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BRIEF COMMUNICATION

Sensitivity of Detecting Alpha-Synuclein Accumulation in the Gastrointestinal Tract and Tissue Volume Examined

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ABSTRACT

Objective This study aimed to evaluate whether a larger tissue volume increases the sensitivity of detecting alpha-synuclein (AS) pathology in the gastrointestinal (GI) tract.

Methods Nine patients with Parkinson's disease (PD) or idiopathic rapid eye movement sleep disorder (iRBD) who underwent GI operation and had full-depth intestinal blocks were included. All patients were selected from our previous study population. A total of 10 slides (5 serial sections from the proximal and distal blocks) per patient were analyzed.

Results In previous studies, pathologic evaluation revealed phosphorylated AS (+) in 5/9 patients (55.6%) and in 1/5 controls (20.0%); in this extensive examination, this increased to 8/9 patients (88.9%) but remained the same in controls (20.0%). The severity and distribution of positive findings were similar between patients with iRBD and PD.

Conclusion Examining a large tissue volume increased the sensitivity of detecting AS accumulation in the GI tract.

Keywords Alpha-synuclein; Synucleinopathy; Gastrointestinal tract; Immunohistochemistry; Volume.

Lewy pathology of the gastrointestinal (GI) tract has potential as a biomarker for the diagnosis of synucleinopathies such as Parkinson's disease (PD) and idiopathic rapid eye movement sleep disorder (iRBD). However, low sensitivity limits its use in clinical practice.[1] In a previous autopsy study, evaluation of multiple sections increased the positivity rate,[2] and earlier in vivo studies reported high sensitivity using a method called 'whole-mount staining.'[3-5]

Therefore, this study aimed to determine whether evaluation of a larger tissue volume increases the sensitivity of detecting alpha-synuclein (AS) pathology in the GI tract in patients with synucleinopathy.

MATERIALS & METHODS

Participants and specimen selection

Patients in this study were selected from those who partici-

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pated in previous studies of PD[1] and iRBD.[6] We selected patients with PD or iRBD, and controls who had both proximal and distal marginal blocks of the GI tract archived in the pathology bank. Therefore, two formalin-fixed paraffin-embedded blocks were collected per participant. This study was approved by the Institutional Review Board of Seoul National University Hospital (IRB No. H-1409-043-608). The requirements for a waiver of informed consent were met, and a waiver was granted.

Immunohistochemistry

A total of five serial 3-μm sections were obtained from each surgical block for immunohistochemistry (IHC). The paraffin sections were mounted on a glass slide, dewaxed, rehydrated, and incubated with primary antibodies on automated machines as previously described.[1,6] A primary antibody against phosphorylated AS (pAS) (1/1,000 anti-pAS at serine 129 monoclonal Ab [EP1536Y]; Abcam ab51253, Cambridge, UK) was used in conjunction with the Leica Bond Max (A33030) system, in accordance with the manufacturer's instructions. Bound antibodies were detected using the Bond Polymer Refine Detection system (Leica Biosystems, Wetzlar, Germany).

Pathologic evaluation

To avoid bias, the raters were blinded to the clinical information of the participants. All stained slides were scanned with a Leica Slide Scanner (Aperio AT2, Leica Biosystems). Anonymized digital slides were evaluated using the Pathologic Slide Viewing Software (Aperio ImageScope ver. 12.4, Leica Biosystems).

pAS positive findings were defined conservatively as in the previous study:[6] 1) pAS IHC showing definite and clear staining such as 'dots and fiber' or 'Lewy body-like staining' pattern, as the consensus paper suggested[7] and 2) localization in neural structures confirmed with anatomic inspection.[8] pAS-positive findings were semiquantitatively rated as grade 1, 2, or 3, which corresponded to sparse, moderate, or frequent in the multicenter study.[6,7]

A neuropathologist (S.K) and neurologist (C.S) were blinded to the anonymization procedure and independently examined the slides. The neurologist (C.S) participated in previous studies regarding GI synucleinopathy,[1,6,8] and both raters underwent a training program in the microscopic reading of peripheral AS pathology of the Systemic Synuclein Sampling Study.[9] Any discrepancy between the two raters was resolved in a consensus meeting with independent investigators (S.P and B.J).

Statistical analysis

This study is an additional analysis of two previous studies, and the subjects were not randomly selected. Therefore, descriptive analyses were mainly conducted. For group comparison, nonparametric tests were used because of the small number of participants. All statistical analyses were conducted with SPSS ver. 26.0.0.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Nine patients (4 PD and 5 iRBD) and five controls were selected. In previous studies, pAS was positive in 5/9 patients (55.6%) and 1/5 controls (20.0%). In an extensive evaluation of 10 slides per patient, the positivity rate increased to 8/9 patients (88.9%), but the rate remained the same (20.0%) in controls ($p = 0.023$; Table 1 and Figure 1).

In the detailed analysis of 5 slides from the proximal and distal blocks in both the patient and control groups, samples from 6 of 9 subjects with pAS positivity (66.7%) were positive in both proximal and distal blocks (Supplementary Table 1 in the online-only Data Supplement and Figure 1). Moreover, positivity was present in all 5 serial sections in 80.0% of positively stained blocks (12/15); three blocks of P02 and C02 were exceptions.

Regarding the severity of pAS positivity, severe density (3+) was found in both PD and iRBD patients. When the results were discordant between proximal and distal blocks (P06, P08, and C02), only mild density (1+) was found in the positive block. Similarly, when pAS-positive findings were not present in all 5 serial sections (P02 and C02), only mild density (1+) was seen. Finally, there was a severity gradient in the distal block of P01.

DISCUSSION

This study demonstrates that pAS positivity increases with increasing tissue volume examined, which is consistent with previous studies. Beach et al.[2] reported that the pAS positivity rate in the GI tract increased from 11/17 (64.7%) to 14/15 (93.3%) when multiple slides and 80-μm frozen sections were examined in their large-scale autopsy study. Lebouvier et al.[3-5] also reported good results (72%–80%, pAS positivity) using whole-mount staining, which involves microdissecting the submucosa from the mucosa in the biopsied colon tissue and mounting and staining the whole submucosa at once. The method allowed us to evaluate a large submucosal tissue volume. This study also showed that mild AS accumulation with the semiquantitative grade 1+ could be inconsistently rated within the proximal and distal sites of one organ and consecutive sections taken from a single site. These findings could be explained by the multifocal

Table 1. pAS immunostaining results of extensive tissue (5 slides per block) compared with original staining findings

Group	ID	Diagnosis	PD subtype	Duration of disease onset to operation (yr)	Specimen	pAS result		Distal block			Proximal block			
						Initial*	Extensive	Initial*	Extensive	Highest grade	N of positive slides	Initial*	Extensive	Highest grade	N of positive slides
Patient	P01	PD	TD	1	Stomach	(+)	(+)	(-)	(+)	3+	5	(+)	(+)	3+	5
Patient	P02	PD	TD	-2	Proximal colon	(+)	(+)	(-)	(+)	1+	1	(+)	(+)	1+	4
Patient	P03	iRBD		-2	Stomach	(+)	(+)	(-)	(+)	3+	5	(+)	(+)	2+	5
Patient	P04	iRBD		-1	Esophagus	(+)	(+)	(+)	(+)	3+	5	(+)	(+)	3+	5
Patient	P05	iRBD		0	Stomach	(+)	(+)	(+)	(+)	2+	5	(+)	(+)	3+	5
Patient	P06	PD	TD	5	Proximal colon	(-)	(+)	(-)	(+)	1+	5	(-)	(-)	0	0
Patient	P07	PD	PIGD	1	Stomach	(-)	(+)	(-)	(+)	3+	5	(-)	(+)	3+	5
Patient	P08	iRBD		-1	Stomach	(-)	(+)	(-)	(-)	0	0	(-)	(+)	1+	5
Patient	P09	iRBD		-3	Stomach	(-)	(-)	(-)	(-)	0	0	(-)	(-)	0	0
Control	C01				Proximal colon	(+)	(-)	(+)	(-)	0	0	(-)	(-)	0	0
Control	C02				Proximal colon	(-)	(+)	(-)	(-)	0	0	(-)	(+)	1+	2
Control	C03				Stomach	(-)	(-)	(-)	(-)	0	0	(-)	(-)	0	0
Control	C04				Stomach	(-)	(-)	(-)	(-)	0	0	(-)	(-)	0	0
Control	C05				Stomach	(-)	(-)	(-)	(-)	0	0	(-)	(-)	0	0

*initial result is retrieved from immunostaining data of previous studies which stained one slide per block. pAS, phosphorylated alpha-synuclein; PD, Parkinson's disease; iRBD, idiopathic rapid eye movement sleep disorder; TD, tremor-dominant; PIGD, postural instability gait disturbance.

distribution of AS accumulation in the GI tract, which has a greater influence on the pathologic evaluation of milder disease stages. Therefore, an extensive volume of large full-depth pathologic specimens is required to achieve the maximal positivity rate, but this is not feasible in clinical practice. These results further support the fundamental limit of biopsies that contain only a small portion of mucosal and submucosal layers from the intestinal wall.[1]

There were no definite differences in the distribution and severity between patients with iRBD and PD, although iRBD is a prodromal stage of PD. Previous studies have reported pAS-positive rates of 62.5% and 58.3% in the stomach specimens of patients with iRBD and PD, respectively.[1,6] Several studies have reported that the sensitivity of detecting AS accumulation is higher in patients with iRBD than in PD using specimens from the colon,[10] submandibular gland,[11] and skin.[12] These results support the notion that AS accumulation in the enteric nervous system precedes the progression of Lewy pathology from the periphery. Therefore, it supports the gut-to-brain progression model presented in Braak's hypothesis.[13]

In contrast, one patient with iRBD (P09) showed no positivity on extensive examination. There should be pathologic changes in the brain because iRBD was confirmed with polysomnography. This result suggests an alternative progression pathway for synucleinopathy that does not follow the gut-to-brain progression model. One study examined AS accumulation in the stomach and vagus nerve from an autopsied series of normal controls, patients with incidental Lewy body disease and patients with PD.[14] There was no AS accumulation in the stomach or vagus nerve in normal elderly subjects in that study, which indicated that AS accumulation in the enteric nervous system was present only in patients who had Lewy pathology in their brain. Therefore, the result questioned the so-called "body-first" origin of synucleinopathy. Recently, a "brain-first" PD hypothesis, in which pathologic AS descends from the brain to the gut, has been suggested based on multimodal imaging data.[15,16] Taken together, this study provides evidence for complexity in the origin and progression of Lewy pathology in synucleinopathy. Another possibility is that some patients with iRBD do not progress to neurodegenerative diseases for a long period of time[17] or develop non-Lewy body diseases, such as multiple system atrophy.[18] Therefore, this patient might have had non-Lewy pathology in the brain.

The negative result of one control subject (C01) whose initial result was positive can be explained by the different definitions of pAS positivity in the previous study.[1] The previous definition of pAS positivity allowed a 'diffuse staining' pattern that the initial staining result for this subject showed. Later, we used a more conservative definition[6] based on evidence from subsequent

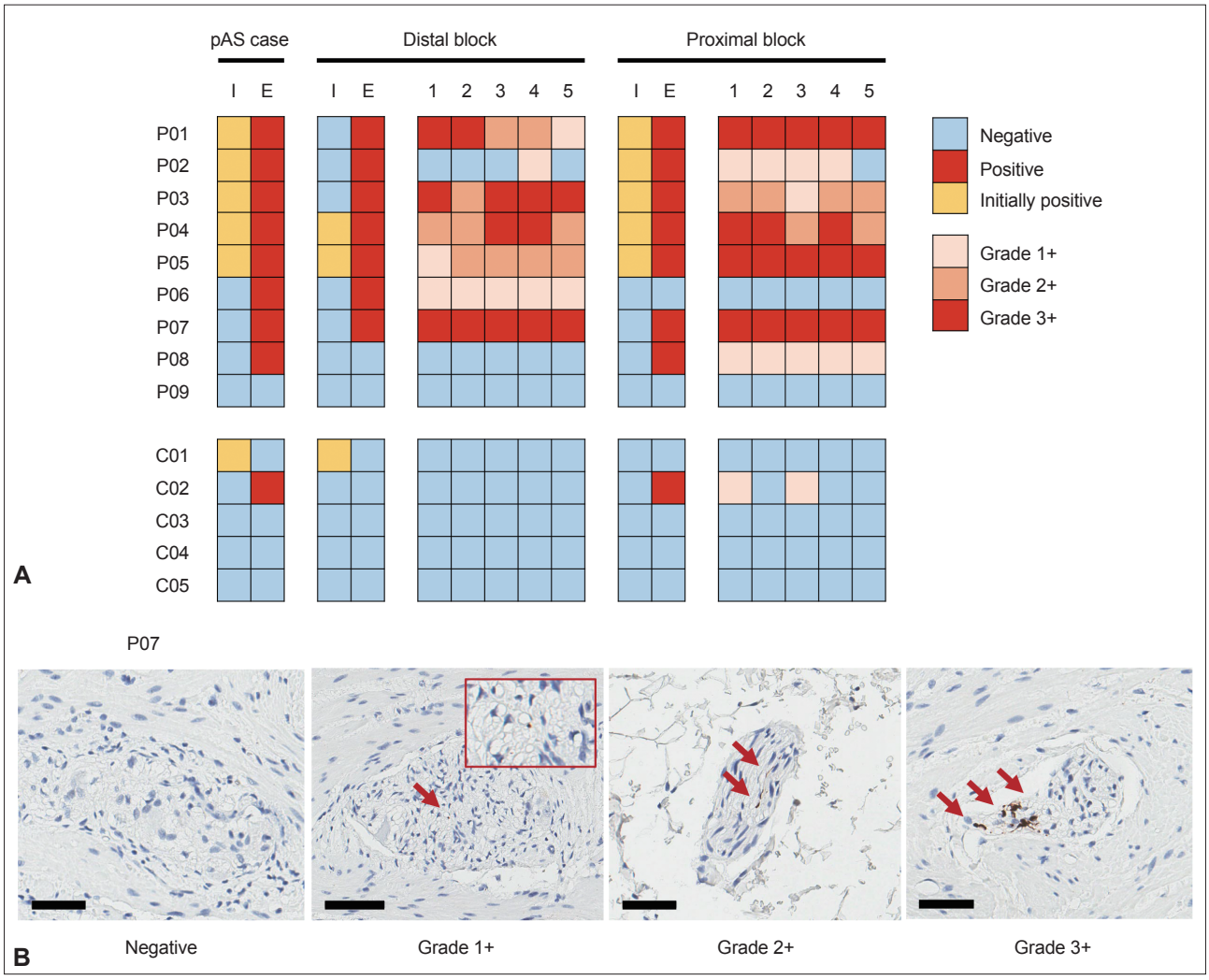

Figure 1. pAS immunostaining results of extensive tissue evaluation (five sequential slides per block) compared with original staining findings. A: The first and second columns compare the pAS positivity results from previous studies (I)[6,8] with the current extensive evaluation (E). The pAS staining results of distal and proximal blocks are also presented. When discordant results were obtained between proximal and distal blocks, only mild positive findings (semiquantitative grade 1) were present (i.e., the three subjects P06, P08, and C02). Moreover, pAS positivity was not found in all five sequential slides in two subjects (P02 and C02), among whom the semiquantitative grade was also 1+. B: Representative figures of multifocally distributed and differently rated nerve plexuses in one patient (P07). Red arrows indicate pAS-positive findings. Calibration bars: figures subtitled Negative, Grade 2+, and Grade 3+ = 50 μm; figure subtitled Grade 1+ = 80 μm. pAS, phosphorylated alpha-synuclein.

studies.[7,8] The staining results for this subject confirmed that the current definition of pAS positivity used in this study was more reliable than the previous one because there were no positive findings in this subject, including the 'diffuse staining' pattern.

In conclusion, examination of a large tissue volume increased the sensitivity of detecting AS accumulation in the GI tract. This study implies a fundamental limit of biopsied tissue and highlights the complexity of pathologic progression of synucleinopathy.

Supplementary Materials

The online-only Data Supplement is available with this article at https://doi.org/10.14802/jmd.22042.

Conflicts of Interest

The authors have no financial conflicts of interest.

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Author Contributions

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Supplementary Table 1. Detailed pAS immunostaining results with semi-quantitative grades of extensive tissue (5 slides per block)

Group	ID	Distal block						Proximal block					
		Extensive	Distal 1	Distal 2	Distal 3	Distal 4	Distal 5	Extensive	Proximal 1	Proximal 2	Proximal 3	Proximal 4	Proximal 5
Patient	P01	(+)	3+	3+	2+	2+	1+	(+)	3+	3+	3+	3+	3+
Patient	P02	(+)	0	0	0	1+	0	(+)	1+	1+	1+	1+	0
Patient	P03	(+)	3+	2+	3+	3+	3+	(+)	2+	2+	1+	2+	2+
Patient	P04	(+)	2+	2+	3+	3+	2+	(+)	3+	3+	2+	3+	2+
Patient	P05	(+)	1+	2+	2+	2+	2+	(+)	3+	3+	3+	3+	3+
Patient	P06	(+)	1+	1+	1+	1+	1+	(-)	0	0	0	0	0
Patient	P07	(+)	3+	3+	3+	3+	3+	(+)	3+	3+	3+	3+	3+
Patient	P08	(-)	0	0	0	0	0	(+)	1+	1+	1+	1+	1+
Patient	P09	(-)	0	0	0	0	0	(-)	0	0	0	0	0
Control	C01	(-)	0	0	0	0	0	(-)	0	0	0	0	0
Control	C02	(-)	0	0	0	0	0	(+)	1+	0	1+	0	0
Control	C03	(-)	0	0	0	0	0	(-)	0	0	0	0	0
Control	C04	(-)	0	0	0	0	0	(-)	0	0	0	0	0
Control	C05	(-)	0	0	0	0	0	(-)	0	0	0	0	0

pAS, phosphorylated alpha-synuclein.