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CASE REPORT

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An Italian case of CADASIL with mutation CGC-TCG in codon 1006, exon 19 *Notch3* gene

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Abstract Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is commonly overlooked or misdiagnosed owing to its recent identification. It is characterized clinically by recurrent cerebral infarcts, usually appearing between the ages of 30 and 50 years, subcortical dementia, and pseudobulbar palsy. It begins with migraine with aura in approximately one-third of patients. The pathological hallmark of angiopathy is the presence of characteristic granular osmiophilic material (GOM) within the basal lamina of smooth muscle cells. The defective gene in CADASIL is *Notch3*, which encodes a large transmembrane receptor, and 70% of missense mutations are in exons 3 and 4. Each gene defect leads to either a gain or loss of a cysteine residue in the extracellular N-terminal domain of the molecule. We report the case of a 53-year-old woman admitted to the hospital for transient ischemic attack and stroke-like episodes recurrent since age 43 years. The patient had pseudobulbar palsy, pyramidal signs, and cognitive impairment but not frank dementia. Cerebral MRI showed periventricular diffuse and confluent ischemic lesions. Ultrastructural study revealed an abnormal deposition of granular osmiophilic material (GOM) within the basal lamina in skin capillaries. Direct sequence analysis of the *Notch3* gene was performed. Since no mutation was detected in exons 3 and 4, the remaining exons were sequenced and a missense mutation, CGC-TGC in codon 1006 of exon 19 was found. The mutation led to a gain of a cysteine residue. This is the first missense mutation in codon 1006 of exon 19 of the *Notch3* gene to be described in Italy and the second reported in the literature.

Key words Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy • CADASIL • *Notch3* gene • Leukoencephalopathy

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Introduction

In 1955, Van Bogaert described two sisters with an autosomal, dominantly inherited form of arteriopathy characterized by an onset at 30–40 years of age with recurrent brain infarcts [1]. Thereafter, families with similar clinical pictures and pathological findings were described in the European literature [2, 3]. In 1991, Tournier-Lasserve et al. [4] described a large pedigree and defined the clinical, neuroimaging, and genetic characteristics of this disorder, now known by the acronym CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) [4, 5]. The disorder most commonly manifests as recurrent ischemic strokes, which occur at a mean age of 45 years. Subcortical dementia, associated with pseudobulbar palsy, is the second most common clinical manifestation of CADASIL. Detailed clinical analysis of 200 families showed that other commonly observed symptoms were attacks of migraine with aura (about 30%

of patients) and mood disorders (about 20%) [6–8]. All individuals with symptoms have prominent signal abnormalities on brain magnetic resonance imaging (MRI) suggestive of small infarcts within the deep white matter and basal ganglia. Ultrastructural examination of small cerebral arteries reveals abnormal patches of a granular osmiophilic material (GOM) within the basal membranes of vascular smooth-muscle cells [9]. The GOMs are a specific hallmark for the disease and are also observed in extracerebral arteries, including skin arterioles [10, 11].

In 1996, Jutel et al. [12] identified *Notch3* as the defective gene in CADASIL. Members of the *Notch3* gene family encode evolutionarily conserved transmembrane receptors and are involved in cell fate specification during embryonic development [13]. The *Notch3* gene includes 33 exons encoding a protein of 2321 amino acids. The missense mutations of *Notch3* gene are strongly stereotyped: all are predicted to replace the wild-type amino acid with a cysteine residue or to replace one of the six highly conserved cysteine residues with another amino acid. Jutel et al. [13] found strong evidence of mutation clustering within exons 3 and 4, which encode the first five epidermal growth factor (EGF) domains (seen in 70% of patients) [14].

Case report

A 53-year-old woman was admitted to the Division of Neurology, Santa Maria Nuova Hospital (Reggio Emilia, Italy) in 1999 for suspected chronic progressive multiple sclerosis. She had had a first episode of right hemiparesis at the age of 40 years, and in the years that followed she experienced recurrent stroke-like episodes. At the age of 51 years, the patient was admitted to the Department of Internal Medicine of a provincial hospital for suspected rheumatological disease with cerebral vasculitis (systemic lupus erythematosus). The patient presented a change in speech, described as "slurred speech", and difficulty in walking due to ataxia and intermittent dizziness. Blood tests showed positivity for anticardiolipin antibody and circulating immunocomplexes. However, these tests, which were repeated a number of times, were not significant on controls. Cerebral computed tomography (CT) at this time showed extensive abnormality of the hemispheric white matter, with leukoaraiosis and lacunar ischemic areas.

In 1997, due to repeated episodes of dysarthria and ataxia, she was hospitalized again in the Department of Internal Medicine and underwent temporal artery biopsy, which resulted normal. The patient was hospitalized again in 1998 for blurred vision episodes, subjective dizziness and worsened speech with clear residual dysarthria. She was sent to us by her attending physician in April 1999 for a cerebrospinal fluid examination and evoked potential studies for suspected multiple sclerosis.

A neurological examination in April 1999 revealed normal visual and extraocular functions, and normal visual field. Language was impaired with dysarthria. Mild right hemiparesis was present, as was hypertonia of the upper and lower limbs, pyramidal signs including extensor plantar response and exaggerated muscle stretch reflexes on the right. In addition, there was decreased vibratory and pin perception on the right side of the body, instability in a standing position and an enlarged base gait. Clinical laboratory studies yielding normal results included thyroid function tests, complete chemistry panel (blood count, blood glucose level, creatinine, uric acid, cholesterol, HDL, LDL, triglycerides), syphilis serology, antinuclear antibody titer, serum rheumatoid factor, anticardiolipin antibody titer, serum lactate, protein C, protein S, fibrinogen, antithrombin III, activated protein C resistance (factor V Leyden), serological analyses for Lyme disease, homocysteine level, serum vitamin B12 and folate levels. Erythrocyte sedimentation rate was mildly elevated in the first hour (24 mm/h; normal values, 0–20 mm/h). Cerebrospinal fluid (CSF) was acellular with normal protein and glucose concentrations. Tests for oligoclonal band production and immunoglobulin synthesis showed two oligoclonal bands both in CSF and serum.

Transthoracic echocardiography and ultrasound examination of the carotid and vertebral arteries were unremarkable. In addition, evoked potential studies using visual, somatosensory and auditory modalities were normal. Only an asymmetry of the P100 between the left (95.2) and right (98.8) was found but both were in the normal range. Electroretinographic evoked responses such as flash electroretinogram or pattern electroretinogram were not performed. All electrodiagnostic studies were normal.

The patient had no history of hypertension, diabetes, heart disease, migraine, or smoking. The patient's mother, who died at the age of 66 years, demented, had lost the ability to walk 6–7 years earlier and had begun to present repeated episodes of cerebral ischemia at the age of 43 years. Two maternal uncles with a similar clinical history died at age 60 years due to repeated strokes (Fig. 1*)*. For the moment, the patient's children, brother, and families of the two maternal uncles have not accepted to undergo testing, and no other family member is symptomatic.

Neuropsychological assessment showed normal performance on memory tests, sustained attention, and letter and category fluency. The patient has pathological performances of reasoning with abstract categories (such as color, shape and number), with functional fixity without preservation (Wisconsin Card Sorting Test), and pathological performances of the left-right orientation ability of own and examiner's body (Benton's Right-Left Test). The patient presented moderate levels of anxiety and depression. Behavioral scales (e.g. functional Instrumental Activities of Daily Living and intellectual correlates scale) showed global and good preservation both of everyday instrumental ability and individual self-government. The neuropsy-

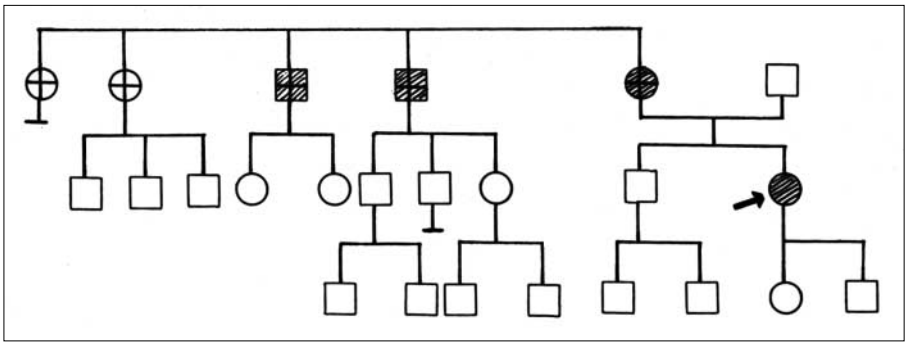

Fig. 1 Genealogy of an Italian woman with CADASIL (*arrow*). Symptomatic family members are shown in black

chological assessment, revealing visuospatial and executive control deficits associated with depression, was compatible with subcortical cognitive disorder.

Neuroradiological study

Magnetic resonance imaging (MRI) was performed using a 1.0 T scanner with acquisition of T1- and T2-weighted sequences. A first study, performed in April 1999, showed numerous partially confluent T2-hyperintense lesions involving the pons on the left side and the white matter of the centrum semiovale, corpus callosum, periventricular white matter, thalamus, basal ganglia, and external capsula on both sides. The lesions were predominantly distributed in the frontal and parietal lobes adjacent to the cella media and trigone of the lateral ventricles, and in the basal ganglia region; the occipital subcortical white matter and the white matter of the basal and orbital parts of the frontal lobe were completely spared (Fig. 2). On T1 sequences, lesions in the frontal and parietal periventricular white matter and in the basal ganglia regions, but not in the brainstem, were suggestive of tissue destruction. Lesions seen on T2-weighted images but not recognizable on T1 sequences were probably areas of edema, gliosis or demyelination.

A second study, performed 2 years later, in April 2001, revealed the appearance of a further lesion in the right parietal white matter, visible only on T2 sequences; other previous findings remained unchanged. During both studies, no lesion showed contrast enhancement after gadolinium administration.

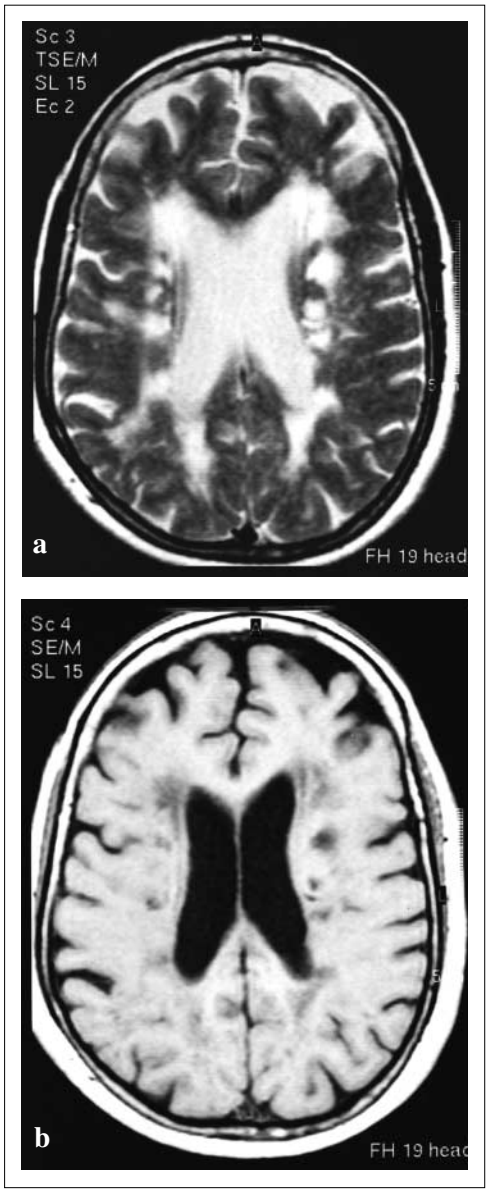

Fig. 2a,b *MR images at lateral ventricular level.* **a** T2-weighted image shows multiple partially confluent hyperintense lesions involving the white matter, mainly in the periventricular region. **b** T1-weighted image reveals part of the lesion, suggesting areas of tissue destruction

Histopathological characteristics

The dermal blood vessels did not reveal specific pathologic abnormalities. Vascular changes consisted of thickening and fibrosis of the wall of small- and medium-sized arteries. Fragmentation of the elastic lamina was also present but the most consistent alteration was the smudged granular degeneration within the intima associated with loss of smooth

muscle cells of the media. The eosinophilic granular material was slightly positive with periodic acid and Schiff's reagent (PAS). Sparse perivascular infiltration with lymphocytes and rare polymorphous leukocytes were observed.

The ultrastructural analysis revealed a marked destruction of the small arteries, sometimes with the complete disappearance of the tunica media. The endothelial cells appeared swollen, filling the vascular lumen: the cytoplasm contained an enlarged rough endoplasmic reticulum. The basal lamina was focally thickened or reduplicated but intercellular tight junctions were unaffected. The elastic lamellae showed marked fragmentation associated with the presence of many small calcified spherules. Some extracellular granulofilamentous electron-dense deposits were observed, especially embedded in vacuoles in the basal lamina-like material. These deposits seemed to modify the smooth muscle cell profile.

Genetic study

Genomic DNA was extracted from buffy coat previously prepared from whole peripheral blood after the patient gave informed consent. Polymerase chain reaction (PCR) amplification was performed with primers specific for exons of the *Notch3* gene. Because most previously described mutations occurred in exons 3 and 4, these regions were screened first. When no mutations were detected in these exons, we designed primer pairs (Table 1) comprising the intron-exon boundaries and sequenced the remaining exons. We screened the exons in which mutations were previously reported first.

Genomic DNA (500–600 ng) was used in a 50 µl reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM of $MgCl_2$, 200 µM of dNTPs, 24 pmol each primer, and 1.5 U Taq DNA polymerase (storage buffer A; Promega, Madison, USA). The samples were amplified on a DNA Thermal Cycler (model 9600; Perkin Elmer, Foster City, USA). Exons 3 and 4 were amplified for 35 cycles of 30 seconds at 94° C, 30 seconds at 66° C and 40 seconds at 72° C. All the other exons were amplified for 30 cycles of 30 seconds at 94° C, 45 seconds at 60° C and 1 minute at 72° C.

Table 1 The sequence of PCR products of *Notch3* gene, in forward and reverse directions

CAD2F	5'-GGCTGATCCTCCACCTTCCTTC-3'
CAD2R	5'-GCCAGGCACTCACAGGCAGGCA-3'
CAD3F	5′-CAAGCCATCTCTGCCCACAG-3′
CAD3R	5′-CAGACTCTTCCCCTCTCACC-3′
CAD4F	5′-AAACTCACCCTGTCCTGGTC-3′
CAD4R	5′-TCCTGAGTAGGGCTCACTCA-3′
CAD5F	5'-CGGTGACCATCCTTGCCC-3'
CAD5R	5'-TGCCTCCCCGCTCCCTCT-3'
CAD6F	5'-GTGGCTGGACTGCTGCATCTGT-3'
CAD6R	5'-AAACGGCCACTCACCAGTCTTG-3'
CAD11F	5'-CAGGCCCCTGGCAAGTGG-3'
CAD11R	5'-CACCATTCCCAAACCCTCTGTG-3'
CAD14F	5'-TTCCCCTGCCAGGTTCCG-3'
CAD14R	5'-GGGGCTGCAGAGGGAAGGTGAG-3'
CAD18F	5'-GATCCTCCCTCCCACTCCTTCC-3'
CAD18R	5'-GTCCCCAGTAACTCCACCCACC-3'
CAD19F	5'-TGACTCTGAGTGCTTCCCCTCC-3'
CAD19R	5'-CACGCCCGCCCACATGCTCCCA-3'

The PCR products from exons 3 and 4 were sequenced in both forward and reverse directions on a Beckman Coulter CEQ2000XL DNA Analysis System using the CEQ DTCS Quick Start Kit (Beckman Coulter). Results were confirmed on a second independent DNA sample and compared with the sequence from a healthy control and with the reference sequence (U97669, AF058883). The other PCR products were sequenced using the forward primers with ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster City, USA) (Table 1). The sequences of the remaining exons were determined, and a missense mutation, CGC-TGC in codon 1006 of exon 19, was observed. This mutation, which led to the gain of a cysteine residue, had been described previously only in one patient [14].

Discussion

CADASIL is a poorly known pathology and therefore its prevalence is probably underestimated due to diagnostic difficulties. There are no incidence or prevalence rates for the disease. It is thus essential that diagnostic cases be described.

New diagnostic possibilities come from molecular genetics. The missense mutations of CADASIL are strongly stereotyped: they were located in exons 3 or 4 in 32 of Jutel et al.'s 45 patients [14] and in 8 of Oberstein et al.'s 11 patients [15]. These mutations are all located within the EGF-like repeats in the extracellular domain and all are predicted to replace a wild-type amino acid with a cysteine residue or to replace one of the six highly conserved cysteines with another amino acid. The molecular diagnostics strategy has been successful in 90% of patients and may benefit patients with clinical and MRI features consistent with a diagnosis of CADASIL, even in the absence of a strong family history and in pre-symptomatic patients.

In the British CADASIL study [16] of 83 potential cases, 48 families were identified with fifteen different point mutations: 73% in exon 4, 8% in exon 3, and 6% in of exons 5 and 6 each. Moderate or severe involvement of the anterior temporal pole on MRI had a sensitivity of 89% and specificity of 86% for diagnosis [16]. However, these patients were less than 60 years of age with recurrent lacunar stroke with leukoaraiosis on neuroimaging. Migraine, psychiatric disorders, dementia or family history could occur but were not essential to the diagnosis. In another study, genetic testing of the same exons was performed on patients with simple cerebral infarcts; in this case a single mutation in exon 4 was found among the 218 consecutive cases studied [17].

The electron microscopic demonstration of GOM within the basal lamina of vascular smooth-muscle cells in arterioles is specific for the diagnosis of CADASIL as they are present in 100% of symptomatic patients but not in presymptomatic patients, according to a French group [18]. But in the British study [16], skin biopsy had a sensitivity of 45% and a specificity of 100%. A genetic study cannot, therefore, be substituted by a histological study, especially in patients with initial symptoms. In addition, in patients for whom GOMs have been demonstrated by skin biopsy but who lack a missense mutation in exon 3 or 4 of the *Notch3* gene, it is important to carry out a direct sequence analysis of other *Notch3* gene exons in order to further our knowledge of the gene and its pathologic mutations.

Immunohistochemistry studies have recently demonstrated that the immunoreactive material that accumulates in CADASIL brains is identical to *Notch3* cleavage product. The mutant *Notch3* ectodomain probably undergoes an improper oligomerization aided by the odd number of cysteine residues, leading to its accumulation in microvessel walls. It has thus been definitively demonstrated that the material accumulated on vessels walls, making up the GOMs, derives from the accumulation of the extracellular part of the receptor produced by the *Notch3* gene. We hope that these studies will soon result in the possibility of diagnosing the disease by means of immunohistochemical methods. Unfortunately, semiquantative analysis of *Notch3* gene product using immunocoloring techniques on skin biopsies does not have a sensitivity greater than 90% [19].

In clinically and neuroradiologically selected cases, the best diagnostic method may be the genetic study of exons 3, 4, 5, 6, 11 and 19, followed by a skin biopsy in those cases who are negative on genetic testing. Genetic testing is not economically feasible in cases where there is doubt, without familiarity, and with simple phenotypes and atypical neuroradiological characteristics, given the limited possibility of identifying cases of CADASIL in this manner [20]. While a positive skin biopsy is considered sufficient to make a diagnosis, it is wise to proceed to genetic testing on the remaining exons in the gene in order to gather the most data possible on the frequency of the mutations and their phenotypes. This would allow us to assess the existence of a possible relationship between genotype and phenotype, even though the data to date do not demonstrate this relationship. The previous case described in the literature of a CGC-TGC mutation in codon 1006 of exon 19 was reported in a genetic study of 50 patients; as there was no phenotype description, it is likely that this was a case that presented typically [14]. Our patient's clinical characteristics are essentially typical of CADASIL, as are the lesions shown on MRI. However, the patient did not have migraine, which is present in only 20%–48% of documented cases (when present, migraine is often the first symptom of disease onset). The patient's modest cognitive impairment, in spite of her age, is not common. A family history of repeated stroke-like episodes with onset at a young age and dementia around the age of 60 years are important to directing diagnostic study [8, 10]. In addition, the current patient had repeated episodes of blurred vision at the onset of symptoms, with no alteration of evoked potentials; these characteristics are typical of the disease and explainable by vascular retinal involvement at onset [21].

The description of this clinical case adds to the Italian case histories and underlines the importance of CADASAL even in cases of multilacunar vasculopathy in only one of the parents. In addition, this case allows us to gather data on the genetics of CADASIL in Italy. We stress the importance of a complete study of the *Notch3* gene in those cases where there is no mutation of exons 2 and 3.

To date, only ten Italian families with CADASIL have been described and, of these, only two have undergone a genetic study for missense mutations by sequential analysis of the *Notch3* gene. These two studies have shown a classic mutation (C635T) in exon 4 in the family with only migraine described by Ceroni et al. [15] and a new mutation (C332T) in exon 6 described by Mazzei et al. [22].

The patient we have described is the third case ever to be published with a deletion TCG-CGC in codon 1006, exon 19, and the second with a mutation in codon 1006 of this exon. The case is the first in Italy with this deletion and with the involvement of this exon. Genetic studies in families with CADASIL from the Central and Southern Italy are underway [23, 24].

Sommario *Si pensa che il CADASIL sia una patologia sottostimata e non diagnosticata, per la sua recente identificazione, ma che vede un interesse clinico e di ricerca crescenti. È caratterizzata clinicamente da infarti cerebrali lacunari ricorrenti, che tipicamente insorgono all'età di 30–50 anni, da deterioramento cognitivo di tipo sottocorticale, che può anche essere tardivo, e da sintomi tipici per paralisi pseudobulabare. In circa un terzo dei casi è presente emicrania vasomotoria, tipicamente con aura visiva. La caratteristica istologica della malattia consiste nell'accumulo di GOM (*granular osmiophilic material*) nella lamina basale delle cellule muscolari dei capillari cerebrali. Il gene coinvolto nella patologia è il* Notch3*, che codifica un recettore transmembrana. Gli esoni più frequentemente portatori di mutazione sono il 3 e il 4, che da soli identificano circa il 70%–80% dei pazienti. Riportiamo il caso di una paziente di 53 anni, ricoverata per TIA ed episodi ripetuti di stroke, iniziati all'età di 43 anni. La paziente al momento della diagnosi presentava segni clinici di paralisi pseudobulbare, segni piramidali, e deficit cognitivo, ma non demenza conclamata. La RMN dimostrava lesioni ischemiche periventricolari confluenti. Lo studio ultrastrutturale della biopsia di cute aveva confermato il sospetto diagnostico dimostrando la presenza di GOM. È stata eseguito una analisi sequenzia-*

le del gene Notch3*, ma non sono state trovate mutazioni a carico degli esoni 3 e 4. Per questo, vista la positività della biopsia di cute, si è proceduto allo studio dei restanti esoni del gene e si è trovata una mutazione non senso, CGC-TGC, nel codone 1006 dell'esone 19 caratterizzata dal guadagno di una cisteina. Si tratta della seconda mutazione segnalata a carico dell'esone 19 nel codone 1006 descritta in letteratura e la prima in Italia.*

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