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LRRK2 I2020T mutation is associated with tau pathology

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ABSTRACT

Mutations in *the leucine-rich repeat kinase 2 (LRRK2)* gene are the most common cause of autosomal-dominant familial Parkinson's disease (FPD). The variable pathological features of *LRRK2*-linked FPD include Lewy bodies, degeneration of anterior horn cells associated with axonal spheroids, neurofibrillary tangles (NFTs) and TAR DNA-binding protein of 43 kDa (TDP-43) positive inclusion bodies. Furthermore, abnormal hyperphosphorylation of microtubule associated protein tau, in part generated by catalysis of protein kinases, has been reported to be involved in progressive neurodegeneration in a number of diseases, including FPD. Thus, we examined six patients carrying the *LRRK2 I2020T* mutation, a pathogenic mutation associated with PARK8, and found abnormal tau phosphorylation depositions in the brainstem. Additionally, we found LRRK2 I2020T enhanced tau phosphorylation in cultured cells co-expressing LRRK2-I2020T and 3 or 4-repeated tau. This is the first report describing the relationship between hyperphosphorylation of tau and LRRK2 I2020T.

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1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disease, characterized by rigidity, bradykinesia, resting tremor and postural instability. Mutations in *leucine-rich repeat kinase 2* (*LRRK2*) have been identified as the causative gene for *PARK8*-linked PD [1,2]. LRRK2, also known as PARK8, is a large protein of 2527 amino acids, with a molecular weight of approximately 280 kDa. LRRK2 contains multiple protein domains, including a leucine-rich repeat (LRR) domain, a ROC domain, a COR domain, a MAPKKK domain and a WD40 domain [2,3]. Various intracellular functions of LRRK2 have been reported, with alterations in its kinase activities thought to be critical for neuronal degeneration [4–7]. Interestingly, the *LRRK2 I2020T* mutation is located within the kinase domain and is also associated with altered kinase activity [6,8,9]. However, molecular studies have not shown a robust association between neuronal cell death and altered LRRK2 kinase activity, and the pathogenic mechanism of the *LRRK2 I2020T* mutation remains unknown.

Patients with *LRRK2* mutations show pleomorphic neuropathologies, which are not unique to PD and show overlap with other neurodegenerative diseases. These include nigral degeneration with or without Lewy bodies (LB) [2,10–14], also observed in diffuse LB disease [2,12,13], anterior horn cell degeneration associated with axonal spheroids, similar to amyotrophic lateral sclerosis [2], and neurofibrillary tangles (NFTs), also observed in progressive supranuclear palsy (PSP) [2,11,14,15] and Alzheimer's disease (AD) [2,12,13]. Notably, PD cases with G2019S [15], Y1699C [11] or I1371V [16] *LRRK2* mutations, have shown varied tau pathology. Similarly, Li et al. reported that tau was hyperphosphorylated in brain tissues from LRRK2-R1441G overexpressing mice, compared with LRRK2 wild type (WT) mice [17]. In addition, G2019S overexpressing mice [18] and Drosophila [19], exhibited tau alterations including mislocalization and hyperphosphorylation. Therefore, we investigated the relationship between the *LRRK2 I2020T* mutation and tau phosphorylation. We examined brain tissue from the Sagamihara family, a Japanese kindred originally reported to be linked to the *PARK8* locus [20], and found abnormally increased deposits of phosphorylated tau in the brainstem. Additionally, we showed that LRRK2 I2020T enhances tau phosphorylation in cultured cells co-expressing both LRRK2-I2020T and 3 or 4-repeated tau.

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However, there was no direct interaction between mutant LRRK2 and tau proteins. Our results indicate that the presence of the pathological I2020T mutation causes hyperphosphorylation of tau and may participate in the pathogenesis of PD and other tau-associated neurodegenerative diseases. Our findings contribute to the understanding of PARK8 pathogenesis.

2. Material and methods

2.1. Subjects

We examined the brains of six patients who came to autopsy. The clinical findings of patients A-E have been reported previously [20,22,23]. In this report patient A represents case 3, B case 4,C case 5 D case 9 E case 10 from the previous report [23]. All patients showed a good response to levodopa developing motor complications in the later stages of their disease, consistent with idiopathic PD. None had marked autonomic or cognitive dysfunction.

Patient F was a 68-year-old female. At 51 years of age, she developed clumsiness in the legs and gait disturbance, and was diagnosed with PD. Treatment with levodopa resulted in a marked improvement of her symptoms. She developed "wearing-off" motor fluctuations at age 57. By 64 years, she had developed visual hallucinations; by age 65, she was unable to walk without assistance. At She died at age 68 of multiple organ failure caused by pneumonia. You have said this already above. This patient was genetically determined to have the I2020T amino acid substitution in LRRK2.

2.2. Immunohistochemistry

Autopsy was performed within 6 h after death in each case. Brain sections were fixed in formalin and representative areas were embedded in paraffin and sectioned. Brain sections were stained with hematoxylin-eosin (H&E) for histological examination. For immunohistochemistry, sections of all patients were deparaffinized and incubated with the following primary antibodies: rabbit polyclonal antibody against ubiquitin (Dako; 1:800), and mouse monoclonal antibodies against phosphorylated α-synuclein (#64; Wako; 1:10,000) and phosphorylation-dependent tau (AT8; Innogenetics, 1:10,000). Primary antibodies were incubated overnight at 4 °C and then visualized by the avidin-biotin-peroxidase complex method. In addition, brain sections were stained with three repeat (3R) or four repeat (4R) tau-specific antibodies (RD3; 1:3000 or RD4; 1:1000 respectively; Upstate) [24], after pretreatment with potassium permanganate and oxalic acid to eliminate non-specific staining [25].

2.3. Construct preparation

pRK5-FLAG-LRRK2-WT and LRRK2-I2020T mutant vectors were prepared as described previously [21]. Three or 4 repeat tau cDNA was amplified from human adult brain using reverse transcript PCR and cloned into Myc-pcDNA 3.1(−). The rabbit polyclonal anti-LRRK2 antibody with synthetic peptides at the C-terminal end (2510-2527 aa) of human LRRK2 was generated as described previously [21]. Monoclonal mouse anti-human PHF-tau antibodies (clone AT-180 and clone AT-270), and tau antibody (clone HT-7) were from Innogenetics. Secondary antibodies conjugated to horseradish peroxidase were from GE HealthCare Bio-Sciences.

2.4. Cell Culture and transfection

COS-1 cells were grown in Dulbecco's modified Eagle's medium (Sigma–Aldrich) supplemented with 10% fetal bovine serum (Sigma–Aldrich) and 1% penicillin/streptomycin (Invitrogen) under an atmosphere of 5% CO_2 at 37 °C. COS-1 cells were transiently transfected with LRRK2 and tau vectors using FuGENE HD Transfection Reagent (Roche Diagnostics) according to the manufacturer's protocol.

2.5. Immunoblotting

After 96 h, cells were lysed in lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% nonidet P-40, 0.25% DOC, 400 μM Na_3VO_4, 400 μM EDTA, 1 mM EGTA, 10 mM NaF, 10 mM sodium pyrophosphate and protease inhibitors (Complete Mini, EDTA-free; Roche Diagnostics). To detect LRRK2, the samples were resolved on 3–8% NuPAGE Tris-acetate polyacrylamide gels (Invitrogen) in 1 × NuPAGE Tris-Acetate SDS running buffer and transferred onto polyvinylidene fluoride (PVDF) membrane. The membranes were blocked for 1 h in PBS containing 0.05% Tween-20 (PBS-T) and 5% non-fat milk (BD Difco) and then incubated overnight at 4 °C with the primary antibody. The membranes were washed with PBS-T three times followed by incubation for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit IgG (1:4000) and immunoreactivity assessed by chemiluminescence reaction using Western Lightning ECL (Perkin Elmer-Cetus). To detect tau, samples were resolved on 10% NuPAGE Bis-Tris polyacrylamide gels (Invitrogen) in 1 × NuPAGE MOPS SDS running buffer and transferred onto PVDF membrane. The membranes were blocked for 1 h in TBS containing 0.05% Tween-20 (TBS-T) and 5% non-fat milk (BD Difco) and then incubated overnight at 4 °C with the primary antibody. The membranes were washed with TBS-T buffer three times followed by incubation for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse IgG (1:2000). The remaining steps were as described above. Blots were quantified using Image J software analysis.

2.6. Immunoprecipitation

Cell lysates were centrifuged at 15,000× g for 20 min at 4 °C and the resulting supernatant fluid was incubated with Anti-FLAG M2 Agarose (Sigma–Aldrich) overnight at 4 °C. The resin was separated by centrifugation, washed three times with lysis buffer and then boiled in Laemmli sample buffer. Finally, each sample was analyzed by SDS-PAGE followed by immunoblotting.

2.7. Statistical analysis

Three group comparisons were analyzed by UNI-ANOVA followed by Turkey's multiple comparison tests (SPSS). All values were expressed as mean ± SEM. A P value less than 5% denoted a statistically significant difference among the groups.

3. Results

3.1. Variable tau pathology in PD associated with LRRK2 I2020T mutation

A previous pathological study of *LRRK2 I2020T* patients reported an apparent loss of nigral neurons without LBs, with the exception of onecase with LBs. However extensive immunohistochemical analysis of phosphorylated tau was not performed.

The pathological features of patients A-E have been described previously [23]. The additional new patient (patient F) shared neuropathological features with patients A-E, as follows [23]. Macroscopic examination revealed marked discoloration of the substantia nigra (SN) (Fig. 1a), with a well preserved locus coeruleus (LC) (Fig. 1b). This region-specific contrast in neuropathology was confirmed following microscopic examination, with marked neuronal loss, gliosis and extraneuronal melanin present in SN (Fig. 1c), in contrast to well preserved neurons with minimal gliosis in LC (Fig. 1d). Of note, the dorsal motor nucleus of the vagus nerve (DVN) appeared predominantly normal. In addition, we observed Marinesco bodies, ubiquitin-positive intranuclear inclusions, in the surviving neuromelanin-containing SN neurons (Fig. 1e).

Characteristics of the tau-positive lesions are summarized in Table 1. Patient B and E had tau-positive lesions restricted to the brainstem, namely SN, LC and the trochlear nucleus (Fig. 2a). In patients C and D, abnormal phosphorylated tau depositions were observed not only in the brainstem but also in the hippocampus and amygdala. Senile plaques were not found in any regions. In patients A and F, there were no tau-positive lesions. Immunohistochemistry with isoform-specific antibodies, determined that the tau-positive lesions contained both 3R and 4R tau (Fig. 2b, c). Overall, these results show that the I2020T mutation causes autosomal-dominant PD with a pleomorphic pathology, as observed with other LRRK2 mutations.

3.2. LRRK2 is associated with hyperphosphorylation of tau

Based on our pathological findings in *LRRK2 I2020T* patients, we hypothesized that mutant LRRK2 may be involved in hyperphosphorylation of tau. To determine the effect of LRRK2 I2020T on tau phosphorylation, we co-transfected COS-1 cells with LRRK2-WT or I2020T and 4R tau. Levels of phosphorylated tau and total tau were assessed by western blotting using antibodies, which recognize tau phosphorylation, AT-180 at Thr231 and AT-270 at Thr181 (Fig. 3c, d). Neither LRRK2-WT nor I2020T changed expression levels of total tau protein (Fig. 3c, d). However, significantly increased levels of phosphorylated 4R tau were detected in cells with overexpressed LRRK2-I2020T, but not WT (AT-180: 100.0 ± 1.2% [mean ± SEM] with WT vs. 118.5 ± 1.5% with I2020T, $p < 0.001$; AT-

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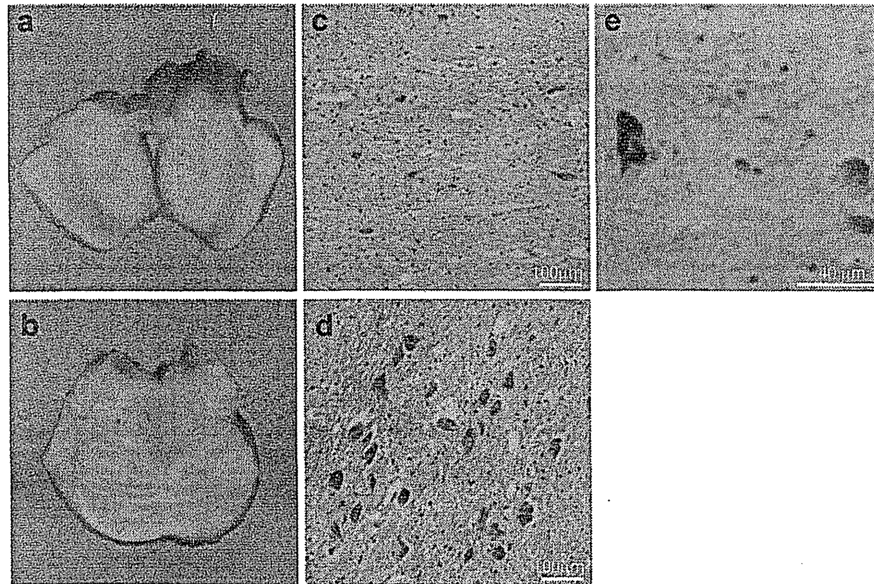

Fig. 1. Neuropathology of patient F, a LRRK2 I2020T carrier from the original Japanese Sagamihara family. Marked discoloration of the substantia nigra (SN, a) and relative preservation of locus coeruleus (LC, b). Marked neuronal loss with gliosis in the SN (c, H&E) is in contrast with preserved neurons in LC (d, H&E). Marinesco bodies are abundant in the SN (e, ubiquitin immunostain). Bars: c, d:100 μm; e: 40 μm.

270: 93.7 ± 4.0% with WT vs. 113.8 ± 5.3% with I2020T, $p < 0.001$; Fig. 3c, d). Next, we determined if I2020T affects expression levels of phosphorylated 3R tau. LRRK2-I2020T induced a significant, albeit modest, increase in the level of phosphorylated 3R tau protein compared with WT (AT-180: 94.9 ± 2.4% with WT vs. 100.5 ± 6.5% with I2020T, n.s.; AT-270: 93.5 ± 1.2% with WT vs. 104.1 ± 2.5% with I2020T, $p < 0.01$; Fig. 3a, b). To investigate further the interaction between LRRK2 and tau, we performed immunoprecipitation experiments. There was no evidence of a direct interaction between either LRRK2-WT or I2020T mutant with 4R tau (Fig. 3e).

4. Discussion

Tau pathology has been identified in the brains of PD patients with *LRRK2* mutations, with reports of various forms of tau depositions of, for example PSP-like or AD-like distribution and pattern of age related changes [26,27]. In this study, we identified tau pathology in four patients with *LRRK2 I2020T* mutation; an increased amount of phosphorylated tau was associated with LRRK2 I2020T mutation compared to wild type in cultured cell models. In addition, we found that affected members of the Sagamihara family display a homogeneous pattern of neuronal loss, namely degeneration of the SN with relative preservation of LC and DVN. This is in sharp contrast to idiopathic PD, where involvement of LC and DVN is observed. We also identified Marinesco bodies in our patients. The presence of Marinesco bodies has been described in other *LRRK2*-linked PD patients with R1441C [2] and G2019S mutations [14]. Thus, mutant LRRK2 may possibly affect dopaminergic neurons by accelerating the formation of Marinesco bodies.

In contrast to the homogeneity of neuronal degeneration that we observed, deposits of α-synuclein were confirmed only in patient E, and tau-positive deposits in the brainstem nuclei also varied? among the subjects. In previous reported pathological findings of *LRRK2*-linked PD, the presence of LBs and tau deposits did not overlap, even in the same family, which is in agreement with our observations in the Sagamihara family. Cookson et al. reported that although clinical features of *LRRK2*-linked PD were similar to sporadic PD, the pathological findings varied, confounding the correlation between etiology and disease expression [29]. Similarly, all examined members of the Sagamihara family showed typical PD features irrespective of pathological deposits. In addition, we did not find a direct correlation between tau deposits and clinical symptoms. Tau-positive deposits were seen in the

Table 1
Summary of tau pathology in LRRK2 I2020T carriers from the Sagamihara family

	Patient A	Patient B	Patient C	Patient D	Patient E	Patient F
Hippocampus	−	−	+	+	−	−
Meynert	−	−	−	−	+	−
Amygdala	NA	−	−	++	NA	NA
IV	−	+++	++	−	NA	NA
LC	−	+	++	+	+	−
Central gray matter	−	−	++	−	−	−
SN	−	−	+	−	+	−
Braak stage	<1	<1	2	3	<1	<1

The severity and distribution of NFT pathology was estimated using Braak staging (Braak and Braak 1991) (− none; + mild; ++ moderate; +++ severe; n/a not applicable). Tau pathology was observed in 4 out of 6 patients. Two individuals (patient B and E) had tau-positive lesions restricted to the brainstem, with another two individuals (patient C and D), showing tau-positive lesions in the hippocampus as well as the brainstem. The remaining two patients (patient A and F) did not show tau-positive lesions in any brain regions. IV; trochlear nucleus, LC; locus coeruleus, SN; substantia nigra.

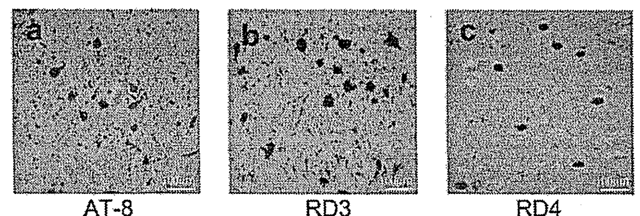

Fig. 2. Tau pathology in patient B, a LRRK2 I2020T carrier. Representative immunohistochemical analysis of tau in the trochlear nerve nucleus from Patient B. Sections are labeled with AT8 (a), RD3 (b) and RD4 (c). Bars: c, d:100 μm; e: 40 μm.

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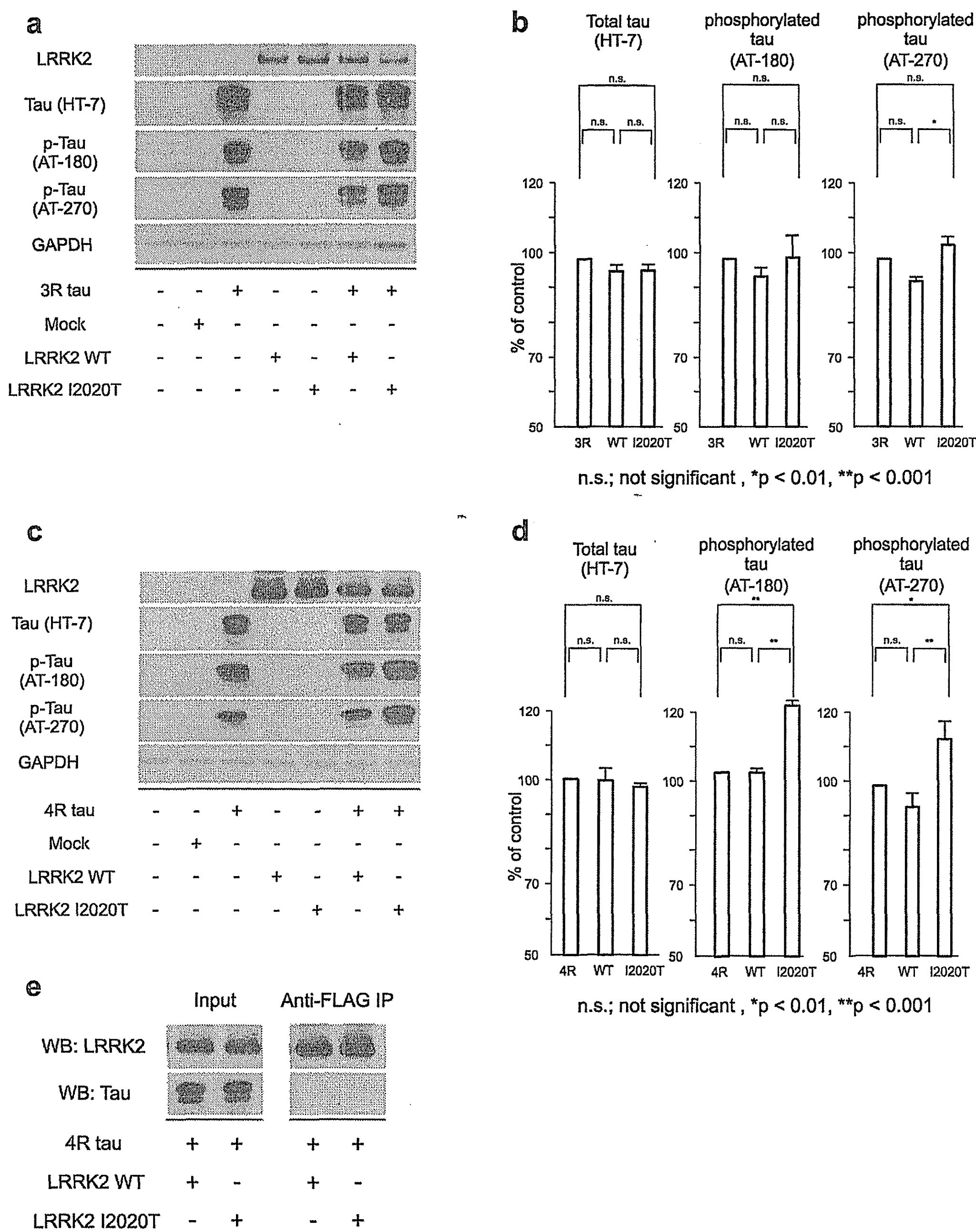

Fig. 3. LRRK2-I2020T induces increasing levels of phosphorylated tau compared with LRRK2-WT or mock transfected cells. (a, b) Lysate prepared from COS-1 cells co-expressing 3R tau and LRRK2-WT or I2020T, were subjected to anti-tau (HT-7) or anti-phosphorylated tau (AT-180 and AT-270) immunoblotting. LRRK2-I2020T increased expression levels of phosphorylated tau compared to LRRK2-WT, albeit modestly. (HT-7; 96.3 ± 1.8% with WT vs. 96.5 ± 1.9% with I2020T [mean ± SEM]; n.s., AT-180; 94.9 ± 2.4% with WT vs. 100.5 ± 6.5% with I2020T; n.s., AT-270; 93.5 ± 1.2% with WT vs. 104.1 ± 2.5% with I2020T; $p < 0.01$) (c, d). Lysate prepared from COS-1 cells co-expressing 4R tau and LRRK2-WT or I2020T, were subjected to anti-tau (HT-7) or anti-phosphorylated tau (AT-180 and AT-270) immunoblotting. LRRK2-I2020T significantly increased expression levels of phosphorylated tau compared to LRRK2-WT. (HT-7; 99.7 ± 3.5% with WT vs. 97.8 ± 1.1% with I2020T; n.s. AT-180; 100.0 ± 1.2% with WT vs. 118.5 ± 1.5% with I2020T; $p < 0.001$, AT-270; 93.7 ± 4.0% with WT vs. 113.8 ± 5.3% with I2020T; $p < 0.001$). (e) Lysate prepared from COS-1 cells transfected with Myc-4 repeats tau and FLAG-LRRK2-WT or FLAG-LRRK2-I2020T, were subjected to immunoprecipitation with anti-FLAG antibody followed by anti-tau (HT-7) immunoblotting. In the left panel, cell lysates were used to detect the expression of LRRK2 and tau. In the right panel, FLAG-LRRK2 was immunoprecipitated using FLAG antibody. Upper lanes show LRRK2 detected with anti-LRRK2 antibody. Lower lanes show that no bands were obtained with anti-HT-7 antibody. As a result, LRRK2 does not directly interact with 4R tau.

nucleus of the trochlear nerve in patients B and C, neither exhibited ophthalmoparesis. Consistent with these findings, Vitte et al. reported that LRRK2 protein is present throughout the human brain, with intense immunoreactivity in the neurons of several midbrain nuclei, including the nucleus of the trochlear nerve [28].

We then demonstrated the association between LRRK2 and tau hyperphosphorylation by using cultured cell models. Compared to LRRK2-WT or mock transfected, overexpression of LRRK2-I2020T in cultured cells resulted in increased levels of phosphorylated tau proteins. Furthermore, this increase in phosphorylated tau was

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associated with upregulation of both 3R and 4R tau isoforms. These findings could provide support for abnormal hyperphosphorylated tau deposition in the pathological findings of patients with *LRRK2 I2020T* mutation.

Based on neuropathological findings and cultured cell models, we hypothesized that LRRK2 is able to enhance tau phosphorylation. Our immunoprecipitation studies showed no evidence of a direct interaction between either LRRK2-WT or I2020T mutant with tau, indicating that tau phosphorylation by LRRK2-I2020T involves the association of an intermediate, genetic, or environmental factor. Smith et al. also reported that LRRK2 failed to bind tau protein [30]. Furthermore, LRRK2 mutations have been reported to be associated with tau hyperphosphorylation without direct interaction in animal models. Li et al. reported that tau is hyperphosphorylated in brain tissues from LRRK2-R1441G overexpressing mice compared with LRRK2-WT mice [17]. Mice and drosophila overexpressing *LRRK2*-G2019S also exhibited tau alterations, including mislocalization and increased tau phosphorylation [18,19]. Therefore, we believe that LRRK2 mutations can be involved in the tau phosphorylation pathway.

How LRRK2 can participate in the tau phosphorylation pathway remains unclear. In addition, we failed to find that these abnormal tau deposits have any apparent spatial correlation with our observed region-specific neuronal degeneration in the Sagamihara family. Therefore, future work will need to evaluate the association between neurodegeneration and the tau hyperphosphorylation due to *LRRK2 I2020T* mutation.

Conflicts of interest

None declared.

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Pharmacokinetics

Pharmacokinetics and effect of food after oral administration of prolonged-release tablets of ropinirole hydrochloride in Japanese patients with Parkinson's disease

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Keywords: food effect, patients with Parkinson's disease, pharmacokinetics, prolonged-release tablets, ropinirole

SUMMARY

What is known and Objectives: Ropinirole hydrochloride, a dopamine receptor agonist with a non-ergot alkaloid structure, is highly selective for the dopamine D_2/D_3 receptors. This study was conducted to evaluate the steady-state pharmacokinetics, safety and efficacy after repeated oral administration of prolonged-release tablets of ropinirole hydrochloride in the absence of L-dopa preparations in Japanese patients with Parkinson's disease (PD).

Methods: This was a multicenter, open-label, uncontrolled study. The total duration of participation in the study ranged from 56 to 63 weeks. In the study, the plasma concentrations of ropinirole, its major metabolite SK&F104557 (*N*-depropyl ropinirole) and another metabolite SK&F89124 (ropinirole hydroxylated at the seventh position of the indole ring) were assessed. Safety based on adverse events, haematology, biochemistry, urinalysis and electrocardiography (ECG) (standard 12-lead ECG) were evaluated, and vital signs (blood pressure/pulse rate) were measured. Efficacy based on the Japanese version of Unified Parkinson's Disease Rating Scale (UPDRS) Parts III (motor) and II [activities of daily living (ADL)] as well as tolerability was evaluated.

Results: After repeated oral administration of prolonged-release tablets of ropinirole hydrochloride in Japanese patients with PD, ropinirole, SK&F104557 and low levels of SK&F89124 were detected in plasma. The trough concentrations of ropinirole and the two metabolites increased in proportion to the dose when ropinirole hydrochloride prolonged-release tablets were administered at doses ranging from 2 to 16 mg/day. The plasma exposure to ropinirole and its two metabolites after intake of normal diet was comparable to that in the fasting state. The most common adverse events (10% or more) were somnolence, nausea, constipation, hallucination and nasopharyngitis. Most adverse events were mild or moderate in severity, and with no death. During the treatment period, serious adverse events were reported in five patients. Efficacy analysis (LOCF) at the final endpoint up to week 16 demonstrated a mean (SD) change from baseline in the Japanese UPDRS III (motor) and II (ADL) scores of −11·3 (8·21) and −3·9 (3·22), respectively, and thereafter remained at similar levels until week 52.

What is new and Conclusions: After administration of prolonged-release tablets of ropinirole hydrochloride in the absence of L-dopa preparations in Japanese patients with PD, the plasma pharmacokinetics of ropinirole and its metabolites was linear and not affected by food. Compared with the immediate-release (IR) tablet, the prolonged-release tablet can be administered to Japanese patients with PD at a reduced daily dose frequency and adjusted to the maintenance dose after fewer dose changes with a smaller diurnal variation in the plasma ropinirole concentration.

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INTRODUCTION

Ropinirole hydrochloride, a dopamine receptor agonist with a non-ergot alkaloid structure and highly selective for the dopamine D_2/D_3 receptors,[1–4] was developed by GlaxoSmithKline K.K. Ropinirole is metabolized by two routes to SK&F89124 and SK&F105447. SK&F89124 is pharmacologically as active as ropinirole *in vivo*, but only traces of free SK&F89124 is found, and the pharmacological activity of SK&F104557 is <1/150 of that of ropinirole.[5] The prolonged-release/extended-release (PR/XR) tablet of ropinirole hydrochloride[6] has a three-layer structure consisting of an inner active layer containing ropinirole hydrochloride between two outer inactive barrier layers. The tablet exhibits sustained release over approximately 24 h. Ropinirole hydrochloride is widely used with or without L-dopa preparations for the treatment of PD. In Japan, the immediate-release (IR) tablet formulation has been commercially available since December 2006 under the brand name of ReQuip tablets® with a thrice-daily regimen. The PR/XR tablet formulation of ropinirole hydrochloride is administered at the initial dose of 2 mg/day once daily in the morning. The dose is increased by 2 mg/day/week to 8 mg/day at week 4 of treatment and then by 2 mg/day/week with due attention to the safety and tolerability until a satisfactory clinical effect is obtained. This study was conducted to evaluate the steady-state pharmacokinetics, safety and efficacy after repeated oral administration of PR/XR tablets of ropinirole hydrochloride once daily in the absence of L-dopa preparations in Japanese patients with PD. Although a clinical pharmacology study in

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non-Japanese showed that the IR tablet formulation was associated with delayed absorption of ropinirole in the presence of food,[7] this pharmacokinetic effect was not seen with the PR/XR tablets after intake of a high-fat diet[8] (as defined by the US Food and Drug Administration). Therefore, the effect of food on the pharmacokinetics of ropinirole hydrochloride PR/XR tablets was also evaluated after repeated oral administration once daily in Japanese patients with PD.

MATERIALS AND METHOD

Patients

All patients in this study were diagnosed with PD stage 1–3 according to the modified Hoehn & Yahr staging and were 20 years old or older at the time of informed consent. Previous treatment was restricted to 3 months or less of low or moderate doses of L-dopa preparations, or 6 months or less of dopamine receptor agonists in total, as long as treatment had been discontinued at least 4 weeks prior to the start of screening. Those who had orthostatic hypotension with subjective symptoms (dizziness, syncope, etc.), serious psychiatric symptoms (confusion, hallucination, delusion, abnormal behaviour, drug/alcohol dependence, etc.) within 26 weeks prior to informed consent, severe dementia with a Japanese UPDRS Part I (Mentation, Behaviour and Mood) score of 3 or 4, past or current history of cancer or malignant tumour, or past history of surgery for treatment of PD (pallidotomy, deep brain stimulation, etc.) were excluded. During the treatment period, concomitant use of L-dopa preparations, IR tablet formulation of ropinirole hydrochloride, other dopamine receptor agonists, drugs that act on dopamine receptors and other investigational products was prohibited. Patients receiving anticholinergics, amantadine hydrochloride, droxidopa, citicoline, selegiline hydrochloride, zonisamide, oestrogen preparations and CYP1A2 inhibitors were permitted to enter the study as long as they could be maintained on a stable regimen.

The study protocol was approved by the institutional review boards in every institution and was in full compliance with the principle of the 'Declaration of Helsinki' (current version) and the 'Good Clinical Practice' guidelines. Written informed consent was obtained from each patient prior to the start of the study.

Study design and dosing regimen

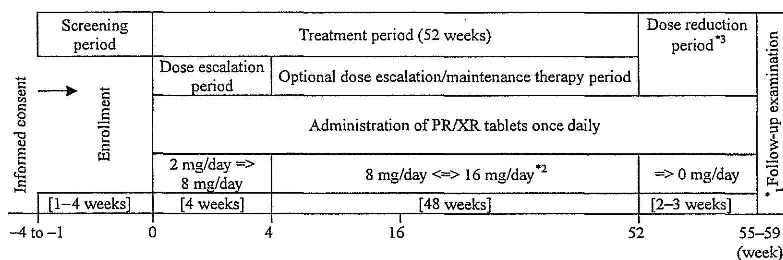
This was a multicenter, open-label, uncontrolled study. The study design is shown in Fig. 1. The total duration of participation in the study ranged from 56 to 63 weeks (screening period: 1–4 weeks, dose escalation period: 4 weeks, optional dose escalation/maintenance therapy period: 48 weeks, dose reduction period: 2–3 weeks, follow-up examination: 1–4 weeks). The formulation was then administered at the achieved maintenance dose for 52 weeks. The maximum dose was 16 mg/day.

Assessment of plasma trough concentrations of unchanged compound and metabolites

The plasma pharmacokinetics of ropinirole, its major metabolite SK&F104557 (*N*-depropyl ropinirole), and another metabolite SK&F89124 (ropinirole hydroxylated at the seventh position of the indole ring) were evaluated. Blood was collected from all patients to assess the trough concentration (concentration at 24 h post-dose) in the dose escalation and maintenance therapy periods immediately before dosing in the morning. During the dose escalation period, blood sampling was performed approximately 24 h after the final dosing at doses of 2, 4 and 8 mg/day. During the maintenance therapy period, blood sampling was performed approximately 24 h after treatment at the maintenance dose of ≥10 mg/day for at least 1 week.

Effect of food on the pharmacokinetics

During the maintenance therapy period, blood sampling over 24 h was performed in 11 patients. Blood sampling was performed from weeks 5–16 of treatment after at least 7 days of maintenance therapy. The treatment status was recorded over 1 week prior to the day before blood sampling. To evaluate the effect of food on the pharmacokinetics, the patients received the study drug after breakfast (normal diet: approximately 500 kcal) and in the fasting state in a crossover manner. Blood sampling was performed at eleven time points: before dosing and 1, 2, 4, 6, 8, 10, 12, 14, 16, and 24 h after dosing. During the crossover period, an interval of at least 2 days was maintained between blood sampling during the time when



*1: The follow-up examination was performed within 1 to 4 weeks after the final dosing.
*2: The dose could be reduced down to 6 mg/day if dose reduction was considered to be required because of adverse events, etc.
*3: No dose reduction was required if the PR/XR tablet formulation was replaced with the IR tablet formulation overnight upon the completion of the study.

Fig. 1. Study design.

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study drug administration was switched to either after breakfast or the fasting state. Blood sampling from each patient was performed after administration of the same dose on the two occasions.

Preparation of plasma samples

To measure the plasma concentrations of ropinirole and its metabolites, blood was collected into a heparinized test tube and centrifuged at 4 °C (1500 *g*) to separate plasma rapidly, which was then stored frozen at or below −20 °C until measurement. Approximately 5 mL of blood (at least 2 mL of plasma) was collected at each time point. The stability of ropinirole and the two metabolites in human plasma at −20 °C is at least 507 days.

Measurement method

The plasma concentrations of ropinirole and its metabolites were measured by HPLC-MS/MS after solid-phase extraction from acidic plasma. In 0·5 mL of human plasma, the lower limit of quantification was 0·02, 0·05 and 0·02 ng/mL for ropinirole, SK&F104557 and SK&F89124, respectively. When a calibration curve was constructed at each time of measurement, QC samples were also analysed to assess the diurnal variation in the measurement. The calibration curves of ropinirole and SK&F89124 were linear at a concentration range of 0·02–5 ng/mL plasma, at a concentration range of 0·05–5 ng/mL plasma for SK&F104557, with a correlation coefficient $r = 0{\cdot}998$. At quantification limit, accuracy and precision (inter-day) were −0·9% and 6·6% for ropinirole, −1·2% and 7·0% for SK&F104557, 3·9% and 9·7% for SK&F89124, respectively.

Pharmacokinetic analysis

The plasma trough concentrations of ropinirole, SK&F104557 and SK&F89124 in individual patient were plotted against dose to visually assess dose linearity. The steady-state pharmacokinetic parameters including the C_{max}, $AUC_{0–24}$, C_{min} and t_{max} were calculated from the plasma concentrations of ropinirole, SK&F104557 and SK&F89124 using a model-independent method. For the dose-normalized pharmacokinetic parameters following administration in the fasting state and after food, the point estimate and its 90% confidence interval were calculated to evaluate the effect of food on the pharmacokinetics of ropinirole and its metabolites.

Safety and efficacy

The investigator/subinvestigator recorded information on adverse events/serious adverse events in the case report form. To collect information on adverse events in a standardized manner, non-leading questions such as 'has there been any change since the start of the study or the previous visit?' were asked. In addition, haematology, biochemistry, urinalysis, electrocardiography (ECG) (standard 12-lead ECG) and measurement of vital signs (blood pressure/pulse rate) were performed. Assessments using the Japanese UPDRS Parts III and II and modified Hoehn & Yahr severity scale were made at the start of the treatment period (week 0) and at weeks 1, 2, 3, 4, 6, 8, 10, 12, 16 to 48 (every 4 weeks), and 52 (or at the time of discontinuation) during the treatment period.

RESULTS

Of 62 patients with PD treated with the PR/XR tablets, 61 were included in the analysis of trough concentrations (C_{min}). One patient from whom blood could not be collected was excluded. The effects of food on C_{max}, t_{max} and $AUC_{0–24}$ were analysed in 10 of the 11 patients. One patient whose blood could not be collected after a meal was excluded. Safety and efficacy data were analysed in all of the 62 patients treated with PR/XR tablets (2–16 mg/day). The majority of patients were female (60%), with a mean age of 67·2 years.

Plasma trough concentrations of unchanged compound and metabolites

After repeated oral administration of the PR/XR tablets, ropinirole, SK&F104557 and SK&F89124 plasma levels were measured. Table 1 and Fig. 2 demonstrate that the plasma trough concentration of ropinirole was similar to that of SK&F104557 in the dose range from 2 to 16 mg/day. On the other hand, the plasma trough concentration of SK&F89124 was consistently about 22–31 times lower than that of ropinirole or SK&F104557 at all doses. SK&F104557 and SK&F89124 are therefore not thought to contribute significantly to the pharmacological activity of ropinirole.

Effect of food on the pharmacokinetics

Steady-state plasma pharmacokinetic parameters of ropinirole, SK&F104557 and SK&F89124 following administration after intake of normal diet in Japanese patients with PD are shown in Table 2. The time courses of plasma concentrations of ropinirole over 24 h following administration of a meal or in the fasting state are shown in Fig. 3, and the pharmacokinetic parameters for ropinirole, SK&F104557 and SK&F89124 are shown in Table 3.

Ropinirole hydrochloride was administered at doses of 8, 10, 12, 14 and 16 mg/day in 2, 3, 2, 1 and 2 patients, respectively. After administration of the PR/XR tablets, the plasma concentrations of ropinirole, SK&F104557 and SK&F89124 increased slowly, with a t_{max} of 7 to 9 h. For ropinirole, SK&F104557 and SK&F89124, the least-squares geometric mean dose-normalized C_{max}, $AUC_{0–24}$ and C_{min} were similar in both fed and fasted condition. These results show that food delayed the t_{max} of SK&F104557 but had no effect on the plasma C_{max}, $AUC_{0–24}$ and C_{min} of ropinirole, SK&F104557 or SK&F89124.

Adverse events on therapy were observed in 60 patients (97%), the most common (10% or more) of which were somnolence (25 patients, 40%), nausea (18 patients, 29%), constipation (16 patients, 26%), hallucinations (13 patients, 21%) and nasopharyngitis (12 patients, 19%) (Table 4). Most adverse events were mild or moderate in severity. Somnolence was mild in 20 patients and moderate in five patients. All hallucination events were mild or moderate in severity and all occurred at doses higher than 8 mg/day except for one event at 2 mg/day. There were no deaths. During the treatment period, serious adverse events were reported in five patients (8%), including small intestine carcinoma, intestinal obstruction and cerebral infarction in three patients for which a causal relationship to the study drug could not be ruled out. No clinically relevant important changes were observed in the laboratory parameters, vital signs or ECG.

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Table 1. Trough levels of ropinirole and its metabolites (SK&F104557 and SK&F89124) following repeat oral doses of ropinirole PR/XR tablet in Japanese patients with PD (monotherapy)

		C_{min} (ng/mL)		
Dose (mg/day)	*n*	Ropinirole	SK&F104557	SK&F89124
2	61	1·80 (1·76)	1·56 (0·78)	0·07 (0·04)
4	58	3·53 (1·75)	3·22 (1·18)	0·14 (0·06)
6	1	3·82 (–)	4·17 (–)	0·16 (–)
8	61	7·60 (5·51)	6·35 (2·17)	0·28 (0·12)
10	17	9·77 (3·24)	7·94 (1·89)	0·32 (0·08)
12	12	12·0 (7·21)	11·8 (3·92)	0·48 (0·19)
14	9	12·6 (5·59)	11·6 (4·28)	0·56 (0·29)
16	12	15·5 (8·29)	12·8 (2·53)	0·63 (0·27)

Values given are mean (standard deviation).

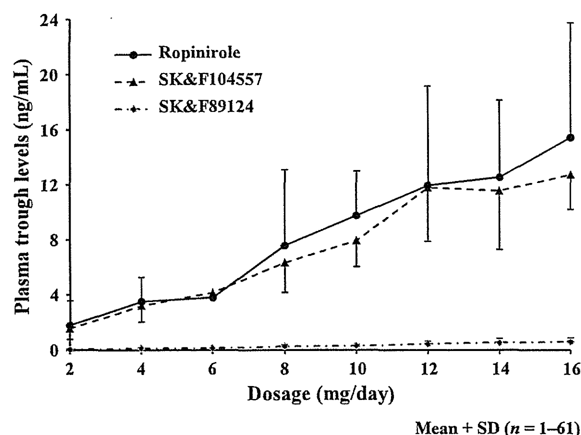

Fig. 2 Plasma trough levels of ropinirole, SK&F104557 and SK&F89124 following repeated once daily oral administration of a 2–16 mg/day for ropinirole PR/XR tablet.

Efficacy analysis (LOCF) at the final endpoint of up to week 16 demonstrated a mean (SD) change from baseline in the Japanese UPDRS III (motor) and II (ADL) scores of –11·3 (8·21) and –3·9 (3·22), respectively. The scores remained at similar levels until week 52. The time courses for the changes in the Japanese UPDRS III (motor) and II (ADL) are shown in Fig. 4. The modified Hoehn & Yahr severity decreased after treatment with the study drug. The mean dose was 11·1 ± 2·77 mg/day during the optional dose escalation/maintenance therapy period, and the most common dose at the final dosing was 10 mg/day (16 patients, 26%), followed by 12 and 16 mg/day (11 patients, 18%).

Table 2. Summary of dose-normalized (to 1 mg) steady-state pharmacokinetic parameters for ropinirole PR/XR tablet in Japanese patients (fed condition) with PD (monotherapy, *n* = 10)

Parameter	Ropinirole	SK&F104557	SK&F89124
Dose-normalized $AUC_{0–24}$ (ng*h/mL/mg)	27·2	23·1	1·04
Dose-normalized C_{max} (ng/mL/mg)	1·56	1·16	0·06
Dose-normalized C_{min} (ng/mL/mg)	0·73	0·80	0·03
t_{max} (h)[a]	7·00	9·00	9·00

Values given are geometric mean, unless otherwise indicated.
[a]Median.

Studies in non-Japanese have shown that ropinirole is primarily metabolized to SK&F104557[9] and that the production of SK&F89124 and its glucuronic acid conjugate was as low as <10% of the dose, with free SK&F89124 corresponding to 1% of the dose.[10] SK&F104557 and ropinirole were detected at similar levels in Japanese patients, but the SK&F89124 concentration was low, indicating that the metabolites would not contribute to

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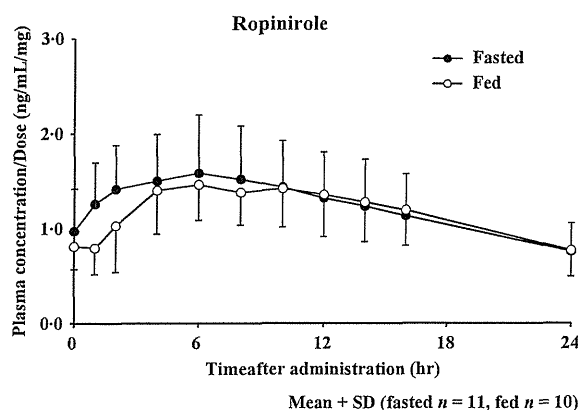

Fig. 3. Dose-normalized plasma concentration-time profiles of ropinirole after oral administration of Ropinirole PR/XR tablet during the 'fed' and 'fasted' sessions.

Table 3. Summary of dose-normalized (to 1 mg) steady-state pharmacokinetic parameters for ropinirole PR/XR tablet in Japanese patients with PD (monotherapy, n = 10)

Parameter	Fed	Fasted	Ratio fed/fasted	90% CI
Ropinirole				
Dose-normalized AUC_{0-24} (ng*h/mL/mg)	27·2	27·2	1·00	0·77, 1·31
Dose-normalized C_{max} (ng/mL/mg)	1·56	1·54	1·01	0·78, 1·33
Dose-normalized C_{min} (ng/mL/mg)	0·73	0·73	1·01	0·75, 1·34
t_{max} (h)[a]	7·00	6·00	2·00[b]	−0·10, 5·00
SK&F104557				
Dose-normalized AUC_{0-24} (ng*h/mL/mg)	23·1	24·5	0·94	0·82, 1·08
Dose-normalized C_{max} (ng/mL/mg)	1·16	1·25	0·93	0·80, 1·07
Dose-normalized C_{min} (ng/mL/mg)	0·80	0·79	1·02	0·87, 1·19
t_{max} (h)[a]	9·00	7·00	3·9[b]	2·00, 6·00
SK&F89124				
Dose-normalized AUC_{0-24} (ng*h/mL/mg)	1·04	1·09	0·96	0·78, 1·17
Dose-normalized C_{max} (ng/mL/mg)	0·06	0·06	0·91	0·73, 1·14
Dose-normalized C_{min} (ng/mL/mg)	0·03	0·03	0·99	0·80, 1·23
t_{max} (h)[a]	9·00	6·00	1·00[b]	−0·10, 5·00

Values given are geometric mean, unless otherwise indicated.
[a]Median.
[b]Difference = fed − fasted.

the clinical efficacy of ropinirole. In this study, it was also shown that the plasma trough concentrations of ropinirole and its two metabolites were highly variable among individuals. An *in vitro* study using human liver microsomes showed that

Table 4. Summary of frequent adverse events (period: on-treatment)

N = 62			
Any event 60 (97)			
Somnolence	25 (40)	Muscle spasms	3 (5)
Nausea	18 (29)	Oedema	3 (5)
Constipation	16 (26)	Sudden onset of sleep	3 (5)
Hallucination	13 (21)	Anaemia	2 (3)
Nasopharyngitis	12 (19)	Asthenopia	2 (3)
Dizziness	5 (8)	Cough	2 (3)
Headache	5 (8)	Dizziness postural	2 (3)
Vomiting	5 (8)	Dyspepsia	2 (3)
Back pain	4 (6)	Dystonia	2 (3)
Bronchitis	4 (6)	Hallucination, visual	2 (3)
Oedema peripheral	4 (6)	Insomnia	2 (3)
Stomach discomfort	4 (6)	Malaise	2 (3)
Anorexia	3 (5)	Nightmare	2 (3)
Blood creatine phosphokinase increased	3 (5)	Orthostatic hypotension	2 (3)
Dermatitis contact	3 (5)	Pain in extremity	2 (3)
Diarrhoea	3 (5)	Pharyngitis	2 (3)
Eczema	3 (5)	Pollakiuria	2 (3)
Fall	3 (5)	Upper respiratory tract inflammation	2 (3)
Gastritis	3 (5)	Urticaria	2 (3)

Adverse events occurring >1 patient.

CYP1A2 was primarily involved in the metabolism of ropinirole.[11] CYP1A2 activity is known to show wide inter-subject variability.[12] Accordingly, the high inter-individual variability in plasma concentrations of ropinirole and its metabolites probably reflects this. *In vivo* metabolism studies showing that CYP1A2 activity is similar in East Asians and Caucasians[13–15] suggest that the time course of plasma concentrations of ropinirole and its metabolites in Japanese would be comparable to that in Caucasians.

In patients with PD outside Japan, it was reported that the plasma $t_{1/2}$ of ropinirole was approximately 5–6 h after administration of IR tablets, and the exposure following oral administration of PR/XR tablets at a dose of 8 mg once daily was similar to that following oral administration of IR tablets at a dose of 2·5 mg thrice daily.[7] In the study, the degree of fluctuation in the plasma ropinirole concentration [DF in the plasma concentration: $DF = (C_{max} - C_{min})/C_{avg}$, $C_{avg} = AUC_{0-\tau}/\tau$ (τ, dosing interval)] was 0·66 for PR/XR tablets and 0·85 for IR tablets, showing that the PR/XR tablet formation was associated with lower peak-to-trough variability than the IR tablet formulation. Although the DF in the plasma ropinirole concentration after administration of IR tablets in Japanese is unknown, the DF following administration of PR/XR tablets in Japanese patients in this study was 0·73 and appeared to be comparable to that in Caucasians (0·66).[7]

In Japan, ropinirole hydrochloride is commercially available only as the IR tablet formulation with a thrice-daily regimen, requiring step-by-step weekly dose increase from 0·75 to 3·0 mg/day during the early dose escalation period. Multistep dose escalation is associated with concerns over treatment compliance. It is

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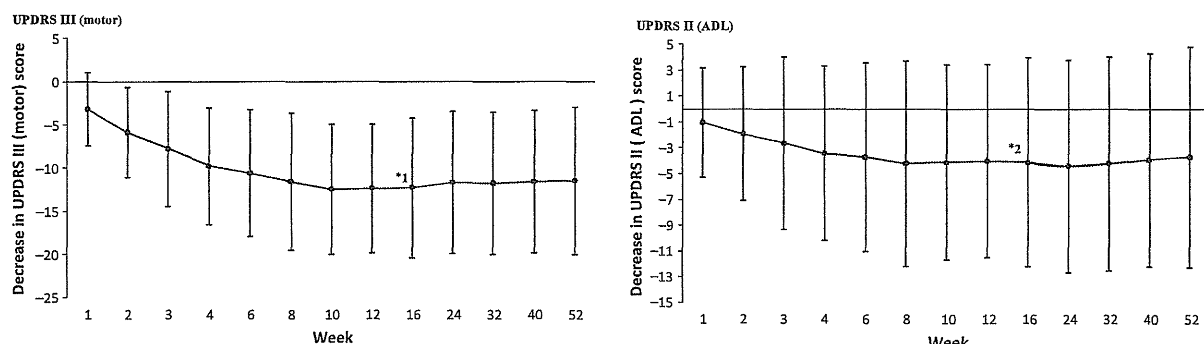

*1: Efficacy analysis (LOCF) at the final endpoint up to week 16 demonstrated a mean (SD) change from baseline in the Japanese UPDRS III (motor) score of −11·3 (8·21).

*2: Efficacy analysis (LOCF) at the final endpoint up to week 16 demonstrated a mean (SD) change from baseline in the Japanese UPDRS II (ADL) scores of −3·9 (3·22).

Fig. 4. The ordinate represents decrease in the Japanese UPDRS III (motor) and UPDRS II (ADL) score and the abscissa weeks. The mean value and standard deviation (SD) of each visit are shown.

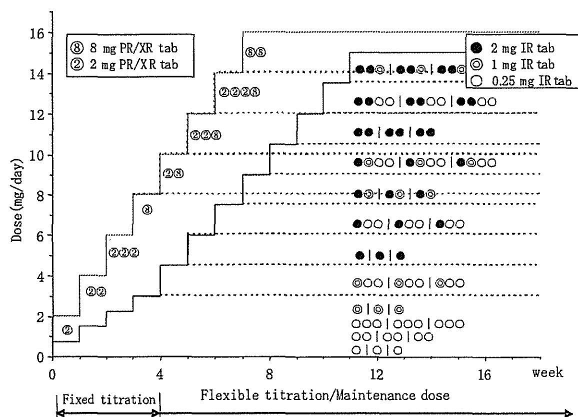

Fig. 5. Dosage comparison between PR/XR tablets and IR tablets.

known that increased daily dose frequency is generally associated with decreased compliance.[16] It has been reported that drugs are not properly used in 20% of patients with PD because of compliance issues[17] and that there is a high frequency of errors in terms of timing or dosage.[18] The IR tablet formulation requires 11 dose changes to reach the maximum dose of 15 mg/day whereas the PR/XR tablet formulation requires seven dose changes to reach the maximum dose of 16 mg/day (Fig. 5). In addition, the PR/XR tablet formation is associated with lower inter-individual variability in the plasma ropinirole concentration than the IR tablet formulation in non-Japanese subjects. In this study, the plasma C_{max} and AUC_{0-24} of ropinirole, SK&F104557 and SK&F89124 were not affected by intake of food after administration of PR/XR tablets. Food also did not affect the production of metabolites SK&F104557 or SK&F89124. Compared with a previous study of Japanese patients with PD treated with IR tablets (0·75–15 mg/day) thrice daily[19] and a non-Japanese clinical study involving patients with PD treated with PR/XR tablets,[20] the safety profile of PR/XR tablets was similar in this study with no new safety signal identified.

What is new and Conclusion: This study shows that compared with the IR tablets, the PR/XR tablets can be administered to Japanese patients with PD at a reduced daily dose frequency and adjusted to the maintenance dose with fewer dose changes. The PR/XR product also produces a smaller diurnal variation in the plasma ropinirole concentration.

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Brief communication

Analysis of *C9orf72* repeat expansion in 563 Japanese patients with amyotrophic lateral sclerosis

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Abstract

Recently, a hexanucleotide repeat expansion in *C9orf72* was identified as the most common cause of both sporadic and familial amyotrophic lateral sclerosis (ALS) and frontotemporal dementia in Western populations. We analyzed 563 Japanese patients with ALS (552 sporadic and 11 familial) using fluorescent fragment-length analysis of *C9orf72* and repeat-primed polymerase chain reaction analysis. Haplotype analysis was performed for 42 single nucleotide polymorphisms in patients with *C9orf72* repeat expansion. *C9orf72* repeat expansion was found in 2 patients with sporadic ALS (2/552 = 0.4%) and no patients with familial ALS (0/11 = 0%). In the probands' families, 1 primary progressive aphasia patient and 1 asymptomatic 76-year-old individual exhibited *C9orf72* repeat expansion. All of the patients with the *C9orf72* repeat expansion carried the 20-single nucleotide polymorphism consensus risk haplotype. The frequency of the *C9orf72* repeat expansion among Japanese patients is much lower than in Western populations. The existence of a 76-year-old asymptomatic carrier supported the notion of incomplete penetrance. The *C9orf72* mutation should be analyzed in sporadic ALS patients after determining their family histories not only of frontotemporal dementia but also of primary progressive aphasia.
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Keywords: Amyotrophic lateral sclerosis; *C9orf72*; Incomplete penetrance; Sporadic; Aphasia; Frontotemporal dementia

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder that primarily affects motor neurons in the spinal cord, brain stem, and cerebral cortex, typically leading to death within a few years. Five to ten percent of ALS cases are familial, and the remaining cases are believed to be sporadic (Valdmanis et al., 2009). A number of genes causing ALS with a dominant mode of inheritance have

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been discovered, such as *SOD1*, *TARDBP*, *FUS*, *VAPB*, *ANG*, *VCP*, *OPTN* (Ticozzi et al., 2011), and *UBQLN2* (Deng et al., 2011). Moreover, there is increasing clinical and pathological evidence for the hypothesis that ALS and frontotemporal dementia (FTD) constitute an overlapping continuum of diseases (Lomen-Hoerth et al., 2002; Neumann et al., 2006). Recently, the expansion of a noncoding GGGGCC hexanucleotide repeat in the *C9orf72* gene has been reported to be a major cause of both ALS and FTD (DeJesus-Hernandez et al., 2011; Gijselinck et al., 2012; Renton et al., 2011) and the most common genetic abnormality in familial and sporadic forms of both ALS and FTD, particularly in Western populations (Chiò et al., 2012; DeJesus-Hernandez et al., 2011; Gijselinck et al., 2012; Renton et al., 2011; Sabatelli et al., 2012; Stewart et al., 2012). In the present study, we describe the incidence and demographic and clinical features associated with the *C9orf72* mutation in a large cohort of Japanese ALS patients. We also perform haplotype analysis to investigate whether Japanese patients have the same risk haplotype as European patients (Gijselinck et al., 2012; Laaksovirta et al., 2010; Mok et al., 2012).

2. Methods

2.1. Subjects

We obtained a total of 760 DNA samples from the Japanese Consortium for Amyotrophic Lateral Sclerosis Research (JaCALS; Appendix A). A total of 563 (11 familial and 552 sporadic) patients were diagnosed with ALS according to the El Escorial revised criteria (Brooks et al., 2000) and classified as bulbar-onset, spinal-onset, FTD-ALS, or other (see Supplementary Table 1 for details). We had determined the family histories of ALS but not FTD or primary progressive aphasia (PPA) in all of the patients when they were enrolled as patients with sporadic ALS (SALS). We recruited 197 control subjects, none of whom had a medical or family history of neurodegenerative disorders. The mean age at onset of the patients with ALS was 60.4 ± 11.7 years (range 20–86), and the mean age at sampling of the controls was 60.6 ± 10.3 years (range 26–83). All of the subjects were unrelated Japanese individuals. Written informed consent was obtained from all of the subjects. The ethical committees at the participating institutions approved this study.

2.2. Fluorescent fragment-length analysis of C9orf72 and repeat-primed PCR analysis

The normal repeat number of the GGGGCC hexanucleotide was determined in all of the patients and control subjects using genotyping primers, as previously described (DeJesus-Hernandez et al., 2011). To provide a qualitative assessment of the presence of *C9orf72* repeat expansions, we performed repeat-primed polymerase chain reaction (PCR), as previously described (DeJesus-Hernandez et al., 2011).

2.3. Haplotype analysis

We genotyped 42 single nucleotide polymorphisms (SNPs) across 232 kilobase of Chromosome 9p21, which were first described as the founder haplotype in the Finnish ALS population (Laaksovirta et al., 2010), using primers (Supplementary Table 2) to determine whether our Japanese patients carried the haplotype associated with a risk of ALS. These 42 SNPs included the 20-SNP consensus risk allele that had recently been detected in genome-wide association studies in several populations (Mok et al., 2012). We also performed haplotype analysis with 4 microsatellites (D9S1121, D9S169, D9S270, and D9S104) flanking the *C9orf72* GGGGCC repeat, as previously described (Gijselinck et al., 2012) (Fig. 1).

3. Results

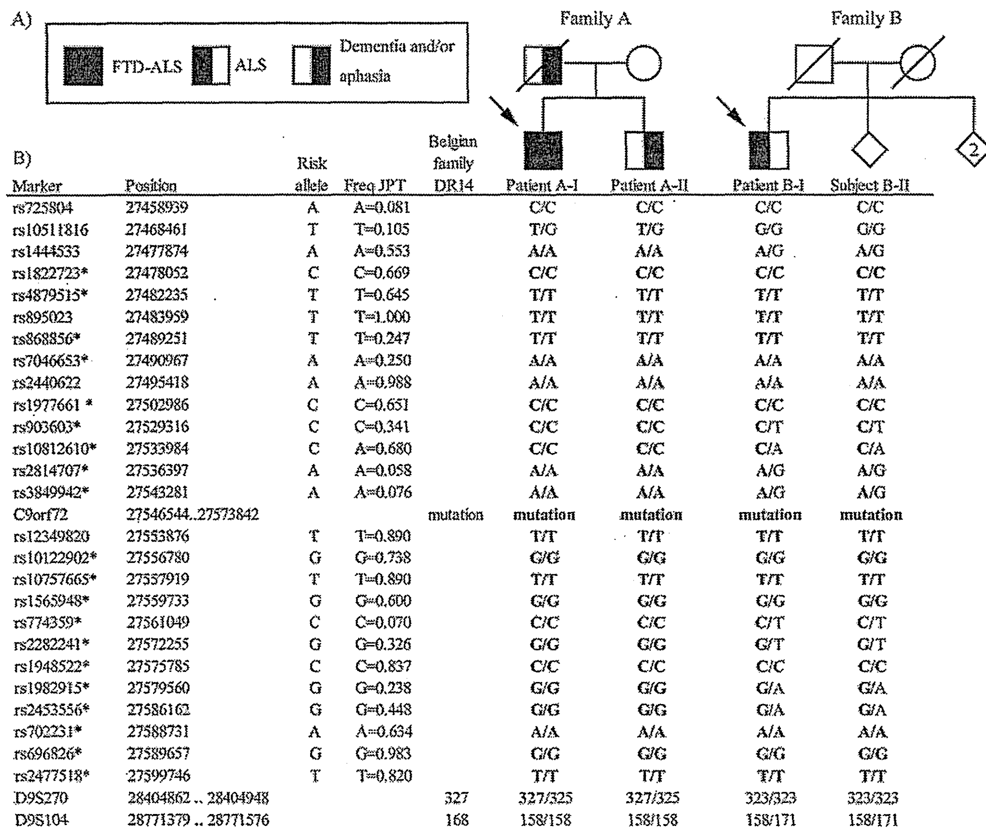
3.1. Detection of C9orf72 repeat expansion

The *C9orf72* repeat expansion was found in 2 of 522 Japanese patients (2/552 = 0.4%) with SALS and none of the 11 patients (0/11 = 0%) with familial ALS (FALS) using repeat-primed PCR (Table 1). Patient A-I with a *C9orf72* mutation was classified as SALS in this study, but after detecting the mutation, we found that patient A-II (a brother of patient A-I) developed aphasia and dementia and had a *C9orf72* mutation (Fig. 1). The average repeat number based on fluorescent fragment-length analysis was 3.65 ± 2.43 (range 2–13 repeats) in 561 ALS patients without the *C9orf72* mutation. A subsequent analysis of 197 healthy controls did not detect any *C9orf72* mutation. The average repeat number was 3.69 ± 2.46 (range 2–14 repeats) in the 197 controls. The mean age at disease onset in patients with *C9orf72* mutation, including patient A-II, was 64.7 ± 6.1 years (range 57–72). The genotypes of all individuals with the *C9orf72* mutation were detected for the 20 SNPs spanning a 140-kilobase segment concordant with the recently identified risk haplotype on chromosome 9p (Mok et al., 2012) and 24 or 25 consecutive SNPs in the 42-SNP Finish risk haplotype (Laaksovirta et al., 2010) (Fig. 1, Supplementary Table 3).

3.2. Clinical presentations of individuals with C9orf72 mutation

3.2.1. Patient A-I (family A)

Patient A-I was a 65-year-old man who reported weakness in the left leg. The weakness progressed, and he developed fasciculation. At age 66, a neurological examination revealed dementia. His Mini Mental State Examination score was 23/30, and his Frontal Assessment Battery score was 13/18. He also exhibited dysarthria and weakness, atrophy, and fasciculation in the tongue and all 4 modalities. His tendon reflexes were diminished, and the plantar re-



Marker	Position	Risk allele	Freq JPT	Belgian family DR14	Patient A-I	Patient A-II	Patient B-I	Subject B-II
rs725804	27458939	A	A=0.081		C/C	C/C	C/C	C/C
rs10511816	27468461	T	T=0.105		T/G	T/G	G/G	G/G
rs1444533	27477874	A	A=0.553		A/A	A/A	A/G	A/G
rs1822723*	27478052	C	C=0.669		C/C	C/C	C/C	C/C
rs4879515*	27482235	T	T=0.645		T/T	T/T	T/T	T/T
rs895023	27483959	T	T=1.000		T/T	T/T	T/T	T/T
rs868856*	27489251	T	T=0.247		T/T	T/T	T/T	T/T
rs7046653*	27490967	A	A=0.250		A/A	A/A	A/A	A/A
rs2440622	27495418	A	A=0.988		A/A	A/A	A/A	A/A
rs1977661 *	27502986	C	C=0.651		C/C	C/C	C/C	C/C
rs903603*	27529316	C	C=0.341		C/C	C/C	C/T	C/T
rs10812610*	27533984	C	A=0.680		C/C	C/C	C/A	C/A
rs2814707*	27536397	A	A=0.058		A/A	A/A	A/G	A/G
rs3849942*	27543281	A	A=0.076		A/A	A/A	A/G	A/G
C9orf72	27546544..27573842			mutation	**mutation**	**mutation**	**mutation**	**mutation**
rs12349820	27553876	T	T=0.890		T/T	T/T	T/T	T/T
rs10122902*	27556780	G	G=0.738		G/G	G/G	G/G	G/G
rs10757665*	27557919	T	T=0.890		T/T	T/T	T/T	T/T
rs1565948*	27559733	G	G=0.600		G/G	G/G	G/G	G/G
rs774359*	27561049	C	C=0.070		C/C	C/C	C/T	C/T
rs2282241*	27572255	G	G=0.326		G/G	G/G	G/T	G/T
rs1948522*	27575785	C	C=0.837		C/C	C/C	C/C	C/C
rs1982915*	27579560	G	G=0.238		G/G	G/G	G/A	G/A
rs2453556*	27586162	G	G=0.448		G/G	G/G	G/A	G/A
rs702231*	27588731	A	A=0.634		A/A	A/A	A/A	A/A
rs696826*	27589657	G	G=0.983		G/G	G/G	G/G	G/G
rs2477518*	27599746	T	T=0.820		T/T	T/T	T/T	T/T
D9S270	28404862 .. 28404948			327	327/325	327/325	323/323	323/323
D9S104	28771379 .. 28771576			168	158/158	158/158	158/171	158/171

Fig. 1. (A) The pedigrees of the 2 families with *C9orf72* repeat expansion. To maintain confidentiality, several unaffected individuals who died early in families A and B are not shown. Probands are indicated by arrows. (B) The genotyping data of the single nucleotide polymorphisms (SNPs) and microsatellites. Twenty SNPs, which comprised a recently identified consensus risk haplotype (Mok et al., 2012), are shown with an asterisk. See Supplementary Table 3 for details of the analyses of 42 SNPs (Laaksovirta et al., 2010) and microsatellites (Gijselinck et al., 2012). Alleles possibly shared between our subjects and patients in Western populations are shown in bold. The genotypes of all 4 subjects with respect to the 20 SNPs were found to be concordant with the risk haplotype (Mok et al., 2012). All of the positions of SNPs and microsatellites were from NC_000009.11. Abbreviations: ALS, amyotrophic lateral sclerosis; Freq JPT, Frequency in Japanese in Tokyo from International HapMap project (International HapMap Consortium, 2003); FTD, frontotemporal dementia.

sponse was extensor on the left. He had neither dysphagia nor dyspnea. No sensory abnormalities were noted. Extensive screening for causes of motor neuropathy was negative. The diagnosis was clinically probable ALS-laboratory supported (Brooks et al., 2000) and FTD-ALS.

3.2.2. Patient A-II (family A)

This patient was a 57-year-old man who presented with difficulty speaking. He was believed to have suffered from a mental disease after being imprisoned because of his involvement in a fatal car accident. At age 64, he was severely dysfluent and could barely speak. Logoclonia was particularly prominent. However, he did not exhibit any violent behavior or other behavioral abnormalities. He also did not display any clinical features of motor neuron disease. Brain magnetic resonance imaging revealed severe frontotemporal lobar atrophy. PPA was considered the most likely diagnosis.

3.2.3. Patient B-I (family B)

Patient B-I was a 72-year-old man who presented with gait disturbance and weakness in the proximal lower extremity muscle. His family history was negative for motor neuron disease and dementia (Fig. 1). The muscle weakness and atrophy progressed and spread to the other parts of his body despite treatment with intravenous gamma globulin. At age 74, he could not roll over while sleeping. A neurological examination showed marked muscle atrophy in his arms and shoulders and prominent fasciculation in his legs. The deep tendon reflexes were decreased in his limbs, and he had no pathological reflexes. Sensations in all 4 modalities were intact. At age 75, he developed dyspnea and dysphagia and started noninvasive positive pressure ventilation and intravenous hyperalimentation. He died of respiratory insufficiency at age 76. An autopsy was not performed. The diagnosis was clinically suspected ALS (Brooks et al., 2000).

Table 1
Frequencies of ALS patients with *C9orf72* and *SOD1* mutations in different countries

Study	Population	*C9orf72*			*SOD1*	
		Familial ALS	Sporadic ALS	Mean AAO (range), years	Familial ALS	Sporadic ALS
This study, 2012	Japanese (JaCALS)	0% (0/11)	0.4% (2/552)	64.7 (57–72)	NA	NA
Akimoto et al. (2011)	Japanese (JaCALS)	NA	NA	NA	NA	1.6% (4/255)
DeJesus-Hernandez et al. (2011)	Mixed[a]	23.5% (8/34)	4.1% (8/195)***	54.5 (41–72)	11.8% (4/34)	0% (0/195)
Renton et al. (2011)	Finish	46.4% (52/112)**	21.0% (61/290)***	53 (30–71)	NA	NA
Gijselinck et al. (2012)	Flanders-Belgian	46.7% (7/15)*	4.9% (6/122)***	54.5 (38–64)	0% (0/16)	0% (0/125)
Stewart et al. (2012)	Unknown[b]	27.4% (17/62)	3.6% (6/169)**	58.2 (39–82)	Total 8.2% (19/231)	
Byrne et al. (2012)	Ireland	40.8% (20/49)*	4.9% (19/386)***	56.3 (NA)	Total 0% (0/191)	
Cooper-Knock et al. (2012)	Northern England	42.9% (27/63)*	7.0% (35/500)***	57.3 (27–74)	Total 2.5% (14/563)	
Chiò et al. (2012)	Italian	37.5% (45/120)*	NA	59.0 (NA-80)	0% (0/141)	NA
	Sardinian	57.1% (12/21)**	NA	60.4 (NA)	NA	NA
	German	22.0% (9/41)	NA	56.4 (NA)	NA	NA
Majounie et al. (2012)	England	45.9% (45/98)**	6.8% (62/916)***	NA	NA	NA
	German	21.7% (15/69)	5.2% (22/421)***	NA	NA	NA
	Italian	37.8% (34/90)*	4.1% (19/465)***	NA	NA	NA
	Sardinian	57.9% (11/19)**	7.8% (10/129)***	NA	NA	NA
	USA White	US total 36.2% (59/163)*	5.4% (48/890)***	NA	NA	NA
	USA Hispanic		8.3% (6/72)***	NA	NA	NA
	USA Black		4.1% (2/49)	NA	NA	NA
	Australian	NA	5.3% (14/263)***	NA	NA	NA
	Israeli	21.4% (3/14)	NA	NA	NA	NA
	Indian	NA	0% (0/31)	NA	NA	NA
	Asian	5.0% (1/20)	0% (0/238)	NA	NA	NA
	Pacific islander/Guam	NA	0% (0/90)	NA	NA	NA
Sabatelli et al. (2012)	Italian	NA	3.7% (60/1624)***	58.6 (49–65)	NA	NA
	Sardinian	NA	6.8% (9/133)***	62.9 (58–63)	NA	NA

Key: AAO, age at onset; ALS, amyotrophic lateral sclerosis; JaCALS, Japanese Consortium of Amyotrophic Lateral Sclerosis Research; NA, not available.
[a] Mixed included 229 ALS patients from Mayo Clinic, Florida: White (212), Asian (1), Pacific Islander (1), and Black or African American (15).
[b] Unknown included 231 ALS patients from the ALS Clinic of Vancouver Coastal Health and the University of British Columbia (Vancouver General Hospital and GF Strong Rehabilitation Centre sites).
* $p < 0.05$, compared with our results (2-tailed, Yates's χ^2 test).
** $p < 0.01$, compared with our results (2-tailed, Yates's χ^2 test).
*** $p < 0.001$ compared with our results (2-tailed, Yates's χ^2 test).

3.2.4. *Subject B-II (family B)*

Subject B-II, a sibling of Patient B-I, had a *C9orf72* mutation but did not have symptoms of dementia or motor neuron disease until age 76 (Fig. 1).

4. Discussion

We began this study considering patients without family histories of ALS to be SALS because our cohort included only family histories of ALS but not FTD or PPA. Although it may be difficult to describe the real frequency in SALS because 1 of the SALS patients had a family member who developed PPA, the frequencies of the *C9orf72* mutation in Japanese patients were 0.4% (2/552) in SALS and 0% (0/11) in FALS according to this classification. In contrast, the frequencies of the *C9orf72* mutation fall within the ranges of 21%–57% in FALS and 3%–21% in SALS in Western populations (Table 1), and the *C9orf72* mutation has been reported as the most common genetic cause of FALS and SALS in Western populations (Byrne et al., 2012; Chiò et al., 2012; Cooper-Knock et al., 2012; DeJesus-Hernandez et al., 2011; Gijselinck et al., 2012; Majounie et al., 2012; Renton et al., 2011; Sabatelli et al., 2012; Stewart et al., 2012). However, the *C9orf72* mutation in this study was not more frequent than the *SOD1* mutation in Japanese SALS patients (0.4% and 1.6%, Table 1) (Akimoto et al., 2011). Considering these data, the *C9orf72* mutation is more common than the *SOD1* mutation in Western populations but not in Japan, suggesting different genetic backgrounds. Our results may explain the association study of rs2814707 on 9p21.2, which was reported to be the most significantly associated SNP with SALS in Caucasian but not in Japanese and Chinese populations (Iida et al., 2011). A recent report revealed that the rate of expansion in Asian FALS and SALS was 5% (1/20) and 0% (0/238), respectively (Majounie et al., 2012). An analysis of the SNPs on chromosome 9p revealed that all 4 subjects with the *C9orf72* mutation and another Japanese subject from the previously mentioned report (Majounie et al., 2012) share a shorter region of the risk haplotype

than Western populations. Thus, the haplotype bearing the *C9orf72* mutation was only shared in a narrow region between Western and Asian populations, suggesting that the *C9orf72* mutation may be an old mutation in human migration history from Western to East Asia. This mutation was estimated to be approximately 1500 years old (Majounie et al., 2012).

Bulbar onset and cognitive impairment have been reported to be more common in ALS patients with the *C9orf72* repeat expansion (Chiò et al., 2012; Cooper-Knock et al., 2012; DeJesus-Hernandez et al., 2011; Sabatelli et al., 2012; Stewart et al., 2012). We did not find any patients with bulbar onset, but we identified 2 patients with dementia. Although the age at onset has been known to be lower in SALS patients with the *C9orf72* mutation than in those without this mutation (Sabatelli et al., 2012), our patients exhibited a relatively older age at onset (Table 1).

Although apparently sporadic patients with *C9orf72* mutation have been detected worldwide (Byrne et al., 2012; Cooper-Knock et al., 2012; Sabatelli et al., 2012), it was not known whether this phenomenon was due to incomplete penetrance or to spontaneous expansion of the GGGGCC hexanucleotide repeat from a nonpathogenic parental form (ie, a de novo expansion). In this study, we found a 76-year-old healthy individual with a *C9orf72* mutation (Subject B-II), as described in previous studies (Majounie et al., 2012; Renton et al., 2011). This discovery suggests not de novo expansion but incomplete penetrance, which explains the existence of apparently sporadic patients with the *C9orf72* mutation. Although it has been reported that the penetrance of the *C9orf72* mutation is almost full by 80 years by Kaplan–Meier analysis of 603 mutant gene carriers and 5 neurologically healthy individuals, further studies of family members of patients with the *C9orf72* mutation will be required to calculate the true penetrance and to improve genetic counseling.

Finally, we found a PPA patient with the *C9orf72* mutation after detecting the mutation in a SALS patient, suggesting the importance of collecting information regarding whether SALS patients have a family history of dementia or aphasia. Therefore, the possibility of *C9orf72* mutation should be investigated when clinicians meet with SALS patients after determining their family histories of FTD or PPA. Furthermore, our data supported Byrne and colleagues' suggestion that a family history of FTD should also be included in the revised definition of FALS (Byrne et al., 2012).

Disclosure statement

All of the authors disclose no conflicts of interest. The study was approved by the ethical committees of the participating centers. All participants gave written informed consent.

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Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2012.05.011.

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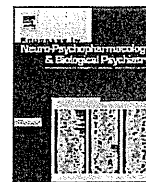

Long-term efficacy and safety of gabapentin enacarbil in Japanese restless legs syndrome patients

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ABSTRACT

Several short- and long-term studies conducted in Europe/North America have demonstrated good efficacy and tolerability of 600–1800 mg gabapentin enacarbil (GEn). However, no studies have evaluated the efficacy of long-term treatment with GEn in Asian patients. Therefore, the objective of this study was to evaluate the efficacy and safety of long-term treatment with GEn in Japanese patients with restless legs syndrome (RLS) in a multicenter open-label study.

RLS patients aged 20–80 years were allocated to receive oral GEn 1200 mg/day for a treatment period of 52 weeks. International Restless Legs Syndrome Scale (IRLS) score, investigator- and patient-rated Clinical Global Impression (CGI) scores, Pittsburgh Sleep Quality Index (PSQI) total scores and subscores, and short form (SF)-36 subscores were assessed, and adverse events (AEs) were monitored. In 181 patients (mean age, 54.9 ± 12.2 years; BMI, 23.0 ± 2.6 kg/m^2) IRLS score decreased from 24.4 ± 0.4 at baseline to 6.3 ± 0.6 at week 52, with a reduction of -18.0 ± 0.6. The IRLS responder rate was 80.3% at week 52. ICGI and PCGI responder rates were 87.1% and 87.1%, respectively. PSQI and SF-36 also showed significant improvements. AEs were reported in 96.2% of patients but remained mild-to-moderate in nearly all the cases. Serious AEs occurred in 1.6%. Dizziness and somnolence were noted in 46.2% and 41.2% of patients, respectively, and mostly occurred during the first 4 weeks. No episodes of augmentation were reported.

In conclusion, long-term treatment with GEn improved RLS symptoms as well as investigator- and patient-reported outcomes in Japanese patients with moderate-to-severe RLS, with an acceptable safety profile. Randomized, double-blind, placebo/active-controlled trials are desirable to confirm these preliminary results.
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1. Introduction

Restless legs syndrome (RLS) is a neurological disorder characterized by a variety of unpleasant sensations, most commonly in the legs, which provoke an irresistible urge to move the affected areas. The prevalence of RLS is 5–10% in white populations (Ohayon and Roth, 2002; Winkelman et al., 2006a) and 2–4% in Japanese populations (Nomura et al., 2008a,b). The irritable sensations in RLS are most commonly experienced while the affected individual is at rest, especially during the evening or at nighttime. Consequently, RLS is associated with insomnia, depression and deterioration of quality of life (QoL) (Allen et al., 2005; Kushida et al., 2007; Rothdach et al., 2000).

In terms of its pathophysiology and treatment, RLS is accepted as a disorder of the central nervous system (Trenkwalder and Paulus, 2010). Although its etiology is not fully understood, dopaminergic dysfunction, genetic background, and abnormal brain iron metabolism seem to be critical factors for the development of RLS (Trenkwalder and Paulus, 2010). Dopaminergic agonists (DAs) are the most commonly used treatments against RLS. However, these drugs are associated with a high risk of adverse events (AEs) including compulsive behaviors, nausea, dizziness, and somnolence, which affect considerable numbers of patients, leading to discontinuation of therapy in many cases (Earley and Silber, 2010). Furthermore, treatment with DAs might cause augmentation–expansion of the affected body areas and earlier onset and paradoxical worsening of RLS symptoms despite increasing the dose-limiting long-term maintenance therapy (Högl et al., 2010; Winkelman and Johnston, 2004).

Abbreviations: AE, adverse event; DA, dopaminergic agonist; GEn, gabapentin enacarbil; ICGI, Investigator-rated Clinical Global Impression; IRLS, International Restless Legs Syndrome Scale; PCGI, Patient-rated Clinical Global Impression; PSQI, Pittsburgh Sleep Quality Index; QoL, quality of life; RLS, restless legs syndrome; SF, short-form.

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To avoid these limitations of dopaminergic treatment, other anti-RLS therapies have been considered, including opioids and anticonvulsants.

Because of its anticonvulsant and antineuralgic properties, the GABA derivative drug gabapentin is regarded as an important candidate drug for the treatment of RLS. Indeed, gabapentin was recommended in the treatment algorithms for RLS developed by the Restless Legs Syndrome Foundation (Silber et al., 2004) and the Movement Disorder Society (Trenkwalder et al., 2008b). Several randomized double-blind and open-label studies have demonstrated that gabapentin improves the symptoms of RLS (Garcia-Borreguero et al., 2002; Happe et al., 2003; Micozkadioglu et al., 2004; Thorp et al., 2001). However, the intestinal absorption of orally administered gabapentin shows marked interpatient variability rendering unpredictable treatment responses. Therefore, to improve plasma exposure, the gabapentin prodrug gabapentin enacarbil (GEn) was developed (Cundy et al., 2008). GEn is absorbed via high-capacity nutrient transporters throughout the gastrointestinal tract and is rapidly converted to gabapentin, providing dose-proportional exposure (Cundy et al., 2004).

Several short-term randomized controlled trials have been conducted to determine the clinical efficacy of GEn in RLS (Merlino et al., 2009). These studies demonstrated greater efficacy of GEn versus placebo in terms of improvements in RLS symptoms and measures of sleep quality over 2 weeks in moderate-to-severe patients (Kushida et al., 2009; Walters et al., 2009). In a longer-term study with a 24-week, single-blind phase followed by a 12-week, randomized, double-blind, placebo-controlled phase, GEn (1200 mg/day) maintained improvements of RLS symptoms for up to 9 months (Bogan et al., 2010). Similarly, Ellenbogen et al. (2011) reported that 600–1800 mg doses of GEn were generally safe and well tolerated in a 52-week open-label extension following a 12-week randomized double-blind placebo-controlled phase. However, no studies have evaluated the efficacy of long-term treatment with GEn in Asian patients.

Therefore, the primary objective of this study was to evaluate the efficacy and safety of long-term (≥1-year) treatment with GEn in Japanese patients with RLS. The second aim was to evaluate the effects of GEn on QoL and subjective sleep disturbances of the patients. In addition, we tried to identify the factor(s) associated with the improvements in RLS symptoms following GEn treatment.

2. Methods

2.1. Study design

This was a multicenter, open-label study to evaluate the efficacy and safety of GEn (GEn is licensed from XenoPort, Inc.) for long-term treatment of RLS. The study was approved by institutional review boards/independent ethics committees at each participating institution, and the protocol conformed to the Declaration of Helsinki. All patients provided written informed consent prior to enrollment. This study was registered with Astellas Pharma protocol no. 8825-CL-0005.

2.2. Patients

Male and female outpatients aged 20–80 years who had been diagnosed with RLS according to the diagnostic criteria established by the International RLS Study Group (Allen et al., 2003; American Academy of Sleep Medicine, 2005), including International Restless Legs Syndrome Scale (IRLS) score ≥15, presence of RLS symptoms on ≥15 days per month and ≥4 days per week preceding inclusion in this study, were enrolled. The target number of subjects was 120, based on the Long-Term Treatment Guideline of the Pharmaceutical Affairs Bureau, Ministry of Health, Labour and Welfare, Japan, which states that a minimum of 100 patients should be treated for 52 weeks for appropriate safety evaluation, with no upper limit. Patients using DAs or gabapentin within 1 week before or any anti-RLS treatment within 2 weeks before the start of the pretreatment observation period were excluded, as were those with an estimated creatinine clearance <60 mL/min determined using the Cockcroft–Gault formula. Pregnant or lactating women and individuals with serum ferritin <20 ng/mL were also excluded from the trial. Moreover, although neurophysiological tests (e.g. nerve conduction studies) were not performed, individuals with movement disorders and/or abnormal neurological findings were excluded.

2.3. Treatment

After completing the 1-week pretreatment observation period, GEn was administered orally once daily after the evening meal at an initial dose of 600 mg/day for 3 days and then uptitrated to 1200 mg/day for a total treatment period of 52 weeks. At week 12, the dose could be increased to 1500 mg/day in patients with an inadequate clinical response to 1200 mg/day or decreased to 900 mg/day for patients showing poor tolerance to GEn. After the 52-week treatment period, patients were administered GEn 600 mg/day for 7 days (dose-tapering period), which was followed by a 1-week treatment-free follow-up period to monitor withdrawal symptoms.

2.4. Safety and efficacy endpoints

Efficacy was assessed by determining the IRLS score at weeks 0, 1, 2 and 4, and every 4 weeks thereafter through to week 52, as well as during the 1-week follow-up period. Patients who achieved an IRLS total score ≤10 were defined as "IRLS responders", and the IRLS responder rate was determined. We also assessed improvements in Investigator-rated and Patient-rated Clinical Global Impression (ICGI and PCGI), Pittsburgh Sleep Quality Index (PSQI) total score and subscores, and short-form (SF)-36 subscores. Patients whose improvements based on ICGI and PCGI were rated as "much improved" or "very much improved" were defined as ICGI/PCGI responders, and the responder rates were determined.

Safety assessments were performed throughout the treatment period and included AEs, changes in vital signs, laboratory tests, and ECG. Assessments of AEs and vital signs were made in weeks, 0, 1, 2, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, 53 and 54 as well as at discontinuation. Laboratory tests were performed at weeks 0, 2, 4, 8, 12, 20, 28, 36, 44 and 52 as well as at discontinuation. ECG was performed during the pre-treatment observation period and at weeks 12, 28 and 52 as well as at discontinuation. Dizziness and somnolence were defined as specific AEs and subjected to further analysis.

2.5. Statistical analysis

The full analysis set (FAS) comprised patients who received investigational product and were assessed for at least one efficacy evaluation variable. The safety analysis set (SAS) consisted of patients who received at least one dose of investigational product.

Summary statistics were calculated for the change in IRLS score, PSQI and SF-36 at each time-point versus baseline with paired t-tests. The change in IRLS score was assessed by multiple regression analysis using baseline IRLS total score, sex, age, BMI and duration of RLS morbidity as covariates. Logistic regression analysis on the factors associated with IRLS responder was also performed using baseline IRLS total score, sex, age, and BMI as independent variables.

AEs and adverse drug reactions were evaluated, and the rates of the events were calculated. Summary statistics were calculated for clinical laboratory values, 12-lead ECG findings, blood pressure and pulse rate, while frequencies were calculated for discrete data.

All statistical analyses were performed using PC-SAS software system version 8.2 (SAS Institute, Cary, NC).