

SHORT REPORT

Description of a simple test for CADASIL disease and determination of mutation frequencies in sporadic ischaemic stroke and dementia patients

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Abstract

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a rare inherited adult onset disease characterised most commonly by cerebral ischaemic events and dementia. It is caused by mutations in the *Notch3* gene with most clustering in exons 3 and 4. Whether these mutations have any influence on common sporadic ischaemic stroke or dementia cases has not been investigated, partly hampered by the lack of a readily usable genetic test.

An easy to use diagnostic array for CADASIL was designed using various restriction endonucleases for the known mutations in exons 3 and 4 and novel mismatch primers were designed where no such enzymes existed. This array was used to identify the allele frequencies of CADASIL mutations and polymorphisms in selected disease cohorts. Seventy patients with radiologically established sporadic ischaemic stroke and 77 patients from a specialist young dementia clinic were recruited. One hundred and seventeen age and sex matched asymptomatic controls were also identified. The diagnostic array was found to work well. None of the 14 known mutations and three previously identified polymorphisms (C474A, A587G, and C594A) in exons 3 and 4 were present in 140 stroke, 110 dementia, or 234 control chromosomes. Molecular variant C381T occurred with a higher frequency of 0.13, whereas G684A occurred with a lower frequency (0.09) than previously reported, although there were no statistical differences between selected cohorts.

In conclusion, a readily usable genetic test for CADASIL has been devised that was used to determine allele frequencies in well characterised cohorts of sporadic stroke and dementia patients. The data suggest that despite the clinical resemblance, CADASIL is not a common masquerading cause of stroke or dementia. The test will enable units locally to rapidly screen patients with suspected CADASIL. (*J Neurol Neurosurg Psychiatry* 2000;69:652-654)

Keywords: Cadasil; allele frequencies; polymorphisms

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a rare inherited adult onset disease characterised most commonly by cerebral ischaemic events (84%) and dementia (31%), but also migraine with aura and mood disturbances.¹ The clinical picture is accompanied by associated magnetic imaging abnormalities.² The disease is due to mutations in the transmembrane receptor of the *Notch3* gene, which either create or destroy a cysteine residue.³ Mutations clustering in exons 3 and 4 account for about 65% of all patients with CADASIL.^{4,5} Thus, exons 3 and 4 should be screened as a first stage but, if no mutations are detected and clinical suspicion remains high the remainder of this gene should be sequenced. Whether mutations in such a rare monogenic syndrome account for, or have any effect on, more common phenotypes has not been investigated, partly hampered by the lack of a quick and reliable screening test. Indeed, the absence of a readily accessible screening test along with the need to determine the frequency of *Notch3* mutations in common related diseases masquerading as CADASIL may slow our ability to understand the pathophysiology of cerebrovascular disease.⁵

We sought to establish a simple and reliable genetic test for the *Notch3* variants clustering in exons 3 and 4, and to determine the allele frequencies of *Notch3* mutations and polymorphisms in patients with stroke and dementia. For these purposes, we designed an array of restriction enzymes and mismatch primers.

Methods

CLINICAL

Seventy (40 men) well characterised relatively young patients with radiologically established sporadic ischaemic stroke were recruited (mean age of disease onset 68.8 (SD 13.6) years; range 20-90). In addition, seventy seven patients (36 men) from a specialist young dementia clinic were also recruited (mean age of disease onset 64.4 (SD 9.2) years; range 46.7-89.6) comprising 55 with Alzheimer's disease (AD) with or without vascular dementia, and 22 with frontotemporal dementia (FTD). The FTD group (in which the dementia was hypothesised to be aetiologically unrelated to CADASIL) was used as a

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Table 1 Newly created mismatch primers for detecting *Notch3* gene molecular variants

Mismatch primers	Restriction enzyme	PCR Product sequence	Fragment size (post-digestion)
Exon 3, G291T, 200 bp 5'-TGCCCACAG GTGCCCGCCTG <u>ACT</u> -3'	BsrI	5'-CTGACTGGG↓TG-3' 5'-CTGACTGTGTG-3'	177 bp
Exon 4, C499T, 154 bp 5'-AGGTGGGACAG GAGCAGAGACAGC-3'	Tth111 I	5'-GGACG↓CTGTCTC-3' 5'-GGATGCTGTCTC-3'	131 bp
Exon 4, G749A, 403p 5'-TAGGGCTCAC TCACCAGGT <u>CG</u> -3'	SalI	5'-CCTG↓TCGACCTGG-3' 5'-CCTATCGACCTGG-3'	381 bp

Italic letters=mismatched bases, Bold letters=mutation sites.

↓=restriction enzyme cutting sites, Underline=recognised sequences of restriction enzyme.

“positive disease” control group for the AD cohort. All patients with dementia were fully investigated using standard psychometry and neuroimaging techniques. Clinical diagnoses were based on established criteria.⁶ Finally, 117 (49 men) age and sex matched (mean age 60.1 (SD 15.0) years; range 35–91) asymptomatic normotensive controls were also recruited. All subjects were white and from the United Kingdom.

Laboratory

Genomic DNA was extracted from peripheral blood leucocytes. Flanking intron primer sequences for amplification of the *Notch3* coding regions were:

exon 3 sense: 5'-TGTGCTGCCCAACCAAGCCA-3'

exon 3 antisense: 5'-ACTGACCACACCCCGACTA-3'

exon 4 sense: 5'-TAGTCGGGGGTGTGTGTCAGT-3'

exon 4 antisense: 5'-CCTCTGACTCTCCTGAGTAG-3'

Polymerase chain reaction conditions used in this study were 95°C for 3 minutes, and 35 cycles of 94°C for 1 minute, 62–65°C for 1 minute, 72°C for 1.5 minutes, and 72°C for 10 minutes. Polymerase chain reaction products of exon 3 (224 bp) and exon 4 (420 bp) were initially confirmed by sequencing on an ABI377. Restriction enzymes were identified for all but three of the molecular variants in

exons 3 and 4 of the *Notch3* gene. The remaining three variants (G291T, C499T, and G749A) were amplified with newly created mismatch primers (table 1). All DNA samples were then screened using the restriction enzyme diagnostic analysis array in table 2. Samples were digested alongside positive controls where appropriate. Restriction enzymes were deliberately chosen to recognise and cut the common wild-type sequence. This reduced the need to obtain positive controls for many of the rare mutations. Products were resolved using a 3% Metaphor gel.

STATISTICS AND POWER CALCULATIONS

Our study was designed to detect a clinically significant 2% occurrence of CADASIL mutations in stroke, dementia, and control groups with over 94%, 89% and 99% power, respectively. Allele frequencies were calculated using χ^2 tests. In all cases $p < 0.05$ was considered to be statistically significant.

Results

The diagnostic array was found to work well (table 2).

None of the 14 known mutations in exons 3 or 4 was present in 140 stroke, 110 dementia, or 234 control chromosomes.

Known polymorphisms C474A, A587G, and C594A did not show any frequency variations. The remaining polymorphism frequencies are shown in table 3. Exon 3 polymorphism C381T occurred in greater frequencies in all cohorts than previously reported.⁴ Exon 4 polymorphism G684A occurred with lower frequency than previously reported.⁴ The two polymorphisms C381T and G684A were not in linkage disequilibrium with each other ($D=0$, $p > 0.05$; $\chi^2=0.68$, 4 df).

There were no statistical differences in the allele frequencies between the control and stroke cohorts. In addition, there were no allele

Table 3 Allele frequencies of *Notch3* polymorphisms in different cohorts

	Controls	Stroke	Dementia	FTD
Chromosomes	234	140	110	44
C381T	0.13	0.15	0.19	0.11
G684A	0.09	0.12	0.09	0.19

C381T

Control v stroke $\chi^2=0.18$ (1 df) $p > 0.05$.

Dementia v FTD $\chi^2=1.34$ (1 df) $p > 0.05$.

Control+FTD v stroke+dementia $\chi^2=1.26$ (1 df) $p > 0.05$.

G684A

Control v stroke $\chi^2=0.79$ (1df) $p > 0.05$.

Dementia v FTD $\chi^2=3.14$ (1df) $p > 0.05$.

Control+FTD v stroke + dementia $\chi^2=0.06$ (1df) $p > 0.05$.

Table 2 Diagnostic array chart of the molecular variants of exons 3 and 4 of the *Notch3* gene by restriction enzyme

Enzyme	Mutation/polymorphism* site	Exon	Digested fragments (bp)	
			Wild type	Mutation/ polymorphism
<i>Bsa</i> I	C346T	3	103, 60	163
	C406T	3	"	69
<i>Bsr</i> I	G291T§	3	177	200
	T631C	4	259, 161	420 (uncut)
<i>Aci</i> I	C381T*	3	95, 71	166
	C474A*	4	106, 60, 48	166†
	C475T	4	"	166†
	T514C	4	"	106, 48
	C583T	4	"	106, 87, 60‡
	C622T	4	"	106, 86, 60‡
<i>Tth</i> 111 I	C499T§	4	131	154
<i>Hae</i> III	C535T	4	118, 44	162
<i>Nla</i> III	G589T	4	221, 106	327
	A587G*	4	"	327
<i>Nla</i> IV	T742G	4	80, 59, 61	80, 78, 59
<i>Pst</i> I	T712A	4	347, 73	420 (uncut)
<i>Mwo</i> I	C594A*	4	107, 61	75
	G684A*	4	"	168
<i>Sal</i> I	G749A§	4	381	403

*Polymorphism.

†Distinguished from each other by *Nla* IV (C474A) or *Msp*A1 I (C475T) digestion.

‡Distinguished from each other by *Msp*A1 I (C622T) digestion. §Mismatch primers.

frequency differences between the control groups (control and FTD) and disease groups (stroke and dementia). Finally, no differences were seen between the dementia cohort and FTD group (table 3).

Discussion

We have developed a rapid and reliable test for screening the most common genetic mutations in the *Notch3* gene using an array of restriction enzymes and mismatch primers that can be utilised by most laboratories. We have used this test to answer the question of whether CADASIL mutations occur more often than would be predicted in patients with sporadic stroke and dementia.⁵

None of the 14 CADASIL mutations screened were present in a well characterised cohort of patients with sporadic stroke and dementia. The allele frequencies determined here do not show significant variation between selected cohorts, although one polymorphism (C381T) was present in greater frequency and another (G684A) with lower frequency than previously reported.⁴ Other known polymorphisms did not show variation (A587T is a known rare polymorphism⁴). The differences in frequencies of molecular variants compared with previous reports⁴ may be accounted for by ancestral population differences. Interestingly, C381T and G684A were not in linkage disequilibrium with each other and will therefore need to be investigated independently in future studies, unless an appropriate haplotype can be identified.

The search for genes underlying complex disorders such as ischaemic stroke⁷ and dementia⁸ is currently topical with various strategies available for their identification.⁹ Although the rare monogenic disorders do not pose a major health problem, their importance rests with being able to extrapolate data to understand mechanisms underlying the more common phenotypes. At first sight the *Notch3* gene seems a prime candidate gene for these diseases. However, our data suggest that the *Notch3* gene is not a likely candidate gene for either sporadic stroke or dementia. However, it should be noted that this was not the primary purpose of this study and a larger affected population may need to be studied to detect a small attributable risk, as demonstrated by the angiotensin-1 converting enzyme gene.¹⁰

There may be shortcomings with our work that should be documented. Firstly, as with any study the question of power arises. Our study was well powered to detect a clinically significant (taken as $\geq 2\%$) occurrence of CADASIL in the common phenotypes. Even if a more conservative clinically significant occurrence of, say, 1% was chosen, the power of our study was 78%. Secondly, patients with stroke

and dementia were deliberately chosen with a relatively low age of disease onset in an attempt to match the average disease onset of CADASIL. However, it is possible that in an even younger diseased cohort with stroke and dementia, *Notch3* allele frequencies may be different. Finally, as our test for the most common of the CADASIL mutations used previously documented allele variants, the possibility exists that there are other unidentified *Notch3* molecular variants not tested by us which may occur more often in sporadic stroke and dementia. However, we think that this is unlikely because had they occurred often they are likely to have been detected earlier.⁴ In addition, during the early testing of our diagnostic protocol direct fluorescent based sequencing failed to identify any new variants.

Clearly, the extent of the clinical phenotype for CADASIL can only be determined by assessing the frequency of mutations in patients with sporadic ischaemic stroke and dementia. Restriction enzyme array with mismatch primers allows both the majority and the most frequent of the mutations of the *Notch3* gene to be rapidly screened and regional disease allele frequencies to be easily established, which is critical before any local diagnostic test can be set up.⁵

CADASIL is probably an underdiagnosed condition,¹ and our test will ensure that the diagnosis can be quickly confirmed or excluded whenever there is a clinical suspicion. On the other hand, our data suggest that, despite the clinical resemblance, CADASIL is neither a frequent underlying masquerading cause of sporadic stroke or dementia nor a likely candidate gene for these conditions.

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