Spectrum of Mutations in Biopsy-Proven CADASIL
Implications for Diagnostic Strategies

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Background: Mutations in the NOTCH3 gene are the cause of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), which is an important cause of stroke in young adults. Mutations are typically located within epidermal growth factor–like repeat domains in the extracellular part of the Notch3 receptor. Identification of the mutation is critical for genetic counseling and testing of relatives at risk.

Objectives: To identify the spectrum of NOTCH3 mutations in CADASIL and to discuss the implications for diagnostic strategies.

Design: Screening for NOTCH3 mutations was performed in 125 unrelated German CADASIL patients with biopsy-proven disease by direct sequencing of exons coding for epidermal growth factor–like repeats. Results were compared with those of previously published studies.

Results: We detected 54 distinct mutations (117 missense mutations and 3 in-frame deletions) in 120 (96.0%) of the 125 patients. Of the mutations, 58.3% were located in exon 4 and 85.8% in exons 2 through 6. In 5 patients (4.0%), no mutation was identified.

Conclusions: Almost 90% of mutations could be detected within a few exons (exons 2-6). Thus, genetic testing should initially be focused on these exons, with some variation depending on the population in whom it is being performed. Yet, genetic testing for CADASIL is associated with a nameable proportion of false-negative results. Cases with a high index of clinical suspicion should be investigated by skin biopsy if genetic testing is negative.

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Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is an important cause of stroke in young adults.1,2 The disease is caused by mutations in the NOTCH3 gene, which encodes a transmembrane receptor expressed in vascular smooth muscle cells. CADASIL mutations affect highly conserved cysteine residues within epidermal growth factor (EGF)–like repeat domains in the extracellular part of the receptor.

CADASIL may be suspected based on the clinical syndrome, a positive family history, and a typical cranial magnetic resonance image with T2-weighted hyperintense signals in the temporopolar white matter or the external capsule.3 Final proof, however, requires genetic testing or pathological examination of skin biopsy specimens.4-7 Besides ultrastructural examination of biopsy specimens with detection of typical granular osmiophilic deposits, immunohistochemical analysis with a monoclonal Notch3 antibody has been reported to be a sensitive diagnostic procedure in CADASIL.5,7 However, identification of the mutation is critical for genetic counseling and testing of relatives at risk.

Previous studies4,8,9 have explored the spectrum of mutations in patients from different countries. However, the criteria for selecting patients and screening protocols largely varied between studies, and in none of them had the diagnosis been systematically confirmed by biopsy.4,8,9 Thus, it is difficult to draw a conclusion about the sensitivity of genetic testing for CADASIL.

In the present study, we analyzed the NOTCH3 mutational spectrum in a large series of German patients with biopsy-proven disease. The implications of our findings for diagnostic strategies are discussed.

METHODS

We analyzed 125 unrelated German patients with biopsy-proven CADASIL who had been referred to our service between 1994 and 2003.
Patients were selected based on a positive skin biopsy result, with characteristic osmiophilic deposits on ultrastructural examination of small dermal blood vessels.  

Genomic DNA was extracted from peripheral blood leukocytes using standard protocols. Polymerase chain reaction of exons 2 through 24 of NOTCH3 was performed using primers designed to amplify the respective exons, including exon/intron boundaries (details regarding primer sequences and polymerase chain reaction condition are available from the authors on request). Following purification of polymerase chain reaction products, DNA was sequenced using an automated sequencer.  

The NOTCH3 mutations identified in 120 biopsy-proven CADASIL patients are listed in Table 1. The mutations were spread across the entire coding sequence, and represented in the first three exons. Only two mutations, each in exon 4 (C472G and A473C), occurred multiple times and were observed in 20% of patients. The remaining mutations were observed only once.

### Table. NOTCH3 Mutations in 120 Biopsy-Proven CADASIL Cases

<table>
<thead>
<tr>
<th>Exon</th>
<th>Nucleotide Exchange</th>
<th>AA Exchange</th>
<th>EGF Repeat</th>
<th>Frequency of Mutation</th>
<th>Mutations per Exon, No. (%)</th>
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<tr>
<td>2</td>
<td>205T&gt;G</td>
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<tr>
<td>3</td>
<td>306T&gt;G</td>
<td>C76V</td>
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<tr>
<td>6</td>
<td>1033_1034GC&gt;TG</td>
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Abbreviations: AA, amino acid; CADASIL, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy; EGF, epidermal growth factor.

*Percentages do not total 100 because of rounding.
reaction products, bidirectional sequencing was performed using BigDye (Applied Biosystems, Foster City, Calif) on an automated sequencer (ABI 310; Applied Biosystems). Initially, direct sequencing of exons 3 and 4 was performed. If there was no mutation in these 2 exons, all remaining exons coding for EGF repeats (exons 2 and 5-24) were analyzed. Sequences were compared with the NOTCH3 consensus sequence (GenBank U97669). Samples of mutation-negative cases were resequenced a second time.

**RESULTS**

NOTCH3 mutations were detected in 120 (96.0%) of the 125 patients with biopsy-proven CADASIL (Table). Of these mutations, 116 (96.7%) were heterozygous missense mutations caused by a single nucleotide exchange and 1 (0.8%) was a dinucleotide substitution. In 3 cases, there were in-frame deletions. Overall, there were 54 distinct mutations, including 27 novel mutations (50.0%). Seventy-nine mutations (65.8%) led to a gain and 41 (34.2%) led to a loss of 1 or more cysteine residues.

Most mutations were found in exon 4. The second most common location was exon 3, followed by exons 6, 2, and 5 (Table and Figure). Thus, 85.8% of the mutations were located in exons 2 through 6. The remaining mutations were broadly distributed across the remaining exons coding for EGF repeats. Some mutations were found multiple times, with R182C being particularly frequent.

On the protein level, most mutations were detected in EGF repeat 4 (32.5%), followed by EGF repeats 3 (24.2%) and 2 (10.8%) (Figure). Five mutations were detected in EGF repeats 10/11, the region known to be critical for interaction of Notch3 with its ligands.10,11

This study describes the spectrum of NOTCH3 mutations in a large number of otherwise unselected patients with biopsy-proven CADASIL.

Our findings confirm and extend previous studies in other populations, all of which found exon 4 to be the most common site of mutations (Figure).4,8,9 In agreement with the French8 and British4 series, we found exon 3 to be the second most common site of mutations. In contrast, exon 11, which was frequently affected in the Dutch9 and French series, harbored few mutations in our series. Possible explanations for these disparities include founder effects,12 different inclusion criteria (eg, linkage data vs referrals for diagnostic screening), and different screening strategies (eg, single-strand conformation polymorphism analysis vs direct sequencing). In the present study, inclusion was based on a definite diagnosis established by ultrastructural analysis of skin biopsy specimens. The sensitivity of this technique is less than 100%.4 However, there is no evidence for a differential influence of single mutations on skin biopsy results. Thus, we consider a selection bias unlikely.

We found a strong clustering of mutations in EGF repeats 3 and 4, in terms of absolute numbers and the number of distinct mutations. In fact, in all previous studies, EGF repeats 3 and 4 were the most frequently affected do-

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**Table**

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<th>No. of Mutations</th>
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</table>

**Figure.** Distribution of NOTCH3 mutations in exons 2 through 24 in previous series from France (clinical or linkage data),4 the United Kingdom (clinical criteria),4 and the Netherlands (diagnostic series)9 and in the present series (biopsy-proven cases). A schematic drawing of the Notch3 receptor and the distribution of mutations across epidermal growth factor-like repeat domains (1-34) in the present series are also given.
mains. There is no obvious explanation for this, since EGF repeats 3 and 4 do not play a particular role with regard to the structure, function, or metabolism of Notch3.10,11 The multiple occurrence of single mutations may be due to the hypermutability of specific DNA sequences (in particular, CpG dinucleotides) or founder effects.12 Like all previous studies, we found a substantial proportion of novel mutations. This finding is in keeping with the occurrence of neo-mutations, which have been documented in several families13 (and own results, data not shown).

Despite direct sequencing of all EGF-coding exons, no mutations could be found in 4.0% of our patients. For comparison, Joutel et al14 detected mutations in only 90% of their patients screened by single-strand conformation polymorphism analysis. Apart from having characteristic vascular deposits, 3 of our mutation-negative patients had a clear positive family history and all exhibited typical clinical and magnetic resonance imaging findings.1 Thus, we consider it unlikely that these patients’ conditions had been misdiagnosed. Obviously, mutations may have been missed in some of our patients. On the other hand, mutations in these cases may be located outside exons 2 through 24 or may not correspond to the typical pattern of mutations. Sequence variants not corresponding to the typical pattern have been described.14 However, it will remain difficult to decide whether those sequence variants are in fact causative.

In conclusion, given that almost 90% of mutations can be detected when focusing on a few exons (exons 2-6), performing genetic testing should be initially focused on these exons, with some variation depending on the population in which it is being performed. Furthermore, our data demonstrate that genetic testing for CADASIL is associated with a nameable proportion of false-negative results despite performing direct sequencing of exons 2 through 24 of the NOTCH3 gene. The latter is important, as several laboratories offer complete sequencing of all 24 exons. We suggest that cases with a high index of clinical suspicion should be investigated by skin biopsy if the result of genetic testing is negative.

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REFERENCES