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図 1. 東京都高齢者ブレインバンクリソースセンター
図書館形式で書架にブロックと大切片標本を，標本箱に標本を番号順に整理し，研究所内 LAN に，パスワードでアクセス可能とした．東京都高齢者ブレインバンク臨床神経病理データベースにアクセス可能環境を構築，参照可能の体制を構築した．

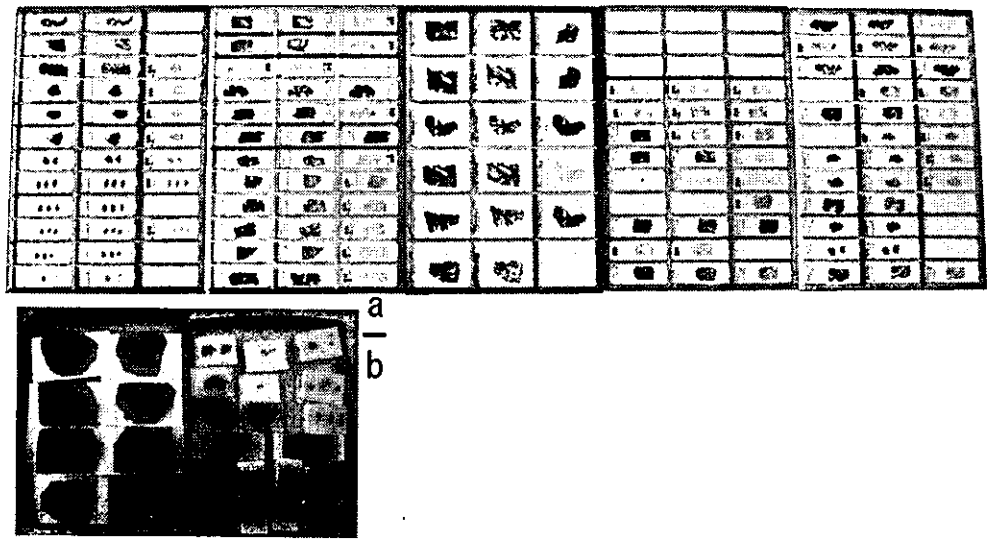


図 2. 東京都高齢者ブレインバンクリソースセンター内資源

a. 組織標本．部位・染色を一定，自動免疫染色プロトコールの公表，国際学会での発表，国際的視察による国際標準化で，一般性の高い資源を提供することを前提とした．

b. 組織ブロック 7,504 例について，同じ形の箱の中に整理し，東京都高齢者ブレインバンク臨床神経病理データベースと連動させ，検索参照システムを構築した．

されたことを背景に，老人総合研究所からの全面的支援を受け，これまでの標本とブロックを系統的に整理することで，東京都高齢者ブレインバンクリソースセンターを整備することが可能となった．

蓄積された資源は，多数の国際誌への報告貴重例を含んでいるだけでなく，たとえばその中の動脈硬化の程度をとりあげるだけで，この30年間にわたる東京の都市部において，頭蓋内動脈硬化が軽快し，頭蓋外動脈硬化が不変ないし増悪という，欧米化が起きていることを示す重要な資源としても活用が可能である．さらに，臨床病歴が全て保存されている点も，後方視的に研究する上で極めて貴重な資源となりうる．また，進行性核上性麻痺60例，百寿齢48例等，系統的に疾患を検索する上でも，重要な資源となりうる．

## 3. 東京都高齢者DNAリソースの構築

1995年度より，東京都老人医療センター病理部が後頭極を含む全身臓器の凍結材料保存を開始し，これを引き継ぐかたちで蓄積を行った．開頭剖検例は2003年12月の時点で1,341例であるが，これら全例のApoE遺伝子多型解析を，DNAの品質管理の意味も含め，施行した．

これらDNA保存症例については，通常染色として，ヘマトキシリンエオジン，クリュバーバレラ染色を基準とし，高感度嗜銀染色である改良メセナミン銀染色，ガリアスブラーク染色を適宜追加した．免疫染色としては，各種抗tau (AT8, Alz50, PHF1, APP422, exon10 specific)，Aβ (11-28, 1-42, 1-40)，シヌクレインン（N末，中間部：LB509，C末，リン酸化部位），ユビキチン（多クローン，単クローン）抗体を採用，グリオーシスの評価に抗glial fibrillary acidic protein(GFAP)，HLA-DR抗体(CD68)，中枢神経系の構成内容の評価には，抗ミエリン塩基蛋白抗体，抗神経細糸抗体を用い，Ventana NX20を用いて自動免疫染色を行った．これらの結果をデータベース化し（表2），遺伝子発現との参照が可能な状況に構築した．この資源は，ゲノム発現と表現型ををみるのに，極めて重要な資源を形成すると考えられる．

この資源に関しては，ゲノムをめぐる倫理的な側面から，使用に関して極めて厳密な制限が医療センター側から提唱されている．一方，我が国においては，このような資源を使用するにあたって，十分な倫理的土壌が，ユーザーであ

表2.

| A/S | CDR | PMI | NFT | SP | Grain | AA | Lewy | τ-astro | ubq | apoE | NPD |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 92F | 3 | 15:35 | 6 | 3 | 0 | 3 | 2L | 0 | 1 | 44 | AD/AA |

| | | |
|---|---|---|
| A/S: | 年齢，性 | |
| CDR: | clinical dementia rating | 0-3 |
| PMI: | 死後時間 | |
| NFT: | 神経原線維変化，Braakステージ | 0-6 |
| SP: | 老人斑，Braakステージ | 0-3 |
| Grain: | 嗜銀顆粒，我々のステージ | 0-3 |
| AA: | アミロイドアンギオパチー，我々のステージ | 0-3 |
| Lewy: | レヴィー小体病，我々のステージ | 0-5 |
| t-astro: | タウ免疫染色陽性アストロサイト | 0-3 |
| ubq: | 抗ユビキチン抗体陽性顆粒 | 0-3 |
| apoE: | 遺伝子多型 | |
| NPD: | 神経病理学的所見 | |

(AD: アルツハイマー病，AA: アミロイドアンギオパチー)

る研究者側に培われていない背景がある．米国においては，ブレインバンクを用いたゲノム検索を行うにあたり，申請用途以外に用いることは，契約違反として研究者倫理において問題となり，研究費の支給が以後なされないかたちでのしばりがある．またヒト材料の供給を受けて，一定の期間に成果を示せない場合も同様である．しかし日本の場合，これが守れる環境がない．この資源に関しては，疾患とコントロールの遺伝子の一定数を欲しい，現在臨床で問題となっている多型について確認したい，しかしその細かい内容に関しては論文が通っていないから明かせないとか，あるいは疾患とコントロールの遺伝子の差をみることで，疾患の本質に迫りたいので，できるかぎり多数例が欲しいという依頼が基本的なパターンで，よほど公的な基盤に基づいていない限り，非特定研究に多数のゲノムを供与することになり，コンセンサスをえることは困難である．以上の背景より，このDNAリゾースに関しては現段階においては，相手が原則としてNPOであること，老人医療センターの事前審査に十分耐える倫理性と達成可能性を有していること，研究内容が老人総合研究所の研究審査委員会の承認を得るレベルを持つこと，目的外使用に関して監視ができる高い倫理性を貫く環境が存在することを前提にしている．

## 4. 東京都高齢者ブレインバンク（狭義）の創設

1999年9月より，センター・研究所双方の倫理委員会の承認を得つつ，凍結部位の拡大を行った．右海馬のみより，右前頭・側頭・頭頂葉の一部を含め，さらに右側頭葉全体に，そして中脳黒質を含め，さらに基底核と凍結部位を順次拡大し，2001年7月より原則として半脳凍結を開始した．それぞれ凍結資源としては，アルツハイマー病解析のための海馬（現在まで616例），パーキンソン病解析のための黒質（同434例），嗜銀顆粒性痴呆解析のための前頭・側頭葉（423例），Aβ定量のための頭頂葉（532例），パーキンソン病解析のための基底核（363例），半脳（310例）が蓄積された（2004年2月時点）．

凍結方法として，全開頭剖検例に神経病理医が立ち会い，臨床受持医よりの情報と神経放射線画像をもとに，剖検担当医と採取と凍結の方法を事前に協議し決定．脳は，写真撮影後，萎縮・動脈硬化の程度を含め外表観察の上，凍結側（原則として右）の脳を大脳は7 mm厚冠状断（図3），脳幹は5 mm厚水平断，小脳は7 mm厚矢状断割面を作製．この時点で肉眼診断を，臨床・病理担当医に説明した．これには月〜土まで当番制をしいた．

凍結側より，前頭・側頭・頭頂・後頭葉，扁桃核・前方海馬，後方海馬，中脳を採取，4% paraformaldehydeに48時間固定し，パラフィン包埋．6 μm連続切片を，通常染色，鍍銀染色，免疫組織化学染色を施行．一部症例については，電顕，免疫電顕標本を作製した．また症例により後方海馬はtissue compoundに埋め迅速固定した．固定前の割面の評価で，標本にすべき病変がある場合は適宜追加した．

凍結は，バイオハザード内のブレインバンクルームの安全キャビネット内で（図4），携帯用冷凍庫にドライアイスを敷き，ディープリーザー内で冷やした銅板を乗せ，脳をその上に載せたあと，ドライアイスパウダーを上からかけて凍結した（図5）．これらには，技師が月〜土までオンコールで対応する体制を強いた．それに伴い，ブレインバンクの構成を整えた（表3）反対脳は20%中性緩衝ホルマリンに7日から13日固定後，神経内科と当研究グループ合同ブレインカッティングカンファランスで肉眼的に検討の上，組織学的検索に，代表的部位を切り出した．

現在までに，AD資源として，海馬74例，側頭葉48例，半脳36例が蓄積された．PDとして，黒質34例，基底核30例，半脳14例，嗜銀顆粒性痴呆として海馬48例，側頭葉37例，半

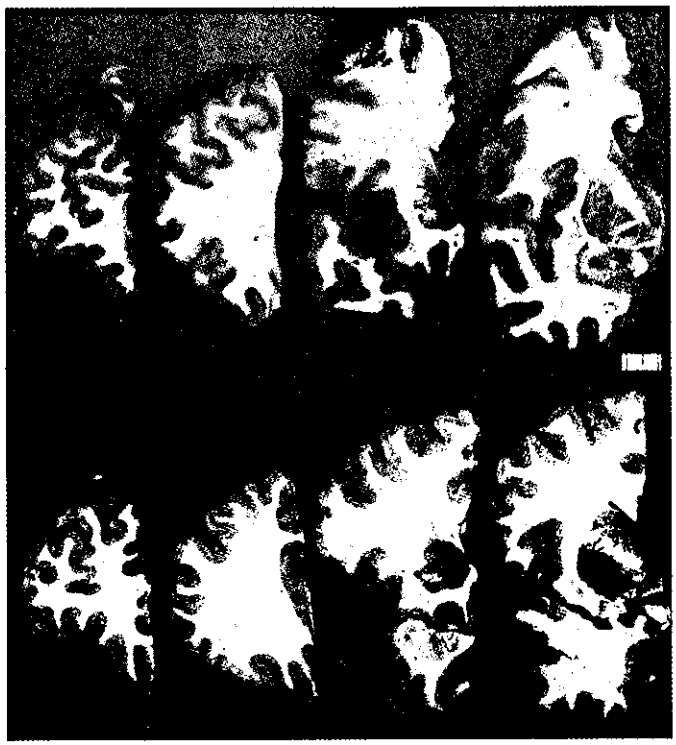

図 3. 新鮮脳のブレインカッティングによる評価．全例に神経病理担当者が立ち会い，生で半脳をブレインカッティングし，評価．撮影写真は提供する標本の部位を明示するのに使用．a: Huntington 病，b: 正常コントロールで，固定前の状況でも十分評価可能である．

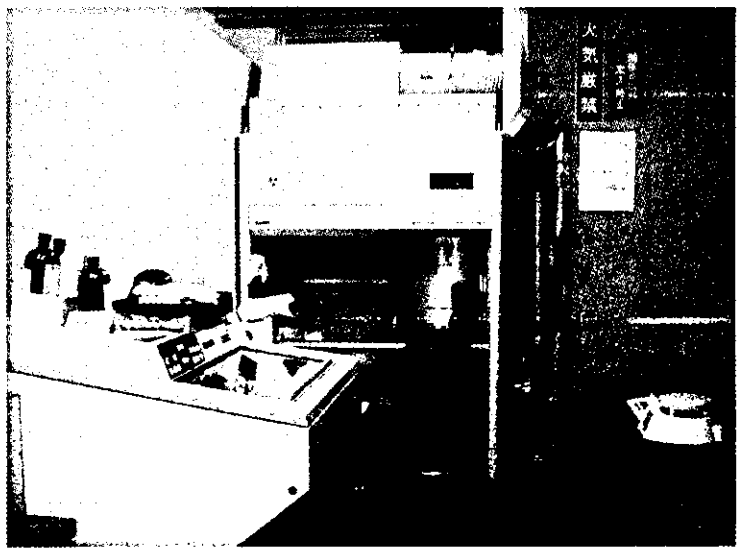

図 4. ブレインバンクルーム．バイオハザードエリアとして，安全キャビネット内で凍結操作を行い，クリオスタット内で，凍結材料の切り出しを行う．

表 3.

ブレインバンク構成員
　ブレインバンクディレクター
　ブレインバンクドクター
　ブレインバンクテクニシャン
　ブレインバンクセクレタリー
事前審査委員
　ブレインバンクディレクター
　東京都老人総合研究所長（兼東京都老人医療センター院長）
　東京都老人医療センター側責任者（副院長）
　同　　病理責任医長
　同　　神経内科責任医長
協力者：東京都老人医療センター兼務研究員
　　（神経内科全員・病理科・リハビリテーション科）
　　東京都老人総合研究所ポジトロン医学研究施設
外部アドバイザリーボード

表 4.　東京都高齢者ブレインバンク（狭義）

あらゆるヒト脳研究の資源
　詳細な臨床情報が独自（共同研究をベースにする）
　死後時間 4：00 未満が 1/4
　医師・患者（遺族）の信頼関係がかけがえのない資源

| | |
|---|---|
| アルツハイマー病とその初期： | 36 例 |
| 嗜銀顆粒性痴呆とその初期： | 30 例 |
| パーキンソン病関連とその初期： | 14 例 |
| 神経原線維変化優位型痴呆： | 9 例 |
| 進行性核上性麻痺： | 6 例 |
| 血管障害性痴呆： | 12 例 |
| 正常コントロール： | 19 例 |

1. 老化研究を志す，若い研究者に貢献する
2. 「養育院」の哲学：高齢者の運動・認知障害の，予防・治療につながる研究には最大限の協力を行う


図5. 迅速凍結法.
a. −20度の携帯用冷凍庫に，ドライアイスの板を引き，超低温漕内で冷やした銅板をその上におき，アルミホイルをしき，その上にスライス脳を並べる.
b. 細かく砕いたドライアイスを上にかけ，瞬時に凍結する.

脳30例，神経原線維変化優位型痴呆として海馬14例，側頭葉11例，半脳9例，進行性核上性麻痺として海馬11例，側頭葉10例，半脳6例，正常コントロールとして海馬38例，半脳19例が蓄積された．半脳分について，表4に示す.

実際の供給部位の同定には，固定前の脳の割面(図3)をマークすることで部位を明示し，切り出しはやはりブレインバンクルームのクリオスタット内(図4)で，ドライアイスを敷き，厳密な対応をとりながら，彫刻刀と木槌を使いながら，原則として共同研究者の立ち会いの元に行っている.

## 5. 現時点での総括と今後の展望

在宅を基本とし，開放病棟しか有さず，死因のほとんどが一般内科的疾患であることより，当施設の脳には正常コントロール，並びに変性疾患とした場合には早期病変が多い点が特徴的である．この意味で，欧米のブレインバンクにはない独自性がある．しかし，ブレインバンクとしての同意がとれないため，剖検承諾書の範囲内で共同研究のかたちで行わざるを得ないこと，在宅高齢者が中心なので，重度痴呆例が少ないことが問題となる．また，欧米型のブレインバンクに移行させる上での問題として，本施設では病歴が全て保管されており，後方視的研究が可能であるのに，ブレインバンクの場合は匿名化が原則なので，資源を本当の意味で有用に活用できなくなる点も憂慮される.

ブレインバンクの試みとして，現在福島医科大学精神科が福島県内で構築しているものがある．これは分裂病をはじめとする精神疾患克服のためとして，患者団体の同意のもと，医師の熱意により運営されている格好をとっている．全剖検は大学病院で行い，精神科にバンクを置くことを前提としている．また，ドナーカードにならって，献脳の意志の有無を示すカードを置いている．ただ症例数が極めて少ないのが問題である．この生前同意システムの構築は，当施設でも検討すべきと考え，現在患者遺族の方にコーディネーターとして協力を得たので，今後展開する予定である.

運用規定（表5）として，共同研究申し込み，事前審査承認後，共同研究施設，研究所・センター双方の倫理委員会の承認，かつ共同研究として行うにふさわしいかを研究所の研究審査会で審査の上，研究所協力研究員として委嘱するにふさわしいかどうかを研究所幹部会（最高議決機関）で承認を受けることを前提とし，定期的研究状況報告を行うこと，要請があれば直ちに資源を返却することを，条件としている．現

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表 5. 運用規定

- 共同研究申し込み
- 事前審査（研究所・センター合同）
- 倫理委員会の承認
  - 共同研究者の所属施設
  - 東京都老人総合研究所
  - 東京都老人医療センター
- 共同研究内容審査
- 共同研究者に対し，研究内容を含め，研究所幹部会（最高決定機関）で承認し，協力研究員として委嘱
- 研究過程の定期的報告
- 要請があった場合の組織の返却

時点で，研究所内4研究グループ，外部18研究施設と共同研究中である．

米国においては，ブレインバンクの存在のため，ヒト材料を用いて神経科学研究を行うことが定着している．ただ，先ほど述べた目的外使用の厳禁以外に，半年ごとの使用状況報告，1年半ごとのサイトビジトによる研究遂行の状況チェック等，厳しい審査が常識であり，日本の場合のように，一応ためておいて，そのうちデータを出そうというかたちでの依頼はそもそも存在しない．さらに，日本の神経科学研究者の多くが，ヒトの脳を使う研究の割り振りになれておらず，あまりに厳密な方法論をあてはめてヒト脳は使い物にならないと結論したり，逆に解剖・疾患に関する知識の欠除から，とんでもない部位，対照疾患及び対象数を要求してくることが多い．当初我々は，データベースを匿名化し，症例の選択を研究者に任せるかたちをとっていたが，現段階でこの方法は誤りであったと反省している．バイオインフォマティクスの観点から，高齢者の臨床・病理の両方の専門家である我々のグループが，研究計画の申し入れに対し，どのような疾患のどの部位の組合せが最も成果を得ることが可能かを十分討論していくことが，最も実りある成果を生みうるというのが，現時点の結論である．

倫理性に関しては，基本的に欧米のブレインバンクと連絡をとりながら，それとほぼ等しいレベルをキープすること，ただし研究の遂行度に関しても，同様の厳しさでの適応を標準としている．これは，日本の多くの倫理委員会が余りに厳しい基準をおいて，研究そのものが行えなくなっている実情に，欧米からの批判が寄せられている点に呼応している．

最近問題となっている点に，知的資産の問題がある．ブレインバンク自身は公的ドメインに属し，公共の福祉に属するとしても，バイオインフォマティクスに関しては，知的資産が生じる．さらに問題なのは，ブレインバンクの運営費用をどこが負担するかである．欧米においてはコンセンサスのもとに，公的資金が導入されているので問題はないが，我々のところのブレインバンクの構成員で，東京都からの派遣職員は，2004年4月からは責任者ひとりとなる予定である．これは，全く新しい体制を持ち込んだため，研究所内にありながら臨床サービスと同様の忙しい状況を作っているため，旧来の人的資源を用いることができないことが原因である．さらに日本の宿命であるが，研究所自体このようなインフラストラクチャーにさける技師を，常勤として確保することが困難であることに起因している．さらにNPOの位置づけであるが，国立大学独立行政法人化のため，この定義付けもあいまいとなり，厚生労働省管轄下の研究所のみが，この基準を満たすことになりかねない．逆に営利企業に使用権がないとすると，

研究の発展と資源の活用と言う意味ではこれも大きな問題となる．我々の立場は，1. 公的な研究費の援助申請を行うことで，公共性を保つ努力をすること，2. 営利企業からの申し込みに関しては，このブレインバンクが役に立つという認識があるなら，コンソーティウムのようなかたちでこのバンクを支える努力をお願いしたいこと，3. 知的資産に関しては，そのような成果があがった段階で協議することを前提にすること，以上の三点を基準とすることを，現時点では考えている．

## 6. 結　語

老化に伴う運動・知的機能低下の予防・治療のための研究資源としての，高齢者ブレインバンク設立の努力と現在までの到達点を述べた．痴呆克服のためのインフラストラクチャーとしては必須であり，かつそこで得られた病理学的所見は，在宅での痴呆対策の基礎をなすと考えられる．当初目的とした資源提供の面は守りつつ，我々の有するバイオインフォマティクスを生かすかたちで，共同研究を前提とした運用を行うことが，最も実りある運用と考える．

## 謝　辞

東京都高齢者ブレインバンクは，下記の方々の協力なしには運営が不可能であるので，氏名のみ列挙する．東京都老人総合研究所ポジトロン医学研究施設：石井賢二；東京都老人医療センター病理：沢辺元司，新井冨生，笠原一郎；東京都老人医療センター神経内科：山之内博，金丸和富，三谷和子，榎本武郎，吉野正俊，小宮正，椎名盟子，渡邊睦房，村上喜生，仁科一隆，砂川昌子，久保田暁，各位(敬称略)．またブレインバンクドクター（非常勤）として，日本医科大学第二内科（神経内科）山崎峰雄博士，東京大学大学院医学系研究科人体病理，柴原純二博士の協力を得た．さらに30年以上にわたるハートカッティングに基づく循環器データベースを提供下さった，千田宏司循環器部長，谷口泰医師各位，感染症科として痴呆患者の終末ケアに積極的に取り組む増田義重医師，高齢者血液疾患に積極的に取り組んでおられる太田雅嗣血液内科部長をはじめとする，東京都老人医療センター医師団，家族のご希望による剖検目的の転入院にご理解を示して下さった東京都老人医療センター看護団，さらにブレインバンクの意義を高く評価して下さっている東京都老人医療センター首脳陣，最後にブレインバンクの構築に積極的援助を常に賜って下さっている，東京都老人総合研究所首脳陣並びに事務担当官諸氏に，深謝する．

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# *In situ* detection of apolipoprotein E ϵ4 in archival human brain

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Received 28 January 2004; accepted 27 February 2004

DOI: 10.1097/01.wnr.0000125781.58139.3b

A monoclonal antibody specific to apolipoprotein E 4 (apoE4) was applied immunohistochemically to archival human brain tissue. The examined 30 cases comprised four ϵ/ϵ4, 10 ϵ3/ϵ4, one ϵ2/ϵ4, 10 ϵ3/ϵ3 and five ϵ2/ϵ3 genotypes. The anti apoE4 antibody visualized senile plaques, neurofibrillary tangles and reactive astrocytes, as well as serum in the blood vessels and vascular smooth muscle cells in the cases of ϵ4. Moreover, the staining intensity was stronger in the cases carrying the ϵ4 homozygosity than in those cases of ϵ4 heterozygosity. Specific immunoreactivity was not obtained in those cases not carrying the ϵ4 allele. This method will allow *in situ* detection of apoE ϵ4 and contribute to studies of the effect of ϵ4 on Alzheimer's disease. *NeuroReport* 15:1113–1115 © 2004 Lippincott Williams & Wilkins.

**Key words:** Apolipoprotein E; Apolipoprotein E4; Alzheimer's disease; Immunohistochemistry; Neurofibrillary tangle; Senile plaque

## INTRODUCTION

The involvement of the apolipoprotein E (apoE) ϵ4 allele in Alzheimer's disease (AD) has now been established [1]. ApoE ϵ4 has also been reported to promote Alzheimer-type pathology in various settings [2]. However, only a limited number of pathological studies concerning apoE ϵ4 are available, partly because of the difficulty in obtaining autopsy materials from patients whose apoE genotyping is available. In this study, we employed a monoclonal antibody that specifically recognizes apoE4 [3] in immunoblots. We applied this antibody to human brain tissue in order to establish an immunohistochemical method to identify not only the presence of apoE4, but also the homo- and heterozygosity of apoE4. This method will contribute to further morphological studies of the biological role of the apoE ϵ4 allele in the progression of Alzheimer-type senile changes.

## MATERIALS AND METHODS

*Tissue source:* Four ϵ4/ϵ4, 10 ϵ3/ϵ4, one ϵ2/ϵ4, ten ϵ3/ϵ3 and five ϵ2/ϵ3 patients were selected serially from our archives. Three ϵ4/ϵ4, three ϵ3/ϵ4, three ϵ3/ϵ3 and two ϵ2/ϵ3 patients had AD. ApoE genotype was determined from genomic DNA that was extracted from the kidney, which was snap-frozen at autopsy, as reported previously [4]. The right hippocampus was fixed in 4% paraformaldehyde for 48 h and the left was fixed in 20% buffered formalin for 7–13 days. Both tissues were embedded in paraffin with an automatic tissue processor. Serial sections (6 μm were obtained from each block and stained immunohistochemically with antibodies to amyloid β (Aβ) (122B2, monoclonal, aa. 11-28 and Aβ1-42, polyclonal, IBL, Maebashi, Japan), phosphorylated τ (ptau) (AT8: monoclonal, Ser/Thr 202/205, Innogenetics, Temse, Belgium; and AP422, polyclonal, Ser-422, a kind gift from Dr. Y. Ihara), phosphorylated α-synuclein (psyn) (psyn#64: monoclonal, Ser 129 [5]; and Pser129, polyclonal, Ser129 [6], a kind gift from Dr. T. Iwatsubo) and glial fibrillary acidic protein (polyclonal, Dako) as reported previously [4]. For light microscopic observation, an automatic apparatus (Ventana 20NX, Tucson, AZ, USA) was used for single and double immunostaining as reported previously [4].

*ApoE ϵ4 immunocytochemistry:* A monoclonal antibody (Ab412-1-7) was previously raised against a synthetic peptide, ADMEDVRGRLV, that corresponds to amino acid residues 106–116 of apoE ϵ4. The antibody specifically recognizes apoE ϵ4 in immunoblots [3]. Anti-apoE antiserum (IBL, Maebashi, Japan) was employed for comparison. For confocal microscopic observation (Zeiss, LSM 5, PASCAL), the anti-apoE4 antibody was visualized with anti-mouse IgG Alexa 488 (Molecular Probes, Eugene, OR) and the polyclonal antibodies were visualized with anti-rabbit Alexa 568.

## RESULTS

The anti-apoE4 antibody stained the sections from the apoE ϵ4 homozygous patients intensely enough to be interpretable with semi-macroscopic observation (Fig. 1a). Light-microscopically the anti-apoE ϵ4 antibody visualized serum in blood vessels (Fig. 1d inset), vascular smooth muscle cells and atheromatous plaques (Fig. 1d). Senile plaques (SPs), neurofibrillary tangles (NFTs; Fig. 1d) and amyloid angiopathy were also visualized intensely. Reactive astrocytes were also detected around these lesions. Some Lewy bodies

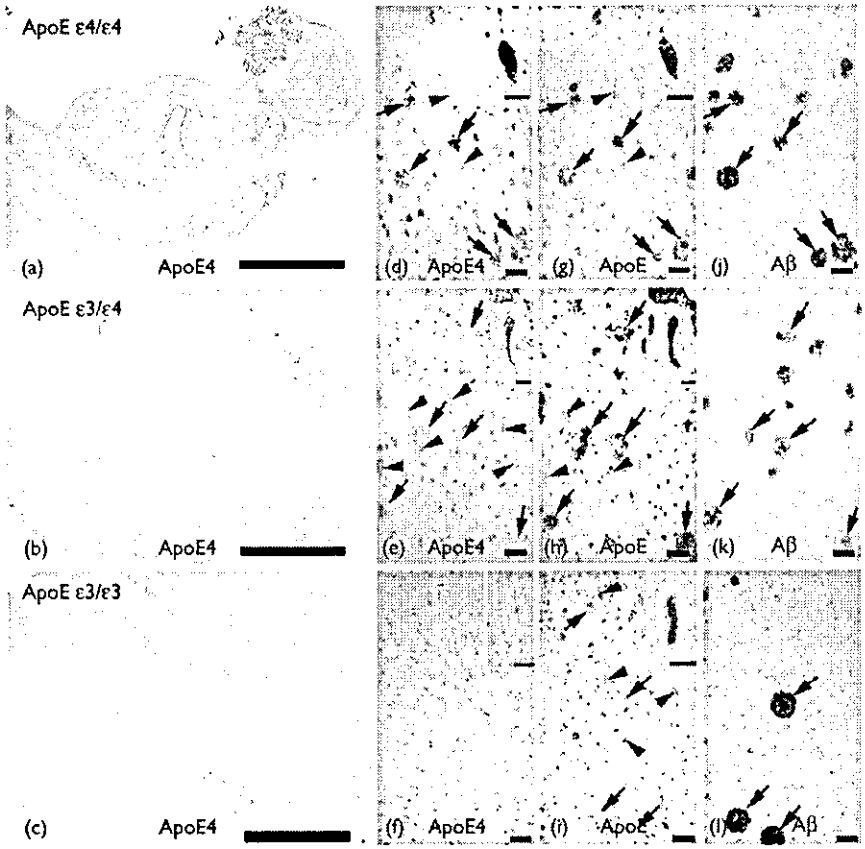
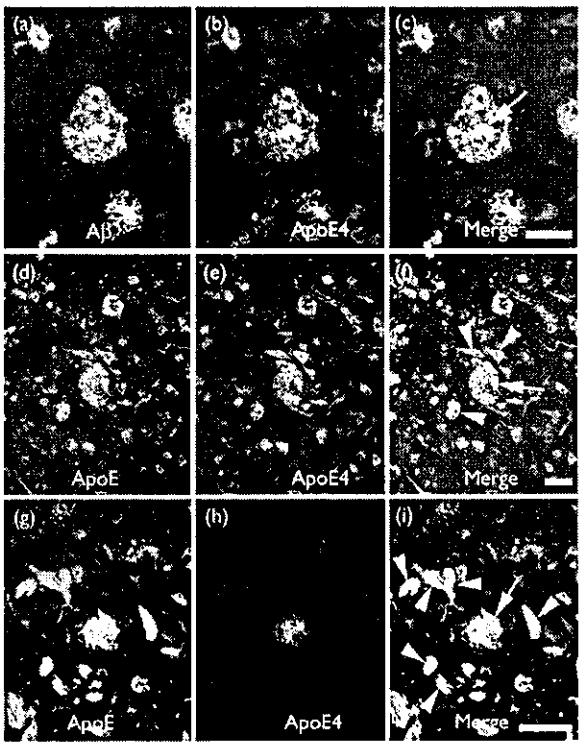



**Fig. 1.** *In situ* immunohistochemical detection of apolipoprotein E4 phenotype. Anti-apoE 4 antibody discerns ε4 homozygosity (**a**), heterozygosity (**b**) and absence of the allele (**c**) at the semimicroscopic level (bar=1 cm). At higher magnification, neurofibrillary tangles (NFTs; arrowheads) and senile plaques (SPs; arrows) as well as serum in the blood vessel (inset) were intensely (**d**), weakly (**e**) and not stained (**f**), respectively. Anti-apoE antibody intensely stained serum in the blood vessel of each case (inset), but the staining intensity of NFTs and SPs in the serial sections was stronger in cases carrying the apoEε4 allele (**g**,**h**), than in cases without the ε4 allele (**i**). Anti-Aβ antibody vizualized SPs in the next serial section (**j**–**l**). Bar=50 μm.




were visualized (not illustrated) but argyrophilic grains were never stained.

In apoE ε4 heterozygous patients the staining intensity was usually weaker than that in the homozygotes (Fig. 1b). The staining intensity of serum, NFTs and SPs was generally weaker in heterozygotes than in homozygotes (Fig. 1e inset). There was no apparent difference between the ε2/ε4 patient and the 10 ε3/ε4 patients. No specific immunoreactivity was obtained from the patients without apoEε4 allele (Fig. 1f), including serum in the blood vessel (inset).

The anti-apoE4 antibody recognized apoE4 both in tissues fixed in 4% paraformaldehyde and in those fixed in 20% buffered formalin, but the former showed more intense and discrete staining and could reliably distinguish the homozygous from heterozygous cases. On the other hand, no specific immunoreactivity was found with anti-apoE4 immu-

**Fig. 2.** Confocal studies of the epitope of apolipoprotein E (apoE) 4 with amyloid β (Aβ, 1–42; **a**–**c**) and apoE (**d**–**l**). Bar=50 μm. (a–c) The Aβ epitope was almost 100% co-localized with apoE4 in cores (arrow) and crowns of the senile plaques (patient I). (d–f) Co-localization of apoE4 with apoE in an ε4 homozygous patient. One hundred percent co-localization was observed with similar staining intensity of neurofibrillary tangles (arrowheads) and dystrophic neurites of senile plaques (arrow) among the moderately stained neuropils. (g–l) Co-localization of the epitope of apoE4 with apoE in an ε4 heterozygous patient. The staining intensity of neurofibrillary tangles (arrowheads) and the periphery of senile plaque was distinctly weaker than that of the center of senile plaques (arrow) among the very weak staining neuropils.

nostaining in any of the patients without the apoE ε4 allele, irrespective of the method of fixation.

The anti-apoE antiserum visualized serum in the blood vessel intensely in all the patients examined (Fig. 1g–i, inset). The staining intensity of NFTs and SPs was stronger in patients carrying the apoE e4 allele (Fig. 1g, h) than in patients without the allele (Fig. 1i).

*Confocal laser-microscopic observation:* The epitope of the anti-apoE ε4 antibody was co-localized with the Aβ epitope in cores and dystrophic neurites of senile plaques (Fig. 2a–c), with that of ptau in neurofibrillary tangles and occasionally in neuropil threads, and with that of psyn in some Lewy bodies, Lewy threads and Lewy dots (not illustrated). The co-localization was almost 100% with Aβ, followed by ptau and then psyn. There was 100% co-localization between apoE and apoE4. However, the staining intensity of NFTs and neuropil threads as well as the rims of the senile plaques with the anti-apoE4 antibody was distinctly weaker in the heterozygotes than in the homozygotes, although the cores of the senile plaques showed similarly strong immunoreactivity (Fig. 2d–i).

## DISCUSSION

We first established a method for *in situ* detection of apoE ε4. The method is quite simple and highly reliable. To keep the conditions of each brain as constant as possible, all bodies for autopsy were kept at four degrees centigrade within 2 h after death. The period of fixation in paraformaldehyde was strictly controlled and an automatic tissue processor was employed to achieve consistent processing. Furthermore, an auto-immunostainer was employed in order to avoid technical variation. Keeping these conditions constant, differentiation between homo- and heterozygosity of apoE ε4 may be possible.

Regarding the sections fixed in buffered formalin, anti-apoE4 antibody specifically detected patients carrying the apoE ε4 allele, without exception. However, a staining difference between the anti-apoE antiserum and the anti-apoE4 antibody did not always support apoE ε4 heterozygosity, probably because the monoclonal antibody requires more optimal fixation for the appropriate staining.

From the biological point of view, it is important to know how apoE ε4 promotes the progression of Alzheimer-type senile changes. The study of the co-localization of the epitope of apoE4 and senile plaques, neurofibrillary tangles and Lewy bodies showed the strongest correlation with Aβ, followed by phosphorylated tau and then phosphorylated α-synuclein.

The staining intensity of serum with anti-apoE antiserum did not differ among the patients examined, but that of NFTs and SPs was stronger in those carrying the apoE ε4 allele. The staining intensity of NFTs and neuropil threads as well as the rims of the senile plaques with the anti-apoE4 antibody was distinctly weaker in the ε4 heterozygotes than in the ε4 homozygotes, although the staining intensity of the core of the senile plaques was no different [9]. This may indicate stronger affinity of apoE4 for amyloid beta and ptau than for apoE3. In the amyloid cascade hypothesis, there is no explanation for how the burden of Aβ induces neocortical extension of neurofibrillary tangles, which can appear independently from Aβ in the limbic system. It is possible that apoE4 may play a role in this mechanism by anchoring ptau in the neocortical neurons more effectively.

With the method reported here, it is possible to detect apoE ε4 in archival human brain tissue. Although it is difficult to determine the heterozygosity or homozygosity of apoE ε4 with routine fixation, detection of the presence or absence of apoE ε4 in the histological sections is quite helpful for studying the pathological contribution of apoE ε4 to the promotion of Alzheimer-type senile changes. The application of this antibody to carefully fixed tissues combined with apoE genotyping may help to clarify the mechanism by which apoE ε4 enhances Alzheimer-type senile changes.

## CONCLUSION

We report here an immunohistochemical method for *in situ* detection of the apoE ε4 allele in archival human brain using a monoclonal antibody raised against a specific sequence of apoE4. A comparative immunohistochemical study with the anti-apoE4 monoclonal antibody and an anti-apoE antiserum suggested that apoE4 may have stronger affinity to senile plaques and phosphorylated tau than apoE3, indicating a possible promoting role of apoE ε4 in the progression of aging-associated limbic tauopathy to AD-related neocortical tauopathy. This method is quite simple and reliable and will contribute to further elucidation of the role of apoE4 in exacerbating Alzheimer-type pathology.

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Acknowledgements: We thank Ms. Hiroko Matsuoka, Nobuko Naoi and Mr. Naoo Aikyo for technical support. This work was supported in part by grants-in-aid from the Ministry of Education and Scientific Technology, Japan.

Journal of Neuropathology and Experimental Neurology

Vol. 63, No. 7
July, 2004
pp. 742–749

# Lewy Body-Related α-Synucleinopathy in Aging

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**Abstract.** To clarify the significance of Lewy body (LB)-related α-synucleinopathy in aging, we investigated the incidence of LBs in 1,241 consecutive autopsy cases (663 males and 578 females). LB pathology was identified histologically in sections stained with hematoxylin and eosin and with anti-ubiquitin and anti-α-synuclein antibodies. Cases without LBs were classified as LB stage 0 (987 cases). Cases with LBs were classified as follows: LB stage I = incidental LBs (149 cases); LB stage II = LB-related degeneration without attributable clinical symptoms (47 cases); LB stage III = Parkinson disease without dementia (10 cases); LB stage IV = dementia with Lewy bodies (DLB) transitional (limbic) form (25 cases); and LB stage V = DLB neocortical form (23 cases). The average age at death was greater for those cases with LBs. There were no gender differences in the LB pathology. G842A polymorphism in the paraoxonase 1 gene was associated with men in LB stage II or above and suggests a gender-specific risk factor. LB stage V had higher stages of neurofibrillary tangle and senile plaque involvement and also had a higher frequency of apolipoprotein E ε4. Our findings indicate that LBs are associated with cognitive decline, either independently or synergistically with neurofibrillary tangles and senile plaques.

**Key Words:** Alzheimer disease; Apolipoprotein E; Dementia with Lewy body; Neurofibrillary tangle; Paraoxonase 1; Parkinson disease; Senile plaque.

## INTRODUCTION

Lewy body (LB)-related α-synucleinopathy is one of the most important post-translationally modified protein accumulations in the aging human brain. However, unlike senile plaques (SPs) or neurofibrillary tangles (NFTs), only limited studies are available on the incidence and biological significance of LBs in age-related motor and cognitive decline (1).

Tokyo Metropolitan Geriatric Hospital (TMGH) serves as a community-based care facility for the elderly in the Tokyo metropolitan area and performs postmortem examinations on a relatively high percentage of hospital cases, irrespective of their clinical symptoms and cause of death. The brains from these cases are ideal for evaluating the incidence of pathological processes in the aging population. As a routine procedure at TMGH, the brain is bisected at the time of autopsy, one hemisphere is deep-frozen and other hemisphere is sampled for light and electron microscopic examination. In this study, we investigated the incidence of LB changes, their contribution to parkinsonism and dementia, and their association with apolipoprotein E (ApoE) and paraoxonase 1 (PON1) genotypes in the most recent 1,241 autopsy cases at TMGH. Our findings indicate that LBs may independently or synergistically contribute to cognitive decline.

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Grants in aid: Tokyo Metropolitan Institute of Gerontology.

## MATERIALS AND METHODS

### Tissue Source

One thousand two hundred forty-one consecutive autopsy brains at TMGH over the past 5 years were the basis of the present work. The patients' ages ranged from 48 to 104 years, with a mean age of 80.6 ± 8.9 years, and a male to female ratio of 663:578.

### Clinical Information

Clinical information, including parkinsonism and cognitive state, was obtained from medical charts and interviews with the patients' personal physicians and caregivers. The Mini-Mental State Examination (MMSE) (2) or the Hasegawa dementia scale (3) was employed for evaluation of cognitive function, and a clinical dementia rating (CDR) (4) was used for grading of dementia. Almost all cases of suspected degenerative dementia received a clinical diagnosis of "senile dementia" based on the recognition that the final diagnosis should be made after postmortem examination of the brain.

### Neuropathology

Formalin-fixed (20% neutral buffered formalin), paraffin-embedded sections of representative areas of the brain were examined, following the recommendations of the Consortium to Establish a Registry for Alzheimer Disease (CERAD) (5) and the consensus guidelines for the diagnosis of dementia with Lewy bodies (DLB) (6). Areas examined included frontal pole, cingulate gyrus, amygdala, temporal neocortex, anterior and posterior hippocampus with entorhinal and transentorhinal cortex, motor cortex, parietal lobe including the intraparietal sulcus, visual cortex, basal ganglia and hypothalamus at the level of the mamillary body, subthalamic nucleus, thalamus at the

TABLE 1
Correlation Between Lewy Body Stage, Lewy Body Score and Clinical Symptoms

| | LB score | Parkinsonism | Dementia | Total cases |
|---|---|---|---|---|
| Stage I | 0–1 | NA* | NA* | 149 |
| Stage II | 0–10 | NA* | NA* | 47 |
| Stage III | NA** | 10 cases | 0 cases | 10 |
| Stage IV | 3–6† | 10 cases | 25 cases | 25 |
| Stage V | ≥7†† | 10 cases | 23 cases | 23 |

LB: Lewy body; LB score: Lewy body score by consensus guidelines (6); NA: not applicable.

* By definition, stage I and stage II cases have neither parkinsonism nor dementia attributable to LB-related neuronal degeneration.

** LB scoring was originally developed for dementia with Lewy bodies (DLB) and not for Parkinson disease without dementia. However, if our LB stage III cases (PD without dementia) were scored, their LB score would be 3 to 6.

† Lewy body score of 3 to 6 or greater than 6 with at least 1 neocortical score of zero.

†† Lewy body score of 7 or greater and no neocortical score of zero.

TABLE 2
Relationship of Lewy Body Stages to Parkinson Disease Without Dementia (PD), Parkinson Disease With Dementia (PDD), and Dementia With Lewy Bodies (DLB), Following the Nomenclature of the 1996 Consensus Guidelines for Dementia With Lewy Bodies (6)

| | PD | PDD | DLB |
|---|---|---|---|
| LB Stage III | 10 cases | | |
| LB Stage IV | | 8 cases | 17 cases |
| LB Stage V | | 5 cases | 18 cases |
| Average age (years) | 77.2*‡ | 82.3* | 85.1‡ |

The average age at death in PD without dementia is significantly younger than that in PDD or DLB.

* $p = 0.031$.

‡ $p = 0.0014$.

level of the red nucleus, midbrain, upper and middle pons, medulla oblongata, cerebellar vermis, dentate nucleus, and the cervical, thoracic, and lumbar spinal cord.

Six-μm-thick sections were routinely stained with hematoxylin and eosin (H&E) and the Klüver-Barrera method. Selected sections were stained with the modified methenamine silver (7) and Gallyas-Braak silver methods (8) for senile changes, with Congo red for amyloid deposition, and with elastica Masson trichrome for vascular changes.

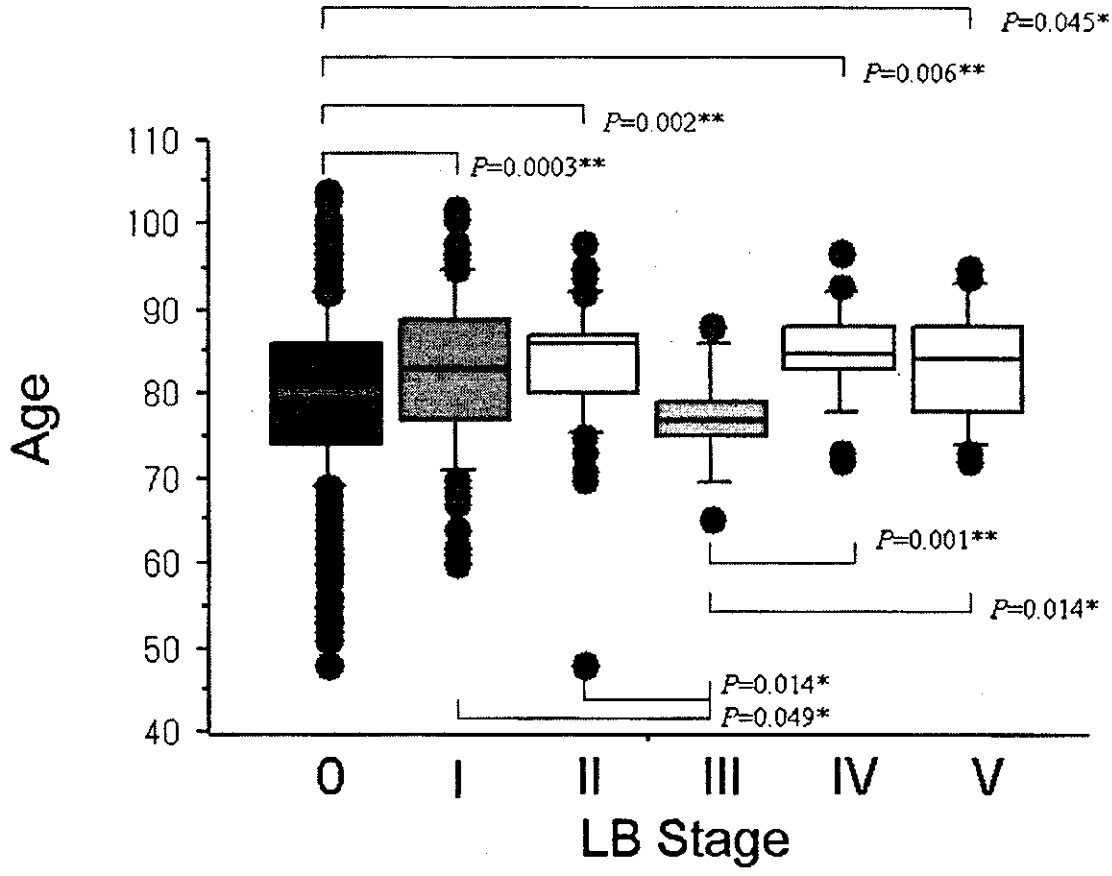


**Fig. 1.** Age distribution in each stage. The average age at death in Lewy body (LB) stages I, II, IV, and V was significantly greater than in LB stage 0 or LB stage III.

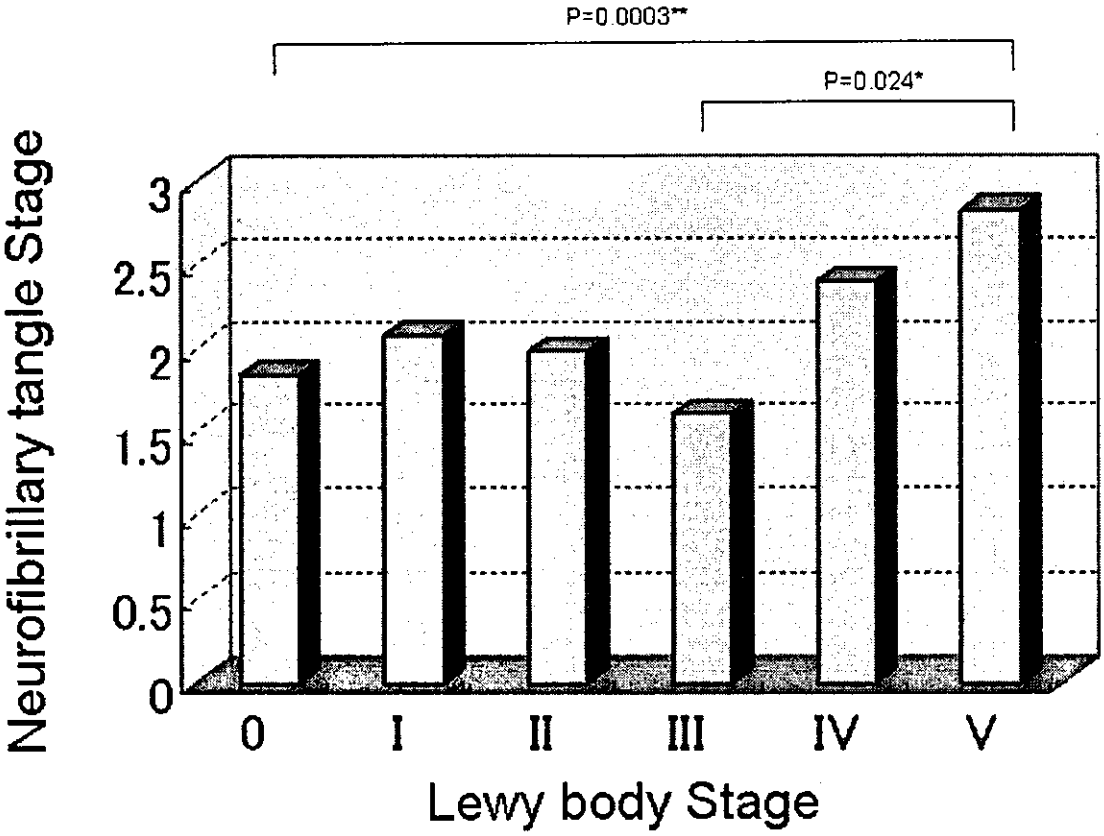


**Fig. 2.** Lewy body stage versus neurofibrillary tangle (NFT) stage. NFT stage is significantly higher in Lewy body (LB) stage V than in LB stage 0 or LB stage III.

### Immunohistochemistry

Six-μm-thick serial paraffin sections were immunohistochemically stained using a Ventana 20NX autostainer (Ventana, Tucson, AZ), as previously described (9). The antibodies employed were as follows: anti-α-synuclein (LB509, monoclonal, kind gift from Dr. T. Iwatsubo); phosphorylated α-synuclein (psyn) [psyn#64 (10) and Pser129 (11)]; phosphorylated tau (ptau) (AT8, monoclonal, Innogenetics, Temse, Belgium); amyloid β (Aβ)11–28 (12B2, monoclonal, IBL, Maebashi, Japan); Aβ1–42 (polyclonal, IBL); ubiquitin (polyclonal, Sigma-Aldrich, St. Louis, MO); glial fibrillary acidic protein (GFAP)(polyclonal, DAKO, Glostrup, Denmark); and HLA-DR (monoclonal, CD68, DAKO). Sections of midbrain and amygdala from all cases were stained with anti-ubiquitin and anti-α-synuclein antibodies. Additionally, in the most recent 600 cases, sections of medulla oblongata at the level of dorsal motor nucleus of vagus, upper pons at the level of locus ceruleus, midbrain, basal ganglia, entorhinal cortex, amygdala, and the anterior cingulate, second frontal, temporal, and supramarginal gyri were stained with anti-α-synuclein and anti-psyn antibodies.

### Evaluation of Lewy Body-Related Neuropathology

Histologic sections of brain were initially evaluated for LB pathology with H&E staining and with anti-ubiquitin immunohistochemistry. The presence of LB pathology was confirmed by immunohistochemistry with anti-α-synuclein and anti-psyn antibodies, and the "LB score" for each case was calculated following consensus guidelines (6).

### Evaluation of Other Disorders Presenting with Dementia and/or Parkinsonism

Our modification (12) of the NIA-Regan criteria (13) was used for the diagnosis of Alzheimer disease (AD). The diagnoses of "dementia with grains" (DG) and "neurofibrillary tangle-predominant form of dementia" (NFTD) were based on Jellinger's criteria (14, 15). The diagnosis of vascular dementia was based on NINDS-AIREN criteria (16).

### Semiquantitative Analysis

LB pathology was classified into 6 LB stages according to our previously published criteria (10). These 6 stages are as follows: LB stage 0 = no LBs; LB stage I = scattered LBs without cell loss; LB stage II = abundant LBs with macroscopic loss of pigmentation in substantia nigra and locus ceruleus and/or gliosis demonstrated by GFAP immunohistochemistry in areas containing LBs but without attributable parkinsonism or dementia; LB stage III = PD without dementia; LB stage IV = DLB, transitional (limbic) form (DLBT); and LB stage V = DLB, neocortical form (diffuse Lewy body disease) (DLBN). Because of controversy surrounding the definition of PD with dementia (PDD), we included PDD as a subgroup in LB stages IV and V.

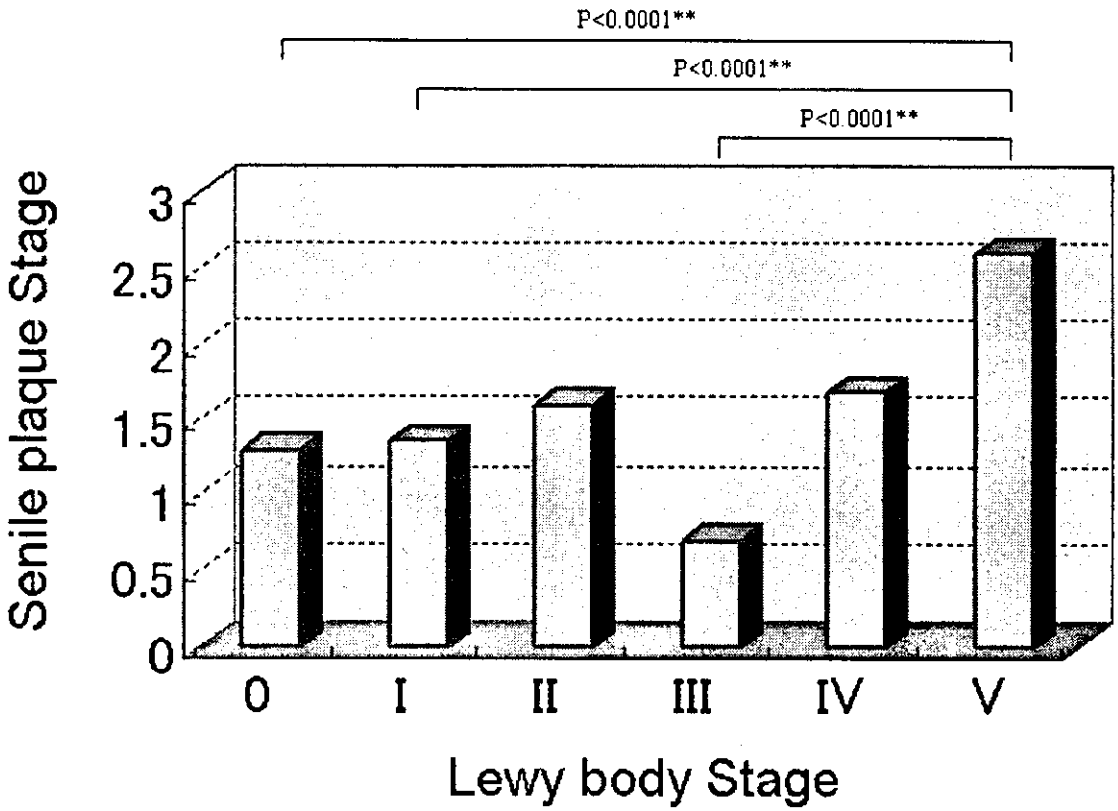


**Fig. 3.** Lewy body stage versus senile plaque (SP) stage. SP stage is significantly higher in Lewy body (LB) stage V than in LB stage 0, LB stage I or LB stage III.

The presence of NFTs and SPs was evaluated with H&E, Klüver-Barrera, Gallyas-Braak, and modified methenamine silver stains and confirmed immunohistochemically with anti-ptau and Aβ antibodies. NFT pathology was classified into 7 NFT stages, and SP pathology was classified into 4 SP stages, based on the Braak criteria (17).

### Molecular Pathology

Genomic DNA was extracted from frozen kidney obtained at autopsy. The genotyping of ApoE was done as previously reported (9) in 1,114 cases from January 1997 to September 2003. The genotyping of the PON1 gene was determined on Q191R, L54M, G(−907)C, G(−824)A, T(−107)C, G(−161)A, and G(−125)C polymorphisms (18–21) in 511 cases from January 1997 to August 2000. The interval of the study of each genotyping was determined separately by the legal committee of Tokyo Metropolitan Institute of Gerontology and TMGH.

### Statistic Analysis

Statistical analysis was performed using chi-square test or Fisher exact test for comparisons of categorical data, Student *t*-test for comparison of means for continuous outcomes, Mann-Whitney *U*-test for nonparametric analysis, and Spearman correlation coefficient by rank for correlation of discrete scores. Statistical significance was established at the $p < 0.05$ level.

## RESULTS

### Clinical Profiles

Parkinsonism was reported in 66 (5.3 %) of 1,241 cases. Clinical dementia ratings were available in 1,105 cases as follows: CDR0 = 436 cases, CDR 0.5 = 190 cases, CDR 1 = 193 cases, CDR 2 = 124 cases, and CDR3 = 162 cases.

### Neuropathology

The morphological changes in cases with dementia were as follows: 218 cases had a neurodegenerative etiology, 104 cases had a vascular etiology, and 11 cases had combined neurodegenerative and vascular etiologies. The neurodegenerative dementias included 97 cases of AD, 53 cases of DG, 33 cases with DLB (of which 20 cases were DLBT and 13 cases were DLBN), 13 cases of NFTD, and 8 cases of progressive supranuclear palsy. Dementia cases with both LB pathology and other neurodegenerative pathology included 9 cases of DLBN plus AD, 4 cases of DLBT plus AD, 1 case of DLBT plus DG, and 1 case of DLBN plus progressive supranuclear palsy.

### Lewy Body Pathology

LBs were found in 254 (20.5%) of the 1,241 cases. Of these 254 cases, 58 (22.8%) had clinical parkinsonism or

TABLE 3
Dementia With Lewy Bodies (DLB) and Alzheimer-type Senile Changes

| DLB, Transitional Form | | | | |
|---|---|---|---|---|
| | SP stage | | | |
| | **0** | **A** | B | C |
| NFT stage | | | | |
| **0** | **0** | **0** | 0 | 0 |
| **I** | **3** | **3** | 3 | 1 |
| **II** | **1** | **4** | 0 | 0 |
| III | 0 | 0 | 3 | 2 |
| IV | 0 | 0 | 1 | *0* |
| V | 0 | 0 | 0 | *3* |
| VI | 0 | 0 | 0 | 1 |

| DLB, Neocortical Form | | | | |
|---|---|---|---|---|
| | SP stage | | | |
| | **0** | **A** | B | C |
| NFT stage | | | | |
| **0** | **0** | **0** | 0 | 0 |
| **I** | **0** | **2** | 2 | 1 |
| **II** | **0** | **0** | 3 | 3 |
| III | 0 | 0 | 0 | 3 |
| IV | 0 | 0 | 0 | *6* |
| V | 0 | 0 | 0 | *3* |
| VI | 0 | 0 | 0 | *0* |

Boldfaced numerals indicate the pure form of DLB or DLB without significant Alzheimer changes. Italicized numerals indicate DLB plus Alzheimer disease.

TABLE 4
Apolipoprotein E Genotyping and Lewy Body Stage

| | Lewy Body Stage | | | | | |
|---|---|---|---|---|---|---|
| | 0 | I | II | III | IV | V |
| Genotyping | | | | | | |
| 23 | 72 | 12 | 1 | 0 | 2 | 1 |
| 33 | 673 | 103 | 28 | 7 | 18 | 8 |
| 34 | 133 | 15 | 13 | 1 | 2 | 10* |
| 44 | 13 | 2 | 0 | 0 | 0 | 1 |
| Allelic Frequency | | | | | | |
| 2 | 72 | 12 | 1 | 0 | 2 | 1 |
| 3 | 1,551 | 233 | 70 | 15 | 40 | 27 |
| 4 | 159 | 19 | 13 | 1 | 2 | 12** |

* $p < 0.0001$, compared with LB stage 0.
** $p < 0.001$, compared with LB stage 0.

cognitive decline. The LB staging of these 1,241 cases was as follows: LB stage 0 = 987 cases (male:female = 528:459); LB stage I = 149 cases (male:female = 86:63); LB stage II = 47 cases (male:female = 22:25); LB stage III = 10 cases (male:female = 4:6); LB stage IV = 25 cases (male:female = 10:15); and LB stage V = 23 cases (male:female = 13:10) (Table 1). No significant gender difference was observed in the LB stage, in the frequency of LBs, or in the frequency of LB-related clinical symptoms.

Because our LB staging did not distinguish Parkinson-associated "primary" α-synucleinopathy from AD- or tauopathy-associated "secondary" α-synucleinopathy (10), we categorized the LB stages I and II cases into primary and secondary types. LB stage I contained 144 cases of primary α-synucleinopathy and 5 cases of secondary α-synucleinopathy. LB stage II contained 44 cases of primary α-synucleinopathy and 3 cases of secondary α-synucleinopathy. The cases of primary α-synucleinopathy showed progressive involvement of the brainstem, limbic system, and neocortex, as previously reported (10).

The cases of primary α-synucleinopathy from our LB stages I through V were also staged using the criteria for staging of PD proposed by Braak et al (1). With one exception, all of our LB stage I cases belonged to Braak PD stage 1. The one exception had LBs only in the locus ceruleus. Our LB stage II cases were scored over Braak PD stages 3 to 6. All of our LB stage III cases had involvement of the temporal neocortex to a minor degree and would be classified as Braak PD stage 5. Our LB stage IV cases had involvement of frontal and temporal neocortex and would be classified as Braak PD stage 5. Our LB stage V cases had involvement of parietal and occipital cortex, as well as mild but constant involvement of primary motor and sensory cortex, and would be classified as Braak PD stage 6.

### Aging and Lewy Bodies (LBs)

Average age at death in cases with LBs was 83.0 ± 8.3 years and was significantly greater (Student t-test, $p < 0.0001$) than the average age at death in cases without LBs (79.9 ± 8.8 years). The average age at death in each LB stage was as follows (Fig. 1): stage 0 = 80.0 ± 8.9 (years); stage I = 82.8 ± 8.8; stage II = 84.1 ± 8.1; stage III = 77.2 ± 6.1; stage IV = 84.9 ± 5.6; and stage V = 83.7 ± 6.8. The average age at death in LB stages I, II, IV, and V was significantly greater than in LB stage 0 (Student *t*-test, p = 0.0003, 0.002, 0.006, and 0.045, respectively). The average age at death in LB stage III was significantly less than in LB stages I, II, IV, and V (Student *t*-test, p = 0.049, 0.014, 0.001, and 0.014, respectively). The results were the same if LB stages IV and V were subclassified into PDD and DLB, following consensus guidelines (6) (Table 2).

### Lewy Body (LB) Stage and Neurofibrillary Tangle (NFT) Stage

The average NFT stage in each LB stage was as follows: LB stage 0 = 1.84; LB stage I = 2.08; LB stage II = 1.98; LB stage III = 1.60; LB stage IV = 2.40; and LB stage V = 2.83. The average NFT stage was significantly higher in LB stage V than in LB stage 0 (Mann-Whitney *U*-test, p = 0.0003) or LB stage III (p = 0.024) (Fig. 2).

TABLE 5
Genotype Distributions of the Paraoxonase 1 (PON1) Polymorphisms

| | | | Men | | | Women | | |
|---|---|---|---|---|---|---|---|---|
| Genotypes | n | Frequency | LB stage 0–I (n = 237) | LB stage II≤ (n = 24) | p* | LB stage 0–I (n = 230) | LB stage II≤ (n = 20) | p* |
| G(-907)C | | | | | | | | |
| GG | 122 | 0.24 | 56 | 8 | 0.2844 | 57 | 1 | 0.1056 |
| GC | 263 | 0.51 | 122 | 13 | | 114 | 14 | |
| CC | 126 | 0.25 | 61 | 3 | | 57 | 5 | |
| G (-824)A | | | | | | | | |
| GG | 272 | 0.53 | 129 | 7 | 0.0246 | 123 | 13 | 0.372 |
| GA | 198 | 0.39 | 89 | 12 | | 90 | 7 | |
| AA | 41 | 0.08 | 19 | 5 | | 17 | 0 | |
| G (-161)A | | | | | | | | |
| GG | 416 | 0.81 | 192 | 18 | 0.7733 | 188 | 18 | 0.5805 |
| GA | 70 | 0.14 | 31 | 4 | | 33 | 2 | |
| AA | 25 | 0.05 | 12 | 5 | | 8 | 0 | |
| G (-125)C | | | | | | | | |
| | 41 | | | | | | | |
| GG | 9 | 0.81 | 195 | 18 | 0.8857 | 188 | 18 | 0.5512 |
| GC | 67 | 0.14 | 29 | 3 | | 33 | 2 | |
| CC | 25 | 0.05 | 14 | 2 | | 9 | 0 | |
| T (-107)C | | | | | | | | |
| | 21 | | | | | | | |
| TT | 4 | 0.42 | 102 | 9 | 0.5753 | 92 | 11 | 0.1171 |
| | 17 | | | | | | | |
| TC | 3 | 0.34 | 80 | 7 | | 78 | 8 | |
| | 12 | | | | | | | |
| CC | 4 | 0.24 | 54 | 11 | | 58 | 1 | |
| 55pol | | | | | | | | |
| | 43 | | | | | | | |
| TT (LL) | 2 | 0.84 | 201 | 18 | 0.2383 | 195 | 18 | 0.8557 |
| TA (LM) | 76 | 0.15 | 37 | 6 | | 30 | 3 | |
| AA (MM) | 3 | 0.01 | 0 | 0 | | 3 | 0 | |
| 192pol | | | | | | | | |
| | 17 | | | | | | | |
| GG | 9 | 0.35 | 71 | 8 | 0.5074 | 94 | 6 | 0.492 |
| | 29 | | | | | | | |
| AG | 0 | 0.57 | 152 | 13 | | 114 | 11 | |
| AA | 42 | 0.08 | 16 | 3 | | 20 | 3 | |

* Fisher exact probability test, LB stages 0–I versus LB stages II–V.

### Lewy Body (LB) Stage and Senile Plaque (SP) Stage

The average SP stage in each LB stage was as follows: LB stage 0 = 1.3; LB stage I = 1.36; LB stage II = 1.6; LB stage III = 0.7; LB stage IV = 1.68; and LB stage V = 2.61. The average SP stage was significantly higher in LB stage V than in LB stage 0 (Mann-Whitney *U*-test, $p < 0.0001$), LB stage I ($p < 0.0001$), and LB stage III ($p < 0.0001$) (Fig. 3).

### Senile Changes in LB Stage IV and LB Stage V

Senile changes in LB stage IV (DLBT) and LB stage V (DLBN) were compared. The pure form of DLB (22) (defined as minimal senile changes, such as NFTs in the entorhinal stage and SPs in Braak stages 0 or A) was found in 11 of the 25 cases of DLBT and in 2 of the 23 cases of DLBN. Combined AD pathology was seen in 4 of the 25 cases of DLBT and in 9 of the 23 cases of DLBN. The pure form of DLB was preferentially seen in DLBT, and combined AD pathology was preferentially seen in DLBN (Table 3).

### ApoE Genotyping and the Lewy Body (LB) Stages

ApoE genotyping was available in 1,114 of the 1,241 cases. ApoE genotyping and allelic frequency in each LB stage are summarized in Table 4. The incidence of genotype ApoE ε3/ε4 and the allelic frequency of ε4 were

significantly higher in LB stage V than in LB stage 0 (chi-square test, $p < 0.0001$ and $p < 0.001$).

### PON1 Gene Polymorphism

The distribution of the PON1 genotypes is listed in Table 5. Statistical analysis was done for PON1 gene polymorphism in each gender and stage. Significance differences in G(−824)A polymorphism were found when male cases in LB stage II or above were compared with male cases less than LB stage II. The proportion of male cases with LB stage II or above was highest in the AA genotype (20.8%), less in the GA genotype (11.9%), and least in the GG genotype (5.1%). This difference in genotypic distribution was significant ($p = 0.024$). The allelic frequencies of A(−824) and G(−824) were also significantly different between male cases in LB stage II or above and male cases in less than LB stage II ($p = 0.007$).

## DISCUSSION

Our study of 1,241 consecutive autopsy brains from a geriatrics hospital revealed the following findings: 1) LBs were present in approximately 20% of this elderly population; 2) the incidence of LBs increased with age but was not influenced by gender; 3) Alzheimer-type pathology and ApoE ε4 genotype were associated with the neocortical form of DLB; and 4) PON1 G(−824)A polymorphism was associated with LB pathology in men.

Our series of consecutive autopsy cases reasonably represents the aging general population, as previously reported (10). Cases with LBs were significantly older than cases without LBs, implying that LBs are an age-associated change like NFTs and SPs. Our staging of cases with LB pathology roughly paralleled Braak PD staging (1), but there were a few differences. One of our early cases (LB stage I) had LBs only in locus ceruleus, a finding also reported by others (23, 24). Our staging criteria separated PD with dementia (our LB stage IV) from PD without dementia (our LB stage III), whereas the Braak criteria lump them into one stage (Braak PD stage 5). We believe that the separation of these 2 clinicopathologic entities may be advantageous for the study of LB-related cognitive decline.

The average age at death in cases with LB stage III (PD without dementia) was not significantly different from the age at death in cases without LBs and was less that the average age at death in other stages with LB pathology. It is possible that PD patients without dementia died of causes other than PD before manifesting dementia.

The presence of a pure form of DLB (22) indicates that neither NFTs nor SPs are required for DLB. In our autopsy series, the pure form of DLB was more frequent in the transitional (limbic) form of DLB than in the neocortical form of DLB. There was a significant increase in both the NFT stage and the SP stage in the neocortical form of DLB, but not in the transitional form of DLB, which suggests a synergistic effect of these 3 types of abnormally accumulating, post-translationally modified proteins in the neocortex.

There is controversy over whether ApoE ε4 is a risk factor for DLB (25–27). Our data revealed that ApoE ε4 was associated with DLBN, but that this association may be due to concomitant AD-type senile changes (28).

PON 1 is an esterase associated with a high-density lipoprotein in serum. The esterase has antioxidant properties, but its natural substrate is unknown. There have been no consistent findings of an association between PD and 2 polymorphisms in the coding region of PON1. However, we found that the G(−824)A polymorphism showed a correlation with LB stage II and above in men, raising the possibility that LB-related neuronal degeneration is influenced by PON1 in men.

In conclusion, our study provides evidence that LBs are a form of age-associated neuronal change and contribute to cognitive decline independently, as in the pure form of DLB, or synergistically with SPs and NFTs, as in DLB plus AD. Elucidation of the mechanisms by which these 3 types of abnormally deposited, post-translationally modified proteins cause brain dysfunction may help clarify the relationship among PD, AD, and DLB.

## ACKNOWLEDGMENTS

The authors thank Drs. Takeshi Iwatsubo (Department of Neuropathology and Neuroscience, Graduate School of Pharmaceutical Science, University of Tokyo) and Yasuo Ihara (Department of Neuropathology, Graduate School of Medicine, University of Tokyo) for donation of their antibody; Mr. Naoo Aikyo, Ms. Mieko Harada, and Ms. Nobuko Naoi for technical support; Ms. Hiroko Matsuoka for molecular work; Dr. Noriko Tanaka (Dept. Biostatistics, Graduate School of Medicine, University of Tokyo) for statistical advice; Professor Kinuko Suzuki (Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill) for helpful discussion and comments; and Professor Thomas W. Bouldin (Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill) for editing the manuscript.

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Received January 5, 2004
Revision received March 29, 2004
Accepted April 7, 2004

Journal of Neuropathology and Experimental Neurology

Vol. 63, No. 9
September, 2004
pp. 911–918

# Staging of Argyrophilic Grains: An Age-Associated Tauopathy

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**Abstract.** We have reported that the ambient gyrus is the site with the greatest accumulation of argyrophilic grains (AGs) and that the degeneration of the ambient gyrus is responsible for dementia with grains. Here we analyzed 1,405 serial autopsy cases from 2 hospitals and detected AGs only in cases older than 56 years of age. The distribution of AGs followed a stereotypic regional pattern. Thus, we propose the following staging paradigm: stage I: AGs restricted to the ambient gyrus and its vicinity; stage II: AGs more apparent in the anterior and posterior medial temporal lobe, including the temporal pole, as well as the subiculum and entorhinal cortex; and stage III: abundant AGs in the septum, insular cortex, and anterior cingulate gyrus, accompanying spongy degeneration of the ambient gyrus. Sixty-three of 65 (96.9%) argyrophilic grain stage III cases without other dementing pathology were classified as 0.5 or higher in the clinical dementia rating. Forty-seven of 50 dementia with grains cases (94%) were stage III and 3 were stage II. No association with apoE genotyping was detected. Our study further confirms that dementia with grains is an age-associated tauopathy with relatively uniform distribution and may independently contribute to cognitive decline in the elderly.

**Key Words:** Alzheimer disease; Clinical dementia rating; Dementia with Lewy bodies; Medial temporal lobe; Neurofibrillary tangle-predominant form of dementia; Progressive supranuclear palsy.

## INTRODUCTION

We have reported that the ambient gyrus, which is situated between the amygdala and the anterior medial temporal lobe, is the site with the greatest propensity to accumulate argyrophilic grains (AGs), and that the degeneration of the ambient gyrus is responsible for dementia with grains (1, 2). There is only 1 report discussing the correlation between the grade of cognitive decline and the distribution and amount of grains (3). Also, as far as we know there has been no report of attempts to stage the grains.

By screening serial autopsy cases from Tokyo Metropolitan Geriatric Hospital and Yokohama Rosai Hospital, we observed a relatively uniform distribution of AGs in a given brain region as follows: the localized presence of grains in the ambient gyrus in earlier stages; the more apparent existence of grains in the anterior and posterior medial temporal lobe in intermediate stage; and the involvement of the septal area, the anterior cingulate gyrus, and the insular cortex beyond the boundaries of the temporal lobe in the later stage. There was a strong correlation between the distribution of AGs and the grade of cognitive impairment. Argyrophilic grains were never observed in subjects younger than the mid-fifties. Further analysis using this staging method provided additional new information about the significance of AGs in the human aging process.

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Sources of support: Grants in aid from the Tokyo Metropolitan Institute of Gerontology (S.M.) and the Ministry of Education, Culture, Sports, Science and Technology (S.M.).

## MATERIALS AND METHODS

### Tissue Source

In the present work, 1,241 serial autopsy brains from Tokyo Metropolitan Geriatric Hospital (TMGH) (Group A) and 164 serial autopsy cases younger than 65 years of age from Yokohama Rosai Hospital (YRH) (Group B) were studied. YRH is a community center general hospital and neuropathological diagnosis there was carried out by two of the authors (YS and SM). The patients' ages ranged from 48 to 104 years in Group A and 0 to 64 years in Group B. The mean age and the male to female ratio were 80.6 ± 8.9 and 663:578 for Group A and 45.0 ± 20.2 years and 101:63 for Group B.

### Cognitive State

Clinical information was retrospectively obtained from the medical charts as well as interviews with the patients' attending physicians and caregivers. The Mini-Mental State Examination (MMSE) (4) or Hasegawa dementia scale (5, 6) was used for evaluation of cognitive function, and the clinical dementia rating scale (CDR) (7) was employed for the grading of cognitive decline as previously reported (1).

### Neuropathology

Representative areas in the central nervous system were examined as previously reported (1). Briefly, the areas recommended by CERAD (8) and Braak (9) were stained by the Gallyas-Braak modified methenamine silver (10) and Bielschowsky