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Submandibular gland is a suitable site for alpha synuclein pathology in Parkinson disease

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ABSTRACT

Objective: To validate the role of α-synuclein (AS) pathology in submandibular gland (SMG) as a biomarker for Parkinson disease (PD).

Methods: We performed ultrasonography (USG) guided core needle biopsy of SMG in PD patients and procured SMG biopsy tissues or surgical excision specimens from non-PD patients as controls. Then, we compared AS deposition in the SMG tissues between the PD patients and the controls. We recruited 16 PD patients in this study. In each individual, two core needle biopsy tissues were obtained from the left submandibular gland under USG guidance. Fourteen sex and age-matched controls who did not have PD and dementia but received a core needle biopsy or surgical resection of the SMG due to SMG diseases were procured from the pathology archive. Biopsy tissues and surgical specimens were immuno-stained with serine 129 phosphorylated AS (pAS) antibody for microscopic examination. pAS deposition in neural structures such as ganglion cells and neurites was considered as positive.

Results: No serious complication occurred during and after the SMG biopsy. We found glandular parenchyma and neural structures in all biopsied SMG tissues from the patients and the controls. Nine out of 16 PD patients (56.2%) were positive for pAS staining, while none of the controls were positive (0%).

Conclusions: SMG core needle biopsy can reliably and safely obtain sufficient glandular parenchyma and neural structures to evaluate the α-synuclein pathology. AS pathology in SMG has high specificity and good sensitivity as a biomarker for PD.

1. Introduction

The gold standard for diagnosing Parkinson disease (PD) is to confirm the loss of dopaminergic neurons and Lewy body pathology in the substantia nigra [1]. For practical reasons, diagnosis of Parkinson disease is made based on clinical features, but previous studies have reported rather low diagnostic accuracy for PD when compared with the autopsy [2–4]. However, from comprehensive autopsy studies, Lewy type α-synuclein (AS) deposition was found not only in the central nervous system but also in the peripheral nervous system of multiple organs including the endocrine glands, colon, stomach and skin in PD patients [5,6]. Thus, clinico-pathologic confirmation through peripheral nervous tissue examination is being extensively studied.

A previous autopsy study revealed a rostrocaudal gradient of Lewy body pathology in the GI tract of PD patients [5]. Of note is that SMG was found to express the highest percentage of AS deposition among the digestive tract in patients with PD [5]. Further autopsy studies have shown promising results with very high sensitivity (100%) and specificity (100%) for AS deposition in the SMG of PD patients [7,8]. Because access to the SMG tissue is feasible and safe with a core needle biopsy, there have been attempts to confirm the AS pathology of the SMG in living PD patients. Recent studies using SMG biopsy in living PD patients showed good but varying sensitivities (67–75%) and specificities (78%–100%) [9–11]. Of note is that these studies in living subjects suffered from having only a modest yield (47–80%) of glandular tissue from the biopsy raising concern over the efficiency of this method as a biomarker for PD.

Therefore, we performed USG guided core needle biopsy of the SMG

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Table 1
Clinical characteristics of the PD patients and controls.

	Parkinson disease	Controls	p-value
Number	16	14	
Age at biosy	56.6 (8.22)	61.7 (9.17)	0.136
Sex (M/F)	6/10	4/10	0.268
Age of PD onset	51.1 (9.33)	NA	–
MMSE	28.31 (2.15)	NA	–
LEDD	817.50 (414.62)	NA	–
H & Y stage		NA	–
1–1.5	7	NA	–
2–2.5	9	NA	–

AbbreviationsMMSE = Mini-mental status examination; HY = Hoehn & Yahr (HY) stage; LEDD = Levodopa equivalent daily doses; Digits are presented as mean (standard deviation).

in PD patients and procured core needle biopsies of the SMG or excisional surgical specimens from age and sex matched non-PD controls from the pathologic archive at SNUH. We compared the AS deposition of the PD patients and controls to validate the sensitivity and specificity of the AS pathology in the SMG as a biomarker for PD.

2. Methods

2.1. Subjects

The study was performed at the Seoul National University Hospital Movement Disorder Clinic between January and September of 2014 after approval by the Institutional Review Board of Seoul National University Hospital. Patients with PD were consecutively and non-selectively recruited during their routine visit to our outpatient clinic. Patients with severe dementia, anticoagulation and known SMG diseases were excluded. Clinical information including age, gender, age at onset, disease duration, Mini-mental status examination (MMSE), Hoehn & Yahr (HY) stage and Levodopa equivalent daily dose (LEDD) were obtained. We procured SMG tissues from age and sex-matched controls (Table 1) from the pathologic archive of Seoul National University Hospital. A review of medical records was done to make sure that controls were free of any neurological disease including PD and dementia. Biopsied tissues of controls were selected from those performed between January and November of 2013.

2.2. Tissues

After signing the informed consent, PD patients underwent a USG guided core needle biopsy (CNB) of the SMG by a radiologist (T.J.Y. with over 10 years of experience in performing CNB). We obtained 2 SMG tissues from the left SMG from each patient. A high-resolution USG machine with a 10–12 MHz linear transducer (AixPlorer, Supersonic Imagine, Aix en Provence, France) was used for the core needle biopsy (CNB). CNB procedures were performed after administration of subcutaneous and local anesthesia with 1% lidocaine. In the CNB procedures, the operator attempted to acquire specimens from the center of the SMG using a disposable 18-gauge, double-action, spring-activated needle (1.1 cm excursion; TSK Ace-cut, Create Medic, Yokohama, Japan) up to three times. All tissue cores were placed immediately in 10% buffered formalin solution. All procedures were continuously monitored with real-time USG to evaluate complications such as the development of a hematoma or parenchymal edema. Each patient was observed with local compression of the biopsy site for 20–30 min. In the controls, SMG core needle biopsy tissues (n = 6) were also obtained with an 18G needle under USG guidance, and surgical specimens (n = 8) were obtained from the excised whole SMG.

2.3. Tissue processing

Tissues were formalin-fixed and paraffin embedded. 4 μm sections from the tissue blocks were immunostained for pAS and neurofilament (NF). We screened 4–11 serial sections (4 μm) with the pAS antibody for each patient. In patients who were negative on the initial screening, we further sectioned all the remaining tissue to minimize the false negative rate. Patient 7, 8 and 9 (Table 2) were found to be positive after the additional screening. In the surgical specimens (n = 8) from the controls, a single section was immuno-stained with NF and another with pAS (Fig. 1I). In the controls, we avoided sections containing underlying pathology and examined sections with normal tissue. Total scanned areas of the sections from the patients and controls are described in Table 2 and Supplementary Table 1.

2.4. Immunohistochemistry (IHC)

The paraffin sections were mounted on a glass slide, de-waxed,

Table 2
Clinical characteristics and pathologic results of the PD patients.

Patient number	Age	Sex	Disease Duration[a]	Disease Duration[b]	MMSE	HY	LEDD	pAS	Number of sections	Total scanned area of tissue (mm2)
1	57	F	6	9	27	2.5	500	+	5	23.82
2	69	M	7	10	30	2	600	+	5	20.41
3	58	F	6	9	29	2	1275	+	5	22.38
4	60	M	6	9	29	2	750	+	5	19.69
5	66	F	7	10	29	1.5	750	+	2	7.49
6	66	M	7	10	28	2.5	1695	+	10	51.64
7	62	F	7	10	24	1	350	+	15	31.07
8	40	F	6	9	30	1	700	+	20	34.72
9	43	F	6	9	30	1.5	737.5	+	25	45.42
10	63	M	5	8	29	2.5	1230	–	25	93.82
11	49	F	3	6	30	2	500	–	50	159.09
12	58	F	6	9	28	1.5	1000	–	25	84.55
13	54	F	2	5	30	2	375	–	30	114.86
14	53	M	23	26	27	2.5	1630	–	25	51.54
15	49	F	3	6	23	1.5	600	–	25	25.45
16	58	M	3	6	30	1	1050	–	25	76.61
Total	56.6 (8.22)	M/F (6/10)	6.44 (4.72)	9.44 (4.72)	28.31 (2.15)		858.91 (417.09)			53.91 (41.59)

AbbreviationsMMSE = Mini-mental status examination; HY = Hoehn & Yahr (HY) stage; LEDD = Levodopa equivalent daily doses; pAS = immunoreactive phosphorylated α-synuclein deposition; Digits in row ('total') are presented as mean (standard deviation).

[a] Disease duration until the SMG biopsy.

[b] Disease duration including additional follow up.

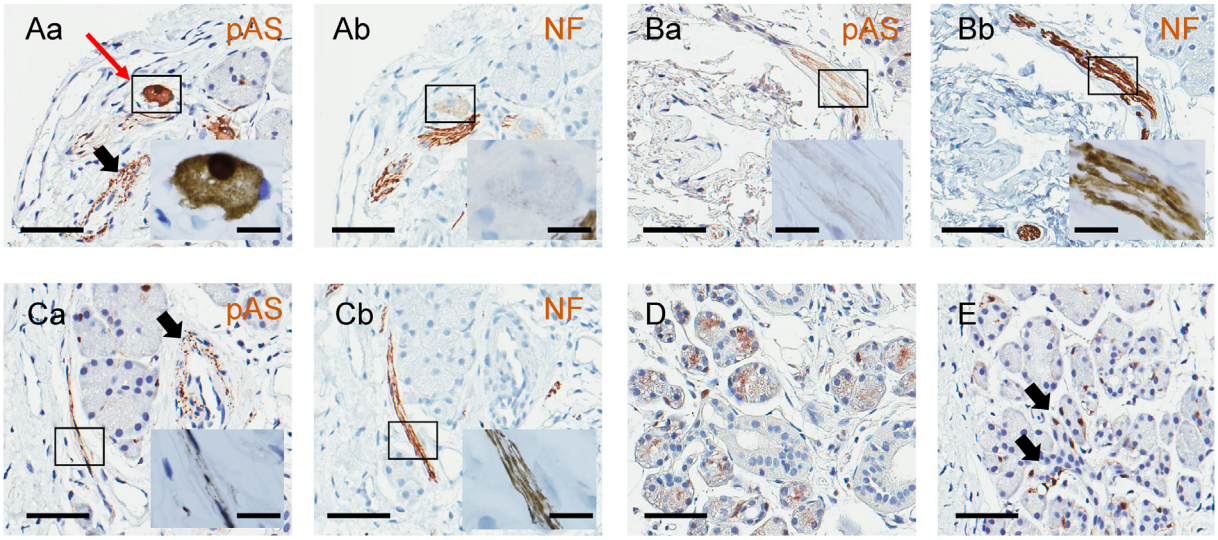

Fig. 1. Immunohistochemical staining of the submandibular gland with pAS in PD patients. The positive immunostain of pAS is presented with the NF staining of the adjacent section (A–B). **A.** Positively stained ganglia cell and neural branches with pAS (Aa) and NF (Ab) in stroma connective tissue. Ganglia cell is annotated with a red arrow, and nonspecific staining of pAS in the media of the blood vessel is annotated with a black arrow (Aa). Higher magnification of area covered by black box is shown as an inserted image. **B.** Neural branch positively stained with pAS (Ba) and NF (Bb) in stroma connective tissue. **C.** Neurites interweaving glandular acinar cells positively stained with pAS (Ca) and NF (Cb). Nonspecific staining of pAS in the media of the blood vessel is annotated with a black arrow (Ca) **D.** Nonspecific staining of pAS in the cytoplasm of acinar cells. **E.** False positive intranuclear staining of pAS. (annotated with a black arrow). Scale bars are 50 μm and 12.5 μm in the main and inserted image, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

rehydrated and incubated with primary antibodies on automated machines as previously described [12]. A primary antibody to pAS (1/1000 anti-pAS at serine 129 monoclonal Ab [EP1536Y]; Abcam ab51253, Cambridge, UK) with the Leica Bond Max (A33030) system was used according to the manufacturer's instructions. Bound antibodies were detected using the Bond polymer Refine Detection system. IHC of the adjacent, parallel section was performed with an antibody for NF (1/2000 anti-NF monoclonal Ab; DAKO clone 2F11, California, US) with the Ventana BenchMark XT system.

2.5. Histological examination

All sections were reviewed by one pathologist (S.H.P) and two neurologists (C.W.S and J.H.S) blinded to the clinical information. All sections were also scanned and reviewed with the Aperio Imagescope (Leica, v12.0) for tissue area calculations and figures. The scanned area of tissue was calculated by summing the area of each section. AS deposition within a neural structure was confirmed by morphological examination and NF staining of the adjacent section (Fig. 1B and C).

2.6. Statistical analysis

Group comparisons were made using the *t*-test for continuous variables and the chi-square test for categorical variables. When the required assumptions for the parametric tests were not satisfied, non-parametric tests such as Fisher exact test were performed. All statistical analyses were performed using the SPSS software (version 21.0, SPSS Inc., Chicago, IL.) with two-sided significance set at 0.05.

3. Results

Sixteen patients with PD were recruited. They had been followed up for more than two years at the time of the SMG biopsy and had 3 years of additional follow-up at the outpatient clinic. All patients maintained their clinical diagnosis as PD with a good levodopa response. In patients with an onset age younger than 40 years (patient 8, 9 and 14 in Table 1), PARK2 was excluded in patient 9 and 14. There were no serious complications such as hematomas or local infections in the patient group after the biopsy. The controls were 14 subjects without PD and dementia who underwent SMG excision (n = 8) or USG guided core needle biopsy (n = 6) because of SMG diseases (supplementary Table 1) between January and November 2014.

There were no significant differences in age and sex between the patients and the controls (Table 1). The mean age ± standard deviation of patients and controls was 56.6 ± 8.22 and 61.7 ± 9.17, respectively. The percentage of males for the patients and controls was 37.5% (6/16) and 28.5% (4/14), respectively.

Glandular parenchyma and neural structures (ganglion cells/neurites) were identified in every patient and control (100%) by morphological examination and NF immunostaining (Fig. 1). Nine of the 16 (56%) patients were pAS positive in the neural structures of the SMG; while none of the 14 controls showed pAS positivity. Examples of positive cases are shown in Fig. 1. Deposition of AS was found in the neural structures such as ganglion cells/neurites in the connective tissue stroma (Fig. 1A and B) or neurites interweaving in the glandular cells (Fig. 1C). Staining in non-neural structures such as the acinar cell cytoplasm (Fig. 1D), nucleus of the acinar cells (Fig. 1E) or media of the blood vessels (Fig. 1Aa and Fig. 1Ca) were also seen in the controls and considered as non-specific [8,9]. There were no demographic or clinical differences between the PD patients with positive and negative staining (Supplementary Table 2).

Because we examined additional sections from patients with negative staining on the initial screening, the total number of sections and scanned areas with the extended examination (Patient No. 7–16) is larger than that found to be positive on the initial screening (*t*-test, $p = 0.006$ and $p = 0.0013$, respectively; supplementary Table 2). The scanned area of the SMG tissue was significantly higher in the controls than in the PD patients (53.91 and 190.01 mm^2 for the PD patients and the controls, respectively; *t*-test, $p = 0.0049$) because excisional surgical specimens were included in the control group.

4. Discussion

In this study, we showed that SMG core needle biopsy is safe and technically feasible to obtain sufficient neural structures to examine AS deposition. To our knowledge, this is the first study to examine pAS

deposition in a sizeable excisional specimen in living controls.

We obtained glandular parenchyma from every patient with the core needle biopsy. Furthermore, we confirmed the neural structures (ganglion cells/neurites) in every patient (100%) by both morphological examination and NF staining. In previous studies [9–11], glandular tissues were not obtained in 20–53% of the participants, which significantly lowered the overall positive rate and raised concern for using this biopsy method to be used as a biomarker study. In fact, the high rate of failure in obtaining glandular parenchyma and neural tissues is considered as a possible limitation of the SMG biopsy as a biomarker for PD [13]. However, our study shows that USG guided biopsy in the hands of an expert rather than relying on palpations [9,10] can have an excellent yield in obtaining glandular SMG tissue for the examination. Moreover, none of the participants experienced serious complications.

Therefore, we showed that SMG core needle biopsy is a safe tool to reliably obtain sufficient glandular parenchyma and neural structures in living individuals. It should be noted that endoscopic biopsies of the stomach or colon are limited in acquiring sufficient neural tissue thus significantly lowering the feasibility and sensitivity of the techniques [5,14].

Our result showed pAS staining in SMG tissue is highly specific (100%). High (100%) specificity was also reported in a previous study [11] in living controls using core needle biopsy. However, Arizona group reported a false positivity of 22% with core needle biopsy [10]. In this study, we included large excisional sections of the SMG in living controls thus reducing the likelihood of false negativity in the controls. Therefore, our result supports the high specificity of pAS deposition in the SMG, which is in line with previous autopsy studies showing a very high specificity using whole SMG sections [7,8].

Regarding sensitivity, our result is low (56.2%) compared with previous studies. Studies from the Arizona group [9,10] showed a sensitivity of 74 and 75% in studies with early PD patients (less than a 5-year disease duration) and late PD patients (more than a 5-year disease duration), respectively. Additionally, a recent report from the Barcelona group [11] showed a 67% positive rate. There may be several reasons for the low sensitivity compared with previous studies. First, there may be concerns regarding the accuracy of the PD diagnosis. A previous prospective pathologic study showed that clinical diagnosis within 5 years of the disease duration was incorrect in 35% of cases for PD and a longer follow-up yielded a better diagnostic rate [15]. Mean disease duration in the pAS negative group was 3.67 years when patient No. 14 with 23 years for the disease duration was excluded. Specifically, four patients (Patient No. 11, 13, 15 and 16, Table 1) in the pAS negative group had disease duration lower than 5 years at the time of the biopsy; thus, an additional clinical observation was required. Therefore, we followed up every PD patient for an additional 3 years at the outpatient clinic after the biopsy, and their diagnoses remained as PD after a mean disease duration of 9.4 years (Table 1). Therefore, we believe the odds of a misdiagnosis of PD is low in our study. Second, there may be an argument that a positive pAS stain may not be observed in patients with low disease duration. The pAS negative group had a lower disease duration compared with the pAS positive group (3.67 years vs. 6.44 years, respectively. $p < 0.001$, t-test) when the pAS negative patient No.14 with 23 years of disease duration was excluded. However, SMG biopsy studies with idiopathic rapid-eye-movement sleep behavior disorder [11] and early PD [10] showed similar sensitivity as that of late PD patients. Furthermore, a recent autopsy study [16] failed to find a correlation between disease duration and the Lewy type synucleinopathy density score of the SMG in PD patients. Overall, the pAS positivity seems to have no correlation with disease duration in clinically developed PD. Third, a recent study [17] with 5 PD patients and 5 controls reported that different staining methods for the primary pAS antibody, epitope exposure and signal development might show varying sensitivity in colon tissue. Our study used the same primary antibody (EP1536Y, abcam) that showed a relatively low sensitivity (Method 3) among the various methods applied in that study; however, other previous SMG biopsy studies [8–10] used a different primary antibody (Method 2) which yielded a higher sensitivity. Therefore, the primary antibody with its low sensitivity may have caused the low pAS positivity in our study. However, the details of the staining method other than the primary antibody are different from our study. Thus, it is premature to conclude the primary antibody as the cause of the low sensitivity.

Clinical trials require accurate diagnosis, which can be challenging especially in the early stage of PD. Supporting diagnosis by examination of peripheral tissue is an attractive and feasible idea based on recent studies. We showed that with the proper technique in the hand of an expert, the core needle biopsy of SMG could yield a sufficient amount of neural tissue to examine the pAS positivity. Furthermore, the pAS positivity in the SMG has shown high sensitivity and specificity in the PD diagnosis among other peripheral tissues obtained from the skin, stomach and colon. Overall, we suggest the submandibular gland as a suitable site for studying AS pathology in PD.

Author's contributions

Study concept and design: JH Shin, B Jeon.

Data acquisition: JH Shin, SH Park, JH Kim, TJ Yun.

Data analysis and interpretation: JH Shin, B Jeon, SH Park, CW Shin, HJ Kim.

Drafting the manuscript: JH Shin, B Jeon.

Revising the manuscript: SH Park, CW Shin, JH Kim, TJ Yun, JH Kim, B Jeon.

Conflicts of interest

Authors report no conflict of interest to this study.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.parkreldis.2018.04.019.

Full financial disclosure (past 12 months)

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