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ORIGINAL ARTICLE

Spine Topographical Distribution of Skin α-Synuclein Deposits in Idiopathic Parkinson Disease

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Abstract

Phosphorylated α-synuclein (p-syn) in skin nerves mainly in the proximal sites is a promising neurodegenerative biomarker for idiopathic Parkinson disease (IPD). However, the p-syn spine distribution particularly in patients with unilateral motor dysfunctions remains undefined. This study aimed to investigate in IPD p-syn differences between left and right cervical spine sites in patients with prevalent unilateral motor symptoms, and cervical and thoracic spine sites in patients with bilateral motor symptoms. We enrolled 28 IPD patients fulfilling clinical diagnostic criteria associated with abnormal nigro-striatal DatScan and cardiac MIBG: 15 with prevalently unilateral motor symptoms demonstrated by DatScan; 13 with bilateral motor symptoms and DatScan abnormalities. Patients underwent skin biopsy searching for intraneural p-syn deposits: skin samples were taken from C7 paravertebral left and right sites in unilateral patients and from cervical (C7) and thoracic (Th12) paravertebral spine regions in bilateral patients. Unilateral patients displayed 20% of abnormal p-syn deposits in the affected motor site, 60% in both sites and 20% only in the non-affected site. P-syn was found in all patients in C7 but in only 62% of patients in Th12. Our data showed that cervical p-syn deposits displayed a uniform distribution between both sides not following the motor dysfunction in unilateral patients, and skin nerve p-syn deposits demonstrated a spine gradient with the cervical site expressing the highest positivity.

Key Words: Diagnostic test assessment, Idiopathic Parkinson disease, Phosphorylated α-synuclein, Skin biopsy, Topography.

INTRODUCTION

The correct diagnosis of idiopathic Parkinson disease (IPD) is important as only a probable level of certainty is currently possible in vivo whereas definite IPD diagnosis requires neuropathologic confirmation (1). In addition, recent clinicopathological surveys have disclosed a clinical error rate of up to 20% (2). Phosphorylated α-synuclein (p-syn) deposits in skin nerves are among the promising neurodegenerative biomarkers for IPD and may help to improve the accuracy of in vivo IPD diagnosis (3–6). Abnormal p-syn deposits in skin nerves may be explored by skin biopsy, a straightforward, inexpensive and minimally invasive technique with minor discomfort for the patient. However, neuritic p-syn inclusions vary widely in IPD according to specific skin sites (4–6), with the highest positivity found in the proximal sites, i.e. spine. Nevertheless, the reported p-syn positivity in the spine differs between the cervical C7 site (100%) (5) and the thoracic Th12 region (35%) (4).

Another important unsolved question concerns p-syn deposits in IPD patients with unilateral motor symptoms. It is not known whether or not p-syn deposits show a preferential side following the motor dysfunctions in these patients. P-syn variability in skin nerves represents an important obstacle for the use of skin biopsy as a diagnostic IPD biomarker. The skin p-syn standardization may help to target the analysis to the most sensitive site. Thus, the specific aims of this study in IPD patients were to (1) ascertain possible differences in p-syn deposits between left and right cervical sites in patients with unilateral motor symptoms; and (2) define the distribution of p-syn deposits along spine skin nerves in patients with bilateral motor dysfunction.

MATERIALS AND METHODS

We studied 28 well-defined IPD patients fulfilling diagnostic criteria of the National Institute of Neurological Disorders and Stroke (1) supported by abnormal nigrostriatal dopamine transporter ligand [123I]ioflupane-DatScan (7) and cardiac uptake of [123-I])-MIBG in the majority of them (8). Table 1 summarizes patients' demographic data and clinical profiles. IPD patients included 15 showing exclusively or prevalently motor symptoms in 1 body site with DatScan demonstrating abnormal findings prevalently in the opposite nigrostriatal site (unilateral IPD),

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TABLE 1. Clinical and Demographic Data of Idiopathic Parkinson Disease (IPD) Patients

Unilateral	Age (Years)	Sex Male:female	Disease Duration (Years)	UPDRS-off	Hoehn and Yahr Stage	Daily L-Dopa (mg)
1	74	m	14	36	3	550
2	77	f	12	37	4	950
3	59	m	7	18	2	500
4	77	f	6	37	4	950
5	65	m	1	14	1	125
6	55	f	7	32	2	400
7	72	f	1	10	0.5	150
8	78	m	1	16	1	150
9	82	m	4	26	1	350
10	68	m	1	12	0.5	150
11	69	m	2.5	19	1	250
12	72	f	2	18	1	250
13	60	f	2.5	29	1	275
14	78	f	3	20	1.5	350
15	53	m	1	15	0.5	125
Mean ± SD	68 ± 9	08:07	3 ± 2	20 ± 8	1 ± 1	310 ± 225
Bilateral						
16	75	m	5	34	2	600
17	78	f	5	35	2	800
18	60	f	10	38	2	1000
19	72	f	2	37	1.5	900
20	64	f	14	42	2.5	800
21	78	m	14	40	3	750
22	68	m	20	53	4	450
23	75	m	15	51	4	750
24	77	m	4	19	2	350
25	72	f	4	29	2	400
26	74	m	3	28	2	400
27	75	m	5	22	2	500
28	65	m	15	53	4	1200
Mean ± SD	71 ± 6	08:05	**10 ± 6***	**37 ± 12*****	**3 ± 1***	**680 ± 282*****

*$p < 0.05$.
***$p < 0.001$ (symmetric vs asymmetric IPD), significant values in bold.

and 13 with bilateral motor symptoms and DatScan abnormalities (bilateral IPD) (Table 2).

The procedures used followed the Helsinki Declaration regarding international clinical research involving human beings. The local Human Ethics Committee approved the study and all subjects gave their written informed consent to the study.

Skin Biopsy

We took 3-mm punch biopsies from the right and left cervical C7 paravertebral area in unilateral patients and from the same cervical C7 and thoracic Th12 paravertebral side (~5 cm from the vertebral apophysis) in bilateral patients. We randomly chose the paravertebral side in bilateral patients: right side in 6 patients and left side in 7 patients. Two skin samples were taken in each site to improve the diagnostic ability to find abnormal synuclein deposits (5, 6). According to previously published procedures (9), we fixed skin samples immediately in cold Zamboni's fixative and kept them at 4 °C overnight.

Using a freezing sliding microtome (HM550, Thermo Scientific, Waltham, MA) we obtained 10-μm-thick sections. Because of previous data showing a likely irregular deposition into the skin innervation in IPD (6) we decided to analyze 4 sections at 50 μm apart for each sample as a representative analysis of the whole skin sample. Skin samples were then double-immunostained overnight with a panel of primary antibodies including rabbit monoclonal p-syn at Ser 129 (p-syn; 1:500, Abcam, Cambridge, UK, catalog number ab-51253) and pan-neuronal marker mouse protein gene product 9.5 (1:750; Abcam, Cambridge, UK, cat. num. ab72911). We then washed the sections and added secondary antibodies for an incubation of 1 hour. We used an anti-rabbit Jackson cyanine dye fluorophores 3.18 (1:200) and anti-mouse Alexa Fluor 488 (1:400; Jackson ImmunoResearch, West Grove, PA) as secondary antibodies. p-Syn staining was rated in each skin site as the percentage of autonomic structures or nerve bundles

TABLE 2. Laboratory Findings in Idiopathic Parkinson Disease (IPD) Patients

	MIBG	Motor side Dysfunction	DatScan	P-Syn Positivity			
Unilateral				C7 Right	Positive Samples	C7 Left	Positive Samples
1	AB	Right	Both > left	+	**1**	0	0
2	AB	Right	Both > right	0	0	+	**1**
3	AB	Left	Both > right	+	**1**	+	**1**
4	AB	Right	Both > left	+	**1**	0	0
5	AB	Right	Both > left	0	0	+	**1**
6	AB	Left	Both > right	0	0	+	**1**
7	ND	Right	Both > left	+	**2**	+	**1**
8	ND	Left	Both > right	+	**2**	+	**2**
9	ND	Left	Both > right	+	**1**	+	**2**
10	AB	Right	Both > left	+	**1**	+	**1**
11	AB	Left	Both > right	+	**1**	+	**1**
12	ND	Left	Both > right	+	**1**	0	0
13	ND	Right	Both > left	+	**2**	+	**1**
14	AB	Left	Both > right	+	**2**	+	**1**
15	AB	Left	Both > right	+	**2**	+	**2**
Bilateral				C7	Positive Samples	Th12	Positive Samples
16	AB	Both	Both	+	**2**	+	**2**
17	AB	Both	Both	+	**1**	0	0
18	AB	Both	Both	+	**2**	0	0
19	AB	Both	Both	+	**1**	0	0
20	AB	Both	Both	+	**1**	+	**1**
21	AB	Both	Both	+	**1**	+	**1**
22	ND	Both	Both	+	**1**	0	0
23	AB	Both	Both	+	**2**	+	**2**
24	AB	Both	Both	+	**2**	+	**1**
25	AB	Both	Both	+	**2**	+	**2**
26	ND	Both	Both	+	**2**	+	**2**
27	AB	Both	Both	+	**2**	+	**1**
28	AB	Both	Both	+	**2**	0	0

Phosphorylated α-synuclein = p-syn; ND = not done; MIBG = cardiac sympathetic innervation; DatScan = nigro-striatal innervation.

showing a positive staining at high magnification (×40). To be classified as positive the patient had to show at least 1 single nerve protein gene product-positive fiber showing a p-syn co-staining (for further details see Donadio et al, 2013 (10), 2014 (5) and 2016 (6)).

Statistical Analysis

We performed statistical analyses using SPSS 15.0 for Windows. We used the Mann–Whitney test

to compare clinical and demographic data between: (1) unilateral versus bilateral IPD patients; and (2) IPD subgroups (unilateral vs bilateral cervical p-syn distribution in unilateral IPD patients and diffuse vs localized spine p-syn distribution in bilateral IPD patients). $p < 0.05$ was considered significant.

RESULTS

Unilateral IPD Patients

We found cervical p-syn deposits in all patients when both nearby skin samples were taken into account: 3 patients (20%) showed abnormal deposits only in the affected motor side, 9 patients (60%) displayed p-syn in both sides, whereas 3 patients (20%) presented abnormal p-syn only in the non-affected side (Figs. 1, 2A). In 6 patients (40%) only 1 cervical skin sample was positive for p-syn, whereas 2 samples were positive in 3 patients (20%), 3 samples in 4 patients (27%), and all 4 skin samples in only 2 patients (13%) (Table 2). Abnormal p-syn deposits were mainly found around arterioles (39 of 178 analyzed: 22%) usually deeper in the derma and inside small dermal nerve bundles (37 of 263 analyzed: 14%), and less in the nerve fibers around sweat gland (4 of 77 analyzed: 5%) and muscle arrector pilorum (2 of 72 analyzed: 3%). The number of skin structures positive for p-syn did not differ between patients with unilateral and bilateral positivity ($p > 0.9$). Similarly, there was no difference in disease duration, UDPRS-off and L-dopa dosage between the 2 groups ($p > 0.09$).

Bilateral IPD Patients

As expected, bilateral patients showed a higher disease duration, UPDRS-off score, Hoehn and Yahr stage, and L-dopa dosage than unilateral patients (Table 1). Considering

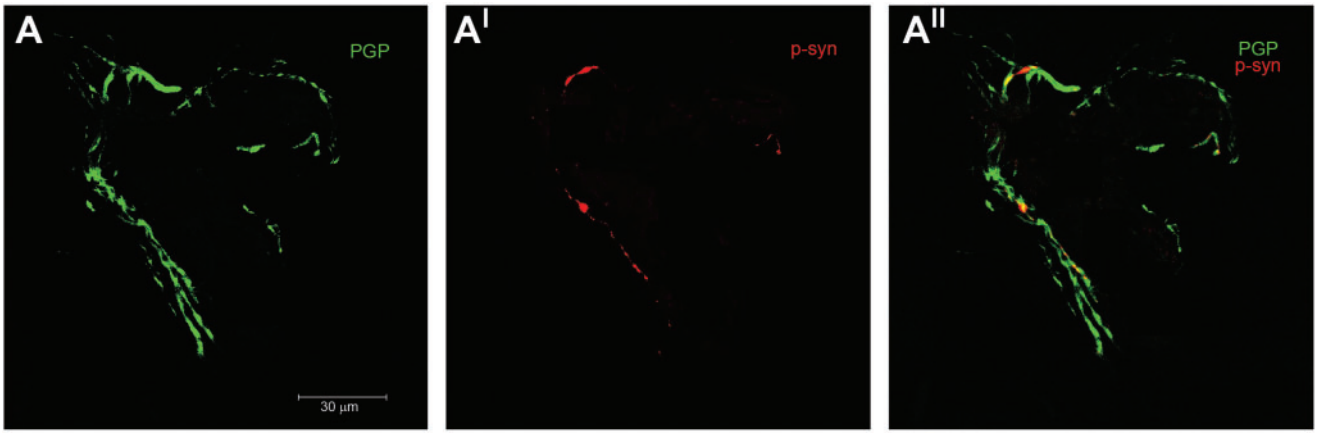

FIGURE 1. Phosphorylated α-synuclein deposition in skin nerves. Confocal microscope (×40) study of intraneural phosphorylated α-synuclein deposits around arterioles in the deep dermis in a bilateral IPD patient. Arterioles usually showed a large network of protein gene product-positive nerve fibers **(A)**. Few of these fibers showed positive phosphorylated α-synuclein **(A^I)** as neuritic inclusions demonstrated by the merged image **(A^{II}).**

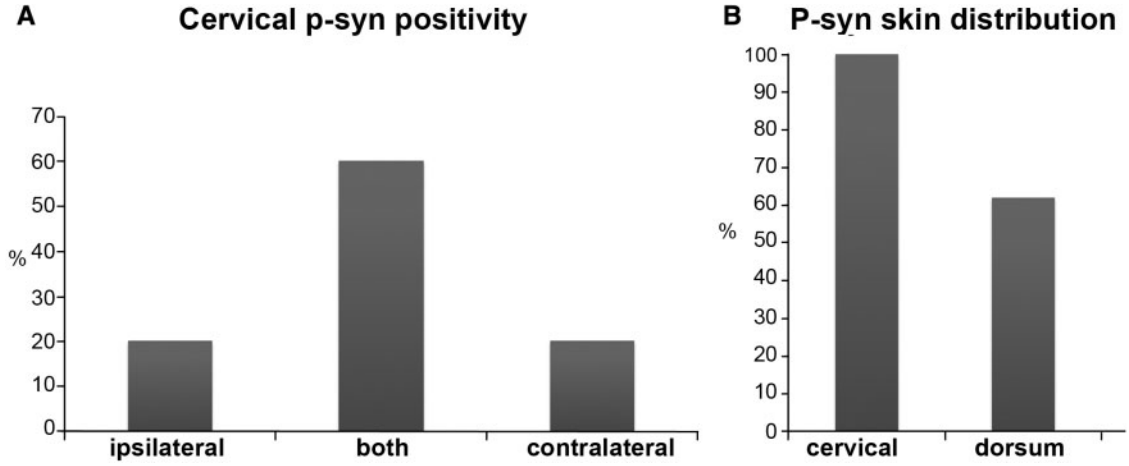

FIGURE 2. Distribution of phosphorylated α-synuclein among different spine skin sites in IPD patients. Percentage of 2 nearby p-syn positive skin samples in unilateral and bilateral IPD patients. **(A)** P-syn positivity between right and left cervical sites in unilateral patients. P-syn deposits displayed a uniform distribution between ipsilateral and contralateral affected motor sides. These data suggest that abnormal p-syn deposits may not follow the motor side dysfunction. **(B)** Positive p-syn staining among cervical and thoracic paravertebral skin sites in bilateral patients. The distribution of samples positive for abnormal p-syn deposits demonstrated a spine gradient with all patients positive in the cervical site but 62% in Th12, supporting a spread of p-syn from brainstem centers.

both nearby skin samples, we found p-syn deposits in all patients with the highest p-syn positivity in the cervical site (all patients positive for p-syn deposits), whereas thoracic region showed a p-syn positivity in 62% of patients (Fig. 2B). In 3 patients (23%) only 1 cervical skin sample was positive for p-syn, whereas 2 samples were positive in 4 patients (31%), 3 samples in 2 patients (15%), and all 4 skin samples in 4 patients (31%) (Table 2). We found p-syn mainly around arterioles (47 of 194 analyzed: 24%) usually deeper in the dermis and inside small dermal nerve bundles (51 of 299 analyzed: 17%), and less in the nerve fibers around sweat gland (8 of 95 analyzed: 8%) and muscle arrector pilorum (1 of 70 analyzed: 1%). There were no differences in disease duration, UDPRS-off and L-dopa dosage between patients with diffuse (both C7 and Th12) or localized (only C7) spine p-syn deposits ($p > 0.1$).

DISCUSSION

Our main results in IPD patients are that (1) cervical p-syn deposits displayed a uniform distribution between both sides not following the motor dysfunction in unilateral patients; and (2) skin nerve p-syn deposits demonstrated a spine gradient with the cervical site expressing the highest positivity. These data may contribute to the standardization of the use of skin biopsy for the in vivo diagnosis of IPD, helping to define the optimal site for searching for skin nerve p-syn deposits.

P-Syn Cervical Side in Patients With Unilateral Motor Symptoms

To define a preferential side spread of p-syn in peripheral nerves we analyzed right and left cervical sides in IPD patients with prevalent unilateral motor symptoms confirmed by nigrostriatal DatScan. To this end, the cervical skin area was chosen because of the highest p-syn positivity usually found in this site (5, 6). Our data showed that cervical p-syn deposits were evenly distributed between affected and non-affected motor sides suggesting that skin nerves p-syn could be independent from the motor and nigrostriatal damage. This finding supported the hypothesis that abnormal p-syn deposits likely spreading from brain to peripheral nerves do not follow a specific neural pathway. Combined DatScan and MIBG scanning in the same patients supported the independence between motor (i.e. nigrostriatal) dysfunction and peripheral autonomic (i.e. cardiac innervation) damage in IPD (11). Thus, the extent of nigrostriatal dopaminergic denervation can be unrelated to the extent of autonomic peripheral involvement and cardiac noradrenergic denervation (12), and patients without motor dysfunctions may show cardiac denervation (13) or, vice versa, other patients may present with cardiac denervation only years after the onset of motor dysfunctions (14). These conclusions are in line with our findings showing no differences of UPDRS-off or disease duration between patients with localized or diffuse p-syn deposits in skin nerves. In fact, patients with long disease duration (and high UPDRS-off) may display localized p-syn deposits (patients 1 and 2 in Table 1), whereas patients with very short disease duration (and low UPDRS-off) presented with bilateral p-syn deposits (patients 7, 8 and 10 in Table 1). The wide diffusion of p-syn deposits may be explained by several α-synuclein seeding mechanisms involved in transferring the α-synuclein pathology along the nervous system (15) with cell-to-cell transfer (16, 17), axonal transport in both directions in the central nervous system (18), or possibly by secretion in extracellular biological fluids including the CSF and blood plasma (19). However, our findings point to the need to perform skin biopsy on both cervical sides when searching for p-syn in patients with prevalent unilateral motor dysfunctions. This is particularly important in the early IPD disease stages when patients often present with unilateral motor dysfunctions (1) likely expressing confined pathological damage (20). In contrast, widespread motor symptoms with diffuse underlying pathological damage and nigrostriatal DatScan abnormalities, as found in bilateral patients, imply that selection of the cervical side to search for p-syn deposits is not relevant. This was suggested by the positivity of p-syn deposits in all patients belonging to the bilateral group in whom the cervical side was randomly chosen.

Spine P-Syn Gradient in Patients With Bilateral Motor Symptoms

Our data showed a rostrocaudal gradient of p-syn staining in the spine from cervical to thoracic paravertebral regions. These data are similar to autopsy studies describing a craniocaudal gradient of α-synuclein pathology burden involving the paravertebral sympathetic chain (21, 22). This finding suggests a p-syn spread from brainstem centers to peripheral nerves. This conclusion was supported by recent studies demonstrating that abnormal α-synuclein may spread throughout the central and peripheral nervous system by means of a prion-like mechanism (15, 23–27). However, our findings may also have important practical implications in the search for p-syn deposits in skin nerves in IPD, suggesting that the site of analysis is critical due to a variable occurrence of these abnormal deposits along skin nerves. According to our data, the optimal skin site with the highest probability of finding p-syn deposits in IPD is the cervical site, provided that 2 nearby skin samples are examined. As previously reported, the probability of detecting abnormal p-syn deposits was decreased in the thoracic Th12 region (4). These data discourage the p-syn search in the thoracic site for diagnostic purposes, and because thoracic skin biopsy often causes discomfort to the patient, particularly when sitting.

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