Research Article

Detection of Phosphorylated Alpha-Synuclein in the Muscularis Propria of the Gastrointestinal Tract Is a Sensitive Predictor for Parkinson’s Disease

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Background. Parkinson’s disease (PD) is a neurodegenerative disorder characterized by motor and nonmotor impairments, including constipation. Lewy bodies and neurites, the pathological hallmarks of PD, are found in the enteric nervous system (ENS) as well as the central nervous system. Constipation is a well-documented premotor symptom in PD, and recent reports have demonstrated Lewy pathology in gastrointestinal (GI) tissues of PD patients prior to the onset of motor symptoms. Objective. In the present study, we assessed Lewy pathology in the GI tracts of seven PD patients who had undergone a gastrectomy, gastric polypectomy, or colonic polypectomy prior to the onset of motor symptoms in order to assess whether the presence of pathological αSyn in the ENS could be a predictor for PD. Methods. GI tissue samples were collected from control patients and patients with premotor PD. Immunohistochemistry was performed using primary antibodies against α-synuclein (αSyn) and phosphorylated αSyn (pαSyn), after which Lewy pathology in each sample was assessed. Results. In all control and premotor PD patients, accumulation of αSyn was observed in the myenteric plexus in both the stomach and colon. In 82% (18/22) of control patients, mild-to-moderate accumulation of αSyn was observed in the submucosal plexus. However, there was no deposition of pαSyn in the ENS of control patients. In patients with premotor PD, abundant accumulation of αSyn was observed in the myenteric plexus, similar to control patients. On the other hand, pαSyn-positive aggregates were also observed in the nerve fibers in the muscularis propria in all examined patients with premotor PD (100%, 3/3), while the deposition of pαSyn in the submucosal plexus was only observed in one patient (14%, 1/7). Conclusion. Our results suggest that the detection of pαSyn, but not αSyn, especially in the muscularis propria of GI tracts, could be a sensitive prodromal biomarker for PD.

1. Introduction

Parkinson’s disease (PD), one of the most prevalent neurodegenerative disorders, is characterized by the progressive degeneration not only of the dopaminergic nigrostriatal system, which is responsible for the core motor symptoms including tremor at rest, bradykinesia, and rigidity [1, 2], but also by the involvement of many other neuronal systems and organs affected by different nonmotor deficiencies, including olfactory dysfunction, cardiac involvement, and REM (rapid eye movement) sleep behavior disorder [3]. Moreover, PD patients often experience symptoms that span the entire alimentary tract including dysphagia, delayed gastric emptying, constipation, and defecatory dysfunction [4, 5].

The postmortem diagnosis of PD requires not only evidence of dopaminergic cell loss in the substantia nigra but also Lewy pathology, or the widespread occurrence of intracytoplasmic depositions of phosphorylated α-synuclein (αSyn), the major protein marker and biological hallmark of PD and other synucleinopathies [6]. αSyn can undergo
several posttranscriptional modifications, including nitration [7], ubiquitination [8], and SUMOylation [9]. However, more than 90% of the αSyn that accumulates in PD brains is phosphorylated at Ser129, and immunohistochemistry using an anti-phosphorylated αSyn (pαSyn) antibody is the strongest tool to detect Lewy pathology [6, 10].

Besides the central nervous system, Lewy pathology is observed within the sympathetic and parasympathetic ganglia [11], adrenal glands [12], enteric nervous system (ENS) [13–16], and cutaneous nerves [17]. The clinical diagnosis of PD depends on the appearance of cardinal motor symptoms, which are signs that do not appear before the loss of an estimated 70–80% of striatal dopamine [2, 18]. It is important to diagnose the disease earlier in order to maximally benefit from the numerous therapies targeting this disease.

PD patients often experience prodromal symptoms such as olfactory dysfunction, constipation, fatigue, and behavioral and mood changes [3, 19, 20]. It is now generally accepted that a variety of nonmotor features of PD are part of the evolving disease spectrum and commonly occur prior to the evaluation of the defining motor signs [3, 20]. Moreover, postmortem studies of incidental Lewy body disease suggest that αSyn pathology may begin in tissues of the gastrointestinal (GI) tract, salivary gland, and olfactory system [14, 15, 21, 22]. These studies lead to the proposal that, in a large proportion of PD cases, the substantia nigra is involved only after the olfactory system and lower brainstem regions [3].

In 2012, Shannon et al. demonstrated the accumulation of αSyn in the colonic mucosa and submucosa in PD patients before the development of characteristic motor symptoms [23], and more recently, Stokholm et al. have demonstrated the presence of Lewy pathology (aggregated pαSyn) in GI tissues of premotor PD patients [24], suggesting that deposition of αSyn in the ENS could be a useful predictor for PD. However, these studies have not considered the difference in Lewy pathology between the submucosa and muscularis propria. The objective of this study is to discover a more sensitive biomarker for premotor PD patients. To this end, we focused on Lewy pathology in the muscularis propria of GI tracts and compared it with that in the submucosa.

2. Materials and Methods

2.1. Subjects. Seven PD patients aged 53–79 years were recruited from the PD database of the Department of Neurology of Osaka University Hospital. A diagnosis was made according to the United Kingdom Parkinson Disease Research Society Brain Bank criteria [1]. The criteria for their recruitment were as follows: (1) a distal gastrectomy, gastric polypectomy, or colonic polypectomy was performed at the Osaka University Hospital before they exhibited any motor symptoms and (2) tissue samples taken by surgery were available. The clinical profiles of these patients are summarized in Table 1. Patient P1 developed dysphagia as the initial symptom of PD three months after the distal gastrectomy. Three patients (P1, P2, and P7) had constipation at the time of operation.

Control cases were selected randomly. Control samples were taken from four autopsy subjects, four patients with advanced gastric cancer, four patients with colon cancer, five patients with early gastric cancer or gastric polyps, and five patients with colonic polyps without a history of neurological or psychiatric diseases, respectively, from Osaka University Hospital. The eighteen patients with advanced gastric cancer, early gastric cancer, gastric polyps, colon cancer, or colon polyps (patients C5–C22) showed no neurological signs for at least six years after the operation. The profiles of the control patients are summarized in Table 2.

This study was approved by the Ethics Committee of Osaka University Hospital (no. 12148) and conducted in accordance with the Declaration of Helsinki (1964). The experiment was conducted with the human subjects’ understanding and consent.

2.2. Immunohistochemistry. Tissue samples were fixed in 10% formalin and then dehydrated and embedded in paraffin blocks, and five-micrometer-thick paraffin serial sections were prepared. Consecutive slices were considered as the same site in each sample. Deparaffinized sections were incubated for 30 min with 0.3% H2O2 to quench any endogenous peroxidase activity, after which they were washed with PBS. The primary antibodies used were a rabbit polyclonal antibody against αSyn (Sigma-Aldrich (S3062), St. Louis, MO), a mouse monoclonal antibody against pαSyn (Wako Pure Chemical Corp. (pSyn #64), Osaka), and a rabbit polyclonal antibody against protein gene product 9.5 (PGP9.5, neuronal marker, Abcam (ab10404), Cambridge, UK). Autoclave treatment was performed for 15 min before incubation with all the antibodies. Goat anti-rabbit and anti-mouse immunoglobulins conjugated to peroxidase-labeled dextran polymer (Dako Envision+, Dako Corp., Carpinteria, CA) were used as secondary antibodies. Reaction products were visualized with 3,3′-diaminobenzidine tetrahydrochloride (Vector Laboratories, Burlingame, CA), and hematoxylin was used to counterstain the cell nuclei.

The staining pattern of αSyn was evaluated according to the following four-grade system: (1) strong, with more than half of the myenteric and/or submucosal plexus in each section and intramuscular nerve fibers strongly immunopositive for αSyn; (2) moderate, with an intermediate level between strong and weak immunoreactivity, with weakly positive intramuscular nerve fibers; (3) weak, with only a few plexuses in each section positive for αSyn and intramuscular nerve fibers negative; and (4) absent, with no immunostaining for αSyn. The expression level of αSyn was scored according to the following system: strong = score 3, moderate = score 2, weak = score 1, and absent = score 0. Three sections from each patient were examined by two specialists of pathology. The scores between the two groups were statistically compared by t-test, and statistical significance was determined at p < 0.05. The intraclass correlation coefficients (ICC) were calculated using Bell Curve for Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan). The staining pattern of pαSyn was divided into two
groups according to whether pαSyn-positive aggregates were detected (positive) or not (negative).

### 3. Results

The results of immunohistochemistry are summarized in Table 3.

In all control patients whose muscularis propria could be analyzed (patients C1–C12), αSyn immunoreactivity was detected in the myenteric plexus in both the stomach (Figure 1(a)) and colon. Six samples from six patients showed strong αSyn immunoreactivity, eight samples showed moderate αSyn immunoreactivity, and one showed weak αSyn immunoreactivity, respectively, in the muscularis propria (Table 3). In eleven cases, the accumulation of αSyn was observed in the intramuscular nerve fibers (Figure 1(d)) in addition to the myenteric plexus. However, phosphorylated αSyn (pαSyn) immunoreactivity was not detected in the muscularis propria in any of the control patients (Figures 1(b) and 1(e)). The components of the ENS were confirmed by immunohistochemical staining with PGP9.5 in the serial sections (Figures 1(c) and 1(f)). In the submucosa, weak-to-moderate αSyn immunoreactivity was visible in four out of seven (57%) PD patients (P1–P4).

### Table 1: Clinical profiles of PD patients.

| Patient | Diagnosis/operation | Age at operation | Digestive symptoms* | Age at PD onset (initial symptoms) | Biopsy site |
|---------|---------------------|------------------|---------------------|------------------------------------|-------------|
| P1      | M                   | 75               | Constipation        | 75 (dysphagia)                     | Descending colon |
| P2      | M                   | 78               | Constipation        | 79 (gait disturbance)              | Descending colon |
| P3      | M                   | 59               | Nausea              | 71 (resting tremor)                | Descending colon |
| P4      | F                   | 64               | Fecal occult blood  | 73 (resting tremor)                | Descending colon |
| P5      | M                   | 58               | Fecal occult blood  | 60 (resting tremor)                | Descending colon |
| P6      | F                   | 45               | Nausea              | 53 (gait disturbance)              | Corpus      |
| P7      | F                   | 52               | Constipation        | 53 (gait disturbance)              | Corpus      |

GC, gastric cancer; DG, distal gastrectomy; CP, colon polyp; GP, gastric polyp; EMR, endoscopic mucosal resection. *Digestive symptoms shown before the operation. The initial motor symptoms of PD are also described.

### Table 2: Clinical profiles of the control patients.

| Patient | Diagnosis | Age | Sex | Age at operation | Digestive symptoms | Biopsy site |
|---------|-----------|-----|-----|------------------|--------------------|-------------|
| C1      | Sepsis    | 74  | F   | (Autopsy)        |                    | None        |
| C2      | MI        | 76  | M   | (Autopsy)        |                    | None        |
| C3      | HCC       | 75  | M   | (Autopsy)        |                    | None        |
| C4      | GC        | 71  | M   | (Autopsy)        |                    | None        |
| C5      | GC        | 89* | F   | 80, DG           | None               | None        |
| C6      | GC        | 76* | F   | 70, DG           | None               | None        |
| C7      | GC        | 79* | M   | 72, DG           | None               | None        |
| C8      | GC        | 74* | M   | 67, DG           | None               | None        |
| C9      | CC        | 79* | F   | 73, Sig          | Bloody stool       | None        |
| C10     | CC        | 73* | F   | 67, RH           | Bloody stool       | None        |
| C11     | CC        | 66* | M   | 60, Sig          | Fecal occult blood | None        |
| C12     | CC        | 62* | M   | 55, Sig          | None               | None        |
| C13     | EGC       | 62* | M   | 55, ESD          | None               | Corpus      |
| C14     | EGC       | 61* | F   | 56, ESD          | Heartburn          | Corpus      |
| C15     | EGC       | 68* | M   | 62, ESD          | None               | Antrum      |
| C16     | EGC       | 67* | M   | 61, ESD          | None               | Corpus      |
| C17     | GP        | 82* | F   | 76, EMR          | Hematemesis        | Corpus      |
| C18     | CP        | 79* | M   | 71, EMR          | Fecal occult blood | Descending colon |
| C19     | CP        | 64* | F   | 58, EMR          | None               | Ascending colon |
| C20     | CP        | 75* | M   | 69, EMR          | None               | Ascending colon |
| C21     | CP        | 69* | M   | 56, EMR          | Diarrhea           | Descending colon |
| C22     | CP        | 74* | F   | 67, EMR          | Fecal occult blood | Sigmoid colon |

MI, myocardial infarction; HCC, hepatocellular carcinoma; GC, gastric cancer; CC, colon cancer; EGC, early gastric cancer; GP, gastric polyp; CP, colonic polyp; DG, distal gastrectomy; Sig, sigmoidectomy; RH, right hemicolectomy; ESD, endoscopic submucosal dissection; EMR, endoscopic mucosal resection. *Age at the latest consultation without neurological symptoms.
Table 3: Immunohistochemical findings in patients.

| Patient | α-Synuclein | Phosphorylated α-synuclein |
|---------|-------------|-----------------------------|
|         | MP | SM | Mucosa | MP | SM | Mucosa |
| C1 Stomach | ++ | + | – | | | |
| C2 Stomach | ++ | + | – | | | |
| C3 Stomach | ++ | ++ | – | | | |
| C4 Stomach | ++ | + | – | | | |
| C5 Stomach | +++ | ++ | – | | | |
| C6 Stomach | ++ | ++ | – | | | |
| C7 Stomach | ++ | + | – | | | |
| C8 Stomach | ++ | + | – | | | |
| C9 Stomach | +++ | ++ | – | | | |
| C10 Colon | +++ | ++ | – | | | |
| C11 Colon | +++ | ++ | – | | | |
| C12 Colon | +++ | ++ | – | | | |
| C13 Stomach | NE | – | – | NE | – | – |
| C14 Stomach | NE | + | – | NE | – | – |
| C15 Stomach | NE | – | – | NE | – | – |
| C16 Stomach | NE | + | – | NE | – | – |
| C17 Stomach | NE | + | – | NE | – | – |
| C18 Colon | NE | – | – | NE | – | – |
| C19 Colon | NE | – | – | NE | – | – |
| C20 Colon | NE | + | – | NE | – | – |
| C21 Colon | NE | – | – | NE | – | – |
| C22 Colon | NE | – | – | NE | – | – |
| P1 Stomach | +++ | ++ | – | + | – | – |
| P2 Stomach | +++ | + | – | + | – | – |
| P3 Stomach | +++ | + | – | + | – | – |
| P4 Colon | NE | + | – | NE | – | – |
| P5 Colon | NE | – | – | NE | – | – |
| P6 Stomach | NE | – | – | NE | – | – |
| P7 Stomach | NE | – | – | NE | – | – |

Staining pattern: strong (+++), moderate (+), weak (+), and absent (–) for α-synuclein and positive (+) or negative (–) for phosphorylated α-synuclein. MP, muscularis propria; SM, submucosa; NE, not examined because MP was not included in the tissue samples.

Figure 1: Continued.
was indistinguishable from the control group. These results suggest that the accumulation of αSyn-positive aggregates in the muscularis propria would predict the onset of PD.

The accumulation of αSyn was not detected in the ENS in four premotor PD patients (P4–P7). This may be because the operation had been performed at a stage that was too early, without patients having any evidence yet of constipation (P4–P6). Previous studies have reported that, within the GI tract, the lower esophagus has the highest frequency of αSyn histopathology, followed by the stomach, while the colon and rectum have the lowest [13, 15, 29]. In contrast, recent reports have revealed that the colon is more sensitive for the detection of Lewy pathology in the premotor phase of PD [26, 27]. In support of our results, recent meta-analyses have shown that the combined use of anti-αSyn antibody and neuronal markers can increase the sensitivity of Lewy pathology detection [26] and, moreover, that biopsied samples often do not contain the muscularis propria/myenteric plexuses, leading to a decrease in sensitivity [26, 28].

The control of GI motility and secretion also depends on both extrinsic parasympathetic and sympathetic innervation [16, 30]. Extrinsic parasympathetic inputs originate in the
Figure 2: Immunohistochemical analyses of patients with premotor PD. Immunohistochemistry for α-synuclein (αSyn) (a–d, f), and phosphorylated α-synuclein (pαSyn) (e, g–i). (a, b, d, e, g) Sections of the stomach of patient P1; (c, h) sections of the stomach of patient P2; (f) section of the colon of patient P5; (i) sections of the stomach of patient P3. (b) High magnification view of the dotted square in (a, d, e) are serial sections. Black circles indicate myenteric plexus (c) and submucosal plexus (d, e). MP, muscularis propria; SM, submucosa. Accumulations of αSyn are observed in the nerve bundle in the MP (arrow in (a)), intramuscular nerve fibers ((b), small arrows), myenteric plexus (c), and submucosal plexus (d), but not in the mucosa (f). pαSyn-positive aggregates are observed in the nerve bundles in the MP (arrows in (g, h)) and submucosal plexus (arrow in (i)). In patient P1, the submucosal plexus with high accumulation of αSyn (d) shows no staining for pαSyn (e). Scale bar: 100 μm (a, c), 50 μm (d–g), and 20 μm (b, h, i).

Figure 3: Scores of αSyn expression. In both the (a) muscularis propria (MP) and (b) submucosa (SM), there was no statistically significant difference in αSyn-expression scores between the control and PD groups. Data are shown as mean ± SEM. n.s, not significant.
dorsal motor nucleus of the vagus nerve and in the sacral parasympathetic nucleus, both of which control the motility of the upper GI tract and the distal colon and rectum [31]. The myenteric plexus primarily controls the activity of the smooth muscle of the gut and thus intestinal motility, whereas the submucosal plexus is involved in the regulation of mucosal functions such as secretion and blood flow [32]. It has been reported that dopaminergic defects are seen in the muscularis propria, but not in the mucosa, in PD patients with chronic constipation [33]. Our results suggest that neuronal dysfunction due to the accumulation of pαSyn may initially occur in the myenteric plexus, which could lead to alimentary tract dysfunction and induce delayed gastric emptying constipation due to slow peristalsis. Finally, pathological aSyn would propagate from the ENS into the brainstem through the vagus nerve, as shown in animal experiments [34].

In the present study, we did not find any relationship between the etiology of gastrointestinal diseases, biopsy sites, and accumulation of pαSyn. This was likely because the number of patients was small and all the samples containing muscularis propria originated from the stomach. Moreover, it remains unclear whether the digestive symptoms were induced by gastrointestinal diseases or the premotor symptoms of PD. Further study in a larger population is necessary.

5. Conclusion
In conclusion, the deposition of pαSyn was observed in the ENS in both the stomach and colon in PD patients prior to the onset of motor symptoms, which could thereby be used as a biomarker for prodromal PD. More importantly, our results suggest that the investigation of GI mucosa and submucosa by immunohistochemistry for aSyn might overlook Lewy pathologies and lead to misdiagnoses, but suggest that the detection of pαSyn-positive aggregates in the GI muscularis propria could be more sensitive in the prediction of the onset of PD. This study lays the foundation for future research aimed at the development of further useful clinical implementations of these results.

Data Availability
The data used to support the findings of this study are available from the corresponding author upon request, following approval by the responsible ethical committee.

Conflicts of Interest
The authors declare that there are no conflicts of interest regarding the publication of this paper.

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