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Neuroimaging, genetic, and enzymatic study in a Japanese family with a *GBA* gross deletion

Yuta Ichinose[a], Hiroyuki Ishiura[b], Masaki Tanaka[c], Jun Yoshimura[d], Koichiro Doi[d], Takako Umeda[e], Hajime Yamauchi[f], Mai Tsuchiya[a], Kishin Koh[a], Nobuo Yamashiro[a], Jun Mitsui[g], Jun Goto[h], Hiroshi Onishi[e], Toshihisa Ohtsuka[f], Kazumasa Shindo[a], Shinichi Morishita[d], Shoji Tsuji[c,g], Yoshihisa Takiyama[a,*]

[a] Department of Neurology, Graduate School of Medical Sciences, University of Yamanashi, Yamanashi, Japan
[b] Department of Neurology, The University of Tokyo, Tokyo, Japan
[c] Institute of Medical Genomics, International University of Health and Welfare, Chiba, Japan
[d] Department of Computational Biology, Graduate School of Frontier Sciences, The University of Tokyo, Chiba, Japan
[e] Department of Radiology, Graduate School of Medical Sciences, University of Yamanashi, Yamanashi, Japan
[f] Department of Biochemistry, Graduate School of Medical Sciences, University Of Yamanashi, Yamanashi, Japan
[g] Department of Molecular Neurology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
[h] Department of Neurology, Mita Hospital, International University of Health and Welfare, Tokyo, Japan

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ABSTRACT

Introduction: Glucocerebrosidase gene (*GBA*) variants are associated with Parkinson's disease (PD) and dementia with Lewy bodies (DLB). The molecular mechanisms underlying these diseases with *GBA* variants, however, are not well understood. In order to determine the effect of a deletion mutation in *GBA*, we performed a neuroimaging, genetic, and enzymatic study in a Japanese family with a gross deletion of exons 3 to 11 in *GBA*.
Methods: We performed [^{123}I] FP-CIT SPECT and [^{123}I] *N*-isopropyl-*p*-iodoamphetamine SPECT (IMP-SPECT), and determined *GBA* expression and glucocerebrosidase (GCase) activity in leukocytes in two *GBA*-associated PD patients and nine unaffected individuals (including four mutation carriers) in a Japanese family with a heterozygous gross deletion mutation in the *GBA* gene.
Results: The two PD patients and two of the four clinically unaffected carriers showed decreased [^{123}I] FP-CIT uptake. IMP-SPECT showed a pattern like that in DLB in one patient. When we compared PD patients with *GBA* mutations with clinically unaffected carriers, there was a poor correlation between the development of PD and the expression level of *GBA* or GCase activity.
Conclusion: We confirmed the gross deletion mutation in the *GBA* gene, which appeared to be associated with the PD or reduced [^{123}I] FP-CIT in this family. However, since we cannot conclude whether a reduction of GCase activity is directly correlated with the pathogenesis of PD or not, longitudinal follow-up of this family is needed.

1. Introduction

Alterations in the glucocerebrosidase gene (*GBA*), the causative gene for Gaucher's disease (GD), appear to affect the risk of developing Parkinson's disease (PD). Heterozygous variants of *GBA* responsible for GD are known to be the most influential variants contributing to PD risk and dementia with Lewy bodies (DLB). The odds ratio of any *GBA* mutation with PD versus controls is 28.0 in Japan [1], but 5.43 worldwide [2]. In addition, *GBA* mutations are significantly more frequent in PD patients with family histories of PD than in isolated cases [1,3]. There are many fPD cases showing autosomal dominant inheritance with reduced penetrance in the *GBA* gene. The molecular mechanisms underlying *GBA*-associated PD (*GBA*-PD), however, remain to be elucidated.

We encountered three generations of a Japanese PD family in which we found a heterozygous gross deletion of exons 3 to 11 in *GBA* on gene analysis. In order to elucidate the pathogenesis of *GBA*-PD, we conducted gene analysis, quantitative reverse transcription PCR, enzyme measurement, and radioisotope imaging in this family.

* Corresponding author.
E-mail address: ytakiyama@yamanashi.ac.jp (Y. Takiyama).

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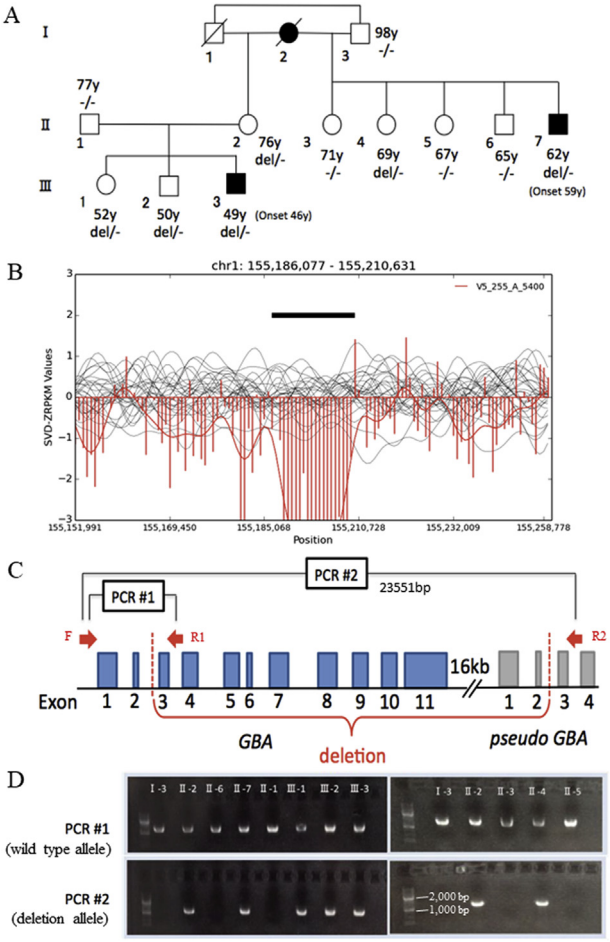

Fig. 1. A - The pedigree of the present family. There are two living PD patients and four clinically unaffected carriers. Age at examination is shown.B - CONIFER data. X-axis: Exons/probes positions on the chr1. Y-axis: SVD-ZRPKM value for each exon. Red bars and line: SVD-ZRPKM values for the exons from the sample with the call. The smooth continuous line is gaussian-smoothed representation of the SVD-ZRPKM values at each exon. Thin black in background: All other samples in the analysis. Black bar: the extent of calls above the threshold value. **C -** We found a gross deletion from intron 2 of the *GBA* gene to intron 2 of the *GBAP1* gene in the two PD patients and four clinically unaffected carriers.**D -** Amplified PCR products were detected on PCR #2 in the patients (II-7 and III-3) and four clinically unaffected carriers (II-2, II-4, III-1, and III-2). PCR products were not detected on PCR #2 in the wild type situation (I-3, II-1, II-3, II-5, and II-6) because the locations of forward primer F and reverse primer R2 were too far apart to amplify. The size of the PCR #1 product was estimated to be 1,633bp by in-silico PCR. The size of the PCR #2 product was estimated to be about 1330bp from the primer and breakpoint sequences.

2. Methods

2.1. Clinical findings in the present family

The pedigree of the present family is shown in Fig. 1A. We performed standard neurological examination in all family members.

The patients with PD are a 62-year-old man (II-7) and a 49-year-old man (III-3), the onset ages being 59 and 46, respectively. Both patients showed the primary symptoms of bradykinesia and muscle pain in the lower limbs. They also developed difficulty in walking and mild resting tremor. II-7 has gradually developed mild cognitive impairment. Patient II-7 had been treated with levodopa/carbidopa, 150mg/15 mg/day, trihexyphenidyl hydrochloride, 1 mg/day orally, and rotigotine, 4.5 mg/day percutaneously. Patient III-3 had been treated with pergolide mesilate, 1 mg/day, trihexyphenidyl hydrochloride, 1 mg/day, and zonisamide, 25 mg/day orally. Brain MRI showed no remarkable changes in either patient. [^{123}I] MIBG myocardial scintigraphy was performed about six months and about one year after the onset, respectively. On [^{123}I] MIBG myocardial scintigraphy, the delayed heart to mediastinal (H/M) ratios were 1.13% and 2.89%, respectively (normal, > 2.2%).

Nine individuals (I-3, II-1, II-2, II-3, II-4, II-5, II-6, III-1, and III-2) did not present parkinsonism.

2.2. Exome sequencing and copy number variation (CNV) analysis

Exome sequencing of genomic DNA from blood from the two PD patients (II-7 and III-3) was performed, using SureSelect V5 + UTRs (Agilent Technologies, CA, USA) with a HiSeq 2500 (Illumina, CA, USA) following the manufacturers' instructions. CNV analysis of exome data was performed using CoNIFER software (Copy Number Inference From Exome Reads, http://conifer.sourceforge.net). We referred to dbSNP (https://www.ncbi.nlm.nih.gov/projects/SNP/) to filter variants for frequency.

2.3. Sanger sequencing to determine the breakpoint of the gross deletion of the GBA gene and agarose gel electrophoresis

Sanger sequencing of the PCR products to determine the deletion breakpoint was performed in 11 individuals (I-3, II-1, II-2, II-3, II-4, II-5, II-6, II-7, III-1, III-2, and III-3) (Fig. 1C). We referred to the NCBI Reference Sequence (NM_000157.3) for *GBA*. The PCR conditions and sequencing of the primers are available on request.

2.4. Quantitative reverse transcription PCR (qPCR)

Lymphoblastoid cell lines were developed from the peripheral blood, and total RNA was extracted from the lymphoblastoid cell lines using TRIzol® Reagent (Invitrogen, CA, USA). qPCR was performed to analyze the *GBA* gene expression in 11 individuals through fluorescence detection using the intercalator method with GeneAce SYBR® qPCR Mix α Low ROX (Nippon Gene Co., Tokyo, Japan). The *GBA* gene was normalized as to the endogenous control β-actin gene (*ACTB*). Comparative determination of gene expression was performed using the comparative C_T method. The primers used for *GBA* in this study were 5′—CCA AGC CTT TGA GTA GGG TAA GCA-3′ and 5′-AGA CAC ACA CCA CCG AGC TGT AG-3′, which are specific for *GBA* exons 2 and 3, respectively (Takara Bio Inc., Shiga, Japan). qPCR was performed with a 7500 Real-Time PCR System (Applied Biosystems, MA, USA) and universal cycling conditions (10 min at 95 °C, and 45 cycles of 30 s at 95 °C and 1 min at 60 °C). A dissociation curve was recorded after each run to confirm the specificity of the analysis. Also, we confirmed that there was no contamination by *GBAP1* transcripts of the qPCR products by Sanger sequencing. The qPCR result for I-3, who is the oldest unaffected individual, was used as a reference, and we performed qPCR six times and calculated the average gene expression level for each sample. Furthermore, we compared the average value for six *GBA* mutation carriers (II-2, II-4, II-7, III-1, III-2 and III-3) with that for three non-mutation carriers (II-1, II-5 and II-6) normalized as to I-3. Statistical analysis of the results of qPCR was performed with Welch's *t*-test after confirmation of unequal variances. *P* values of < 0.05 were considered to be significant.

2.5. [^{123}I] FP-CIT SPECT

We performed [^{123}I] FP-CIT SPECT to evaluate dopaminergic neuronal death in seven individuals (II-2, II-3, II-4, II-7, III-1, III-2, and III-3). An intravenous injection of 167 MBq [^{123}I] FP-CIT (Nihon Medi-Physics, Tokyo, Japan) was administered. Four hours later, a SPECT scan was obtained using a digital gamma camera (GCA-9300R; Toshiba Medical Systems, Tochigi, Japan). SPECT acquisition was performed

using a 360-degree orbit and a 128 × 128 matrix, and scatter collection was performed using the triple-energy-window (TEW) method. Quantitative analysis, i.e., calculation of the specific binding ratio (SBR) in the striatum by Bolt's method [4], was performed using DaTView software (Nihon Medi-Physics, Tokyo, Japan). The reference value was based on the results for 256 Japanese healthy controls, as previously reported [5].

2.6. [^{123}I] N-isopropyl-p-iodoamphetamine SPECT (IMP-SPECT)

We performed IMP-SPECT to evaluate cerebral blood flow in seven individuals (II-2, II-3, II-4, II-7, III-1, III-2, and III-3). An intravenous injection of 167 MBq [^{123}I] -IMP (Nihon Medi-Physics, Tokyo, Japan) was administered. Fifteen minutes later, a SPECT scan was obtained using the GCA-9300R. SPECT acquisition was performed using a 360-degree orbit and a 128 × 128 matrix, and scatter collection was performed using the TEW method. We assessed the reduction of the cerebral blood flow in some regions of interest (ROI), i.e., the parietal lobe, precuneus, posterior cingulate gyrus, and medial and lateral occipital lobes, by the computer-assisted diagnostic system Z-score summation analysis method (Z-SAM) using three-dimensional stereotactic surface projections (3D-SSP) [6,7].

2.7. GCase activity in leukocytes

Peripheral leukocytes were obtained from seven individuals (II-2, II-3, II-4, II-7, III-1, III-2, and III-3). Unfortunately, II-5 and II-6 did not consent to this measurement. GCase activity measurement using 4-MU-D-β-glucopyranoside as a substrate was performed once at an external commercial laboratory (SRL, Tokyo, Japan). The assay details are proprietary and have not been disclosed. The reference value was based on the test results for 100 healthy volunteers from the SRL Company (4.1–9.7 nmol/mg/protein/hour).

The present study was approved by the respective institutional review boards, and written informed consent was obtained from all individuals participating in the present study.

3. Results

The clinical information and results of each analysis are summarized in Table 1.

3.1. Exome sequencing and copy number variation (CNV) analysis

No single nucleotide or short insertion/deletion mutations in the causative genes for PD, *SNCA, UCH-L1, LRRK2, HTRA, VPS35, EIF4G1, DNAJC13, CHCHD2, PARK2, PINK1 PARK7, ATP13A2, PLA2G6, FBXO7, DNAJC6, SYNJ1, VPS13C, GIGYF2, RAB39B, and RAB7L1,* were detected on exome sequencing in the two patients. Variants that, after bioinformatic filtering, were found to be present in both affected family members are shown in the supplemental table. We did not sequence any of these variants in other family members. We found a heterozygous gross deletion from exon 3 of *GBA* to exon 2 of the neighboring pseudogene in the two patients on CNV analysis using the CoNIFER software (Fig. 1B).

3.2. Sanger sequencing to determine the breakpoint of the gross deletion of the GBA gene and agarose gel electrophoresis

The PCR primers for PCR #1 and PCR #2 were placed as shown in Fig. 1B. Sanger sequencing was performed in 11 individuals, and if they had a gross deletion in *GBA*, amplified PCR products were detected electrophoretically on PCR #2. If they did not, PCR products were not detected because the locations of forward primer F and reverse primer R2 were too far apart to amplify on PCR #2.

As a result, six individuals including the two patients (II-2, II-4, II-7, III-1, III-2, and III-3) were found to carry a heterozygous gross deletion in the *GBA* gene (Fig. 1C and D). Four individuals (II-2, II-4, III-1, and III-2) were *GBA* mutation carriers without any neurological deficits. Sanger sequencing revealed that the breakpoint of this gross deletion is located somewhere in the homologous 151 bp sequences (5′-AAAATC CTCACCCCAAAGTTGGTCTCAGTCACTCAAAAGGATTTATTGAGCACC TACTAAAAGTCTACCACCAGCTTACTGGAAGGCTACCAAAGGACTATG AGGCAGAAGGGAGGCTCTGTGCTACCTCCCCACTGCCTTGACTCAC TCA-3′) in intron 2 of *GBA* and that of *GBAP1*.

The size of the PCR #1 product was estimated to be 1,633bp by in-silico PCR. The size of the PCR #2 product was estimated to be about 1330bp from the primer and breakpoint sequences.

3.3. qPCR

We compared *GBA* expression between the *GBA* mutation carriers and unaffected individuals. The *GBA* expression in four clinically unaffected carriers and two patients was approximately half of that in five non-carriers ($p = 0.036$) (Fig. 2A).

3.4. [^{123}I] FP-CIT SPECT

The visual results with the DaTView software are shown in Fig. 3A. It was found that [^{123}I] FP-CIT uptake was markedly reduced in the two patients (II-7 and III-3), their SBR being 1.57 and 0.94, respectively. Furthermore, the uptake was slightly reduced in two clinically unaffected carriers (3.98 and 4.92 in III-1 and III-2, respectively) in their 50s. Signal loss was predominantly detected in the posterior dorsal putamen. The SBR in one clinically unaffected carrier (4.59 in II-2) in her 70s was at about the lower limit of the reference value range for her age, however, no signal loss was observed visually. Meanwhile, another clinically unaffected carrier (II-4) showed unremarkable findings. An unaffected non-mutation carrier (II-3) showed normal uptake.

Table 1
The clinical information and results of each analysis.

	I-3	II-1	II-2	II-3	II-4	II-5	II-6	II-7	III-1	III-2	III-3
Age at examination	98	77	76	71	69	67	65	62	52	51	49
Clinical phenotype	no PD	no PD	no PD	no PD	no PD	no PD	no PD	PD	no PD	no PD	PD
Age at onset	–	–	–	–	–	–	–	59	–	–	46
GBA genotype	wt/wt	wt/wt	del/wt	wt/wt	del/wt	wt/wt	wt/wt	del/wt	del/wt	del/wt	del/wt
123I-FP-CIT	–	–	lower part of reference range	normal	normal	–	–	markedly decreased	decreased	decreased	markedly decreased
GBA expression	normal/ reference	normal	reduced	high/ outliner	reduced	normal	normal	reduced	reduced	reduced	reduced
Enzyme activity	–	–	3.4	5.8	4.1	–	–	3.5	4.7	4.1	3.7

Reference value for enzyme activity, 4.1–9.7 (nmol/mg protein/hour).

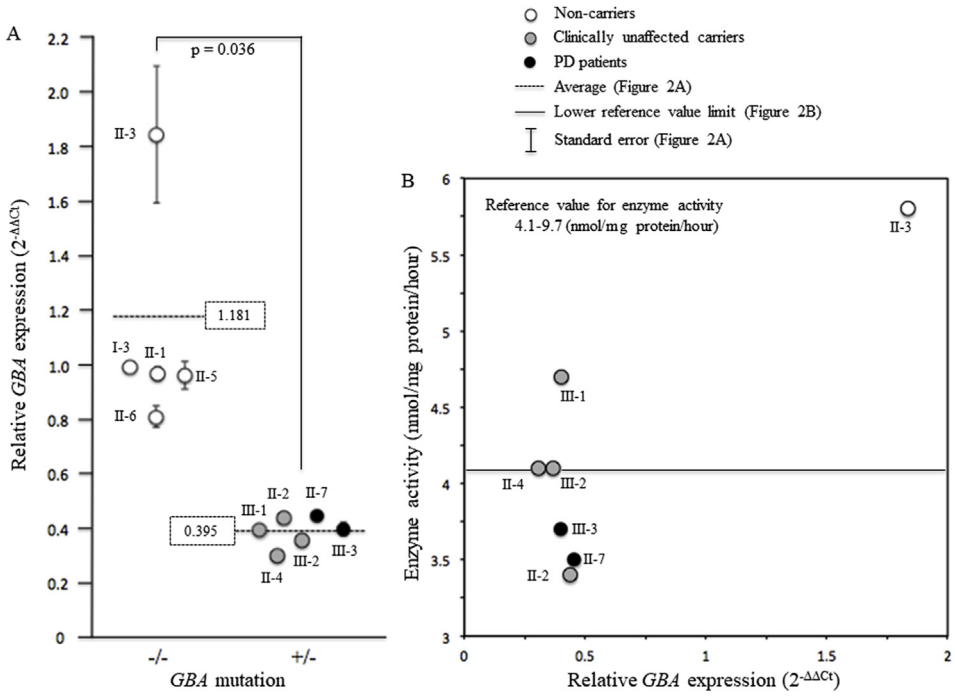

Fig. 2. A - *GBA* expression in I-3, who is the oldest unaffected individual, was used as a reference. Statistical analysis of the qPCR results was performed with Welch's *t*-test after confirmation of unequal variances. *P* values of < 0.05 were considered to be significant. *GBA* expression is significantly reduced in the two PD patients and five clinically unaffected carriers than in three non-mutation carriers ($p = 0.036$). We also performed re-calculation after omission of the value for II-3, that we considered an outliner. The difference between mutation carriers and non-mutation carriers remained significant ($p = 0.0032$), and the average relative expression for the non-carriers was 0.93. **B -** A scatter plot comparing enzyme activity to gene expression. The two PD patients (II-7 and III-3) and one clinically unaffected carrier (II-2) showed lower enzyme activity levels than the reference value (4.1–9.7 nmol/mg protein/hour).

3.5. IMP-SPECT

The visual results on 3D-SSP analysis (decrease) are shown in Fig. 3B. One PD patient (II-7) showed occipital hypoperfusion like that in DLB on 3D-SSP analysis. Six other individuals (one PD patient and five unaffected individuals) showed normal findings (data not shown).

3.6. GCase activity in leukocytes

A scatter plot comparing enzyme activity to gene expression is shown in Fig. 2B. GCase activity measurement was performed only once, so we only have one value per individual. A non-carrier (II-3) showed the highest enzyme activity. The two patients (II-7 and III-3) and one clinically unaffected carrier (II-2) showed lower activities than the reference value. One clinically unaffected carrier (II-4) showed the lowest gene expression in the present family, her enzyme activity was slightly low but within normal range.

4. Discussion

This is the first report of *GBA*-PD patients with a gross deletion in the *GBA* gene, and assessment of radioisotope imaging in *GBA*-PD patients and clinically unaffected carriers. We conducted these analyses to determine if the *GBA* gene is directly related to PD.

To date, the molecular mechanisms underlying *GBA*-PD have not been completely elucidated. In the present family, the gene expression in the *GBA* mutation carriers was decreased by about 50% compared to in healthy individuals. Both mutant *GBA*-mediated haploinsufficiency and toxic gain-of-function hypotheses have been proposed based on knowledge obtained so far [8], it has also been reported that both PD patients and clinically unaffected carriers who have heterozygous *GBA* mutations (e.g., p.N370S, p.L444P, etc.) or variants have lower GCase activity than non-carriers [9]. Although there is no enzymatic data for Japanese *GBA* mutation carriers, we expected that a gross deletion of the *GBA* gene would result in a reduction of GCase activity and thereby contributes to the *GBA*-PD pathogenesis through haploinsufficiency. Although there is limitation as to discussion of the relation between the *GBA* gross deletion and reduction of GCase activity in the present family because the only intra-familial control (II-3) has an outlying result, the results might show that the heterozygous *GBA* gross deletion was directly correlated with GCase activity in leukocytes. However, the reduction of GCase activity is not directly correlated the abnormality of [^{123}I] FP-CIT uptake. There are two clinically unaffected carriers (II-2 and II-4) who have lower enzyme activity levels without any abnormality of [^{123}I] FP-CIT uptake, although we found reduction of GCase activity and marked reduction of [^{123}I] FP-CIT uptake in the two *GBA*-PD patients in this family. It is known that reduction of the GCase activity in the brain, and also in the blood, may promote the aggregation of α-synuclein [10,11]. However, judging from our results, there may not be a direct relationship between GCase activity and α-synuclein.

In the present family, the GCase activity and gene expression in peripheral leukocytes tended to be lower in the two *GBA*-PD patients (II-7 and III-3) and one unaffected carrier (II-2) than in the other unaffected family members, and there were three clinically unaffected carriers (II-4, III-1 and III-2) in whom GCase activity in leukocytes was almost within the normal range but whose gene expression was lower than that in the non-mutation carriers. It is supposed that expression of the *GBA* gene on the normal allele maintained the enzyme activity in the reference value range in these three clinically unaffected carriers (II-4, III-1 and III-2), and that there might be other genetic factors that are involved in the development of PD because II-2 was still unaffected though her enzyme activity was the lowest in this family. Reduction of GCase activity alone has been reported to be insufficient for the aggregation of α-synuclein and cell death [12]. It is known that there are some genes that modify GCase activity (i.e., *SCARB2*) [13], and that saposin C, an activator of *GBA*, antagonizes the interaction between α-synuclein and GCase [14]. So we suspect that there might be some additional unknown factors that modify the residual GCase activity related to the pathogenesis of *GBA*-PD. Although some genetic risk factors for PD are known from genome wide association studies [15–17], and some components of food in nature (i.e., cuminaldehyde and pyrroloquinoline quinone) confer neuroprotection to inhibit α-synuclein fibrillation [18,19], this is not relevant to the present family.

Interestingly, three clinically unaffected carriers (II-2, III-1 and III-2) showed mild decreases in SBR compared with their ages. The binding affinity of [^{123}I] FP-CIT for presynaptic dopamine transporters decreases by 0.5–2.5% a year with aging, and by 6–13% a year in PD cases [20–22]. Furthermore, the aging-related change of the binding affinity

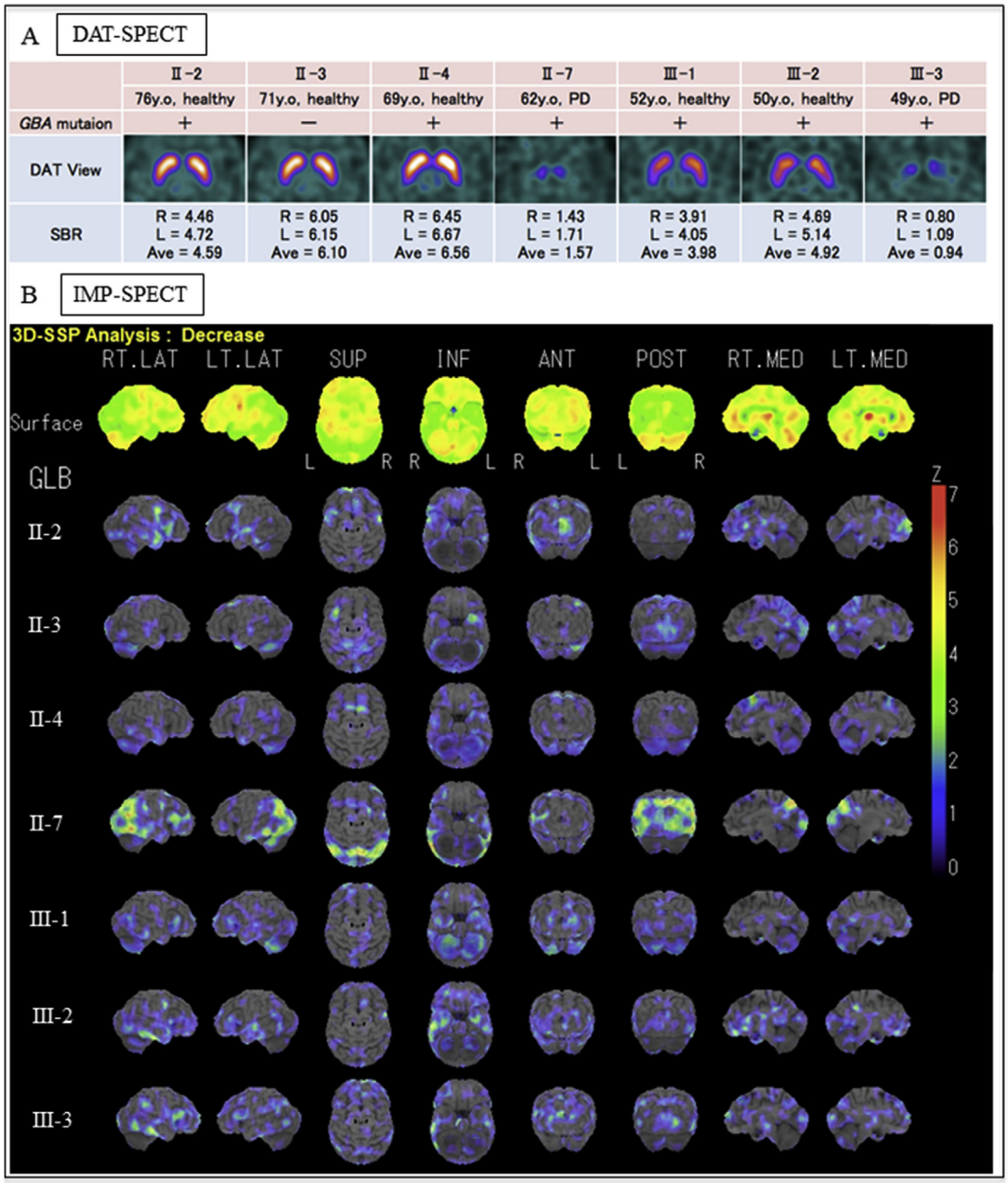

Fig. 3. A - The visual results with DaTView software. [^{123}I] FP-CIT uptake was markedly reduced in the two patients (II-7 and III-3), and was slightly reduced in two clinically unaffected carriers (III-1 and III-2) in comparison with their ages. Signal loss was predominantly detected in the posterior dorsal putamen. [^{123}I] FP-CIT uptake was about the lower reference value limit in one clinically unaffected carrier (II-2). On the other hand, [^{123}I] FP-CIT uptake was normal in one unaffected carrier (II-4).**B -** The results of IMP-SPECT revealed the decreased blood flow in the occipital lobes on 3D-SSP analysis in one PD patient (II-7).

is 5.5% every 10 years [23]. Therefore, we referenced the control data considering aging-related changes [5], and interpreted the results considering the important feature of the posterior putamen-predominant reduced uptake of [^{123}I] FP-CIT in the early stages of PD [4,5,24]. The finding that III-1 and III-2 showed degradation in the posterior putamen may reflect degeneration of dopaminergic neurons in view of their age and there is a possibility that they may have already developed dopaminergic dysfunction subclinically. Their SBR were lower than the 95% lower limit for SBR in healthy Japanese controls (95% lower limit of SBR = 5.95 in women and 5.41 in men in their 50s) [5]. It is very important to follow them because they might develop PD clinically in the near future. Although the SBR in II-2 was at the lower normal value limit for her age, there was no remarkable change in the posterior putamen visually. On the other hand, the SBR in II-4 was high enough for her age, and we thus conclude that she is a true unaffected carrier who will not develop PD in her lifetime. Judging from the results of the radioisotope imaging, a heterozygous *GBA* gross deletion might not necessarily lead to reduction of dopamine uptake observable on [^{123}I] FP-CIT SPECT. Although, to the best of our knowledge, there have been no reports of pathological investigation of healthy *GBA* mutation carriers, the present [^{123}I] FP-CIT SPECT results might indicate that the degeneration of dopaminergic neurons does not occur in every *GBA* mutation carrier, and that some other genetic factors might affect the development of *GBA*-PD.

The number of individuals enrolled in this study was very limited, so we are planning to conduct analysis of unrelated controls in the future. Further investigation to analyze other genetic factors is required to elucidate the pathological mechanism underlying *GBA*-PD.

5. Conclusion

This is the first report of *GBA*-PD patients with a gross deletion in the *GBA* gene, and of [^{123}I] FP-CIT SPECT being performed in clinically unaffected heterozygous *GBA* mutation carriers. The major limitation of our study is the small number of clinically unaffected carriers and there is a possibility that some of them will develop PD in the future. However, we found the gross deletion mutation in the *GBA* gene appears to be associated with the PD or reduced [^{123}I] FP-CIT in this family, but cannot conclude if reduction of GCase activity is directly correlated with the pathogenesis of PD.

Two PD patients and two of four clinically unaffected carriers showed decreased [^{123}I] FP-CIT uptake, whereas not all the *GBA* mutation carriers showed decreased it. [^{123}I] FP-CIT SPECT may be helpful for discriminating lifelong unaffected carriers from carriers who have the potential to develop PD, which will further facilitate investigations of risk factors other than *GBA* mutations in *GBA*-PD. Longitudinal follow-up is needed in this family.

Financial disclosure/Conflict of interest

None.

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Relevant conflicts of interest/financial disclosures

The authors declare no conflicts of interest.

Funding agencies

The authors declare that there are no financial agencies.

Author contributions

Y. I., H. I., S. T., and Y. T. designed the study; Y.I., H. I., M. T., H. Y., M. T., K. K., N. Y., J. M., J. G., and T. O. performed the experiments and analyzed the data; Y. I., J. Y., K.D., and S. M. performed the computational analyses; Y.I., T. U., and H. O. performed the neuroimaging analyses; Y. I., M. T., N. Y., K. S., and Y. T. collected and analyzed the clinical data; and Y. I., H. I., S. T., and Y. T. wrote the manuscript, with contributions from all authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parkreldis.2018.11.028.

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