cr or increases in lactate in the brains of these patients, suggesting that neither active myelin breakdown nor substantial inflammation or mitochondrial impairment should be expected in pa-

Figure 3. Proton brain magnetic resonance imaging and magnetic resonance spectroscopy in a normal subject (A) and a patient (III:3) with cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (B). Conventional transverse magnetic resonance image of the patient with CADASIL shows diffuse white matter abnormalities (B, top). Proton spectra from representative voxels located in the deep white matter (black square) of a normal control subject (A, bottom) and the patient with CADASIL (B, bottom) show a significant decrease of N-acetylaspartate (NAA) in the latter. Cho indicates choline-containing phospholipids; Cr, creatine.
tients with CADASIL, at least far from clinically relevant attacks.

In conclusion, our findings emphasize the role of direct DNA sequence analysis for the diagnosis of CADASIL. Moreover, the results of $^1$H-MR spectroscopy indicate that widespread axonal damage might be an early finding of the disease.

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