G.研究発表

（発表雑誌名巻号・頁・発行年なども記入）

1. 論文発表

なし

2.学会発表

なし

H.知的所有権の取得状況（予定を含む）

1.特許取得

なし

2.実用新案登録

なし

3.その他

なし

厚生労働科学研究費補助金（難治性疾患等克服研究事業（難治性疾患克服研究事業））
三重県南部に多発する家族性認知症-パーキンソン症候群 発症因子の探索と治療介入研究班
(分担)研究報告書

三重県南部に多発する家族性認知症-パーキンソン症候群患者からのiPS細胞の樹立

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研究要旨

人工多能性幹細胞（iPS 細胞）は、皮膚由来線維芽細胞に４つの初期化因子(Sox2, KLF4, Oct3/4, cMyc)を発現させ作製する。この細胞は試験管内で容易に増幅可能であり、神経細胞を誘導し作り出すことができるので、神経疾患の発症機序や治療法の開発へ利用できると期待されている。iPS 細胞樹立には、国内で開発されたセンダイウイルスベクター（SeV ベクター）を用いた。この方法では、iPS 細胞作製に用いる初期化因子が染色体に組み込まれないために、疾患研究により有用な iPS 細胞を作製できる。本研究班の協力のもと、三重県南部に多発する家族性認知症-パーキンソン病５症例の皮膚生検サンプルを現在までに提供していただき、そのうちの４例から皮膚由来線維芽細胞を樹立した。H24 年度までに、このうちの２症例から iPS 細胞を樹立した。H25 年度は、さらに１例からの iPS 細胞の樹立した。さらに樹立した iPS 細胞が神経細胞へ誘導可能であること確認した。

A.研究目的

人工多能性幹細胞（iPS 細胞）は、皮膚由来の線維芽細胞や末梢血液細胞に４つの初期化因子(Sox2, KLF4, Oct3/4, cMyc)を発現させ作製した多能性幹細胞である。この細胞は試験管内で容易に増幅可能であり、様々な細胞を誘導し作り出すことができる。そこで患者由来の iPS 細胞を樹立し、その細胞から病気の標的細胞を作り出して研究することで、病気の発症機序や治療法の開発へ利用できると期待されている。神経疾患においても、通常患者から得ることが困難な神経細胞を誘導し得ることで、疾患解析や治療薬開発に貢献できると期待されている。本研究では、難治性疾患の１つである三重県南部に多発する家族性認知症-パーキンソン病患者から iPS 細胞を樹立することを目的とする。

B.研究方法

1. iPS 細胞樹立のための皮膚線維芽細胞の樹立

三重県南部に多発する家族性認知症-パーキンソン病患者の皮膚生検から、皮膚由来初代線維芽細胞を樹立する。

2. SeV ベクターを使った iPS 細胞の確立

SeV ベクターによって患者由来線維芽細胞へ初期因子（Oct3/4, Sox2, KLF4, c-Myc）を一過性に発現させ iPS 細胞の樹立を行う。

樹立した iPS 細胞については、1)アルカリフォスファターゼ染色 2)Nanog, Oct3/4, SSEA-4, TRA-1-60 の免疫染色による iPS 細胞の確認を行う。さらに、未分化マーカーの発現を PCR にて確認する。

3. 樹立した iPS 細胞から神経細胞への分化を誘導し、神経細胞マーカー（Nestin 等）の発現を調べ、神経細胞であることを確認する。

(倫理面への配慮)

1) 倫理審査

疾患由来の iPS 細胞作製とその解析については倫理委員会ですでに承認済みである。また患者サンプルの提供については、提供機関の倫理審査委員会の承認があることを確認した後、研究を行う。

2) 人権擁護上の配慮

本研究は、個人ゲノムそのものの情報を得るわけではない。作製した iPS 細胞等を用いた病因解析・治療薬開発研究は本研究では行わない。また、研究の成果を学術雑誌に投稿することや、学会等で発表する場合、個人が特定される個人情報は公表されることはない。本研究のために特別に用意した番号によって管理し、人種・性別・年齢・診断名以外の患者情報はサンプル提供を行う臨床機関にて管理を行う。作製した iPS 細胞は所属機関において施錠できる研究室にて管理し、一般の人々やこの研究に関係ない他の研究者の目に触れることはない。したがって、iPS 細胞から個人の特定の情報につながることはない。また、ヒト iPS 細胞から個体を作製すること、ヒト胚への導入、ヒト胎児への導入、生殖細胞の作製は、行わない。

3) 不利益・危険性の排除や説明と同意

サンプル採取には、研究目的・予想される成果、患者情報の保護、予想される不利益等を同意書に記述している内容に準じて、担当医からの十分な説明の後(必要であれば代表申請者も同席して)、同意(インフォームド・コンセント) を得て行う。

皮膚由来線維芽細胞を得るための皮膚生検は通常の医学診療の範囲で行われている方法に準じて行う。痛みは、局所麻酔注射の時のみである。瘢痕は普通のけがの場合と同じである。以上より、危険性はほとんどない。

本研究による成果が知的財産権の対象になる場合もあるが、提供者に権利が帰属したり、利潤を得ることはない。サンプル提供者にご負担していただく必要経費はなく、また、サンプル提供による謝金・交通費の支給もない。研究にかかる費用については、研究費から支出する。

C.研究結果

1. 皮膚由来線維芽細胞の樹立

患者からの同意が得られた 5 例の症例において皮膚生検を行い、うち 4 例から iPS 細胞作成に必要な皮膚由来の線維芽細胞を樹立した。残り 1 例は培養中に汚染があり破棄した。

2. iPS 細胞の樹立

症例から樹立した皮膚由来線維芽細胞を用いて iPS 細胞の作製を行った。線維芽細胞に初期化因子 (Oct3/4, Sox2, KLF4,c-Myc) を持つセンダイ・ウイルスを感染させ、感染後 1 週間目にマイトマイシンで処理したマウス胎仔初代線維芽細胞(MEF)上へまきなおした。

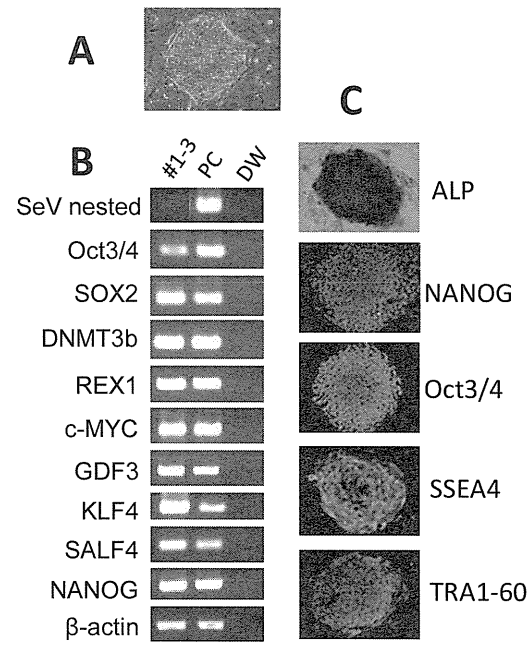

図1 樹立したiPS細胞

A. iPS細胞のコロニー(明視野)、
B. 未分化マーカーの発現(RT-PCR)、
C. 未分化マーカーの発現(免疫染色)とアルカリフォスファターゼ染色(ALP)
PC: positive control, DW: negative control

感染から 14 日後ぐらいからコロニーが出現した。感染から 25 日目にコロニーを顕微鏡下にてピックアップしそれぞれのクローンを培養、増幅した。その後、ウイルス除去のために培養の温度を 38 度へ

シフトさせた。用いたセンダイウイルスベクターは温度感受性株のために 38 度では増殖が停止し、結果としてウイルスベクターフリーの iPS 細胞を得ることができる。PCR にてウイルス除去を確認した後、未分化マーカーの発現を免疫染色と RT-PCR にて調べ、iPS 細胞であることを確認した。H25 年度は、1 症例から 10 数株のウイルスベクターフリーの iPS 細胞株を樹立した(図 1)。

3. 樹立した iPS 細胞からの神経細胞への誘導

これまでに樹立した iPS 細胞のうち症例 1 から樹立した iPS 細胞を神経細胞へと誘導した(図 2)。約 2 週間あまりで形態学的にも神経細胞へと誘導するに成功した。神経細胞特異的マーカーである Nestin の免疫染色を行い、陽性であることから神経細胞であることを確認した。

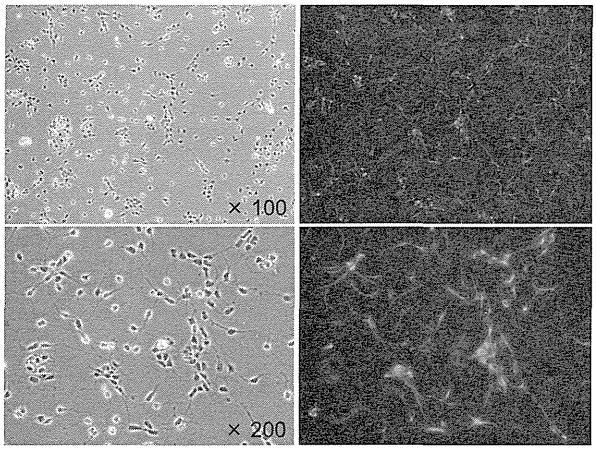

図2 疾患由来iPS細胞から神経細胞への誘導
iPS細胞から神経細胞への分化を誘導し、神経細胞特異的マーカーであるNESTINの抗体にて免疫染色を行った。

D.考察

これまでに、患者 5 例より皮膚由来の線維芽細胞を樹立した。H25 年度は、そのうち 1 例から iPS 細胞を樹立した。この症例も加えてこれまで行った 3 例からの iPS 細胞樹立の効率は特に健常者と変わりはなかった。この結果より、三重県南部に多発する家族性認知症-パーキンソン病では、この疾患の異常は、細胞のリプログラミングや iPS 細胞の増殖には影響を与えないことが示唆された。線維芽細胞樹立のためには生検が必要であり、樹立まで 1 ヶ月かかる。そこで、血液細胞あるいは血液細胞由来の細胞を iPS 細胞作製のソースとすることで、生検を行わずに末梢血の採血で iPS 細胞作製が可能となるために安全かつ容易に行える。

H25 年度は、作製した iPS 細胞から神経細胞を誘導し神経細胞を得ることができた。神経細胞の誘導効率では、健常者由来の iPS 細胞の場合と比較して特に差はなかった。したがって、この疾患の異常が神経細胞の分化に与える影響は、少ないと考えられる。

E.結論

患者 4 例の皮膚生検より皮膚由来線維芽細胞を樹立した。これまでの 2 例に加えて H25 年度は、新たに 1 例より iPS 細胞を樹立した。樹立した iPS 細胞は形態的にも、また、未分化マーカーの発現でも iPS 細胞に矛盾することがなく、iPS 細胞が樹立されたと言える。さらに神経細胞を樹立した iPS 細胞から誘導することに成功した。以上より、樹立した iPS 細胞は今後、三重県南部に多発する家族性認知症-パーキンソン病を研究する解析ツールをして有用である。

F.健康危険情報

特になし。

G.研究発表

（発表雑誌名巻号・頁・発行年なども記入）

1. 論文発表

特になし。

2.学会発表

1. Hamasaki M, Hashizume Y, Yamada Y, Katayama T, Hojoh H, Fusaki N, Nakashima Y, Furuya H, Haga N, Takami Y and Era T. Analysis of iPS cells derived from Fibrodysplasia ossificans progressive. Oral presentation. The 11th Stem Cell Research Symposium. Tokyo, 17th May 2013.
2. Hamasaki M, Hashizume Y, Yamada Y, Katayama T, Hojoh H, Fusaki N, Nakashima Y, Furuya H, Haga N,

Takami Y and Era T. Analysis of iPS cells derived from Fibrodysplasia ossificans progressive. Poster presentation. 11th Annual Meeting of International Society for Stem Cell Research. Boston, 14th June, 2013.

3. 江良　択実　難治性疾患由来 iPS 細胞の樹立、解析とそのバンク化　第 130 回　熊本小児科学会総会　特別講演　熊本　2013 年 6 月 16 日
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5. Era T, Soga M, Fusaki N, Hamasaki M, Yoneda K, Nakamura K, Matsuo S, Irie T, and Endo F. Model of Niemann-Pick disease type C using iPS cells. Oral presentation. The 3rd Asian Congress for Inherited Metabolic Diseases and The 55th Annual Meeting of The Japanese Society for Inherited Metabolic Diseases. Chiba, 28th November, 2013.

H.知的所有権の取得状況（予定を含む）

1. 特許取得

特になし。

2. 実用新案登録

特になし。

3. その他

特になし。

厚生労働科学研究費補助金（難治性疾患等克服研究事業（難治性疾患克服研究事業））
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(分担)研究報告書

Kii ALS/PDC（牟婁病）における病態解析および創薬を目的とした疾患特異的ヒトiPS細胞の作成

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研究要旨

Kii ALS/PDC（牟婁病）患者より疾患特異的iPS細胞を作成・品質管理を行うこと、さらに神経系細胞への分化を行い、疾患の病態解析並びに診断・治療のターゲット探索を行うことを目的とする。現在、Kii ALS/PDC患者5名の血液検体を用いて、疾患特異的iPS細胞を樹立し、解析に適したクローンの選抜中である。各症例間でT細胞やiPSCの樹立効率・増殖速度は異なっていた。今後、神経系解析に用いるiPSCクローンの選抜を進め、慶應拠点技術を用いて各種神経細胞やグリア細胞といった神経系細胞への分化誘導を行い、病態の解明や新たな治療に迫る検討を行っていく。

A.研究目的

Kii ALS/PDC(牟婁病)患者より疾患特異的iPS細胞を作成・品質管理を行う。さらに神経系細胞への分化を行い、疾患の病態解析並びに診断・治療のターゲット探索を行う。

B.研究方法

採血及びヒトiPS細胞（induced pluripotent stem cell; iPSC）の作成（Fig.1）

① 平成25年度に三重大学において承認された「牟婁病(Kii ALS/PDC)患者からのヒトiPSCの樹立とそれを用いた疾患解析および創薬に関する研究」計画書に基づいて、十分なインフォームドコンセントを得た上で、Kii ALS/PDC患者の協力のもと、(現在）5名の患者において採血を試行。

② 慶應義塾大学にて、末梢血単核球又はT細胞を調製。続けてエピソーマルベクターを用いて、SOX2, OCT3/4, KLF4, L-MYC, ドミナントネガティブp53, LIN28, EBNA1といった初期化因子を導入し、STOフィーダー細胞上で3〜5週間培養を行った。具体的には、京都大学iPS研究所(CiRA)より公開されている、「エピソーマルベクターを用いた末梢血からのiPS細胞樹立 Ver.1」プロトコールに準じた。iPSCコロニーの単離後は拡大培養を行い、未分化性を維持した状態で継代と凍結保存を行う。

③ 各々の患者由来iPSCについて、解析に用いることの可能なクローンの選抜を試行（エピソーマルベクターの残存およびゲノム挿入が起きていないこと、未分化マーカー発現、テラトーマ形成能の確認、神経系分化能の確認)。(Fig.2)

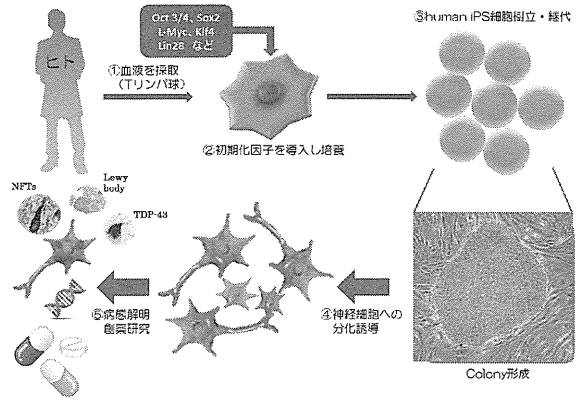


Figure 1. Overview of iPSC

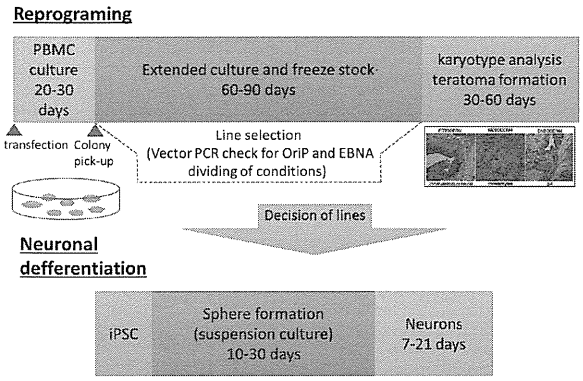


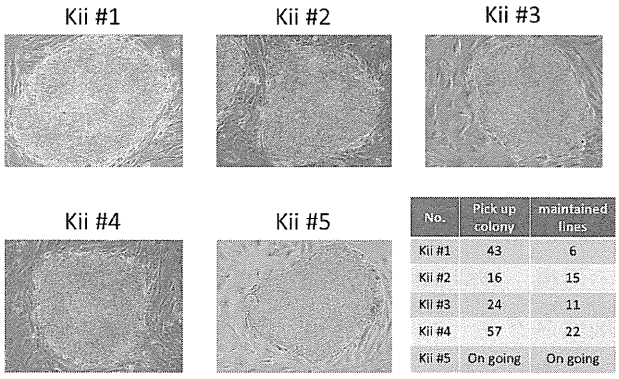
Figure 2. Time course of human iPSC establishment

C.研究結果

現在、Kii ALS/PDC 患者５名（Table.1）の血液検体を用いて、疾患特異的 iPS 細胞を樹立し、解析に適したクローンの選抜中である（Fig.3)。各症例間で T 細胞や iPSC の樹立効率・増殖速度は異なっていた。単離した iPSC クローンに関して、PCR を用いたエピソーマルベクターの残存確認を行った。(Fig.4)

Cases	Gender	Age	Onset	Birth	FH	Clinical phenotype		
						Parkinsonism	Dementia	MND
Kii #1	Male	66	59	Hohara	+	+	-	-
Kii #2	Female	74	60	Hohara	+	+	+	+
Kii #3	Female	83	72	Hohara	+	+	+	+
Kii #4	Female	74	58	Hohara	+	+	+	+
Kii #5	Male	79	74	Hohara	+	-	+	-

Table 1. Characteristics of five patients.



No.	Pick up colony	maintained lines
Kii #1	43	6
Kii #2	16	15
Kii #3	24	11
Kii #4	57	22
Kii #5	On going	On going

Figure 3. Colonies picked up in Kii #1-Kii #5.

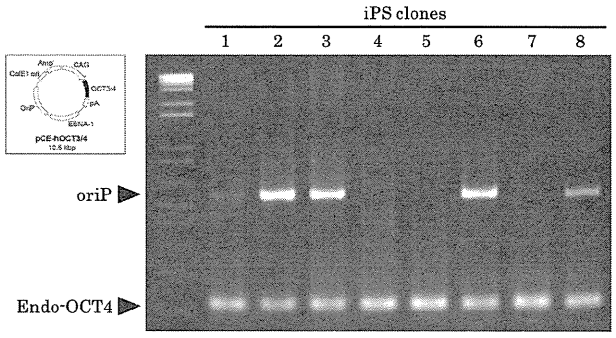
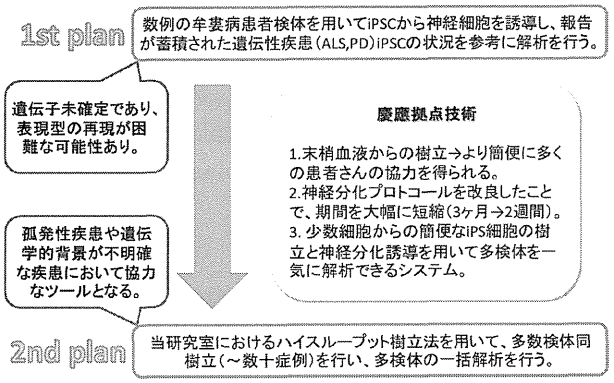


Figure 4. Check for residual plasmid vectors.

D.考察

現在疾患特異的 PSC の樹立、選別を行っている段階であり、今後、ｉ）アルカリフォスファターゼ活性の確認、ii）RT-PCR・免疫染色を用いた未分化マーカーの発現確認を行った後に、iii）SCID マウスへの iPSC 移植を用いたテラトーマ形成能（多分化能）の確認、iv）核型解析を行い、異常の認められないクローンを解析用として最終的に選別する。本疾患 iPSC 作成時の問題点としては、疾患の Heterogeneity が挙げられる。Kii ALS/PDC は家族性に発症するものの、遺伝子が同定されておらず、作製した iPSC を扱う上では、依然として孤発性疾患の要素が色濃く残る。→可能であれば autopsy による確認が必要。しかしながら、本疾患における iPSC 作製及び表現型の再現や病態解析が成功すれば、他の孤発性疾患解明の糸口ともなる。また、iPS 細胞作成におけるゲノム変異も問題となるが、東京大学神経内科（辻省次先生）が分担拠点として品質管理を行っている。次年度以降の展望として、疾患特異的ヒト

iPSC から各種神経系細胞への分化誘導と病態解析（樹立したヒト iPSC を用いて、運動神経、ドパミン作動性神経、興奮性神経、抑制性神経、グリア細胞へと分化誘導後、生化学的、遺伝学的並びに電気生理学的手法により、異常タンパク質蓄積をはじめとした in vivo で確認されている病態の再現、神経細胞の活動性や代謝能の解析）及び創薬スクリーニング（細胞内活性酸素種の軽減、グルタミン酸による興奮毒性の抑制、異常構造物の抑止などを指標に、リルゾール、フリラジカルスカベンジャー、抗酸化ビタミン群といった既存の薬剤に加え、神経保護作用や異常凝集物除去能を有する新たな治療薬を探索）を行っていく予定である。



E.結論

Kii ALS/PDC 患者 5 例から iPSC 樹立を行った。今後、神経系解析に用いる iPSC クローンの選抜を進め、神経系細胞への分化誘導を行い、病態の解明や新たな治療に迫る検討を行っていく。

F.健康危険情報

特記事項なし

G.研究発表

1. 論文発表

特記事項なし

2.学会発表

特記事項なし

H.知的所有権の取得状況（予定を含む）

1.特許取得

2.実用新案登録

3.その他

いずれも特記事項なし

Ⅲ. 研究成果に関する一覧表

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著　者　名	論　文　題　名	雑　誌　名	巻	頁	出版西暦年	GRANTへの謝辞の有無
Yui Nakayama, Satoru Morimoto, Misao Yoneda, Shigeki Kuzuhara, and Yasumasa Kokubo	Cerebrospinal Fluid Biomarkers for Kii Amyotrophic Lateral Sclerosis/Parkinsonism-Dementia Complex	Journal of Neurodegenerative Diseases	Volume 2013	Article ID 679089, 4 pages	2013	無
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著　者　名	論　文　題　名	雑　誌　名	巻	頁	出版西暦年	GRANTへの謝辞の有無
小久保康昌	線条体黒質変性症の治療とその具体的臨床事例	脊髄小脳変性症マニュアル決定版！（監：西澤正豊/編：月刊難病と在宅ケア）		初め頁-終り頁	2013	無

Ⅳ. 研究成果の刊行物・別刷

Neuropathology 2013; **33**, 47–58 doi:10.1111/j.1440-1789.2012.01329.x

Original Article

Neuropathologic analysis of Lewy-related α-synucleinopathy in olfactory mucosa

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We analyzed the incidence and extent of Lewy-related α-synucleinopathy (LBAS) in the olfactory mucosa, as well as the central and peripheral nervous systems of consecutive autopsy cases from a general geriatric hospital. The brain and olfactory mucosa were immunohistochemically examined using antibodies raised against phosphorylated α-synuclein. Thirty-nine out of 105 patients (37.1%) showed LBAS in the central or peripheral nervous systems. Seven patients presented LBAS (Lewy neurites) in the olfactory lamina propria mucosa. One out of the seven cases also showed a Lewy neurite in a bundle of axons in the cribriform plate, but α-synuclein deposits were not detected in the olfactory receptor neurons. In particular, high incidence of α-synuclein immunopositive LBAS in the olfactory mucosa was present in the individuals with clinically as well as neuropathologically confirmed Parkinson's disease and dementia with Lewy bodies (6/8 cases, 75%). However, this pathologic alteration was rare in the cases with incidental or subclinical Lewy body diseases (LBD) (one out of 31 cases, 3.2%). In the olfactory bulb, the LBAS was usually present in the glomeruli and granular cells of most symptomatic and asymptomatic cases with LBD. Our studies further confirmed importance of the olfactory entry zone in propagation of LBAS in the human aging nervous system.

Key words: α-synuclein, Lewy body, neuropathology, olfactory mucosa, Parkinson's disease.

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INTRODUCTION

Sporadic Parkinson's disease is a neurodegenerative disorder characterized clinically by resting tremor, rigidity, bradykinesia and gait disturbance, as well as neuropathologically by the loss of neurons in several brainstem nuclei and the presence of Lewy bodies formed by abnormal accumulation of α-synuclein.[1–5] Of the many types of neurons in the central and peripheral nervous systems, a specific subset of neurons is vulnerable to accumulation of α-synuclein, which takes the form of aggregates such as Lewy bodies and Lewy neurites (LBs/LNs).[6–8]

Based on studies of a large number of autopsy cases, the initial sites involved in Lewy-related pathology are reported to be the dorsal motor nucleus of the vagus, the intermediate reticular zone in the lower brainstem and olfactory bulb.[9,10] We previously reported that in the earliest stage of Lewy-related α-synucleinopathy (LBAS), abnormal α-synuclein accumulation extends from the peripheral part of the olfactory bulb to the anterior olfactory nucleus as well as the amygdala.[11] From a clinical standpoint, impaired olfactory function constitutes one of the earliest symptoms of sporadic Parkinson's disease.[12,13] Therefore, the olfactory system may be one of the vital regions in the development of Lewy body disease (LBD).

In the olfactory bulb, α-synuclein accumulation is observed in the anterior olfactory nucleus as well as the mitral, tufted, and granular cells of individuals with clinical Parkinson's disease or dementia with Lewy bodies (DLB). Even in the early stages of these diseases, LNs, LBs or both, can be seen in the olfactory bulbs.[11,14,15] Based on the results of a neuropathologic study, Beach *et al.* suggested that the olfactory bulb may be a candidate region of biopsy study to

confirm the diagnosis of LBD.[16] However, the biopsy of olfactory bulb is too invasive and difficult to carry out for patients without risk.[17,18]

The olfactory epithelium is composed of paraneurons and neurites from which the glomeruli of the olfactory bulb originate. However, a neuropathologic analysis of LBAS has not been carried out adequately for LBD. Duda *et al.* reported that normal α-synuclein is expressed in the basal cells, olfactory receptor neurons, supporting cells, and Bowman's glands of the olfactory epithelium in normal controls, as well as patients with Parkinson's disease, Alzheimer disease and multiple system atrophy.[19] However, pathologic α-synuclein accumulation is rare (3.7%) among both normal controls and individuals affected by DLB, Alzheimer disease or Parkinson's disease.[20] According to a biopsy study of the olfactory epithelium in individuals with Parkinson's disease and younger hyposmic controls, no specific pathologic alteration was found.[21]

Therefore, it is still controversial whether abnormal α-synuclein accumulation in the olfactory epithelium precedes the formation of LBs/LNs in the olfactory bulb and contributes to olfactory dysfunction in sporadic Parkinson's disease. The aim of this study was to clarify the neuropathologic alterations of the olfactory mucosa in LBD by immunohistochemical analysis of a series of autopsied individuals.

MATERIALS AND METHODS

Tissue source

Tissue samples were obtained from autopsy materials that were collected at the Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology between October 2008 and August 2010. This hospital is located at the center of Tokyo city and is a geriatric general emergency hospital with 579 beds. This hospital provides community-based medical service to the aged population 24 h/day in cooperation with local general practitioners. The number of autopsy cases was 162 in the above duration. In addition to the general organs, we could obtain the brains and spinal cords from 105 cases in that period, that were registered to the Brain Bank for Aging Research (BBAR) with the deceased's relatives' informed consent. The BBAR is approved by the ethics committee of the Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology to carry out comprehensive research.

Clinical information

All clinical information, including the presence or absence of Parkinsonism as well as dementia, was retrospectively obtained from medical charts and reviewed by two board-certified neurologists.[11,22–26] First, we evaluated Parkinsonism such as bradykinesia, resting tremor, rigidity and postural instability. In this study, when individuals had two or more of these four clinical symptoms, we defined them as having Parkinson's disease-related symptoms.[27] Second, we analyzed scores for the Mini-Mental State Examination[28] or the Hasegawa Dementia Scale (or its revised version),[29,30] the Instrumental Activities of Daily Living,[31] and the Clinical Dementia Rating (CDR).[32] When individuals were not assigned to a category of CDR, we retrospectively determined CDR using medical records, including the battery of cognitive tests above, as well as interviews with attending physicians and caregivers when necessary. Based on these results, we assigned a clinical diagnosis to each patient. The clinical diagnosis of Alzheimer disease was carried out based on the criteria of the National Institute of Neurological and Communication Disorders and Stroke-Alzheimer Disease and Related Disorders Association.[33] The diagnosis of DLB and Parkinson's disease with dementia conformed to the third report of the DLB consortium.[34]

Histology

We examined the brain and olfactory epithelium, olfactory bulb, esophagogastric mucosal junction, sympathetic ganglia, thoracic spinal cord, adrenal glands, anterior wall of the left ventricle of the heart, and abdominal skin.[22,26] The brains and spinal cords were examined as previously reported.[22,24,25] Briefly, the cerebral and cerebellar hemispheres as well as brainstem were dissected in the sagittal plane at the time of autopsy. In each case, half of the brain was preserved at −80°C for further biochemical and molecular analyses. The other half of the brain and abdominal skin were fixed in 20% buffered formalin (WAKO, Osaka, Japan) for 7–13 days and sliced in the same manner as the contralateral hemisphere. The adrenal gland and anterior wall of the left ventricle of the heart were fixed in 20% formalin. The representative areas were embedded in paraffin. Six-micrometer-thick serial sections were cut and stained with HE and KB. Sections of the amygdala, hippocampus, parahippocampal gyrus and temporal cortex were stained with the modified Gallyas-Braak method for senile plaques, NFTs and argyrophilic grains.[35]

Immunohistochemistry

Sections were immunostained using the following antibodies raised against phosphorylated tau protein (p-tau) (AT8, monoclonal; Innogenetics, Temse, Belgium): synthetic peptide corresponding to amino acids 11–28 of amyloid-beta protein (12B2, monoclonal; IBL, Maebashi, Japan); phosphorylated α-synuclein (pSyn#64, monoclonal[25] and

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Table 1 Antibodies used for immunohistochemistry

Antibody	Epitope	Source	Clone	Dilution ratio	Antigen method	Retrieval (min)
pSyn#64	α-synuclein phosphorylated ser 129	T. Iwatsubo	Monoclonal	1:20000	99% formic acid	5
PSer129	α-synuclein phosphorylated ser 129	T. Iwatsubo	Polyclonal	1:100	None	
PGP9.5	PGP9.5	Biomol	Polyclonal	1:5000	microwave	30
SMI31	phosphorylated neurofilament	Sternberger	Monoclonal	1:20000	None	
Tyrosine hydroxylase	Anti-tyrosine hydroxylase, rat	CALBIOCHEM	Monoclonal	1:10	microwave	30
AT8	Phosphorylated tau protein	Innogenetics	Monoclonal	1:1000	None	
12B2	Aβ11–28	IBL	Monoclonal	1:50	99% formic acid	5

PSer129 polyclonal[36]), ubiquitin (polyclonal, Sigma-Aldrich, St. Louis, MO), Protein Gene Product 9.5 (PGP9.5, polyclonal; ENZO Life Sciences International, Farmingdale, NY USA); phosphorylated neurofilament (SMI31, monoclonal; Sternberger Immunochemicals, Bethesda, MA, USA); and tyrosine hydroxylase (Anti-Tyrosine Hydroxylase, Rat, monoclonal; Calbiochem-Novabiochem Corporation, Darmstadt, Germany) (Table 1). The signals from monoclonal and polyclonal antibodies were detected by using the automatic system on a VENTANA NX20 with the I-View DAB Universal Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. Sections were counter-stained with hematoxylin.

LBAS

CNS

In order to analyze LBAS,[22] we carried out immunohistochemical analysis with phosphorylated α-synuclein antibodies for the following sections: the medulla oblongata at the level of the dorsal motor nucleus of the vagus, the upper pons at the level of the locus coeruleus, and the midbrain including the substantia nigra, amygdala, anterior hippocampus and the peripheral nervous system from all cases (described in the next section). When immunopositive deposits were observed in these anatomic regions, we carried out additional immunohistochemical analysis for sections of the basal nucleus of Meynert, anterior cingulate gyrus, entorhinal cortex, the second frontal and temporal gyri and the supramarginal gyrus, using antibodies raised against phosphorylated α-synuclein.

Peripheral nervous system

To analyze LBAS of the peripheral nerve, tissue sections from epicardium and epicardial fat of the left ventricle of the heart, sympathetic ganglia, esophagogastric mucosal junction, adrenal gland[22] and abdominal skin[26] were examined by using antibodies raised against phosphorylated α-synuclein.

Olfactory mucosa

At the time of autopsy, the olfactory mucosa, bony septae and contiguous cribriform plate were removed en bloc (Fig. 1). The cribriform plate was dissected in the sagittal plane of the midline by using an electric jigsaw. The left side was fixed for 24 h in 4% paraformaldehyde. After fixation, the olfactory mucosa was removed, dehydrated in a graded alcohol series, cleared in xylene and embedded in paraffin. The right side was fixed for 24 h in 4% paraformaldehyde, decalcified with EDTA for 2 weeks, and dehydrated and embedded in paraffin. Serial 6-μm-thick sections were stained with HE and immunolabeled with antibodies against phosphorylated α-synuclein, PGP9.5, phosphorylated neurofilament, tyrosine hydroxylase, phosphorylated tau and amyloid β (Table 1). In particular, the olfactory receptor neurons of the olfactory epithelium were identified by using PGP9.5 immunohistochemistry.[19] The normal anatomical appearance of the olfactory system is shown in Figure 2.

Olfactory bulb

The olfactory bulbs were prepared for histologic sections to analyze the presence of LBAS. By using HE stain and α-synuclein antibodies, LBAS were identified in the glomeruli, mitral cells, tufted cells and granular cells as previously reported.[11] Mitral and tufted cells were distinguished by their specific shapes. Each neuron was identified when it had an apparent nucleus containing a prominent nucleolus and Nissl substance.

Semiquantitative scoring system of Lewy-related pathology

For each section, we semi-quantitatively graded the immunohistochemical staining with antibody raised against phosphorylated α-synuclein. Our grading system was modified based on the scoring system of the third report of the DLB consortium[34] because we used both the HE stain and immunohistochemistry using monoclonal antibody for phosphorylated α-synuclein to identify LBAS.

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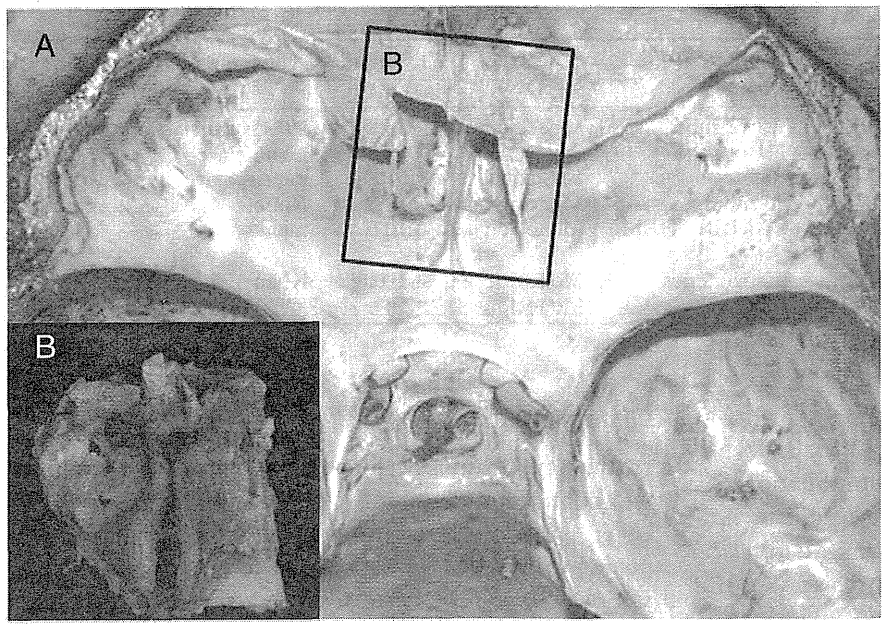

Fig. 1 (a) The anterior cranial fossa after removal of the brain. In order to obtain the olfactory mucosa, the bony septae and contiguous cribriform plate (the rectangular area) were dissected using an electric jigsaw. (b) An inset shows the olfactory mucosa and cribriform plate from the opposite side of the rectangular area.

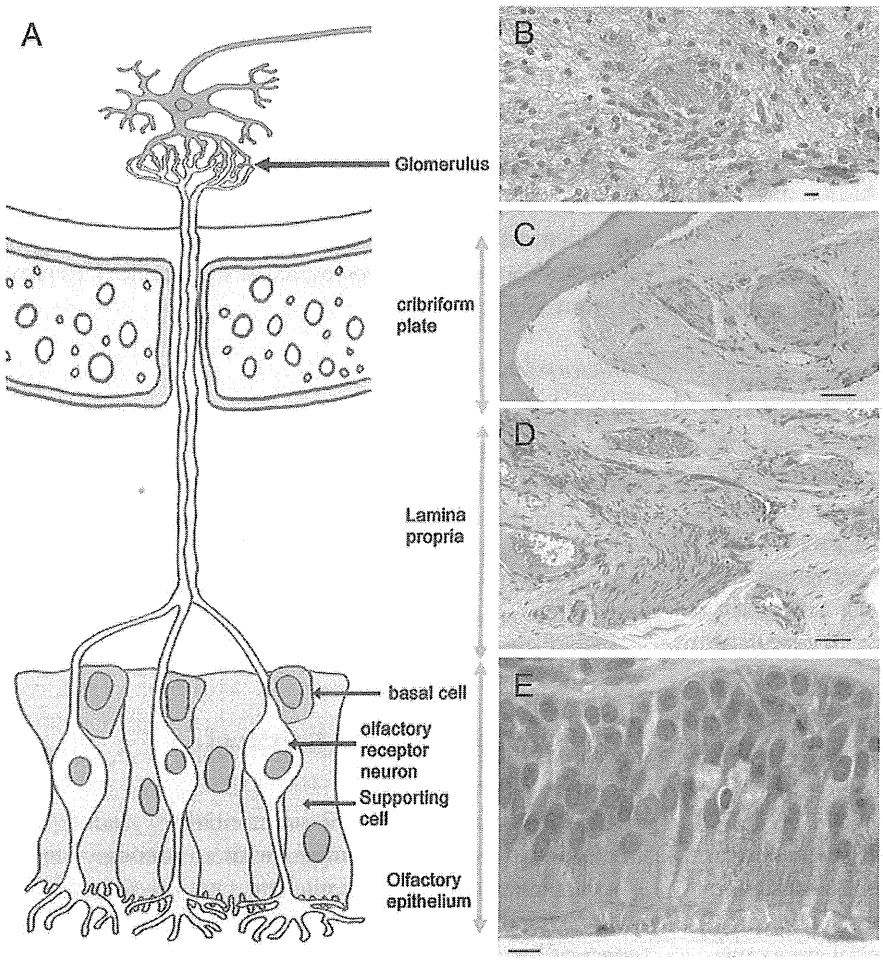

Fig. 2 Scheme of the normal olfactory pathway (a) and photomicrographs of representative histologies of each region (b–e). The olfactory epithelium is composed of three cell types: the basal cells, olfactory receptor neurons and supporting cells (a). The basal cells are the progenitor of the olfactory receptor neurons (a, e). In general, the turnover rate of the olfactory receptor neurons is approximately 30–90 days. Nerve fibers are present in the lamina propria and cribriform plate (c and d, respectively). They consist of either the axons of the olfactory receptor neurons or postganglionic sympathetic nerve fibers. There are glomeruli in the olfactory bulb (b). Glomeruli are the synaptically connected structures of the axons of the olfactory receptor neurons and mitral/tufted cells in the olfactory bulb. (b, e), scale bar = 10 μm; (c), scale bar = 50 μm; (d), scale bar = 100 μm.

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For example, 'Stage 1' of the original scoring system was defined as 'sparse Lewy bodies or neurites.' On the other hand, 'Grade 1'of our methodology is defined as 'sparse Lewy neurites without Lewy bodies.'

Grade 0 = neither LNs nor LBs detected using anti-phosphorylated α-synuclein antibody.

Grade 1 = sparse phosphorylated α-synuclein immunopositive dots or neurites, or diffuse granular cytoplasmic stain in the neuron, neither LBs nor phosphorylated α-synuclein-immunopositive neuronal intracytoplasmic dense aggregations.

Grade 2 = 1–3 LBs or phosphorylated α-synuclein-immunopositive intracytoplasmic dense aggregations and scattered LNs in a low-power field (×10).

Grade 3 = more than four LBs and scattered LNs in a low-power field (×10).

Grade 4 = numerous LBs and neurites with severe immunoreactivity for phosphorylated α-synuclein in the neuropil or background.

LB staging system of our BBAR (BBAR LB stage)

In order to assess the clinical and neuropathologic alterations of LBD, we applied the following rating system to our BBAR for all autopsy cases (Table 2, Fig. 3). The original BBAR LB staging system was developed in order to track the individual data of our brain bank.[24,25] This rating system requires clinical symptoms, gross and microscopic neuropathologic alterations, and LB scores used in the consensus guidelines for the clinical and pathologic diagnosis of DLB.[27] In this staging system, Parkinson's disease with

Table 2 Lewey body stage of Brain Bank for Aging Research

Stage	Psyn-IR	LB	SN: loss of pigmentation	LB score	Dementia	Parkinsonism	Diagnosis
0	–	–	–				
0.5	+	–	–				
1	+	+	–				Incidental LBD
2	+	+	+	0–10	–†	–†	Subclinical LBD
3	+	+	+	0–10	–	+	PD
4	+	+	+	3–6	+	+	PDDL
	+	+	+	3–6	+	+ or –	DLBL‡
5	+	+	+	7–10	+	+	PDDN
	+	+	+	7–10	+	+ or –	DLBN‡

†Neither dementia nor Parkinsonism associated with Lewy body-related α-synucleinopathy. ‡Differential diagnosis of PDD and DLB was based on the '1-year rule' according to the consensus guidelines (34). DLBL, dementia with Lewy bodies and a Lewy body score corresponding to the limbic form; DLBN, dementia with Lewy bodies and a Lewy body score corresponding to the neocortical form; LB, Lewy body; LBD, Lewy body disease; PD, Parkinson's disease; PDDL, Parkinson's disease with dementia and a Lewy body score corresponding to the limbic form; PDDN, Parkinson's disease with dementia and a Lewy body score corresponding to the neocortical form; Psyn-IR, phosphorylated alpha-synuclein immunoreactivity; SN, substantia nigra.

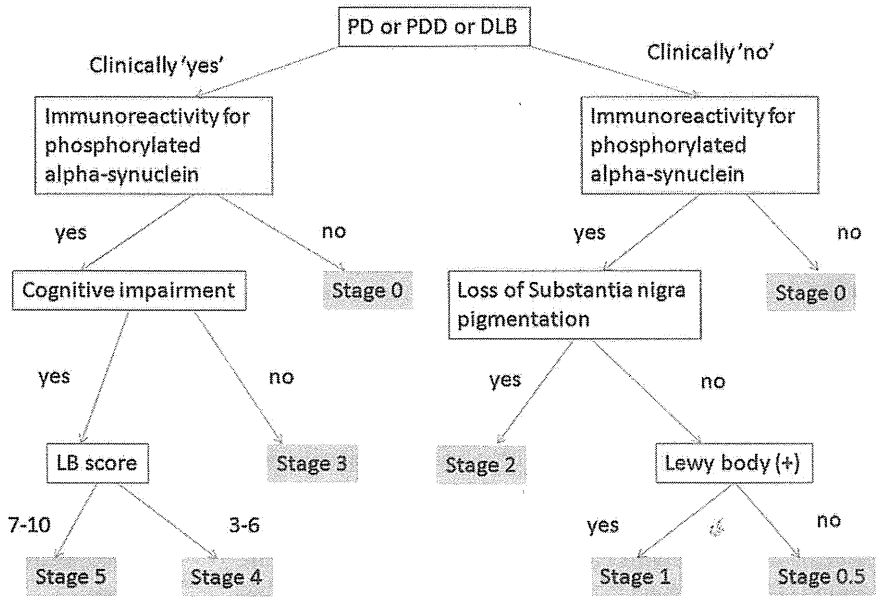

Fig. 3 Flow-chart of the Lewy body staging system of the Brain Bank for Aging Research (BBAR). PD, Parkinson's disease; PDD, Parkinson's disease with dementia; DLB, dementia with Lewy bodies; SN, substantia nigra; LB score, Lewy body score. See Table 2 for detailed description of each stage.

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dementia was differentiated from DLB by applying the '12-month (1-year)' rule noted in the Consensus Guidelines (i.e., 'dementia appears more than one year after the onset of Parkinsonism').[27]

Evaluation of senile changes and neuropathologic diagnosis

NFTs were classified according to Braak and Braak's staging system using modified Gallyas-Braak staining[37] and AT8 immunohistochemistry.[38] The staging system for senile plaques (SPs) comprises four stages (0–C). Argyrophilic grains were classified into our four stages (0–III), as reported previously.[23] The neuropathologic diagnosis of Alzheimer disease was based on our previous definition,[39] which proposed a modification of the National Institute on Aging and Reagan Institute criteria.[40,41] The diagnoses of dementia with grains and NFT-predominant forms of dementia were based on the previously described definitions.[42,43]

Statistical analysis

Fisher's exact test was carried out to compare the number of cases having LBAS pathology in the olfactory mucosa.

RESULTS

Clinical information

Of the 105 consecutive autopsy patients, 58 were men and 47 were women. The patient ages at death ranged from 65 to 104 years (82 ± 37, mean ± SD). Twelve patients showed Parkinson's disease-related symptoms according to the clinical criteria in this study. Six out of 105 patients were clinically diagnosed as LBD including Parkinson's disease, Parkinson's disease with dementia and DLB.

Neuropathologic diagnosis

The neuropathologic diagnoses consisted of Alzheimer disease ($n = 15$), dementia with grains ($n = 11$), NFT-predominant form of dementia ($n = 8$), Parkinson's disease ($n = 2$), Parkinson's disease with dementia ($n = 2$), and DLB ($n = 1$), as well as one case each of dentatorubral-pallidoluysian atrophy, neuronal hyaline inclusion body disease, frontotemporal lobar degeneration with transaction response (TAR) DNA-binding protein-43 kDa-immunoreactive inclusions, and progressive multifocal leukoencephalopathy. Patients with combined pathologies, included Alzheimer's disease plus DLB ($n = 2$), dementia with grains plus NFT-predominant form of dementia ($n = 3$), and one patient each of diffuse NFTs with calcification (DNTC)[44] plus DLB and dementia with grains plus Alzheimer's disease. The remaining patients did not fulfil the clinical and/or pathological criteria for neurodegenerative diseases.

Eight out of 105 patients (8/105 = 7.6%) were clinically and neuropathologically diagnosed as having LBD, including Parkinson's disease (2 patients), Parkinson's disease with dementia (2 patients) and DLB (4 patients).

Incidence, distribution and extent of LBAS

BBAR staging

Based on clinical and neuropathologic analyses, the BBAR LB stages were as follows: stage 0 = 66 cases, stage 0.5 = 6 cases, stage 1 = 21 cases, stage 2 = 4 cases, stage 3 = 2 cases, stage 4 = 3 cases and stage 5 = 3 cases. All of the stage 5 cases had DLB, with an LB score corresponding to the value for the neocortical form (DLBN).

LBAS in CNS and peripheral nervous system

We identified 39 (37.1%) out of the 105 individuals with α-synuclein immunopositive LBAS in the CNS or peripheral nervous system (Table 3). Therefore, we focused on these 39 cases in the present study. Here, LBAS was identified by using α-synuclein immunohistochemistry. In LBAS, LBs were confirmed with HE stains and α-synuclein immunohistochemistry. Out of the 39 cases, 33 showed LBAS in the olfactory bulb, 15 in the enteric nerve plexus, 23 in the sympathetic ganglia, and 16 in the pericardial nerve fibers of the left ventricle (Tables 3 and 4).

Olfactory mucosa

The olfactory epithelium is a pseudostratified columnar epithelium lying deep within the recess of the superior nasal cavity; it is composed of a mixture of multipotential stem cells (basal cells), supporting cells and olfactory receptor neurons (Fig. 2). Mature neurons are reported to give rise to fine and unmyelinated axons that ascend through the cribriform plate to synapse at glomeruli in the olfactory bulb.[20,45]

LBAS were found in the olfactory mucosa of seven (17.9%) out of 39 cases (Tables 3 and 4). These seven also had LBAS in the olfactory bulb. LBAS was present in the lamina propria mucosa of the seven cases (Fig. 4a–c). In addition, one case showed LBAS in a bundle of axons in the cribriform plate (Fig. 4d). None of the cases showed LBAS in the olfactory epithelial paraneuron. We summarized the demographic results of these seven individuals with LBAS in the olfactory mucosa in Table 5. Neither phosphorylated tau-positive deposits nor amyloid β immunopositive deposits were detected in the olfactory mucosa.

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Table 3 The distribution of α-synuclein deposits in various anatomical regions of 39 cases with Lewy body disease

Age at death/gender	Parietal lobe	Frontal lobe	Temporal lobe	Cingulate gyrus	Entorhinal cortex	Amygdala	Olfactory bulb	Nucleus basalis of Meynert	Substantia nigra	Locus coeruleus	Dorsal motor nucleus of the vagus	Spinal Cord	Gastrointestinal system	Olfactory Mucosa	Sympathetic ganglion	Adrenal gland	Pericardial nerve	Skin	BBAR LB stage	NFT stage	SP stage
104/F																			5	4	C
70/F																			5	4	C
86/F																			5	6	C
84/M																			4	2	A
79/F																			4	2	A
80/F																			4	2	A
81/M																			3	2	A
88/M																			3	3	A
79/M																			2	1	A
68/F																			2	2	B
79/F																			2	6	C
77/F																			2	6	C
78/M																			1	2	A
75/M																			1	2	A
89/F																			1	3	C
93/F																			1	4	C
86/M																			1	4	C
81/M																			1	2	A
90/F																			1	2	A
86/M																			1	2	A
97/F																			1	2	A
78/M																			1	1	A
92/M																			1	3	C
94/M																			1	4	A
85/M																			1	3	A
81/F																			1	5	C
96/F																			1	2	0
87/F																			1	3	A
101/F																			1	4	A
69/F																			1	4	0
83/F																			1	3	A
72/M																			1	1	A
77/M																			1	2	A
83/M																			0.5	4	C
71/M																			0.5	2	A
89/M																			0.5	2	A
85/F																			0.5	3	A
85/F																			0.5	2	A
96/F																			0.5	3	A

Grade 0 = blank, grade 1 = light grey, grade 2 = light blue, grade 3 = blue, grade4 = navy blue. The number in each cell indicates a score based on the semiquantitative scoring system of Lewy-related pathology. 0 = neither Lewy neurites nor bodies detected by using anti-phosphorylated α-synuclein antibody. 1 = sparse phosphorylated α-synuclein immunopositive dots or neurites, neither Lewy bodies nor phosphorylated α-synuclein immunopositive intracytoplasmic aggregations. 2 = one to three Lewy bodies or phosphorylated α-synuclein immunopositive intracytoplasmic aggregations in a low-power field (×10). 3 = more than four Lewy bodies and scattered Lewy neurites in a low-power field (×10). 4 = numerous LBs and neurites with severe immunoreactivity for phosphorylated α-synuclein in the neuropil or background. Individuals of BBAR LB stages 3–5, with clinical Parkinsonism and neuropathologically numerous LBASs in the CNS, showed high incidence (75%, 6/8 individuals) of LBASs in the olfactory mucosa. In contrast, individuals of BBAR LB stages 1–3 without Parkinsonism showed extremely low incidence of Lewy body-related α-synucleinopathy (LBAS) (3%, 1/31) in the olfactory mucosa. LBAS was found in the olfactory mucosa mostly in advanced BBAR LB stages 3–5. BBAR LB Brain Bank for Aging Research Lewy body staging, NFT stage, Braak's stages for neurofibrillary tangles; SP stage, Braak's stages for senile plaques.

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Table 4 Regional frequency of Lewy body-related α-synucleinopathy (LBAS) in various anatomical regions

The BBAR LB stage	Olfactory epithelium	Olfactory mucosa	Olfactory bulb	Spinal cord	GI tract	Sympathetic ganglia	Adrenal gland	Pericardial nerve	Skin
0.5	0/6	0/6	2/6	0/6	0/6	2/6	0/6	1/6	0/6
1	0/21	1/21	19/21	7/21	6/27	10/21	1/21	6/21	1/21
2	0/4	0/4	4/4	3/4	1/4	3/4	0/4	2/4	0/4
3	0/2	1/2	2/2	2/2	2/2	2/2	2/2	1/2	2/2
4	0/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	2/3
5	0/3	2/3	3/3	3/3	3/3	3/3	1/3	3/3	0/3
All	**0/39**	**7/39**	**33/39**	**18/39**	**15/39**	**23/39**	**7/39**	**16/39**	**5/39**

BBAR LB Brain Bank for Aging Research Lewy body staging.

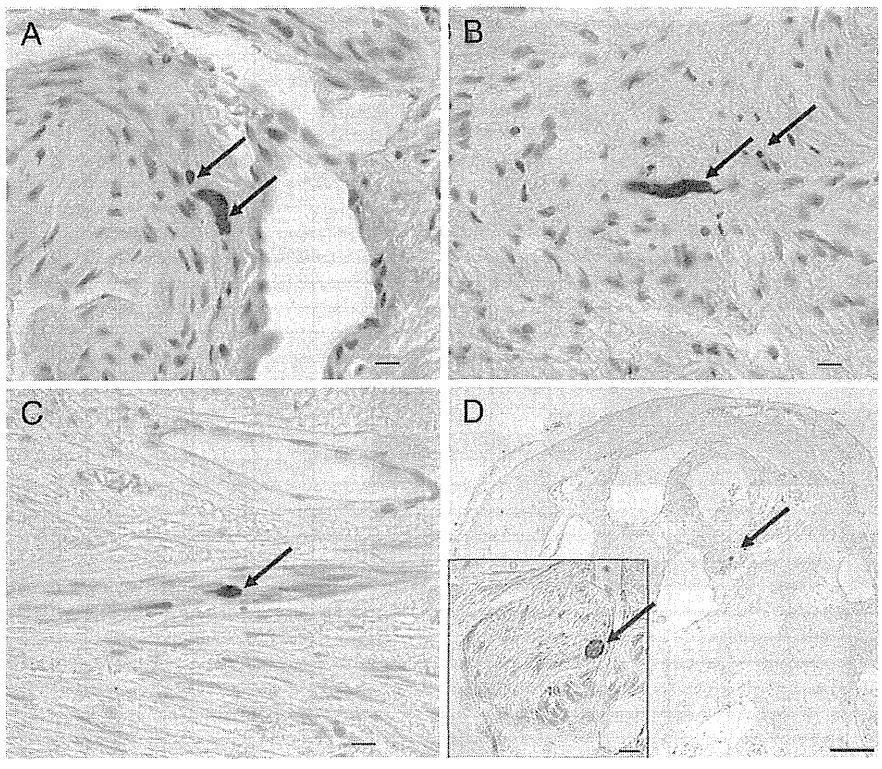

Fig. 4 Photomicrographs show α-synuclein immunopositive deposits (arrows indicate Lewy neurites) in the axonal bundle of the lamina propria (a–c) and cribriform plate (d). The inset in figure (d) shows a higher magnification image of α-synuclein immunopositive deposits in the axonal bundle of the cribriform plate. Immunohistochemistry using monoclonal antibody against phosphorylated α-synuclein (pSyn#64). Photomicrographs (a, b, c and d) were obtained from cases 4, 5, 7 and 3, respectively, in Table 5. (a–c), scale bar = 10 μm; (d) scale bar = 100 μm (inset, 10 μm).

Correlations between α-synuclein immunopositive LBs or LNs in the olfactory mucosa and CNS

Alpha-synuclein immunopositive LBs or LNs in the olfactory mucosa were detected in seven cases, including three with DLB, three with Parkinson's disease or Parkinson's disease with dementia, and one with incidental LBD (Tables 3–5). LBAS in the olfactory mucosa was compared with those in other locations of the CNS (Table 3). Individuals of BBAR LB stages 3–5, clinical and neuropathological diagnosis of LBD, showed a high incidence (75%, 6/8 individuals) of α-synuclein immunopositive LBAS in the olfactory mucosa (Table 6, Fig. 5). Six individuals with Parkinson's disease also showed a high incidence of α-synuclein accumulation (66%, 4/6 individuals) in the olfactory mucosa. In contrast, individuals of BBAR LB stages 0.5–2 (here we classified them into asymptomatic group) showed a low incidence of LBAS (3%, 1/31) in the olfactory mucosa.

Olfactory bulb

There is neural connectivity among olfactory receptor neurons and nuclei in the olfactory bulbs.[45] Hence, we analyzed the frequency of LBAS in the glomeruli, tufted cells, mitral cells and granular cells between LBAS-positive and LBAS-negative groups in the olfactory epithelium.

In individuals of BBAR LB stages 3–5 (symptomatic stage), LBAS was frequently observed in the glomeruli (8/8 cases, 100%), granular cells (8/8, 100%) and tufted cells (7/8, 87.5%). In contrast, there were low numbers of cases with LBAS in the mitral cells (2/8, 25%). Asymptomatic stage cases of LBD, corresponding to BBAR stage 0.5–2,

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