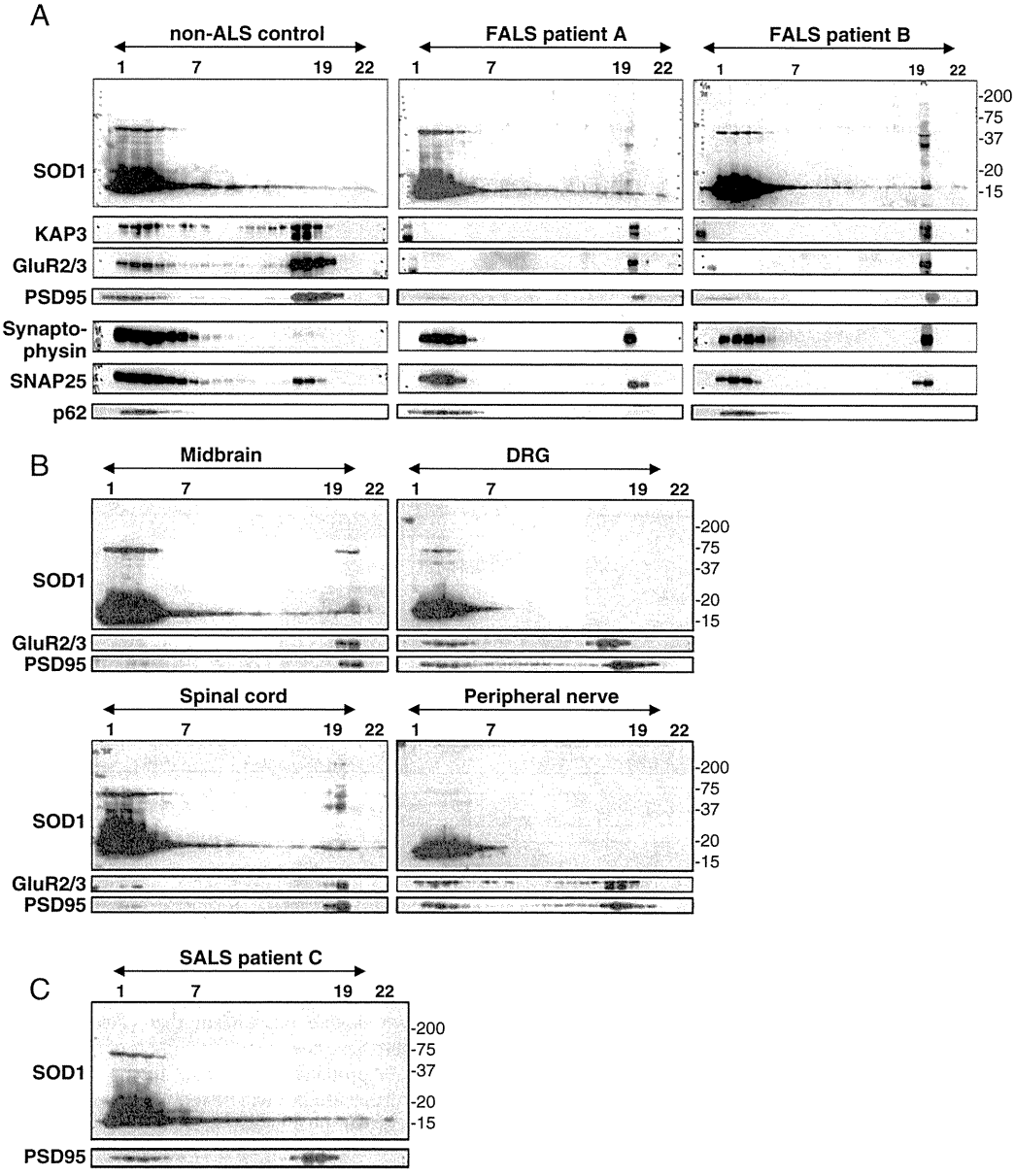

Fig. 1. Synaptic molecules are associated with misfolded SOD1 (SOD1(L126S)) in motor regions of mutant SOD1-associated FALS brain tissues. Fractions obtained from Nycodenz density-gradient centrifugation of lysates of precentral gyrus of cerebral cortex tissue from a non-ALS control and two mutant SOD1-associated FALS cases (both cases have L126S mutation in SOD1) (A), or indicated tissues lysates from a mutant SOD1-associated FALS brain (B), or lysate of precentral gyrus of cerebral cortex from a sporadic ALS case (C) were analyzed for the presence of indicated proteins (SOD1, KAP3, GluR2/3, PSD95, Synaptophysin, and SNAP25) by immunoblot analysis. Each lane (left to right) represents an aliquot collected from the top. Note that misfolded SOD1 species migrated around fraction No.19, together with synaptic components, only when motor regions of mutant SOD1-linked FALS brain were analyzed.

SOD1-linked FALS cases and excitotoxic mechanisms, we employed a simplified cellular FALS model that we developed previously. In this model, NG108-15 cells overexpressing FALS-related mutant SOD1 become differentiated to cholinergic neuron-like cells in response to elevated levels of cAMP and dexamethasone [5]. Misfolded SOD1 is detected in the cells by exposing the cells to oxidative stress. Here we applied glutamate to the cells to examine if hyperstimulation by glutamate causes formation of protein aggregates containing misfolded SOD1 and synaptic proteins. We found, by a density gradient centrifugation approach similar to what we used for patient tissue analysis, that increased amount of synaptotagmin, a synaptic vesicle protein, and SNAP25, a presynaptic terminal molecule, showed shifted mobility in the density gradient by applying glutamate in culture (Fig. 2). The shifted mobility was observed together with increased levels of misfolded SOD1. We found these results in cells expressing SOD1 bearing L126 mutation identified in the FALS cases that we analyzed in the current study, but not wild-type SOD1 expressing cells (Fig. 2). These results suggested that excitotoxicity caused by glutamate can increase misfolding of mutated SOD1 and promote formation of protein aggregation involving misfolded SOD1 together with synaptic molecule.

4. Discussion

In this study, we tried to identify molecules that associate with misfolded SOD1 in mutated SOD1-linked human FALS patient tissues

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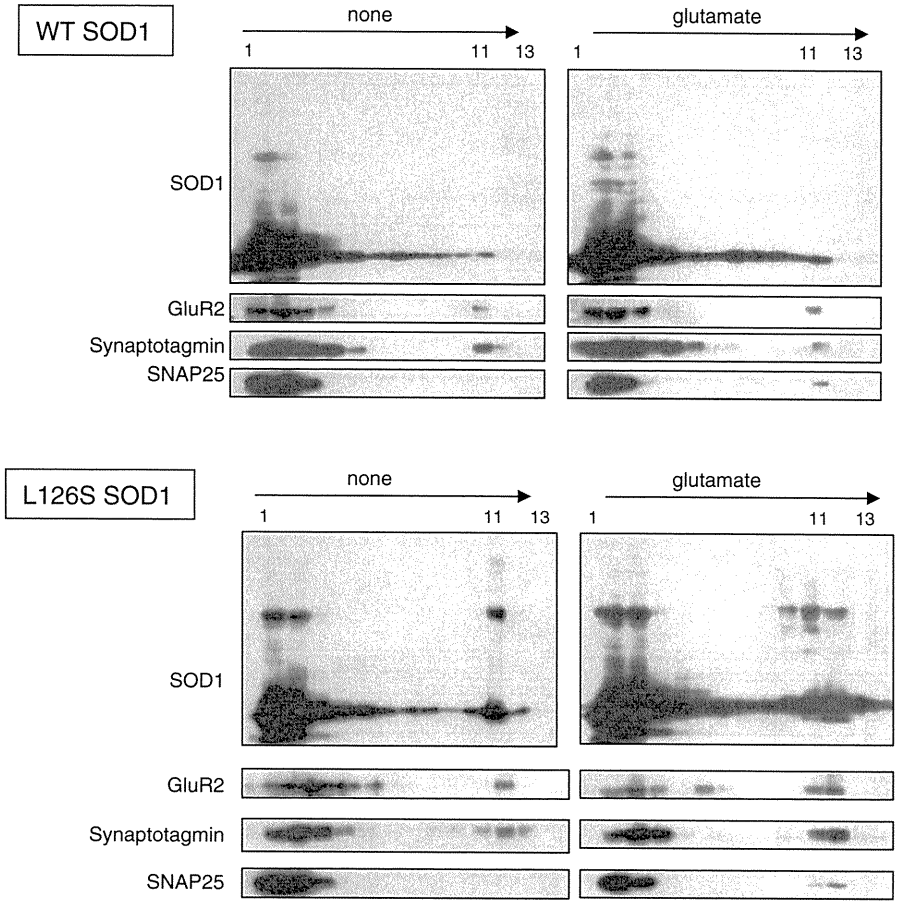

Fig. 2. Association of misfolded SOD1 with synaptic components can be induced by excitotoxic hyperstimulation. Fractions obtained from Nycodenz density-gradient centrifugation of lysates of NG108-15 cells stably expressing WT SOD1 or SOD1 bearing L126S mutation were analyzed for the presence of indicated proteins (SOD1, GluR2/3, Synaptotagmin, and SNAP25) by immunoblot analysis. Note that increased amount of misfolded SOD1 species, together with synaptic components, migrated around fraction No. 11 after hyperstimulation by glutamate.

to find that misfolded SOD1 forms very high density molecular complexes together with pre- and post-synaptic proteins in the synaptic terminal region. Surprisingly, this phenomenon seems specific to mutated SOD1-linked FALS cases, since we did not observe similar molecular complexes formed in SALS patient tissues. We further showed that membrane depolarization that mimics synaptic hyperactivation/excitotoxicity can promote formation of protein aggregates containing synaptic components. While we did observe the formation of molecular complexes containing synaptic components in our cellular FALS model, the size of the molecular complexes seemed smaller compared with those observed in patient tissue lysates. This may be due to the facts that the differentiated NG108-15 cell we used here do not form differentiated synaptic structures or synaptic contacts with neighboring cellular processes, and that the cells were exposed only to experimentally possible transient excitotoxic stresses.

We have previously shown that misfolded SOD1 associates with KAP3, a kinesin-2 motor complex component [5] Kinesin-2 is required for anterograde axonal transport of various molecules including choline acetyltransferase [13,14], and disturbed Kinesin-2 function may therefore explain a part of early pathogenesis of mutated SOD1-linked FALS. Here we identified another pathogenic mechanism, i.e., dysfunction of synaptic molecules directly caused by mutant SOD1. While synaptic dysfunction is observed during pathogenesis of not only mutant SOD1-linked FALS but also ALS of other etiologies [1], the large molecular complex involving various synaptic components which we identified this time seems specific to mutated SOD1-linked human FALS cases. This may suggest that mutant SOD1-linked FALS has its own unique mechanism that causes synaptic dysfunction. Thus far there have been limited numbers of reports analyzing molecular details of proteins accumulated in the synaptic region [15] and therefore further studies will be required for understanding of mechanistic diversity of synaptic pathology in ALS.

5. Conclusion

Here we report identification of various pre- and post-synaptic molecules forming molecular complexes with misfolded SOD1 in mutant SOD1-associated FALS patient tissues as well as in cellular FALS models. The results suggest that inhibition of synaptic release mechanism by association of misfolded SOD1 with synaptic molecules plays a role in the dysfunction of FALS.

Acknowledgements

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References

[1] Boillee S, Vande Velde C, Cleveland DW. ALS: a disease of motor neurons and their nonneuronal neighbors. Neuron 2006;52:39–59.

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[2] Pasinelli P, Brown RH. Molecular biology of amyotrophic lateral sclerosis: insights from genetics. Nat Rev Neurosci 2006;7:710–23.
[3] Shaw BF, Valentine JS. How do ALS-associated mutations in superoxide dismutase 1 promote aggregation of the protein? Trends Biochem Sci 2007;32:78–85.
[4] Barber SC, Mead RJ, Shaw PJ. Oxidative stress in ALS: a mechanism of neurodegeneration and a therapeutic target. Biochim Biophys Acta 2006;1762:1051–67.
[5] Tateno M, Kato S, Sakurai T, Nukina N, Takahashi R, Araki T. Mutant SOD1 impairs axonal transport of choline acetyltransferase and acetylcholine release by sequestering KAP3. Hum Mol Genet 2009;18:942–55.
[6] Hirokawa N, Takemura R. Molecular motors and mechanisms of directional transport in neurons. Nat Rev Neurosci 2005;6:201–14.
[7] Tateno M, Sadakata H, Tanaka M, Itohara S, Shin RM, Miura M, et al. Calcium-permeable AMPA receptors promote misfolding of mutant SOD1 protein and development of amyotrophic lateral sclerosis in a transgenic mouse model. Hum Mol Genet 2004;13:2183–96.
[8] Kruman II, Pedersen WA, Springer JE, Mattson MP. ALS-linked Cu/Zn-SOD mutation increases vulnerability of motor neurons to excitotoxicity by a mechanism involving increased oxidative stress and perturbed calcium homeostasis. Exp Neurol 1999;160:28–39.
[9] Gurney ME, Fleck TJ, Himes CS, Hall ED. Riluzole preserves motor function in a transgenic model of familial amyotrophic lateral sclerosis. Neurology 1998;50:62–6.
[10] Rothstein JD, Van Kammen M, Levey AI, Martin LJ, Kuncl RW. Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis. Ann Neurol 1995;38:73–84.
[11] Shaw PJ, Eggett CJ. Molecular factors underlying selective vulnerability of motor neurons to neurodegeneration in amyotrophic lateral sclerosis. J Neurol 2000;247(Suppl. 1):I17–27.
[12] Van Damme P, Leyssen M, Callewaert G, Robberecht W, Van Den Bosch L. The AMPA receptor antagonist NBQX prolongs survival in a transgenic mouse model of amyotrophic lateral sclerosis. Neurosci Lett 2003;343:81–4.
[13] Baqri R, Charan R, Schimmelpfeng K, Chavan S, Ray K. Kinesin-2 differentially regulates the anterograde axonal transports of acetylcholinesterase and choline acetyltransferase in *Drosophila*. J Neurobiol 2006;66:378–92.
[14] Ray K, Perez SE, Yang Z, Xu J, Ritchings BW, Steller H, et al. Kinesin-II is required for axonal transport of choline acetyltransferase in *Drosophila*. J Cell Biol 1999;147:507–18.
[15] Sasaki S, Takeda T, Shibata N, Kobayashi M. Alterations in subcellular localization of TDP-43 immunoreactivity in the anterior horns in sporadic amyotrophic lateral sclerosis. Neurosci Lett 2010;478:72–6.

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BRIEF RESEARCH COMMUNICATION

AMERICAN JOURNAL OF medical genetics PART B
Neuropsychiatric Genetics

TRPM7 Is Not Associated With Amyotrophic Lateral Sclerosis-Parkinsonism Dementia Complex in the Kii Peninsula of Japan

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Amyotrophic lateral sclerosis-parkinsonism dementia complex (ALS/PDC) is a distinct neurodegenerative disorder characterized by ALS pathology with neurofibrillary tangles (NFTs) in the spinal cord and brain. Recent clinical studies have revealed a high incidence and a high familial occurrence of ALS/PDC in both Guam and the Kii peninsula of Japan, suggesting a strong genetic predisposition to this disorder. The T1482I variant (rs8042919) of *TRPM7* gene which is suggested to play roles in regulating the cellular homeostasis of Ca^{2+}, Mg^{2+}, and trace metals, has recently been reported to be associated with Guamanian patients with ALS/PDC. To investigate whether *TRPM7* is associated with Kii ALS/PDC, we conducted parametric linkage analyses of the *TRPM7* locus in a large extended family with ALS/PDC. Linkage analysis did not reveal any evidence supporting the linkage to the *TRPM7* locus. Resequencing of the entire coding region of *TRPM7* did not reveal any pathogenic mutations in an affected individual in this family. The allele frequencies of the T1482I in affected individuals in this family or in those from other families are not significantly different from those in regional controls or those in HapMap-JPT samples. These results indicate that *TRPM7* is not associated with ALS/PDC in the Kii peninsula of Japan. © 2009 Wiley-Liss, Inc.

Key words: ALS/PDC; extended family; *TRPM7* gene; linkage analysis; resequencing

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Am J Med Genet Part B 153B:310–313.

INTRODUCTION

Amyotrophic lateral sclerosis and parkinsonism-dementia complex (ALS/PDC) is a unique form of ALS highly prevalent in the island of Guam, southern West New Guinea, and the Kii peninsula of Japan [Kimura, 1961; Elizan et al., 1966; Gajdusek and Salazar, 1982]. Both Guamanian and Japanese patients with ALS/PDC are pathologically characterized by neurofibrillary tangles (NFTs) in the brain and spinal cord in addition to the ALS pathology affecting the upper and lower motor neurons [Hirano et al., 1961]. High incidences of ALS in Guam and the Kii peninsula of Japan have been reported since the 1950s [Kurland and Mulder, 1954] and 1960s [Kimura, 1961], respectively. Recent studies have indicated that the PDC type is still common, but also that the incidence of the ALS type is decreasing in both Guam and the Kii peninsula of Japan [Plato et al., 1969; Kuzuhara, 2007]. Because the disease focus occurs in a restricted area among three genetically different populations, genetic and/or environmental factors have been proposed as the etiologies of this disorder. A recent epidemiological study of Kii ALS/PDC has revealed that approximately 70% of patients have a family history of ALS/PDC [Kuzuhara et al., 2001;

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Kuzuhara, 2007]. Furthermore, families with multiple cases of ALS/PDC are common in Guam [Kurland and Mulder, 1955; Morris et al., 2004]. These observations made in both Guam and the Kii peninsula of Japan strongly suggest the involvement of genetic factors in ALS/PDC.

A comprehensive mutational analysis of 19 candidate genes including ALS/FTLD-related genes (*SOD2, SOD3, ALS2/alsin, SMN1, PGRN, ANG, VEGF, VCP, VAPB, DCTN1, CHMP2B,* and *TARDBP/TDP-43*), the tauopathy-related gene (*GSK3β*), and parkinsonism-related genes (*α-synuclein, LRRK2, parkin, DJ-1, PINK1,* and *ATP13A2*) did not reveal any mutations in these genes in the patients with ALS/PDC [Tomiyama et al., 2008]. The T1482I variant of transient receptor potential melastatin 7 gene (*TRPM7*) has recently been reported to be associated with five Guamanian ALS/PDC patients [Hermosura et al., 2005]. *TRPM7* is a member of the TRP superfamily of ion channels that has been suggested to play roles in the homeostatic regulation of Ca^{2+} and Mg^{2+}. The association of the *TRPM7* variant with ALS/PDC may support the environmental factor hypothesis that prolonged exposure to low level of Ca^{2+} and Mg^{2+} contributes to the high incidence of development of ALS/PDC [Garruto, 1991]. To explore the implication of *TRPM7* in Kii ALS/PDC, we have conducted parametric linkage analyses of the *TRPM7* locus, and resequenced the entire coding region of *TRPM7* of an affected individual using a large extended family with ALS/PDC in the Kii peninsula of Japan. We further compared the frequencies of T14821 in the affected individuals in this family or in those from other families with those in regional controls or HapMap-JPT samples to investigate the potential association of T1482I with ALS/PDC in the Kii penisula.

MATERIALS AND METHODS

Samples

Genomic DNA was extracted from peripheral leukocytes according to standard protocol after obtaining informed written consent from patients. The clinical and pathological evaluations of the family members are described elsewhere [Tomiyama et al., 2008]. The research project was approved by the ethics committee of Niigata University, Mie University School of Medicine, and the University of Tokyo.

Linkage Analysis of the TRPM7 Locus on Chromosome 15q21.2

Parametric pair-wise linkage analysis of the TRPM7 locus was performed using the Superlink program (http://bioinfo.cs.technion.ac.il/superlink/) [Fishelson and Geiger, 2002] with the 23 family members including the 8 affected individuals (Supplementary Figure, A family). The pedigree information was updated based on information obtained after publication of our previous study [Tomiyama et al., 2008]. Pair-wise lod scores at D15S978 and D15S1016 flanking the TRPM7 locus at 1.6 Mb upstream and 2.6 Mb downstream, respectively, were obtained using autosomal dominant (AD) and autosomal recessive (AR) models. A disease gene frequency of 0.01 and penetrance rates of 0.9 and 1.0 were used.

Resequencing of Entire Coding Regions of TRPM7

Coding regions of *TRPM7* were amplified by polymerase chain reaction (PCR) using TaKaRa LA Taq (TaKaRa, Tokyo, Japan) for one patient (V-10) clinically diagnosed with ALS/PDC. The primers were designed using ExonPrimer (http://ihg2.helmholtz-muenchen.de/ihg/ExonPrimer.html; see Supplementary Data). The PCR products were purified using ExoSAP-IT (USB), and subjected to direct nucleotide sequence analysis using a BigDye terminator Cycle Sequencing kit v3.1 and an ABI3100 sequencer (Applied Biosystems, Foster City, CA). The obtained sequence data were analyzed by Variant Reporter™ Software v1.0 (Applied Biosystems).

Association Analysis of T1482I

The allele frequencies of T1482I was investigated in other 7 patients with ALS/PDC included in the linkage study (VI-2, VI-7, VI-9, VI-17, VI-18, VI-21, and VII-4) and another 1 recently diagnosed patient (VI-20) in the A family (Supplementary Figure), 16 Kii-ALS/PDC index patients from other families (6 multiplex families and 10 apparently sporadic patients), and 27 control subjects living in the same region by resequencing exon 28 in *TRPM7* using a primer pair of 5′-TGGTGTCCAGGTAGAATAAAG-3′ and 5′-TTCACTGCTCATGTGTTTGAC-3′, or that described in the Supplementary Table (Exon28F and Exon28R).

Association analysis of T1482I variant was conducted with χ^2 analysis of the allele frequencies of 9 patients in family A, or of 16 index patients in other families, and those in 27 regional controls, or HapMap-JPT samples.

RESULTS

Pair-wise LOD scores were −infinity at both D15S978 and D15S1016 for AD and AR models with complete penetrance. We obtained LOD scores of 0.64/−0.62 and 0.51/−1.65 for AD and AR

TABLE I. Pair-Wise LOD Scores at D15S978 and D15S1016

Mode of inheritance	AD model		AR model	
Penetrance	0.9	1.0	0.9	1.0
D15S978	0.64	−infinity	0.51	−infinity
D15S1016	−0.62	−infinity	−1.65	−infinity

TABLE II. Comparison of Allele Frequencies of T1482I in Patients With Those in Controls

	No. of subjects (frequency)			
	Affected in A family	Affected in other families	Control in the region	HapMap-JPT
Genotype				
T/T	0 (0.0%)	1 (6.3%)	0 (0.0%)	2 (4.4%)
C/T	2 (22.2%)	4 (25.0%)	11 (40.7%)	16 (35.6%)
C/C	7 (77.8%)	11 (68.8%)	16 (59.3%)	27 (60.0%)
	No. of alleles (frequency)			
	Affected in A family	Affected in other families	Control in the region	HapMap-JPT
Allele				
T	2 (11.1%)	6 (18.8%)	11 (20.4%)	20 (22.2%)
C	16 (88.9%)	26 (81.3%)	43 (79.6%)	70 (77.8%)
P-value	0.38[a] [0.29][b]	0.86[a] [0.68][b]		

[a]*P* values of χ^2 tests obtained by comparison of allele frequencies of T1482I between affected individuals and the regional controls are shown.
[b]*P* values of χ^2 tests obtained by comparison of allele frequencies of T1482I between affected individuals and HapMap-JPT samples are shown in parenthesis. There was no significant difference in the allele frequencies of T1482I between the regional controls and HapMap-JPT samples ($P = 0.79$).

models with incomplete penetrance (0.9), respectively (Table I). These results suggest that the linkage of Kii ALS/PDC to the *TRPM7* locus is unlikely in this family.

Resequencing of the entire coding regions of TRPM7 of a patient (V-10) from family A revealed two homozygous SNPs in introns (IVS3-26G>C [rs2063011] and IVS22-41T>A [rs675011]). Because both are present in the majority of the HapMap samples, they are unlikely to be pathogenic for Kii ALS/PDC.

Allele frequencies of T1482I variant (rs8042919) in the nine affected individuals in the A family are similar to those in regional controls ($P = 0.38$; Table II). We further extended the analysis to the 16 index patients from other families, but we did not observe any significant association of T1482I with ALS/PDC ($P = 0.86$). Allele frequencies of T1482I in the regional controls are similar to those in the HapMap-JPT samples ($P = 0.79$; dbSNP: http://www.ncbi.nlm.nih.gov/SNP/). Resequencing of exon 28 of TRPM7 revealed a previously described polymorphism (IVS28+15 C/T, rs3109894), which also did not show any significant association with ALS/PDC. Taken together, we conclude that the T1482I variant is not associated with Kii ALS/PDC.

DISCUSSION

In this study, we did not obtain any supportive evidence for the genetic linkage of Kii ALS/PDC to the *TRPM7* locus and resequencing analysis of the entire *TRPM7* of the affected individual did not reveal any causative mutations. Furthermore, the allele frequencies of T1482I in the affected individuals are not significantly different from those in regional controls or those in HapMap-JPT samples. Thus, it is unlikely that T1482I or *TRPM7* is associated with the Kii-ALS/PDC.

The structure of a large extended family with ALS/PDC in the Kii of Japan is complex for explicitly determining the inheritance mode [Tomiyama et al., 2008]. There are three consanguineous marriages in this pedigree, suggesting an AR pattern, whereas the disease occurs partially in the successive generations, suggesting an AD pattern with reduced penetrance. The complexity of the inheritance pattern has also been discussed with regard to ALS/PDC families in Guam. Formal segregation analysis of Guamanian ALS/PDC families rejected both dominant and recessive models, but were consistent with a 2-allele major locus model [Bailey-Wilson et al., 1993]. Indeed, a genome-wide association study of Guamanian ALS/PDC using 834 microsatellite markers did not provide any associated markers with a genome-wide significant level ($P < 0.0001$). Furthermore, pair-wise linkage analysis of 17 microsatellite markers in which they determined the threshold for further study ($P < 0.015$) has shown some interesting loci such as D3S2406 (LOD score = 0.78) and D20S103 (LOD score = 1.82) but failed to identify a convincing single locus [Morris et al., 2004]. These results suggest that familial ALS/PDC is not caused by a mutation of a single gene but is a complex disease involving genetic and environmental factors. Further extended association and linkage studies employing high-density single nucleotide polymorphisms (SNPs) and a larger sample size may be useful to identify susceptibility genes for the Kii ALS/PDC.

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REFERENCES

Bailey-Wilson JE, Plato CC, Elston RC, Garruto RM. 1993. Potential role of an additive genetic component in the cause of amyotrophic lateral sclerosis and parkinsonism-dementia in the western Pacific. Am J Med Genet 45:68–76.

Elizan TS, Hirano A, Abrams BM, Need RL, Van Nuis C, Kurland LT. 1966. Amyotrophic lateral sclerosis and parkinsonism-dementia complex of Guam. Neurological reevaluation. Arch Neurol 4:356–368.

Fishelson M, Geiger D. 2002. Exact genetic linkage computations for general pedigrees. Bioinformatics 18(Suppl 1):S189–S198.

Gajdusek DC, Salazar AM. 1982. Amyotrophic lateral sclerosis and parkinsonian syndromes in high incidence among the Auyu and Jakai people of West New Guinea. Neurology 32:107–126.

Garruto RM. 1991. Pacific paradigms of environmentally-induced neurological disorders: clinical, epidemiological and molecular perspectives. Neurotoxicology 12:347–377.

Hermosura MC, Nayakanti H, Dorovkov MV, Calderon FR, Ryazanov AG, Haymey DS, Garruto RM. 2005. A TRPM7 variant shows altered sensitivity to magnesium that may contribute to the pathogenesis of two Guamanian neurodegenerative disorders. Proc Natl Acad Sci USA 102:11510–11515.

Hirano A, Malamud N, Kurland LT. 1961. Parkinsonism-dementia complex, an endemic disease on the island of Guam. II. Pathological features. Brain 84:662–679.

Kimura K. 1961. Endemiological and geomedical studies on amyotrophic lateral sclerosis and allied diseases in Kii Peninsula, Japan (preliminary report). Folia Psychiatr Neurol Jpn 15:175–181.

Kurland LT, Mulder DW. 1954. Epidemiologic investigations of amyotrophic lateral sclerosis. I. Preliminary report on geographic distribution, with special reference to the Mariana Islands, including clinical and pathologic observations. Neurology 4:355–378.

Kurland LT, Mulder DW. 1955. Epidemiologic investigations of amyotrophic lateral sclerosis. 2. Familial aggregations indicative of dominant inheritance I. Neurology 5:182–196.

Kuzuhara S. 2007. Revisit to Kii ALS—The innovated concept of ALS-Parkinsonism-dementia complex, clinicopathological features, epidemiology and etiology. Brain Nerve 59:1065–1074.

Kuzuhara S, Kokubo Y, Sasaki R, Narita Y, Yabana T, Hasegawa M, Iwatsubo T. 2001. Familial amyotrophic lateral sclerosis and parkinsonism-dementia complex of the Kii Peninsula of Japan: clinical and neuropathological study and tau analysis. Ann Neurol 49:501–511.

Morris HR, Steele JC, Crook R, Wavrant-De Vrièze F, Onstead-Cardinale L, Gwinn-Hardy K, Wood NW, Farrer M, Lees AJ, McGeer PL, Siddique T, Hardy J, Perez-Tur J. 2004. Genome-wide analysis of the parkinsonism-dementia complex of Guam. Arch Neurol 61:1889–1897.

Plato CC, Cruz MT, Kurland LT. 1969. Amyotrophic lateral sclerosis-Parkinsonism dementia complex of Guam: further genetic investigations. Am J Hum Genet 21:133–141.

Tomiyama H, Kokubo Y, Sasaki R, Li Y, Imamichi Y, Funayama M, Mizuno Y, Hattori N, Kuzuhara S. 2008. Mutation analyses in amyotrophic lateral sclerosis/parkinsonism-dementia complex of the Kii peninsula, Japan. Mov Disord 23:2344–2348.

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No evidence for pathogenic role of *GIGYF2* mutation in Parkinson disease in Japanese patients

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ABSTRACT

Grb10-Interacting GYF Protein-2 (*GIGYF2*) is a candidate gene for *PARK11* locus. To date, seven different *GIGYF2* missense mutations have been identified in patients with familial Parkinson disease (PD) of European descent. To clarify the pathogenic role of *GIGYF2* in PD, we analyzed the frequency of *GIGYF2* mutations in 389 Japanese patients with PD (including 93 patients with late-onset familial PD, 276 with sporadic PD, and 20 with a single heterozygous mutation in the PD-associated genes), and 336 Japanese normal controls, by direct sequencing and/or high-resolution melting analysis. None of the reported *GIGYF2* mutations or digenic mutations were detected. Two novel non-synonymous variants were identified (p.Q1211delQ and p.H1023Q), however, we could not determine their roles in PD. In summary, we found no evidence for PD-associated roles of *GIGYF2* mutations. Our data suggest that *GIGYF2* is unlikely to play a major role in PD in Japanese patients, similar to other populations.

Parkinson disease (PD) is a common neurodegenerative movement disorder characterized by selective loss of dopaminergic neurons in the substantia nigra. The clinical features of PD include resting tremor, rigidity, bradykinesia, and postural instability. PD is estimated to affect 1% of people over 60 years of age [3]. Although the etiology of PD remains unknown, increasing evidence from genetic studies suggests that genetic factors contribute to the development of PD [20,27]. Family-based genome-wide linkage analyses and case-control association studies have identified 16 chromosomal loci (*PARK1–PARK16*) for PD and familial parkinsonism [1,8,9,12,16,17,19,21,22,24,25,30,33]. In recent reports, *GRB10 interacting GYF protein 2* (*GIGYF2*) was suggested to be the causative gene for *PARK11*-linked parkinsonism in Italian and French populations [11,18]. Furthermore, digenic mutations of *GIGYF2* and *LRRK2* could be associated with severe clinical phenotypes [11]. However, more recent studies demonstrated that *GIGYF2* mutations are rare and have a weak relationship to the development of PD [2,4,7,13,14,23,26,28,31,32,34].

The aim of the present study was to determine the distribution of *GIGYF2* mutations in the Japanese population. For this purpose, we analyzed the *GIGYF2* sequence in 93 Japanese patients with late-onset familial PD, and 276 Japanese patients with sporadic PD. In addition, to examine the frequency of digenic mutations in *GIGYF2* and other PD-associated genes, we also analyzed 20 PD patients with a single heterozygous mutation in PD-associated genes (*SNCA*, *LRRK2*, *parkin*, and *PINK1*).

A total of 93 Japanese patients (87 index cases) with late-onset familial parkinsonism (onset ≥ 45 years) were selected from the PD DNA bank of Juntendo University School of Medicine (Group A). Patients of this group had PD with putative autosomal dominant inheritance (ADPD), who were defined as the least affected family members in two consecutive generations without mutations, in the following PD-associated genes: *LRRK2*, *parkin*, *DJ-1*, *PINK1* (by direct sequencing), *SNCA*, and *parkin* (by gene dosage analysis). In addition, we also analyzed 20 Japanese patients (20 index cases) with a single heterozygous mutation in the following PD-associated genes (Group B; *SNCA* duplication: 6, *LRRK2*: 4, *parkin*: 6, and *PINK1*: 4 patients) [9,10,15,29]. The age and sex distribution of the subjects are shown in Table 1. To confirm the detected variants were associated with PD, we analyzed another group of 276 Japanese patients with sporadic PD (SPD) and 336 healthy control subjects (Table 1). Each subject gave a signed informed consent. This study was approved by the Ethics Committee of Juntendo University School of Medicine.

DNA samples were isolated from peripheral blood using standard protocols. All exons and intron/exon boundaries of the *GIGYF2*

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Table 1
Clinical characteristics of the subjects.

	Number of subjects (index cases)	Males/females	Age at onset mean ± SD (range)	Age at sampling mean ± SD (range)
Group A	93 (87)	1/1.5	57.8 ± 8.7 (45–77)	63.8 ± 9.8 (46–85)
Group B	20 (20)	1/1	48.7 ± 12.6 (32–74)	58.2 ± 12.4 (40–79)
Sporadic PD	276 (276)	1/1.1	58.5 ± 9.8 (21–88)	65.9 ± 8.8 (37–92)
Control	336 (336)	1/1.5	–	57.9 ± 12.4 (23–98)

Group A: familial PD patients without known mutations. Group B: familial PD patients with a single heterozygous mutation in known PD-associated genes.

were amplified from genomic DNA using PCR primers, which were prepared based on the report of Lautier et al. [11] or originally designed by Primer3 (http://frodo.wi.mit.edu/primer3/).

The PCR products were sequenced directly by standard protocols for ABI BigDye Terminator chemistry (Applied Biosystems, Foster City, CA). The sequencing reaction products were electrophoresed on an ABI Prism 3130 genetic Analyzer (Applied Biosystems). We used variant 2 (NM_015575.3) of *GIGYF2* as a reference sequence. We also analyzed another single exon, which was referred to variant 1 (NM_001103147.1) as reported previously [2].

When the candidate variants were detected, we performed high-resolution melting analysis using LightScanner (Idaho Technology, Salt Lake City, ID) to examine the frequencies among SPD patients and control subjects.

The Fisher's exact test and odds ratio were used to evaluate difference in allele frequencies. In all tests, P values ≤ 0.05 were considered statistically significant.

We identified 12 variants of *GIGYF2* in 113 Japanese patients with PD (Groups A and B; Table 2). Seven variants were presented in dbSNP (http://www.ncbi.nlm.nih.gov/snp/) and two repeat variants (p.Q1021_Q1022insQ and p.Q1216_Q1217insQQ) were reported previously [2]. Interestingly, one of these repeat variants (p.Q1021_Q1022insQ) was present in three of the patients of Group A, while it had been reported in only a single healthy control subject [2]. The remaining three were unreported variants, including two synonymous variants (p.A99A and p.V341V) and one repeat variant (p.Q1211delQ). In these novel variants, p.Q1211delQ was found in a heterozygous state, p.V341V was found in a homozygous state, and p.A99A was found in both homozygous and heterozygous state.

To investigate the frequency of the repeat variant (p.Q1021_Q1022insQ), which was previously reported in one control subject [2], we screened 276 patients with SPD and 336 healthy controls by high-resolution melting analysis and/or direct sequencing. The variant was detected in one patient with SPD and three control subjects. Although four patients harbored this variant among our subjects, the odds ratio (OR) of this variant was not statistically significant (OR = 1.15, 95% confidence interval 0.26–5.19, $P = 0.85$).

In addition, we found one novel non-synonymous variant (p.H1023Q) in a single patient with SPD, but none in the controls, or patients of Groups A and B. The amino acid H1023 was highly conserved across species (Fig. 1). The frequency of the reported variants was not significantly different between our patients and Japanese data from The Asian HapMap and dbSNP database (Table 2). However, the frequencies of all of the comparable variants were significantly different from previous European and North American studies [2], but no significant differences were detected in allele frequencies between our Japanese patients and Asian patients with PD (Table 2) [28]. Moreover, there were no differences in the frequencies between Groups A and B.

The aim of the present study was to determine the frequency of mutations in *GIGYF2* in Japanese patients with PD. *GIGYF2* was reported recently to be the causative gene for *PARK11* in Italian and French populations, however, most of the replication studies could not conclude whether *GIGYF2* mutation was pathogenic in the development of PD [2,4,7,13,14,23,26,28,31,32,34]. Based on this background, we performed sequencing analysis of *GIGYF2* in 389 Japanese patients with PD. The results showed no definite pathogenic mutations or PD-associated mutations including digenic mutations. This finding suggests that *GIGYF2* mutation is also rare in Japanese patients with PD similar to the results of previous replication studies [2,4,7,13,14,23,26,28,31,32,34]. The allele frequencies of the variants in Asians were different from Caucasians, suggesting different distributions of *GIGYF2* variants in some populations, whereas they are not common in patients with PD.

We identified two rare novel non-synonymous variants (p.Q1211delQ and p.H1023Q), however, the results could not establish their roles in PD. Concerning p.Q1211delQ, this amino acid is located in proline and glutamine-rich repeat region, the site of many insertion/deletion polymorphisms. Although the variant p.Q1211delQ might not play a pathogenic role in PD, the existence of this variant in the poly Q region is interesting because polyglutamine expansion causes neurodegenerative disorders such as spinocerebellar ataxia. Intriguingly, spinocerebellar ataxia 2 can show a PD phenotype in addition to spinal, brainstem, and cerebellar degeneration [8]. The other novel variant, p.H1023Q, was only found in one patient with sporadic PD but not in the other patients with PD and the controls. Although the amino acid H1023 was highly conserved from birds to mammals, we could not conclude whether this rare variant was pathogenic or not. If pathogenic mutations or PD-associated mutations in *GIGYF2* are found in Asian populations, these mutations would be novel ones. Further large-scale genetic and functional in vitro and in vivo studies are needed to clarify the pathogenicity of p.Q1211delQ and p.H1023Q variants.

GIGYF2 is thought to play a role in insulin/insulin-like growth factor signaling interaction with the Grb10 protein [5]. Interestingly, heterozygous GIGYF2-knockout mice develop age-related neurodegeneration with the appearance of α-synuclein-positive neuritic plaques in the brainstem and cerebellum [6]. On the other hand, it seems that GIGYF2 in zebrafish is not related to the development of dopaminergic neuronal system and suscepti-

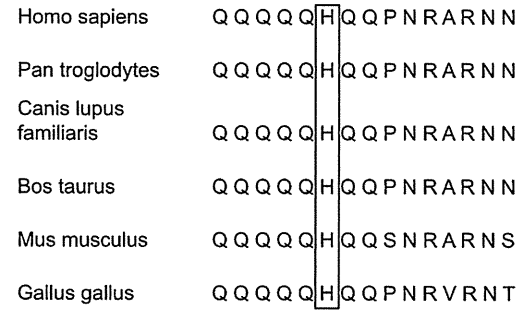

Fig. 1. Sequence homology of GIGYF2 between human and other species. His1023 in the human GIGYF2 and aligned amino acids at this position are boxed. NCBI/GenBank accession numbers are as follows: NP_056390.2 [*Homo sapiens*], XP_001147956.1 [*Pan troglodytes*], XP_861818.1 [*Canis familiaris*], NP_001095347.1 [*Bos taurus*], NP_666224.2 [*Mus musculus*], XP_422565.2 [*Gallus gallus*].

Table 2
Comparison of frequencies of *GIGYF2* variants.

Variant				Allele frequency (%)									
	rs number	cDNA	Protein	This study			SPD Japanese (n=550)	Japanese Controls (n=672)	Asian HapMap/dbSNP data	References			
				Total (n=226)	Group A (n=186)	Group B (n=40)				[11]	[2]	[2]	[28]
5′UTR	rs11555646	-	-	25.7 (58)	25.8 (48)	25.0 (10)	-	-	33.3	-	32.2	33.6#	-
Exon 6	-	c.297T > A	p.A99A	2.2 (5)	2.7 (5)	0 (0)	-	-	-	-	-	-	-
Exon 11	-	c.1022A > T	p.V341V	1.0 (2)	0 (0)	5.0 (2)	-	-	-	-	-	-	-
Exon 13	rs2289912	c.1378C > A	p.P460T	32.3 (73)	33.9 (63)	25.0 (10)	-	-	29.5	1.8##	0.7##	1.5##	42.2
Exon 14	rs2305138	c.1554G > A	p.E518E	15.5 (35)	13.4 (25)	25.0 (10)	-	-	15.8	5.4##	5.1##	-	21.1
Exon 24	rs3816334	c.2940A > G	p.Q980Q	73.5 (166)	73.1 (136)	75.0 (30)	-	-	74.3	28.1##	31.8##	31.2##	94.4
Exon 24	-	c.3064_3065 insCAG	p.Q1021_Q1022insQ	1.3 (3)	1.6 (3)	0 (0)	0.2 (1)	0.4 (3)	-	-	0	-	-
Exon 24	-	c.3069C > G	p.H1023Q	0 (0)	0 (0)	0 (0)	0.2 (1)	0 (0)	-	-	-	-	-
Intron 26	rs2305137	-	-	76.5 (173)	75.3 (140)	82.5 (33)	-	-	74.1	-	33.0##	36.0##	-
Exon 27	rs10555297	c.3630_3632delACA	p.P1210_Q1211delQ	-	-	-	-	-	-	46.4##	39.5##	47.5##	-
Exon 27	-	c.3631_3633delCAG	p.Q1211del	0.4 (1)	0.5 (1)	0 (0)	-	-	-	-	-	-	-
Exon 27	-	c.3649_3650insCAGCAG	p.Q1216_Q1217insQQ	-	-	-	-	-	-	-	0.6##	0.4##	-
Exon 27	rs12328151	c.3651G > A	p.P1217P	4.9 (11)	4.8 (9)	5.0 (2)	-	-	-	22.7##	29.0##	29.2##	-

Bras et al. (Portuguese) [2], Lautier et al. (Italian and French) [11], Bras et al. (US) [2], Tan et al. (Asian) [28].
# $P < 0.05$ compared with our results.
$P < 0.01$ compared with our results.

bility to neurotoxin [7]. More functional data using these animal models would provide more evidence and define the pathological consequences of GIGYF2 mutation in PD and neurodegenerative disorders.

In summary, extensive genetic analysis of *GIGYF2* sequence variations did not find any evidence for the pathogenic role of *GIGYF2* mutation in Japanese patients with PD. Furthermore, no digenic mutations were detected in our cohort. Our data on Japanese patients further suggest that *GIGYF2* variants are not frequent causes or risk factors in PD.

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References

[1] V. Bonifati, P. Rizzu, M.J. van Baren, O. Schaap, G.J. Breedveld, E. Krieger, M.C. Dekker, F. Squitieri, P. Ibanez, M. Joosse, J.W. van Dongen, N. Vanacore, J.C. van Swieten, A. Brice, G. Meco, C.M. van Duijn, B.A. Oostra, P. Heutink, Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism, Science 299 (2003) 256–259.
[2] J. Bras, J. Simón-Sánchez, M. Federoff, A. Morgadinho, C. Januario, M. Ribeiro, L. Cunha, C. Oliveira, A.B. Singleton, Lack of replication of association between GIGYF2 variants and Parkinson disease, Hum. Mol. Genet. 18 (2009) 341–346.
[3] L.M. De Lau, M.M. Breteler, Epidemiology of Parkinson's disease, Lancet Neurol. 6 (2006) 525–535.
[4] A. Di Fonzo, E. Fabrizio, A. Thomas, E. Fincati, R. Marconi, M. Tinazzi, G.J. Breedveld, E.J. Simons, H.F. Chien, J.J. Ferreira, M.W. Horstink, G. Abbruzzese, B. Borroni, G. Cossu, A. Dalla Libera, G. Fabbrini, M. Guidi, M. De Mari, L. Lopiano, E. Martignoni, P. Marini, M. Onofrj, A. Padovani, F. Stocchi, V. Toni, C. Sampaio, E.R. Barbosa, G. Meco, Italian Parkinson Genetics Network, B.A. Oostra, V. Bonifati, GIGYF2 mutations are not a frequent cause of familial Parkinson's disease, Parkinsonism Relat. Disord. 15 (2009) 703–705.
[5] B. Giovannone, E. Lee, L. Laviola, F. Giorgino, K.A. Cleveland, R.J. Smith, Two novel proteins that are linked to insulin-like growth factor (IGFI) receptors by the Grb10 adapter and modulate IGF-I signaling, J. Biol. Chem. 278 (2003) 31564–31573.
[6] B. Giovannone, W.G. Tsiaras, S. de la Monte, J. Klysik, C. Lautier, G. Karashchuk, S. Goldwurm, R.J. Smith, GIGYF2 gene disruption in mice results in neurodegeneration and altered insulin-like growth factor signaling, Hum. Mol. Genet. 23 (2009) 4629–4639.
[7] I. Guella, A. Pistocchi, R. Asselta, V. Rimoldi, A. Ghilardi, F. Sironi, L. Trotta, P. Primignani, M. Zini, A. Zecchinelli, D. Coviello, G. Pezzoli, L. Del Giacco, S. Duga, S. Goldwurm, Mutational screening and zebrafish functional analysis of GIGYF2 as a Parkinson-disease gene, Neurobiol. Aging (2010), Epub ahead of print.
[8] K. Gwinn-Hardy, J.Y. Chen, H.C. Liu, T.Y. Liu, M. Boss, W. Seltzer, A. Adam, A. Singleton, W. Koroshetz, C. Waters, J. Hardy, M. Farrer, Spinocerebellar ataxia type 2 with parkinsonism in ethnic Chinese, Neurology 55 (2000) 800–805.
[9] T. Kitada, S. Asakawa, N. Hattori, H. Matsumine, Y. Yamamura, S. Minoshima, M. Yokochi, Y. Mizuno, N. Shimizu, Mutations in the parkin gene cause autossomal recessive juvenile parkinsonism, Nature 392 (1998) 605–608.
[10] R. Kumazawa, H. Tomiyama, Y. Li, Y. Imamichi, M. Funayama, H. Yoshino, F. Yokochi, T. Fukusako, Y. Takehisa, K. Kashihara, T. Kondo, B. Elibol, S. Bostantjopoulou, T. Toda, H. Takahashi, F. Yoshii, Y. Mizuno, N. Hattori, Mutation analysis of the PINK1 gene in 391 patients with Parkinson disease, Arch. Neurol. 65 (2008) 802–808.
[11] C. Lautier, S. Goldwurm, A. Durr, B. Giovannone, W.G. Tsiaras, G. Pezzoli, A. Brice, R.J. Smith, Mutations in the GIGYF2 (TNRC15) gene at the PARK11 locus in familial Parkinson disease, Am. J. Hum. Genet. 82 (2008) 822–833.
[12] E. Leroy, R. Boyer, G. Auburger, B. Leube, G. Ulm, E. Mezey, G. Harta, M.J. Brownstein, S. Jonnalagada, T. Chernova, A. Ehejia, C. Lavedan, T. Gasser, P.J. Steinbach, K.D. Wilkinson, M.H. Polymeropoulos, The ubiquitin pathway in Parkinson's disease, Nature 395 (1998) 451–452.
[13] B. Meeus, K. Nuytemans, D. Crosiers, S. Engelborghs, P. Pals, B. Pickut, K. Peeters, M. Mattheijssens, E. Corsmit, P. Cras, P.P. De Deyn, J. Theuns, C. Van Broeck-

hoven, GIGYF2 has no major role in Parkinson genetic etiology in a Belgian population, Neurobiol. Aging (2009), Epub ahead of print.
[14] W.C. Nichols, D.K. Kissell, N. Pankratz, M.W. Pauciulo, V.E. Elsaesser, K.A. Clark, C.A. Halter, A. Rudolph, J. Wojcieszek, R.F. Pfeiffer, T. Foroud, Parkinson Study Group-PROGENI Investigators, Variation in GIGYF2 is not associated with Parkinson disease, Neurology 72 (2009) 1886–1892.
[15] K. Nishioka, S. Hayashi, M.J. Farrer, A.B. Singleton, H. Yoshino, H. Imai, T. Kitami, K. Sato, R. Kuroda, H. Tomiyama, K. Mizoguchi, M. Murata, T. Toda, I. Imoto, J. Inazawa, Y. Mizuno, N. Hattori, Clinical heterogeneity of alpha-synuclein gene duplication in Parkinson's disease, Ann. Neurol. 59 (2006) 298–309.
[16] C. Paisan-Ruiz, S. Jain, E.W. Evans, W.P. Gilks, J. Simon, M. van Der Brug, A. Lopez de Munain, S. Aparicio, A.M. Gil, N. Khan, J. Johnson, J.R. Martinez, D. Nicholl, I.M. Carrera, A.S. Pena, R. de Silva, A. Lees, J.F. Martí-Massó, J. Pérez-Tur, N.W. Wood, A.B. Singleton, Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease, Neuron 44 (2004) 595–600.
[17] C. Paisan-Ruiz, K.P. Bhatia, A. Li, D. Hernandez, M. Davis, N.W. Wood, J. Hardy, H. Houlden, A. Singleton, S.A. Schneider, Characterization of PLA2G6 as a locus for dystonia-parkinsonism, Ann. Neurol. 65 (2008) 2–3.
[18] N. Pankratz, W.C. Nichols, S.K. Uniacke, C. Halter, A. Rudolph, C. Shults, P.M. Conneally, T. Foroud, Parkinson Study Group, Significant linkage of Parkinson disease to chromosome 2q36–37, Am. J. Hum. Genet. 72 (2003) 1053–1057.
[19] N. Pankratz, T. Foroud, Genetics of Parkinson disease, Genet. Med. 9 (2007) 801–811.
[20] H. Payami, S. Zareparsi, Genetic epidemiology of Parkinson's disease, J. Geriatr. Psychiatry Neurol. 11 (1998) 98–106.
[21] M.H. Polymeropoulos, C. Lavedan, E. Leroy, S.E. Ide, A. Dehejia, A. Dutra, B. Pike, H. Root, J. Rubenstein, R. Boyer, E.S. Stenroos, S. Chandrasekharappa, A. Athanassiadou, T. Papapetropoulos, W.G. Johnson, A.M. Lazzarini, R.C. Duvoisin, G. Di Iorio, L.I. Golbe, R.L. Nussbaum, Mutation in the alpha-synuclein gene identified in families with Parkinson's disease, Science 276 (1997) 2045–2047.
[22] A. Ramirez, A. Heimbach, J. Grundemann, B. Stiller, D. Hampshire, L.P. Cid, I. Goebel, A.F. Mubaidin, A.L. Wriekat, J. Roeper, B. Stiller, A. Al-Din, A.M. Hillmer, M. Karsak, B. Liss, C.G. Woods, M.I. Behrens, C. Kubisch, Hereditary parkinsonism with dementia is caused by mutations in ATP13A2, encoding a lysosomal type 5 P-type ATPase, Nat. Genet. 38 (2006) 1184–1191.
[23] L. Samaranch, E. Lorenzo, M.A. Pastor, M. Riverol, M.R. Luquin, M.C. Rodríguez-Oroz, J.A. Obeso, P. Pastor, Analysis of the GIGYF2 gene in familial and sporadic Parkinson disease in the Spanish population, Eur. J. Neurol. 17 (2010) 321–325.
[24] W. Satake, Y. Nakabayashi, I. Mizuta, Y. Hirota, C. Ito, M. Kubo, T. Kawaguchi, T. Tsunoda, M. Watanabe, A. Takeda, H. Tomiyama, K. Nakashima, K. Hasegawa, F. Obata, T. Yoshikawa, H. Kawakami, S. Sakoda, M. Yamamoto, N. Hattori, M. Murata, Y. Nakamura, T. Toda, Genome-wide association study identifies common variants at four loci as genetic risk factors for Parkinson's disease, Nat. Genet. 41 (2009) 1303–1307.
[25] J. Simón-Sánchez, C. Schulte, J.M. Bras, M. Sharma, J.R. Gibbs, D. Berg, C. Paisan-Ruiz, P. Lichtner, S.W. Scholz, D.G. Hernandez, R. Krüger, M. Federoff, C. Klein, A. Goate, J. Perlmutter, M. Bonin, M.A. Nalls, T. Illig, C. Gieger, H. Houlden, M. Steffens, M.S. Okun, B.A. Racette, M.R. Cookson, K.D. Foote, H.H. Fernandez, B.J. Traynor, S. Schreiber, S. Arepalli, R. Zonozi, K. Gwinn, M. van Der Brug, G. Lopez, S.J. Chanock, A. Schatzkin, Y. Park, A. Hollenbeck, J. Gao, X. Huang, N.W. Wood, D. Lorenz, G. Deuschl, H. Chen, O. Riess, J.A. Hardy, A.B. Singleton, T. Gasser, Genome-wide association study reveals genetic risk underlying Parkinson's disease, Nat. Genet. 41 (2009) 1308–1312.
[26] G.T. Sutherland, G.A. Siebert, J.R. Newman, P.A. Silburn, R.S. Boyle, J.D. O'Sullivan, G.D. Mellick, Haplotype analysis of the PARK 11 gene, GIGYF2, in sporadic Parkinson's disease, Mov. Disord. 24 (2009) 449–452.
[27] S. Sveinbjörnsdottir, A.A. Hicks, T. Jonsson, H. Pétursson, G. Guğmundsson, M.L. Frigge, A. Kong, J.R. Gulcher, K. Stefansson, Familial aggregation of Parkinson's disease in Iceland, N. Engl. J. Med. 343 (2000) 1765–1770.
[28] E.K. Tan, C.H. Lin, C.H. Tai, L.C. Tan, M.L. Chen, R. Li, H.Q. Lim, R. Pavanni, Y. Yuen, K.M. Prakash, Y. Zhao, R.M. Wu, Non-synonymus GIGYF2 variants in Parkinson's disease from two Asian populations, Hum. Genet. 126 (2009) 425–430.
[29] H. Tomiyama, Y. Li, M. Funayama, K. Hasegawa, H. Yoshino, S. Kubo, K. Sato, T. Hattori, C.S. Lu, R. Inzelberg, R. Djaldetti, E. Melamed, R. Amouri, N. Gouider-Khouja, F. Hentati, Y. Hatano, M. Wang, Y. Imamichi, K. Mizoguchi, H. Miyajima, F. Obata, T. Toda, M.J. Farrer, Y. Mizuno, N. Hattori, Clinicogenetic study of mutations in LRRK2 exon 41 in Parkinson's disease patients from 18 countries, Mov. Disord. 21 (2006) 1102–1108.
[30] E.M. Valente, P.M. Abou-Sleiman, V. Caputo, M.M. Muqit, K. Harvey, S. Gispert, Z. Ali, D. Del Turco, A.R. Bentivoglio, D.G. Healy, A. Albanese, R. Nussbaum, R. González-Maldonado, T. Deller, S. Salvi, P. Cortelli, W.P. Gilks, D.S. Latchman, R.J. Harvey, B. Dallapiccola, G. Auburger, N.W. Wood, Hereditary early-onset Parkinson's disease caused by mutations in PINK1, Science 304 (2004) 1158–1160.
[31] C. Vilariño-Güell, O.A. Ross, A.I. Soto, M.J. Farrer, K. Haugarvoll, J.O. Aasly, R.J. Uitti, Z.K. Wszolek, Reported mutations in GIGYF2 are not a common cause of Parkinson's disease, Mov. Disord. 24 (2009) 618–619.
[32] Y. Zhang, L. Zheng, T. Zhang, Y. Wang, Q. Xiao, Q.Z. Fei, P.J. Cui, L. Cao, S.D. Chen, GIGYF2 Asn56Ser mutation is rare in Chinese Parkinson's disease patients, Neurosci. Lett. 463 (2009) 172–175.
[33] A. Zimprich, S. Biskup, P. Leitner, M. Farrer, S. Lincoln, J. Kachergus, M. Hulihan, R.J. Uitti, D.B. Calne, A.J. Stoessl, R.F. Pfeiffer, N. Patenge, I.C. Carbajal, P. Vieregge, F. Asmus, B. Müller-Myhsok, D.W. Dickson, T. Meitinger, T.M. Strom, Z.K. Wszolek, T. Gasser, Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology, Neuron 44 (2004) 601–607.
[34] A. Zimprich, C. Schulte, E. Reinthaler, D. Haubenberger, J. Balzar, P. Lichtner, S. El Tawil, S. Edris, T. Foki, W. Pirker, R. Katzenschlager, G. Daniel, T. Brücke, E. Auff, T. Gasser, PARK11 gene (GIGYF2) variants Asn56Ser and Asn457Thr are not pathogenic for Parkinson's disease, Parkinsonism Relat. Disord. 15 (2009) 532–534.

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Polypyrimidine tract-binding protein 1 regulates the alternative splicing of dopamine receptor D_2

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Abstract

Dopamine receptor D_2 (DRD2) has two splicing isoforms, a long form (D2L) and short form (D2S), which have distinct functions in the dopaminergic system. However, the regulatory mechanism of the alternative splicing of *DRD2* is unknown. In this study, we examined which splicing factors regulate the expression of D2L and D2S by over-expressing several RNA-binding proteins in HEK293 cells. In a cellular splicing assay, the over-expression of polypyrimidine tract-binding protein 1 (PTBP1) reduced the expression of D2S, whereas the knockdown of PTBP1 increased the expression of D2S. We also identified the regions of *DRD2* that are responsive to PTBP1 using heterologous minigenes and deletion mutants. Our results indicate that PTBP1 regulates the alternative splicing of *DRD2*. Considering that DRD2 inhibits cAMP-dependent protein kinase A, which modulates the intracellular localization of PTBP1, PTBP1 may contribute to the autoregulation of DRD2 by regulating the expression of its isoforms.

Keywords: alternative splicing, dopamine, dopamine receptor D_2, PTBP1.

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Dopamine is the predominant neurotransmitter in the CNS, where it plays a leading role in the regulation of such physiological functions as locomotor activity, cognition, positive reinforcement, and hormone secretion. The effects of dopamine are mediated by its binding to five G-protein-coupled receptors, which are divided into two subclasses: D_1-like (D_1 and D_5) and D_2-like (D_2, D_3, and D_4). Dopamine receptor D_2 (DRD2) is the main autoreceptor of the dopaminergic system (Centonze *et al.* 2002); however, it is also critical for post-synaptic transmission (Usiello *et al.* 2000).

Alternative gene splicing generates two distinct isoforms of DRD2, a long form (D2L) and short form (D2S), which differ in the presence of a 29-amino-acid insert in the third cytoplasmic loop. D2L is expressed mainly in post-synaptic regions, whereas D2S is expressed mainly in pre-synaptic regions (Khan *et al.* 1998; Usiello *et al.* 2000). These isoforms differentially contribute to the pre-synaptic inhibition of glutamate and GABA transmission (Centonze *et al.* 2004); moreover, they exhibit specific G_i protein preferences (Senogles 1994; Guiramand *et al.* 1995; Senogles *et al.* 2004) and have distinct roles in the regulation of protein phosphorylation (Lindgren *et al.* 2003). Furthermore, behavioral studies of D2L-deficient mice have shown that D2L and D2S contribute differentially to the regulation of certain motor functions (Usiello *et al.* 2000; Wang *et al.* 2000) and emotional responses (Hranilovic *et al.* 2008). Similarly, human genetic studies have shown that the intronic single nucleotide polymorphism rs1076560, which has a significant effect on the expression ratio of the DRD2 isoforms, is associated with cognitive processing (Zhang *et al.* 2007) and emotional processing (Blasi *et al.* 2009). These results suggest that the expression ratio of the DRD2 isoforms is important for their functions.

However, little is known about the regulatory mechanism that mediates the alternative splicing of *DRD2*. Although it has been reported that haloperidol, sex steroid hormones, and ethanol affect the expression of splice variants (Arnauld *et al.* 1991; Guivarc'h *et al.* 1995, 1998; Oomizu *et al.* 2003), the molecular basis for these differences is unclear. In general, changes in splicing patterns are directed by regula-

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Abbreviations used: D2L, long form of DRD2; D2S, short form of DRD2; DRD2, dopamine receptor D_2; nPTB, neural PTB; PTBP1, polypyrimidine tract-binding protein 1; Tpm2, tropomyosin 2.

tory proteins that bind the pre-mRNA sequence and enhance or silence particular splicing choices (Li *et al.* 2007). Thus, in this study, we searched for proteins that regulate the alternative splicing of *DRD2* using a cellular splicing assay and identified the involvement of the splicing factor polypyrimidine tract-binding protein 1 (PTBP1).

Materials and methods

Plasmid construction

The region from exon 5 to exon 7 of *DRD2* was amplified from human genomic DNA and cloned into the *Xho*I-*Hind*III site of pEYFP-C1 (Clontech, Mountain View, CA, USA) (Fig. 1a). The open reading frames that encode SF2/ASF, PTBP1, nPTB, NOVA1, HuB, FOX2, hnRNP A1, and Tra2b were amplified by PCR from a human fetal brain cDNA library (Clontech) and cloned into pcDNA3.1/V5-His (Invitrogen, Carlsbad, CA, USA) using conventional biological techniques. Primer sequences are listed in Table S1. Plasmid constructions of NAPOR and FOX1 are gifts from Dr. Yoshihiro Kino, RIKEN Brain Science Institute, and hnRNP H from Dr. Kinji Ohno, Nagoya University. Heterologous minigenes were generated by inserting *DRD2* fragments containing exon 6, exon 7 and flanking regions into pEGFP-Tpm2-ex1-2 (a gift from Dr. Kino, RIKEN Brain Science Institute). *DRD2* deletion mutants were generated by inverse PCR from the wild-type plasmid using primers flanking the deleted regions. The nucleotide sequences of the DNA inserts were confirmed by sequencing.

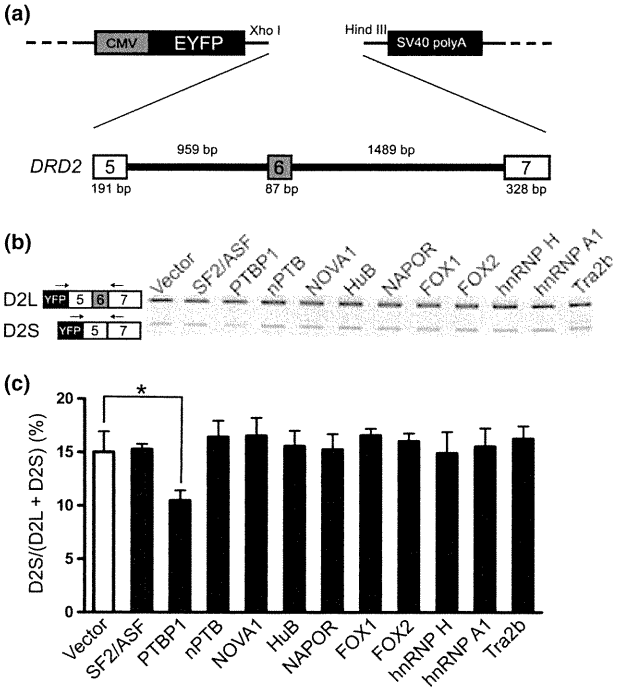

Fig. 1 The over-expression of PTBP1 reduced the alternative splicing of D2S. (a) Structure of the *DRD2* minigene. (b) Representative result from RT-PCR assays in which the *DRD2* minigene and plasmids for expressing RNA-binding proteins were transfected into HEK293 cells. The upper bands correspond to the splice product containing exon 6 (D2L), while the lower bands correspond to the splice product lacking exon 6 (D2S). (c) Bar chart showing the quantified percentage of D2S (Mean + SEM, $n = 3$). The statistical significance was analyzed by Dunnett's multiple-comparison test (*$p < 0.05$).

Cell culture and transfection

HEK293 and SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and incubated at 37°C with 5% CO_2. For the minigene assays, HEK293 cells were transfected with plasmids for the expression of minigene and V5-tagged proteins using Fugene 6 (Roche Diagnostics, Basel, Switzerland). In our RNAi experiments, HEK293 cells were transfected with the minigene plasmids and an siRNA for *PTBP1* (Invitrogen, Stealth™ Select RNAi HSS143520, and Negative Control Hi GC) and *nPTB* (Invitrogen, Stealth™ Select RNAi HSS126818, and Negative Control Lo GC) using Lipofectamine 2000 (Invitrogen), and SH-SY5Y cells were transfected with the siRNA using Lipofectamine RNAiMAX (Invitrogen) and the Reverse Transfection protocol. The efficacy of the RNAi-mediated knockdown of endogenous PTBP1, nPTB, and actin expressions was determined by western blot analysis using anti-PTBP1 (Invitrogen, catalog No. 32-4800), anti-nPTB (Abnova, Taipei City, Taiwan, catalog No. H00058155-A01), and anti-actin (Sigma-Aldrich, St. Louis, MO, USA, catalog No. A2066) antibodies.

Identification of DRD2 splice variants

Forty-eight hours after transfection, total RNA was isolated from the cells using a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). cDNA synthesis was performed using a PrimeScript First Strand cDNA Synthesis Kit (TAKARA BIO, Shiga, Japan) using oligo dT primer. The DRD2 minigene fragments were amplified by PCR (20 cycles) using a forward primer specific for the 3′ region of *EYFP* (AAGTCCGGACTCAGATCTCG) and a *DRD2*-specific reverse primer (DRD2-Ex7-Rv) that annealed to the 5′ region of exon 7 (CATCTCCATCTCCAGCTCCT). To detect endogenous *DRD2* fragments, a forward primer specific for exons 4 and 5 (CAATAACGCAGACCAGAACG) and DRD2-Ex7-Rv were used (40 cycles). For tropomyosin 2 (Tpm2)-based minigenes, primers green fluorescence protein (GFP)-Fw (CATGGTCCTGCTGGAGTTCGTG) and Tpm2-ex2-splicing-Rv2 (GGAGGGCCTGCTGCTCTTC) were used (Kino *et al.* 2009). The amplified products were resolved by 6% polyacrylamide gel electrophoresis and visualized using ethidium bromide. The intensities of the bands corresponding to the long and short forms were quantified by LAS-3000 and MultiGage software (Fuji Film, Tokyo, Japan). The quantified values were divided by the number of base pairs.

Results

PTBP1 regulates the alternative splicing of DRD2

To identify trans-acting factors that regulate the alternative splicing of *DRD2*, we used RT-PCR to detect splice variants. We constructed a gene fragment encompassing exons 5 through 7 of human *DRD2* in the vector pEYFP (Fig. 1a). This minigene was then transfected into HEK293 cells, and the expression ratios of D2L and D2S were analyzed by

RT-PCR. When the *DRD2* minigene was transfected with empty pcDNA3.1, the percentage of D2S was about 15% (Fig. 1b and c). Next, we expressed V5-tagged versions of several proteins known to regulate pre-mRNA splicing in the nervous system (SF2/ASF, PTBP1, nPTB, NOVA1, HuB, NAPOR, FOX1, FOX2, hnRNP H, hnRNP A1, and Tra2b); notably, SF2/ASF was previously proposed to regulate the alternative splicing of *DRD2* (Oomizu *et al.* 2003). Among the proteins tested, only when PTBP1 was transfected with the *DRD2* minigene was the percentage of D2S significantly reduced (to about 10%; Fig. 1b and c). We have confirmed the expressions of each RNA-binding proteins by western blot analysis and noted that the abundance of nPTB, NAPOR, and FOX1 are low (Figure S1). In addition, we showed the effects of PTBP1 were concentration dependent (Figure S2).

Next, we knocked down endogenous PTBP1 expression using an siRNA to confirm the effect of PTBP1 on *DRD2* splicing. We first confirmed the efficacy of the siRNA in modulating the expression of the target protein by western blot analysis (Fig. 2b). The presence of two PTBP1 bands rather than one is most likely because of phosphorylation. (Grossman *et al.* 1998). When the *DRD2* minigene was transfected with an siRNA for *PTBP1*, the percentage of D2S was significantly increased compared to transfection with a control siRNA (Fig. 2a). We also examined the effect of the knockdown of nPTB, a homologue of PTBP1, because it was reported that appearance of some exons are affected by both PTBP1 and nPTB (Boutz *et al.* 2007). The knockdown of PTBP1 increased the expression of nPTB (Fig. 2b), consistent with the previous reports (Boutz *et al.* 2007; Makeyev *et al.* 2007). While endogenous nPTB level was remarkably low and the knockdown of nPTB by siRNA was not observed, the increase in nPTB expression by the knockdown of PTBP1 was clearly inhibited by a siRNA for nPTB (Fig. 2b). Even when the increase in nPTB was inhibited, the knockdown of PTBP1 still increased the production of D2S splice variant (Fig. 2a), suggesting that the increase in nPTB has little or no effect on the alternative splicing of DRD2. Furthermore, we examined whether PTBP1 regulates the alternative splicing of endogenous *DRD2* in human neuroblastoma SH-SY5Y cells. When the siRNA for *PTBP1* was transfected into SH-SY5Y cells, the percentage of endogenous D2S fragments was also increased (Fig. 2c and d).

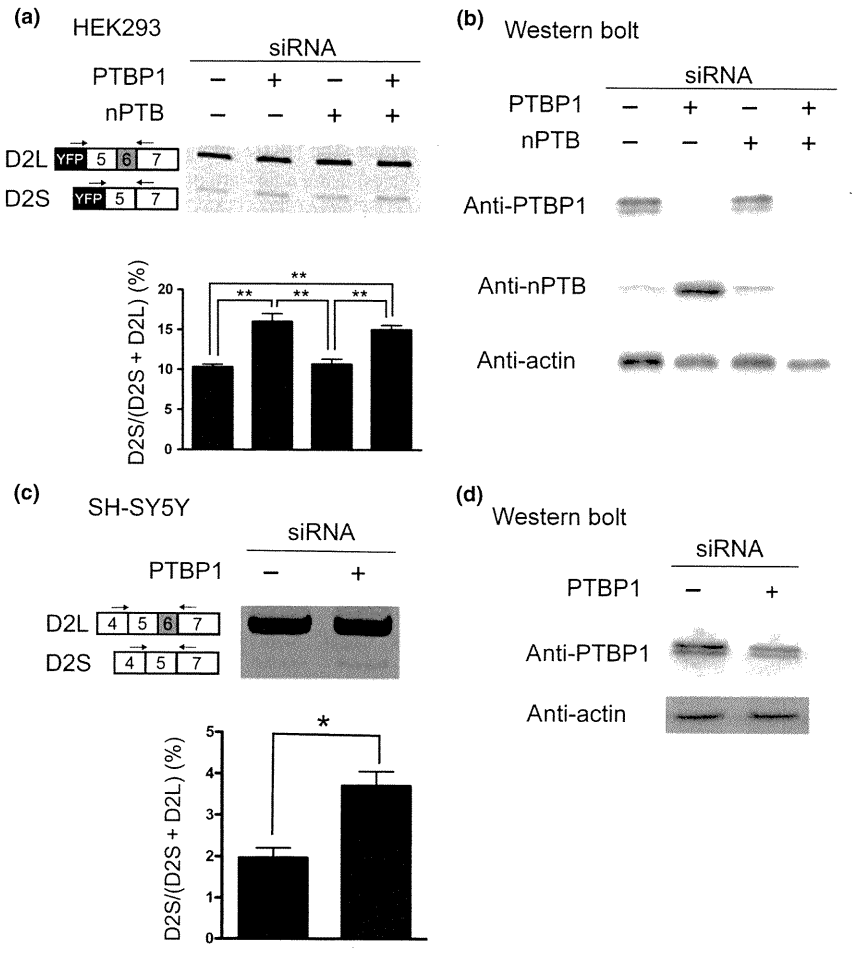

Fig. 2 The knockdown of PTBP1 increased the production of D2S splice variant. (a) Representative result from our cellular splicing assay using the *DRD2* minigene and siRNA for *PTBP1* and *nPTB* in HEK293 cells. Bar charts show the quantified percentages of D2S (Mean + SEM, $n = 3$). The statistical significances were analyzed using Tukey's multiple comparison test (**$p < 0.01$). (b) Representative result of western blot analysis of PTBP1 and nPTB in HEK293 cells. (c) Representative result of endogenous *DRD2* splicing using a siRNA for *PTBP1* in SH-SY5Y cells. Bar charts show the quantified percentages of D2S (Mean + SEM, $n = 3$). The statistical significance was analyzed using *t*-tests (*$p < 0.05$). (d) Representative result of western blot analysis of PTBP1 in SH-SY5Y cells.

Intronic regions flanking exon 6 are required for the PTBP1-mediated regulation of DRD2 splicing

To define the regions of *DRD2* that are required for its regulation by PTBP1, we utilized several previously generated heterologous minigenes (Kino *et al.* 2009). In these minigenes, the regions of interest were inserted in the context of constitutive exons of mouse *Tpm2*, which is distinct from *DRD2*. A reference fragment containing exon 9 of *Tpm2* and its flanking intronic regions or a *DRD2* fragment containing exon 6 or exon 7 and their flanking regions were inserted into a *Tpm2* fragment covering exons 1 and 2 (Fig. 3a). First, we predicted branch sites by a web-based program called ESEfinder 3.0 (Table S2, http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi). Then, using our *Tpm2*-based heterologous minigenes, we found that 14 bp upstream and 5 bp downstream of exon 6 are necessary for proper splicing (Fig. 3b). When exon 7 of DRD2 was inserted into Tpm2 cassette, a shorter exon 7 lacking the first 140 nucleotides was the main product. It was shown that 17 bp upstream of exon 7 is necessary for the splicing of full-length exon 7 (Fig. 3c). Because the primary elements regulating alternative splicing are thought to be located up to 200–300 nucleotides upstream and/or downstream of the regulated exon (Cooper 2005), a *DRD2* fragment stretching from 200 bp upstream of exon 6 (760 bp downstream of exon 5) to 200 bp downstream of exon 6 was used to examine the binding sequence of PTBP1 (Fig. 4a). PTBP1 had no effect on the inclusion of *Tpm2* exon 9 in HEK293 cells (Fig. 4b, left). In contrast, PTBP1 repressed *DRD2* exon 6 inclusion of the heterologous minigene, demonstrating that the inserted fragment of *DRD2* was sufficient for the response to PTBP1 (Fig. 4b, right). Next, to examine which region is necessary for the gene's responsiveness to PTBP1, we constructed *DRD2* deletion mutants lacking 200 bp upstream of exon 6 (Δint5_760–945), downstream of exon 6 (Δint6_6–200) or upstream of exon 7 (Δint6_1290–1487) (Fig. 4c). These deletion mutants were designed to include the regions that are necessary for splicing of exon 6 and exon 7. As shown in Fig. 4(d), Δint5_760–945 and Δint6_6–200 mutations altered the basal splicing pattern. Both deletion mutants exhibited markedly increased exclusion of exon 6 (from 15% to about 60% with vector transfection), suggesting the presence of elements in the deleted regions that enhance the inclusion of exon 6. Further, the over-expression of PTBP1 had no effect on either deletion mutant, indicating that both mutants had impaired responsiveness to PTBP1 (Fig. 4d). On the other hand, the over-expression of PTBP1 reduced D2S in the Δint6_1290–1487 mutant as well as a wild-type minigene, suggesting that PTBP1 affects the alternative splicing of DRD2 in regions other than the 3′ end of intron 6.

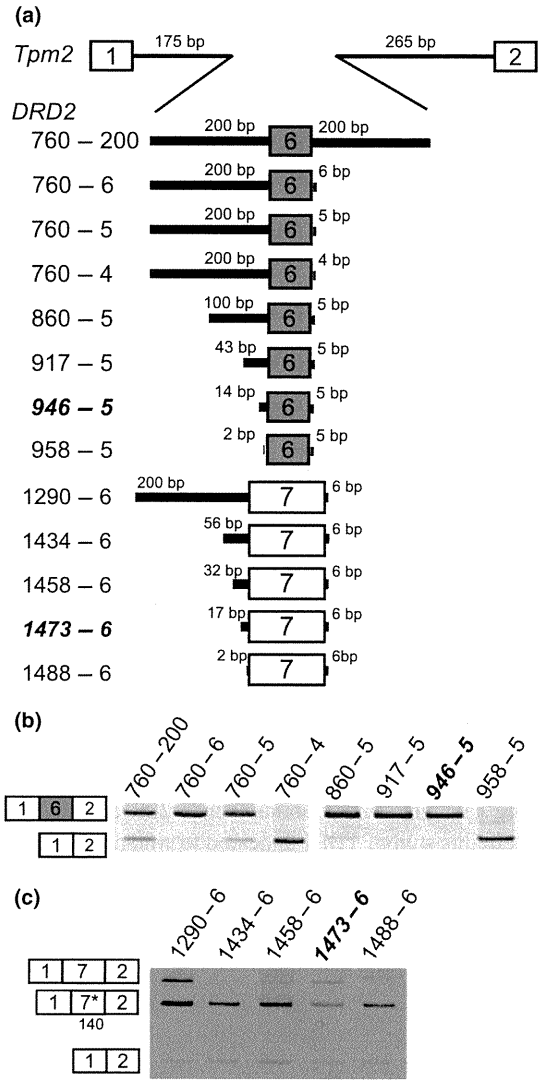

Fig. 3 Identification of DRD2 intronic regions which are necessary for the splicing of exon 6 and exon 7. (a) Structure of the *Tpm2*-based heterologous minigenes. The positions of the inserted nucleotides in introns 5, 6 and 7, as well as the numbers of base pairs in the fragments, are indicated. (b, c) Representative results from identification of splice variants using *Tpm2*-based heterologous minigenes in HEK293 cells. The white box 7* shows a shorter exon 7 lacking the first 140 nucleotides.

Discussion

Previous studies have shown that the functions of two splice variants of DRD2, D2L and D2S, differ in their biochemical properties and physiological functions (Senogles 1994; Guiramand *et al.* 1995; Khan *et al.* 1998; Usiello *et al.* 2000; Wang *et al.* 2000; Centonze *et al.* 2002, 2004; Lindgren *et al.* 2003; Senogles *et al.* 2004; Hranilovic *et al.* 2008); however, it is unclear what regulates the expression ratio of these isoforms. In this study, we identified PTBP1 as a splicing regulatory protein that reduces the expression of the D2S isoform.

Among the eleven proteins that we over-expressed with the *DRD2* minigene in HEK293 cells, only PTBP1

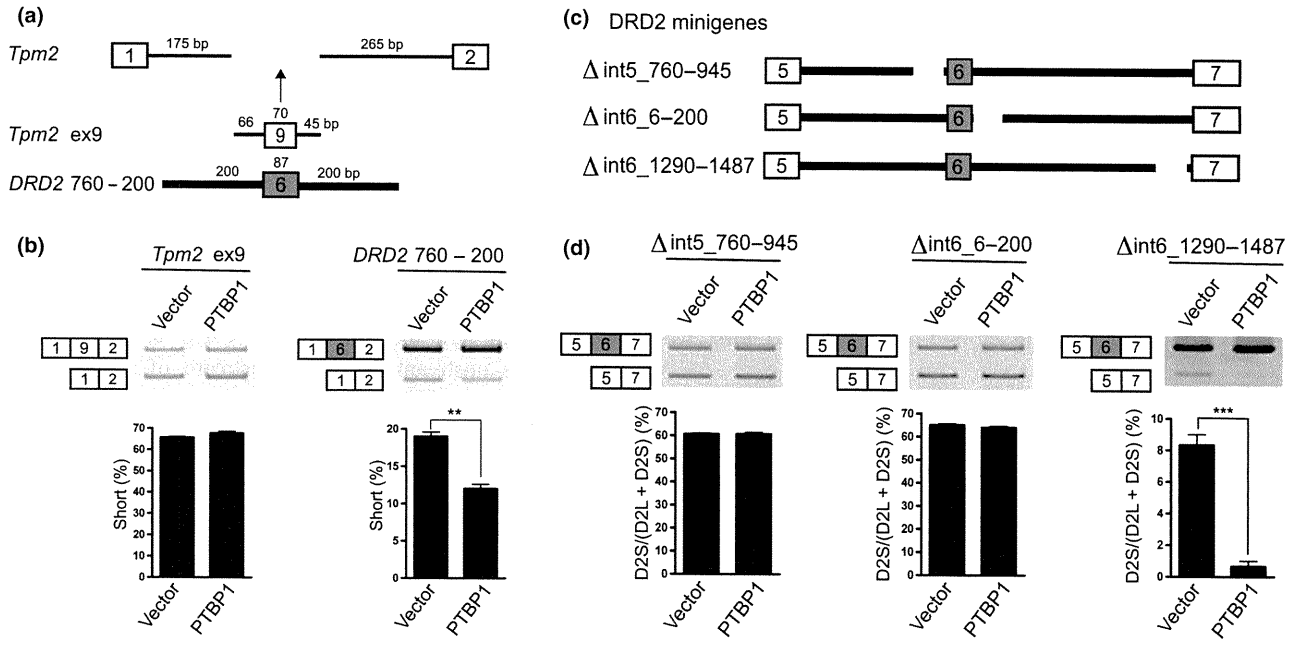

Fig. 4 Splicing regulation by PTBP1 in heterologous minigenes and *DRD2* deletion mutants. (a) Structure of the *Tpm2*-based heterologous minigene. Intronic fragments derived from *DRD2* are indicated by thick lines, whereas those derived from *Tpm2* are indicated by thin lines. (b) Splicing assay results using *Tpm2*-based heterologous minigenes and PTBP1 in HEK293 cells. Bar charts show the quantified percentages of exon exclusion (Mean + SEM, $n = 3$). (c) Structure of the *DRD2* deletion mutants. The positions of the inserted nucleotides in introns 5 and 6 are indicated. (d) Splicing assay results using the *DRD2* deletion mutants and PTBP1 in HEK293 cells. Bar charts show the quantified percentages of D2S (Mean + SEM, $n = 3$). The statistical significance was analyzed using *t*-tests (**$p < 0.01$, ***$p < 0.001$).

produced an altered splicing pattern (Fig. 1b and c). The reduction in the percentage of D2S suggests that PTBP1 enhances the inclusion of the alternative exon 6. Although the effect of PTBP1 was relatively small, this effect was shown to be concentration dependent (Figure S2). We also demonstrated that endogenous PTBP1 regulates DRD2 splicing by knockdown experiments in HEK293 cells with the *DRD2* minigene and in SH-SY5Y cells with the endogenous *DRD2* gene (Fig. 2a and c). Even though the effect of PTBP1 was statistically significant, it was quantitatively small in our splicing assay. Therefore, some other splicing factors may be involved in the splicing regulation of *DRD2*. In addition, the double knockdown of PTBP1 and nPTB suggested that nPTB, a homolog of PTBP1, has little or no effect on the alternative splicing of DRD2 (Fig. 2a). However, because the expression levels of exogenous and endogenous nPTB were remarkably lower than PTBP1 in HEK293 cells, it is still unclear whether nPTB regulates the splicing of DRD2.

Next, we identified the regions responsive to PTBP1, using *Tpm2*-based heterologous minigenes and *DRD2* deletion mutants. Using our heterologous minigenes, the splicing of a *DRD2* fragment containing exon 6 as well as 200 bp-upstream and -downstream intronic regions was altered by PTBP1 (Fig. 4b), similar to the results obtained using the *DRD2* minigene (Fig. 1b and c). In the *DRD2* deletion mutants, PTBP1 had no effect on the splicing of deletion mutants lacking exon 6-flanking regions in intron 5 or 6, whereas PTBP1 still affected the splicing of a deletion mutant lacking the 3′ end of intron 6 (Fig. 4d). These results indicate that exon 6-flanking regions are sufficient for the response to PTBP1, and that both regions in introns 5 and 6 are necessary.

Although PTBP1 is known to bind cytosine and uracil (CU)-rich intronic elements flanking an exon and repress splicing (Wagner and Garcia-Blanco 2001; Sharma *et al.* 2008), in this study PTBP1 appeared to enhance the inclusion of *DRD2* exon 6 rather than repressing the splicing from exon 5 to exon 7. It is noted that intron 5 contains UCUCU (849–853) and intron 6 contains UCUUUCU (32–38) sequences, but we have no evidence that PTBP1 directly binds the pre-mRNA of *DRD2*. Therefore it is possible that PTBP1 may indirectly affect the alternative splicing of *DRD2*.

It was reported that a DRD2 antagonist, haloperidol, increased the expression of D2S (Arnauld *et al.* 1991). The activation of DRD2 is coupled to the inhibition of adenylyl cyclase and cAMP-dependent protein kinase A, and cAMP-dependent protein kinase A has been shown to modulate the nucleocytoplasmic translocation of PTBP1 (Xie *et al.* 2003; Knoch *et al.* 2006). Together with these reports, our results suggest that DRD2 regulates the expression of its isoforms by modulating the localization of PTBP1.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1. The expressions of RNA-binding proteins were confirmed by western blot analysis.

Figure S2. The concentration dependency of the PTBP1 effects.

Table S1. Primer sequences used for cloning.

Table S2. Branch site prediction by ESEfinder 3.0.

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References

Arnauld E., Arsaut J. and Demotes-Mainard J. (1991) Differential plasticity of the dopaminergic D2 receptor mRNA isoforms under haloperidol treatment, as evidenced by in situ hybridization in rat anterior pituitary. *Neurosci. Lett.* **130**, 12–16.

Blasi G., Lo Bianco L., Taurisano P. *et al.* (2009) Functional variation of the dopamine D2 receptor gene is associated with emotional control as well as brain activity and connectivity during emotion processing in humans. *J. Neurosci.* **29**, 14812–14819.

Boutz P., Stoilov P., Li Q. *et al.* (2007) A post-transcriptional regulatory switch in polypyrimidine tract-binding proteins reprograms alternative splicing in developing neurons. *Genes Dev.* **21**, 1636–1652.

Centonze D., Usiello A., Gubellini P., Pisani A., Borrelli E., Bernardi G. and Calabresi P. (2002) Dopamine D2 receptor-mediated inhibition of dopaminergic neurons in mice lacking D2L receptors. *Neuropsychopharmacology* **27**, 723–726.

Centonze D., Gubellini P., Usiello A. *et al.* (2004) Differential contribution of dopamine D2S and D2L receptors in the modulation of glutamate and GABA transmission in the striatum. *Neuroscience* **129**, 157–166.

Cooper T. A. (2005) Use of minigene systems to dissect alternative splicing elements. *Methods* **37**, 331–340.

Grossman J., Meyer M., Wang Y., Mulligan G., Kobayashi R. and Helfman D. (1998) The use of antibodies to the polypyrimidine tract binding protein (PTB) to analyze the protein components that assemble on alternatively spliced pre-mRNAs that use distant branch points. *RNA* **4**, 613–625.

Guiramand J., Montmayeur J., Ceraline J., Bhatia M. and Borrelli E. (1995) Alternative splicing of the dopamine D2 receptor directs specificity of coupling to G-proteins. *J. Biol. Chem.* **270**, 7354–7358.

Guivarc'h D., Vernier P. and Vincent J. (1995) Sex steroid hormones change the differential distribution of the isoforms of the D2 dopamine receptor messenger RNA in the rat brain. *Neuroscience* **69**, 159–166.

Guivarc'h D., Vincent J. and Vernier P. (1998) Alternative splicing of the D2 dopamine receptor messenger ribonucleic acid is modulated by activated sex steroid receptors in the MMQ prolactin cell line. *Endocrinology* **139**, 4213–4221.

Hranilovic D., Bucan M. and Wang Y. (2008) Emotional response in dopamine D2L receptor-deficient mice. *Behav. Brain Res.* **195**, 246–250.

Khan Z., Mrzljak L., Gutierrez A., de la Calle A. and Goldman-Rakic P. (1998) Prominence of the dopamine D2 short isoform in dopaminergic pathways. *Proc. Natl Acad. Sci. USA* **95**, 7731–7736.

Kino Y., Washizu C., Oma Y., Onishi H., Nezu Y., Sasagawa N., Nukina N. and Ishiura S. (2009) MBNL and CELF proteins regulate alternative splicing of the skeletal muscle chloride channel CLCN1. *Nucleic Acids Res.* **37**, 6477–6490.

Knoch K., Meisterfeld R., Kersting S., Bergert H., Altkrüger A., Wegbrod C., Jäger M., Saeger H. and Solimena M. (2006) cAMP-dependent phosphorylation of PTB1 promotes the expression of insulin secretory granule proteins in beta cells. *Cell Metab.* **3**, 123–134.

Li Q., Lee J. and Black D. (2007) Neuronal regulation of alternative pre-mRNA splicing. *Nat. Rev. Neurosci.* **8**, 819–831.

Lindgren N., Usiello A., Goiny M., Haycock J., Erbs E., Greengard P., Hokfelt T., Borrelli E. and Fisone G. (2003) Distinct roles of dopamine D2L and D2S receptor isoforms in the regulation of protein phosphorylation at presynaptic and postsynaptic sites. *Proc. Natl Acad. Sci. USA* **100**, 4305–4309.

Makeyev E., Zhang J., Carrasco M. and Maniatis T. (2007) The microRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing. *Mol. Cell* **27**, 435–448.

Oomizu S., Boyadjieva N. and Sarkar D. (2003) Ethanol and estradiol modulate alternative splicing of dopamine D2 receptor messenger RNA and abolish the inhibitory action of bromocriptine on prolactin release from the pituitary gland. *Alcohol. Clin. Exp. Res.* **27**, 975–980.

Senogles S. (1994) The D2 dopamine receptor isoforms signal through distinct Gi alpha proteins to inhibit adenylyl cyclase. A study with site-directed mutant Gi alpha proteins. *J. Biol. Chem.* **269**, 23120–23127.

Senogles S., Heimert T., Odife E. and Quasney M. (2004) A region of the third intracellular loop of the short form of the D2 dopamine receptor dictates Gi coupling specificity. *J. Biol. Chem.* **279**, 1601–1606.

Sharma S., Kohlstaedt L., Damianov A., Rio D. and Black D. (2008) Polypyrimidine tract binding protein controls the transition from exon definition to an intron defined spliceosome. *Nat. Struct. Mol. Biol.* **15**, 183–191.

Usiello A., Baik J., Rougé-Pont F., Picetti R., Dierich A., LeMeur M., Piazza P. and Borrelli E. (2000) Distinct functions of the two isoforms of dopamine D2 receptors. *Nature* **408**, 199–203.

Wagner E. and Garcia-Blanco M. (2001) Polypyrimidine tract binding protein antagonizes exon definition. *Mol. Cell. Biol.* **21**, 3281–3288.

Wang Y., Xu R., Sasaoka T., Tonegawa S., Kung M. and Sankoorikal E. (2000) Dopamine D2 long receptor-deficient mice display alterations in striatum-dependent functions. *J. Neurosci.* **20**, 8305–8314.

Xie J., Lee J., Kress T., Mowry K. and Black D. (2003) Protein kinase A phosphorylation modulates transport of the polypyrimidine tract-binding protein. *Proc. Natl Acad. Sci. USA* **100**, 8776–8781.

Zhang Y., Bertolino A., Fazio L. *et al.* (2007) Polymorphisms in human dopamine D2 receptor gene affect gene expression, splicing, and neuronal activity during working memory. *Proc. Natl Acad. Sci. USA* **104**, 20552–20557.

原　著

大阪府下筋強直性ジストロフィー患者の受療動向調査

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要旨：筋強直性ジストロフィー患者の受療動向を検索する目的で，大阪府下の循環器・糖尿病・産婦人科・眼科全専門医に無記名アンケートを実施し，20.7% から回答をえた．33.8% の専門医は診療経験を有し，10.1% は診断経験があり，患者が様々な科を受診していること，神経内科以外の科を先に受診する症例も多いことが確認された．周産期・周術期トラブルでの発見も多く，非専門科での診断能力向上が課題だが，診断経験を持つ医師は筋強直現象や顔貌など特徴的症状への関心が高く，簡易スクリーニング法の開発が有効と思われた．患者の病識不足や専門医との連携困難を指摘する意見もあり，患者・医療者双方への啓発と情報共有が重要である．

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Key words：筋強直性ジストロフィー，受療動向，集学的治療，無記名アンケート

はじめに

Myotonic dystrophy type 1（DM1）は myotonin protein kinase（*DMPK*）の 3'非翻訳領域に存在する CTG 繰返し配列の延長によって生じる triplet repeat 病の一つである[1)]．本症は筋ジストロフィーの中でも頻度の高いものの一つであるが，骨格筋障害に加え様々な内分泌・代謝障害，心伝導障害，若年性白内障，種々の臓器の良性・悪性腫瘍など多彩な合併症を呈する多臓器疾患である[2)]．このため，本症の生命予後・QOL の改善には，集学的管理が重要である．一方，われわれが以前おこなった本症患者の子宮筋腫合併検索で，骨格筋症状発現前に子宮筋腫が発見された症例が 40% 以上を占めていた[3)]ように，骨格筋障害が軽微な症例では合併症により神経内科や小児神経科などの専門科以外の科を最初に受診することも多いため，非専門科における診断能力向上と専門科への連携も重要な課題である．しかし，本邦で本症患者の医療受療動向の実態を地域レベルで検索した報告は，われわれのしらべたかぎりこれまでにない．そこで，本症患者の受療動向と医療連携に向けた課題を明らかにする目的で，本症患者の受診機会が多いと推測される循環器科，糖尿病，産婦人科，眼科について，大阪府下の各専門医に往復葉書による無記名アンケート調査を実施した．

対象・方法

調査票作成時点（2009 年 9 月）の日本循環器学会，日本糖尿病学会，日本産科婦人科学会，日本眼科学会の専門医名簿に基づき，大阪府下の各専門医全員（住所不明者は除く，送付数は循環器専門医 927 名，糖尿病専門医 357 名，産婦人科専門医 882 名，眼科専門医 915 名）に，往復葉書による無記名アンケート調査をおこなった．調査期間は 2009 年 10 月から同年 12 月である．

質問内容は，各科共通のものとして所属機関の特性（診療所，病院：神経内科診療の有無），卒後年数（卒後 10 年以下，10～20 年，21 年以上），DM1 診療経験の有無と経験症例数，DM1 を診断した経験の有無とその数，本症をうたがう症状（筋力低下，握った手が開きにくい，顔貌，CK 高値，本症の家族歴），該当科以外に受診している診療科，DM1 の診療において困った経験を挙げた．各科別の設問として，受診契機となった病態，循環器科ではペースメーカー・除細動器の適応（適応無し，条件付き，一般と同様，積極的に考慮）を，糖尿病では本症に実施する耐糖能検査（75g-oral glucose tolerance test：OGTT，homeostasis model assessment of insulin resistance：HOMA-IR，食後血糖/インスリン，hemoglobin A1c：HbA1c），本疾患の耐糖能異常に対する治療方針（一般の糖尿病より厳密，同様，緩やか），本症で積極的に使用する耐糖能障害治療薬を，産婦人科では周産期合併症を挙げた．

有効回答数は循環器科 172 名（18.6%），糖尿病 85 名

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（受付日：2011 年 2 月 26 日）

Table 1　Summary of common questionnaire.

	Cardiologist	Diabetologist	Gynecologist	Ophthalmologist
Number of objective specialists	927	357	882	915
Institutions				
Hospitals: Neurology (+)	381	149	242	233
Hospitals: Neurology (−)	258	73	179	87
Clinics	281	130	453	590
Unclassified	7	5	8	5
Number of responders	172	85	220	154
Institutions				
Hospitals: Neurology (+)	74	37	58	46
Hospitals: Neurology (−)	39	13	46	0
Clinics	59	34	108	108
No answer	0	1	0	0
Years of experience as medical specialist				
Under10 years	7	2	21	10
11-20 years	37	29	47	58
Over 21 years	127	54	152	83
No answer	1	0	0	3
Experience to treat patients with myotonic dystrophy				
Yes	63	36	65	49
No	107	48	154	104
No answer	2	1	1	1
Experience to diagnose myotonic dystrophy				
Yes	13	16	29	6

（23.8%），産婦人科220名（25.7%），眼科154名（16.8%）であった．

統計学的検討は，各科専門医全体と回答者の所属機関比率の比較はχ二乗検定を，各科毎や勤務先毎など3群間以上の比較はKruskal-Wallis rank testを，DM発見経験有無など2群間の比較はMann-Whitney U testをもちいておこなった．

結　果

所属機関および卒後年数

各専門医の所属先および卒後年数をTable 1に示した．回答者の所属先比率は概ね専門医全体の所属先比率を反映していたが，眼科では神経内科診療をおこなっていない病院の勤務者からの回答が無かったため回答者と専門医全体の間に有意差をみとめた（$p<0.001$）．回答者の卒後年数についてはすべての科で21年以上が半数以上を占めたが，循環器科でその割合が高かった（$p=0.005$）．

診療経験の有無と経験症例数

本症の診療経験は213名（33.8%）の医師が有しており，診療科による有意差はなかったものの（$p=0.126$）糖尿病専門医が43.5%ともっとも高かった．経験症例数は1例が100名，2例47名，3例28名で，4例以上は18名であった．施設別では神経内科診療をおこなっている病院の医師の経験率が44.0%ともっとも高く，神経内科診療をおこなっていない病院（39.4%），診療所（25.2%）の順に低下した（$p<0.001$）．

診断経験の有無と診断症例数，本症をうたがう症状

本症を診断した経験を持つ医師は64名（10.1%）あり，診療経験を有する医師の中での割合は，循環器科20.6%，糖尿病43.2%，産婦人科44.6%，眼科12.2%で糖尿病と産婦人科が高かった（$p<0.001$）．施設別では神経内科診療をおこなっている病院の医師の診断率が15.6%ともっとも高く，神経内科診療をおこなっていない病院（11.1%），診療所（6.4%）の順に低下した（$p=0.007$）．本症をうたがう症状について診断経験の有る医師と無い医師で比較すると，筋強直現象（$p=0.001$），顔貌（$p=0.02$），CK高値（$p<0.001$）において，診断経験を有する群が有意に高かった（Fig. 1）．設問以外の症状として，産婦人科では先天性患児の出産（25.1%），眼科では若年性白内障（28.1%）を挙げた医師が多かった．

当該科以外の受診科

当該科以外の受療科をわかる範囲で列挙してもらったところ，神経内科が回答者の80.2%を占め，内科16.6%，整形外科6.4%，小児科6.4%と続いたが，神経内科・小児科のどちらも受診していない患者が17.6%あり，「困ったこと」の項目に，神経内科や小児神経科の受診を勧めても消極的とのコメントもあった．

各科別項目

循環器科

受療疾患としては心不全29名，伝導障害23名，心房細動・粗動11名，洞不全症候群8名，期外収縮6名，発作性上室性頻拍5名，弁膜症3名，QT延長症候群2名，虚血性心疾患1名であった．その他として在宅胃瘻管理や呼吸不全，肺炎，糖脂質代謝異常などの非循環器疾患を挙げた医師も12名あった（Fig. 2A）．ペースメーカー・除細動器の適応については無回答71名，一般と同じが69名，条件付き23名，適応無し5

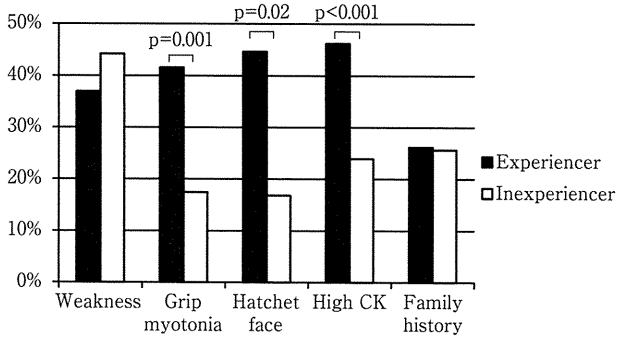

Fig. 1 Clinical signs which remind myotonic dystrophy.
Abbreviation: CK: creatine kinase
The doctors having experiences of diagnosis of DM paid more attention for grip myotonia, hatchet face and serum high CK.

名，積極的に適応4名であった．条件付きの内容としては，ADLや原疾患の予後を考慮する，原疾患の治療が優先，年齢や心機能を考慮する，致死性不整脈や完全房室ブロックの存在，心臓電気生理検査をおこなって判断する，などに加え明らかなガイドラインが無いとする意見やペースメーカーが心機能におよぼす悪影響を懸念する意見もあった．困ったこととして，本人・家族の疾患に対する受容・理解不足で治療への協力がえにくい，疾患・予後・遺伝の説明が難しい，心疾患以外の合併症管理が困難，突然死の経験がある，などのコメントがあった．

糖尿病

耐糖能障害検索のための検査としては，OGTTが20名，HbA1cが18名，食後血糖・インスリンが10名，HOMA-IRが7名であったが，食後高脂血症の評価としてクッキーテストを推奨する意見もあった．耐糖能障害への治療方針としては，一般と同じが45名，より緩やかは12名でより厳密の3名より多かった．本症に優先的に使用する薬剤としては，チアゾリン誘導体が12名ともっとも多く，ビグアナイド薬10名，インスリン5名，αグルコシダーゼ阻害剤3名の順で，スルホニル尿素（SU）製剤を挙げた医師はいなかった．

産婦人科

受診疾患は異常妊娠・出産が44名，不妊症9名，正常妊娠・出産4名と産科が多数を占め，婦人科疾患としては子宮筋腫16名，悪性腫瘍12名，良性腫瘍7名，子宮内膜症4名，その他3名であった（Fig. 2B）．周産期合併症を33名（15%）の医師が経験しており，羊水過多9名の他，抜管困難，呼吸器合併症，新生児仮死・管理困難が各8名，横紋筋融解症・筋痛6名，不整脈3名，その他3名が挙げられた（Fig. 3）．塩酸リトドリンによるトラブルを挙げた医師も4名あった．それ以外の困ったこととして，病識が乏しく治療に非協力的である，遺伝相談への対応が困難，切迫早産の際に使用できる薬剤が制限されること，先天性患児によりNICUのベッドが長期占有される問題や，未診断例が緊急手術で術後抜管不能となって発見された，体外受精採卵時の静脈麻酔によるトラブル，相

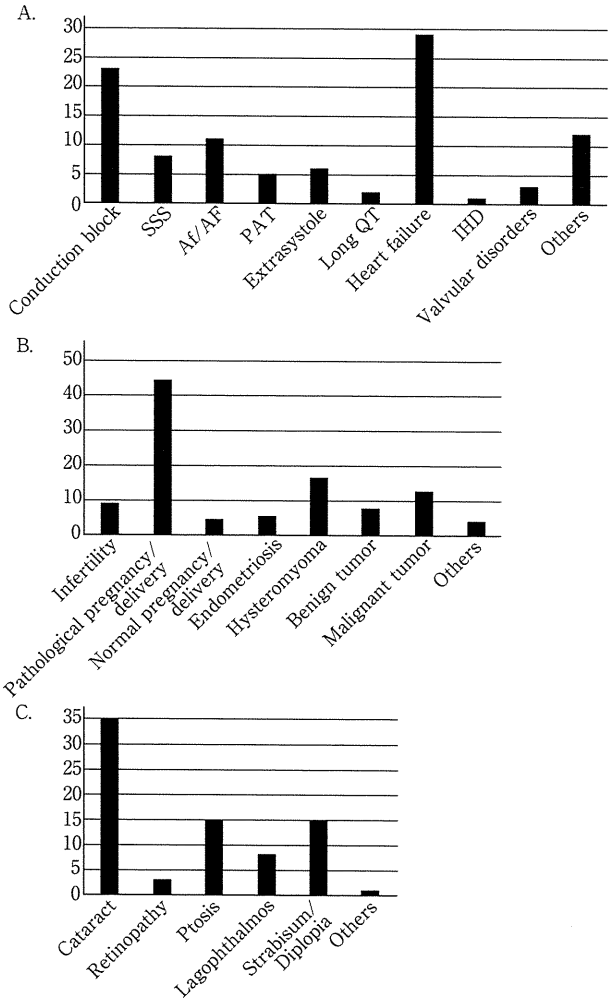

Fig. 2 The consulted disorders of myotonic dystrophy patients.
The disorders which made patients visit medical agency were asked in a multiple choice manner.
A. Cardiologist, B Gynecologist, C. Ophthalmologist
Abbreviations: SSS: sick sinus syndrome, Af: atrial fibrillation, AF: atrial flatter, PAT: paroxysmal atrial tachycardia, IHD: ischemic heart disorders

談できる専門医が少ないなどが挙げられ，コメントの記載率は11.4%と他の科（循環器5.8%，糖尿病0%，眼科0.6%）にくらべ高かった．

眼科

受診疾患は白内障35名，眼瞼下垂15名，斜視・複視15名，兎眼8名，網膜症3名，その他1名であった（Fig. 2C）．

考　察

今回の調査では，回答率が全体で20.1%（16.8～25.7%）であったが，協力いただいた各専門医にとっては専門外の疾患に対するアンケートであったことを考慮すると，低いものではないと考える．また，眼科を除き回答者の勤務先比率は専門