

SCA14 (Chen et al. 2003), TATA-binding protein for SCA17 (Koide et al. 1999), and FGF14 for ADCA with FGF14 mutation (van Swieten et al. 2003). Puratrophin-1 is the first known protein related to intracellular signaling and cytoskeleton that is associated with ADCA. Our preliminary results suggest that the frequency of chromosome 16q22.1-linked ADCA is relatively high, since it was ranked the third most frequent ADCA in Japan, after MJD/SCA3 and SCA6. Since chromosome 16q22.1-linked ADCA had a strong founder effect, it will be important to clarify whether this specific C→T change is seen in other ethnic groups. Particularly, it is extremely important to clarify whether original patients with SCA4 reported from North American and German populations harbor mutation within the *puratrophin-1* gene (Flanigan et al. 1996; Hellenbroich et al. 2003).

A heterozygous, single-nucleotide substitution in the 5' UTR that is associated with aggregation of the gene product is a unique feature as a cause of human disease. Generally, a single-nucleotide substitution in the 5' UTR may result in expression of aberrant mRNA, abnormal trafficking of mutant transcripts due to conformational changes, or reduced transcription of mRNA, as in  $\beta$  thalassemia intermedia (Sgourou et al. 2004). In the present study, no aberrant transcripts were cloned from patient brains. Instead, the result from the in vitro study suggested that the transcription efficiency could be reduced significantly by the C→T substitution in the 5' UTR of the *puratrophin-1* gene. Consistent with this in vitro finding, levels of *puratrophin-1* mRNA tended to be lower in cerebella of patients with 16q22.1-linked ADCA than in those of AD-affected controls. Further studies comprising a larger number of samples that include "neurologically normal" controls will be necessary to confirm that reduced *puratrophin-1* mRNA expression is associated with the C→T change in the 5' UTR. Although aggregations of mutated proteins in most neurodegenerative diseases are due to conformational changes produced by amino acid alterations, reduced protein expression may also cause protein aggregation, such as the neurofilament light-chain aggregation in anterior horn cells of patients with amyotrophic lateral sclerosis (ALS) (Bergeron et al. 1994). It is currently not known how the C→T change in the *puratrophin-1* gene leads to puratrophin-1 aggregation. However, it is possible that alteration in the stoichiometry or changes in the protein stability, observed in neurofilament proteins in ALS (Ge et al. 2003), also exist for puratrophin-1 in chromosome 16q22.1-linked ADCA. In addition, it should be noted that since reduction of *puratrophin-1* mRNA levels in patient cerebella was small, that may not be a sufficient explanation of the formation of aggregation. It will be important to assess the *puratrophin-1* mRNA levels specifically in Purkinje cells, where the protein aggregates.

Puratrophin-1 is a novel protein with four important domains: the spectrin repeat, CRAL/TRIO, Rho GEF/DH, and PH domains. Therefore, the *puratrophin-1* gene would be the fifth gene that encodes Rho GEF protein to be associated with human disease, after the *FGD1GEF* gene for faciogenital dysplasia (Pasteris et al. 1994), the *ARHGEF6* gene for X-linked nonsyndromic mental retardation (Kutsche et al. 2000), the *ARHGEF10* gene for autosomal dominant neuropathy with thin myelinated fibers (Verhoeven et al. 2003), and the *ALS2* gene for the autosomal recessive juvenile motor neuron disease ALS2 (Hadano et al. 2001; Yang et al. 2001). GEFs exert diverse functions in intracellular-membrane trafficking and microtubule dynamics and ultimately regulate numerous cellular responses—such as proliferation, differentiation, and movement—by activating small G-protein GTPases (Rossmann et al. 2005). Although Rho GEF/DH and PH domains are typical for Rho GEF proteins, the presence of a spectrin repeat suggests that puratrophin-1 could be targeted to the Golgi-apparatus membrane, where it may regulate certain membrane dynamics through modulating actin (Godi et al. 1998; Lemmon et al. 2002). The CRAL/TRIO domain is implicated in the nerve growth factor (NGF) pathway that leads to neurite outgrowth through activating Rho G (Estrach et al. 2002), which indicates that puratrophin-1 may also have a role in cell differentiation signaling through NGF. In the present study, we showed that the Golgi-apparatus membrane protein and spectrin both form aggregation in the Purkinje cells of patient brains, which strongly supports the hypothesis that puratrophin-1 indeed interacts with these essential cytoskeletons. Formation of these aggregations, which seems to be a new phenomenon implicated in pathogenesis of human disease, could be deleterious for cells, since Golgi-apparatus membrane proteins and spectrin are important in maintaining cellular architecture, as noted for autosomal dominant polycystic kidney diseases (Charron et al. 2000). In addition, it also seems rational to speculate that disturbance of puratrophin-1 may affect Purkinje-cell morphology and eventually cause the peculiar Purkinje-cell atrophy that characterizes chromosome 16q22.1-linked ADCA, since Rho GEFs are implicated particularly in neural morphogenesis and connectivity by regulating actin dynamics (Godi et al. 1998).

Finally, the association of progressive hearing impairment in patients with chromosome 16q22.1-linked ADCA may suggest a role of puratrophin-1 in hearing. As far as we were able to examine by auditory tests, 42.8% of study families had hearing impairment. Audiometric configurations showing mid-frequency U-shaped or flat-shaped pattern also suggested that the hearing impairment of these patients was not a simple age-related hearing loss. However, it should be noted

that some families had moderate hearing impairment, whereas other families had only mild hearing impairment, which may indicate the presence of other modifying genetic factors for this phenotype. Further clinical analyses with use of detailed auditory tests are needed to ascertain how strongly hearing impairment is associated with ataxia. If hearing impairment is confirmed as being complicated by chromosome 16q22.1–linked ADCA, puratrophin-1 will be the first deafness/hearing-impairment protein related to Rho GEF. Since mutations in the  $\gamma$ -actin gene (*ACTG1*; DFN20/26) (Zhu et al. 2003), genes coding proteins that interact with actin (e.g., the myosins *espin* and *harmonin*), or a gene coding proteins that coordinate actin polymerization (Mburu et al. 2003) are known to cause deafness, it is possible that puratrophin-1 disruption causes hearing impairment by disturbing actin dynamics in the cochlea. In support of this hypothesis, expression of puratrophin-1 was seen in mouse cochlear hair cells, in which actin is also expressed (Mburu et al. 2003). Rho GTPases are also important regulators of actin cytoskeleton in stereocilia development, which is crucial for auditory transduction (Kollmar 2001). We hypothesize that the *puratrophin-1* gene mutation ultimately causes hearing impairment by dysregulation of actin in the cochlea. Further studies, such as targeted disruption of puratrophin-1 in mouse, will be important for clarifying whether puratrophin-1 has a role in hearing.

In summary, we have identified that a single-nucleotide C→T substitution in the 5' UTR of the gene *puratrophin-1* is strongly associated with chromosome 16q22.1–linked ADCA. If patients with SCA4 are found to harbor mutations in this gene, it would suggest that the chromosome 16q22.1–linked ADCA in Japan is allelic with SCA4. Identification of the mechanism of puratrophin-1 expression, the upstream signaling cascade that activates puratrophin-1, and the actual Rho GTPase activated by puratrophin-1 would be the next key steps for understanding the molecular mechanisms that underlie cellular degeneration of chromosome 16q22.1–linked ADCA and hearing impairment.

## Acknowledgments

We thank the doctors who participated in this work by recruiting families with chromosome 16q22.1–linked ADCA: Drs. Hidenao Sasaki, Department of Neurology, Graduate School, Hokkaido University; Masashi Aoki, Department of Neurology, Graduate School, Tohoku University; Yoshihisa Takiyama, Department of Neurology, Jichi Medical School; Kazuo Yoshizawa, National Mito Hospital; Kazuko Mitani and Yu-ichi Fumimura, Department of Neurology, Tokyo Metropolitan Geriatric Hospital; Hirohiko Murakami, Department of Neurology, Tokyo Women's Medical School; Satoshi Orimo, Department of Neurology, Kanto Central Hospital; Sou-

chiro Mochio, Department of Neurology, Jikei Medical School, The Third Hospital; Kunihiro Yoshida, Department of Clinical Genetics, Shinshu University; Isao Sahashi, Fourth Department of Internal Medicine, Aichi Medical University; Masanori Nakagawa, Department of Neurology, Kyoto Prefectural Medical College; Akihumi Goto, Department of Neurology, Nagasaki Medical Center of Neurology; Hideki Kida, Kida Hospital; Eiichiro Uyama, Department of Neurology, Kumamoto University of Medicine; Jun Goto and Shoji Tsuji, Department of Neurology, Graduate School, University of Tokyo; and Miho Murata and Ichiro Kanazawa, National Center of Neurology and Psychiatry, Ministry of Health, Labor and Welfare. For human and mouse control specimens, we also thank Professors Morio Koike, Department of Pathology, and Ken Kitamura, Department of Audio-Vestibular Neuroscience, Graduate School, Tokyo Medical and Dental University. We thank Ms. Iku Sudo and Ms. Minori Kono for technical assistance. We deeply acknowledge Professors Ichiro Kanazawa, Director of National Center of Neurology and Psychiatry, Ministry of Health, Labor and Welfare, and Shoji Tsuji, Department of Neurology, Graduate School, University of Tokyo, for supporting this study. This study was supported by Grant-in-Aids for Scientific Research on Priority Areas—Advanced Brain Science Project—from Ministry of Education, Culture, Sports, Science and Technology, Japan (to K.I. and H.M.), as well as grants from Research on Intractable Disorders (to H.M.) and from Human Genome and Regenerative Medicine, Ministry of Health, Labor and Welfare, Japan (to K.I. and H.M.).

## Web Resources

Accession numbers and URLs for data presented herein are as follows:

- Celera Discovery System, <http://www.celeraDiscoverySystem.com/index.cfm> (for candidate genes)
- Ensembl, <http://www.ensembl.org/> (for genetic map, genomic sequences, ESTs, and 21 candidate genes, including *Q9H7K4* and *SLC9A5*)
- GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for microsatellite DNA markers *GGAA10* [accession number AB13610], *TTCC01* [accession number AB13611], *TA001* [accession number AB13612], *GA001* [accession number AB197662], and *AAT01* [accession number AB13613] and full-length and short-form *puratrophin-1* mRNAs [accession numbers AB197663 and AB197664])
- NCBI, <http://www.ncbi.nlm.nih.gov/> (for DKFZP434I216 [accession numbers BC054486 and AK024475])
- Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for 16q-linked ADCA type III, or SCA4)
- The Hereditary Hearing Loss Homepage, <http://webhost.ua.ac.be/hhh/>
- UniGene, <http://www.ncbi.nlm.nih.gov/UniGene/> (for candidate genes and ESTs)

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# A clinical, genetic, and neuropathologic study in a family with 16q-linked ADCA type III

**Abstract**—Presented is the new kindred with autosomal dominant cerebellar ataxia linked to chromosome 16q22.1 (16q-ADCA type III) associated with progressive hearing loss. By haplotype analysis, the critical interval was slightly narrowed to three megabase regions between GATA01 and D16S3095. Neuropathologic study of 16q-ADCA type III demonstrated characteristic shrinkage of Purkinje cell bodies surrounded by synaptophysin-immunoreactive amorphous material containing calbindin- and ubiquitin-positive granules.

NEUROLOGY 2005;65:629–632

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Autosomal dominant cerebellar ataxia (ADCA) type III is characterized by purely cerebellar ataxia throughout different generations.<sup>1</sup> We previously described families with ADCA type III that map to chromosome 16q13.1-q22.1<sup>2</sup> (16q-ADCA type III; OMIM: %600223), the same region to which maps spinocerebellar ataxia type 4 (SCA4).<sup>3</sup> We subsequently found that families with 16q-ADCA type III harbor a common, ‘founder’ haplotype for the 3.8 mega-base (Mb) region between GGAA05 and D16S3095.<sup>4</sup>

We describe here the new five-generation kindred with 16q-ADCA type III and slightly narrowed candidate region. We also report the first neuropathologic data of 16q-ADCA type III.

**Methods.** *Clinical analysis.* The index patient (Patient III-1; figure 1) first consulted our clinic because of unsteadiness of gait.

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Supported by Grant-in-Aids for Scientific research on Priority Areas, Advanced Brain Science Project, from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (K.I. and H.M.), as well as Grants for Research on Intractable Disorders, Ministry of Health, Labor and Welfare, Japan (H.M.).

Disclosure: The authors report no conflicts of interest.

Received June 26, 2004. Accepted in final form April 18, 2005.

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Multiple family members over five generations were documented to have progressive ataxia (see figure 1). Interview and neurologic examination were performed in 12 individuals (Patients II-11, III-1, III-16, III-18, III-20, IV-1, IV-2, IV-3, IV-6, IV-7, V-1, and V-2). Patient II-11 was also examined neuropathologically. This patient presented with gait ataxia at age 70 and experienced hearing difficulties from age 75. Both symptoms slowly progressed. At age 90, she had prominent ataxia and could not walk without assistance. She became bed-ridden at age 92 and died of natural causes at age 96.

*Genetic analysis.* After obtaining informed consent, genomic DNA was extracted from peripheral blood from 12 individuals (see figure 1). After excluding spinocerebellar ataxia (SCA) 1, 2, 3, 6, 7, 12, and 17 and dentatorubral and pallidolysian atrophy, genotypes were determined for 12 informative markers on chromosome 16q22.1<sup>4,5</sup> (see table E-1 on the *Neurology* Web site at [www.neurology.org](http://www.neurology.org)). Allele frequencies in general population were analyzed in 50 unrelated individuals without histories of ataxia or other neurodegenerative diseases. Two-point and multipoint lod scores were calculated using software LINKAGE (ver 5.1) and GENEHUNTER.

*Neuropathological analysis.* The brain and spinal cord were fixed in 10% neutral buffered formalin for two weeks. Paraffin-embedded sections were stained by hematoxylin and eosin (H&E), Klüver-Barrera (KB), and modified Bielschowsky methods. Immunohistochemistry was undertaken using standard avidin-biotinylated peroxidase complex (ABC) method and developed with diaminobenzidine (DAB). Primary antibodies used were mouse monoclonal antibodies against expanded polyglutamine (1C2; Chemicon; diluted into 1:4000), calbindin D28k (Sigma; 1:200), synaptophysin (Sigma; 1:50), phosphorylated neurofilament (SMI31, Steinberger; 1:5000), and rabbit polyclonal antibody against ubiquitin (Sigma; 1:400). Specimens from two individuals, one without neurological diseases and the other with SCA6, served as controls.

**Results.** *Clinical features.* By neurologic examination, seven individuals were confirmed as having pure cerebellar ataxia (see figure 1). The individual IV-6 was not confirmed ‘affected’ but showed very mild imbalance. Cardinal features in the seven patients were truncal ataxia (100%), limb ataxia (71.4%), cerebellar speech (71.4%), and reduced muscle tonus (57.1%). Tendon reflexes were normal but were reduced at ankles in 28.6% of patients. Gaze nystagmus was not obvious. Only Patient II-11 had mild positional and action tremor in hands. Decreased sensation, absence of tendon reflexes, and Babinski signs, distinctively seen in SCA4 patients,<sup>3,6</sup> were all absent.

The average age at onset of ataxia was 52.1 years, ranging from 8 to 70 years. Patients IV-1 and V-1 documented their ages at onset at younger than 20 years. Phenomenon

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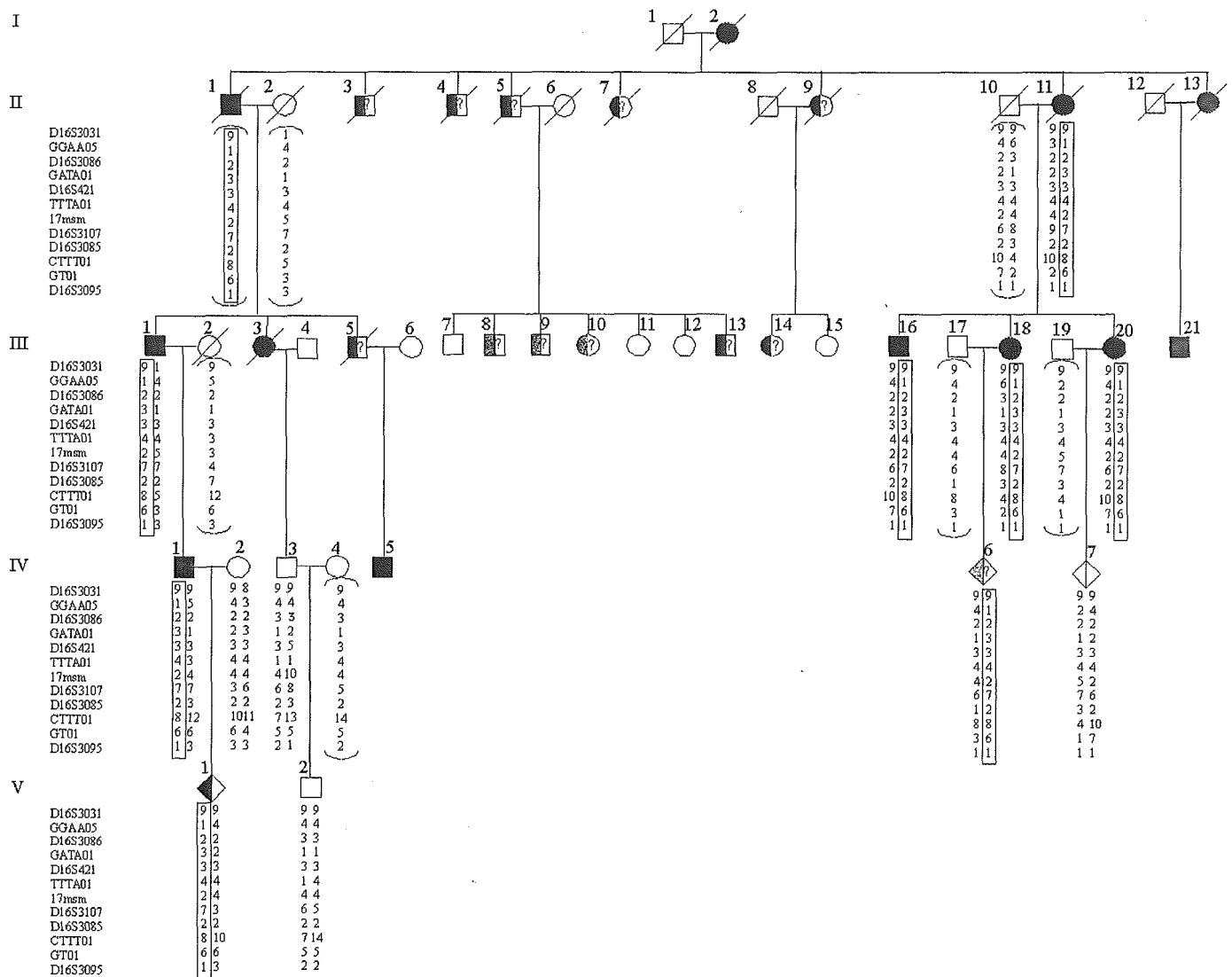


Figure 1. Pedigree of the present family with reconstructed haplotypes for markers at 16q22.1. Women are shown with a circle, and men with a square. Deceased are slashed. Rhombes indicate the sex is not specified because of confidentiality. Individuals with ataxia are filled in the left half of their symbols, and individuals with definite hearing impairment are filled in their right half of their symbols. The individual IV-6 who was suspected of having ataxia was filled gray. The symbol “?” indicates presence of hearing impairment was suspected, but was not confirmed. Individuals III-8, III-9 and III-10 are suspected of having ataxia, since lurching gait and dysarthria have been noticed by several family members. Haplotypes for chromosome 16 markers are shown. The haplotypes cosegregating with ataxia are boxed. Deduced haplotypes are shown in braces.

of anticipation was generally not obvious but was seen in transmission through Patients III-1, IV-1, and V-1. Brain MRI revealed isolated cerebellar atrophy. Peripheral nerve conduction study and somatosensory evoked potentials in two individuals (Patients III-1 and IV-1) were normal.

Six of seven individuals with ataxia documented hearing difficulties (Patients II-11, III-1, III-16, III-18, III-20, and IV-1). Several other members also seemed to have both ataxia and hearing impairment (I-2, II-1, II-3, III-3, and IV-5). Audiograms, brainstem auditory evoked potentials, and otoacoustic emissions of Patients III-1 and IV-1 indicated that the hearing impairment had a cochlear origin (figure 2). The average age at onset of hearing impairment was 59.4 years.

**Genetic features.** The highest two-point lod score was obtained with marker CTTT01 with a maximum lod score of 2.2 at  $\theta = 0$ , supporting linkage to chromosome 16q22.1 (see table E-2). Maximum multipoint lod score reached 2.3, suggesting linkage. Comparing the haplotypes of the current and previous families, different alleles are seen at GATA01: The current family had allele 3, whereas the previous eight families had allele 2<sup>4</sup> (see figure 1; also see table E-1). This would indicate that the gene for with autosomal dominant cerebellar ataxia linked to chromosome 16q22.1 (16q-ADCA type III) lies in the 3-Mb region between GATA01 and D16S3095.

**Neuropathologic features.** The brain including the brainstem and cerebellum weighed 1,200 g after fixation.

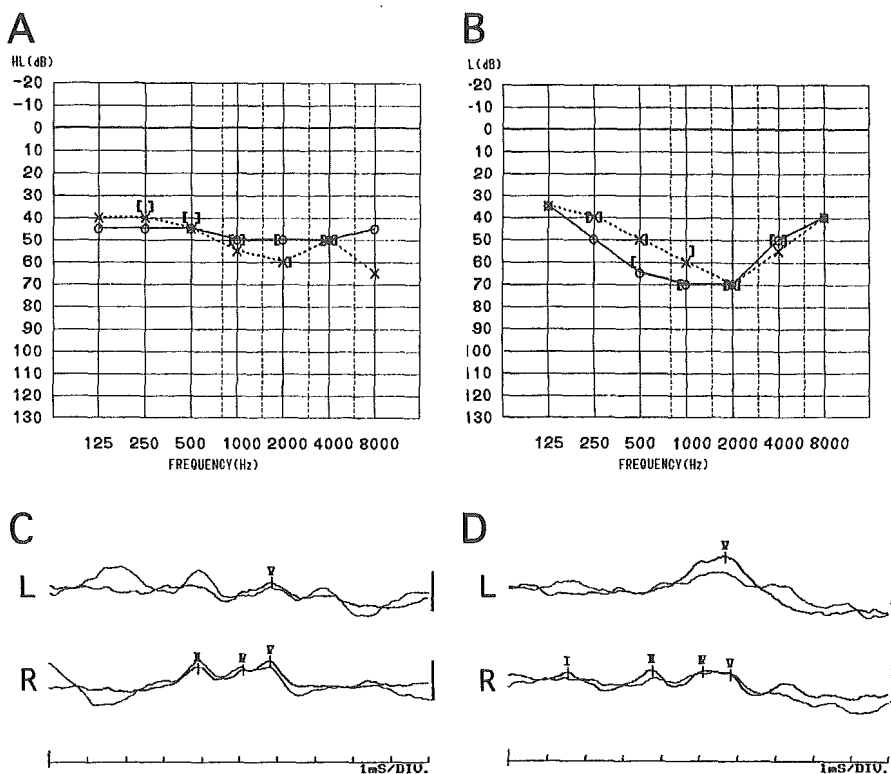


Figure 2. Audiograms of the Patients III-1 (A) and IV-1 (B). Open circle indicates air conduction of the left ear, cross indicates that of the right ear. Symbols [ indicate bone conduction of right ear, ] indicate that of left ear. Both audiograms show sensorineural hearing impairment, slightly worse in Patient IV-01. BAEPs of the Patients III-1 (C) and IV-1 (D). "L" indicates responses (CZ-A1) elicited by left ear stimulation (90dB, 9.5Hz), and "R" indicates responses (CZ-A2) elicited by right ear stimulation. The BAEPs show poor I-wave formation in both patients, consistent with their internal ear impairments.

Macroscopically, the brain and spinal cord appeared normal except for the moderate cerebellar atrophy in the upper vermis.

Microscopically, the most prominent feature was the Purkinje cell degeneration with relative preservation of molecular and granule cell layers (figure 3A). Generally, the Purkinje cell loss was severe in the anterior lobe such as culmen, moderate in the upper aspect of the posterior lobe, and mild in the rest of the regions such as tonsil. The remaining Purkinje cells were often atrophic and were surrounded by thick amorphous materials (figure 3B). The dentate nucleus had moderate astrogliosis. No obvious pathologic change was seen in the cerebrum, brainstem, including the inferior olivary nucleus or in the spinal cord, although mild myelin pallor was seen in the fasciculus gracilis and the spinocerebellar tracts (figure 3, C and D).

1C2-immunoreactive aggregates were not seen (figure 3E). Ubiquitin-positive granules were seen within the amorphous material, also sometimes within the Purkinje cell body (figure 3F). A few Purkinje cells showed processes stemming from their cell bodies resembling 'somatic sprout' on calbindin D28k immunohistochemistry (figure 3G). Within the amorphous materials, a zone with weak calbindin-immunoreactivity or calbindin-positive granules were seen (figure 3, H and I). The amorphous materials were intensely immunoreactive for synaptophysin (figure 3J), while such finding was not seen in control brains. Immunoreactivity for SMI31 was seen only in the periphery of the amorphous material (figure 3K).

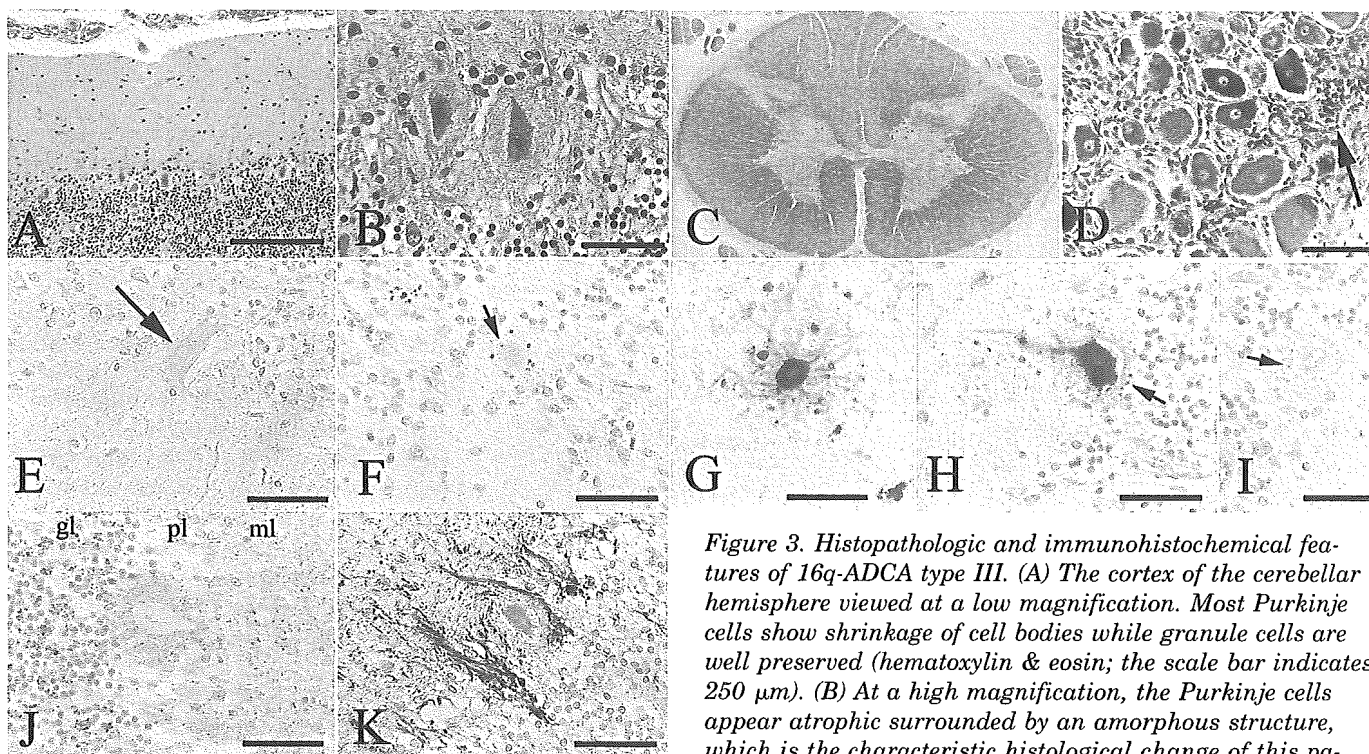
**Discussion.** We described a new family of 16q-ADCA type III, showing co-segregation of pure cerebellar ataxia and hearing impairment. It is possible that the two phenotypes are caused by a single gene mutation. Since genes for deafness are not known within the candidate region, discovering the 16q-

ADCA type III gene would be important. Audiogram and BAEPs are necessary to clarify whether hearing impairment is indeed associated with 16q-ADCA type III.

Neuropathology of 16q-ADCA type III was characterized by shrunken cell bodies, abnormal dendrites, somatic sprouts of Purkinje cells, and the amorphous materials surrounding the Purkinje cell. Although somatic sprouts are described in other diseases such as Menkes' disease,<sup>7</sup> the amorphous materials have not been described.<sup>8</sup> Therefore, presence of amorphous materials around shrunken Purkinje cells could be diagnostic for 16q-ADCA type III. The amorphous materials resemble "grumose degeneration" of the dentate nucleus in DRPLA and progressive supranuclear palsy (PSP).<sup>9</sup> Presence of calbindin- and ubiquitin-immunoreactive granules within the amorphous materials would indicate that the amorphous materials are formed in association both with the degeneration of Purkinje cell processes and the increase of presynaptic terminals innervated either from basket cells, inferior olivary neurons, or other neurons.<sup>10</sup> Alteration of calbindin-immunoreactivity in Purkinje cells may also indicate that the intracellular calcium buffering system, which is one of the important roles of calbindin, may be altered in 16q-ADCA type III.

**Note added in proof.** After acceptance of this article, a genetic change tightly associated with the 16q-linked ADCA type III including the present family was discovered in the gene encoding a protein with spectrin and Rho guanine-nucleotide exchange-





**Figure 3.** Histopathologic and immunohistochemical features of 16q-ADCA type III. (A) The cortex of the cerebellar hemisphere viewed at a low magnification. Most Purkinje cells show shrinkage of cell bodies while granule cells are well preserved (hematoxylin & eosin; the scale bar indicates 250  $\mu$ m). (B) At a high magnification, the Purkinje cells appear atrophic surrounded by an amorphous structure, which is the characteristic histological change of this patient (hematoxylin & eosin; the scale bar indicates 50  $\mu$ m).

(C) The thoracic spinal cord. Myelin pallor is seen in the dorsal column and spinocerebellar tracts (Klüver-Barrera staining). (D) The dorsal root ganglia of the 7th thoracic spine. Neuronal loss is not apparent. A Nageotte's nodule is seen (arrow) (Klüver-Barrera staining; the scale bar indicates 10  $\mu$ m). (E) Immunohistochemistry for expanded polyglutamine using mouse monoclonal antibody 1C2 does not demonstrate microscopic aggregations in the Purkinje cell (arrow) (immunoperoxidase, counter stained with methyl green; scale bar indicates 50  $\mu$ m). (F) There are some ubiquitin-immunoreactive granules in the amorphous materials. A granule is also seen within the cell body (small arrow) (immunoperoxidase, counter stained with hematoxylin; scale bar indicates 50  $\mu$ m). (G) Immunohistochemistry for calbindin D28k shows a bizarre-shaped Purkinje cell, called "somatic sprouts" (immunoperoxidase; scale bar indicates 50  $\mu$ m). (H) A fuzzy and weak immunoreactivity against calbindin D28k is seen at the amorphous structure. Immunoreactive granules are also seen outside the Purkinje cell body (small arrow) (immunoperoxidase, counter stained with hematoxylin; scale bar indicates 50  $\mu$ m). (I) No obvious immunoreactivity for calbindin is seen in an atrophic Purkinje cell, except for granules within amorphous material (small arrow) (immunoperoxidase, counter stained with hematoxylin; scale bar indicates 50  $\mu$ m). (J) Synaptophysin immunoreactivity is increased around the Purkinje cell body (pl: Purkinje cell layer). The immunoreactivities at the molecular layer (ml) and at the granulus in the granular layer (gl) are not obviously reduced (immunoperoxidase, counter stained with hematoxylin; scale bar indicates 50  $\mu$ m). (K) The SMI31-immunoreactive basket cell axons attach to the outer margin of the amorphous structure. The Purkinje cell body is weakly immunoreactive for SMI31 (immunoperoxidase, counter stained with methyl green; scale bar indicates 50  $\mu$ m).

factor domains by the authors (Am J Hum Genet 2005;77:280–296).

### Acknowledgment

The authors thank Drs. Jiro Kumagai and Toshihiko Kuroiwa (Department of Pathology, Tokyo Medical and Dental University) for help with the neuropathology and Dr. Hiroto Fujigasaki (Department of Neurology, Tokyo Medical and Dental University) for support.

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SHORT COMMUNICATION

## Somatosensory-evoked cortical potential during attacks of paroxysmal dysesthesia in multiple sclerosis

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**Keywords:**

multiple sclerosis, paroxysmal dysesthesia, somatosensory-evoked potential

Received 21 August 2003

Accepted 28 December 2003

Paroxysmal dysesthesia is considered to be one of the characteristic symptom of multiple sclerosis (MS), but the lesion responsible and the pathophysiology of this dysesthesia are not known. We report the interesting finding of somatosensory-evoked potentials (SEPs) in a patient with MS during a paroxysmal dysesthesia attack.

### Case report

A 43-year-old woman developed paroxysmal dysesthesia of the left arm and leg. Four months before she experienced bilateral optic neuropathy, symptoms of transverse myelopathy at the Th5 segment, and dysesthesia in the C5 to C8 area in the left arm. She was diagnosed as MS by her recurrence and remission of the symptoms. During the recovery stage, attacks of paroxysmal dysesthesia occurred more than 20 times a day. These were induced by voluntary or passive, quick anteroflexion of the neck and lasted for 60–90 s. The attacks started in the left hand with an unpleasant tickling or electrical sensation that radiated to the left upper extremity immediately and to the left lower extremity approximately half a minute later. Most attacks were accompanied by tonic spasm in the muscles of the left extremities.

T2-weighted magnetic resonance imaging showed three small irregular areas of high signal intensity; in the base of the right pons, dorsal part of cervical spinal cord from C5 to C7, and center of the thoracic spinal cord from Th5 to Th7.

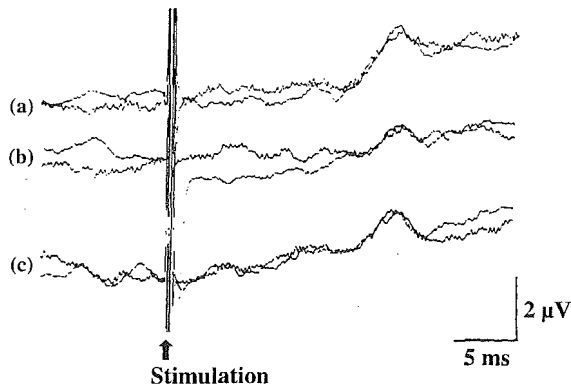
Somatosensory-evoked potentials elicited on left and right median nerve stimulation showed no abnormality when there was no attack. SEPs were recorded before

and after the attacks, whilst the patient mimicked the dystonic posture and muscle contraction of a tonic spasm to exclude the effect of proprioceptive impulses from the activated muscles. The median nerve was stimulated at the wrist, and the intensity adjusted to the level that evoked a 2 mV compound muscle action potential (CMAP) from the abductor pollicis brevis muscle. CMAP size was monitored to maintain constant stimulation. The SEP data was recorded on digital audiotape before the attack induced by neck flexion until the end of the attack. More than 100 responses during attack were averaged for one test. The average N20 amplitude from the baseline during the attack was smaller (1.1  $\mu$ V) than before (1.9  $\mu$ V) and after (1.7  $\mu$ V) the attack (Fig. 1). The reduction of N20 amplitude was statistically significant in four independent examinations ( $P < 0.05$ , Mann–Whitney *U*-test). There was no discernible difference in the N20 latencies. N13 could not be evaluated due to artifacts produced by muscle contraction.

### Discussion

The pathophysiology of paroxysmal dysesthesia is proposed to be the generation of ectopic impulses and ephaptic transmission in a focal demyelinated lesion in a tract of the central nervous system (Osterman and Westerberg, 1975; Rasminski, 1981). Ectopic impulses were shown to proceed from a lesion both rostrally and caudally in an experimental animal model (Smith and McDonald, 1982). We think this impulse is generated at axons of the posterior column of the cervical cord because (i) there is a lesion in the posterior part of the cervical cord on MRI; (ii) the attack was induced by neck flexion; and (iii) dysesthesia begins in the hand and does not involve the area innervated by cranial nerves. We speculate that three mechanisms are responsible for the attenuation of N20 size during an attack. First,

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**Figure 1** Somatosensory-evoked cortical potentials by left median nerve stimulation before (a), during (b) and after (c) a dysesthesia attack. Recording site: 2 cm posterior to C4. Reference site: Fz (International 10–20 system).

descending impulses are generated abnormally in dorsal column axons of a cervical lesion and collide with afferent impulses evoked by stimulation of the nerve. Secondly, ectopic impulses produce presynaptic modification on synaptic transmission of posterior column

cells or thalamic neurons, which is similar to the gating mechanism seen in the spinal cord. Thirdly, the ectopic impulses have a direct or indirect inhibition of N20 generation (Abbruzzese *et al.*, 1980).

We consider that the attenuation of N20 on SEPs during a dysesthesia attack supports the hypothesis that the ectopic impulse generated in the posterior column causes paroxysmal dysesthesia in MS.

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## Brief Communication

# New RNAi Strategy for Selective Suppression of a Mutant Allele in Polyglutamine Disease

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and HIDEHIRO MIZUSAWA<sup>1,2</sup>

### ABSTRACT

**In gene therapy of dominantly inherited diseases with small interfering RNA (siRNA), mutant allele-specific suppression may be necessary for diseases in which the defective gene normally has an important role. It is difficult, however, to design a mutant allele-specific siRNA for trinucleotide repeat diseases in which the difference of sequences is only repeat length. To overcome this problem, we use a new RNA interference (RNAi) strategy for selective suppression of mutant alleles. Both mutant and wild-type alleles are inhibited by the most effective siRNA, and wild-type protein is restored using the wild-type mRNA modified to be resistant to the siRNA. Here, we applied this method to spinocerebellar ataxia type 6 (SCA6). We discuss its feasibility and problems for future gene therapy.**

### INTRODUCTION

**R**NA INTERFERENCE (RNAi) is a powerful tool for posttranscriptional gene silencing. Small interfering RNA (siRNA) binds and cleaves the targeted RNA in a sequence-specific manner, thereby preventing translation of the encoded protein (Elbashir et al., 2001a). One possible therapeutic application of siRNA is the silencing of mutant genes that cause dominantly inherited diseases. We and others demonstrated that it is possible to design siRNA that selectively suppresses the expression of the mutant protein in amyotrophic lateral sclerosis (ALS), Alzheimer's disease, polyglutamine disease, and DYT1 dystonia (Gonzalez-Alegre et al., 2003; Miller et al., 2003, 2004; Li et al., 2004; Yokota et al., 2004). Recently, adeno-associated virus expressing siRNA injected into the cerebellum improved the polyglutamine-induced phenotype in a transgenic mouse model (Xia et al., 2004).

Although siRNA can discriminate even a single nucleotide alternation (Elbashir et al., 2001b), selectivity is not complete (Gonzalez-Alegre et al., 2003; Miller et al., 2003, 2004; Li et al., 2004; Yokota et al., 2004), and at a higher concentration of siRNA, the wild-type allele is more inhibited (Miller et al., 2004). In addition, the cleavage efficiency of the mutant allele is not necessarily excellent because selection of the mutant allele-specific siRNA has a restriction; that is, the siRNA target sequence should include the mismatch. In diseases caused by an expanded trinucleotide repeat, such as polyglutamine diseases, it is impossible to design siRNA that can recognize the expanded CAG repeat. In spinocerebellar ataxia type 3 (SCA3), a C/G polymorphism related to CAG repeat expansion has been used to design siRNA to discriminate the expanded allele (Miller et al., 2003; Li et al., 2004). We reported on siRNA with relative discrimination of the expanded allele of the SCA3 gene, which is possibly due to a

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change in the RNA secondary structure (Li et al., 2004). In SCA6, however, the CAG repeat length in the mutant allele is within the normal range of other polyglutamine diseases, so that the secondary structure of RNA does not alter greatly even in the mutant. Here, we show a new alternate method for allele-specific suppression by siRNA. After suppressing both mutant and wild-type proteins by the most effective siRNA, wild-type protein is returned by coexpression of siRNA-resistant wild-type mRNA. This new strategy can be applied to any mutation.

## MATERIALS AND METHODS

### Plasmid construction and siRNA synthesis

Construction of expression plasmids of the  $\alpha 1A$  calcium channel gene (CACNA1A) was reported previously (Kubodera et al., 2003). The CACNA1A cDNA in the plasmid was truncated at the 3'-terminal region containing 13 (normal, pQ13C) or 28 (expanded, pQ28C) CAG repeats. Each construct corresponds to nucleotide positions 6727–7521 and 6727–7566 of the full-length CACNA1A cDNA. Modified pQ13C, in which CACNA1A cDNA is mutated not to be cleaved by siRNA7493 but translated to the same amino acids as those of pQ13C, was made by PCR amplification using pQ13C as a template (Fig. 1). The reverse primer included the modified mutations (light gray in Fig. 1), and the PCR fragment was subcloned into pcDNA3.1(+). (Invitrogen, San Diego, CA).

Selection of an siRNA target site was made according to the reported protocol (Reynolds et al., 2004). Sense and antisense strands of siRNA oligonucleotides (ODNs) were synthesized and prepared as described previously (Yokota et al., 2003). siRNA for an unrelated target, hepatitis C virus (HCV) gene, was used as a negative control.

### Transfection and Western blotting

To see the effect of siRNA for CACNA1A mRNA, we cotransfected both siRNA and expression plasmids of CACNA1A to human embryonic kidney 293T cells. Transfection was done with Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's protocol.

For Western blotting at 24 hours posttransfection, cells were solubilized in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100), separated on a 10%–20% gradient of SDS-PAGE, immunoblotted with rabbit polyclonal antibody specific for the C-terminal portion of the  $\alpha 1A$  calcium channel protein (A6PRT-C) (Ishikawa et al., 1999), then made visible by enhanced chemiluminescence (ECL) (Amersham Bioscience, Buckinghamshire, England).

## RESULTS

Using the synthetic siRNA (siRNA7493; 5'-ACAGC-GAGAGUGACGAUGAdTdT-3' in sense sequence), expressions of both wild-type (Q13C) and mutant (Q28C)

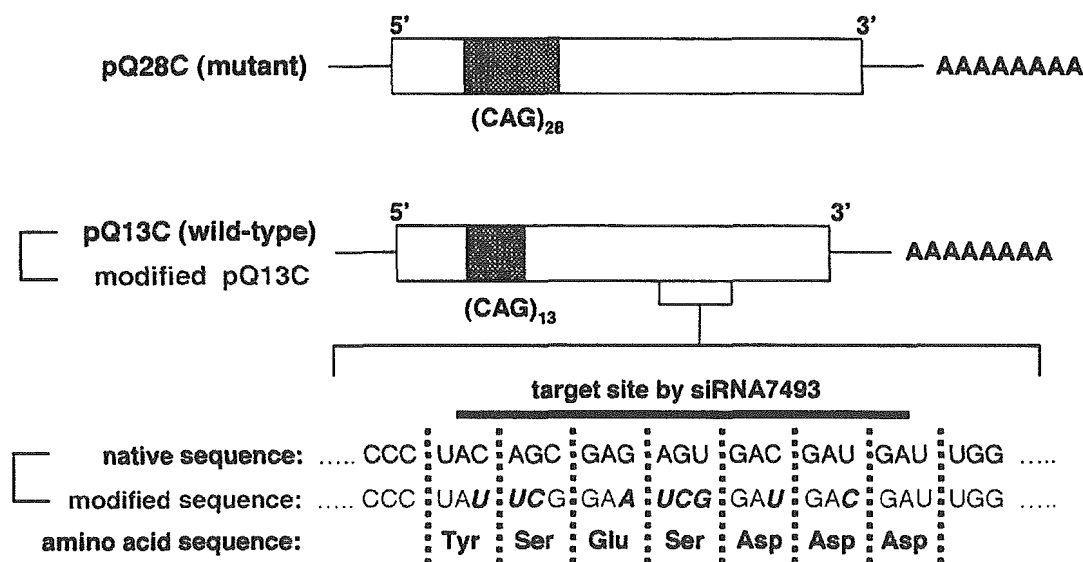
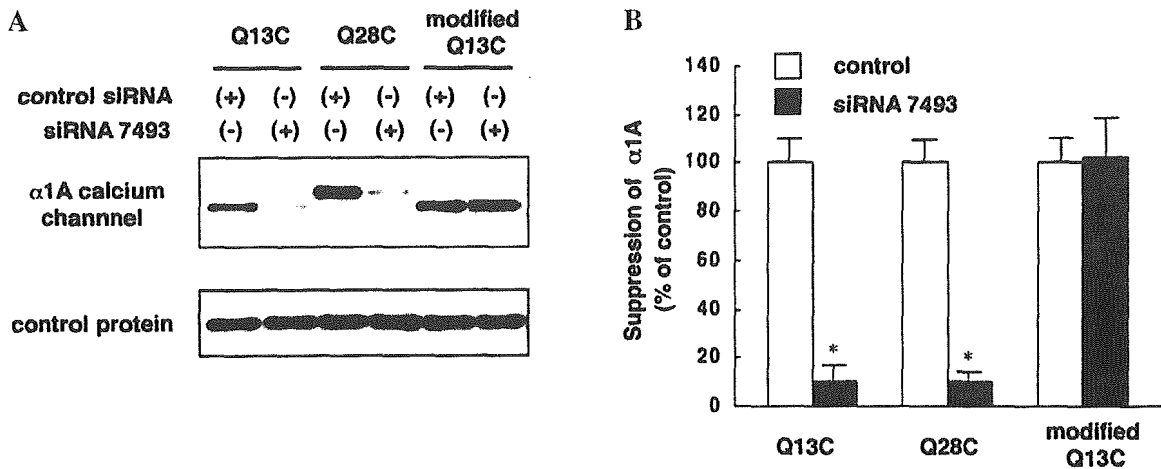


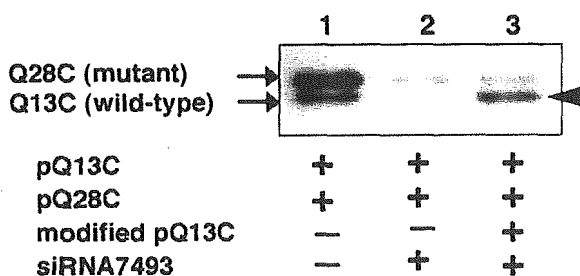
FIG. 1. Schema of mRNAs transcribed by expression plasmids of truncated CACNA1A. The RNA sequence around the target site of siRNA7493 is shown at bottom. The bold bar indicates the targeted sequence by siRNA7493. Characters in boldface italics are RNA nucleotides that are mutated from the wild-type. There is no change in amino acid sequences expressed by pQ13C or modified pQ13C.



**FIG. 2.** Effect of siRNA7493 on expression of pQ13C, pQ28C, and modified pQ13C. (A) Western blot analysis of cells transfected with the indicated expression plasmids and control siRNA/siRNA7493 at 25 nM. The superoxide dismutase 1 (SOD1) protein staining was used as a loading control. (B) Quantitation of signal intensities. The suppression level of target protein was compared with transfection of control siRNA. siRNA7493 could markedly silence pQ13C and pQ28C but not modified pQ13C. Values are means  $\pm$  SEM. \* $p < 0.001$  (Student's *t*-test).

$\alpha$ 1A calcium channel proteins were markedly decreased by >90% on Western blot analysis (Fig. 2).

Because the siRNA7493 did not discriminate wild-type and mutant allele, we tried to restore the wild-type  $\alpha$ 1A calcium channel protein expressed by modified pQ13C, in which CACNA1A cDNA sequence is resistant to siRNA7493. We modified wild-type CACNA1A cDNA construct (modified pQ13C). The amino acid sequence encoded by modified pQ13C was the same as that of native pQ13C, but the nucleotide sequence targeted by siRNA was altered (Fig. 1). In fact, expression of modified pQ13C was not suppressed at all by siRNA7493 (Fig. 2).



**FIG. 3.** Effect of siRNA7493 in cells cotransfected with pQ13C, pQ28C, or modified pQ13C (25 nM). Expressions of both pQ13C and pQ28C were markedly decreased by siRNA7493 (lane 2), but wild-type  $\alpha$ 1A calcium channel was restored to the same intensity level as that of lane 1 by expression of modified pQ13C (arrowhead) (lane 3). siRNA7493 (-) indicates no siRNA.

Next, we cotransfected modified pQ13C with pQ13C, pQ28C, and siRNA7493 to 293T cells. Modified pQ13C restored the expression of the wild-type  $\alpha$ 1A calcium channel that had been markedly inhibited by siRNA7493 (Fig. 3). Consequently, the mutant allele of CACNA1A was selectively silenced, whereas the wild-type protein was unchanged.

## DISCUSSION

SCA6 is an autosomal dominant cerebellar ataxia characterized by late onset, pure cerebellar ataxia (Ishikawa et al., 1999). The causative gene for SCA6 has been identified as CAG repeat expansion in the  $\alpha$ 1A voltage-dependent calcium channel gene (Zhuchenko et al., 1997). The  $\alpha$ 1A subunit mediates  $\text{Ca}^{2+}$  influx across presynaptic and somatodendritic membranes, thereby triggering fast neurotransmitter release and other key neuronal responses.  $\alpha$ 1A-deficient mice develop ataxia and dystonia and die before 4 weeks of age (Jun et al., 1999), and the natural mutant mice of *cacna1a*, *leaner*, in which channel function is severely reduced, produce severe ataxia (Lorenzon et al., 1998). In gene therapy of SCA6 with siRNA, therefore, reduction of endogenous  $\alpha$ 1A calcium channel expression may produce an undesirable effect, and preservation of wild-type  $\alpha$ 1A calcium channel expression is necessary.

Our new method for allele-specific suppression by siRNA has the following advantages. (1) Any type of mutation can be applied by our method. (2) Only one set of the siRNA and wild-type protein-expressing vector in

our strategy works in many different mutants in a single gene; there are more than 100 mutations of superoxide dismutase 1 (SOD1) gene for familial ALS (*alsod1.iop.kcl.ac.uk/*) and presenilin 1 (PS1) gene for familial Alzheimer's disease (*www.molgen.ua.ac.be/ADMutations/*). (3) Greater than 90% suppression efficiency of mutant allele expression usually can be achieved using the recent prediction program of siRNA site (Reynolds et al., 2004) because the best siRNA site can be selected from the whole sequence of the target mRNA. In contrast, suppression efficiency of conventional siRNA should include the mutation, so that the target region is limited.

On the other hand, this method has the following important problems. (1) The expression level of the restored wild-type protein is difficult to control. If it is much greater than the endogenous level, it may produce an unexpected side effect. (2) Both siRNA and restored wild-type protein should be delivered to every cell, preferably by putting both expressing cassettes of short-hairpin RNA and siRNA-resistant wild-type cDNA into a single vector. To date, however, ODN is better than expression vectors for *in vivo* delivery of siRNA by systemic intravenous injection (Anton et al., 2002; Soutscheck et al., 2004). (3) There may be unknown differences of endogenous and exogenously expressed protein functions.

The efficacy of this strategy should be confirmed in an *in vivo* model, and the cited problems must be further addressed. Our new approach promotes the feasibility of using siRNA-based gene therapy for dominantly inherited diseases.

## ACKNOWLEDGMENTS

We thank Toshinori Unno for his help. This work was supported by grants from the Ministry of Education, Science and Culture of Japan and from the Ministry of Health, Labor and Welfare of Japan.

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Received April 21, 2005; accepted in revised form  
June 28, 2005.