의학박사 학위논문

파킨슨병 환자의 위장관의 수술 조직과 생검 조직을 이용한 알파시누클린 침착에 대한 분석

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서울대학교 대학원

의학과 중개의학 전공

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지도교수 김 종 민
이 논문을 의학박사 학위논문으로 제출함

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신 채 원

신 채 원의 박사학위논문을 인준함
2018년 01월

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ABSTRACT

Analysis of the alpha-synuclein accumulation with surgical and biopsy specimens of the gastrointestinal tract in patients with Parkinson's disease

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Background: Alpha-synuclein (AS) accumulation identified by immunohistochemistry (IHC) of gastrointestinal (GI) tract biopsies is considered as a potential pathologic biomarker for Parkinson's disease (PD). The AS accumulation in the GI tract is hypothetically related to the pathogenesis and clinical characteristics of PD. Moreover, AS staining in non-neural structures has been frequently reported, but whether it is truly non-neural AS accumulation or only non-specific finding is not known.

Objectives: We compared AS IHC findings in biopsy specimens and surgically resected full-depth specimens to examine the reliability of GI tract biopsies. We also evaluated the demographic differences of patients who had phosphorylated AS (pAS) (+) and pAS (-) immunostaining in surgical

specimens of the GI tract.

Methods: We included patients with PD who had undergone operation of the GI tract for treatment of tumors. Controls were matched with age at operation, gender, and surgical resection site. If available, Biopsy specimens obtained for clinical diagnoses of tumors within 1 year of operation were included. We assessed the presence of AS accumulation in neural and non-neural structures in immunostained slides by IHC with pAS monoclonal antibody on the sections from both the rostral and caudal resection margins of the radically resected tissues of the surgical specimen and biopsy tissue. We compared pAS positivity in neural and non-neural structures between patients and controls, and within individuals between surgical and biopsy specimens. Patients were further categorized to pAS (+) or (-) group according to the presence of pAS positivity in the neural structures of the surgical specimen. The demographic characteristics and pAS positivity in biopsy tissues were compared between pAS (+) and (-) groups.

Results: A total of 33 patients with PD were categorized into either the stomach (N=12) or colorectal group (N=21). The frequency of pAS positivity in gastric surgical specimens was 58.3% (7/12) and 8.3% (1/12) in the patient and control groups, respectively (p=0.027). The frequency of pAS positivity in colorectal surgical specimens was identical in the patient and control group (23.8% [5/21] in each). Intriguingly, immunostaining results for biopsy specimens were not concordant with those for surgical specimens. There was no significant difference in the frequency of pAS positivity in biopsy specimens between patients and controls (9.1% [2/22] vs 18.2%

[4/22]; p=0.664). The age at operation and symptom onset of PD were younger in pAS (+) patients in the stomach group. There were no differences in other demographics and pAS positivity in biopsy between pAS (+) and pAS (-) patients. In the non-neural staining patterns, the diffuse staining pattern of the gastric mucosal epithelium and the dotted staining pattern of vessel walls were seen in a considerable number of both patients and controls. These patterns were not related to the pAS positivity in neural structures as well as in patients with PD.

Conclusion: The results of our study clearly demonstrate that AS accumulation identified via pAS IHC of GI biopsies is unreliable due to its low positive rates and poor concordance with surgical specimens, AS accumulation in the stomach is more prevalent in younger patients with PD, and non-neural staining patterns are non-specific findings. Our results indicate that future studies investigating AS accumulation in the GI tract should target the stomach, rather than the colon or rectum.

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keywords: Parkinson's disease, alpha-synuclein, immunohistochemistry, gastrointestinal tract, biopsy, surgical specimen, neural staining, non-neural staining

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LIST OF ABBREVIATIONS

AS = Alpha-synuclein;

EMR = endoscopic mucosal resection;

ENS = enteric nervous system;

GI = gastrointestinal;

IHC = immunohistochemistry;

NF = neurofilament;

pAS = phosphorylated alpha-synuclein;

PD = Parkinson's disease;

SNUH = Seoul National University Hospital

INTRODUCTION

The AS aggregation is observed not only in the central nervous system but also in multiple peripheral tissues such as the skin, salivary glands, and GI tract.[1,2] There has been a growing interest in detecting AS accumulation in peripheral tissues using IHC as a possible *in vivo* pathological and prodromal biomarker of PD. Among such peripheral tissues, GI tract biopsies have shown considerable promise as potential biomarkers.

According to Braak's hypothesis, AS aggregation is first observed in the ENS, following which aggregates travel to the dorsal motor nucleus of the vagus nerve—one of the earliest sites in the central nervous system affected by Lewy pathology in patients with PD.[3,4] Autopsy studies have revealed that AS accumulation in the GI tract is more frequent in patients with PD than in healthy controls.[1,5-7] Furthermore, AS pathology has also been identified in tissue biopsied from the stomach[8] or colon[9-15] of patients with PD, even at the prodromal stage.[16-20] Recently, Stokholm et al. reported that the frequency of pAS positivity in needle biopsies or surgical specimens was higher in patients with prodromal PD than in matched controls.[21] The spread of Lewy pathology in the GI tract to the brain was also confirmed in animal model studies.[22,23] Moreover, clinically constipation is one of the earliest non-motor symptoms before the onset of motor symptoms of PD.[24] The AS accumulation in the GI tract was correlated with age, axial symptoms, levodopa responsiveness, and constipation, but not with olfactory

dysfunction.[12,13] These evidences suggest that AS accumulation in the GI tract would be used as a biomarker for the clinical characteristics of patients with PD.

However, a substantial number of studies argue that positive AS IHC staining in GI tissues does not constitute a biomarker of PD. AS accumulation in the GI tract has been observed in neurologically healthy individuals.[25,26] Furthermore, studies have reported that AS staining is age-related,[27] does not discriminate patients with PD from healthy controls,[28-30] and does not have any correlations with clinical characteristics of PD.[28] Moreover, a recent study reported conflicting results depending on the specific AS antibody used.[31]

Surgically resected GI specimens are ideal for pathologic evaluation, as they provide the largest available area of tissue and allow for complete preservation of the full layers of the gut wall. However, as surgical specimens are not available in most cases, biopsy specimens are considered the best candidate on which to perform AS IHC. However, endoscopic biopsies have fundamental limitations. They vary in size and quality, and cover an area much smaller than that of surgical specimens. Most importantly, endoscopic biopsy specimens include only the mucosal and submucosal layers of the GI wall. Previous studies have reported that the myenteric plexus is the most frequently pAS (+) structure, which cannot be accessed via endoscopic biopsy.[1,5,21,25] One study further reported that 45% of AS accumulation occurs in the myenteric plexus, while only about 15%

occurs in the submucosal plexus.[6] Even in an autopsy study utilizing full-depth specimens, Beach et al. reported that pAS positivity was only 37.5 and 5.9% in the stomach and colon, respectively.[1]

The non-neural AS staining has been frequently reported in previous studies,[6,7,16,21,26,31-33] but whether it is truly non-neural AS accumulation or only non-specific finding is not known. Moreover, recent studies showed conflicting results depending on the specific AS antibody used.[31,33] However, no study has been evaluated non-neural staining quantitatively in sizable patients and controls.

Therefore, we hypothesized that endoscopic biopsy may not be sensitive enough to evaluate AS accumulation in the GI tract. To test this hypothesis, we conducted a case-control study to assess the reliability of AS IHC in the GI tract as a pathological *in vivo* biomarker of PD. We aimed to examine whether pathological finding of biopsy specimens is concordant with that of surgically resected full-depth specimens. We also evaluated the demographic differences of patients who had pAS (+) and pAS (-) immunostaining in surgical specimens of the GI tract.

METHODS

Participants

We identified potentially eligible patients via a review of electronic medical records for patients who had diagnosis of both parkinsonism and tumors of the GI tract between 2004 and 2013 at SNUH. Inclusion criteria were as follows: (1) clinical diagnosis established as either PD or PD dementia by a movement disorder specialist; (2) surgically resected specimen of the GI tract available at the pathology bank of SNUH. Exclusion criteria were as follows: (1) diagnosis of Parkinson plus syndrome or secondary parkinsonism; (2) known genetic cause for parkinsonism; (3) chemotherapy or radiotherapy prior to operation. Finally, included patients were classified into either a stomach or colorectal group based on the site of surgical resection. Patients with colorectal specimens were further sub-categorized into either the proximal colon or distal colon/rectum group prior to matching, as patients with ascending or transverse colon cancer usually receive resection of the proximal colon via right hemicolectomy, while patients with sigmoid or rectal cancer receive resection of the distal colon or rectum via anterior resection or other procedures.

A matched control for each patient was identified from the pathology database. Each control was matched with respect to age at operation, gender, surgical resection site, and surgical procedure. Candidates for controls who

had received endoscopic biopsy in routine clinical practice within 1 year of the operation were given priority. Those who had metastatic cancer or had received chemotherapy or radiotherapy prior to surgery were excluded. To ensure that controls did not have parkinsonism or dementia, we reviewed the medical records and included only those individuals without diagnostic codes or descriptions of parkinsonism, dementia, or other neurodegenerative diseases at the time of the most recent follow-up visit (mean±standard deviation: 4.8±2.2 years). A detailed description of the participant selection process is presented in Figure 1-1. The present study was approved by the Institutional Review Board of SNUH (No. H-1409-043-608). A waiver of informed consent met the requirements and was granted.

Surgical and biopsy specimens

All formalin-fixed surgical and biopsy tissues were paraffin-embedded and archived in the pathology bank at SNUH. For each participant, blocks from both the rostral and caudal resection margins of the radically resected tissues that were most distant from the tumor were used to contain adequate samples of normal tissue. Biopsy specimens obtained for clinical diagnoses of tumors within 1 year of operation were included for analysis. We included specimens from EMR as biopsy tissue. EMR is a procedure for the removal of cancerous or other abnormal tissues from the GI tract. Thus, the specimens generated by EMR are much larger than those obtained via diagnostic biopsy, although they do not include the muscular layers of the gut wall. Two serial 4-μm sections were cut from each surgical or biopsy

block for IHC. A total of four sections from the surgical tissue blocks (two from the rostral resection margin and two from the caudal resection margin) and two sections from the biopsy tissue blocks per person were used for pAS and NF IHC.

Immunohistochemistry

The paraffin sections were mounted on a glass slide, de-waxed, rehydrated, and incubated with primary antibodies on automated machines as previously described.[34] A primary antibody to pAS (1/1000 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). IHC of the adjacent, parallel section was performed with an antibody to NF (1/2000 anti-NF monoclonal Ab; DAKO clone 2F11, California, US) using the Ventana BenchMark XT system.

Neuropathological assessment

Immunostained slides were evaluated by one neuropathologist (S.P) and one neurologist (C.S) blinded to clinical information. Discrepancies in opinion were resolved by discussion with other investigators unaware of clinical information. We excluded slides with predominant cancerous infiltration inadequate for evaluation. We defined pAS positivity as the presence of

definite pAS staining in at least one of the nerve fibers or nerve plexus. Immunohistochemical localization within neural structures was confirmed by evaluating NF staining of the adjacent section. Non-neural staining was defined as the presence of pAS staining without adjacent co-localization of NF positive structures. There were substantial number of slides that showed perivascular dots-like staining in the vascular wall and diffuse mucosal epithelial staining,[33] we considered these patterns as non-specific and did not count as pAS positivity because no NF staining was observed in the adjacent section slide in this study.

Statistical analysis

Patients were further categorized to pAS (+) or (-) group according to the presence of pAS positivity in the neural structures of the surgical specimen. Demographic characteristics were compared between patients and controls as well as pAS (+) and (-) groups. Recognized neural and non-neural staining patterns were described by the anatomic location and staining characteristics. We assessed the normality of the demographic data using Kolmogorov-Smirnov tests. When the required assumptions were met, Student's *t*-tests were used for continuous variables, while Pearson's chi-square tests were used for binominal variables. When the assumption was not satisfied, non-parametric statistical analysis was performed. The frequency of pAS positivity in surgical specimens from patients and controls was compared using Fisher's exact tests. Differences in the frequency of pAS positivity in biopsy specimens between participants with pAS (+) and

pAS (-) surgical specimens were evaluated using Fisher's exact tests. All statistical analyses were performed using SPSS version 21.0 (IBM Corporation, New York, US). A two-sided p-value < 0.05 was considered significant.

RESULTS

A total of 33 patients diagnosed with PD and 33 matched controls were included in the present study (Table 1). Twelve patients underwent gastric operation (stomach group), while the remaining 21 underwent colorectal operation (colorectal group). Within the colorectal group, nine patients underwent resection of the proximal colon, while 12 underwent resection of the distal colon or rectum. Detailed surgical diagnoses and procedures are described in Table 2. No significant differences in sex, mean age at operation, mean age at last follow-up, or the number of participants with biopsy specimens were observed between patients and controls.

Table 1. Clinical characteristics at the last follow-up visit

Characteristic[a]	Total			Stomach group			Colorectal group		
	Patients (n = 33)	Controls (n = 33)	*p* Value[a]	Patients (n = 12)	Controls (n = 12)	*p* Value[a]	Patients (n = 21)	Controls (n = 21)	*p* Value[a]
No. of participants with biopsy tissue available, N (%)	22 (66.7)	22 (66.7)	1.000	5 (41.7)	10 (83.3)	0.089	17 (81.0)	12 (57.1)	0.181
Percentage of men, N (%)	23 (69.7)	23 (69.7)	1.000	10 (83.3)	10 (83.3)	1.000	13 (61.9)	13 (61.9)	1.000
Age at last follow-up visit, mean (SD)	73.6 (9.6)	73.3 (9.8)	0.879	75.3 (8.4)	74.3 (8.5)	0.793	72.7 (10.2)	72.7 (10.6)	0.988
Age at operation, mean (SD)	68.7 (10.2)	68.5 (10.0)	0.923	70.0 (8.0)	70.0 (8.0)	1.000	68.0 (11.4)	67.6 (11.1)	0.913
Range of age at operation (min, max)	48 - 88	48 - 87		52 - 82	52 - 82		48 - 88	48 - 87	
Age at PD onset, mean (SD)	64.7 (11.3)			68.2 (9.7)			62.8 (12.1)		
PD disease duration until operation, mean (SD)	4.0 (6.0)			1.8 (4.8)			5.2 (6.3)		
No. of participants who underwent operation before PD onset, N (%)	6 (18.2)			2 (16.7)			4 (19.0)		

[a]Fisher's exact tests were used for the binominal variables and Student's t-tests were used for the continuous variables.

Table 2. Detailed surgical diagnoses and procedures

	Patients (n = 33)	Controls (n = 33)
Surgical diagnosis of stomach specimen, N (%)	12 (36.4)	12 (36.4)
Early gastric cancer	5 (15.2)	10 (30.3)
Advanced gastric cancer	6 (18.2)	2 (6.1)
Esophageal cancer	1 (3.0)	0 (0.0)
Operation procedure for stomach specimen, N (%)		
Total gastrectomy	4 (12.1)	2 (6.1)
Subtotal gastrectomy	6 (18.2)	5 (15.2)
Distal gastrectomy	1 (3.0)	5 (15.2)
Esophagectomy + Total gastrectomy[a]	1 (3.0)	0 (0.0)
Surgical diagnosis of colorectal specimen, N (%)	21 (63.6)	21 (63.6)
Proximal colon		
Ascending colon cancer	9 (27.3)	7 (21.2)
Transverse colon cancer	0 (0.0)	2 (6.1)
Distal colon/rectum		
Sigmoid colon cancer	5 (15.2)	7 (21.2)
Rectal cancer	7 (21.2)	5 (15.2)
Operation procedure for colorectal specimen, N (%)		
Proximal colon		
Right hemicolectomy	4 (12.1)	7 (21.2)
Extended right hemicolectomy	5 (15.2)	2 (6.1)
Distal colon/rectum		
Anterior resection	4 (12.1)	6 (18.2)
Low anterior resection	5 (15.2)	6 (18.2)
Ultralow anterior resection	1 (3.0)	0
Hartmann operation	1 (3.0)	0
Miles operation	1 (3.0)	0

[a]The proximal margin of the specimen was the distal esophagus.

The selection process of tissue blocks for evaluation is summarized in Figure 1. From the patient group, one surgical block from the rostral margin of the stomach was excluded because there was scarcely any normal tissue. In the control group, one surgical block from the rostral margin of the stomach and one from the caudal margin of the proximal colon were missing. Biopsy blocks were available from 28 patients (four EMR specimens) and 23 controls (six EMR specimens). A total of seven biopsy blocks (five from the stomach and one from the rectum in patients, and one from the stomach in controls) were excluded because only tumor tissues were present.

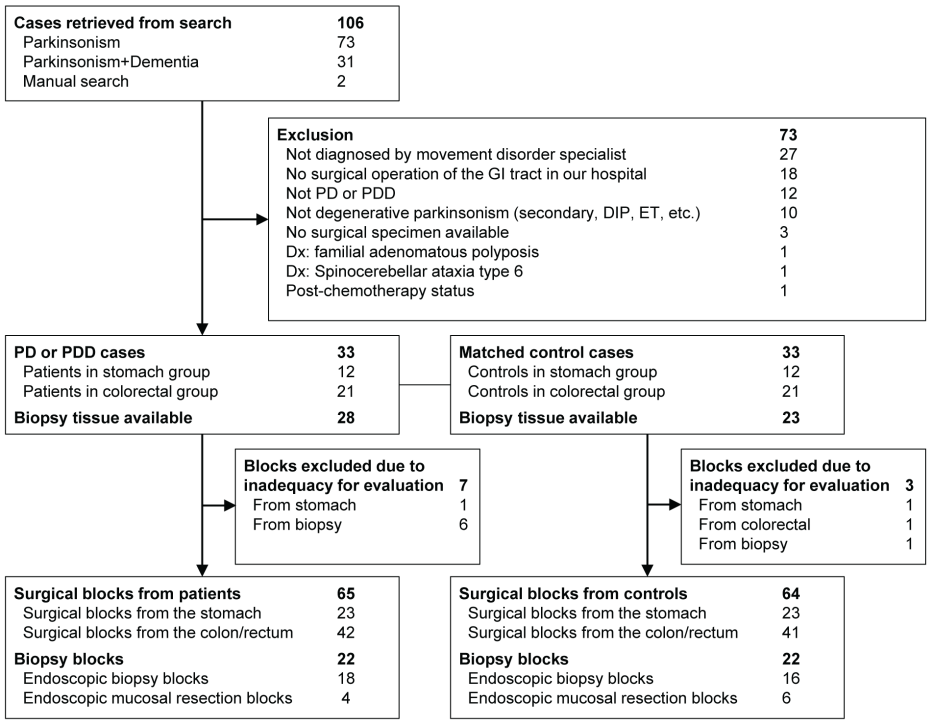

Figure 1. Flow diagram of participant selection process.

Frequency of pAS positivity in the surgical specimens

Overall, 36.4% (12/33) of patients with PD and 18.2% (6/33) of controls had at least one pAS (+) surgical specimen (Figure 2). The frequency of pAS positivity presented in detail in Table 3. The percentage of pAS (+) gastric surgical specimens was significantly higher in patients than matched controls (58.3% [7/12] vs. 8.3% [1/12]; p=0.027); while the percentage of pAS (+) colorectal surgical specimens was identical in both groups (23.8% vs. 23.8% [5/21]; p=1.000).

Table 3. Immunostaining results for phosphorylated alpha-synuclein in patients and controls

Region, N (%)	Surgical specimens			Biopsy specimens		
	Patients (n=33)	Controls (n=33)	*p* Value[a]	Patients (n=22)	Controls (n=22)	*p* Value[a]
Stomach	7/12 (58.3)	1/12 (8.3)	0.027	0/5 (0)	1/10 (10.0)	1.000
Colon/rectum	5/21 (23.8)	5/21 (23.8)	1.000	2/17 (11.8)	3/12 (25.0)	0.622
Ascending/transverse colon	3/9 (33.3)	1/9 (11.1)	0.576	1/8 (12.5)	0/4 (0)	1.000
Distal colon/rectum	2/12 (16.7)	4/12 (33.3)	0.640	1/9 (11.1)	3/8 (37.5)	0.294
Total	12/33 (36.4)	6/33 (18.2)	0.166	2/22 (9.1)	4/22 (18.2)	0.664

[a]Fisher's exact tests were used for statistical analysis.

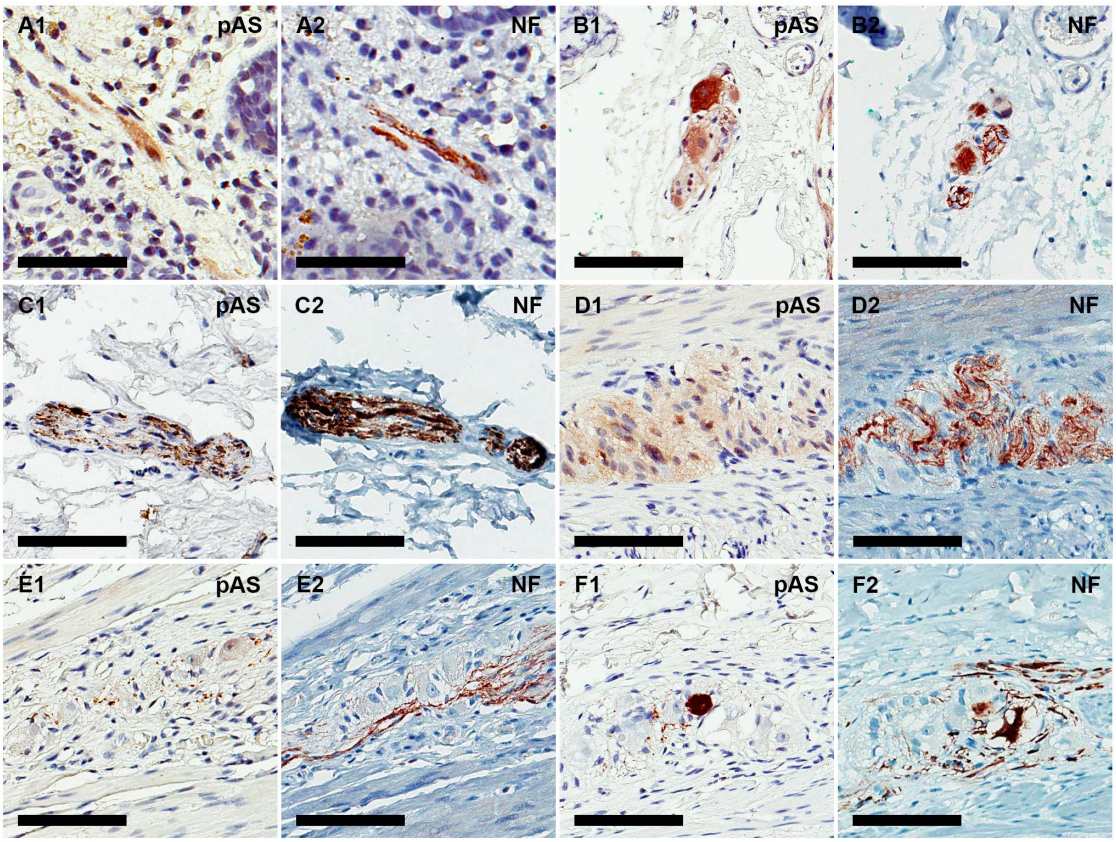

Figure 2. Positive immunostaining patterns within neural structures in the gastrointestinal tract.

Identified patterns of pAS staining are presented alongside NF staining in the adjacent section. **(A1, A2)** Lamina propria, linear pattern. *Calibration bar* 50 µm. **(B1, B2)** Submucosal plexus, diffuse pattern. *Calibration bar* 100 µm. **(C1, C2)** Submucosal plexus, dotted pattern. *Calibration bar* 100 µm. **(D1, D2)** Myenteric plexus, diffuse pattern. *Calibration bar* 100 µm. **(E1, E2)** Myenteric plexus, dotted pattern. *Calibration bar* 100 µm. **(F1, F2)** Myenteric plexus, Lewy body-like inclusion pattern. *Calibration bar* 100 µm.

In the 6 patients who underwent operation before the onset of PD (Table 4), 2 patients (33%) had pAS (+) surgical specimens. The duration from operation to PD onset in 2 pAS (+) patients in prodromal stage was 1 year and 2 years, respectively. In the subgroup analysis excluding these 6 patients, the percentage of pAS (+) gastric surgical specimens was significantly higher in patients than controls (70.0% [7/10] vs. 8.3% [1/12]; p=0.006); while the percentage of pAS (+) colorectal surgical specimens was not different (17.6% [3/17] vs. 23.8% [5/21]; p=0.709).

Table 4. Characteristics of patients who underwent operation before the onset of Parkinson's disease

No.	pAS positivity	Sex	Age at operation (yrs)	Age at PD onset (yrs)	Duration from operation to PD onset (yrs)	Operation group	Surgical diagnosis	Operation procedure
1	0	M	77	85	8	Stomach	Early gastric cancer	Subtotal gastrectomy
2	0	M	69	72	3	Stomach	Esophageal cancer	Esophagectomy + total gastrectomy
3	0	F	65	71	6	Colorectal	Rectal cancer	Low anterior resection
4	1	M	48	50	2	Colorectal	Ascending colon cancer	Right hemicolectomy
5	0	M	60	61	1	Colorectal	Sigmoid colon cancer	Anterior resection
6	1	M	66	67	1	Colorectal	Rectal cancer	Ultralow anterior resection

The distribution of pAS (+) staining in the layers of the GI wall is described in Table 5. Almost all pAS positivity in surgical specimens was observed within the myenteric plexus in both patients (92.3% [12/13]) and controls (100% [7/7]). A rostrocaudal gradient in pAS staining was observed in the patient group, such that the percentage of pAS (+) surgical specimens was highest in the rostral margin of the stomach, but zero in the caudal margin of the distal colon or rectum (Figure 3).

Table 5. Distribution of phosphorylated alpha-synuclein positive staining in the layers of the gastrointestinal wall

Localization, N (%)	Patients (n = 33)				Controls (n = 33)			
	Total surgical specimen (n=65)	Stomach surgical specimen (n=23)	Colorectal surgical specimen (n=42)	Biopsy (n=22)	Total surgical specimen (n=64)	Stomach surgical specimen (n=23)	Colorectal surgical specimen (n=41)	Biopsy (n=22)
Mucosa								
Lamina propria, linear, NF (+)				1 (4.5)				1 (4.5)
Submucosa								
Submucosal plexus, diffuse				1 (4.5)				3 (13.6)
Submucosal plexus, dotted	1 (1.5)	1 (4.3)						
No submucosa				13 (59.1)				10 (45.5)
Muscularis propria				X				X
Myenteric plexus, diffuse	1 (1.5)		1 (2.4)		4 (6.3)	1 (4.3)	3 (7.3)	
Myenteric plexus, dotted	9 (13.6)	4 (17.4)	5 (11.9)		3 (4.7)	1 (4.3)	2 (4.9)	
Myenteric plexus, Lewy body-like inclusion	2 (3.1)	2 (8.7)						

All patterns were described in terms of anatomic localization and the major staining pattern of the structure. When multiple patterns were identified from one slide, all the patterns were counted multiple times.

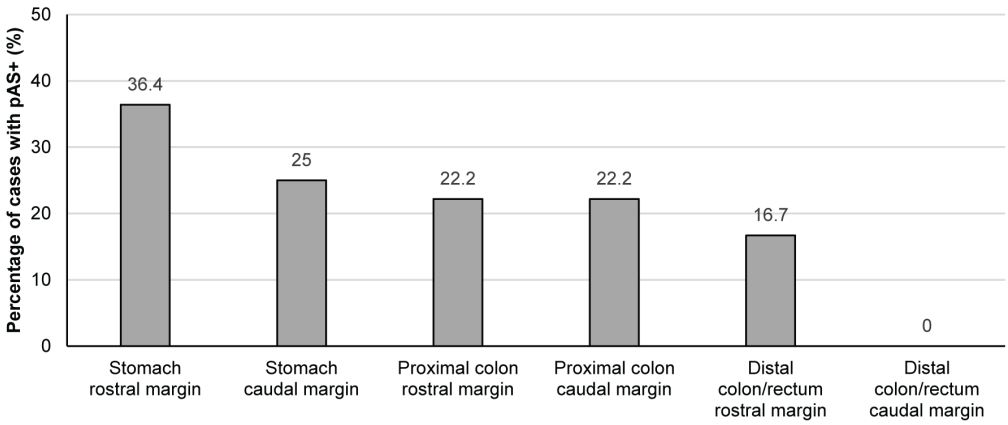

Figure 3. Relative frequency of cases with pAS-positivity arranged from rostral to caudal parts in the gastrointestinal tract of patients.

Frequency of pAS positivity in the biopsy specimens and concordance between surgical and biopsy specimens

The percentage of pAS (+) biopsy specimens (Table 3) did not significantly differ between patients and controls (9.1% [2/22] vs. 18.2% [4/22]; p=0.664). Neither (0%) of the two patients with pAS (+) biopsy specimens had pAS (+) surgical specimens, while only one of the four (25%) controls with pAS (+) biopsy specimens had positively stained surgical specimens.

Correlations of pAS positivity in the surgical specimen with demographics and pAS positivity in biopsy

The age at operation and symptom onset of PD were younger in PD patients with pAS (+) surgical specimens in the stomach group (Table 6). This tendency was also present in colorectal group although it was not statistically significant. There was no difference in duration from symptom onset to surgical operation between pAS (+) and pAS (-) patients. The frequency of pAS positivity was highest (43.8%) in early PD patients (0-5 years after onset of PD symptoms and gradually decreased in longer duration of PD (Figure 4). There was no gender difference between pAS (+) vs pAS (-) patients. In control group, there were no differences in the percentage of men, age at operation between pAS (+) vs pAS (-) subjects.

Table 6. Differences of pAS positivity in biopsy and demographics between patients with or without pAS positivity in the surgical specimen

Characteristic	Patients (n=33)			Stomach group (n=12)			Colorectal group (n=21)		
	pAS (+) (n=12)	pAS (-) (n=21)	*p* Value[b]	pAS (+) (n=7)	pAS (-) (n=5)	*p* Value	pAS (+) (n=5)	pAS (-) (n=16)	*p* Value
pAS positivity in biopsy, N (%)[a]	0/8 (0.0)	2/14 (14.3)	0.515	0/3 (0.0)	0/2 (0.0)		0/5 (0.0)	2/12 (16.7)	1.000
Percentage of men, N (%)	10 (83.3)	13 (61.9)	0.259	6 (85.7)	4 (80.0)	1.000	4 (80.0)	9 (56.3)	0.606
Age at operation, mean (SD)	64.9 (8.1)	70.9 (10.8)	0.106	65.7 (7.0)	76.0 (4.8)	0.015[c]	63.8 (10.2)	69.3 (11.8)	0.457[c]
Age at motor symptom onset of PD, mean (SD)	61.7 (7.2)	66.5 (13.1)	0.251	62.3 (7.0)	76.4 (6.2)	0.015[c]	60.8 (8.3)	63.4 (13.2)	0.534[c]
Duration from motor symptom onset to operation, mean (SD)	3.3 (4.2)	4.4 (6.8)	0.593	3.4 (3.8)	-0.4 (5.5)	0.251[c]	3.0 (5.1)	5.9 (6.6)	0.282[c]
Operation before motor symptom onset, N (%)	2 (16.7)	4 (19.0)	1.000	0 (0.0)	2 (20.0)	0.152	2 (40.0)	2 (12.5)	0.228

All patterns were described as anatomic localization and major staining pattern in the structure. When multiple patterns were recognized in one slide, all the patterns were counted separately. Patterns in bold were considered as phosphorylated alpha-synuclein immunoreactivity in the neural structure.

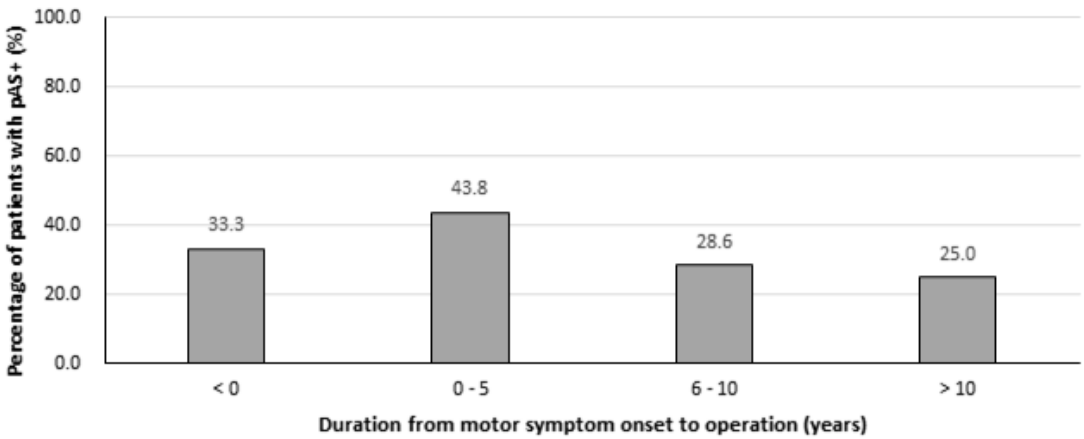

Figure 4. Relative frequency of patients with phosphorylated alpha-synuclein positivity stratified by duration from motor symptom onset of Parkinson disease to surgical operation.

Identified non-neural immunostaining patterns

We identified 4 patterns in non-neural structures (Figure 5). Distribution of the non-neural immunostaining patterns in the gut wall is described in Table 7. Among the staining patterns of non-neural structures, the diffuse staining pattern of the gastric mucosal epithelium (Figure 5A) and the dotted staining pattern of vessel walls (Figure 5D-F) were seen in a considerable number of both patients and controls.

The dotted pattern of vessel walls was observed in almost all patients and controls. We further compared the frequency of the diffuse staining pattern of the gastric mucosal epithelium between patients and controls, and pAS (+) and pAS (-) patients in the stomach group. There were no significant differences between patients (75.0% [9/12]) and controls (50.0% [6/12], p=0.378), and between pAS (+) (85.7% [6/7]) and pAS (-) (60.0% [3/5], p=0.523) patients.

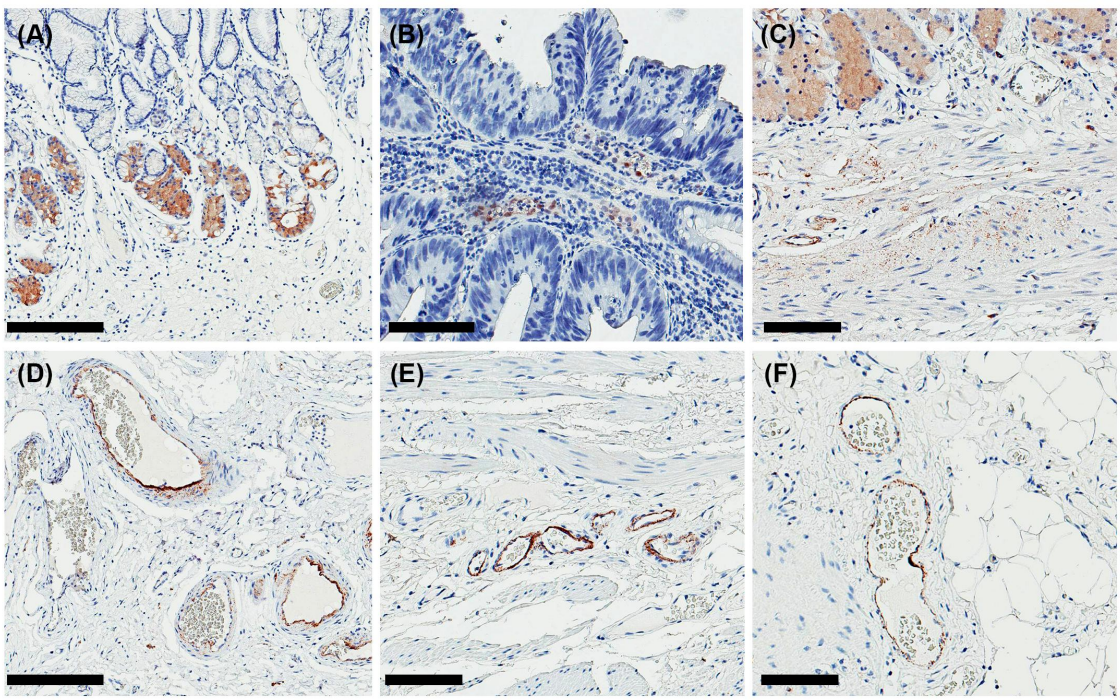

Figure 5. Staining patterns of non-neural structures immunoreactive to phosphorylated alpha-synuclein immunohistochemistry in the gastrointestinal tract.

(A) Mucosal epithelium, diffuse pattern. This pattern was always confined to the basal area of the gastric glands where chief cells exist most frequently. **(B)** Lamina propria, nuclear pattern. This pattern was not considered as immunoreactivity in neural structure because of non-reactivity to the neurofilament immunohistochemistry. **(C)** Muscularis mucosae, dotted pattern. Mucosal epithelial diffuse pattern was also seen below. **(D-F)** Vascular wall, dotted pattern in (D) submucosa, (E) muscularis propria, and (F) serosa. *Calibration bar* (A), (D) = 200 μm, (B), (C), (E), (F) = 100 μm.

Table 7. Detailed non-neural immunostaining patterns of the surgical specimens and biopsies

Pattern, N (%)	Patients (n = 33)				Controls (n = 33)			
	Total surgical specimen (n=65)	Stomach surgical specimen (n=23)	Colorectal surgical specimen (n=42)	Biopsy (n=22)	Total surgical specimen (n=64)	Stomach surgical specimen (n=23)	Colorectal surgical specimen (n=41)	Biopsy (n=22)
Mucosa								
Epithelium, diffuse	10 (15.4)	10 (43.5)		1 (4.5)	7 (10.9)	7 (30.4)		1 (4.5)
Lamina propria, nuclear				1 (4.5)				2 (9.1)
Muscularis mucosae, dotted					1 (1.6)	1 (4.3)		
Submucosa								
Vessel wall, dotted	62 (95.4)	23 (100)	39 (92.9)	1 (4.5)	60 (93.8)	22 (95.6)	38 (92.7)	6 (27.3)
No submucosa				13 (59.1)				10 (45.5)
Muscularis propria				X				X
Vessel wall, dotted	13 (20.0)	7 (30.4)	6 (14.3)		13 (20.3)	4 (17.4)	9 (22.0)	
Serosa				X				X
Vessel wall, dotted	54 (83.1)	19 (82.6)	35 (83.3)		55 (85.9)	22 (95.7)	33 (78.6)	

All patterns were described as anatomic localization and major staining pattern in the structure. When multiple patterns were recognized in one slide, all the patterns were counted separately. Patterns in bold were considered as phosphorylated alpha-synuclein immunoreactivity in the neural structure.

DISCUSSION

Issues inherent in using positive pAS IHC staining of GI tissue biopsies as a pathologic biomarker of PD

In the present study, we aimed to assess the reliability of pAS IHC in the GI tract as a pathological *in vivo* biomarker of PD. Importantly, we observed a low frequency of pAS positivity in the surgical specimens from patients with (overall: 36.4%; stomach group: 58.3%; colon group: 16.7%), in consistent with the low rates of pAS-positivity observed in an autopsy study by Beach et al.[1] As previously mentioned, biopsied GI tissues have fundamental limitations when compared with full-depth surgically resected tissues. In the present study, the mean area of examined tissues was only about 17 mm^2 for conventional endoscopic mucosal biopsies and 105 mm^2 for EMR specimens, though the mean area of surgical specimens was approximately 229 mm^2. Almost all pAS positivity in surgical specimens was observed in the myenteric plexus, in accordance with the findings of previous studies.[1,5,6,21,25] Taken together, these findings confirm that the frequency of pAS positivity in biopsies is low.

Although we included EMR specimens, rates of pAS positivity in GI biopsy tissues were low (9.1% in patients and 18.2% in controls) and failed to discriminate between patients and controls (Table 2). Some early studies including small numbers of patients with PD reported relatively high pAS (+) rates in the biopsy tissues.[9,13] However, this finding was not

substantiated in the larger-scale studies that followed.[16,28] The results of this study question the sensitivity and reliability of conventional pAS IHC of GI biopsies.

In the patient group, all patients with pAS (+) biopsy tissues had pAS (-) surgical specimens. An explanation for this discrepancy is that biopsy tissues may have pAS (+) neural structures in areas not covered by surgically resected tissues. Thus, even two tissue blocks from each surgical specimen may not have covered an area sufficiently large for evaluation, further implying that pAS positivity in GI specimens is not reliable biomarker of PD. An alternative explanation for the discordance rests on the fact that endoscopic biopsies in this study, which were performed as part of routine clinical practice, specifically targeted tumor tissues. The location of the biopsied tissues is closer to abnormal, cancerous tissues than the rostral and caudal resection margins of the surgical specimens, which may have affected pAS accumulation and the immunostaining results of the biopsy specimens. Further large scale studies specifically addressing the concordance of pAS IHC between biopsy and surgical specimens are necessary to reach a definitive conclusion.

The colon and rectum are not suitable anatomic sites for performing pAS IHC

The same frequency of pAS positivity in the colorectal surgical specimens of both patients and controls indicates that the colon and rectum are not appropriate sites on which to perform pAS IHC. Previous studies including

the most recent review article[2] have focused on the use of colorectal biopsies because of their accessibility in clinical practice.[9-14] However, studies have reported the futility of positive pAS staining in colon biopsies as a biomarker.[28,30] Moreover, a study by Visanji et al., which employed paraffin-embedded tissue blot immunoassay to detect aggregated AS, reported no specificity of colonic mucosal biopsies.[29] Another study of colorectal surgical specimens from neurologically healthy individuals demonstrated that pAS positivity increased with age.[27] Our results, in accordance with those of the previous studies, corroborate the idea that pAS accumulation in the colorectal area may not be pathological. Rather, the stomach or esophagus would be the appropriate site for evaluation of AS accumulation in the GI tract.[1,8,35]

A recent study of Beach et al. tried to find out an appropriate staining method for colorectal surgical specimens.[33] In that study, authors found one IHC method with striking 100% diagnostic accuracy by the two most accurate raters in discrimination of 5 patients with PD and 5 controls. The primary antibody, epitope exposure, and signal development in that study were different from the methods of our study, hence study-to-study comparison was not feasible. However, early studies that included small numbers of patients already showed promising accuracy,[3,14] which have not been replicated in subsequent studies that included larger numbers of patients with PD.[16,21,28-30] Therefore, their results should be confirmed in the larger population of patients with PD for generalization.

AS accumulation in the stomach is related to earlier onset

of motor symptoms of PD

In the results of comparison in pAS (+) and pAS (-) patients, motor symptom onset was significantly lower in pAS (+) patients only in the stomach group. This result substantiates that the AS accumulation in the stomach is more pathologic than in colon because the AS accumulation in the GI tract can be present in neurologically normal subjects,[25,26] and age-related.[27] Large-scale validation studies showed that the Braak's pathologic classification was not applicable in 17-43% of autopsied brain with Lewy body pathology.[35,36] Moreover, recently published 4th consensus diagnostic criteria for dementia with Lewy bodies added two pathology categories: amygdala-predominant and olfactory bulb only, which are out of the entero-central pathway of pathologic progression in the Braak's hypothesis.[37] Therefore, results of pAS (+) and (-) patients in the stomach group may reflect different pathologic progression, although this should be proven in larger-scale clinico-pathologic studies.

Non-neural staining of pAS IHC is non-specific finding

Of non-neural staining patterns found in this study, diffuse pattern of the gastric mucosal epithelium (Figure 2A) and dotted pattern of vessel walls (Figure 2D-F) were identified considerably in patients and controls compatible with previous studies.[6,7,16,21,26,31-33] Previous studies considered these patterns as non-specific without evidences. The diffuse staining pattern of the gastric mucosal epithelium and dotted pattern of vessel walls were

corresponded to the epithelial nuclear pattern and perivascular/vascular pattern, respectively, in the multicenter study to select the most appropriate staining method for the GI tract biopsy.[31] In that study, both two patterns did not show good accuracy for prediction of PD in biopsy tissue. Our study further confirmed that non-neural staining patterns had no relationship with not only PD patients but also presence of AS accumulation in neural structures. We recommend using IHC with neural markers to confirm the localization of pAS staining in neural structures.

CONCLUSION

The results of our study clearly demonstrate that AS accumulation identified via pAS IHC of GI biopsies is unreliable due to its low positive rates and poor concordance with surgical specimens, AS accumulation in the stomach is more prevalent in younger patients with PD, and non-neural staining patterns are non-specific findings. Our results indicate that future studies investigating AS accumulation in the GI tract should target the stomach, rather than the colon or rectum.

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국문 초록

배경: 위장관 조직 생검의 면역조직화학검사로 확인할 수 있는 알파시누클린 단백질의 침착은 파킨슨병의 병리학적 바이오마커로 가능성이 높다고 각광받고 있다. 위장관에서의 알파시누클린 축적은 파킨슨병의 병인 및 임상적 특징과 연관이 있다는 가설이 제시되고 있다. 또한, 비신경 구조물에서 알파시누클린 염색이 빈번하게 보고되고 있지만, 실제로 비신경 구조물의 알파시누클린 침착인지 비특이적 소견인지는 알려져 있지 않다.

목적: 본 연구에서는 위장관 벽의 전층을 확인할 수 있는 수술 조직과 생검 조직에서 면역조직화학검사 결과를 비교하여 위장관 조직 생검의 신뢰성을 조사하고자 한다. 또한 위장관 수술 조직에서 인산화 알파시누클린 면역염색 여부에 따른 환자의 인구학적 차이를 평가하고자 한다.

방법: 위장관 종양 치료를 위해 수술을 받은 파킨슨병 환자들이 본 연구의 대상이 되었다. 대조군은 수술 시 연령, 성별, 수술 절제 부위가 일치하여 선별하였다. 수술한 날짜 기준 1년 이내의 진단적 목적으로 얻은 생검 조직이 있을 경우 포함시켰다. 우리는 수술 조직의 근위부와 원위부 절제 경계의 정상 조직과 생검 조직에서 병리 슬라이드를 만들어 인산화 알파시누클린 면역조직화학검사를 실시하였다. 염색한 슬라이드에서 신경 구조물과 비신경 구조물의 알파시누클린 침착 여부를 평가하였다. 이후 환자와 대조군에서 수술 조직과 생검 조직의 침착 여부를 비교하였다. 환자군은 수술 조직의 인산화 알파시누클린 양성 여부에 따라

인산화 알파시누클린 양성군과 음성군으로 분류되었다. 이후 양 군 간의 인구학적 특성과 생검 조직의 알파시누클린 침착 여부를 비교하였다.

결과: 총 33 명의 파킨슨병 환자가 위장군 (N = 12) 과 결장-직장군 (N = 21)으로 분류되었다. 위 수술 조직에서 인산화 알파시누클린 양성률은 환자군과 대조군에서 각각 58.3% (7/12)와 8.3 % (1/12)이었다 (p = 0.027). 결장-직장의 수술 조직에서 인산화 알파시누클린 양성률의 빈도는 환자군과 대조군에서 동일했다 (각각 23.8 % [5/21]). 흥미롭게도 생검 조직에 대한 면역 염색 결과는 수술 조직의 결과와 일치하지 않았다. 환자와 대조군 간의 생검 조직에서 인산화 알파시누클린 양성 빈도에는 유의미한 차이가 없었다 (9.1 % [2/22] vs 18.2 % [4/22]; p = 0.664). 파킨슨병의 증상 발현 연령과 수술시 연령은 위장군에서만 인산화 알파시누클린 양성 환자들에서 더 적었다. 인산화 알파시누클린 양성군과 음성군 사이의 생검에서 인산화 알파시누클린 양성률과 다른 인구학 통계 수치는 차이가 없었다. 비신경 구조물 염색 패턴들 중에서 위 점막 상피세포의 퍼짐 염색 패턴과 혈관 벽의 점 염색 패턴은 상당한 수의 환자 및 대조군에서 나타났다. 이 패턴들은 파킨슨병을 가진 환자 및 신경 구조물에서의 인산화 알파시누클린 침착 양성 여부와 관련이 없었다.

결론: 본 연구 결과에 따르면 위장관 조직 생검의 조직 생검의 면역조직화학검사를 통해 확인하는 알파시누클린 침착은 양성률이 낮고 외과 조직과의 일치도가 낮기 때문에 신뢰할 만하지 않았다. 또한 젊은 파킨슨병 환자에서 위의 알파시누클린 침착이 더 많으며, 비신경 구조물의 알파시누클린 염색은 비특이적인 결과였다. 이 결과들은 종합할 때, 위장관에서의 알파시누클린 침착을 연구하는 향후 연구들은 결장이나 직장보다

는 위를 표적으로 해야할 것이다.

주요어: 파킨슨병, 알파 시누클레인, 면역조직화학, 위장관, 생검, 수술 조직, 신경 염색, 비신경 염색

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