old). Our findings demonstrated a decreasing trend of carcinoma of the upper third of the stomach in the very old group and no significant change in the younger groups. This finding suggests that an age-related decreasing trend of carcinoma of the upper third of the stomach is no longer occurring in the younger groups. These trends have been found in the 1980s and 1990s in the United States and Europe [25–29]. These studies provide support for the idea that the incidence of tumors located in the distal part of the stomach has declined, whereas cancers of the proximal stomach have been rapidly increasing. Age-period-cohort analysis suggested that the proportions of the locations of gastric carcinomas may, to some extent, reflect a birth-cohort phenomenon [25]. This birth-cohort phenomenon influences the time trend of both cardia and distal gastric carcinomas and suggests that the incidence of carcinoma of the proximal stomach will increase in the future because the prevalence of lower esophageal adenocarcinoma and cardia carcinoma is currently increasing worldwide [25,28].

In conclusion, we found a distal shift in cancer location and increased multiplicity of gastric cancer with advancing age. We also found that differentiated-type carcinoma was more common in early disease stages and undifferentiated-type carcinoma was more common in advanced disease stages, which may indicate increased histologic diversity with tumor growth. These findings have important implications for the screening and diagnosis of gastric cancer in the elderly.

Acknowledgments We thank the staff at the Department of Pathology, Tokyo Metropolitan Geriatric Medical Center for their excellent technical assistance. The study was supported, in part, by Novartis Foundation for Gerontological Research 2003.

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Modern Pathology (2004) 17, 172–179

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Hypermethylation of the *hMLH1* promoter with absent hMLH1 expression in medullary-type poorly differentiated colorectal adenocarcinoma in the elderly

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To clarify the significance of *hMLH1* promoter hypermethylation in the development of medullary-type poorly differentiated colorectal adenocarcinoma, we studied the status of promoter methylation and hMLH1 expression in 23 medullary-type and 12 pleomorphic-type carcinomas, as well as the pathology and microsatellite status. In medullary-type carcinomas, the percentages of cases with promoter methylation (83%) and an absence of hMLH1 expression (91%) were significantly higher than in pleomorphic-type carcinomas (14 and 17%), respectively. The rate of microsatellite instability in the medullary type was significantly higher than that of the pleomorphic type (87 *vs* 40%, $P<0.01$). Compared with pleomorphic-type carcinomas, medullary-type carcinomas were significantly associated with *hMLH1* promoter methylation, absent expression of hMLH1 protein, microsatellite instability, as well as a proximal location, a Crohn's-like lymphoid reaction, a low incidence of lymph node metastasis, and a favorable outcome. Medullary-type carcinomas accumulated with advancing age, especially in the female. These results indicated that *hMLH1* hypermethylation, concurrent with a lack of its protein expression, may play an important role in the development of medullary-type poorly differentiated colorectal adenocarcinomas in the elderly.
Modern Pathology (2004) **17,** 172–179, advance online publication, 5 December 2003; doi:10.1038/modpathol.3800018

Keywords: medullary-type poorly differentiated adenocarcinoma; large intestine; hMLH1 protein; hypermethylation; *hMLH1* gene promoter; microsatellite instability and elderly

In the large intestine, adenocarcinoma composed of cuboidal epithelial cells showing minimal or no glandular differentiation is defined as a poorly differentiated adenocarcinoma.[1–3] Poorly differentiated lesions are heterogeneous with respect to their clinicopathologic and molecular features.[4–8] A peculiar type of poorly differentiated colorectal adenocarcinoma has been recently recognized and termed either medullary-type poorly differentiated adenocarcinoma,[4,6,9,10] solid-type poorly differentiated adenocarcinoma,[5] or large cell minimally differentiated carcinoma.[11] Histologically, the tumor is characterized by a small uniform population of tumor cells with fine chromatin and prominent nucleoli. The tumor cells grow in solid sheets and trabeculae, often associated with a prominent intratumoral and/or peritumoral lymphocytic infiltration. The main clinicopathologic features of the tumor are its predominant occurrence in elderly women, a location of the proximal colon, and a relatively favorable prognosis in spite of the high-grade histological features.[4,6,10] Furthermore, recent molecular studies demonstrate that medullary-type carcinomas show a diploid DNA pattern, a high frequency of microsatellite instability, and low p53 expression.[4,10]

Cancers with high levels of microsatellite instability are the hallmark of hereditary nonpolyposis colorectal cancer, and microsatellite instability occurs in 10–15% of sporadic colorectal carcinomas. Colorectal adenocarcinomas with microsatellite instability often show an absence of hMLH1 protein,[12–14] indicating that the tumor with microsatellite

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Received 22 April 2003; revised 28 July 2003; accepted 10 September 2003; published online, 5 December 2003

instability may be induced by an abnormality of the mismatch repair gene. A germline mutation has been detected in the mismatch repair gene in patients with hereditary nonpolyposis colorectal cancer, and hypermethylation has been found in the *hMLH1* gene promoter in sporadic colorectal cancer.[13] The spread of methylation in the *hMLH1* promoter is closely associated with age and the development of sporadic colorectal cancers with microsatellite instability.[15]

Mucinous or poor differentiation and stromal inflammatory reactions are frequent features of hereditary nonpolyposis colorectal cancer in which germline mutations of mismatch repair genes cause genetic instability. A link exists between such histological features and somatic genetic instability, consistent with a mutator phenotype, in nonfamilial colorectal cancer.[16] However, there are few reports investigating a relationship between medullary-type poorly differentiated adenocarcinoma and methylation of the *hMLH1* promoter. In the present study, we focused on investigating this possible relationship and examined 35 cases of poorly differentiated adenocarcinomas, including 23 medullary-type carcinomas in the elderly.

Materials and methods

Patients

In all, 35 cases, aged 65 years or older with poorly differentiated adenocarcinoma of the large intestine, were selected from the list of colorectal cancers at a geriatric hospital. They were composed of 13 men and 22 women, with an average age of 78 years, ranging from 65 to 99 years old. Patients with inflammatory bowel disease or belonging to families with evidence of hereditary nonpolyposis colorectal cancer (according to the Amsterdam criteria), or familial adenomatous polyposis were excluded from the present study. This work was approved by the Ethics Committee of the Tokyo Metropolitan Geriatric Medical Center.

Histopathological Evaluation

All tissue samples were fixed with 10% formalin after resection and then embedded in paraffin in a standard procedure. Serial sections, 3 and 10 μm thick, were prepared for each specimen. The 3 μm-thick section was used for hematoxylin and eosin staining and immunostaining, and the 10 μm-thick section was used for DNA extraction.

All of the tumors were pathologically diagnosed as poorly differentiated adenocarcinomas.[1–3] The 35 cases were classified into two types, medullary and pleomorphic types, according to the description by Ruschoff *et al*.[4] The medullary type of poorly differentiated adenocarcinoma was composed of solid sheets or trabeculae of small- to medium-sized quite uniform cells with a variable amount of eosinophilic or amphophilic cytoplasm (Figure 1a). The nuclei were round to oval, regular, with slight or moderate pleomorphism, and a single nucleolus. Occasional cells with more voluminous nuclei were observed in some tumors. The tumor grew expansively with prominent intra- and peritumoral inflammatory cell infiltration and/or a Crohn's-like lymphoid reaction.[17] On the other hand, the pleomorphic-type poorly differentiated adenocarcinoma was composed of solid sheets of medium- to large-sized variable cells with a variable amount of eosinophilic cytoplasm (Figure 1b). The tumor cells demonstrated large irregular nuclei with a few irregular nucleoli, coarse chromatin, atypical mitoses, and an infiltrative growth pattern.

DNA Extraction

Three 10-μm sections on glass slides were deparaffinized with xylene, rinsed in 100, 90, and 80% ethanol, and briefly stained with hematoxylin. The

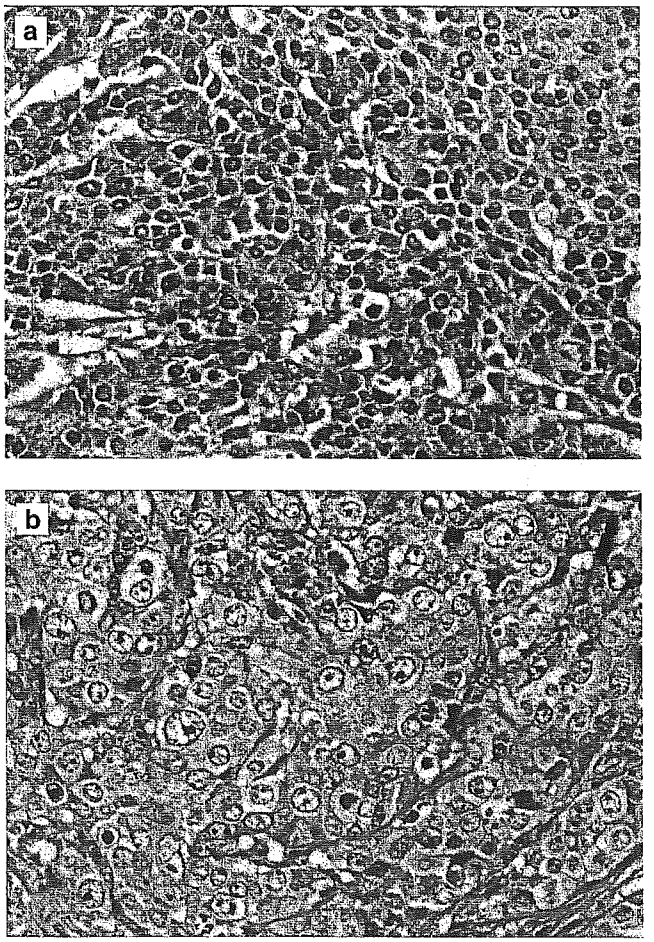

Figure 1 Histology of poorly differentiated adenocarcinoma of the large intestine in the elderly. (**a**) Medullary-type carcinoma (case 2) showing small uniform tumor cells. (**b**) Pleomorphic-type carcinoma (case 10) showing tumor cells with atypia. Both figures show the same magnification. Hematoxylin–eosin staining.

tissues of the tumor were scraped from the semi-dried section with a blade under the stereomicroscope. When a lesion was histologically heterogeneous (ie a tumor containing well or moderately differentiated adenocarcinoma and/or adenoma as well as poorly differentiated adenocarcinoma), DNA was extracted separately from each individual area under the stereomicroscope. DNA samples from normal colorectal tissue were also extracted. The tissue sample was transferred into a sterile 1.5 ml microtube. DNA was extracted from all of these samples by a phenol–chloroform procedure.

Microsatellite Instability

We screened for microsatellite instability using the mononucleotide repeats *BAT-26* and *BAT-40* according to protocols described by other investigators.[18,19] These probes are sensitive and recommended for the detection of microsatellite-unstable tumors, especially in case of high-frequency microsatellite instability. The cases where bands of different molecular weights were observed in the tumor DNA, but not observed in normal DNA, were designated as microsatellite-unstable.

Immunohistochemical Analysis of hMLH1 Proteins

The expression of hMLH1 proteins was evaluated by immunohistochemistry. Endogenous peroxidase was blocked by treatment with 0.3% H_2O_2 in methanol for 15 min. The sections for hMLH1 proteins were heated at 100°C for 10 min to retrieve antigen. Slides were immunostained by the streptavidin–biotin method using an anti-human MLH1 monoclonal antibody (G168-15, PharMingen, San Diego, CA, USA), and developed with diaminobenzidine substrate. Counterstaining was hematoxylin. For hMLH1 protein, adjacent normal tissues were used as internal controls. The intensity of nuclear staining in the entire tumor was classified as negative, weakly positive, and strongly positive in comparison with that of normal tissues. Focal or heterogeneous staining patterns were also recorded. For the statistical analysis, weak or focal staining was counted as negative for hMLH1.

Methylation Study (Combined Bisulfite Restriction Analysis, COBRA Method)

Bisulfite treatment was performed using a CpGenome DNA Modification Kit (Oncor, Gaithersburg, USA) according to the manufacturer. The COBRA protocol was performed as described previously.[20] Bisulfite-modified DNA was amplified by nested PCR with specific primers for the *hMLH1* promoter. The primer sequences used for primary and secondary PCR amplification of *hMLH1* were described previously.[20] Primary PCR was performed in 25 μl reaction mixtures as follows: 94°C for 2 min, followed by 35 cycles at 94°C for 1 min, 55°C for 2 min, and 72°C for 1 min, with a final 10 min extension at 72°C. Secondary PCR was performed in 25 μl reaction mixtures as follows: 94°C for 2 min, followed by 35 cycles at 94°C for 1 min, 63°C for 2 min, and 72°C for 1 min, with a final 10 min extension at 72°C. The PCR products were then digested with a specific restriction enzyme, *RsaI* for at least 2 h and then electrophoresed on 15% polyacrylamide gels.

Statistical Analysis

All data were subjected to statistical analysis. Comparisons among continuous and categorical variables were made using the Students *t*-test, χ^2 or Fisher's exact probability test. A probability value of less than 0.05 (two-sided) was considered to be significant.

Results

Clinicopathologic Findings

Poorly differentiated adenocarcinomas of the proximal colon (proximal to the splenic flexure) in women accounted for 91% of the cases, being significantly higher than in men (62%, $P<0.05$). The incidence rate of proximal colonic cancers increased with advancing age, reaching 100% in patients aged over 85 years. Although no significant difference in site distribution was found among any age groups, a higher proportion of proximal colonic cancer was noted with advancing age. The mean size of the poorly differentiated adenocarcinomas, including the accompanying neoplastic lesion, was 9.1 cm (ranging from 1.5 to 12.5 cm). Macroscopically, all cases showed ulcerative-invasive features.

Histologically, medullary, and pleomorphic types of poorly differentiated adenocarcinomas accounted for 23 and 12 cases, respectively (Figure 1). In medullary-type carcinomas, glandular differentiation was absent in 11 cases and minimal (<5%) in five cases, whereas in seven cases more extensive features of glandular differentiation were observed. Glandular differentiation was mainly represented by the occurrence of a mucosal layer or well-formed glandular structures within the solid sheets. Crohn's-like lymphoid reactions and/or intensive lymphocytic infiltration in the tumor stroma were detected in 18 cases.

On the other hand, in the pleomorphic-type carcinomas glandular differentiation was absent in eight cases and minimal in four cases. None of the tumors showed more extensive features of glandular differentiation. A Crohn's-like lymphoid reaction was found in five cases of the pleomorphic type.

The mean age at diagnosis in the medullary type was significantly higher than that in the pleomorphic

type (80.6 *vs* 73.6 years). Although both types of poorly differentiated adenocarcinoma were staged as pT3 or pT4 at the time of surgery, medullary-type carcinomas exhibited significantly less frequent lymph node metastasis (43.5 *vs* 83.3%, $P=0.03$). The mortality rate (20%) of patients with medullary-type carcinomas was significantly lower than that (71%) with pleomorphic-type carcinomas ($P=0.03$). Corresponding to these data, patients with medullary-type carcinomas had a more favorable outcome.

Immunohistochemistry

In all, 24 cancer specimens (69%) showed an absence of hMLH1 protein expression (Figure 2), while the remaining 11 cancer specimens showed positive nuclear hMLH1 staining. All the adjacent normal tissues, including the 24 cases with absent hMLH1 expression, exhibited nuclear hMLH1 expression. Of the 24 tumors 23 with absent hMLH1 expression were located in the proximal colon.

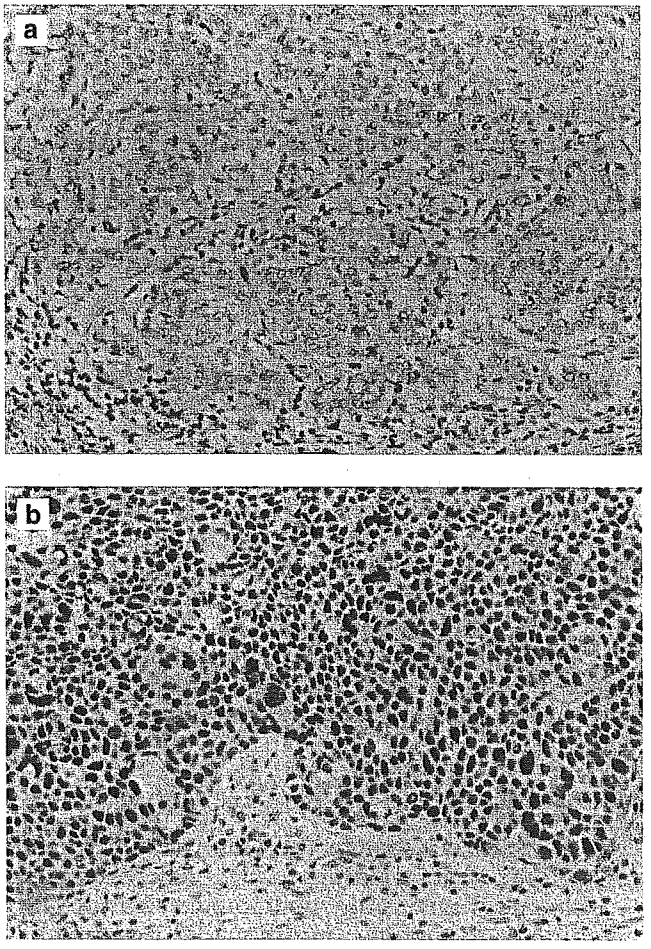

Figure 2 Immunohistochemistry of hMLH1 expression in poorly differentiated adenocarcinoma. (**a**) Poorly differentiated adenocarcinoma showing no hMLH1 protein expression, with peritumoral infiltration of hMLH1-positive lymphocytes (case 8). (**b**) Poorly differentiated adenocarcinoma showing positive nuclear staining of hMLH1 protein in cancer cells (case 9). Counterstaining, hematoxylin.

Microsatellite Analysis

Representative microsatellite instability is shown in Figure 3. Of the 35 cases 24 were microsatellite-unstable (69%), and 11 were microsatellite-stable (31%). Of the 24 microsatellite-unstable cases, 13 cases showed the instability of only one marker and 11 cases showed the instability of both markers. In all, 20 (83%) of the microsatellite-unstable cases were medullary-type carcinomas, while only three (27%) of the microsatellite-stable cases were medullary-type carcinomas ($P<0.01$).

Methylation of hMLH1 Gene Promoter

Of the 35 poorly differentiated adenocarcinomas examined, methylation status was determined in 26 cases. Totally, 16 cancers (62%) presented with *hMLH1* methylation (Figure 4). Methylation of the *hMLH1* promoter was found in 14 of 18 cancers with absent hMLH1 protein expression, but only two in nine cancers with normal hMLH1 expression exhibited methylation, demonstrating a close correlation of transcriptional loss with *hMLH1* methylation ($P=0.009$).

Associations among Microsatellite Instability, hMLH1 Expression and Methylation Status of hMLH1 Promoter

Table 1 demonstrates the hMLH1 expression and methylation status of the *hMLH1* promoter in poorly

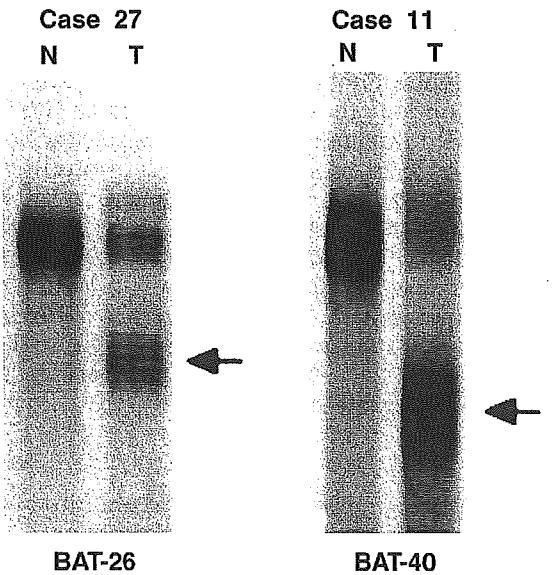

Figure 3 Analysis of microsatellite instability in paired tumor (T) and normal mucosa (N) at loci of *BAT-26*, and *BAT-40*. Abnormal patterns (arrows) indicating mobility alteration of PCR product from tumor DNA compared to normal mucosa DNA are shown at each microsatellite locus.

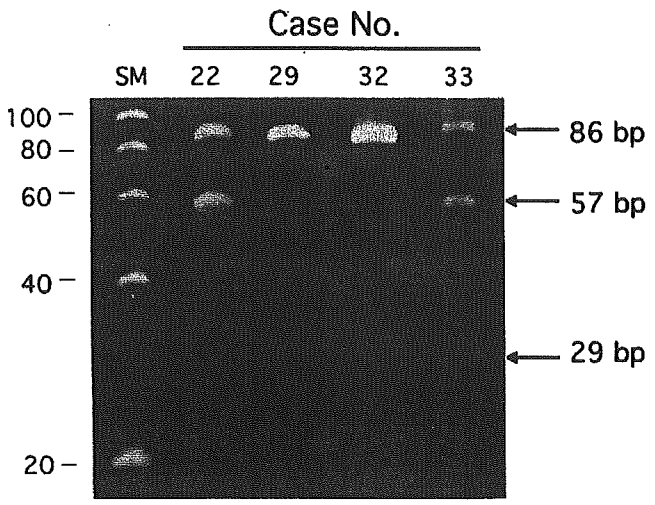

Figure 4 Methylation of the *hMLH1* promoter in four representative poorly differentiated adenocarcinomas. *Rsa*I only cleaves the methylated alleles, yielding 57- and 29 bp bands. Cases 22 and 33 show hypermethylation of the *hMLH1* promoter.

Table 1 Relationship between microsatellite instability, expression of hMLH1 protein, and hypermethylation of the *hMLH1* promoter

Microsatellite instability	*Medullary type*	*Absent expression of hMLH1 protein*	*Hypermethylation of hMLH1 promoter*
Microsatellite-stable	3/11 (27%)	1/11 (9%)	2/8 (25%)
Microsatellite-unstable	20/24 (83%)[a]	22/24 (92%)[a]	12/17 (71%)[b]

[a]$P<0.01$. [b]$P<0.05$, compared with microsatellite-stable cases.

differentiated adenocarcinoma with or without microsatellite instability. The absence of hMLH1 protein expression (92%) concurrent with hypermethylation of the *hMLH1* promoter (71%) in microsatellite-unstable cases was significantly higher than those (9 and 25%) in microsatellite-stable cases ($P<0.01$ and $P<0.05$, respectively). Microsatellite-unstable cases were significantly correlated with medullary-type carcinoma ($P<0.01$). The mortality rate was significantly correlated with microsatellite instability ($P<0.01$), but not with *hMLH1* promoter methylation ($P=0.09$) or hMLH1 expression ($P=0.22$). Microsatellite-unstable cases showed a favorable outcome.

Clinicopathologic Characteristics of Medullary-type and Pleomorphic-type Poorly Differentiated Adenocarcinomas

The clinicopathologic and molecular characteristics of medullary-type carcinomas were compared with those of pleomorphic-type carcinomas (Table 2). Significantly different pathologic and molecular features of medullary-type carcinomas occurred in the elderly such as proximal location, more frequent Crohn's-like lymphoid reaction, low incidence of lymph node metastasis, absent hMLH1 expression, microsatellite instability, *hMLH1* promoter methylation, and less frequent mortality from the colorectal cancer.

Table 2 Clinicopathologic and molecular characteristics of medullary-type poorly differentiated adenocarcinoma in comparison with pleomorphic-type carcinoma

Characteristics	*Medullary type*	*Pleomorphic type*	P-*value*
Age (years, means±S.D.)	80.6±7.8	73.6±5.1	0.008
Gender (male/female)	7/16	6/6	0.22
Tumor location (proximal/distal)	22/1	5/7	<0.001
Tumor size (≧5 cm/<5 cm)	21/2	8/4	0.09
Glandular differentiation (Extensive/minimal/absent)	7/5/11	0/4/8	0.10
Crohn's-like lymphoid reaction (+/−)	18/5	5/7	0.04
Lymph node metastasis (+/−)	10/13	10/2	0.03
Microsatellite instability (+/−)	20/3	4/6	0.011
hMLH1 protein expression (+/−)	2/21	10/2	<0.001
hMLH1 promoter methylation (+/−)	15/3	1/7	0.0012
Mortality rate	20% (2/10)	71% (5/7)	0.03

Age-related Alterations of Clinicopathologic Characteristics of Poorly Differentiated Adenocarcinomas

As a result of the stratification of cases according to their ages, medullary-type poorly differentiated adenocarcinoma, concurrent with absent hMLH1 expression and methylation of the *hMLH1* promoter, was observed to accumulate with advancing age (Figure 5). Patients aged more than 80 years old with poorly differentiated colorectal adenocarcinoma were all female, and patients over 85 years of age all showed medullary-type carcinomas.

Discussion

We have shown that in the elderly, approximately two-thirds of poorly differentiated colorectal adenocarcinomas have clinicopathologic features of a medullary phenotype. Additionally, hypermethylation of the *hMLH1* promoter with an absence of hMLH1 protein expression is closely correlated with medullary-type carcinomas with microsatellite instability in the elderly.

The present study has provided evidence that medullary-type poorly differentiated adenocarcinomas

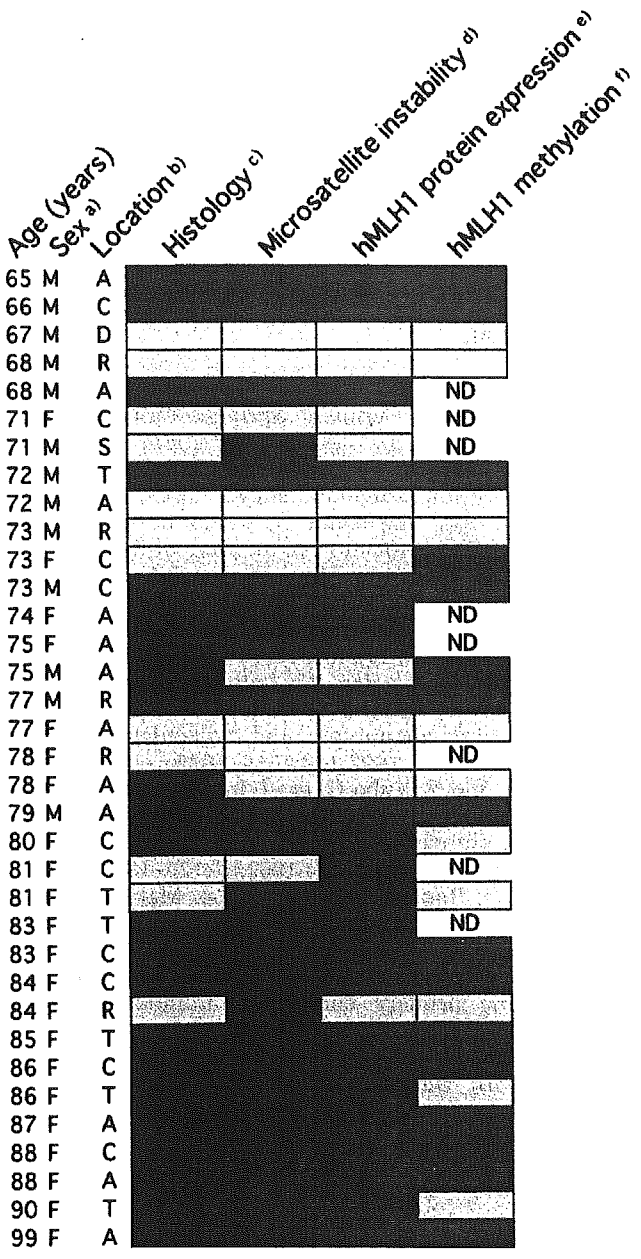

Figure 5 Age-related accumulation of medullary-type carcinoma with microsatellite instability, absent hMLH1 protein expression, and hypermethylation of the *hMLH1* promoter. All cases are listed in order of age. (**a**) M, male; F, female. (**b**) C, cecum; A, ascending colon; T, transverse colon; D, descending colon; S, sigmoid colon; R, rectum. (**c**) ■, medullary-type carcinoma; □, pleomorphic-type carcinoma. (**d**) ■, microsatellite-unstable; □, microsatellite-stable. (**e**) ■, absent expression of hMLH1 protein; □, normal expression. (**f**) ■, methylated *hMLH1* promoter; □, unmethylated *hMLH1* promoter; ND, not determined.

showed microsatellite instability associated with absent hMLH1 expression and hypermethylation of the *hMLH1* promoter. Most of the medullary-type carcinomas showed a high proportion of microsatellite instability in comparison with pleomorphic-type carcinomas. This result is in accord with that of other investigators.[4,10,14] Although the high incidence of absent hMLH1 expression was reported in poorly differentiated colorectal adenocarcinomas,[14] our results indicated that in poorly differentiated adenocarcinoma, the medullary-type carcinoma is primarily affected as compared with pleomorphic-type carcinoma. In view of the histopathological type of tumor, we are the first to describe hypermethylation of the *hMLH1* promoter in medullary-type carcinomas. The molecular event was detected in sporadic colorectal carcinomas with microsatellite instability,[13,21] and caused inactivation of the *hMLH1* gene, resulting in absent expression of its protein.[13] These results suggest that hypermethylation of the *hMLH1* promoter is the underlying cause of the mismatch repair defects observed in medullary-type carcinomas. In contrast, pleomorphic-type carcinomas do not seem to be induced by *hMLH1* methylation. Thus, these findings indicate that medullary- and pleomorphic-type carcinomas differ not only in their clinicopathologic features but also in their molecular findings and therefore we hypothesize that both types of poorly differentiated adenocarcinomas have different pathways in carcinogenesis.[4,14]

Medullary-type carcinomas in the elderly share clinicopathologic and biological features with those occurring in patients with hereditary nonpolyposis colorectal cancer: predominant occurrence in the proximal colon, large-sized tumors, expansive growth, prominent Crohn's-like lymphoid reaction, low incidence of lymph node or hematogenous metastasis, and favorable outcome.[22] Common features are tumor location, histology, and biological behavior while age, gender distribution, and the presence of extra-colorectal primary malignancies are different between the two. Genetically, four human mismatch repair genes, *hMSH2*, *hMLH1*, *hMSH6*, and *hPMS2*, are involved in 47, 47, <5 and $<1\%$ of patients with hereditary nonpolyposis colorectal cancer, respectively. Mutations in such genes result in a 'mutator phenotype' that can be identified by observing genetic instability in the form of deletion and insertion mutations in simple repetitive DNA sequences at microsatellite loci. The present study supports the hypothesis that hypermethylation of the *hMLH1* promoter also results in a 'mutator phenotype'.[13,23] Consequently, medullary-type carcinomas in the elderly may be induced by an epigenetic event within the *hMLH1* gene, whereas hereditary nonpolyposis colorectal cancer results from germ-line mutation of the mismatch repair gene with occasional hypermethylation of the *hMLH1* promoter.[24]

In the present study, medullary-type carcinomas accumulated with advancing age. The spread of methylation in the *hMLH1* promoter in the normal colonic mucosa was closely associated with age and with the development of sporadic colorectal cancers with microsatellite instability.[15] The frequency of methylation in those tumors was significantly correlated with aging.[21,25] Methylation has been

observed not only in the *hMLH1* promoter but also in other gene promoters, such as the *estrogen receptor (ER), N33, MyoD,* etc.[26,27] Furthermore, our previous study showed that age-related methylation was found in gastric cancer as well as colorectal cancer.[20] The absent expression of hMLH1 protein was closely related with aging in gastric and colorectal cancers.[20,28] Since aberrant CpG island methylation is a powerful mechanism for the inactivation of gene activity, age-related methylation of the genes may play a significant role in the increase of malignant neoplasms in the elderly.

Age and gender differences exist in colorectal cancers. The proportion of proximal colonic cancer in women was approximately 10% higher than those in men, and increased with advancing age.[29] Molecular evidence showed colorectal cancer with microsatellite instability was most frequent among younger male and older female patients.[12,30] The frequency of *hMLH1* methylation with lack of its expression was significantly correlated with females and aging.[21,31] These facts may contribute to the observation that medullary-type carcinomas occur more frequently in the older female patient and in the proximal colon.[6,10]

The present study has suggested that medullary-type poorly differentiated carcinomas should be distinguished from other adenocarcinomas with minimal or no glandular differentiation due to its biological behavior. Since approximately 90% of medullary-type carcinomas showed an absence of hMLH1 protein expression together with microsatellite instability, immunohistochemistry for the detection of hMLH1 protein may be useful in predicting the tumor type.[12,32] However, immunohistochemistry cannot replace testing for microsatellite instability to identify microsatellite-unstable sporadic colorectal cancer,[33] and morphological prediction of microsatellite-unstable cancer has low sensitivity.[34] Thus, we emphasize that both hMLH1 immunohistochemistry and a histopathological evaluation can predict medullary-type carcinomas, but they may miss some cases.[32,33] Molecular analysis is required for therapeutic decisions.[34]

Colorectal adenocarcinoma are graded predominantly on the basis of the extent of glandular appearances.[2,3] They are divided into well, moderately, and poorly differentiated, and in general the differentiation may reflect a biological behavior. Although medullar-type carcinoma is classified as poorly differentiated adenocarcinoma or undifferentiated carcinoma,[3] several reports, together with our data, demonstrated that medullary-type carcinoma showed a favorable prognosis.[4,6,10] The mortality rates in medullary-type carcinoma *vs* in well or moderately differentiated adenocarcinoma are unclear, but warrant further examination.

In conclusion, we found an age-related accumulation of medullary-type poorly differentiated adenocarcinomas in the elderly, especially in females, as well as hypermethylation of the *hMLH1* promoter with absent hMLH1 protein expression, which may play an important role in carcinogenesis of the tumor.

Acknowledgements

We thank the staff in the Department of Pathology, Tokyo Metropolitan Geriatric Medical Center for their excellent technical assistance.

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Original Research Article

Dementia
and Geriatric
Cognitive Disorders

Dement Geriatr Cogn Disord 2004;18:145–150
DOI: 10.1159/000079194

Accepted: December 19, 2003
Published online: June 18, 2004

Association of Estrogen Receptor α Gene Polymorphisms with Neurofibrillary Tangles

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Key Words
Estrogen receptor α · Polymorphism · Neurofibrillary tangles · Alzheimer's disease

Abstract
Estrogen receptor α (ERα) may be implicated in the pathogenesis of Alzheimer's disease (AD). The aim of this study was to clarify the association between ERα gene polymorphisms and AD-related pathologic changes. The staging of neurofibrillary tangles (NFT) and senile plaques (SP) was performed according to the method by Braak and Braak and two polymorphisms, *Pvu*II (P or p) and *Xba*I (X or x), of the ERα gene were typed in 551 Japanese cadavers (294 men and 257 women; mean age, 80.8 years). Distributions of the NFT and SP stages significantly correlated with age (NFT: $r = 0.306$, $p < 0.0001$; SP: $r = 0.237$, $p < 0.0001$) and were significantly higher in patients with the apolipoprotein E ε4 allele ($p < 0.0001$). Possession of the P allele showed a trend to be associated with a more serious NFT stage, but had no relationship with the SP stage. In men, a significant association between *Pvu*II polymorphism and the NFT stage ($p = 0.002$) was found, revealing a gene-dose effect of the P allele. Similar results were obtained in the men without the ε4 allele ($p = 0.011$). Multiple regression analyses demonstrated that age was the strongest determinant of the NFT stage, possession of the ε4 allele was the next strongest, and *Pvu*II polymorphism was the third strongest ($p < 0.0001$, $R^2 = 0.144$). The *Xba*I polymorphism did affect neither the NFT stage nor the SP stage. In conclusion, the *Pvu*II polymorphism of the ERα gene is associated with Braak NFT stages and possession of the P allele may act as a risk factor for AD in Japanese men, especially in those without the ε4 allele.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease and a major cause of dementia. The ε4 allele of the apolipoprotein E (APOE) gene is a genetic factor closely related to late-onset AD, but the cause of sporadic AD has not been fully elucidated. Other genetic factors may be associated with the development of AD. One of the candidates is the estrogen receptor α (ERα)

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gene [1]. Recently, estrogen replacement therapy has been reported to reduce the risk of developing AD and to help patients with AD to maintain cognitive function [2]. The distribution of estrogen receptors in the brain overlaps that of neurons known to be affected by the pathological changes in AD [3]. These findings suggest that ERα may be involved in the pathogenesis of AD. To date, two restriction fragment length polymorphisms (*PvuI* and *XbaI*) located in intron 1 of the ERα gene are known [4]. The two polymorphisms, P or p and X or x, are coded so that the capital letter signifies the absence of the restriction site.

To date, several studies have examined the association between AD and these ERα gene polymorphisms. Isoe et al. [5] reported that frequencies of P and X alleles in Japanese patients with sporadic AD were significantly higher than those in the control group. Brandi et al. [6] demonstrated that the risk of AD increased with the presence of the PPXX ERα genotype, especially in ε4-homozygous Italian individuals, suggesting an interaction of the ERα gene with the APOE ε4 allele. Ji et al. [7] also found that frequencies of the ERα gene P allele and PP genotype were significantly higher in Japanese patients with the ε4 allele and late-onset AD. However, these findings were inconsistent. Although Mattila et al. [8] reported a significant association between ERα and AD, the pp or xx genotype frequency was found to be significantly increased only in women with at least one ε4 allele and sporadic AD. Moreover, Maruyama et al. [9] found no significant association between these ERα polymorphisms and the risk of developing AD in Caucasian and Japanese patients. Similarly, Lambert et al. [10] did not detect a significant association between these polymorphisms and the risk of AD, but suggested that the latter might only be modulated when the xp ERα haplotype and the GG ERβ genotype were combined.

AD is characterized neuropathologically by neurofibrillary tangles (NFT), senile plaques (SP), and neuronal and synaptic loss. However, the preceding studies were mostly conducted on the basis of a clinical diagnosis of AD, and pathological confirmation of AD was mentioned in only two papers [8, 9]. In addition, because AD-related neuropathologic changes are in parallel with the accompanying progression of dementia, the case-control design of the preceding studies based on a dichotomy between AD and non-AD would lose much information. Therefore, the aim of this study was to evaluate semi-quantitatively the association of ERα gene polymorphisms with NFT and SP formation, using a neuropathological staging model developed by Braak and Braak [11].

Materials and Methods

Subjects

We examined 551 deceased Japanese adults (294 men and 257 women; mean age 80.8 years, range 60–104 years, SD 8.5) whose bodies, including the brain, were consecutively autopsied between May 1995 and November 1999 at Tokyo Metropolitan Geriatric Hospital, an emergency and general hospital for the aged. Patients who had been treated with hormone replacement therapy were excluded. Written informed consent was obtained either from the patients themselves prior to death or from family members under the Act of Postmortem Examination. The study protocol was reviewed and approved by the ethics committee of Tokyo Metropolitan Geriatric Hospital.

Neuropathological Examination

Brains were fixed in a 10% formalin solution and tissues from various regions were processed for paraffin embedding. Six-micrometer-thick sections were stained as follows: solochrome and eosin, hematoxylin and eosin, Klüver-Barrera, Bodian, Bielschowsky, Gallyas (G-B), and modified methenamine silver methods. Serial 6-μm-thick sections from paraffin-embedded blocks were deparaffinized and immunostained with an automated immunostaining apparatus (Ventana NX20; Ventana, Tucson, Ariz., USA), as previously described [12]. The anti-τ antibodies were used, and their epitopes were as follows: AT8, phosphorylated Ser^{202}/Thr^{205} (monoclonal; Innogenetics, Temse, Belgium); Alz50, amino acids 5–51, 312–322 (monoclonal, a gift from Dr. P. Davis); PHF1, $Ser^{396/404}$ (monoclonal, a gift from Dr. P. Davis). Also used were anti-Aβ1–42 (polyclonal; IBL, Maebashi, Japan), anti-Aβ1–40 (polyclonal; IBL), and anti-Aβ11–28 (monoclonal; IBL). The staging of NFT and SP was performed according to the method by Braak and Braak [11].

DNA Purification for ERα and APOE Gene Polymorphisms

Genomic DNA was purified from frozen kidney tissue according to the standard method [13]. ERα gene polymorphisms were determined by *PvuII* and *XbaI* restriction endonuclease digestion of polymerase chain reaction products, as previously described by Kobayashi et al. [4]. Capital and small letters denoted the absence and presence of the restriction sites, respectively. The APOE gene polymorphisms were determined by *HhaI* restriction endonuclease digestion of polymerase chain reaction products, according to Hixson and Vernier [14].

Statistical Analysis

Quantification was made by transforming ordinal scales into numerical variables (NFT stage: none→0, I→1, II→2, III→3, IV→4, V→5, VI→ 6; SP stage: none→0, A→1, B→2, C→3). Two groups were compared using the unpaired t test. More than two groups were compared using ANOVA. Bonferroni's method was chosen for use in post hoc tests. Correlation coefficients were calculated by Pearson's method. Multivariate analyses were done with forward selection stepwise regression, in which numerical variables were assigned to nominal variables (*PvuII*: pp→0, Pp→1, PP→2; *XbaI*: xx→0, Xx→1, XX→2). These statistical analyses were performed using the SAS software system, version 6.12 (SAS Institute Inc., Cary, N.C., USA). Statistical significance was established at the $p < 0.05$ level. All p values were two-sided.

Table 1. Genotypic distributions of ERα and APOE polymorphisms

	Genotypes	All subjects (n = 551)	Men (n = 294)	Women (n = 257)
ERα				
*Pvu*II	PP	114 (20.7)	55 (18.7)	59 (23.0)
	Pp	255 (46.3)	151 (51.4)	104 (40.5)
	pp	182 (33.0)	88 (30.0)	94 (36.6)
*Xba*I	XX	20 (3.6)	9 (3.1)	11 (4.3)
	Xx	159 (28.9)	87 (29.6)	72 (28.0)
	xx	372 (67.5)	198 (67.3)	174 (67.7)
*Pvu*II × *Xba*I	PPXX	20 (3.6)	9 (3.1)	11 (4.3)
	PPXx	50 (9.1)	25 (8.5)	25 (9.7)
	PPxx	44 (8.0)	21 (7.1)	23 (8.9)
	PpXx	106 (19.2)	60 (20.4)	46 (17.9)
	Ppxx	149 (27.0)	91 (31.0)	58 (22.6)
	ppXx	3 (0.5)	2 (0.7)	1 (0.4)
	ppxx	179 (32.5)	86 (29.3)	93 (36.2)
APOE	ε2/3	39 (7.1)	25 (8.5)	14 (5.4)
	ε3/3	421 (76.4)	216 (73.5)	205 (80.0)
	ε3/4	82 (14.9)	46 (15.6)	36 (14.0)
	ε4/4	9 (1.6)	7 (2.4)	2 (0.8)

Numbers in parentheses represent the frequencies of these genotypes.

Results

Distributions of the ERα and APOE genotypes are listed in table 1 and do not deviate from those predicted by the Hardy-Weinberg equilibrium. Genotypic frequencies of both ERα and APOE polymorphisms are similar to those reported in the literature [3, 14] and did not differ between sexes. Genotypic frequencies of two polymorphic sites of ERα were in linkage disequilibrium ($X = 189.401$, d.f. = 4, $p < 0.0001$), and 7 combined genotypes were present (table 1). Possession of an APOE ε4 allele did not affect the ERα gene polymorphism distribution. The subjects' mean age did not significantly differ among genotypes of ERα or those of APOE.

The distributions of the NFT and SP stages were positively correlated with age (NFT: $r = 0.306$, $p < 0.0001$; SP: $r = 0.237$, $p < 0.0001$). The women were significantly older than the men (82.6 vs. 79.5 years, $p < 0.0001$) and their NFT stages were correspondingly higher than those of the men (1.7 vs. 1.3, $p < 0.001$). However, the SP stages did not differ significantly between sexes. Both NFT and SP stages were significantly higher in ε4 carriers ($p < 0.0001$; fig. 1a, b).

Concerning the *Pvu*II site, possession of the P allele showed a trend to be associated with a more severe NFT stage (fig. 2a), but had no relationship with the SP stage.

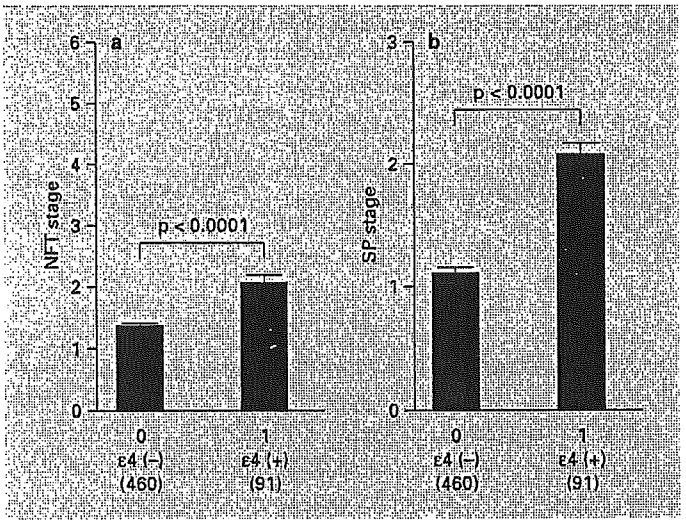

Fig. 1. NFT and SP stages in the subjects with or without the APOE ε4 allele. **a** NFT stage. **b** SP stage. Figures in parentheses represent the subject number. Error bars indicate standard error of the mean. p values by unpaired t test are shown.

The *Xba*I polymorphism did not affect either NFT or SP stage. In analyses of the men only, the association of the *Pvu*II polymorphism with the NFT stage became significant (fig. 2b, $p = 0.002$) and a gene-dose effect of the P

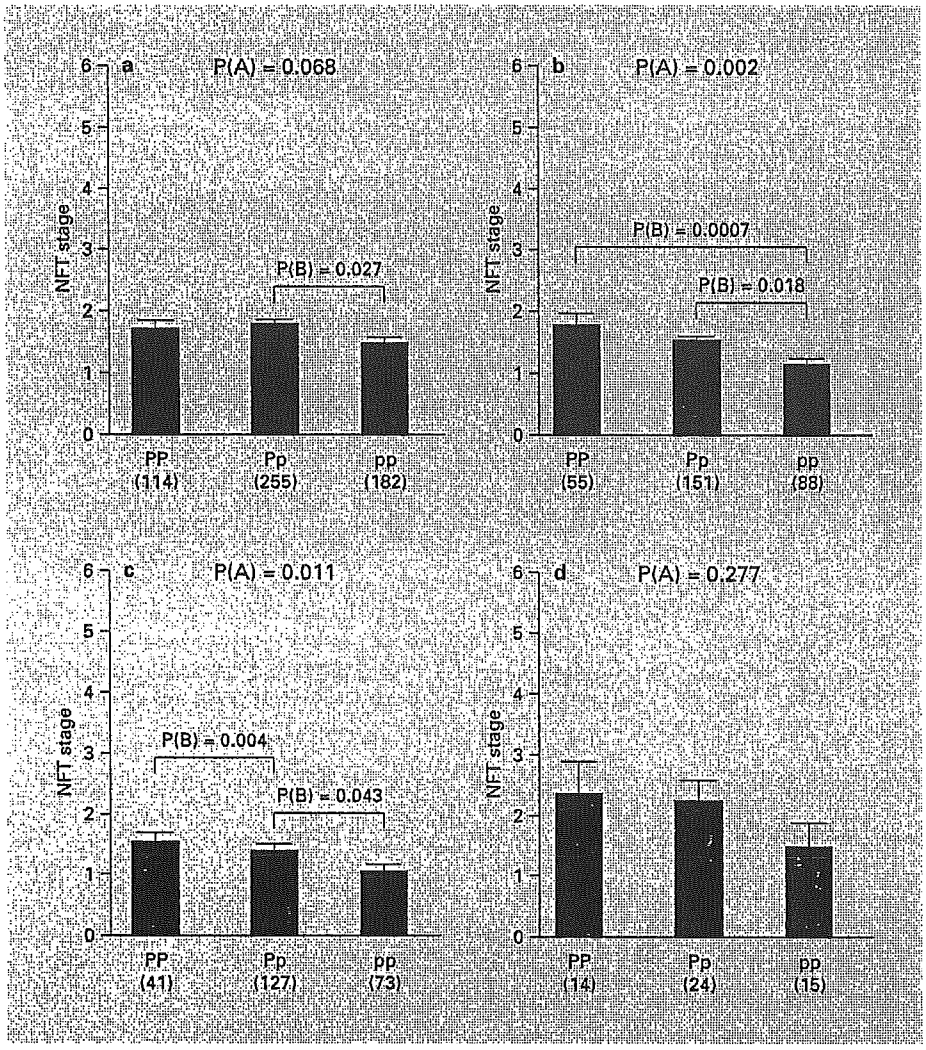

Fig. 2. ERα genotypes and the NFT stage. **a** All subjects. **b** Men. **c** Men without the ε4 allele. **d** Men with the ε4 allele. p(A) and p(B) indicate p values by ANOVA and by Bonferroni's method, respectively. Figures in parentheses represent the subject number. Error bars indicate standard error of the mean.

allele was evident. When the same analyses were performed on data from men that do not carry the ε4 allele, similar results were obtained (fig. 2c, $p = 0.011$). In male ε4 carriers, this trend was also observed but was not significant (fig. 2d). In the women, no significant association was found.

The relative contribution of the ERα gene polymorphisms to the NFT stage in men was evaluated by a stepwise forward selection multiple regression analysis. Age and possession of the ε4 allele were also worked into the model. This analysis demonstrated age to be the strongest determinant of the NFT stage, with possession of the ε4 allele being the second strongest determinant, and *Pvu*II polymorphism the third strongest (table 2, $p < 0.001$, $R^2 = 0.143$). The *Xba*I polymorphism was not selected.

Table 2. Relative contribution of age, APOE ε4, and ERα polymorphisms on NFT stage

	β	r
Age	0.268	0.278**
APOE ε4 (– = 0, + = 1)	0.210	0.219**
*Pvu*II (pp = 0, Pp = 1, PP = 2)	0.159	0.173*
R^2	0.144**	

Relative contribution of age, APOE ε4, and ERα polymorphisms on the NFT stage was evaluated by forward selection stepwise multiple regression. Age, APOE ε4 bearing and two ERα polymorphisms were forced into the model as independent variables and the NFT stages were dependent variables. β = Standardized partial regression coefficient; r = correlation coefficient. * $p < 0.01$; ** $p < 0.0001$.

Discussion

We have demonstrated for the first time that the *Pvu*II polymorphism of the ERα gene is associated with Braak NFT stage and that possession of the P allele may be an independent risk factor for NFT formation and consequently for AD in Japanese men. Although interaction of the *Pvu*II polymorphism with the APOE ε4 allele was not evident, our results were generally compatible with those of three previous studies [5–7], but contradictory to those of others [8–10]. These discrepancies may have arisen mainly from different study designs and subjects.

Our study design has many advantages over the preceding studies. First, the use of autopsy cadavers allowed the presence of NFT and SP to be confirmed. Second, the association of the NFT and SP stages with ERα polymorphisms was separately examined. Because NFT and SP are formed in a different manner, our method is quite reasonable. Third, degrees of NFT and SP formation could be semi-quantitatively evaluated independently of clinical symptoms. Because NFT and SP are present many decades before clinically detectable disease onset, case-control studies based on a dichotomy between clinically diagnosed AD and non-AD may lose information by discarding subclinical AD patients. However, great care must be taken to draw conclusions from the association of the ERα polymorphism with the occurrence of AD, because this study utilized autopsied materials of aged patients, not restricted to individuals with AD. It should also be considered that NFT in the hippocampus are generally related to aging and that those in the neocortex are specific for AD [15]. Therefore, the results of this study could be explained in terms of the aging process rather than by the AD pathological process.

One reason that the interaction of the *Pvu*II polymorphism with the APOE ε4 allele was not clearly demonstrated may also be attributable to differences in study designs. Because ε4 carriers tend to accumulate among patients with AD in case-control studies, the preceding studies may have overestimated the relationship between the *Pvu*II polymorphism and the ε4 allele. Conversely, our sample size may have been too small to detect it. However, as the multiple regression analysis revealed, ε4 is a stronger risk factor than the P allele, which explains the smaller relative contribution of the P allele to NFT formation among ε4 carriers.

The question arises as to whether the subjects of this study represent the general Japanese elderly population. Our subjects were taken from consecutive autopsies at a general hospital located in an urban area. Most of these patients were residents in that neighborhood and autopsies were not limited to a particular medical department. The genotypic distribution of ERα polymorphisms is in the Hardy-Weinberg equilibrium and for the most part comparable to those of previous reports [4, 5, 7], as well as the genotypic distributions of other polymorphic genes, such as angiotensin-converting enzyme and paraoxonase 1 [16, 17]. Thus, we believe our subjects to represent the general Japanese elderly population, at least in terms of genotypic distributions.

We did not consider the cognitive function of the subjects in this study, because it had been evaluated by specialists in only a small number of patients and because it was very difficult to collect information about the previous cognitive function of all autopsied subjects. However, because cognitive function evaluated by psychometric tools, such as clinical dementia rating, is known to be correlated with the NFT and SP stages [18, 19], our study could be regarded to have indirectly considered cognitive function.

At present, it is not clear how polymorphisms of the ERα gene affect NFT development and why there is a gender difference in this association. However, the findings of this study will give us a hint as to the etiological relation of the function of ERα in the formation of NFT and consequently as to the pathophysiology of AD, although our results should be interpreted in terms of the aging process rather than by the AD pathological process as mentioned previously. On the other hand, the *Pvu*II polymorphism may be merely in the linkage disequilibrium with genetic variability in another adjacent gene.

In conclusion, the *Pvu*II polymorphism of the ERα gene is significantly associated with Braak NFT stage in Japanese men. Possession of the P allele may promote NFT formation by influencing the aging process of the brain and act as an independent risk factor for cognitive decline in Japanese men, although age and the APOE ε4 allele are stronger risk indicators. These results must be verified by large-scale population studies in many races.

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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.

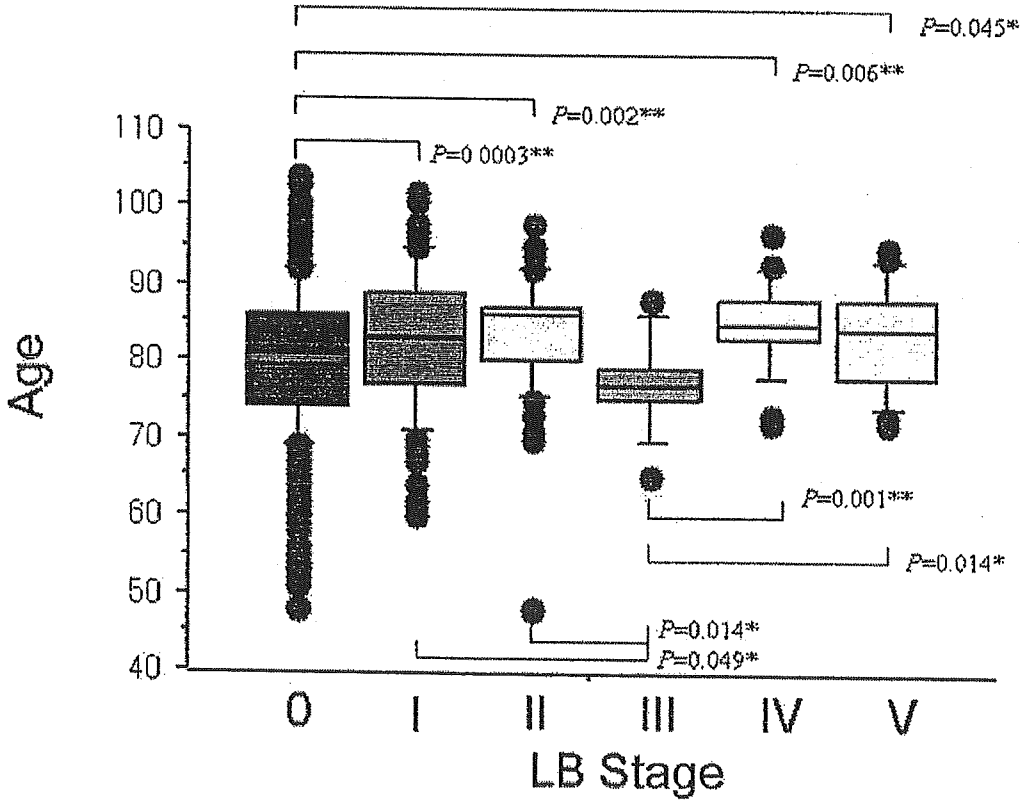

Fig. 1. Age distribution in each stage. The average age at death in Lewy body (LB) stages I, II, IV, and V was significantly greater than in LB stage 0 or LB stage III.

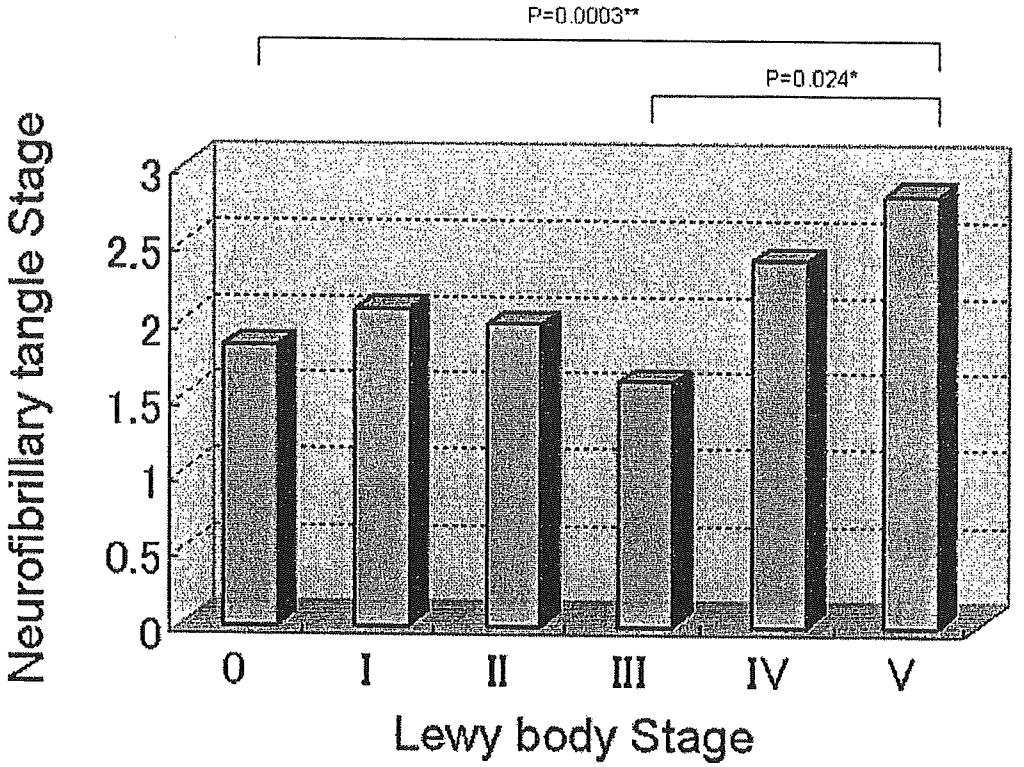

Fig. 2. Lewy body stage versus neurofibrillary tangle (NFT) stage. NFT stage is significantly higher in Lewy body (LB) stage V than in LB stage 0 or LB stage III.

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.

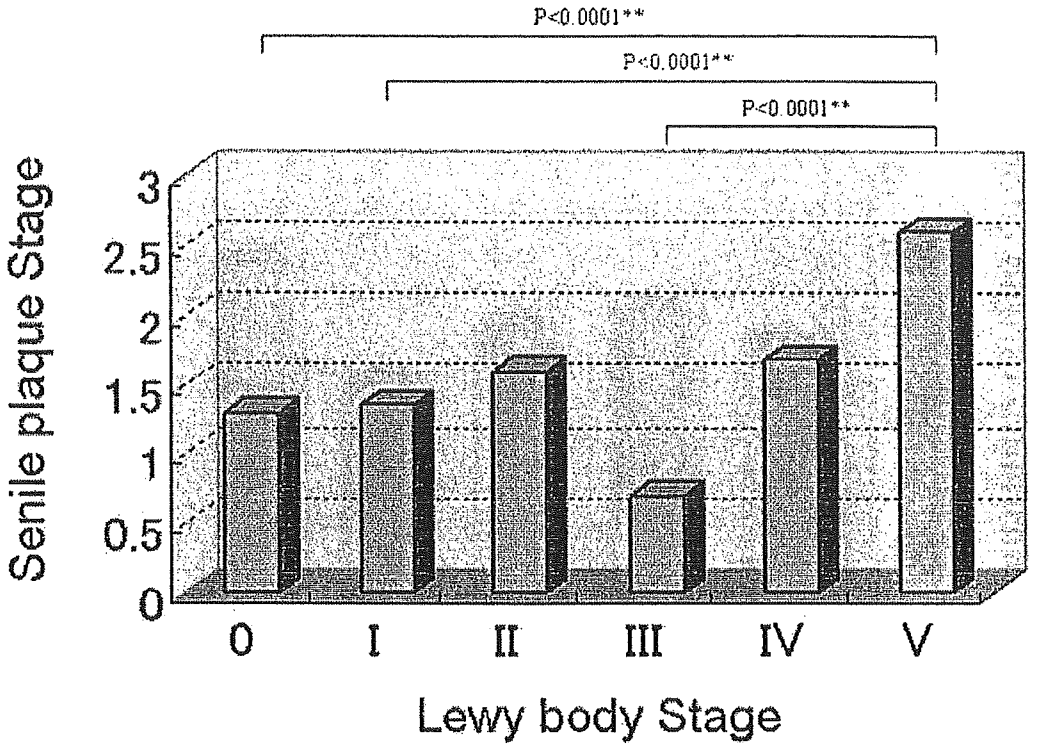

Fig. 3. Lewy body stage versus senile plaque (SP) stage. SP stage is significantly higher in Lewy body (LB) stage V than in LB stage 0, LB stage I or LB stage III.

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).