Short report

Comparative whole transcriptome analysis of Parkinson’s disease focusing on the efficacy of zonisamide

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ABSTRACT
Objective Interindividual variations in responsiveness to zonisamide in patients with Parkinson’s disease (PD) have been observed in clinical settings. To decipher the molecular mechanisms determining the efficacy of zonisamide, we conducted whole transcriptome sequencing analysis of patients with PD.

Methods We selected 23 super-responders (SRs) and 25 non-responders (NRs) to zonisamide from patients with PD who had participated in a previous clinical trial for the approval of zonisamide for the treatment of ‘wearing-off’. Whole transcriptome analysis of peripheral blood was conducted on samples taken before and 12 weeks after zonisamide treatment. We performed differential gene expression analysis to compare between the SRs and NRs at each time point.

Results Differentially expressed genes in the pre-treatment samples were significantly enriched for glutamatergic synapses and insulin-like growth factor binding (FDR=7.8 × 10^{-3} and 0.029, respectively). The gene sets associated with these functions changed more dynamically by treatment in SRs than NRs (p=7.2 × 10^{-3} and 8.2 × 10^{-2}, respectively).

Conclusions Our results suggest that the efficacy of zonisamide in PD patients is associated with glutamate-related synaptic modulation and p53-mediated dopaminergic neural loss. Their transcriptomic differences could be captured before treatment, which would lead to the realisation of future personalised treatment.

INTRODUCTION
Patients with advanced Parkinson’s disease (PD) often experience shortening of beneficial effects of levodopa, termed as ‘wearing-off’.2 Zonisamide, which was originally developed as an antiepileptic drug, is an effective adjunct for PD patients that improves various symptoms, especially ‘wearing-off’.1 Interindividual variations in responsiveness to zonisamide in PD patients are often observed in clinical settings,1 and we previously conducted a genome-wide association study (GWAS) on PD patients to decipher genetic factors influencing their responsiveness to zonisamide. We identified a single-nucleotide variation (SNP) significantly associated with the effect of zonisamide to reduce ‘off’ time in patients with PD, which could alter the expression of MDM4 and downregulate p53 in neural cells.2

Despite the successful identification of the genetic basis, the mechanisms behind the interindividual variation in zonisamide responsiveness have not been fully elucidated. In addition, considering that a genetic testing is time and cost-intensive, a serological biomarker would be helpful in personalised treatment. To provide insight to these issues, we conducted a comparative whole transcriptome sequencing analysis of the peripheral blood samples of patients with PD, which were taken during a clinical trial for the approval of zonisamide in the treatment of PD.1

MATERIALS AND METHODS
Subjects and definition of zonisamide responsiveness
Participants were selected from 220 Japanese patients with PD who participated in a previous clinical trial for the approval of zonisamide as an adjunct to levodopa therapy for ‘wearing-off’.3 They received either 25 mg or 50 mg of zonisamide per day, and the plasma concentration of zonisamide was measured at the week 4 of their treatment. Blood samples for transcriptome sequencing were collected before and 12 weeks after the start of treatment. Responsiveness to zonisamide was evaluated by the changes in ‘off’ time and the total score of Unified Parkinson’s Disease Rating Scale (UPDRS) part III, from baseline (before treatment) to 12 weeks after the start of zonisamide treatment. A subject who showed a decrease of at least 1.5 hour from baseline in ‘off’ time as well as a decrease of at least five points from baseline in the UPDRS part III total score was defined as a super-responder (SR). On the other hand, a subject who showed no improvement or worsening in both ‘off’ time (change ≥0) and UPDRS part III total score (change ≥0) was defined as a non-responder (NR). Only those who met the criteria of SR or NR were considered for subsequent RNA sequencing analysis.

RNA sequencing
RNA was extracted from blood samples using the RNeasy Mini Kit (QIAGEN) following the manufacturer’s protocol. The quality and quantity of RNA samples were examined using an Agilent Technologies 2100 TapeStation. RNA Integrity Number equivalent (RIN) and rRNA ratio were determined and used to evaluate the quality of the
samples. The sequencing library was prepared using the Library Protocol TruSeq RNA Sample Preparation v2 Guide (Illumina). RNA sequencing was performed using the Illumina platform to generate 101bp paired-end sequences. Sequencing data were converted into raw data for subsequent analysis.

**Genotyping, quality control and read counting**
Gene expression data preparation was conducted using the following steps: (1) Alignment: the paired-end sequences were mapped to the human reference genome (hg38) by using STAR aligner with a two-pass method. PCR duplicates were removed using Picard (http://broadinstitute.github.io/picard/). (2) Read counting: the number of reads was obtained at the gene level using Picard (http://broadinstitute.github.io/picard/). (2) Read counting: the number of reads was obtained at the gene level using Picard (http://broadinstitute.github.io/picard/).

**Differential gene expression analysis**
Differential gene expression analysis and other statistical analyses were conducted using the Bioconductor package DESeq2 and R (3.6.1) (https://www.r-project.org/), respectively. In differential gene expression analysis, we fitted a generalised linear model of negative binomial distribution with a logarithmic link and conducted Wald’s test with age, sex and zonisamide dose as covariates. We set a study-wide significance threshold of p=1.9 × 10−6 based on Bonferroni correction of the total number of tested genes (≈0.05/26,475). We analysed the gene expression differences between SRs and NRs before and after treatment. To test whether the expression levels of a gene set changed more dynamically in SRs than in NRs, we calculated the means of absolute logarithm of fold changes by treatment of individual genes, and evaluated the intergroup difference using the Wilcoxon signed-rank test.

**Gene set enrichment analysis**
Gene set enrichment analysis was performed for differentially expressed genes (DEGs) for gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, respectively, using g:Profiler with the ordered query option. The order was based on the statistical significance of DEGs.

### RESULTS

#### Clinical characteristics
Among clinical trial participants with complete records, only 25 and 26 participants met the criteria for SR and NR, respectively. Additionally, of these 51 participants, three failed at RNA sample collection or RNA quality tests. Hence, a total of 48 samples (23 SRs and 25 NRs), were processed for subsequent analyses.

Demographic and clinical characteristics of the patients are summarised in table 1. In general, SRs and NRs have matched demographic and clinical information. SRs achieved a mean decrease of 2.8 hours in ‘off’ time whereas NRs showed a mean increase of 1.5 hour in ‘off’ time, after 12 weeks of zonisamide treatment.

#### Table 1 Clinical characteristics of super-responders and non-responders to zonisamide in this study

<table>
<thead>
<tr>
<th>Variables</th>
<th>Responsiveness</th>
<th>SR (n=23)</th>
<th>NR (n=25)</th>
<th>P value (SR vs NR)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female sex (%)</td>
<td></td>
<td>11/23 (47.8%)</td>
<td>13/25 (52.0%)</td>
<td>0.48</td>
</tr>
<tr>
<td>Age (mean±SD)</td>
<td></td>
<td>63.3±6.7</td>
<td>65.1±6.1</td>
<td>0.48</td>
</tr>
<tr>
<td>BMI (mean±SD)</td>
<td></td>
<td>24.4±2.9</td>
<td>21.8±3.3</td>
<td>0.05</td>
</tr>
<tr>
<td>Onset age of PD (year, mean±SD)</td>
<td></td>
<td>57.4±7.8</td>
<td>55.9±8.7</td>
<td>0.39</td>
</tr>
<tr>
<td>Years with PD (year, mean±SD)</td>
<td></td>
<td>5.9±2.8</td>
<td>8.0±5.2</td>
<td>0.40</td>
</tr>
<tr>
<td>Onset age of ‘wearing-off’ (year, mean±SD)</td>
<td></td>
<td>61.1±8.1</td>
<td>60.6±8.4</td>
<td>0.28</td>
</tr>
<tr>
<td>Years with ‘wearing-off’ (year, mean±SD)</td>
<td></td>
<td>2.2±3.1</td>
<td>2.3±3.3</td>
<td>0.36</td>
</tr>
<tr>
<td>MMSE (mean±SD)</td>
<td></td>
<td>27.8±2.6</td>
<td>28.1±2.1</td>
<td>0.35</td>
</tr>
<tr>
<td>Modified Hoehn and Yahr score (ON) (mean±SD)</td>
<td></td>
<td>2.5±0.3</td>
<td>2.1±0.8</td>
<td>0.16</td>
</tr>
<tr>
<td>Modified Hoehn and Yahr score (OFF) (mean±SD)</td>
<td></td>
<td>3.3±0.6</td>
<td>3.3±0.7</td>
<td>0.44</td>
</tr>
<tr>
<td>Dose of levodopa (mg/day, mean±SD)</td>
<td></td>
<td>350.0±120.2</td>
<td>416.7±181.3</td>
<td>0.48</td>
</tr>
<tr>
<td>LEDD† (mg/day, mean±SD)</td>
<td></td>
<td>491.8±163.5</td>
<td>497.1±203.5</td>
<td>0.20</td>
</tr>
<tr>
<td>Dose of MAO-B inhibitors (mg/day, mean±SD)</td>
<td></td>
<td>4.2±1.4</td>
<td>5.0±2.0</td>
<td>0.33</td>
</tr>
<tr>
<td>No of concomitant drugs (mean±SD)</td>
<td></td>
<td>2.8±0.8</td>
<td>3.0±1.5</td>
<td>0.07</td>
</tr>
<tr>
<td>Plasma concentration of zonisamide at week 4 (µg/ mL)</td>
<td></td>
<td>1.1±0.5</td>
<td>1.1±0.3</td>
<td>0.16</td>
</tr>
<tr>
<td>Total UPDRS part III total score at week 12 (mean±SD)</td>
<td></td>
<td>13.7±10.0</td>
<td>18.3±9.2</td>
<td>5.0×10⁻³</td>
</tr>
<tr>
<td>Total UPDRS part III total score at baseline (mean±SD)</td>
<td></td>
<td>21.4±9.4</td>
<td>14.3±9.8</td>
<td>0.050</td>
</tr>
<tr>
<td>Changes of UPDRS III from baseline (mean±SD)</td>
<td></td>
<td>−7.7±3.0</td>
<td>4.0±7.0</td>
<td>2.8×10⁻³</td>
</tr>
<tr>
<td>Average ‘off’ time at week 12§ (mean±SD)</td>
<td></td>
<td>4.2±2.2</td>
<td>7.3±2.8</td>
<td>1.6×10⁻³</td>
</tr>
<tr>
<td>Average ‘off’ time at baseline (mean±SD)</td>
<td></td>
<td>7.0±1.7</td>
<td>5.8±2.4</td>
<td>0.018</td>
</tr>
<tr>
<td>Changes of ‘off’ time from baseline (mean±SD)</td>
<td></td>
<td>−2.8±1.6</td>
<td>1.5±1.4</td>
<td>2.6×10⁻¹⁵</td>
</tr>
</tbody>
</table>

*P values were determined by using two-tailed unpaired t-test with equal variance.
†Conversion factor for LEDD: bromocriptine mesilate, ×10; cabergoline, ×70; pergolide mesilate, ×100; pramipexole hydrochloride hydrate, ×60; ropinirole hydrochloride, ×16.67; talipexole hydrochloride, ×60.
§ ‘Off’ time was calculated using patients’ diary information for the last 7 days before each visit (excluding screening visit).
BMI, body mass index; LEDD, levodopa-equivalent daily dose; MAO-B, monoamine oxidase-B; MMSE, Mini–Mental State Examination; NR, non-responder; PD, Parkinson’s disease; SR, super-responder; UPDRS, Unified Parkinson’s Disease Rating Scale.
RNA-sequencing data profile

The 96 RNA samples from 48 subjects were successfully sequenced and passed quality control. The summary statistics for raw RNA sequencing data are shown in online supplemental table 1. We further removed one sample from the pre-treatment NR data that was strongly suspected of viral infection from the transcriptomic profile (online supplemental note1 and online supplemental figure 1).

Differential gene expression analysis

There were no individual DEGs between SRs and NRs that satisfied the study-wide significance threshold both before and after treatment (p>1.9 × 10^{-6}; Figure 1A,B). However, we further performed enrichment analysis for genes that satisfied nominal significance (p<0.05) to capture the combined effects of weak but meaningful signals. We identified several significant enrichments at each time point (figure 1C,D).

The 613 DEGs in the pre-treatment comparison were significantly enriched for GO terms of glutamatergic synapse, insulin-like growth factor (IGF) binding, and corticosteroid signalling pathway (P_{adj}=7.8 × 10^{-3}, 0.029, and 0.026, respectively; figure 1C). The 595 DEGs in the post-treatment comparison were significantly enriched for a GO term of positive regulation of T cell migration (P_{adj}=0.041; figure 1D). There was no significant enrichment in the KEGG pathway.

Among the ontologies enriched for DEGs in the pre-treatment, we focused on genes related to glutamatergic synapses because the glutamatergic-mediated pathway was implicated in the mechanism of zonisamide on PD,9 and our previous GWAS suggested its relevance to the responsiveness of zonisamide.2 We then revealed that genes belonging to glutamatergic synapses changed more dynamically in SRs than in NRs (p=7.2 × 10^{-3}).
Similarly, genes belonging to IGF binding changed more dynamically in SRs than in NRs (p = 8.2 × 10⁻³), in contrast to the corticosteroid signalling pathway, which resulted in no significance changes (p = 0.50).

**DISCUSSION**

In the current study, significant enrichment of DEGs between SRs and NRs to zonisamide in PD patients was identified for several biological functions, providing meaningful insights. First, glutamatergic synapses-associated genes were differentially expressed between SRs and NRs before treatment. Zonisamide could increase the release of metabotropic glutamate receptor agonists from astrocytes and inhibit neurotransmission, which may explain the efficacy and tolerability of zonisamide in PD.⁸ ¹⁰

In the current study, zonisamide treatment resulted in increased expression levels of glutamatergic synapses-related genes in SRs and reduced differences in expression levels against NRs. This suggests that this pathway might be dysregulated in SRs and that zonisamide is more effective. Since the current study was conducted on blood samples, validation using brain samples is warranted. Second, genes related to IGF binding were also differentially expressed between SRs and NRs. Our GWAS suggested that an SNP located in MDM4 could determine the efficacy of zonisamide for ‘wearing-off’ through suppression of p53.² Upregulation of p53 is reportedly associated with dopaminergic neuronal apoptosis in PD.¹¹ Several studies supported that zonisamide exerts neuroprotective effects by inhibiting caspase-3, which is regulated by p53,¹² against endoplasmic reticulum stress.¹³ Thus, the caspase-3-mediated effect of zonisamide may be reinforced by suppression of p53 via the overexpression of MDM4 in responders. Since p53 also regulates the IGFl-pathway,¹⁴ the current results suggest that differential expression of genes involved in this pathway may also be present in the blood before treatment. Finally, considering the implications of the immune system in the etiology of PD¹⁵ and the hypothesised antineuropathic and antiiinflammatory effect of zonisamide,¹⁶ the enrichment in immune-related functions may also be significant.

Overall, our results indicate that the efficacy of zonisamide in PD patients is associated with glutamate-related synaptic modulation and p53-mediated dopaminergic neural loss. Their transcriptomic differences could be captured in blood sample treatment; thus, more thorough investigations with larger samples would lead to the development of a molecular biomarker that predicts the responsiveness to zonisamide.

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**Contributors** TN, WS and P-CC analysed the data and wrote the manuscript. WS and KK conducted RNA sequencing. MM and TT conceived the original idea.

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**Patient consent for publication** Not applicable.

**Ethics approval** This study was approved by the Research Ethics Committee of the University of Tokyo with the approval number 2021124G. Participants gave informed consent to participate in the study before taking part.

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