[16], which do not usually develop cerebellar ataxia, but sometimes accompany cognitive and affective symptoms [17,18].

We have reported unique cases with the combination of SCA and MND/ALS symptoms [19,20] and have newly found GGCCTG hexanucleotide repeat expansions within NOP56 gene as the causative mutation in the previous and additional several families [21]. The patients usually develop walking unsteadiness at around age 50 years, soon followed by dysarthria, tongue fasciculation, and atrophy, moderate to severe skeletal muscle atrophy with fasciculation and hyperreflexia, and limb ataxia in subsequent 10–20 years and usually survive until age 80 years or more. Speech disturbance is characterized initially by cerebellar slurred type, which is eventually modified by motor system involvement with tongue atrophy and fasciculation [19–21]. Because most of these cases are originally found along 'Asida' river located in the west part of Japan, we have been calling this mutation as 'Asidan'. Physico-clinical and pathological details will be described elsewhere.

NOP56 is involved in RNA processing with forming RNA foci [21] similar to some hereditary SCAs with pentanucleotide ATTCT repeat expansion (SCA10, ref. [22–23]) and TGGAA repeat expansion (SCA31, ref. [24]) as well as ALS with TAR DNA-binding protein 43 (TDP43, ref. [25]) and fused-in-sarcoma (FUS, ref. [26,27]) gene mutations. However, unlike those hereditary SCAs and ALS, the present Asidan cases show a unique clinical balance to develop a slowly progressive and a relatively pure cerebellar ataxia with moderate to severe motor involvement (both upper and lower motor signs). Based on this unique clinical characteristics having both SCA and MND phenotypes together, Asidan may locate on the crossroad of clinical spectrum between SCA and MND (Ikeda Y, Ohta Y, Kobayashi H, Okamoto M, Takamatsu K, Oota T, Yasuhiro M, Okamoto K, Koizumi A, Abe K, unpublished observation).

We hypothesized that cognitive and affective functions of these Asidan patients may also reside of the crossroad on those functions in SCA and MND/ALS. Therefore, in this report, we focused on their cognitive and affective characteristics, which may also reside on the crossroad of SCA and MND/ALS.

Patients and methods

We found NOP56 gene mutation (=SCA36) in nine unrelated Japanese familial SCA originating from Asida river area in the western part of Japan, thus nicknamed Asidan for this mutation [21]. Because the numbers of patients were small, we took inclusion criteria of as many Asidan patients as possible who were genetically proven, but excluded if they disagree to join the present study. So far 14 patients were clinically examined and genetically confirmed in the nine families. In the present study on cognitive and affective analyses, 12 patients (seven men and five women) agreed to join the examination with average age at onset of 53.1 ± 3.2 years, average duration of 12.1 ± 5.2 years, and current average age at 65.1 ± 6.2 years. Their educations were all 12 years, representing graduation of high school at age 18 (Table 1). Their GGCCTG hexanucleotide repeat expansions were measured and estimated with Southern blot analysis [21]. Clinical severities for SCA and dementia were evaluated with the scale for the assessment and rating of ataxias (SARA, higher worse, [28,29]) and clinical dementia rating (CDR, higher worse) scale, respectively. CDR relies on informant interview, and therefore, CDR scores were obtained from spouses or children for the Asidan patients and from any family members for the control subjects. Standard cognitive function was evaluated with mini-mental state examination (MMSE, lower worse, [30]) and Hasegawa dementia score-revised (HDS-R, lower worse, [31]). Frontal cerebral function was evaluated with frontal assessment battery (FAB, lower worse, [32,33]) and Montreal cognitive assessment (MoCA, lower worse, [34]). The tests of verbal fluency in FAB and five-word recall in MoCA were evaluated at the end of 1 min. Depressive state was evaluated with geriatric depression scale (GDS, higher worse, [35]). Vitality and apathy were evaluated with vitality index (VI, lower worse, [36]) and apathy score (AS, higher worse, [37,38]), respectively. For cognitive and affective assessment, age-, gender-, and education period-matched subjects (n = 94, 65.2 ± 10.0 years old, 56 men/38 women, education period 11.7 ± 1.6 years) were also examined as normal controls.

Magnetic resonance imagings (MRIs) were examined for their brain with T1- and T2-weighted images by axial, coronal, and sagittal slices. Frontal cerebral atrophy was calculated with the ratio of sagittal lengths of intracranial space minus the brain/sagittal length of intracranial space at the slice level of maximum anterior horn in T2-weighted slice. Regional cerebral blood flow (rCBF) of the patients was measured with ^{99m}Tc-ECD-SPECT (single-photon emission tomography), and the data were analyzed with easy Z-score imaging system (eZIS) for obtaining standardized deviation of their rCBF [39]. eZIS score is expressed as SD (standard deviation) from standardized normal control value, and the eZIS score more than two represents an evident decline of rCBF. All the data in the present study are expressed with mean ± SD, and statistical analyses

Table 1 Clinical summary of 12 patients of Asidan (sorted by duration > onset age of the disease) and 94 control subjects

Case No.	1	2	3	4	5	6	7	8	9	10	11	12	Asidan total mean ± SD	Control subjects (n = 94, age-, gender-, and education period-matched)
Onset age (years old)	57	51	52	56	55	51	48	49	52	52	58	55	53.1 ± 3.2	–
Disease duration (years)	6	7	7	7	8	11	13	13	16	17	19	21	12.1 ± 5.2	–
Current age (years old)	63	58	59	63	63	62	61	62	68	69	77	76	65.1 ± 6.2	65.2 ± 10.0
Gender	F	F	M	M	M	M	F	F	F	M	M	M	M7/F5	M56/F38
Education (years)	12	12	12	12	12	12	12	12	12	12	12	12	12.0 ± 0.0	11.7 ± 1.6
SARA (0–40)	13.0	14.0	9.0	14.0	20.5	24.0	28.0	22.0	25.5	34.0	25.0	26.0	21.3 ± 7.4	–
CDR (0–3)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MMSE (0–30)	30	30	28	29	26	29	30	30	27	25	22	28	27.9 ± 2.5	28.2 ± 2.7
HDS-R (0–30)	30	30	29	29	28	29	28	30	30	25	22	28	28.2 ± 2.4	28.1 ± 3.0
FAB (0–18)	15	17	17	17	14	12	15	16	10	11	6	11	13.4 ± 3.4*	15.8 ± 2.7
MoCA (0–30)	29	28	28	23	18	19	26	26	22	16	18	15	22.3 ± 5.0*	25.5 ± 2.4
GDS (0–15)	11	6	6	0	13	10	3	8	2	13	10	5	7.3 ± 4.3**	3.2 ± 3.3
VI (0–10)	9	10	9	9	8	8	9	8	10	10	9	9	9.0 ± 0.7	9.7 ± 0.6
Apathy score (0–42)	21	27	14	4	26	17	14	7	3	17	38	39	18.9 ± 11.9*	8.4 ± 5.2
Frontal lobe atrophy (%)	1.2	1.8	1.6	1.6	2.3	4.5	4.8	4.6	4.4	5.5	4.8	4.6	3.5 ± 1.6	
eZIS (SD decrease)														
Area 24	<2	2.2	<2	< 2	2.4	2.5	2.6	c.n.o.	3.2	2.9	2.8	3.8	2.8 ± 0.5**	
Area 44–46	<2	<2	<2	2.4	3.1	3.4	3.1	c.n.o.	4.7	3.2	3.5	4.3	3.5 ± 0.7**	

CDR, clinical dementia rating; FAB, frontal assessment battery; GDS, geriatric depression scale; MMSE, mini-mental state examination; SARA, scale for the assessment and rating of ataxias; c.n.o.= consent not obtained.
(Bracket in each score represents the range of respective score.)
*P < 0.05 and **P < 0.01 compared with the control.

were performed by nonparametric Dunnet method with $P < 0.05$ as significant for comparison between two groups of the Asidan patients and the control subjects.

The present study was approved by the Ethical Committee of Graduate School of Medicine, Dentistry and Pharmaceutical Science, Okayama University.

Results

As shown in Table 1, the SARA scale ranged from 9.0 to 34.0 with average of 21.3 ± 7.4 (53.3 ± 18.5% of full scale 40). Eight items of SARA scale were analyzed with dividing into four symptom categories relating to gait disturbance (walking, standing, and heel-knee test), speech disturbance (speech), pure truncal ataxia (sitting stability), and upper limb ataxia (finger chasing test, finger-nose test, and diadochokinesis). The four symptom categories of SARA ataxia scale showed decreases in gait disturbance ranging 6–18 (sum full score 18) with 12.3 ± 4.0 (mean ± SD, 68.3% of full score), speech disturbance ranging 1–6 (full score 6) with 3.1 ± 1.2 (51.7% of full score), pure truncal ataxia ranging 0–4 (full score 4) with 1.7 ± 1.2 (41.5% of full score), and upper extremity (UE) ataxia ranging 2–7 (sum full score 12) with 4.2 ± 1.6 (35.0% of full score), respectively (Fig. 1).

Typical physical pictures of Asidan patients are shown in Fig. 2a–d. The patients usually developed gait unsteadiness first, followed by wide-based standing (Fig. 2a) and slurred speech with tongue fasciculation, then developed a swallowing difficulty with a marked tongue atrophy (Fig. 2b), and finally developed leg and hands/forearm atrophies with fasciculations and neurogenic electromyography findings (Fig. 2c and d). Most of the patients showed hyperreflexia in four limbs. On MRI examination, cerebellar vermis atrophy was found in all 12 patients, but was kept mild from the early (Fig. 2e, arrow) to the advanced (Fig. 2g, arrow) stages. Cerebellar hemisphere and brain stem (Fig. 2e and 1g, arrowheads) were usually spared. Frontal lobe atrophy ranged from 1.2 to 5.5%, which was related to the disease duration (Table 1). In cases with advanced stage, frontal lobe atrophies were evident (Fig. 2h, arrows) with corresponding anterior horn dilatation (Fig. 2h, arrowheads).

Analysis of eZIS with ^{99m}Tc-ECD-SPECT showed a decrease in cerebellar vermis and hemisphere from early (Fig. 2i and 1k, arrows) to advanced (Fig. 2l and 1n, arrows) stages. Brodmann cerebral cortical areas 44–46 (area 44, pars opercularis of inferior frontal gyrus; area 45, pars triangularis of inferior frontal gyrus; area 46, dorsolateral prefrontal cortex) showed a slight decrease

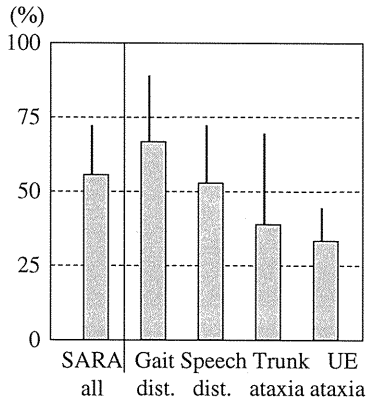

Figure 1 (Left) Average scale for the assessment and rating of ataxias (SARA) ataxia scale in all 12 Asidan patients, and (Right) those with dividing into four symptom categories relating to gait disturbance (walking, standing, and heel-knee test), speech disturbance (speech), pure truncal ataxia (sitting stability), and upper extremity (UE) ataxia (finger chasing test, finger-nose test, and diadochokinesis). Data are expressed as % of respective full scale. Note gait disturbance is the highest symptoms, and UE ataxia the lowest.

in rCBF at the early stage (Fig. 2j, arrowhead) with a progressive decrease to the advance stage (Fig. 2m, arrowhead). Although rCBF was not reduced in Brodmann area 24 (ventral frontal cingulate gyrus) at the early stage (Fig. 2k, arrowhead), it showed an evident decrease at the advanced stage (Fig. 2n, arrowhead) with eZIS score of 2.6-3.8 SD of the control after 13 years of onset (Table 1, bottom). Asidan patients showed a statistically significant decrease in rCBF in eZIS score in Brodmann areas 24 (2.8 ± 0.5, $^{**}P < 0.01$) and 44–46 (3.5 ± 0.7, $^{**}P < 0.01$) (Table 1).

Cognitive and affective functions in the Asidan patients and 94 controls are summarized in the Table 1. Amongst these functions, FAB ($^{*}P < 0.05$), MoCA ($^{*}P < 0.05$), GDS ($^{**}P < 0.01$), and apathy ($^{*}P < 0.05$) scores were significantly decreased in the Asidan patients compared with the controls. The number of hexonucleotide GGCCTG repeat expansion of these patients ranged from 1700 to 2300 (2120 ± 215, mean ± SD). As shown in Fig. 3, there was no correlation between the number of GGCCTG expansion and MMSE (dark circles and solid line, $r = 0.20265$, $P > 0.05$) or FAB (dark squares and dotted line, $r = 0.10909$, $P > 0.05$) cognitive scores. Correlation between the number of GGCCTG expansion and age at onset was not also found ($r = 0.28682$, $P > 005$, data not shown). In contrast, there was slight correlations between MMSE and the disease duration (years, Fig. 4, left panel, dark circles and solid line, $r = -0.43287$, $^{*}P < 0.05$) or SARA scale (Fig. 4, right panel, dark circles and solid line, $r = -0.39144$, $^{*}P < 0.05$) and stronger correlations between FAB and the disease course (years, Fig. 4, left panel, dark squares and dotted line, $r = -0.76475$, $^{**}P < 0.01$) and between MoCA and SARA scale (Fig. 4, right panel, dark diamonds and dotted line, $r = -0.69269$, $^{**}P < 0.01$).

In six FAB subcategories, motor programming was the most impaired to 1.58 ± 1.31 (mean ± SD, 52.7% of full score 3, $^{**}P < 0.01$ vs. control = 2.83 ± 0.45), then verbal fluency to 1.83 ± 0.94 (61.0% of full score 3, $^{*}P < 0.05$ vs. control = 2.37 ± 0.56), similarities/conceptualization to 1.83 ± 1.03 (61.0% of full score 3, $^{*}P < 0.05$ vs. control = 2.78 ± 0.54), Go/no Go selection to 2.42 ± 0.79 (80.7% of full score 3, P = ns vs. control = 2.31 ± 0.97), conflicting instructions to 2.67 ± 0.65 (89.0% of full score 3, P = ns vs control = 2.90 ± 0.41), and prehension behavior to 2.92 ± 0.29 (97.3% of full score 3, P = ns vs. control = 2.99 ± 0.31), respectively (Fig. 5, left panel). Verbal fluency test in FAB took 40–50 s in the control subjects and <35 s in the Asidan patients.

In seven MoCA subcategories, five-word recall was the most impaired to 1.92 ± 1.93 (mean ± SD, 38.4% of full score 5, $^{**}P < 0.01$ vs. control = 3.19 ± 1.48), then language to 1.50 ± 1.00 (50.0% of full score 3, $^{*}P < 0.05$ vs. control = 1.84 ± 0.66), visuospatial executive function to 3.08 ± 1.51 (61.6% of full score 5, $^{*}P < 0.05$ vs. control = 4.33 ± 0.81), attention to 4.58 ± 1.51 (76.3% of full score 6, P = ns vs. control = 5.11 ± 0.96), abstraction to 1.58 ± 0.67 (79.0% of full score 2, P = ns vs. control = 1.85 ± 0.39), naming to 2.67 ± 0.65 (89.0% of full score 3, P = ns vs. control = 2.86 ± 0.38), and orientation to 6.00 ± 0.00 (100.0% of full score 6, P = ns vs. control = 5.97 ± 0.18), respectively (Fig. 5, right panel). The five-word recall test in MoCA took 30–40 s in the control subjects and <30 s in the Asidan patients.

Discussion

The present study first showed that the patients with intronic hexanucleotide GGCCTG expansion with NOP56 mutation (Asidan) demonstrated normal cognitive scores measured by standard screening cognitive tests (MMSE and HDS-R) and CDR, but that their frontal lobe function detected with FAB and MoCA demonstrated a significant decline compared with age-, gender-, and education period-matched controls (Table 1, Fig. 3–5). CDR was insensitive to this type of cognitive impairment (Table 1). Important contributions of cerebellar activity for normal cognitive function have been pointed out by many reports [40–45], and in fact, cognitive reductions have also been reported in

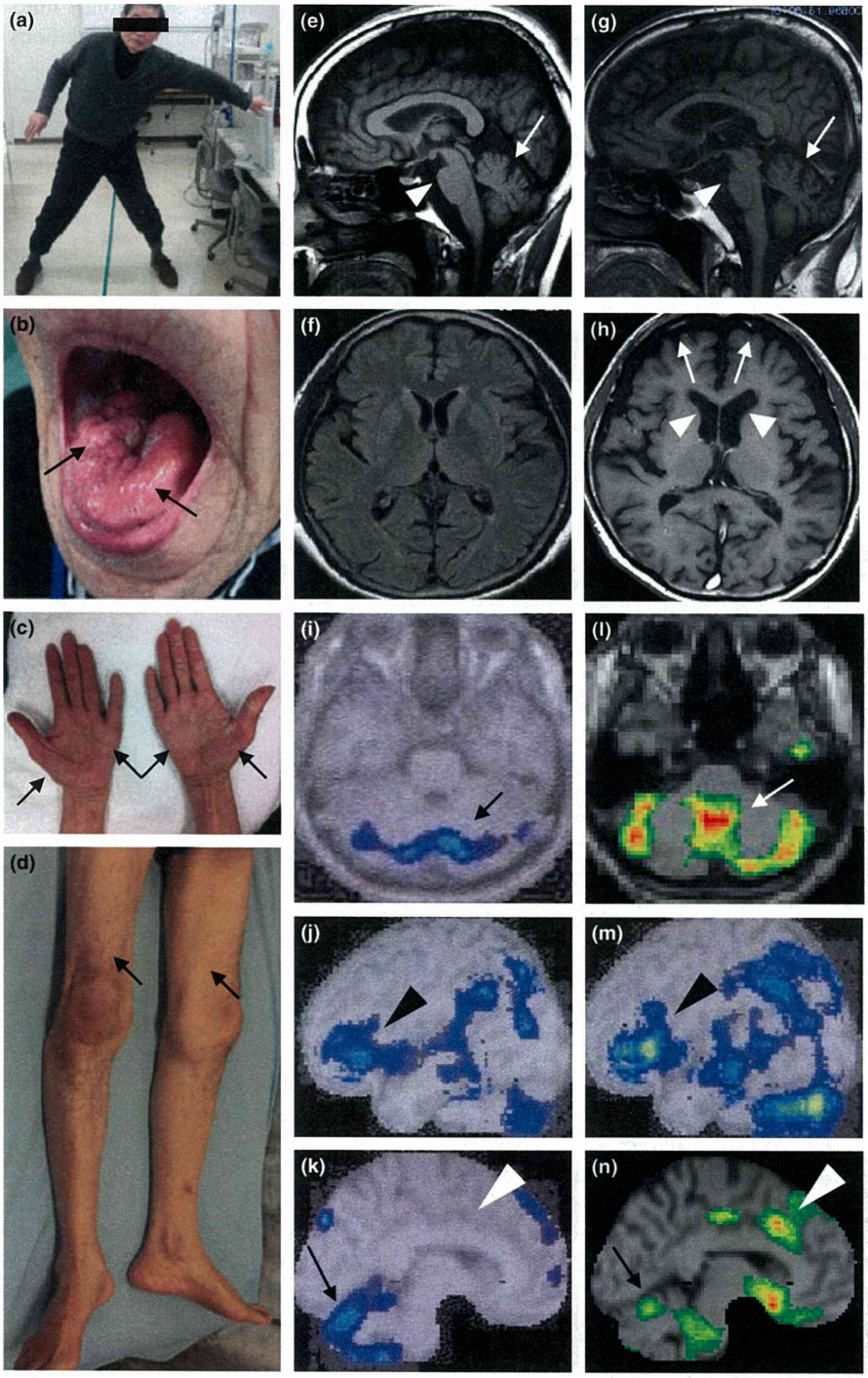

Figure 2 (a–d) Typical physical pictures of Asidan patients with a strong standing/walking disability (a), tongue atrophy with fasciculation (b, arrows), thenar and hypothenar atrophies (c, arrows), and leg atrophy with fasciculation (arrows). Magnetic resonance imaging MRI images with an early stage patient (e, f) and an advanced stage patient (g, h). Note cerebellar atrophies (e and g, arrows) without brain stem atrophy (e and g, arrowheads), and frontal lobe atrophy in the advanced stage (h, arrows) with corresponding anterior horn dilatation (h, arrowheads). Examples of eZIS analysis with ^{99m}Tc-ECD-SPECT in an early stage patient (i–k) and an advanced stage patient (l-m) show rCBF reduction in cerebellar vermis and hemisphere from the early (i and k, arrows) to advanced stage (l and n, arrows) and also show the progressive rCBF decline in Brodmann areas 44–46 (j and m, arrowheads) with decline in Brodmann area 24 only at the advanced stage (k and n, arrowheads).

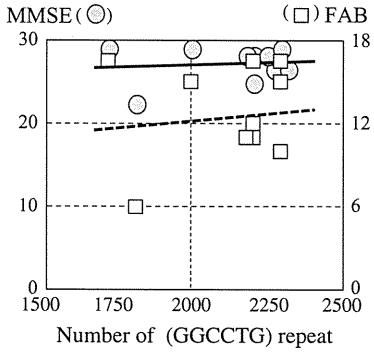

Figure 3 Plots of the number of GGCCTG repeat expansion and the scores of mini-mental state examination (MMSE) (dark circles and solid line) and FAB (dark squares and dotted line). No correlation between number of GGCCTG repeat expansion and MMSE or FAB cognitive score.

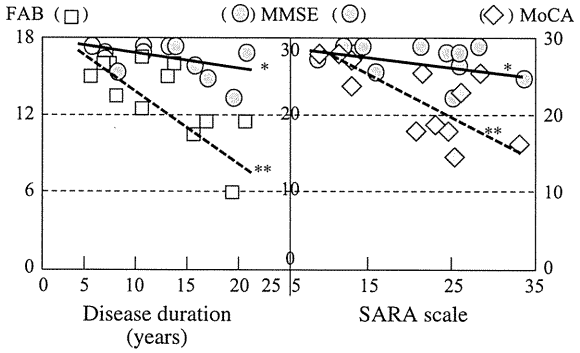

Figure 4 Slight correlations between MMSE and the disease course (left panel, dark circles and solid line, $r = -0.43287$, $^{*}P < 0.05$)) and scale for the assessment and rating of ataxias (SARA) score (right, dark circles and solid line, $r = -0.39144$, $^{*}P < 0.05$), and stronger correlations between FAB and the disease course (left, dark squares and dotted line, $r = -0.76475$, $^{**}P < 0.01$) and between MoCA and SARA score (right, dark diamonds and dotted line, $r = -0.69269$, $^{**}P < 0.01$).

SCA1, 2, 3, and 6 especially in executive frontal function [6,46,47]. A recent report found a decline of frontal attentional and executive functions with mild depressive mood in 4 types of hereditary SCA with a severer reduction in SCA1 > SCA2 > SCA3 > SCA6 in this order [48]. Recent studies suggested a more impact of brain stem (SCA 1–3) on attentional/executive declines and depressive mood than simple cerebellar dysfunction (SCA6) [47,48]. Those studies were mainly performed with hereditary SCA with exonic CAG repeat expansion, but not intronic hexanucleotide expansion such as Asidan.

Although MMSE and FAB scores of the present Asidan patients were not correlated with the numbers of GGCCTG hexanucleotide repeat expansion (Fig. 3),

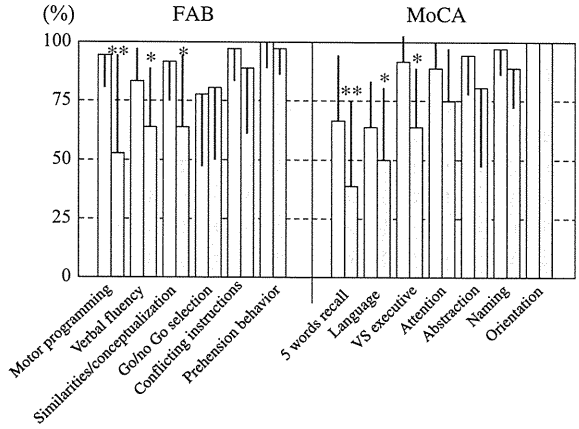

Figure 5 Each six subcategories in FAB (left panel) and seven in MoCA (right panel) of the control (white bars) and Asidan patients (dark bars). Data are expressed as % mean ± SD of the respective full score. Note strong impairments in motor programming, verbal fluency, and similarities/conceptualization as FAB subcategory and in five-word recall, language, and visuospatial (VS) executive function as MoCA subcategory. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs control.

MMSE were slightly correlated with the disease duration and SARA scale (Fig. 4) with age of an alternative covariate (Table 1, case #11, MMSE 22 at age 77). Their frontal lobe dysfunction in FAB and MoCA were well correlated with the disease duration and SARA scale, respectively (Fig. 4). Amongst 6 FAB subcategories, motor programming, verbal fluency, and similarities/conceptualization were decreased to 52.7–61.0% of the respective full score (Fig. 5, left), and five-word recall, language, and visuospatial executive function were decreased to 38.4–61.6% of the respective full score amongst 7 MoCA subcategories (Fig. 5, right). These declines in the frontal lobe function were correlated with the frontal lobe atrophy and respective decline of their rCBF especially in Brodmann areas 24 and 44–46 (Table 1, Fig. 2f–n). These results suggest that the reduction of frontal executive function may be a common cognitive characteristic between exonic CAG repeat expensions (SCA 1, 2, 3, and 6) and intronic hexanucleotide expansion such as Asidan. Because Abrahams *et al.* ([49,50]) indicated an importance of motor control for evaluating verbal fluency in PET activation study, a motor control is preferable when there is any evidence of motor impairment. In the present experiment, the Asidan patients finished verbal fluency test in FAB and five-word recall test in MoCA within 30–35 s with no more words coming out until 1 min of examination, which suggests an actual disturbance of verbal fluency and may partly excuse the lack of motor control in this non-activation study.

On the other hand, affective characteristics of these hereditary SCA have not been fully described. Compared with age-, gender-, and education period-matched controls, GDS score in Asidan patients was worse to 7.3 ± 4.3 (Table 1). We have previously reported a depressive state of SCA1 and 3 patients and their family members detected by self-rating depression scale (SDS, [51,52]) in an occasion of genetic testing [53]. A recent report confirmed such a mild depressive mood in exonic CAG expansion of hereditary SCAs (mainly SCA1, 2, and 3) [48]. Unlike another type of pure cerebellar hereditary ataxia SCA6, the moderate increase in GDS to 48.7% of full score suggests a subclinical involvement of the brain stem (such as Raphe nucleus) in these Asidan patients (Table 1). Our present study also first showed that a slight decrease of VI and a significant increase of AS than the control (Table 1), suggesting a slight unwillingness (apathy) in these Asidan patients. Because such VI and AS were not examined in the past for hereditary SCA, a possible presence of this apathy in other types of hereditary SCAs may be a future subject to be studied.

In addition to pure cerebellar ataxia, Asidan patients also develop typical lower/upper motor neuron involvement clinically identical with ALS. Thus, a comparison of the cognitive and affective functions of Asidan with MND/ALS is also important. Subtle executive deficits are found in a large proportion of ALS, whilst only a small proportion shows fronto-temporal dementia (FTD). Although ALS patients developed affective and behavioral changes [17,18,25,54], they showed only a slight intelligent decline detected by Raven's Coloured Matrices test [55]. Emotional lability in ALS may be related to glutamatergic and serotoninergic neurotransmitter systems [56], and Taylor *et al.* [57] recently established that the prevalence of depression in ALS is not lower than that of patients with other motor disorders. Hereditary ALS revealed reductions of verbal fluency and executive function and high AS [58]. Previous reports showed reductions of rCBF in the fronto-temporal, fronto-parietal, and prefrontal cerebral cortex of ALS [59,60] or no such reductions [49]. Of interest was a subtle rCBF decrease in the anterior cingulate gyrus of ALS without dementia with 3D-SSP [60] similar to our result (Fig. 2n, arrowhead).

Previous studies suggested an active participation of cerebellum both in cognitive and affective functions independent from its dedicated motor control [50,61,62]. PET activation study showed rCBF reduction in areas 4, 8, 9, 10, and 46 [49]. Our Asidan cases did not show general decreases in the fronto-parieto-temporal cerebral cortices, but showed an evident decrease in the particular cerebral cortical areas of Brodmann areas 24 (ventral frontal cingulate gyrus) and 44–46 (pars opercularis of inferior frontal gyrus, pars triangularis of inferior frontal gyrus, dorsolateral prefrontal cortex) (Fig. 2j–n, arrowheads), which suggest that these decreases of rCBF are not simply related to ALS pathology but a unique finding in this Asidan mutation. There are number of limitations in the present study. Several of the FAB and MoCA subscores rely on motor coordination, which may be impaired by the patients' cerebellar deterioration. Therefore, we must be cautious in attributing such changes to purely frontal lobe function, even if there are some subtle frontal neuroimaging changes. We would also conduct future studies either to use tests that control all forms of motor impairment or to perform functional neuroimaging tests such as fMRI simultaneously with neuropsychological testing.

In summary, the present study described cognitive and affective impairments of a novel hereditary cerebellar ataxia with motor neuron involvement (Asidan) in relation to brain imagings. The decline of frontal executive function was related to their disease duration and SARA scale. They also demonstrated mild depression and apathy. These data were not reported before in other hereditary SCAs with exonal CAG expansions or ALS.

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Title page

Distribution of Moyamoya Disease Susceptibility Polymorphism p.R4810K in *RNF213* in East and Southeast Asian Populations

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Abstract

Moyamoya disease is an idiopathic vascular disorder of the intracranial arteries. We previously identified *ring finger 213* (*RNF213*) as the strongest susceptibility gene for moyamoya disease in East Asian people by a genome-wide linkage analysis and exome analysis. The coding variant p.R4810K in *RNF213* was strongly associated with moyamoya disease in Japanese (odds ratio: 338.94, P= 1.05 x 10^{-100}) and Korean (odds ratio: 135.63, P= 7.59 x 10^{-27}) people, and much less strongly associated in Chinese people (odds ratio: 14.70, P= 2.63 x 10^{-5}). In the present study, we investigated the distribution of p.R4810K in *RNF213* in 2,508 participants from East and Southeast Asian countries using a TaqMan probe. p.R4810K was detected at an allele frequency of about 1.00% in 4 of 11 investigated locations in China. In contrast, p.R4810K was detected homogeneously at relatively high frequencies of 1.00–1.72% in all investigated locations in Korea and Japan, including Okinawa. p.R4810K was not detected in Southeast Asian populations. The susceptibility population to moyamoya disease was estimated to be 16.16 million people in East Asian countries, i.e., 11.41 million Chinese, 1.36 million Korean and 3.39 million Japanese people. The number of patients with moyamoya disease, which was estimated at approximately one per 300 carriers of p.R4810K, was considered to be 53,800 in East Asian populations.

Introduction

Moyamoya disease [MIM 607151] is a rare idiopathic progressive disorder characterized by occlusive lesions in the supraclinoid internal carotid artery and its main branches in the circle of Willis. A fine vascular network that resembles "puffs of smoke" ("moyamoya" in Japanese) develops to compensate for the blood flow around the occlusive regions.[12,13] Moyamoya disease has the highest prevalences in East Asian countries such as Japan, Korea and China compared with other countries.[2,7,10]

We had identified a susceptibility locus for familial moyamoya disease on 17q25.3.[11] Significant associations with genes in this region had been reported,[4,8] but rigorous identification awaited further study. The *ring finger 213* (*RNF213*) gene was finally identified as a susceptibility gene for moyamoya disease by the rigorous traditional positional cloning approach, exome analysis and functional analysis using zebrafish.[9] Overall, 74.5% of East Asian patients with moyamoya disease carry the rare founder variant p.R4810K in *RNF213*. However, a gradient of prevalence of patients with moyamoya disease was observed, with 90% in Japanese, 79% in Korean and 23% in Chinese people. On the other hand, no gradient of prevalence was observed in three general populations. In total, 2.4% of East Asian general populations carry p.R4810K. The minor allele frequencies of p.R4810K in general populations were 1.4%, 1.3% and 1.0% for Japanese, Korean and Chinese people, respectively.[9] These similar allele frequencies of p.R4810K in the three East Asian general populations could not explain the lower frequency of Chinese patients with moyamoya disease compared with Japanese and Korean patients with moyamoya disease. In Japan, moyamoya disease occurs in approximately one of 300 carriers.[8] If so, this discrepancy in the allele frequency between cases and controls in Chinese people might be attributable to

selection bias in the general populations in the previous study.[9] The primary aim of the present study was to estimate the susceptibility population to moyamoya disease by conducting a large scale screening of p.R4810K in general populations in East and Southeast Asia. The secondary aim was to estimate the patients with moyamoya disease associated with p.R4810K in *RNF213*.

Materials and Methods

Ethical statement

Ethical approval for this study was given by the Institutional Review Board and Ethics Committee of Kyoto University School of Medicine, Kyoto University, Japan (approval number: G140; approval date: 10/18/2004), by the Seoul National University Hospital Institutional Review Board (approval number: H0507-509-153; approval date: 8/24/2005). The study participants were recruited in these institutes. The subjects, who participated in this study after 2000 and were recruited by School of Medicine, Kyoto University. All subjects gave written informed consent. Those, who donated blood samples before 2000 who were recruited by Tohoku University School of Medicine, or Akita University School of Medicine, or Kyoto University School of Medicine, gave verbal informed consents. The application of blood samples which were donated before 2000 for genetic analysis were also approved by the Institutional Review Board and Ethics Committee of Kyoto University School of Medicine, Kyoto University, Japan (approval number: G140; approval date: 10/18/2004).

Subjects

A total of 2,508 unrelated participants from East Asian and Southeast Asian

countries were recruited, comprising 587 Chinese, 294 Korean, 1474 Japanese, 103 Vietnamese and 50 Filipino people. The Japanese participants were recruited from mainland Japan including Hokkaido, Honshu, Shikoku, Kyushu and Okinawa. The Korean participants were recruited from five locations (Table 1). The Chinese participants were recruited from 11 locations (Table 1). Blood samples were obtained from two sources. One source was the Kyoto University Human Specimen Bank, which collected blood samples from the 1990s and 2000s as previously described.[5,6] These blood samples included Chinese, Korean, Japanese and Philippine samples. The other source involved samples collected in an international collaboration that included Vietnam and Seoul, South Korea.

DNA isolation

Peripheral blood (10 mL) was collected from all participants. Genomic DNA was extracted from the blood samples using a QIAamp DNA Blood Mini Kit (Qiagen, Maryland,USA) according to the manufacturer's protocol. The quality and concentration of the extracted DNA were assessed using an infinite M200 PRO (TECAN, Tokyo, Japan). The DNA was stored in a freezer at –20°C until analysis.

Genotyping of p.R4810K

Genotyping of p.R4810K in all participants was conducted using a TaqMan probe (Custom TaqMan SNP Genotyping Assays; Applied Biosystems, Foster City, CA, USA) and a 7300/7500 Real-Time PCR System (Applied Biosystems) according to the manufacturer's protocols. Briefly, the PCR amplifications were performed with 1–20 ng of purified genomic DNA, 0.1 μL of 40× SNP Genotyping Assay, 6.25 μL of 2×

TaqMan Universal PCR Master Mix, No AmpErase UNG and 5.15 μL of DNase-free water. The final reaction volume was 12.5 μL/well in 96-well plates. The standard protocol for the cycling conditions was hold for 10 min at 95°C for AmpliTaq Gold enzyme activation, followed by 40 cycles of 15 sec at 92°C for denaturation and 1 min at 60°C for annealing/extension. After each PCR amplification, an endpoint plate read was performed using the Real-Time PCR System (Applied Biosystems). The associated Sequence Detection System (SDS) software uses the fluorescence measurements taken during the plate read to plot fluorescence (Rn) values based on the signals from each well. The plotted fluorescence signals indicate which alleles are in each sample.

Results

Demographic characteristics of the participants

The demographic features of all participants in this study are shown in Table 1. The sampled cities covered 234.02 million people. A total of 2,508 participants from three East Asian countries and two Southeast Asian countries were recruited from 1987 to 2009. Briefly, the recruited subjects comprised 587 participants from 11 Chinese locations, 294 from five Korean locations, 1474 from five Japanese locations, 103 from one Vietnamese location and 50 from one Philippine location. Most of the participants were females, with the exception of 608 Japanese males and 80 Korean males. Information on age was only available for the participants from certain locations. Angiography was only conducted for 384 Japanese and 46 Korean participants.

Geographic distribution of p.R4810K in East and Southeast Asian populations

The geographic distributions of p.R4810K in the different ethnicities are shown in Table 2 and Figure 1. p.R4810K was detected in East Asian populations, but not in Southeast Asian populations. p.R4810K was detected at allele frequencies of 0.43%, 1.36% and 1.36% in the Chinese, Korean and Japanese populations, respectively. p.R4810K was detected at an allele frequency of about 1.00% in 4 of 11 investigated locations in China, suggesting that the distribution of p.R4810K was heterogeneous and limited to certain specific locations in China. In contrast, p.R4810K was homogenously spread throughout Korea and Japan at relatively high allele frequencies of 1.00–1.72%. p.R4810K was not detected in 49 Taiwanese, 103 Vietnamese and 50 Filipino participants.

Discussion

In the present study, we confirmed the presence of similar allele frequencies of p.R4810K in *RNF213* in Japanese and Korean general populations. However, the expansion of the population examined revealed that the allele frequency of p.R4810K in Chinese population was 0.43%, being one-third of the frequency in Japanese or Korean populations. Therefore, our previous observation was considered to be attributable to selection bias.[9] The lower prevalence of p.R4810K in the Chinese general population might be proportional to the lower carrier rate (i.e., 23%) in Chinese patients. In accordance with this observation, while a single dominant polymorphism is associated with Japanese or Korean patients, various polymorphisms in *RNF213* may be associated with Chinese patients. In fact, we observed five additional mutations in Chinese patients while no additional mutations were observed in Korean or Japanese patients.[9]

In the present study, magnetic resonance angiography was only conducted for

limited numbers of participants. This limitation did not affect our results because of the very low prevalences of moyamoya disease in the general populations. The estimated total numbers of carriers in the three ethnicities were 11.41 million for Chinese, 1.36 million for Korean and 3.39 million for Japanese people. An assumption that moyamoya disease occurs in one of 300 carriers with p.R4810K[8, 9] yields estimated numbers of patients with moyamoya disease attributable to p.R4810K of 38,000 in Chinese, 4,500 in Korean and 11,300 in Japanese people. Although these numbers provide rough and minimum estimates because only p.R4810K variant is counted, a large number of patients suggest that more attention should be paid to moyamoya disease in East Asian countries.

Differences in the clinical profiles of moyamoya disease have been recognized among the three countries.[1,3] However, the differences between the clinical profiles of Korean and Japanese patients are unlikely to be explained by genetic differences. It is also speculated that additional factor(s) to the genetic factor are needed to explain the variation in the clinical phenotypes and low penetrance among carriers.[8, 9] Therefore, unknown modifier factor(s) may also be attributable to the differences in the clinical phenotypes. Further research is needed to identify such factor(s), which may also be crucial for the development of moyamoya disease among carriers of p.R4810K in *RNF213*.

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Figure legend

Figure 1. Locations of the sampling sites in East and Southeast Asian countries.