NOTCH3 Gene Mutation in a Chilean Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy Family

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Introduction: Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a rare hereditary stroke disorder caused by mutations in the NOTCH3 gene. We report the first Chilean CADASIL family with complete radiological and histological studies. *Methods:* The family tree was constructed from an autopsy-confirmed confirmed patient, and includes 3 generations. We performed clinical, pathologic, genetic, and radiologic examinations on members of a family with CADASIL. *Results:* In the second generation, findings compatible with CADASIL were identified in 6 individuals, all of whom had a missense mutation in exon 3 (c.268C>T) resulting in an arginine to cysteine amino acid substitution at position 90 (R90C). In the third generation, a missense mutation was detected in one of the 4 asymptomatic individuals. *Conclusions:* There are similarities in clinical presentation between this family and previously described Asian and European series with R90C mutations. Detecting genotypes with a gain or loss of cysteine residues opens the door to future gene transfection-based therapies.

Key Words: CADASIL—R90C mutation—NOTCH3—cerebral infarction—migraine

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Introduction

Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) is an adult-onset hereditary disorder characterized by migraine with aura, apathy, mood disorders, subcortical cognitive impairment, and stroke.[1] During the presymptomatic stage, patients show brain magnetic resonance imaging (MRI) abnormalities characterized by white matter lesions in the anterior temporal lobe and external capsule. CADASIL is caused by mutations in the *NOTCH3* gene, which codes for the epidermal growth factor receptor. This protein is mainly expressed in vascular smooth muscle cells.[2] Mutations lead to a systemic vasculopathy involving progressive degeneration of vascular smooth muscle cells, predominantly in the small cerebral arteries. Ultrastructural studies may reveal deposits of granular osmiophilic material (GOM).[3] In approximately 90% of cases, the mutations alter the number of cysteine residues, favoring protein accumulation in the vascular wall.[4]

Over 230 different *NOTCH3* mutations associated with CADASIL have been reported, mainly in European and Asian families.[5] In South America, only 2 studies have characterized the clinical and genetic profile.[6,7] In Chile, 3 individuals with an R141C mutation of the *NOTCH3* gene, associated with dystonic symptoms, have been reported.[8] The aim of this study was to characterize the clinical, imagenological, and genetic features of a Chilean family with CADASIL.

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Methods and Patients

Recruitment of Families with CADASIL and Clinical Assessment

The proband (individual I-1; Fig 1) had a history of recurrent stroke and subcortical dementia that led to his death at the age of 46 years. The individual was diagnosed with CADASIL by a brain biopsy that showed GOM.

Neuroimaging Studies

Every member of the second generation underwent brain MRI in a 1.5 T., FLAIR, diffusion, and magnetic susceptibility sequences were analyzed by a neuroradiologist. MRI was considered compatible with CADASIL if at least 2 of the following characteristic findings were identified: (i) hyperintensities in the periventricular white matter, anterior temporal lobe or external capsule; (ii) ischemic lesion(s) at typical sites of lacunal infarction; and/or (iii) microbleeds.[20]

Histological Studies

Every member of the second generation underwent a skin biopsy. The biopsy was analyzed by a neuropathologist using a transmission electron microscope to evaluate for GOM in the vascular wall.

DNA Extraction and Amplification of Select Mutated NOTCH3 *Regions*

About 3 mL of peripheral blood was collected from each family member. DNA extraction from the blood samples was performed with the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The quality control measures included quantification and integrity analysis of the extracted DNA. High molecular weight DNA extracted from the blood samples was quantified using the Qubit 4 fluorometer system. Agarose gel (1%) electrophoresis was used for integrity analysis of extracted genomic DNA using GeneRuler 1 kb DNA Ladder. Specific targeted regions of exons 3 and 4, which are mutational hotspots in the *NOTCH3* gene, were amplified using polymerase chain reaction (PCR) from 20 nanograms of genomic DNA to prepare next generation sequencing (NGS) libraries and direct Sanger sequencing.

Bioinformatics Analysis

After sequencing, FASTQ files were generated and incorporated into a bioinformatic pipeline to clean the data, analyze variants, and perform biological interpretation of the genetic findings for each individual. The variant discovery software, developed by the GATK 1000 Genomes Project, was used to identify single-nucleotide polymorphisms (SNPs) and INDELs.[9]

Results

The clinical data for all subjects, as well as the brain MRI findings and ultrastructural changes identified in the skin biopsy, are summarized in Table 1.

The SNPs were classified according to functional class as missense or synonymous mutations using GATK, as shown in Table 2. The bioinformatically-obtained SNPs were validated with Sanger sequencing, and the

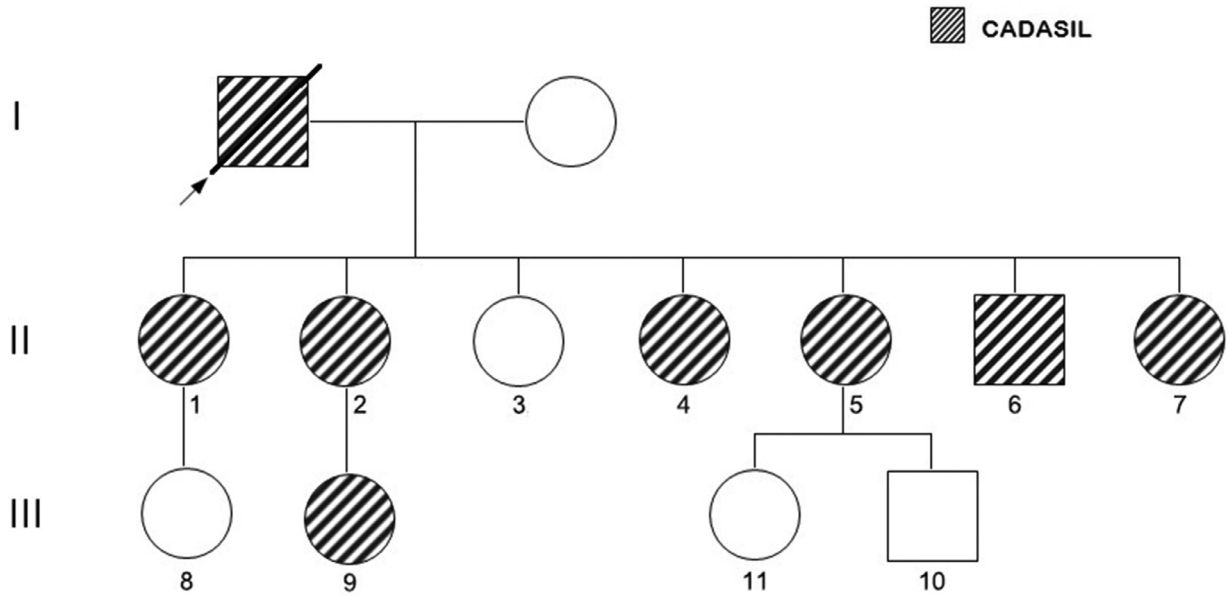

Figure 1. *Pedigree for 3 generations of the Chilean family affected by CADASIL. Abbreviation: CADASIL, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy.*

Table 1. *Clinical information summary and phenotypes of patients included in the studied cohort*

Patient recruitment ID	Clinical information					
	Migraine	Stroke	Mood disorders	Mild cognitive impairment (MCI)	MRI*	Skin biopsy†
1	X	X	–	X	X	X
2	X	X	–	–	X	X
3	–	–	–	–	–	–
4	X	X	–	–	X	
5	X	–	–	–	X	X
6	X	–	–	–	X	X
7	X	X	X	–	X	X
8	–	–	–	–	–	
9	–	–	X	–	X	
10	–	–	–	–	–	
11	–	–	–	–	–	

*MRI results compatible with CADASIL.
†Skin biopsy positive for granular osmiophilic material (GOM).

electropherogram revealed 2 SNPs (Fig 2). A missense SNP was found in 6 of the 7 second-generation subjects and in subject III-2. This SNP consisted of a cytosine-to-thymine substitution at position 268 of exon 3, resulting in an arginine-to-cysteine amino acid substitution at position 90 (R90C).

This change leads to an odd number of cysteine residues in the extracellular portion of the transmembrane receptor structure, altering the electrostatic potential. The R90C *NOTCH3* mutation produces a $\Delta\Delta G$ value of −.75 in the vicinity of the mutated amino acid, and this electronegative change reduces the stability of the folding proteins, affecting the 3-dimensional structure to favor pathogenic accumulation (Fig 3).

Six members of the second generation carrying the mutation showed brain MRI findings characteristic of the pathology. The anterior temporal lobe and external capsule were the most frequently affected locations (Fig 4). Furthermore, the skin biopsies of the same 6 individuals showed the presence of GOM (Fig 5), unlike subject II-3.

The most frequent clinical symptom was migraine with visual aura, with an average age of onset at 26 years. Subject II-5 experienced headache more frequently than the other family members, with an average of 2 episodes per week without prophylaxis. Of the 11 living subjects studied, 4 had experienced at least one stroke during their life, all of which were deep lacunar ischemic lesions.

A cognitive evaluation was performed on the 7 subjects in the second generation. The average Addenbrooke's Cognitive Examination score was 84 points, and individuals II-2 and II-4 showed the lowest performance with 68 and 71 points, respectively.

Table 2. *Genetic variants in exons 3 and 4 of NOTCH3 gene identified in 11 subjects*

Patient recruitment ID	Exon	HGVSC	HGVSP	Consequence	Zygosity
1	3	c.268C>T	p.(Arg90Cys)	Missense variant	Heterozygous
	4	c.606A>G	p.(Ala202=)	Synonymous variant	Heterozygous
2	3	c.268C>T	p.(Arg90Cys)	Missense variant	Heterozygous
	4	c.606A>G	p.(Ala202=)	Synonymous variant	Heterozygous
3	4	c.606A>G	p.(Ala202=)	Synonymous variant	Homozygous
4	3	c.268C>T	p.(Arg90Cys)	Missense variant	Heterozygous
	4	c.606A>G	p.(Ala202=)	Synonymous variant	Heterozygous
5	3	c.268C>T	p.(Arg90Cys)	Missense variant	Heterozygous
	4	c.606A>G	p.(Ala202=)	Synonymous variant	Heterozygous
6	3	c.268C>T	p.(Arg90Cys)	Missense variant	Heterozygous
	4	c.606A>G	p.(Ala202=)	Synonymous variant	Heterozygous
7	3	c.268C>T	p.(Arg90Cys)	Missense variant	Heterozygous
	4	c.606A>G	p.(Ala202=)	Synonymous variant	Heterozygous
8	4	c.606A>G	p.(Ala202=)	Synonymous variant	Homozygous
9	3	c.268C>T	p.(Arg90Cys)	Missense variant	Heterozygous
	4	c.606A>G	p.(Ala202=)	Synonymous variant	Heterozygous
10	4	c.606A>G	p.(Ala202=)	Synonymous variant	Homozygous
11	4	c.606A>G	p.(Ala202=)	Synonymous variant	Homozygous

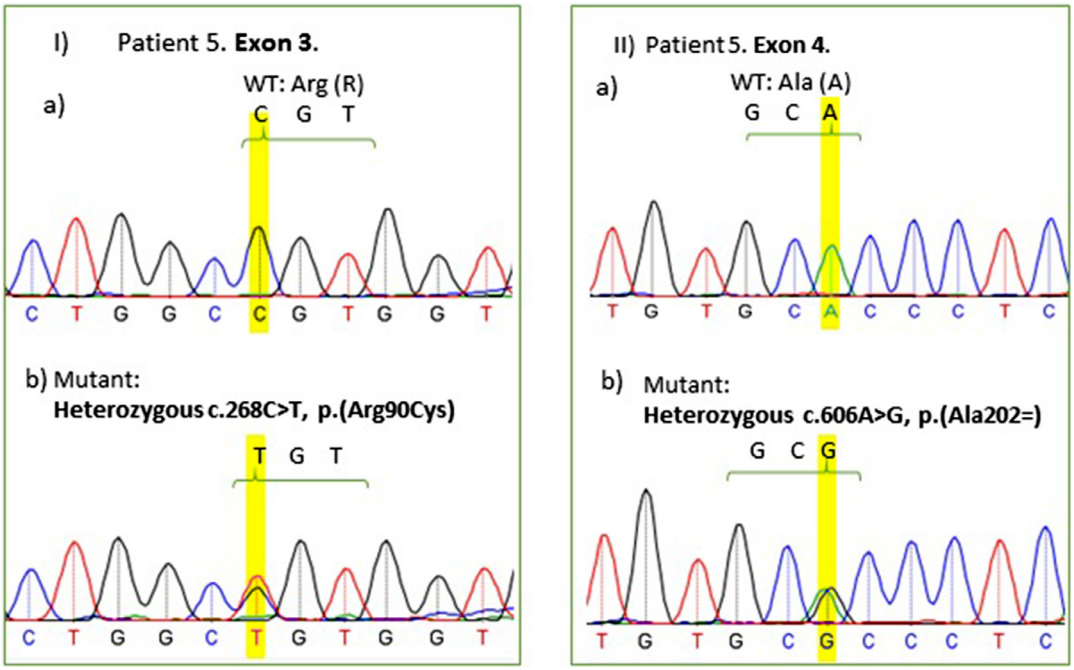

Figure 2. *Sequencing electropherograms of Notch3 gen exons 3 and 4 show SNP validation using Sanger sequencing approach. I. Electropherogram of exon 3 of patient 5. (a) Wild type p.(Arg90) (b) Mutant p. (Arg90Cys). Missense variant. II. Electropherogram of exon 4 of patient 5. (a) Wild type p.(Ala202) (b) Mutant p.(Ala202=). Synonymous variant. Patient 5 is heterozygous for both mutations. Abbreviation: SNP, single-nucleotide polymorphisms.*

Discussion

In this study, we fully characterize the first genetically-confirmed Chilean family with CADASIL.

The affected subjects carried a heterozygous missense mutation located in exon 3 (c.268C>T). This mutation produced a gain of 1 cysteine residue through a p. Arg90>Cys substitution, as previously described in Chinese,[10] Turkish[11] and Israeli[12] families.

The clinical penetrance of the mutation in this family was significant, with a high incidence in subjects over 30 years old, similar to rates reported in Asia[13-15] and

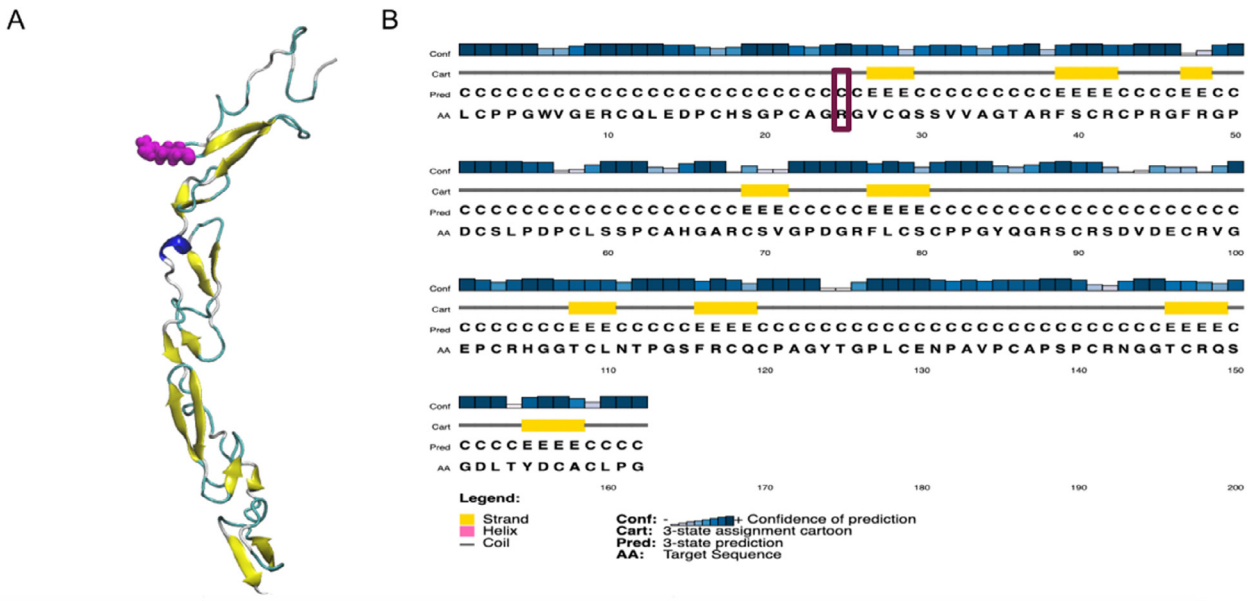

Figure 3. *Protein structural modeling and predictions. (A) Three-dimensional and secondary structure of the Notch1 receptor wild type used as template. (B) Connectivity pattern prediction based on Support Vector Regression (SVR) over Simple Linear Regression (SLR) models.*

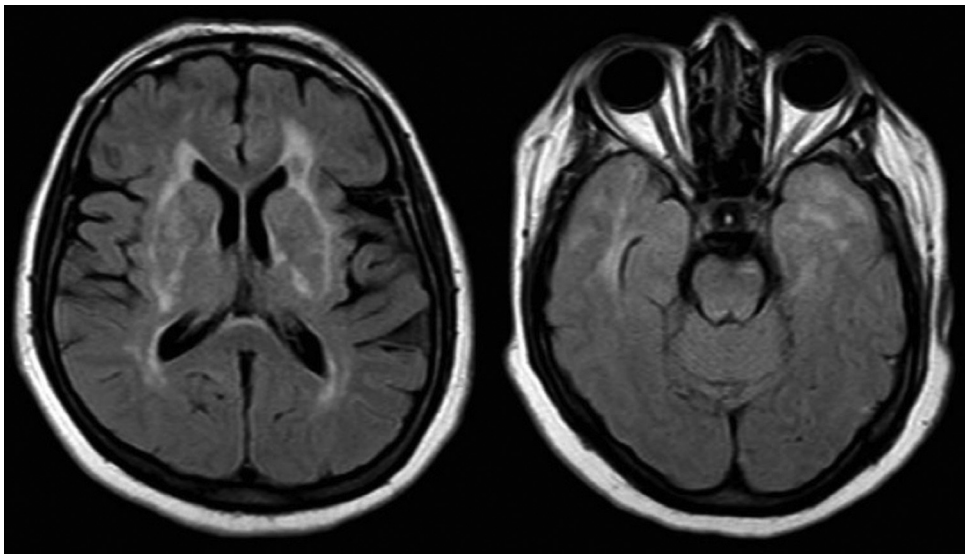

Figure 4. *FLAIR MRI from the patient II-2 showed hyperintensities in external capsule and in temporary poles.*

Europe.[16,17] However, phenotypic expression varied widely within the family, even within the same generation.[18] Migraine with aura was the most prevalent symptom. However, subjects II-5 and II-6 experienced more frequent headaches but had not had a stroke and performed better on the ACE examination, suggesting that migraine is a protective factor against acute and chronic ischemic lesion load.[19]

Genetic testing is the most sensitive and specific method for diagnosing CADASIL.[20] Furthermore, genetic

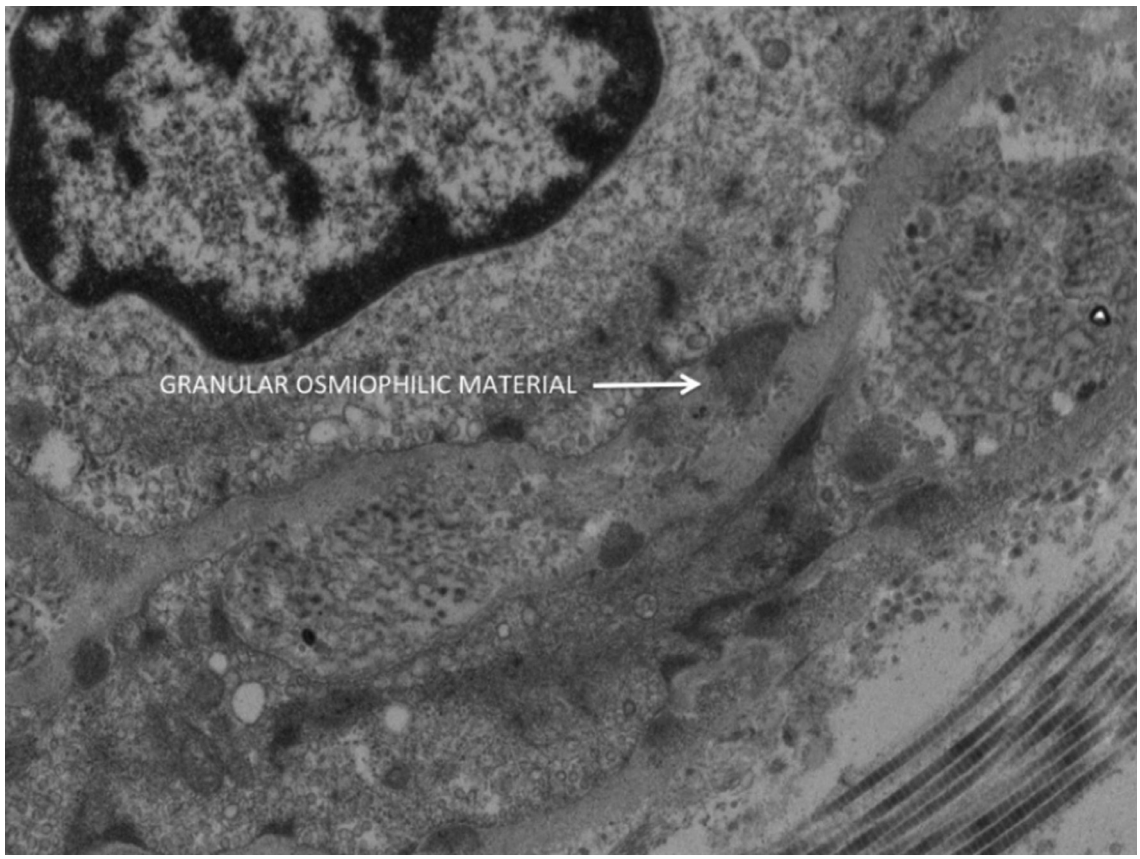

Figure 5. *Ultrastructural study of a skin biopsy from the patient II-7 revealed the presence of granular osmiophilic material (arrow).*

testing has the advantage of providing a diagnosis at an early stage of life in asymptomatic persons and does not require an MRI. In our study, the mutation in subject III-9 was identified during an early, nonspecific clinical stage, in which the only manifestations were incipient brain MRI alterations, mainly temporal pole abnormalities and white matter gliosis that was unusual for her age. Currently, the main purpose of early diagnosis is to allow for appropriate genetic counseling; education on migraine management, especially avoidance of triptans and ergotamine; and management of vascular risk factors to avoid comorbidities that may increase the rate of ischemic brain lesions.

At present, there is no specific treatment for CADASIL.[21] However, transfection of antisense oligonucleotides into the smooth muscle cells of brain vessels to skip the mutated exon may allow for exclusion of the epidermal growth factor receptor domain with an unpaired cysteine residue, without affecting the normal signaling of the *NOTCH3* gene, instead translating a protein that has no tendency to multimerize.[22] This gene therapy strategy may be useful in CADASIL patients whose causal mutation leads to a gain or loss of a cysteine residue, as in the case of the family described above.

Conflict of Interest

The authors report no conflict of interest.

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