Parkinson's disease therapy with Istradefylline and blood biomarkers of epigenetics

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Abstract

Background: Istradefylline (IST), an adenosine A2A receptor antagonist, has been used since 2013 in Japan as an adjunctive therapy to levodopa (L-DOPA) for patients with Parkinson's disease (PD). The long-term use of IST as an adjunct to L-DOPA (IST-LD) was herein investigated to clarify the cooperative potential to keep motor functions, and an epigenetic modification for disease-specific up-regulated A2AR signals.

Methods: The cohort comprising 62 PD patients with diurnal variations in motor function were treated with IST-LD for 36 months, and clinical and biomarker parameters were evaluated. One monozygotic twin pair with juvenile PD and eight healthy control (HC) subjects were recruited for analyses of epigenetic biomarkers with peripheral blood lymphocyte (PBL): the A2AR protein (A2AR-p), A2AR mRNA (A2AR-m), and DNA methylation of the ADORA2A (Chr22q11.23) and DRD1 (Chr5q35.1) genes.

Results: IST-LD partially reversed locomotive dysfunction and motor off time and did not promote the severe dyskinesia (LID) develop. A2AR expression levels of the PBL were higher in IST-naïve PD patients than in HC, which were associated with the effectiveness of IST-LD and clinical global impression improvements. Intrinsic DNA demethylation of the ADORA2A gene were observed in juvenile monozygotic twin pair of the PD and IST-naïve PD patients, which were reversed by IST-LD at 12 months.

Conclusions: IST-LD was cooperative for long term to preserve motor execution controls and global daily activities improvement, through the canonical pharmacodynamics of inhibiting A2AR signaling, and also via the epigenetic modification on the ADORA2A gene.

Keywords

adenosine A2A receptor antagonist, Istradefylline, ADORA2A, epigenetics, Parkinson's disease
1 | INTRODUCTION

Parkinson’s disease (PD) is a neurodegenerative disorder that disables more areas of the body as it progresses. The independence of locomotion and daily activities is an essential target of symptomatic therapy. Motor execution controls are organized by the neural circuitry of the basal ganglia, in which neurons are densely innervated by nigrostriatal dopamine projections. The loss of dopaminergic neurons induces synaptic adaptations in the striatum. Dysfunctions in striatonigral (D1R) MSNs have been linked to LTD, and a disturbance in striatopallidal (D2R) MSNs induces a supersensitive condition accompanied by the excitatory plasticity of LTP by A2AR signaling, which occurs independent of dopamine depletion.\(^1,2\) The prodromal to early stages of PD are characterized by the micro-aggregation of α-synuclein in presynaptic terminals without dopaminergic axonal degeneration, following cell death of the substantia nigra pars compacta (SNc) with disease progression, those changes contribute to the network pathology of PD.

Motor learning is executed by dynamic remodeling of dendritic synapses in the M1, in which they are innervated by mesocortical dopamine projections.\(^3,5\) Dopamine depletion suppresses LTP on both D1R/M1 and D2R/M1, which are involved in dendritic spine elimination and formation, respectively, leading to failed motor skill learning in PD patients.

Treatments with L-DOPA restore LTP by early genes expression change; however, continued treatment is expected to enhance D2R/MSN trafficking at the cell surface with phosphorylation by GPCR kinase and linked activation of the GNα/Gβ-olf, those promote higher signal transduction and synaptic transmission, prone to develop LID.\(^6,10\) L-DOPA also activates c-JNK signaling with downstream ERK activation, which plays relevant role to maintain LID.\(^6,11,12\)

A2AR forms a functional complex with D3R/MSNs and antagonizes its functions reciprocally and independently. The adenosine A2AR antagonist/KW6002/IST suppresses the presynaptic terminals of the globus pallidus by reducing the excessive output of the basal ganglia.\(^1,2,13\) Increased A2AR expressions were observed in the drug-naïve PD putamen and blood lymphocytes using Western blot assays or radioligand binding assays with A2AR antagonist/\[^{[3H]}\]ZM241385, those were regulated by post-transcriptional epigenetic activation of ADORA2A gene.\(^14-17\)

The treatment of PD with IST has been covered by the national health insurance system in Japan since 2013 as non-dopaminergic adjunctive therapy to L-DOPA, and this treatment has been shown to exert robust effects on motor dysfunctions in both the off and on periods.\(^18,19\) Howevet, several issues associated with A2AR antagonists/IST remain unclear in PD patients, such as neuronal protection from the pathological evolution of the disease, preventive effects against dyskinesia evolution, and pharmaceutical actions

### TABLE 1 Baseline patient demographics and clinical characteristics

| Predictor                              | Baseline | 36 mo |
|----------------------------------------|----------|-------|
|                                        | MI       | SI    | NC   | AW   | OR (95% CI) |
| Age (y)                                | 66 ± 11 (41-85) | 62 ± 11 (43-77) | 64 ± 11 (48-83) | 75 ± 8 (61-87) | 67 ± 9 (56-79) | 0.948 (P = .09, 0.05-2.965) |
| Sex (n, female/male)                   | 36/26    | 6/5   | 14/10 | 8/5  | 4/3       | nd            |
| Duration (y)                           | 11.2 ± 6 (1-32) | 8.4 ± 4.9 | 11.4 ± 6.1 | 12.9 ± 6.4 | 6.8 ± 2.8 | 1.017 (0.89-1.09) |
| Modified Hoehn and Yahr stage (on)     | 3.0 ± 1.0 | 4.0 ± 0.8 | 3.3 ± 0.6 | 3.6 ± 0.5 | 2.8 ± 0.5 | 0.676 (0.36-5.0) |
| Modified Hoehn and Yahr stage (off)    | 3.0 ± 0.9 | 4.2 ± 0.4 | 4.3 ± 0.5 | 3.8 ± 0.5 | 0.481 (0.31-3.9) |
| UPDRS Part I                           | 6.1 ± 3.5 (1-18) | (base) 3.2 ± 2.0 | (36 m) 2.6 ± 2.2 | 7.0 ± 3.9 | 9.0 ± 1.2 | 5.0 ± 2.7 | 1.332 (0.3-2.57) |
|                                        | (off) 4.0 ± 0.8 | 6.1 ± 3.4 | 8.9 ± 1.3 | 4.8 ± 2.2 | 0.584 (0.5-6.57) |
| UpDRS Part III                         | 31.7 ± 17 (4-93) | (base) 20.9 ± 16.3 | (36 m) 16.9 ± 13.7 | 36.4 ± 20.9 | 41.2 ± 9.5 | 24.5 ± 7.5 | 0.93 (0.13-9.99) |
|                                        | (off) 27.7 ± 17 (4-93) | 29.4 ± 13.4 | 51.1 ± 10.6 | 32.2 ± 8.8 | 1.06 (0.06-3.30) |
| Dyskinesia (%)                         | 26.1     | 30.8  | 21.1  | 60   | 30       | 0.79 (0.67-1.52) |
| LEDD (mg)                              | 729 ± 255 (200-1758) | 580 ± 322 (200-1170) | 667 ± 328 (345-1758) | 643 ± 230 (370-1290) | 567 ± 214 (200-1210) | 1.00 (1.0-1.34) |
| Concomitant PD medication (%)           | 100      | 100   | 100   | 100  | nd       |
| Levodopa/carbidopa                     | 92       | 85    | 95    | 100  | 83       | 0.552 (0.44-1.16) |
| Dopamine agonist                       | 4.7 ± 1.3 | 5.0 ± 1.2 | 4.6 ± 1.6 | 4.8 ± 1.2 | 4.4 ± 0.9 | 1.228 (0.21-1.25) |
| A2AR/GAPDH                             | 0.62 ± 0.3 | 0.6 ± 0.2 | 0.7 ± 0.2 | 0.6 ± 0.3 | 0.5 ± 03 | 3.29 (0.30-9.5) |

Abbreviations: AW, aggravation/withdraw; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LEDD, Levodopa equivalent daily dose; MI, marked improvement; NC, no change; nd, not determined; OR, odds ratio as NC + AW to MI + SI with a logistic regression analysis; PD, Parkinson’s disease; SI, slight improvement; UPDRS, Unified Parkinson’s Disease Rating scale.

\(^*P < .05\) is significant.
for up-regulated A2AR expression, those have not yet been evaluated in any clinical trials.\textsuperscript{20,21}

In the present study, we examined the effects of the long-term use of IST as an adjunct to L-DOPA (IST-LD) in clinical practice and assessed blood biomarkers of epigenetics, including the A2AR-p, A2AR-m, and DNA methylation of the ADORA2A gene in peripheral blood lymphocytes (PBL).

2 | METHODS

2.1 | Subjects

The cohort included 62 patients with the early to advanced stages of PD with diurnal variations in motor functions. Participants were recruited as part of an open-label trial for 3 years between 2013 and 2016. One monzygotic twin pair with juvenile PD and eight healthy control (HC) subjects were also recruited for the biomarker assay. The present study was approved by the local Ethics Committee of our hospital, and informed consent were obtained from recruited patients and HC subjects.

2.2 | Assessments of clinical parameters

Baseline assessments of disease duration and medication history were quantified. The effects of the IST-LD treatment were prospectively measured at 10 days, 3 months, 12 months, and 36 months according to the Clinical Global Impression-Improvement scale (CGI-I) and the Unified Parkinson's Disease Rating scale (UPDRS). The effectiveness of IST-LD was rated by a physician's global assessment using CGI-I within 5 categories: marked improvement (MI), slight improvement (SI), no change (NC), aggravation/withdraw (AW), and discontinuation (DC). Locomotive function scores were as follows, UPDRS part III items 27 + 28 + 29 + 30 + 31 (total of 20 points); off-time scores, UPDRS part IV items 36 + 37 + 38 + 39 (total of 16 points); and daily off time, UPDRS part IV item 39 (percentage per daytime).

2.3 | Measurement of biomarkers of PBL

Venous blood samples were collected while fasting (to eliminate caffeine) as baseline at the initiation of the IST treatment. Serum levels of uric acid were measured by the enzymatic uricase-peroxidase method (Sekisui Medical, Japan).

Immunoblotting: Membrane proteins were isolated from PBL by Lymphoprep (Cosmobio, Japan) and then assayed by immunoblotting using antibodies for A2AR. Proteins electrotransferred onto polyvinylidene difluoride (PVDF) membranes were immunoblotted with a rabbit polyclonal A2AR antibody (ab3461, Abcam) and reacted with a horseradish peroxidase (HRP)-conjugated secondary antibody (#K4003, Dako), followed by an incubation with chemiluminescence reagent (Thermo Fisher Scientific). The bound antibody was detected using an imaging plate and the BAS-2000 system (Fuji Photo Film Co.). An anti-GAPDH antibody (ab125247, Abcam) was used to load controls.

RT-PCR: Total RNA was isolated from PBL by Isogen (Nippon Gene, Japan) and transcribed into cDNA (RT). A polymerase chain reaction (PCR) was performed using primers specific for A2AR and GAPDH. The primer for A2AR, 559 bp was forward: TCC TAC CGC AGC AGA GAT CTC ATC TT and reverse: TGC AGT CCG GCG CAA GAA AGAAGT.

The primer for GAPDH, 226 bp was forward: AAAGT GAAGG TCAG AGT CTGA and reverse: GAAGAT GTGA GTGGGATTCC.

Measurement of A2AR-p, A2AR-m was conducted under blinding condition.

FIGURE 1 Effects of the IST-LD treatment evaluated with CGI-I. A, 10 days; B, 36 months. Patients were classified into 5 categories. MI, marked improvement; SI, slight improvement; NC, no change; AW, aggravation/withdraw; DC, discontinuation (due to comorbidities)
2.4 Measurement of DNA methylation of ADORA2A and DRD1

DNA was isolated from PBL, and 100 ng of DNA was bisulfite treated with the Methylamp DNA Bisulfite conversion kit (Epigentek Service, NY) according to previous methods.15,22 Eleven primer pairs were used to selectively amplify bisulfite-converted DNA sequences in the following DNA regions: 5′UTR of ADORA2A (Chr22q11.23); 1 primer pair: ADORA2A upstream of exon 1E (404bp); 4 primer pairs: ADORA2A entire exon 1E (1255bp), 5′UTR of DRD1 (Chr5q35.1); 6 primer pairs (1590bp), these regulatory regions have rich CpG islands. PCR products were quantified on a bioanalyzer. A total of 10 nmol/L of sample libraries were subjected to next-generation sequencing using Illumina HiSeq 2500 (Epigentek Service, NY). The controls derived from human Jurkat genomic DNA were used as standards. Surrogate global DNA methylation was compared across the sample of monozygotic twin pair with LINE-1 kit (Epigentek Service, NY).
2.5 Statistical analysis

The Wilcoxon signed-rank test and a binomial logistic regression analysis were used. All statistical analyses were conducted using IBM SPSS statistics version 12.0. *P* values of < .05 were considered to be significant.

3 RESULTS

3.1 Subjects

The demographics and clinical characteristics of 62 PD patients at baseline and 36 months after the IST-LD treatment are shown in Table 1. Effectiveness of IST-LD was compared within CGI-I categories as odds ratio of NC + AW to MI + SI with a logistic regression analysis. UPDRS Part III scores had a significant impact on effectiveness (*P* < .05). Disease duration varied with 32 years being the longest, had no influence on effectiveness. Our cohort showed a lower frequency of dyskinesia at the baseline with 26.1%, which increased to 39.6% after 36 months of IST-LD even though severe LID diminished (data not shown). The 36 months prospective LEDD dose were decreased compared with those of the baseline, but no significant differences were observed within the CGI-I categories.

Regarding biomarkers in blood samples, A2AR-m/GAPDH expressions had a significant influence on effectiveness (*P* < .05).

3.2 Clinical global effectiveness of IST-LD with CGI-I

CGI-I after 10 days showed 11% MI, 45% SI, 32% NC, and 11% AW (Figure 1A). After 36 months of treatment, up to 58% of patients (MI + SI) maintained their motor functions and well-being of daily life (Figure 1B). Discontinuation of IST occurred in 11% of participants due to other incidental comorbidities, such as pneumonia and cerebral infarction.

3.3 Locomotive function and off time

Locomotive function scores improved by 4.2 points over baseline in MI patients (*P* < .05) (Figure 2A). AW patients showed an increase of 3.6 points from the baseline (*P* < .05), which was evaluated at 10 days as aggravated/discontinued (Figure 2A). Off-time scores improved by 2 points over the baseline with 4 hours of daily off time in MI patients (*P* < .05), while AW patients showed aggravation from the baseline, which was significant (*P* < .05) (Figure 2B).

**FIGURE 3** Expression of the A2AR-p and A2AR-m of PBL. A, A2AR-p expression in HC (*n* = 8) and PD-IST naïve (*n* = 62) (representative data are shown). A2AR-p was detected by the immunoblotting of membrane proteins isolated from PBL with the anti-A2AR antibody. Ratios of A2AR-p to the GAPDH protein (loading control) calculated by a densitometric analysis are shown. B, A2AR-m expression in HC (*n* = 8) and PD (*n* = 62) analyzed by semi-quantitative real-time PCR. Expression levels were compared between HC and PD, and among the patient groups before and after the IST treatment.
3.4 | Changes in blood biomarkers

The expression level of the $A_2A$R-p in PBL appeared to be higher in PD patients with IST-naïve than the healthy controls (HC) (Figure 3A).

Since all samples of PBL from PD patients and HC showed the 559-bp band corresponding to $A_2A$R-m by RT-PCR (data not shown), we examined $A_2A$R-m expression levels by semi-quantitative real-time PCR and found that they were significantly higher in PD than HC.
After 12 months of the IST treatment, $A_{2a}$R-m expression levels appeared to be decreased in samples obtained from MI or SI responsiveness except for AW, suggesting positive test may be associated to CGI-I effectiveness.

### 3.5 DNA demethylation for the whole genome in the monozygotic twin with PD

Using surrogate global DNA methylation sequencing, DNA demethylation differences were compared across the samples of juvenile monozygotic twin pair of the naive early stage of PD and NC; since they have the same genome, demethylation differences showed epigenetic modifications (Figure 4A). After 12 months of the IST-LD treatment, the difference was restored to small (Decrement > 5%) in the promoter and CpG islands of the several genomic sites including ADORA2A (Figure 4B).

### 3.6 Chronological changes in the DNA demethylation of ADORA2A and DRD1 after IST-LD

Five PD patients compared across the samples of 12 months of IST-LD treatment, using human Jurkat genomic DNA as normal controls. MI or SI responsiveness (PD2-5) showed demethylation were restored to small at the ADORA2A (Decrement > 10%). DNA demethylation of DRD1 did not change, whereas MI with severe LID (PDS) altered both ADORA2A and DRD1 (Figure 4C).

### 4 DISCUSSION

In the present study, the long-term treatment with IST-LD partially reversed locomotive dysfunction and motor off time, and indicated the potential to preserve global daily activities improvement, expected to decline with disease progression and age-related comorbidities. Although our cohort was small and did not include a placebo arm, it is important to note that another long-term natural history study on Rasagiline (ADAGIO follow-up study) achieved similar outcomes with a slower rate of progression. The overall rate of discontinuation was 22% (aggravation/withdrew 11% and comorbidities 11%), which was similar to the findings of the previous long-term continuation was 22% (aggravation/withdrew 11% and comorbidities 11%).

Taken together, IST-LD has the potential to influence biological off targets of the disease process by restoring intrinsic ADORA2A gene demethylation, resulting in reductions of the target molecules of $A_{2a}$R expressions in the striatum. Although ADORA2A gene single-nucleotide polymorphism (SNP) variants at phosphorylation sites (Thr324 and Thr400) have been associated with the down-regulated expression of intrinsic $A_{2a}$R,$^{27,22}$ ADORA2A gene epigenetics may be strongly influenced by several environmental signals and aging factors.

Concerning about the DNA demethylation, which are small differences in NC (<5%) and in aging process (<10%),$^{33,34}$ our cohort showed large demethylation differences (>10%) of ADORA2A accompanied by the up-regulated $A_{2a}$R-p in PBL. Epigenetics are haplotype-dependent and highly tissue-specific, particularly in the nervous system, so small change of demethylation difference may be associated with the altered expression of neurons and/or glia cells as a hallmark of the neurodegenerative disease. The real-time PET imaging of $A_{2a}$R has been studied as target engagement (TE) biomarker of pharmaceutical researches, and the epigenetic modification of ADORA2A and A2AR molecule (substrate) both in striatum and in the blood sample may be proof of concept (POC) biomarker of PD.$^{25}$

Regenerative medicine will play an essential role in the treatment of PD in the near future.$^{26}$ However, pharmacotherapy will still be crucial for preventing disease-specific pathological evolution and accelerated neurodegeneration.

### ACKNOWLEDGMENTS

We thank Akihisa Mori, PhD (Research fellow of Kyowa Kirin Co., Ltd.), for his helpful discussions.

### CONFLICT OF INTEREST

None.

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How to cite this article: Kanzato N, Nakachi K, Naka T, Mochizuki S, Miyamae Y, Okada Y. Parkinson’s disease therapy with Istradefylline and blood biomarkers of epigenetics. Neurrol Clin Neurosci. 2020;8:276–283. https://doi.org/10.1111/ncn3.12415