Arg133Cys Mutation of *Notch3* in Two Unrelated Japanese Families with CADASIL

Eiichiro UYAMA, Makoto TOKUNAGA, Akihito SUENAGA*, Satoru KOTORII**, Kohei KAMIMURA**,
Keikichi TAKAHASHI**, Takeshi TABIRA** and Makoto UCHINO

Abstract

Objective More than 80 unrelated, but all Caucasian, patients with cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), originating from various communities around the world, have been molecularly identified. To clarify the occurrence of CADASIL in Orientals, we investigated Japanese families presenting as CADASIL.

Methods We performed the PCR-SSCP and sequence analyses using genomic DNA, isolated from venous blood of participants under informed consent.

Patients We identified two unrelated Japanese families with CADASIL, including 5 affected members through 2 generations.

Results Each of the affected individuals developed recurrent strokes without risk factors resulting in progressive dementia, pseudobulbar palsy, and gait disturbances which started after the fifth decade of life. Although affected individuals had no vascular risk factors, they showed various degrees of narrowing of retinal arteries. Their MRI/CTs showed characteristics of the disease; bilateral small infarcts in the thalamus, basal ganglia, brain stem, and deep white matter in addition to the findings of leukoaraiosis. On SPECT imaging, there was severe hypoperfusion in the cortex as well as in the white matter. Ultrastructural studies revealed an abnormal deposition of granular osmiophilic materials (GOM) within the basal lamina of pericytes in muscular capillaries. On PCR-SSCP and sequence analyses, a heterozygous Arg133Cys mutation was present, in the affected individuals, in the exon 4 of *Notch3* gene which is the hot spot region for CADASIL mutations in Caucasian families. None of the non-affected members nor the 50 Japanese normal controls revealed this mutation.

Conclusion Thus, our results confirm that CADASIL is a geographically widespread disorder caused by a *Notch3* mutation.

(Internal Medicine 39: 732–737, 2000)

Key words: hereditary vascular dementia, leukoaraiosis, multiple cerebral infarction, migraine, granular osmiophilic materials

Introduction

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL: OMIM 125310) occurs in mid-adulthood as a result of mutations of the *Notch3* gene on chromosome 19p13.2-p13.1 (1). Initial symptoms include migraine with aura or mood disorders, and recurrent subcortical infarcts without vascular risk factors are common in the course of the disease (2, 3). Pathologically, the fundamental lesion consists of a marked thickening of the media of small muscular and leptomeningeal cerebral arteries. Abnormal patches of a granular osmiophilic material (GOM) within the basal membranes of vascular smooth-muscle cells are a specific hallmark (4). The nature of systemic involvement allows the presence of GOM in the muscle (4, 5), sural nerve (6), and skin (4, 5), but is less evident than that in cerebral small arteries. There have been at least 80 unrelated CADASIL patients with *Notch3* mutations identified from various communities around the world (7–12), however all were Caucasian. The occurrence of molecularly-confirmed CADASIL in other races has not been documented.

We identified two unrelated Japanese families with CADASIL and sharing the same *Notch3* mutation. Related data on clinicopathologic and genetic investigations are described herein.

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Patients and Methods

We identified two affected families living in well separated

From the Department of Neurology, Kumamoto University School of Medicine, Kumamoto, *the Department of Internal Medicine, Kitakyushu City Yahata Hospital, Kitakyushu and **the Department of Demyelinating Disease and Aging, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo

Received for publication October 4, 1999; Accepted for publication January 31, 2000

Reprint requests should be addressed to Dr. Eiichiro Uyama, the Department of Neurology, Kumamoto University School of Medicine, 1-1-1 Honjo, Kumamoto 860-0811

Family 1

Family 2

Figure 1. Family pedigrees of Family 1 (Patients 1 and 2) and Family 2 (Patient 3). Solid black symbols indicate affected persons. A line through a symbol means a deceased subject.

areas: Family 1 in Kumamoto Prefecture (Southern Japan) and Family 2 in Kanagawa Prefecture (Central Japan).

Each member of the two families descended from unrelated Japanese ancestors who had no relationship with Caucasians, and there were no consanguineous marriages. Family 1 included three affected members (2 men, 1 woman, mean age 58 years) through two generations (Fig. 1). The father of Patient 1 (son, the proband) and Patient 2 (daughter) died of aspiration pneumonia at age 57, following recurrent infarctions which had begun when he was 40 years of age. Family 2 included two affected members (Fig. 1). The mother of Patient 3 (the proband) had a history of recurrent infarctions from the age of 41 and died 10 years later. Informed consent was obtained from family members for all studies, including muscle biopsy and DNA analysis.

Patient 1

A 53-year-old man, the proband of Family 1, had difficulty in seeing clearly at age 38. Bilaterally, his visual acuity had decreased to 0.5 without defect of visual field. Several years later, both intelligence and physical movement deteriorated in a stepwise progression. He did not have a history of migraines, but he had had recurrent generalized convulsions since age 48. Head CT showed multiple low density lesions in the deep white matter. By age 51, recurrent subcortical infarctions led to dementia. On admission, he was in an akinetic mutism-like state, and he had severe pseudobulbar palsy, increased deep tendon reflexes in the limbs, Babinski signs, and frontal lobe signs.

Laboratory studies included screening antibodies to DNA and nuclear soluble proteins, detailed analyses of coagulation and hemostasis, ECG, cardiac echogram, and ultrasonographic evaluation for intra- and extra-cerebral vessels were normal. Clear CSF contained protein, 120 mg/dl and IgG, 15.4 mg/dl (12.8%) under a pressure of 200 mmH₂O. T2-weighted MRI showed diffuse brain atrophy, multiple small high-intensity lesions in the thalamus, basal ganglia and deep white matter, and marked leukoariosis (Fig. 2A, B). No gadolinium-enhancing lesions were observed. SPECT imaging using ^{99m}Tc-ethylcysteinate dimer tracer indicated severe hypoperfusion in the cortex as well as in the white matter. Repeated EEG in the post-ictal state showed diffuse slow background activity without paroxysmal waves. Four-vessel angiography showed no abnormal findings. On ophthalmologic evaluation, entirely narrowed retinal arteries were revealed along with pale optic discs with sharp margins in the optic fundi (Fig. 3). Neither occlusion of capillaries nor hemorrhage were disclosed. His poor general state in advanced stage disturbed the performance of fluorescein angiography. Intraocular pressure and slit-lamp examinations were normal.

Patient 2

A 64-year-old woman, an elder sister of Patient 1, was normal until 13 years before the first examination, when her family noticed that she had difficulty using her right hand and walking at a normal speed. From that time on, she had several stroke-like episodes, and she became socially withdrawn. During the clinical course, she had no epileptic seizures or attacks of migraine with aura. On examination, she showed moderate mental deterioration without aphasia or agnosia, pseudobulbar palsy, asymmetric tetraplegia, and bilateral pyramidal tract signs. Although she had no apparent vascular risk factors, her brain CT showed asymmetric small infarctions in the thalamus, putamen, and central semi-ovale accompanied by diffuse low density lesions in the cerebral white matter. The degree of brain atrophy was less evident than in Patient 1. Ophthalmologic evaluations revealed a slight narrowing of the retinal arteries, and moderately decreased visual acuity, but with her visual field was spared.

Patient 3

A 47-year-old woman, the proband of Family 2, had a sudden onset of hemiparesis in the left side with an unsteady gait at age 42. She went on to experience stroke-like episodes approximately once every year, even though she had no risk factors such as hypertension, diabetes mellitus, or hyperlipidemia. Her difficulty in walking had worsened in a stepwise progression, which resulted in her hospitalization. By age 47, she had had neither a history of migraines nor seizure attacks. On examination, she revealed moderate memory impairment with infrequent emotional incontinence, hemiparesis in the left side, pyramidal tract signs in all limbs, and marked truncal ataxia. T2-weighted brain MRI disclosed multiple high intensity lesions in the basal ganglia, thalamic nuclei, pons, and white matter in addition to the pattern of leukoariosis (Fig. 2C, D).

Figure 2. Brain MRI in Patient 1 (A, B) and Patient 3 (C, D). A) T1-weighted imaging shows diffuse brain atrophy and multiple low intensity lesions in bilateral thalamus, basal ganglia, and periventricular white matter in the frontal lobe. B) T2-weighted imaging reveals diffuse high intensity lesions in the white matter. C) T2-weighted imaging shows multiple high intensity lesions in the thalamus, basal ganglia, and white matter. D) Flair-weighted imaging reveals marked leukoaraiosis mixed with multiple small low intensity lesions.

Figure 3. Fundus photograph of the right eye in Patient 1. Marked narrowing of retinal arteries (small arrows) (ratio of diameter, artery : vein = 1 : 2~4, normal, 2 : 3).

Her ophthalmologic examination showed moderately narrowed retinal arteries and slightly pale optic discs with sharp margins in the optic fundi. Fluorescein angiography showed nothing particular changes.

Morphological study

Serial frozen sections from specimens of biopsied biceps muscle in Patient 1 were stained with hematoxylin-eosin, modified Gomori trichrome and other currently used histochemical stains. For the electron microscopic studies, the specimens were fixed in 2.5% glutaraldehyde and post-fixed in 2% osmium tetroxide, embedded in Epon and processed according to standard techniques.

Mutational Analysis

Genomic DNA was extracted from blood leukocytes. In the PCR-SSCP and sequence analyses, intronic primer pairs (S.K. et al., unpublished data) were used to amplify exons 2 to 33 except for 26 of the *Notch3* gene. The PCR products of exons

3–5 were directly cloned into the pUC vector and sequenced by a BcaBest sequencing kit (TaKaRa, Osaka) using α -[^{32}P]-dCTP. The PCR products of the remaining exons were heat denaturated in a solution and analyzed on a GenePhor electrophoresis system (Pharmacia Biotech, Uppsala, Sweden) using GeneGel Excel precast polyacrylamide gels. Exon 4 was amplified using sense primer: 5'-CTAAACTCACCCCTGTCCTGG-3' and antisense primer: 5'-GAGCCAGG-TGTGTTGAGGCAG-3'. Reactions were carried out in 10 μl of mixture with 5 pmoles of ^{32}P labeled primers and 0.25 units of Taq DNA polymerase at 94°C for 60 seconds, 65°C for 40 seconds and 72°C for 50 seconds for 30 cycles.

Results

The clinical features of Patients 1, 2, and 3 are summarized in Table 1. Light microscopic findings of biopsied muscle specimens revealed non-specific changes such as a mild variation in fiber size and some thickening of walls of arteriole, but without PAS or Congo-red reactions. Ragged-red fibers and rimmed vacuoles were absent. Ultrastructural studies disclosed an abnormal deposition of GOM within the basal lamina of pericytes of muscular capillaries (Fig. 4).

On SSCP analysis, PCR products of exon 4 of *Notch3* in each of the affected individuals displayed two different mobility shifts (Fig. 5). On DNA sequence analysis, we identified the same Arg133Cys mutation caused by a C→T transition at nucleotide position 406 as noted in Caucasian families (Fig. 6), but this mutation could not be detected in 5 unaffected mem-

bers of these families and also in the 50 Japanese normal controls. There were no additional mutations in other exons. This mis-sense mutation which created a new cysteine residue was heterozygous and co-segregated with the disease, indicating that the *Notch3* gene is also responsible for CADASIL in our Japanese patients.

Table 1. Summary of the Clinical Features in Japanese CADASIL Patients with Arg133Cys Missense Mutation in *Notch3*

	Family 1		Family 2
	Patient 1	Patient 2	Patient 3
Age when examined (yrs)	53	64	47
Age at onset (yrs)	38	51	42
Sex	M	F	F
Blood pressure (mmHg)	145/85	100/60	132/80
Migraine	—	—	—
Epileptic seizures	++	—	—
Dementia	+++	++	+
Frontal signs	++	+	+
Pseudobulbar palsy	++	+	±
Gait disturbance	+++	++	++
Narrowing of retinal arteries	+++	+	++

M: male, F: female, +++: severe, ++: moderate, +: slight, —: negative.

Figure 4. Electron micrograph of a capillary in the biceps muscle from Patient 1. Abnormal deposition of granular osmiophilic materials within the basement membrane of pericyte is shown (arrows) (original magnification, $\times 1,200$, bar = 1 μm). P: pericyte, L: lumen, E: erythrocyte.

Figure 5. SSCP analysis using genomic DNA for exon 4 of *Notch3*. There are two different mobility shifts in Patient 1 (lane 2) and Patient 3 (lane 3). lane 1: normal control, lane 4: healthy father of Patient 3.

Discussion

The diagnosis of CADASIL in our Japanese family was based on an autosomal dominant inheritance with middle-aged-onset recurrent strokes, stepwise progression of dementia, the pattern of leukoaraiosis on MRI/CT, and the presence of GOM in basal membranes of vascular smooth-muscle cells in arterioles in skeletal muscle. Considering the clinical features, the present Japanese patients did not have attacks of migraine with aura, which was frequently present in the Caucasian patients as the clinical onset (2, 13, 14). Although the presence of epileptic seizures has been documented as a rare neurologic manifestation in French families (2), it was one of the major features in the probands of our Japanese families, as also seen in an Italian family (15). The frequency of seizures in 102 German patients with CADASIL was estimated as 10% (3). Furthermore, cerebral blood flow was markedly decreased not only in the subcortex regions, but also in the cortex, as seen on SPECT imaging. This finding corroborates the result of PET (16) and SPECT (17) studies in demented patients with CADASIL. This evidence lead to the speculation that epileptic seizures may not be so rare a feature in CADASIL.

The electron microscopic demonstration of GOM within the basal lamina of vascular smooth-muscle cells in arterioles is specific for the diagnosis of CADASIL (4, 6, 18–22). As shown in our demonstration, GOM was also present within the basement membrane of pericyte in the capillaries. A recent review of the pathology indicates that the deposition of GOM is usually prominent in tissues with a continuous endothelium with tight junction, such as in skeletal muscle (4). Especially, the involvement of vascular smooth-muscle cells is notable in tissues such as brain, optic nerve, and retina, which are particularly composed of blood-brain barrier endothelium (4). These

Figure 6. Nucleotide sequences of exon 4 from Patient 3 and normal control. The sense strand sequence of the mutant and normal allele are shown.

morphologic aspects entertain the possibility of the occurrence of retinal arteriopathy during the course of CADASIL, as was shown in the present patients. The negative results of fluorescein angiography in our case and the lack of similar cases in previous reports, indicate other possibilities of secondary effects by factors such as overnight blood pressure. Further ophthalmologic evaluations in large numbers of patients with CADASIL seem to be necessary.

As previously confirmed in the European CADASIL families (7, 8, 10, 11), we also identified a single mis-sense mutation within exon 4 of the *Notch3* gene, which is the hot spot region for CADASIL mutations (8, 10, 11). This Arg133Cys mutation has been identified in three unrelated families in a French study, one family in a Dutch study and 10 families including 80 patients in Finland (12), and it has led to an unpaired number of cystein residues probably resulting in the interruption of *Notch3* signaling. To our knowledge, the present patients represent the first fully documented cases of CADASIL in Japanese with *Notch3* mutations. Concerning the origin of our Japanese CADASIL, there are at least two possibilities: it spread from a European founder by way of the many Westerners who came to Japan from the 16th century onward; or it occurred independently by a mutation of *Notch3*. The fact of the negative relationship between our two unrelated Japanese families and the non-Japanese communities on a historical investigation, indicates the possibility of the latter. However, in a previously performed haplotype analysis of Caucasian families with recurrent *Notch3* mutations (8), two unrelated families with Arg133Cys mutation of *Notch3* share the same haplotype. Furthermore, all 10 CADASIL families in Finland share the same Arg133Cys mutation due to a founder effect from a common ancestor from at least the early 1,700s (12). To confirm the hypothesis, that the occurrence of an independent *Notch3* mutation in CADASIL with Arg133Cys mutation origi-

nated in different communities, haplotype analysis of the affected families should be carried out.

In conclusion, our results confirm that CADASIL is a geographically widespread disorder caused by mutation of *Notch3* gene.

Acknowledgements: We thank Drs. Satoshi Yamashita and Yasuhiro Murata for ophthalmologic evaluation, and Drs. Yoichiro Hashimoto, Masayuki Nakao, and Shoichi Sasaki for providing useful patient information.

This work was supported in part by grant from the Science and Technology Agency of Japan (COE).

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