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Mutational and haplotype map of *NOTCH3* in a cohort of Italian patients with cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL)

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ABSTRACT

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), the most common form of familial vascular dementia, is caused by mutations of the *NOTCH3* gene. Approximately two hundred pathogenic mutations have been reported within five exons (exons 3, 4, 6, 11 and 19) which accounted for 78% of known mutations in worldwide series. We reported twenty-one *NOTCH3* pathogenic mutations (including five novel ones) identified in 53 index Italian patients. Exons 4 (28%), 7 (21%) and 19 (24%) were the most frequently involved. To dissect genetic heterogeneity, we analyzed five haplotyped tagging single nucleotide polymorphisms (rs1044009, rs4809030, rs10426042, rs10423702 and rs3815188) in 95 patients, 39 unaffected pedigree members and 50 healthy controls. SNPs were analyzed using the Illumina VeraCode Universal Capture Beads technology by Allele Specific Primer Extension (ASPE). We identified ten different haplotypes named H1–H10; H1 was the most common haplotype in patients and controls and it was associated with at least twelve out of the twenty-one mutations. Detected mutations were not associated to specific haplotypes while genotyping was compatible with a possible founder effect for the novel p.S396C mutation which clustered in a restricted geographical area of northeast Italy. The results added on to the genetic heterogeneity of CADASIL and emphasized difficulties in designing algorithms for molecular diagnosis.

1. Introduction

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL, OMIM# 125310) is the most common form of familial vascular dementia which may manifest by the second decade with migraine, recurrent cerebrovascular events (transient ischemic attacks or lacunar strokes), mood disorders, psychiatric disturbances, cognitive decline and dementia [1–5]. Brain magnetic resonance imaging (MRI) discloses suggestive white matter lesions and signs of multiple lacunar infarctions in the basal ganglia and brainstem [6–8]. Although CADASIL manifests as a brain small vessels disease, the vascular pathology is a systemic degeneration of smooth muscle cells in the tunica media of small arteries and capillaries associated with extracellular deposition of granular osmiophilic material (GOM) [9–12].

NOTCH3, the causal gene, has 33 exons coding for 8089 nucleotide-long transcript and a single-pass transmembrane receptor, which is selectively expressed by vascular smooth muscle cells (VSMC) of arterial walls [13] and involved in their differentiation and maintenance [14,15]. The N-terminal extracellular domain of the protein consists of 34 epidermal growth factor (EGF)-like repeats, each containing six cysteine residues.

Approximately two hundred mutations distributed among the EGF-like repeat coding exons 2–24 have been associated to CADASIL world-widely; most of them are missense mutations, resulting in a gain or loss of a cysteine residue in one of the EGF-like repeats, thus sparing the number of cysteines.

Although exons 4 and 3 appeared as major cumulative mutational hot spots, significant variations in the mutational distribution were reported among series from different countries [16–20].

Here we added on to the genetic heterogeneity of CADASIL by describing 21 *NOTCH3* mutations identified in 53 index Italian patients. We also characterized the *NOTCH3* haplotypes to investigate possible influences of the genetic background on the recurrence of mutations.

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2. Materials and methods

2.1. Patients

Fifty-three index CADASIL patients had been referred to us from northern and central Italy. Twenty-two patients disclosed a clear dominant inheritance as inferred from medical histories or records and, in some cases, from church archives (pedigree probands). For thirty-one patients no information was available for pedigree construction ("isolated" probands).

2.2. Mutation analysis of the NOTCH3 gene

Genomic DNA from probands, available affected or unaffected relatives and healthy controls, was extracted routinely from peripheral blood leukocytes after a written informed consent. *NOTCH3* was analyzed by Denaturing High Performance Liquid Chromatography (DHPLC) using a WAVE® 3500 HT System (TRANSGENOMIC). Coding exons 2–24 and related intron–exon boundaries were amplified by polymerase chain reaction (PCR). Amplicons and oligonucleotides were designed according to GenBank clone NM_000435. PCR products were amplified using GoTaq® DNA Polymerase (Promega) or AmpliTaq Gold® (Roche) by a GeneAmp 9700 thermal cycler (Applied Biosystems) (primer sequences and amplification conditions available online in Supplementary Table 1). Samples with abnormal chromatographic profiles were characterized by automated direct nucleotide sequencing of both sense- and antisense-strands, using the GenomeLab Dye Terminator Cycle Sequencing with the Quick Start Kit (Beckman Coulter) on a Capillary Array Electrophoresis Sequencer CEQ™ 8800 (Beckman Coulter).

2.3. Tagging SNPs of NOTCH3 gene

A group of single nucleotide polymorphisms (SNPs) was selected from the HapMap database (http://www.hapmap.org, rel#24) by a tag selection algorithm that searched for the marker subset presenting maximum entropy (http://www.well.ox.ac.uk/rmott/SNPS/running.shtml) for the *NOTCH3* gene region. The following five SNPs were selected to tag the most common *NOTCH3* haplotypes in the Caucasian population (CEU): rs1044009, rs10426042, rs4809030, rs10423702 and rs3815188. Each SNP was analyzed by DHPLC, nucleotide sequencing and Illumina ASPE Assay. Genotyping was performed on 184 subjects including: 103 individuals from CADASIL pedigrees (64 patients and 39 unaffected), 31 "isolated" probands and 50 healthy controls of Italian origin.

2.4. SNP genotyping by Illumina ASPE assay

The five tagging SNPs were analyzed using Illumina VeraCode Universal Capture Beads technology by ASPE (Allele-Specific Primer Extension) chemistry combined with Illumina BeadXpress Reader System. PCR-primers and ASPE-probes were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/) following VeraCode Assay Guideline. All five selected SNPs were amplified in a multiplex PCR performed on a GeneAmp 9700 thermocycler (Applied Biosystems) (PCR-primer sequences and amplification conditions available online in Supplementary Table 2). After amplification, PCR products underwent a single-step enzymatic clean-up, by using ExoSAP-IT® (usb) reagent. After incubation with both wild-type and variant ASPE probes, PCR products underwent multiple rounds of oligonucleotide extension incorporating biotin (ASPE-probe sequences and elongation conditions available online in Supplementary Table 3). After probes extension, products were hybridized to the VeraCode Beads (that have been previously kitted into a 96-well polypropylene plate) and labeled with a streptavidin-fluorophore conjugate according to the manufacturer's protocol. After this step, the VeraCode Bead plate was scanned by BeadXpress Reader. The automatic allele calling was performed by the Illumina BeadStudio Data Analysis Software with a GeneCall threshold of 0.25. The genotype of the SNP was determined by the ratio of the relative fluorescent levels of the two bead types. ASPE genotyping accuracy was evaluated and validated comparing the SNP genotype obtained using the ASPE Assay with the available results of direct sequencing and DHPLC analysis.

2.5. Haplotype analysis

Haplotypes were reconstructed in twenty-two CADASIL pedigrees using the Genehunter (http://www.broad.mit.edu/ftp/distribution/software/gh2.1/) computer program and further studied by two investigators (G.M. and S.T.) to evaluate possible alternative segregation patterns involving different haplotype configurations, according to the haplotype frequency estimated on the entire family set. Haplotype frequency was also estimated in the fifty-three index patients and in the fifty unrelated healthy controls by a maximum-likelihood computation, based on an expectation-maximization algorithm as implemented in the computer program Gerbil [21].

3. Results

3.1. NOTCH3 mutational analysis

Twenty-one different *NOTCH3* missense mutations were identified (Table 1). To the authors' knowledge, the following five mutations were unreported and excluded in 50 healthy controls: p.Cys108Ser, p.Ser396Cys, p.Cys606Arg, p.Cys939Arg and p.Gly1013Cys. Mutations were scattered across ten exons; exons 4, 7 and 19 were mostly affected accounting together for ~74% of all mutations. Several mutations were identified in patients originating in different major geographical areas of Italy: eleven mutations (p.Arg90Cys, p.Cys146Arg, p.Arg169Cys, p.Cys174Tyr, p.Cys201Arg, p.Ser396Cys, p.Arg592Cys, p.Cys606Arg, p.Arg607Cys, p.Cys939Arg and p.Gly1013Cys) were detected in patients from northeast Italy; four (p.Cys251Arg, p.Cys271Phe, p.Cys366Trp and p.Arg1231Cys) were detected in patients from northwest Italy; three (p.Cys108Ser, p.Gly528Cys and Arg1006Cys) were detected in patients from central Italy.

Table 1
NOTCH3 mutations in 53 CADASIL index patients.

Exon	Nucleotide change	Amino acid change	EGF domain	No. of index cases	No. of mutation for exon (%)
3	c.268 C>T	p.R90C	2	3	4 (7.56%)
	c.322 T>A	p.C108S[a]	2	1	
4	c.397 C>T	p.R133C	3	2	15 (28.3%)
	c.421 C>T	p.R141C	3	3	
	c.436 T>C	p.C146R	3	4	
	c.505 C>T	p.R169C	4	1	
	c.521 G>A	p.C174Y	4	1	
	c.601 T>C	p.C201R	5	1	
	c.619 C>T	p.R207C	5	3	
5	c.751 T>C	p.C251R	6	1	1 (1.9%)
6	c.812 G>T	p.C271R	6	1	1 (1.9%)
7	c.1098 T>G	p.C366W	9	1	11 (20.75%)
	c.1187 C>G	p.S396C[a]	10	10	
10	c.1582 G>T	p.G528C	13	2	2 (3.77%)
11	c.1774 C>T	p.R592C	15	1	3 (5.66%)
	c.1816 T>C	p.C606R[a]	15	1	
	c.1819 C>T	p.R607C	15	1	
18	c.2815 T>C	p.C939R[a]	24	1	1 (1.9%)
19	c.3016 C>T	p.R1006C	26	12	13 (24.53%)
	c.3037 G>T	p.G1013C[a]	26	1	
22	c.3691 C>T	p.R1231C	31	2	2 (3.78%)

[a] Novel mutations.