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ORIGINAL ARTICLE

Effects of a polymorphism in the *GFAP* promoter on the age of onset and ambulatory disability in late-onset Alexander disease

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Alexander disease (AxD) is a rare neurodegenerative disorder. Most patients with AxD have a *de novo* dominant missense mutation in the glial fibrillary acidic protein (*GFAP*) gene. Patients with late-onset AxD exhibit a more variable onset and severity than patients with early-onset AxD, suggesting the existence of factors that modify the clinical phenotype of late-onset AxD. A −250-bp C/A single-nucleotide polymorphism (SNP) of the *GFAP* promoter (rs2070935) in the activator protein-1 binding site is a candidate factor for modification of the clinical phenotype. We analyzed the SNP in 10 patients with late-onset AxD and evaluated the effects of the SNP on the clinical course of late-onset AxD. Three of four cases with the C/C genotype lost the ability to walk in their 30s or 40s, whereas all six cases with the other genotypes retained the ability to walk throughout their 30s. The age of onset in patients with the C/C genotype was significantly earlier than in patients with the other genotypes ($P<0.05$). A more severe phenotype was observed in the patient in whom the C allele of rs2070935 was *in cis* with the *GFAP* mutation compared with the patient in whom the C allele of rs2070935 was *in trans* with the *GFAP* mutation. Our investigation revealed the possibility that the C/C genotype at rs2070935 of the *GFAP* promoter in late-onset AxD was associated with an earlier onset and a more rapid progression of ambulatory disability compared with the other genotypes.
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INTRODUCTION

Alexander disease (AxD) is a rare neurodegenerative disorder characterized by white matter degeneration and the formation of cytoplasmic inclusions known as Rosenthal fibers, which primarily accumulate in the astrocyte end-feet in the subpial and perivascular zones and consist of glial fibrillary acidic protein (GFAP), heat-shock protein 27 and αB-crystallin.[1–3] Since 2001, studies have reported *GFAP* mutations in various clinical types of AxD.[4] Although most of the mutations in the coding region of *GFAP* in patients with AxD are missense mutations, additions and/or deletions of one or a few amino acids and intronic mutations that result in frame shifts have also been identified.[5] These mutations are expected to have dominant gain-of-function and dominant-negative effects.[4,6]

AxD has been classified according to the age of onset into the following three subtypes: infantile AxD (<2 years of age), juvenile AxD (2–12 years of age) and adult AxD (>12 years of age). However, we recently proposed a novel classification of AxD into three distinct types based on neurological and magnetic resonance imaging findings: cerebral (type 1), bulbospinal (type 2) and intermediate (type 3).[7,8] Late-onset AxD, which includes types 2 and 3, exhibits a more variable onset and severity in comparison with early-onset AxD, which includes type 1, suggesting the existence of factors that modify the clinical phenotype, particularly of types 2 and 3.[9–11]

A single-nucleotide polymorphism (SNP) in the activator protein-1 binding site of the *GFAP* promoter, −250 bp upstream from the *GFAP* transcriptional start site (NCBI dbSNP: rs2070935), is a candidate factor for mediating the effects of *GFAP* mutations.[12] The A allele at rs2070935, which is a minor allele with an allelic frequency of 0.43, would primarily modify the site sequence and create a novel activator protein-1 binding site. An investigation of healthy controls

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has revealed that, based on differences in the extent of binding between C and A alleles, the C allele has a greater association with transcriptional activity than the A allele.[12] These results indicate higher levels of GFAP expression in the presence of the C allele than in that of the A allele.[12]

In this study, to evaluate whether rs2070935 affected the clinical course of late-onset AxD, we analyzed the SNP in the *GFAP* promoter and examined the relationship between the SNP, age of onset and ambulatory disability in patients with AxD that was classified as types 2 and 3.

MATERIALS AND METHODS

Gene analysis

Between 2005 and 2012, to analyze *GFAP* mutations, 44 patients suspected of having AxD were referred to Kyoto Prefectural University of Medicine from other hospitals all over Japan. Genomic DNA was extracted from the peripheral blood of all patients after obtaining their informed consent. To detect *GFAP* mutations, sequence analysis of genomic DNA was performed as described previously.[13] Point mutations in *GFAP* were identified in 12 of these patients. Of these, six satisfied the criteria of our proposed novel classification for type 2 AxD, and four satisfied the criteria for type 3 AxD. These 10 patients were included in this study.

The genomic DNA containing rs2070935 was amplified using polymerase chain reaction (PCR) with the following primers: forward, 5′-GTCCCCAGTT-CATAGCAGGA-3′; reverse, 5′-GTGATGCGTCTCCTCTCCAT-3′. Direct sequence analysis was performed using an ABI PRISM 3100 autosequencer (PE Applied Biosystems, Foster City, CA, USA) and Big Dye terminators according to the manufacturer's instructions.

Subcloning

The genomic DNA containing the coding region of rs2070935 and codon 74 of the *GFAP* exon 1, with a PCR product size of 803 bp, was amplified using PCR with the following primers: forward, 5′-ACTCAGCCCTTTCCTTCCTT-3′; reverse, 5′-CAGATTGTCCCTCTCAACCTCC-3′. The PCR products subjected to the attachment of a poly-A tail (10 × A-attachment mix) were ligated into pGEM-T Easy Vector (Promega, Madison, WI, USA), and subsequently transformed into chemically competent *Escherichia coli* cells. After screening for successful insertion using Insert Check Ready (Toyobo, Osaka, Japan), direct sequence analysis of three clones for each PCR product was performed as described above.

Phenotype–genotype correlation

We evaluated the age of onset and the age of ambulatory disability among patients with the C/C, C/A and A/A genotypes. Furthermore, ambulatory disability was evaluated by constructing the Kaplan–Meier ambulatory curves of C/C versus C/A and A/A genotypic patients. The statistical analysis of the relationship between the SNP and age of onset was performed by Ekuseru–Toukei 2010. A P-value <0.05 was considered statistically significant.

RESULTS

In 10 patients with type 2 or 3 AxD, we identified the following rs2070935 genotypes: four C/C homozygotes, five C/A heterozygotes and one A/A homozygote (Table 1). Of four patients with the C/C genotype, three had lost the ability to walk in their 30 s or 40 s; however, all six patients with the C/A or A/A genotypes retained the ability to walk throughout their 30 s (Figure 1). The mean age of onset in the patients with the C/C genotype was 9.5 years and that in patients with C/A and A/A genotypes was 40.2 years old ($P<0.05$). An R79H *GFAP* mutation was observed in three patients with the following rs2070935 genotypes: A/A homozygote (patient 7), C/C homozygote (patient 8) and C/A heterozygote (patient 10). Patient 8 with the C/C genotype exhibited earlier onset than patients 7 and 10

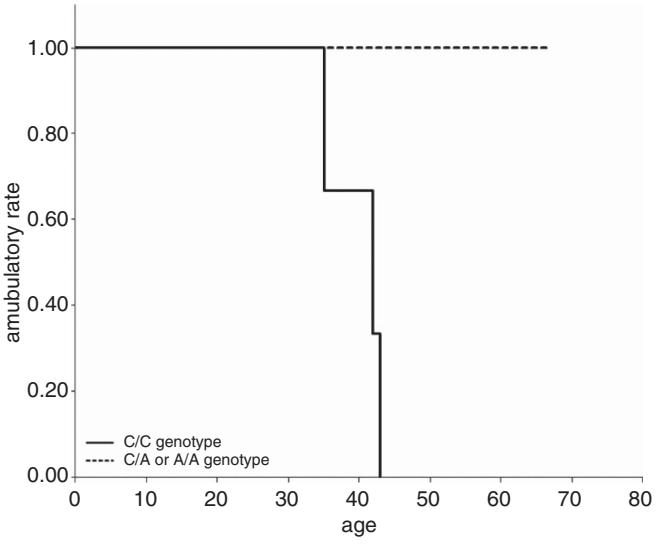

Figure 1 Ambulatory disability was evaluated by constructing the Kaplan–Meier ambulatory curves of C/C versus C/A and A/A genotypic patients.

Table 1 Clinical and genetic data in 10 patients of AxD

Patient number	*Sex*	*Age of gene analysis (y.o.)*	*Age of onset (y.o.)*	*Clinical type*[a]	*First symptom*	*Age of ambulatory disability (y.o.)*	*GFAP mutation (ref.)*	*−250 bp Genotype*
1	M	67	64	Type 2	Dysphagea	None	R70W[13]	C/A
2	M	56	51	Type 2	Gait disturbance	None	M74T[14]	C/A
3	M	53	51	Type 2	Dysarthria	None	M74T[13,30]	C/A
4	M	24	18	Type 2	Gait disturbance	None	L357P[13]	C/C
5	M	58	12	Type 2	Gait disturbance	30s	E362G	C/C
6	F	31	1	Type 3	Convulsion	None	V87L[31]	C/A
7	M	37	36	Type 3	Gait disturbance	None	R79H[13]	A/A
8	F	51	5	Type 3	Gait disturbance	43	R79H	C/C
9	F	47	3	Type 3	Encephalitis	42	A268D	C/C
10	F	40	38	Type 3	Gait disturbance	None	R79H[32]	C/A

Abbreviations: AxD, Alexander disease; F, female; GFAP, glial fibrillary acidic protein; M, male; y.o., years old.
[a]Type 2 = bulbospinal AxD; type 3 = intermediate form AxD.

Table 2 Comparison of clinical data among three patients with R79H AxD

	Patient 7	*Patient 8*	*Patient 10*
−250 bp Genotype	A/A	C/C	C/A
Age of gene analysis (age of onset)	37 (36)	52 (5)	40 (38)
Sex	M	F	F
Convulsion	Infantile only	Infantile only	Once at the age of 1
Mental retardation	+	+	−
Macrocephaly	−	−	−
Dysarthria	+	+	−
Dysphagia	−	+	−
Ataxia	+	?	+
Palatal myoclonus	−	+	−
Muscle weakness	+ (only lower extremities)	+	−
Hyperreflexia	−	+	−
Babinski sign	−/−	+/+	−/−
Sphincter dysfunction	+	+	−
Sleep disorder	−	+	−

Abbreviations: AxD, Alexander disease; F, female; M, male.

Table 3 Comparison of clinical data between two patients with M74T

	Patient 2[a]	*Patient 3*
−250 bp Genotype	C/A	C/A
Age of gene analysis (age of onset)	56 (51)	53 (51)
Sex	M	M
Convulsion	?	−
Mental retardation	−	−
Macrocephaly	−	−
Dysarthria	+	+
Dysphagia	−	+
Ataxia	−	−
Palatal myoclonus	−	−
Muscle weakness	+	−
Hyperreflexia	+	+
Babinski sign	+/+	−/−
Sphincter dysfunction	+	−
Sleep disorder	−	−

Abbreviation: M, male.
[a]Patient 2 required the aid of walker.

with the A/A and C/A genotypes, respectively. Patient 8 exhibited gait disturbance at the age of 5 years and lost the ability to walk at the age of 43 years (Table 2). In addition, in her mid-30 s, she developed bulbar dysfunction, which progressed gradually. She had difficulty in swallowing and underwent a percutaneous endoscopic gastrostomy at the age of 45 years. Patients 7 and 10 did not show gait disturbance until their late 30 s, and at the time of this study, they were able to walk and did not present with difficulties in swallowing. Although two patients (patient 2 and 3) in whom M74T (c.221 −T>C) mutation was observed had the C/A genotype at rs2070935 and similar ages of onset, patient 2 showed spastic tetraparesis with muscle weakness and required the aid of a walker (Table 3).[14] Gene analysis using the subcloning method revealed that the C allele of rs2070935 was *in cis* with the gene containing the mutant allele of codon 221 in patient 2 and *in trans* in patient 3.

DISCUSSION

Investigations on clinical phenotype–genotype correlations and morphological and functional impairment of astrocytes due to *GFAP* mutations using cells or animal models have suggested that the clinical type of AxD is primarily determined by the *GFAP* mutation.[4,5,13,15–28] However, patients with late-onset AxD, which includes types 2 and 3, exhibit variable onset and severity, suggesting the existence of other factors that modify the clinical course.[9–11]

An investigation using a transgenic mouse model that overexpressed wild-type *GFAP* has revealed that the aggregates are similar to Rosenthal fibers and that a shorter lifespan is observed in patients with increased *GFAP* expression,[29] suggesting that *GFAP* overexpression may cause AxD. Although 3% of the patients with AxD did not have detectable *GFAP* mutations,[15] other causative factors such as *GFAP* multiplication[8] have not been detected either.

The rs2070935 in the activator protein-1 binding site of the *GFAP* promoter, which may mediate the effects of *GFAP* mutations, is a candidate factor for the modification of the clinical course of AxD,[12] and differences in the degree of binding between C and A alleles have demonstrated a greater association of the C allele with transcriptional activity compared with the A allele.[12]

Our results suggested that the presence of the C/C genotype at rs2070935 may contribute to the early onset and the severity of ambulatory function in late-onset AxD, supporting the findings of a previous study that demonstrated a greater association of the C allele with transcriptional activity compared with that of the A allele.[12] Our investigation of two M74T mutations at C/A genotype at rs2070935 suggested that the patient in whom the C allele of rs2070935 was *in cis* with the *GFAP* mutation may show a more severe phenotype than the patient in whom the C allele of rs2070935 was *in trans* with the *GFAP* mutation.

Although the minor allelic frequency of the A allele in rs2070935 of the *GFAP* promoter is 0.43, the C/A SNP allelic frequency differs among races, as follows: the A allelic frequency is 0.35 in Asians, 0.44 in Europeans, 0.39 in Americans and 0.54 in Africans (1000 Genomes; http://www.1000genomes.org/). Thus, the severity of AxD may differ among races. Additional studies are needed to clarify the natural history of late-onset AxD among races.

A limitation of our investigation was the extremely small sample size. However, it may be difficult to examine a sufficient number of subjects because AxD is extremely rare, with an estimated prevalence of approximately one in 2.7 million people in Japan.[7] Furthermore, identical *GFAP* mutations in late-onset AxD are much rarer.[7]

In conclusion, our investigation demonstrated the possibility that a C/C genotype at rs2070935 in patients with late-onset AxD may be associated with an earlier onset of motor impairment and more rapid progression of ambulatory disability than the C/A and A/A genotypes at rs2070935, although larger-scale investigation and long-term evaluation are required.

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