Original Article

NOTCH3 Variants in Patients with Suspected CADASIL

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Abstract

Background: Cerebral autosomal dominant arteriopathy with subcortical infarctions and leukoencephalopathy (CADASIL) is the most common hereditary form of cerebral small vessel disease. It is clinically, radiologically, and genetically heterogeneous and is caused by *NOTCH3* mutations. **Methods:** In this study, we analyzed *NOTCH3* in 368 patients with suspected CADASIL using next-generation sequencing. The significant variants detected were reported along with the clinical and radiological features of the patients. **Results:** Heterozygous *NOTCH3* changes, mostly missense mutations, were detected in 44 of the 368 patients (~12%). **Conclusions:** In this single-center study conducted on a large patient group, 30 different variants were detected, 17 of which were novel. CADASIL, which can result in mortality, has a heterogeneous phenotype among individuals in terms of clinical, demographic, and radiological findings regardless of the *NOTCH3* variant.

Keywords: CADASIL, NGS, NOTCH3

Introduction

Cerebral small vessel diseases (CSVDs) are an etiologically heterogeneous group of diseases. Their origin is classified as either sporadic (related to age, immune disorders, or infections) or hereditary. Hereditary CSVDs include cerebral autosomal dominant arteriopathy with subcortical infarctions and leukoencephalopathy (CADASIL); cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL); mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS); Fabry disease; CSVD because of type IV collagen disease; retinal vasculopathy with cerebral leukoencephalopathy; and hereditary cerebral amyloid angiopathies (CAAs).[1]

CADASIL is a vasculopathy that causes neuropsychiatric symptoms including migraine/headache, stroke, epilepsy, and dementia.[2] It is the most common hereditary disease of the small vessels and is caused by *NOTCH3* mutations. Neuroimaging of CADASIL patients shows various combinations of white matter hyperintensities (WMHs), lacunae, cerebral microbleeds, and brain atrophy, depending on the stage of the disease.[3]

A part of CADASIL pathogenesis is the loss of or gain in cysteine residues located in 34 epidermal growth factor-like repeats that make up the extra-cellular domain (ECD) of the type-1 transmembrane receptor, NOTCH3 (N3). This results in an unpaired cysteine residue, which can disrupt the structure of the N3 ECD. Consequently, it can accumulate on the surface of vascular smooth muscle cells, and this is a component of granular osmiophilic material.[4] Deposits of granular osmiophilic material in small blood vessels and the progressive loss of vascular smooth muscle cells are typical histopathological features of CADASIL, which can lead to neurodegeneration.[5,6]

In this study, we performed molecular genetic analysis of *NOTCH3* using next-generation sequencing in 368 patients with symptoms and neuroimaging findings suggestive of CADASIL. All exons, splicing sites, and 5'- and 3'-untranslated regions (UTR) regions were sequenced. Heterozygous variants, mostly missense variants, were detected in 44 patients.

Methods

This study was approved by the local ethics committee (2011-KAEK-25 2019/08-01 and 2022/11-07). Patients suspected of CADASIL and who underwent *NOTCH3* whole gene sequencing between 2015 and 2022 in Medical Genetics Unit of Bursa Yuksek Ihtisas Training and Research Hospital were evaluated retrospectively.[7] DNA was isolated from the peripheral blood of patients using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Next-generation sequencing of *NOTCH3* was performed

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with a custom kit (CleanPlex kit, Paragon Genomics, Hayward, CA, USA) on an MiSeq platform (Illumina, San Diego, CA, USA). All exons, splicing sites, and UTR regions were sequenced. All bioinformatic analyses were performed on the Sophia DDM™ platform (SOPHiA GENETICS SA, Switzerland). Changes with a frequency of less than 1% in the population were considered. Changes that were not reported in the Human Gene Mutation Database (HGMD) at the time of testing were considered novel (https://www.hgmd.cf.ac.uk/ac/index.php). Pathogenicity scoring for novel changes was performed according to the American College of Medical Genetics and Genomics (ACMG) criteria; frequency information was determined according to The Genome Aggregation Database (gnomAD). ACMG and gnomAD were accessed via VarSome (https://varsome.com/). Relevant HGMD numbers were used for variants reported in the literature. Segregation analysis was performed using capillary electrophoresis for family members who agreed to be tested. Primers for the variant-associated target sites were designed using Primer 3 (http://ihg.gsf.de/ihg/ExonPrimer.html). Polymerase chain reaction (PCR) was conducted on a thermal cycler (Applied Biosystems™ Veriti™) using custom primers. Samples were purified using a Zymo Research Sequencing Clean-up Kit (Epigenetic Companies, Irvine, CA, USA). Sanger sequencing was performed with a 3500 Genetic Analyzer (Applied Biosystems®, Foster City, CA, USA), and the results were analyzed using v5.3.1 software (Applied Biosystems). Patients carrying a CADASIL-significant variant in the *NOTCH3* gene are listed in our study.

Results

A total of 368 patients (287 women and 81 men) with suspected CADASIL based on radiological and clinical findings were included in the study. The youngest patient was a 14-year-old male, while the oldest was a 76-year-old female. Except for the 14-year-old, all patients were over the age of 18, and the median age was 40. *NOTCH3* whole-gene sequencing (all exons, splicing regions, and 5'- and 3'-UTR regions) was performed in all patients. We detected variants that were thought to cause CADASIL or similar phenotypes in 44 individuals from 40 families. In total, 30 variants were detected, 17 of which were novel. The remaining 13 variants were previously reported in the literature. Although we found synonymous, small deletion, and splicing mutations, the majority were missense mutations. While one of the variants was a splicing mutation, the others showed a heterogeneous distribution in the exonic regions [Figure 1]. Five of the novel changes were likely benign (LB), six were uncertain significance, five were likely pathogenic, and one was pathogenic. Headache was the most common complaint among patients. WMH was the most frequently observed neuroimaging finding [Figure 2]. The clinical findings, comorbidities, and cranial magnetic resonance (MR) findings of patients with *NOTCH3* variants are presented in Table 1.

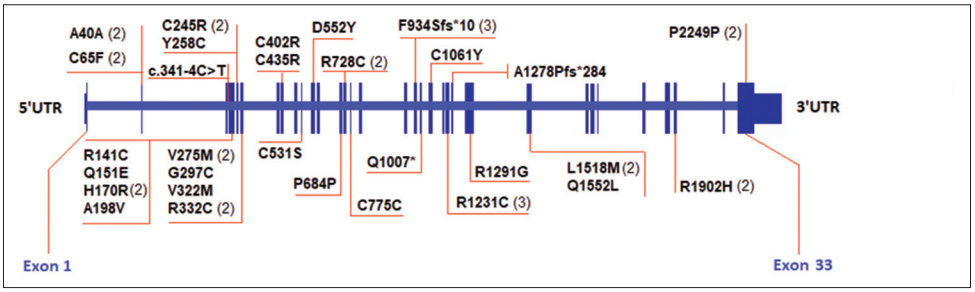

Figure 1: Distributions of variants on the *NOTCH3* gene. *NOTCH3* gene exon regions 1–33 are shown with blue lines (ensemble). Variants are indicated by amino acid symbols using red lines on the relevant exons. The numbers in parentheses represent the number of individuals with the variant

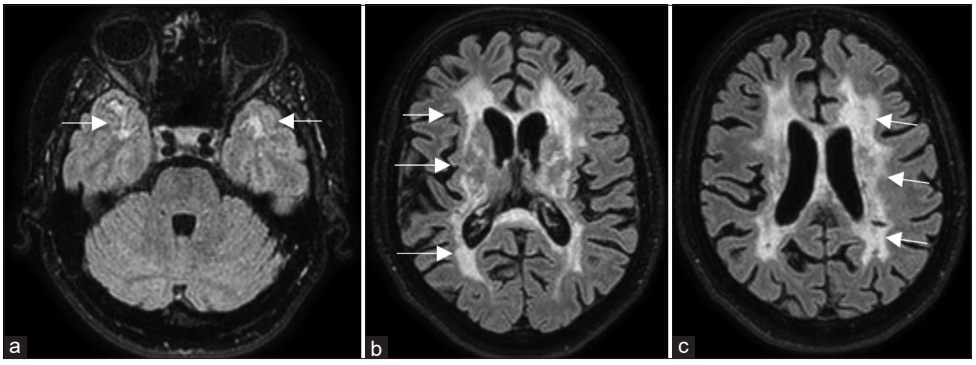

Figure 2: MRIs of patients with CADASIL. Axial fluid attenuation inversion recovery (FLAIR) images showing anterior temporal lobe (a) and diffuse frontal and parietal subcortical white matter and external capsule (b and c) hyperintensities. Lesions are indicated by white arrows. (a), patient 11; (b), patient 24A; (c), patient 24B

Table 1: Demographic, clinical and neuroradiological characteristics of patients with *NOTCH3* variants

Demographic features			Genetic characteristics						Clinical findings					Neuroradiology				
ID	Sex	Age	Variation	Type	Exon	R/N	ACMG	Frequency	Headache, Migraine	Stroke, TIA	Cognitive impairment	Neuro psychiatric symptoms	Other	WMHs	Lacunar lesions	CMBs	Brain atrophy	Family history
1	F	37	c.120C>T p.Ala40 =	Synonym	2	N	LB	0.0000329	+					+				
2	M	46	c.120C>T p.Ala40 =	Synonym	2	N	LB	0.0000329		+				+	+	+	+	
3	F	22	c.194G>T p.Cys65Phe	Missense	2	N	P	0	+	+		+		+				+
4	M	46	c.194G>T p.Cys65Phe	Missense	2	N	P	0		+			Epilepsy	+				
5	F	56	c.341-4C>T	Splicing	intron 3	N	UCS	0	+	+					+			
6	F	47	c.421C>T p.Arg141Cys **	Missense	4	CM971058	P	0	+					+				
7	F	50	c.451C>G p.Gln151Glu *	Missense	4	CM0910786	LP	0.000045	+			+	Epilepsy, and hearing loss	+			+	
8	F	34	c.509A>G p.His170Arg *	Missense	4	CM107598	UCS	0.00184					Epilepsy	+				
9	F	46	c.509A>G p.His170Arg *	Missense	4	CM107598	UCS	0.00184	+	+	+	+		+				
10	F	43	c.593C>T p.Ala198Val	Missense	4	N	LB	0.000032	+	+		+	Dizziness	+			+	
11	F	43	c.733T>C p.Cys245Arg **	Missense	5	CM052275	LP	0	+	+				+				
12	F	51	c.733T>C p.Cys245Arg **	Missense	5	CM052275	LP	0	+					+				
13	M	59	c.773A>G p.Tyr258Cys **	Missense	5	CM971066	P	0		+				+			+	
14A	M	30	c.823G>A p.Val275Met	Missense	6	N	LP	0	+									+
14B	M	57	c.823G>A p.Val275Met	Missense	6	N	LP	0			+	+	The patient died	+			+	
15	F	61	c.889G>T p.Gly297Cys	Missense	6	N	UCS	0	+		+	+		+			+	+
16	F	52	c.964G>A p.Val322Met *	Missense	6	CM150662	LP	0.00014				+	Dizziness	+				
17	F	32	c.994C>T p.Arg332Cys **	Missense	6	CM014070	P	0	+					+				

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Table 1: Contd...

Demographic features			Genetic characteristics						Clinical findings					Neuroradiology				
ID	Sex	Age	Variation	Type	Exon	R/N	ACMG	Frequency	Headache, Migraine	Stroke, TIA	Cognitive impairment	Neuro psychiatric symptoms	Other	WMHs	Lacunar lesions	CMBs	Brain atrophy	Family history
18	M	65	c.994C>T p.Arg332Cys **	Missense	6	CM014070	P	0		+	+	+	The patient died	+				
19	M	35	c.1204T>C p.Cys402Arg	Missense	8	N	UCS	0	+					+				+
20	F	35	c.1303T>C p.Cys435Arg **	Missense	8	CM035646	LP	0	+	+	+		Dizziness	+				+
21	M	51	c.1591T>A p.Cys531Ser	Missense	10	N	LP	0		+				+				
22	F	50	c.1654G>T p.Asp552Tyr	Missense	11	N	UCS	0	+					+				
23	F	36	c.2052G>A p.Pro684 =	Synonym	13	N	LB	0.0000878	+					+				
24A	F	47	c.2182C>T p.Arg728Cys **	Missense	14	CM971067	LP	0.0000082	+			+						+
24B	F	53	c.2182C>T p.Arg728Cys **	Missense	14	CM971067	LP	0.0000082		+				+				+
25	F	76	c.2325C>T p.Cys775 =	Synonym	15	N	LB	0		+		+		+			+	
26A	M	29	c.2799delC p.Phe934SerfsTer10	Frameshift	18	N	LP	0	+					+				+
26B	M	35	c.2799delC p.Phe934SerfsTer10	Frameshift	18	N	LP	0				+	Dizziness, tinnitus, and visual impairment	+				+
26C	F	54	c.2799delC p.Phe934SerfsTer10	Frameshift	18	N	LP	0	+		+			+				+
27	F	33	c.3019C>T p.Gln1007Ter	Nonsense	19	N	LP	0	+			+	Visual impairment,	+				
28	F	46	c.3182G>A p.Cys1061Tyr **	Missense	20	CM127977	P	0		+			Sjogren	+				
29	F	34	c.3691C>T p.Arg1231Cys **	Missense	22	CM971071	UCS	0.000887	+		+	+	Hypothyroidism	+			+	+
30	F	47	c.3691C>T p.Arg1231Cys **	Missense	22	CM971071	UCS	0.000887	+				Dizziness	+				
31	F	69	c.3691C>T p.Arg1231Cys **	Missense	22	CM971071	UCS	0.000887	+	+	+			+			+	

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Table 1: Contd...

Demographic features			Genetic characteristics						Clinical findings					Neuroradiology				
ID	Sex	Age	Variation	Type	Exon	R/N	ACMG	Frequency	Headache, Migraine	Stroke, TIA	Cognitive impairment	Neuro psychiatric symptoms	Other	WMHs	Lacunar lesions	CMBs	Brain atrophy	Family history
32	F	37	c.3831_3832delTG p.Ala1278ProfsTer284	Frameshift	23	N	LP	0		+				+				
33	F	47	c.3871C>G p.Arg1291Gly	Missense	24	N	UCS	0	+			+		+				
34	F	36	c.4552C>A p.Leu1518Met **	Missense	25	CM119551	B	0.00466	+	+		+		+				
35	M	47	c.4552C>A p.Leu1518Met **	Missense	25	CM119551	B	0.00466	+	+	+	+		+				
36	F	22	c.4655A>T p.Gln1552Leu	Missense	25	N	UCS	0.00000414	+			+		+				
37	F	31	c.5705G>A p.Arg1902His	Missense	31	N	LP	0.000008	+			+		+				
38	F	50	c.5705G>A p.Arg1902His	Missense	31	N	LP	0.000008		+			Dizziness, and tinnitus	+				
39	F	39	c.6747C>T p.Pro2249 =	Synonym	33	N	LB	0.0000202		+				+				
40	F	45	c.6747C>T p.Pro2249 =	Synonym	33	N	LB	0.0000202		+				+				

R/N, reported/novel; ACMG, American College of Medical Genetics and Genomics; gnomAD, Genome Aggregation Database; F, female; M, male; P, pathogenic; LP, likely pathogenic; UCS, uncertain significance; LB, likely benign; B, benign; TIA, transient ischemic attack; WMHs, white matter hyperintensities; CMBs, cerebral microbleeds (*), non-cysteine-altering variant; (**), cysteine-altering variant[8-10]. Note: gnomAD was used to determine the population frequency of variants, and novel variants were classified according to ACMG criteria. Human Gene Mutation Database (HGMD) numbers are provided for reported variants

Discussion

Analysis of *NOTCH3*, consisting of 33 exons and localized at 19p13.12, is the gold standard for CADASIL diagnosis.[11,12] Currently, 400 variants of *NOTCH3* that cause various neurological disorders have been identified, the majority of which are missense mutations (https://www.hgmd.cf.ac.uk/ac/index.php). In this study, we detected 30 variants of *NOTCH3* in a large group of patients with neurological symptoms and neuroradiological findings indicative of CADASIL. Consistent with the literature, the majority (22) of these mutations were missense, while four were synonymous variants, two were small deletion variants, and one was a splicing variant. Seventeen of these variants have not been previously reported, enhancing our understanding of pathological *NOTCH3* variants. The majority of mutations reported to cause CADASIL are located between *NOTCH3* exons 2 and 24.[8] Similarly, most of the *NOTCH3* variants detected in this study were between exons 2 and 24. However, recent studies have reported that mutations between exons 25 and 33 may also cause CADASIL,[13] and we detected four variants in exons 25, 31, and 33. Two of these, L1518M and P2249P, were benign and likely benign, respectively, according to ACMG. Lesions consistent with bilateral parietal WMH were observed in a 45-year-old female patient with a history of stroke and a 39-year-old female patient with a history of transient ischemic attacks. Importantly, the synonymous variant P2249P was detected in both patients. In addition, the mother of the 39-year-old female patient complained of migraine and was heterozygous for this change. Schmidt *et al*. (2011) associated the L1518M variant with severe white matter lesions and defined this condition as the CADASIL-like phenotype.[14] A 36-year-old female patient with severe neurological symptoms and a 47-year-old male patient were heterozygous for L1518M. Neuroimaging of these unrelated patients showed extensive white matter involvement. Dunn *et al*. (2022) detected the p.A40A (c.120C>G) variant in the cis position of *NOTCH3* with two different missense variants in a 60-year-old male patient with ischemic changes on a brain MRI. They did not identify the synonym A40A as a pathogenic variant but commented that it may cause CADASIL without altering cysteine residues.[15] A heterozygous A40A (c.120C>T) variant was detected in a 37-year-old female patient who suffered from migraine attacks with visual impairment and a 46-year-old male patient with a history of hemiplegia due to stroke. Both patients had WMH, but the lesions were more extensive in the male patient. The nucleotide change causing A40A was different in our patients; therefore, it is considered novel. We believe that the association of B and LB changes, according to the ACMG criteria, with the disease may cause confusion. For this reason, such changes are discussed in the context of the literature. Apart from those discussed above, the other two LB variants were A198V and P684P. These novel changes detected in our patients with neurological deficits and MRI findings were not homozygous in population studies, and their frequencies were quite low.

CADASIL, which follows an autosomal dominant inheritance pattern, is mainly caused by heterozygous mutations; however, some cases with biallelic *NOTCH3* mutations have also been reported.[16] All variants detected in our patients were heterozygous.

CADASIL is known to affect men more severely than women, but there is no difference in the incidence between the sexes.[17] In our whole patient group and cases with variants, the proportion of females was significantly higher than that of males. Further research is needed on the relationship between the severity of neurological involvement and sex.

White matter pathologies and lacunar infarcts are most frequently observed while neuroimaging patients diagnosed with CADASIL.[18] CADASIL patients show phenotypic variability in symptoms, disease severity, and progression for reasons that are not fully understood. This variability can even be observed in individuals diagnosed within the same family. In addition, CADASIL is a potentially fatal disease that often progresses to dementia and physical disability.[16] Similarly, in our entire patient group, with or without a variant in *NOTCH3*, the most common radiological findings were WMH and infarcts, and the most common symptom was a headache. In addition, there was heterogeneity in disease severity and the radiological and clinical findings. For example, patients 14B and 18 died within a few years of undergoing the molecular test.

The prevalence of CADASIL, which is not known in Turkey, is estimated to be 1.98–4.6 cases per 100,000 people. Although it is a rare genetic disease, the prevalence of cysteine-altering *NOTCH3* mutations was reported to be 2.2–3.4 cases per 1,000 people in studies conducted with reference to exome databases. This apparent discrepancy between the prevalence of CADASIL and the frequency of *NOTCH3* mutations indicates that not all carriers are symptomatic. This situation has been interpreted in different ways, but further research is still needed.[16] Similarly, in the segregation analysis of patients severely affected by CADASIL, we observed that other family members carrying the same mutation had no symptoms. In addition, there was variability in the distribution of significant variants between *NOTCH3* exons 2 and 24 detected for CADASIL in patient groups and population studies.[19] We believe that the number of cases in our study is insufficient to conclude on this aspect. There are a limited number of studies conducted for CADASIL in our country. In a recent study, Rustemoglu *et al*. detected 12 different missense *NOTCH3* variants significant for CADASIL in 18 individuals from 14 different families. Three of these variants were novels. The variants we detected in our study were different except for R141C.[20] Although most of the detected variants were in the region between exons 2 and 24, there was a heterogeneous distribution within this region.

All mutations that cause CADASIL result in an odd number of cysteine residues. Particularly in adulthood, CADASIL presents with signs of brain dysfunction caused by the progressive development of disseminated white matter lesions

with subcortical lacunar infarctions. An increase in the total volume of lesions and the progression of cerebral atrophy increases the frequency and severity of motor difficulties and cognitive dysfunction. In CADASIL, the most common form of hereditary vascular dementia, cognitive impairment, is progressive.[21] The majority of patients in our study became symptomatic in adulthood, and the disease continued to progress over time.

Many neurological deficits, especially multiple sclerosis, progress with symptoms resembling those of CADASIL. While the pathogenesis of MS is multifactorial, most of these diseases develop genetically. Therefore, molecular genetic analysis combined with detailed neurological examinations and imaging will likely clarify the diagnosis.[22] From our group of patients pre-diagnosed with CADASIL, we detected clinically significant variants in 12% of the patients. This low rate can be attributed to the fact that CADASIL is a member of a heterogeneous group of neurological diseases.[22]

Cysteine-altering *NOTCH3* variants cause a broad spectrum of neurological symptoms. Different phenotypes, ranging from asymptomatic to typical CADASIL, can be seen. Non-cysteine-altering variants have also been shown to cause dementia and mild cognitive impairment.[23] We do not know whether the novel variants detected in our study alter the number of cysteine residues in NOTCH3. In conclusion, *NOTCH3* variants and phenotypic findings are discussed in this study.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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