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.

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Development of a geriatric autopsy database and Internet-based database of Japanese single nucleotide polymorphisms for geriatric research (JG-SNP)

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The GEAD and JG-SNP have been created by joint collaboration between the Tokyo Metropolitan Geriatric Medical Center and the Japan Science and Technology Agency (JST)

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Abstract

To facilitate geriatric research on the roles of genetic polymorphisms of candidate genes, two databases were developed based on data obtained from autopsy examinations of elderly subjects: the geriatric autopsy database (GEAD) and the Japanese single nucleotide polymorphisms (SNP) database for geriatric research (JG-SNP) which is accessible on the Internet (http://www.tmgh.metro.tokyo.jp/jg-snp/english/E_top.html). The data for the GEAD were derived from 1074 consecutive autopsy cases (565 male and 509 female cases) with an average age of 80 years. The GEAD was installed on a stand-alone Windows 2000 server using Oracle 8i as the database application. The GEAD contains clinical diagnoses of 26 geriatric diseases, histories of smoking and alcohol consumption, pathological findings (720 items), severity of atherosclerosis, genetic polymorphism data, etc. On the JG-SNP website, case distribution corresponding to a specified SNP or disease can be searched or downloaded. Although there are several Internet-based SNP databases such as dbSNP, no databases are available at present on the web that contain both SNP data and phenotypic data. As autopsy studies can provide large amounts of accurate medical information, including the presence of undiagnosed diseases such as latent cancers, the GEAD is a unique and excellent database for research on genetic polymorphisms.

Keywords: Geriatrics; Pathology; Autopsy; Genetic polymorphism; Single nucleotide polymorphism; Database

Abbreviations: ACE, angiotensin-converting enzyme; DHPLC, degenerative high performance liquid chromatography; GEAD, geriatric autopsy database; JG-SNP, Japanese SNP database for geriatric research; PCR-RFLP, polymerase chain reaction restriction fragment length polymorphism; PON1, paraoxonase 1; SNP, single nucleotide polymorphism.

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1. Introduction

Improvements in health care management have increased the population of the aged in Western and some other countries. Geriatric medicine is thus becoming an important medical field. Most geriatric diseases are multifactorial diseases and are considered to be caused by interactions between hereditary factors such as disease susceptibility of the individuals and external factors such as smoking. The susceptibility and resistance to a particular disease show a wide range of individual differences that are believed to stem from subtle differences of the genome called genetic polymorphism. Genetic polymorphisms, in turn, can be used to identify the genes responsible for diseases (Schneider et al., 2003).

Tokyo Metropolitan Geriatric Medical Center is one of the institutions in Japan where autopsy examination is conducted very frequently. Over the last three decades, the center has performed more than 7000 autopsies, and has registered all the clinical and pathological information collected thus in a pathology database named "ANATOMY" (Ohtsubo et al., 1992). Based on this database, the authors have been studying the relationships between geriatric diseases and genetic polymorphisms of candidate genes. We have already reported associations between an insertion/deletion polymorphism of the angiotensin-converting enzyme (ACE) gene and cardiomegaly, a single nucleotide polymorphism (SNP; the most frequent type of genetic polymorphism) of the paraoxonase 1 (PON1) gene and Parkinson's disease, and a SNP of the estrogen receptor-alpha gene and Alzheimer's disease (Nakahara et al., 2000; Kazama et al., 2002; Kazama et al., 2004).

The objective of the present study was to establish an autopsy database specifically designed to promote further genetic researches on the relationships between geriatric diseases and genetic polymorphisms. The geriatric autopsy database (GEAD) established by the authors contains clinicopathological information and SNP data derived from autopsy examination of elderly subjects. Based on the GEAD, we have developed another database named "Japanese SNP database for geriatric research (JG-SNP)," which is accessible to the public on the Internet. Researchers interested in genetic polymorphisms can access our database on the web to obtain the allele frequencies of SNPs of candidate genes and associations between SNPs and geriatric diseases.

2. Materials and methods

2.1. Materials

The subjects were 1074 consecutive autopsy cases performed at the Tokyo Metropolitan Geriatric Medical Center during the last 5.5 years. They consisted of 565 males and 509 females, and the average age (±S.D.) of the subjects was 79.2 (±8.1) years for males and 81.8 (±9.5) years for females. The subject population also included 167 nanogerians and 7 centenarians. The average autopsy rate at this center during this period was 40%.

2.2. Flow and control of clinicopathological information

At autopsy, all internal organs were extirpated, examined, and fixed in 10% formalin. Two or three weeks after autopsy, macroscopic re-examinations of the extirpated organs were performed at weekly gross conferences. With the results of microscopic examinations, all cases were presented and discussed in details with clinicians at weekly autopsy conferences. All initial data were taken from the gross and microscopic autopsy reports and entered into the free-text type autopsy database, ANATOMY (Ohtsubo et al., 1992). The data on ANATOMY were then translated into the GEAD by the pathologists (M.S., T.A. and I.K.). The GEAD has been installed on a stand-alone Windows 2000 Server, and Oracle 8i, R.8.1.6 (Oracle Corp. Japan, Tokyo) was employed as the database application. The statistical analysis on the data of the GEAD was performed by one of us (N.T.) using the SAS system for Windows, V8 (SAS Institute Japan Ltd., Tokyo). The JG-SNP was installed on an Internet server run by NTT Hokkaido Telemart Co. Ltd., Sapporo, Japan.

2.3. Preparation and Storage of DNA

At the time of autopsy, small specimens from each of the five following organs, namely, the renal cortex, liver, ventricular myocardium, esophageal mucosa, and cerebral cortex were obtained and stored frozen at −80 °C. Brain specimens were obtained in 84% of the cases. Serum specimens collected within a week prior to death were also available for 75% of the cases. DNA used for genetic analysis was extracted from the renal cortex by the phenol–chloroform method, and stored frozen until use.

2.4. Genotyping assays

Depending on each polymorphic site of the candidate genes, one of the three genotyping methods was applied; the degenerative high performance liquid chromatography (DHPLC), polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP), and Taqman method. When none of these methods was applicable, direct sequencing was performed for genotyping. All exons including exon–intron junctions and promoter regions of the candidate genes were amplified by PCR and analyzed by the DHPLC to detect genetic polymorphisms. The DHPLC was performed by the Transgenomic WAVE DNA Fragment Analysis System (Transgenomic, Omaha, NE, USA) according to the manufacture's instructions. When more than one peaks appeared on the chromatography, these amplicons were directly sequenced. If restriction sites were present at

the polymorphic sites, PCR-RFLP was a choice of analysis. The allelic discrimination assay using Taqman fluorogenic probes was performed by ABI Prism 7000 according to the manufacture's instructions. All polymorphic data were validated by the direct sequencing of the PCR amplicons. The direct sequencing was performed by either ABI Prism 377 sequencer (Applied Biosystems, Foster City, CA, USA) or GeneRapid (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The detailed methods of analysis on each SNP will appear on the web pages of SNP data.

2.5. Ethical considerations

Written informed consent was obtained from the bereaved family of each of the patients prior to the autopsy examination. The use of autopsy materials for medical education and research is generally permitted by the Act of Postmortem Examinations of Japan. The database server and software have their own user IDs and passwords to protect the data from unauthorized access. The GEAD also has a provision to provide anonymity, in which a new ID is assigned to the patient ID to conceal the identity in cases of the leakage of data. Approvals for individual genetic researches and the release of the JG-SNP to the public on the Internet were obtained from the Ethical Committee of the Tokyo Metropolitan Geriatric Medical Center.

3. Results

3.1. Two database systems

The autopsy subjects of the GEAD were a specific group from our center, and the number of registered cases was as small as 1074 cases. The GEAD contains highly confidential, personal information, such as disease profile of the subjects. Accordingly, maximum caution to prevent leakage of confidential information is required before releasing the database to the public. Therefore, we established another separate database, namely JG-SNP, for Internet users. The flow-chart of the databases is shown in Fig. 1.

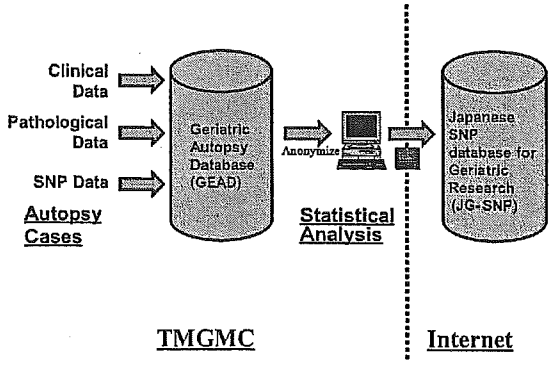

Fig. 1. Flow-chart of information compiled into GEAD and JG-SNP.

3.2. Geriatric autopsy database (GEAD)

The GEAD contains the following items:

1. Patient information: gender, date of birth, date of death, age at the time of death, and postmortem time to autopsy.
2. Clinical information: presence or absence of 26 geriatric diseases, results of clinical dementia rating, and histories of smoking and alcohol consumption. The 26 geriatric diseases included the following diseases/pathological conditions: ischemic heart disease, atrial fibrillation, degenerative valvular diseases, hypertension, aneurysm, arteriosclerosis obliterans, dementia, cerebrovascular disorder, Parkinson's disease, diabetes mellitus, hyperlipidemia, malnutrition, osteoporosis, degenerative osteoarthritis, aspiration, chronic obstructive pulmonary disease, idiopathic interstitial pneumonia, urinary tract infection, prostatic hypertrophy, decubital ulcer, lung cancer, gastric cancer, colon cancer, hematopoietic malignancy, cataract, and glaucoma.
3. Pathological findings: systemic pathological findings consisted of 720 items. The 720 codes used herein were originally created from pathological findings frequently encountered in autopsy examinations of elderly subjects. The data were entered as semi-quantitative values in the database. A code table of pathological findings of the heart is shown as an example in Table 1. Also included were the severity of atherosclerosis and pulmonary emphysema, degree of coronary arterial stenosis, weights of the organs, and volumes of intracavitary fluids.
4. Genetic polymorphism data: the current data included insertion/deletion polymorphism of the ACE gene and SNP of the PON1 gene. A total of 29 SNP data of the 13 candidate genes shown below are scheduled to be open at the next revision of the web pages: ACE, ALPL (alkaline phosphatase, liver/bone/kidney), CYP2C9 (P450, family 2, subfamily C, polypeptide 9), ESR1 (estrogen receptor-alpha), GGCX (gamma-glutamyl carboxylase), IL6 (interleukin 6, interferon, beta 2), KL (klotho), MTHFR (5,10-methylenetetrahydrofolate reductase, NADPH), NOS3 (nitric oxide synthase 3, endothelial cell), PON1, PPARG (peroxisome proliferative activated receptor, gamma), WRN (Werner syndrome), ZNF147 (zinc finger protein 147, estrogen-responsive finger protein). Additional 20 SNPs are scheduled to be open in the near future.

3.3. Japanese SNP database for geriatric research (JG-SNP)

The following items are included in the JG-SNP:

1. Patient information: gender and age group at the time of death.
2. Clinical diagnosis: presence or absence of the above-mentioned 26 geriatric diseases.

Table 1
Code table for pathological findings of the heart

Code number	Pathological diagnosis
1	Miscellaneous
2	Atrial septal defect
3	Ventricular septal defect
4	Bicuspid aortic valve
5	Endocarditis, NOS
6	Nonbacterial thrombotic endocarditis
7	Infectious (bacterial) endocarditis
8	Thrombosis
9	Atrial thrombosis
10	Tricuspid regurgitation (TR)
11	TR with ring-dilatation (relative TR)
12	Pulmonary valvular regurgitation
13	Mitral regurgitation (MR)
14	Mitral stenosis (MS)
15	Mitral stenosis and regurgitation (MSR)
16	Mitral-ring calcification (MRC)
17	MR with ring-dilatation (relative MR)
18	Mitral valve prolapse (hooding)
19	MR with papillary muscle dysfunction
20	MR with rupture of the chordae tendineae
21	Aortic (valvular) regurgitation (AR)
22	Aortic (valvular) stenosis (AS)
23	Calcification of the aortic valve
24	Aortic stenosis and regurgitation (ASR)
25	Degenerative valvular diseases
26	Degenerative aortic valvular diseases
27	Degenerative mitral valvular diseases
28	Rheumatic valvular diseases
29	Status after valvular surgery
30	Myocardial infarction (MI)
31	Acute myocardial infarction (AMI)
32	Old myocardial infarction (OMI)
33	OMI with acute extension
34	MI with thrombosis
35	MI with ventricular aneurysm
36	MI with ventricular/septal rupture
37	Myocardial fibrosis/scars, NOS
38	Myocardial necrosis, NOS
39	Coronary arteriosclerosis
40	Status after coronary angioplasty or surgery
41	Amyloidosis or amyloid deposition
42	Myocarditis, NOS
43	(Brown) atrophy
44	Left ventricular hypertrophy
45	Left ventricular dilatation
46	Right ventricular hypertrophy
47	Right ventricular dilatation
48	Cor pulmonale
49	Atrial dilatation (atriomegaly)
50	Cardiomyopathy
51	Hypertrophic cardiomyopathy
52	Dilated cardiomyopathy
53	Metastatic tumors
54	Status after pacemaker implantation
55	Cardiac tamponade
56	Pericarditis, NOS
57	Purulent pericarditis, NOS
58	Hemorrhagic pericarditis, NOS
59	Fibrous/fibrinous pericarditis, NOS
60	Pericarditis carcinomatosa

3. Pathological findings: presence or absence of 12 pathologically-identified diseases/pathological conditions, including atherosclerosis, gastric ulcer, pneumonia, cholelithiasis, urinary tract infection, and cancers of seven organs (thyroid, lung, stomach, colon, liver, kidney, and prostate).
4. Genetic polymorphism data: genetic polymorphism data of ACE and PON1. Also included in the homepage of the JG-SNP are characteristics of geriatric diseases, outlines of genetic polymorphisms, brief explanations of each geriatric disease, research results on genetic polymorphisms studied by us, ethical considerations, call for joint study, etc.

The major functions of the JG-SNP are outlined as follows:

1. Case distribution of genetic polymorphisms according to the clinical and pathological diagnoses can be searched and downloaded in a comma separated value (CSV) format.
2. Case distribution of clinical and pathological diagnoses corresponding to a specified genetic polymorphism can be searched and retrieved.
3. Gender and age group of the cases can be specified on search.
4. In order to prevent leakage of confidential information during any searches on the web, if there is only one case corresponding to a specific SNP and a specific disease, the JG-SNP is so constructed that the data will not be displayed on the screen. The corresponding data are excluded from the total counts of the table as well.

The followings are the URLs for the home page of the JG-SNP. The "Search by Disease" screen of the JG-SNP home page is shown in Fig. 2. English version: http://www.tmgh.metro.tokyo.jp/jg-snp/english/E_top.html. Japanese

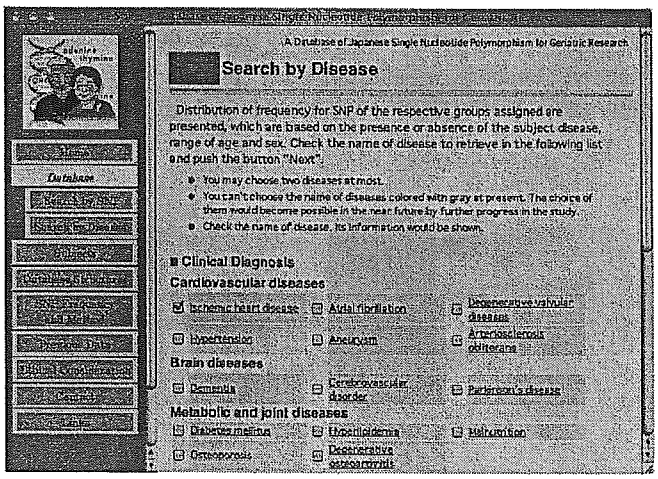

Fig. 2. "Search by Disease" screen of the Japanese SNP database for geriatric research (JG-SNP), which can be seen at http://www.tmgh.metro.tokyo.jp/jg-snp/english/E_top.html.

version: http://www.tmgh.metro.tokyo.jp/jg-snp/japanese/top.html.

4. Discussion

Currently, there are several databases related to genetic polymorphisms that are accessible on the Internet. For example, dbSNP runs under the control of the National Center for Biotechnology Information, USA, and opens at http://www/ncbi.nim.nih.gov/SNP/. A Japanese SNP database (JSNP) has been developed by the Human Genome Center (Institute of Medical Science, University of Tokyo) and the Japan Science and Technology Agency (JST), and opens at http://snp.ims.u-tokyo.ac.jp/index.html. As of December 2003, a huge number of SNPs has been registered: 4,760,000 SNPs under dbSNP and 195,000 SNPs under JSNP. For approximately half of the SNPs in the JSNP, the allele frequency is also available. However, the phenotypes, such as information on individual differences or medical information on the subjects, are not included in these and other SNP databases.

In contrast, the GEAD contains not only the SNP data but also related medical information, and has the following features:

1. Pathological diagnosis is currently recognized as the final diagnosis; thus, the GEAD contains a much more precise diagnosis than the clinical diagnosis.
2. As the entire body is thoroughly examined during autopsy, many undiagnosed diseases such as latent cancers are also discovered and included in the database.
3. The average age of the subject cases, 80 years, matches the average life expectancy of the Japanese people. Therefore, except for some geriatric diseases that occur in only extremely aged people, almost all diseases that may potentially occur during a lifetime are assumed to be included.
4. The GEAD contains many nanogerians and centenarians, which makes it potentially possible to identify the longevity-related genes.

With the progress in the fields of molecular biology and molecular genetics, it has become possible to obtain large amounts of information on the human genome and genetic polymorphisms on the Internet, and management of the information as well as the technology for analysis have been developing at a rapid pace. On the other hand, as for the phenotypic profiles of an individual called the "Phenome" corresponding to the Genome, no consensus has been reached regarding what kind of information should be included in the "Phenome" (Mahner and Kary, 1997; Freimer and Sabatti, 2003; Fredman et al., 2004). Medical information has been frequently chosen in the content of "Phenome", from the perspective of promotion of medical research. In our attempt to create a database for genetic research, the most important data, such as the clinical diagnoses and the pathological findings, were chosen. As the GEAD contains only data obtained from autopsy examinations, the number of subjects is inevitably limited. Thus, it would be necessary to include much more detailed quantitative pathological data for the study of genetic polymorphisms. This issue requires to be solved in future studies.

We have created the JG-SNP database for a wide target audience. The visitors of the JG-SNP website are assumed to be a part of the general public who are interested in geriatric diseases and molecular biology, and researchers on genetic polymorphism. For the general public, the website provides an easy-to-understand explanation on the significance of genetic polymorphisms and brief explanations on each geriatric disease. For researchers on genetic polymorphisms, this database is of the highest value. It is specifically possible for researchers to make comparisons with their own data on SNPs of the candidate genes, and furthermore to obtain the allele frequencies in the elderly population.

In conclusion, we have established an autopsy database for geriatric research, especially focusing on genetic polymorphisms, named GEAD. The GEAD contains both SNP data and medical information derived from autopsy examination of 1074 elderly subjects. Although the number of subjects is limited, it is hoped to open a new field of geriatric research. Based on the GEAD, we have developed another database called the JG-SNP for public use, which is accessible on the Internet.

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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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MATERIALS AND METHODS

Tissue Source

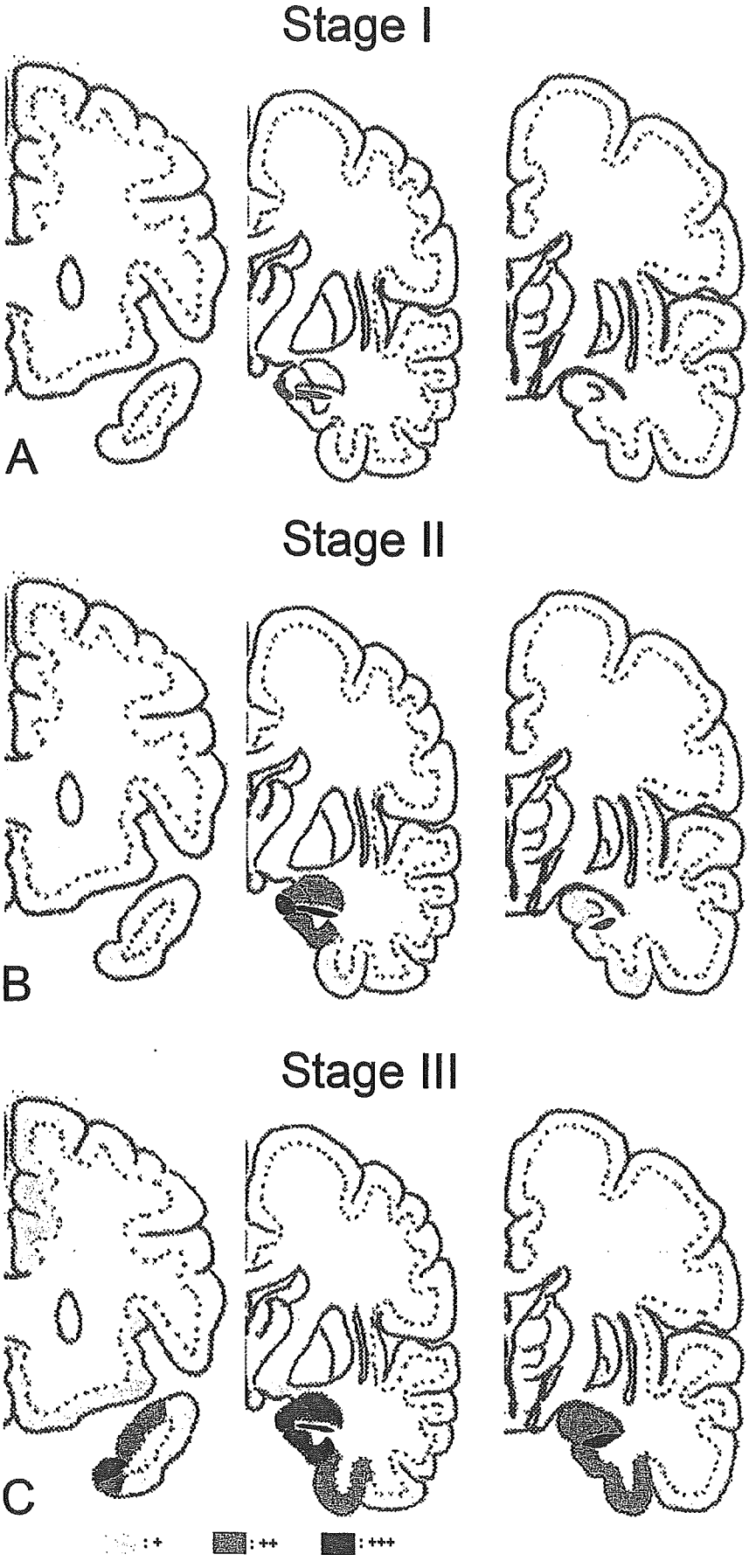
In the present work, 1,241 serial autopsy brains from Tokyo Metropolitan Geriatric Hospital (TMGH) (Group A) and 164 serial autopsy cases younger than 65 years of age from Yokohama Rosai Hospital (YRH) (Group B) were studied. YRH is a community center general hospital and neuropathological diagnosis 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


Stage I
A
Stage II
B
Stage III
C
: +
: ++
: +++

methods, as well as immunohistochemically using anti-phosphorylated tau (AT8, Innogenetics, Themes, Belgium; PHF1, a gift from Dr. P. Davies), anti-tau (anti-human tau, a gift from Dr. Y. Ihara; Alz50, a gift from Dr. P. Davies), anti-4-repeat-specific (4R) tau antibody (a kind gift from Dr. H. Mori), anti-Aβ 11–28 (12B2, IBL, Maebashi, Japan), anti-α-synuclein (LB509, a gift from Dr. T. Iwatsubo), and anti-ubiquitin (Dako, Glostrup, Denmark) antibodies using a Ventana NX20 (Ventana, Tucson, AZ) autoimmunostainer (11).

For the staging of neurofibrillary tangles (NFTs) and senile plaques (SPs), Braak and Braak criterion (9) was applied. For the staging of Lewy bodies, our staging method (12) was employed. Neuropathological diagnosis of degenerative dementia followed the previously reported criteria (1). Briefly, a diagnosis of Alzheimer disease (AD) was based on Braak's tangle stage equal to or above IV combined with plaque stage C, diagnoses of neurofibrillary tangle-predominant form of dementia and dementia with grains on Jellinger's criteria (13, 14), and a diagnosis of dementia with Lewy bodies (DLB) on its consensus guidelines (15). Neuropathological diagnosis of vascular dementia followed clinical (16), radiological (17), or pathological (18) criteria.

ApoE Genotyping

Genomic DNA was extracted from the kidneys (which had been snap-frozen at autopsy) and apoE genotyping was performed by the PCR method (19), as previously reported (1, 2). The results of typing were available for 1,114 of 1,241 cases in Group A.

Statistical Analysis

Statistical analysis was performed using the chi-square test or the Fisher exact test for comparisons of categorical data, Student *t*-test for comparison of means for continuous outcomes, Mann-Whitney *U*-test and Kruskal-Wallis 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

Neuropathology

The cases of degenerative dementia in Group A were neuropathologically classified into 105 cases of AD, 50 cases of dementia with grains, 33 cases of DLB (14 cases of neocortical form and 19 cases of transitional form), 13 cases of AD plus DLB, 13 cases of neurofibrillary tangle-predominant form of dementia, 8 cases of progressive supranuclear palsy, 4 cases of corticobasal degeneration, 4 cases of dementia with grains plus neurofibrillary tangle-predominant form of dementia, and 2 cases of DLB plus dementia with grains. 103 cases were categorized as vascular dementia. Group B did not include either degenerative or vascular dementia, except for a 49-year-old man with Huntington disease and a 57-year-old man with myotonic dystrophy, both of whom may have presented with mild cognitive impairment.

Staging of AGs

The detection of AGs was done by the Gallyas-Braak method and confirmed by immunohistochemical analyses with AT8, PHF1, Alz50, anti-human tau, anti-4R-specific antibody, and anti-ubiquitin antibodies.

The youngest case with AGs was a 56-year-old male from Group B. The incidence of grain-positive cases definitely increased with age. The distribution of AGs followed a stereotypic regional pattern and could be classified into the following stages (Fig. 1):

Stage 0: No grains are detected.

Stage I: Argyrophilic grains are observed in the ambient gyrus, usually forming clusters and frequently affecting the most anterior area of the CA1 of the hippocampus. The cortical and basolateral nuclei of the amygdala may be mildly involved. Oligodendroglial coiled bodies are scattered in the affected gray matter as well as its subcortical white matter. Bush-like astrocytes (20), defined as astrocytes with many thin processes immunoreactive with anti-phosphorylated tau antibodies but not well visualized with the Gallyas-Braak silver staining method, may be seen in the affected areas of the amygdala, but are quite rare.

Stage II: Argyrophilic grains definitely involve the amygdala and accompany ballooned neurons and bush-like astrocytes. Argyrophilic grains are apparent in the more posterior transentorhinal cortex and subiculum and the more anterior medial temporal lobe. A few pretangles, defined as intracytoplasmic neuronal accumulation of the epitope of anti-phosphorylated tau antibody not recognized by authentic silver staining, appear in the dentate gyrus. Bush-like astrocytes appear in the ambient gyrus, but superficial spongy degeneration involving the ambient gyrus is not observed.

Stage III: Argyrophilic grains are more apparent now in the insular cortex, the anterior cingulate gyrus, the nucleus accumbens, the septal nuclei, the hypothalamus,

←

Fig. 1. The topographical distribution of argyrophilic grains (AGs) in each grain stage. Three coronal sections through the genu of corpus callosum, the mammillary body, and the lateral geniculate body. Scoring of the frequency of AGs in sections stained with the Gallyas-Braak silver impregnation method, based on the number of grains in a ×400 field was as follows: + = 20 to 50; ++ = 50 to 100; and +++ = >100, as reported (1). **A:** Stage I: AGs are localized to the ambient gyrus, the anterior CA1 of hippocampus, the anterior entorhinal area, and the amygdala. **B:** Stage II: AGs are more apparent in the medial temporal pole, in the posterior subiculum, and the entorhinal and transentorhinal cortex. **C:** Stage III: AGs involve the anterior cingulate gyrus, septum, accumbens, gyri recti, insular cortex, and hypothalamus in addition to the medial temporal lobe.

and the gyri recti beyond the boundary of the temporal lobe. Pretangles in the fascia dentata increase in number. Tau-immunoreactive ballooned neurons are scattered in the affected area, including the anterior cingulate gyrus and the entorhinal area. Bush-like astrocytes are frequent in the amygdala, the gyrus rectus, and the nucleus accumbens and are scattered in the other affected areas. Superficial spongy degeneration associated with grains is present most prominently in the ambient gyrus, followed by the cortical subnucleus of amygdala, the posterior entorhinal area, and the medial temporal pole. In the terminal stage, marked atrophy of the junction between the amygdala and the anterior temporal lobe is a characteristic feature (2). The size of AGs apparently increases with advanced staging. However, in the terminal stage, the number of grains appears to be decreased in the areas where severe neuronal loss is present (1).

Clinical Correlation with the Staging of AGs

Cases from Group A were categorized as follows: stage 0, 792 cases (63.8%); stage I, 234 cases (18.9%); stage II, 118 cases (9.5%); and stage III, 97 cases (7.8%).

CDR was available in 1,105 out of 1,241 cases in Group A as follows: CDR0, 436 cases; CDR0.5, 190 cases; CDR1, 193 cases; CDR2, 124 cases; and CDR3, 162 cases. Further analysis was done for these CDR cases.

Among the 479 cases of dementia (CDR 1, 2, and 3), 50 cases presented with AGs as the only morphological substrate that might explain the cognitive decline. Forty-seven of these 50 cases were classified as argyrophilic grain stage III and the remaining 3 cases as stage II. The 3 stage II cases presented with sparse NFTs (Braak stage I) and SPs (Braak stage A) without Lewy bodies (stage 0) or any vascular lesions possibly contributing to cognitive decline (17, 18).

Among the 66 stage III cases whose CDR was available and who did not have any other degenerative or vascular dementing lesions, 47 cases had a clinical description of dementia as stated above, 17 cases were classified as CDR0.5, and 2 cases as CDR0. The rate of dementia (CDR> = 1) among argyrophilic grain stage III cases was 71.2%, and the percentage of cases with cognitive impairment (CDR> = 0.5) reached 97%. One of the 2 CDR0 cases had a history of suicide attempt and the degeneration of the ambient gyrus was milder, and the remaining case showed marked right-sided predominance of grains with right-handedness. The difference between the CDR0.5 and CDR3 cases was macroscopically distinct atrophy of the ambient gyrus in the latter.

ApoE Genotyping and AGs

There was no correlation between the staging of AGs and apoE genotyping or apoE allelic frequency (Table 1). However, comparing the average argyrophilic grain stage of each allele, the heterozygotes for the ε2 allele (0.64) tended to have higher stage than the combinations of the other alleles (ε3: 0.54 and ε4: 0.44, Mann-Whitney *U*-test, p = 0.094) and homo- or heterozygotes for ε3 tended to have higher stage than homozygotes for ε4 (p = 0.056), although no statistical significance of these differences was found.

TABLE 1
Apolipoprotein E Status and Staging of Argyrophilic Grains (AGs)

	AG Stage			
Genotype	0	I	II	III
23	4.8	1.7	0.8	0.6
33	48	14.5	7	5.6
34	10.7	3.1	1.3	0.4
44	1.2	0.1	0	0.2
Allelic frequency				
2	2.4	0.9	0.4	0.3
3	55.8	17	8	6.1
4	6.5	1.6	0.6	0.4

The percentage of each apoE genotype and allelic frequency with argyrophilic grain (AG) stage. The total number was 1,114 cases. There was no significant difference among the AG stages.

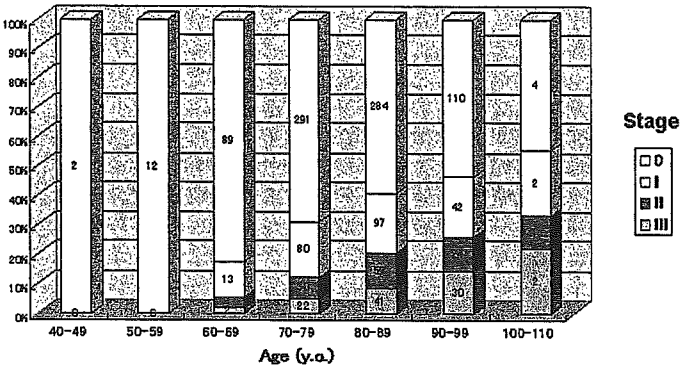


Fig. 2. The correlation between age and the argyrophilic grain stage. The argyrophilic grain stage significantly increased with age (Spearman correlation coefficient by rank, p < 0.0001).

The Influence of Age, Gender, and Brain Atrophy on AGs

The percentage of cases carrying AGs and the average staging both increased with age (Spearman rank correlation coefficient, p < 0.0001, Fig. 2). As for gender difference, the stage was significantly higher (Mann-Whitney *U*-test, p = 0.003) in females (average = 0.69) than in males (average = 0.54) and the frequency of grains was also significantly higher (χ^2 test, p = 0.0049) in females (40%) than in males (32%). However, no gender difference was detected in stage III cases (χ^2 test, p = 0.053) (Fig. 3). The average brain weight from each grain staging was as follows: stage 0, 1,227 ± 139 g; stage I,

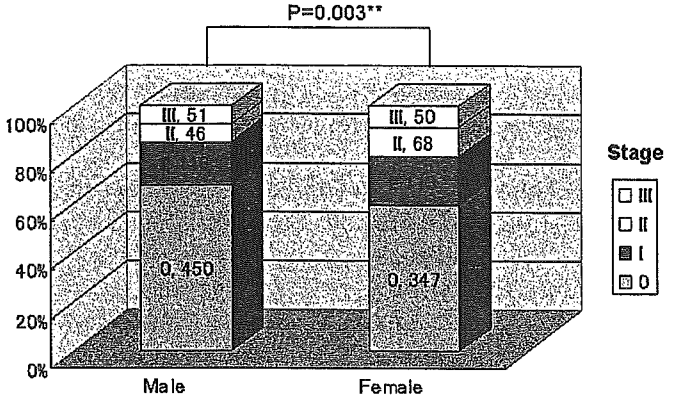


Fig. 3. The correlation between each gender and the stage of argyrophilic grains (AGs). Both average AG stage (Mann-Whitney *U*-test, p = 0.003) and frequency (χ^2 test, p = 0.0041) were higher in females than males. No statistically significant gender difference was found among stage III cases (χ^2 test, p = 0.53).

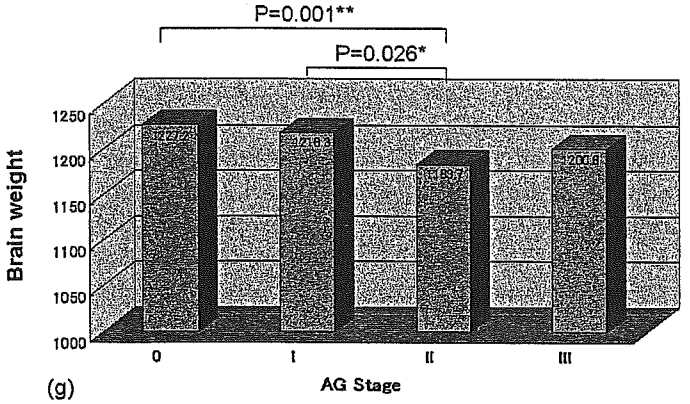


Fig. 4. The stage of argyrophilic grains and the brain weight. The average brain weight was significantly lower in argyrophilic grain stage II than stage 0 (Student *t*-test, p = 0.001) and I (p = 0.026). However, there were no significant differences between stage 0 and I (p = 0.39), stage 0 and III (p = 0.65), stages I and III (p = 0.25), or stages II and III (p = 0.31).

1,218 ± 134 g; stage II, 1,183 ± 136 g; and stage III, 1,201 ± 109 g. The average brain weight in stage II was significantly lower than in stage 0 (Student *t*-test, p = 0.001) and I (p = 0.026) (Fig. 4).

The average NFT stage (Braak) was significantly higher in argyrophilic grain-positive cases than in negative ones (Fig. 5A), but NFT stage was not correlated with argyrophilic grain staging. There was no relationship between the staging of AGs and Braak staging of SPs or our staging of Lewy bodies (Fig. 5B, C).

AGs in Other Neurodegenerative Diseases

The average staging of AGs in several neurodegenerative diseases is shown in Table 2. Both the frequency (Fisher exact probability test) and stage (Mann-Whitney *U*-test) were significantly higher in progressive supranuclear palsy (p < 0.0001 and p < 0.0001, respectively), neurofibrillary tangle-predominant form of dementia (p = 0.0048 and p = 0.0017), and DLB (p = 0.001 and p = 0.0017) compared with those of the background (Table 2).

DISCUSSION

This is the first report proposing a system for the staging of AGs and demonstrating the usefulness of this staging system for examining the contribution of AGs to cognitive decline.

In this report, we confirmed our previous finding (1) that dementia with grains is associated with grain-associated spongy degeneration of the ambient gyrus, spreading to the more anterior medial temporal lobe and to the more posterior parahippocampal gyrus. In this stage, AGs are observed beyond the boundary of the medial temporal lobe to the anterior cingulate gyrus, the gyri recti, the septal nuclei, the nucleus accumbens, the hypothalamus, and the insular cortex. In turn, when the cognitive state of the cases with AGs showing such widespread distribution was examined, 97% of the cases presented with cognitive impairment. Therefore, we categorized this phase as advanced stage (stage III). In more than 50 percent of cases, AGs were found only in the ambient gyrus and its vicinity, confirming the ambient gyrus as an initial site of involvement in argyrophilic grain-related senile change. Thus, we categorized these cases as the early stage (stage I). The relatively uniform progression of AGs may result in progression to the intermediate stage II. This staging method is quite convenient and only requires a section of the ambient gyrus as the minimal requirement, in addition to the sections recommended for use in CERAD and Braak methods.

The age-dependent increase in the incidence and severity of AGs that we observed here is in accordance with a previous report (21), although some exceptional cases of dementia with grains with either younger onset or with neocortical involvement have been reported (2, 22, 23).

Our statistical analysis showed that AGs were independent of SPs or Lewy bodies. NFT stage was significantly higher in argyrophilic grain-positive cases than negative ones, suggesting a mutual interaction in the deposition of tau. However, since NFT stage was not related to argyrophilic grain stage, the interaction may not be strong. The preponderance of AGs in females but a lack of gender difference in dementia with grains was first noted in this study and will require further confirmation in other groups.

The genetic effect of ApoE genotype on AGs is controversial (1, 24–26). In dementia with grains, a higher frequency of apoE ε2 and a lower frequency of apoE ε4

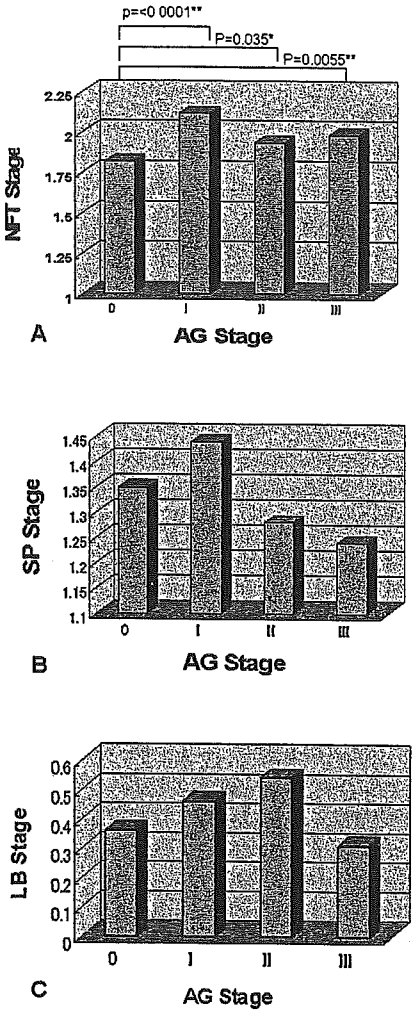


Fig. 5. The correlation between the stage of argyrophilic grains (AGs), and each stage of neurofibrillary tangles (NFTs), senile plaques (SPs), or Lewy bodies (LBs). **A:** The average NFT stage (Braak) was significantly higher in AG-positive cases than in negative cases (Mann-Whitney *U*-test, stage I: $p < 0.0001$, stage II: $p = 0.035$, stage III: $p = 0.0055$), but NFT stage was not related to AG staging. **B:** Average SP stage (Braak) was not different in the different AG stages. **C:** Average LB stage showed no difference among AG stages.

were reported (1, 24–26). We could not find any significant correlation between the staging of AGs and apoE genotyping in this study. Previously, we reported that dementia with grains with minimal senile changes was more common in subjects with apoE ε2 (1). The exclusion of stages B and C of SPs (Braak), which was done to highlight pure cases of dementia with grains in that study, might have contributed to the increase of the ε2 allele rather than dementia with grains itself. In this study, we strictly excluded cases with any vascular lesions possibly contributing to dementia (17, 18) in order to highlight the independent contribution of argyrophilic grain stages to cognitive decline. This difference in the selection of the cases of dementia with grains may also have influenced the difference in the correlation with apoE genotype.

The distinction between argyrophilic grain stages II and III is the involvement of the frontal lobe beyond the boundaries of the medial temporal lobe, as well as the presence of grain-associated spongy degeneration involving the ambient gyrus. For strictly accurate evaluation of this advanced stage, sections of the insular cortex, the anterior cingulate gyrus, the septal nuclei with the nucleus accumbens, and the gyri recti should be investigated with the Gallyas-Braak method for the presence of grains. It is still controversial whether AGs really contribute to cognitive decline. Our study showed that it is highly probable that the pathology in argyrophilic grain stage III contributed to cognitive decline. It is worth noting that approximately one fourth of stage III cases were categorized into CDR0.5 or the mild cognitive impairment level. The macroscopically distinct atrophy of the ambient gyrus separated more advanced dementia with grain cases from these CDR0.5 cases, confirming the importance of the ambient gyrus in cognitive decline associated with grains. However, the distinction between CDR0.5 and CDR1 cases was often very difficult and may indicate the limitation of this type of retrospective study. Prospective studies are now ongoing in our institute. Dementia with grains was the second most common form of degenerative dementia in our series as well as in studies by Braak and Tolnay (personal communication with Drs. Braak and Tolnay). Cases in these series represent data from the general geriatric population and have been analyzed by morphological examination able to detect AGs. Since many of dementia with grains cases present clinically with a milder form of dementia or mild cognitive impairment, prospective studies with special attention to clinical cognitive decline as well as morphological appearance of AGs may confirm the biological significance of AGs in human aging.

Argyrophilic grains have frequently been reported to be associated with other neurodegenerative diseases (27). In this large series, cases of progressive supranuclear palsy, neurofibrillary tangle-predominant form of dementia, and DLB clearly had a higher incidence as well as more advanced staging of grains compared with the background.

In conclusion, our staging method may contribute to better understanding of the role of AGs in the age-associated cognitive decline involving the human central nervous system.

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TABLE 2
Argyrophilic Grains in Neurodegenerative Disorders

	Stage 0	Stage I	Stage II	Stage III	Frequency		Average stage
PSP	0	4	4	2	10/10	(100%)**	1.8++
NFTD	5	5	3	4	12/17	(71%)**	1.35++
DLB	12	12	7	3	22/34	(65%)**	1.029+
MSA	1	1	1	0	2/3	(67%)	1
AD	75	20	7	3	30/105	(29%)	0.4
ALS	7	1	1	0	2/9	(22%)	0.33
PD	8	1	1	0	2/10	(20%)	0.3
CBD	3	1	0	0	1/4	(25%)	0.25
Group A	792	234	118	97	449/1,241	(36%)	0.53

Stage: argyrophilic grain stage, PSP: progressive supranuclear palsy, NFTD: neurofibrillary tangle-predominant form of dementia, DLB: dementia with Lewy bodies, MSA: multiple system atrophy, AD: Alzheimer disease, ALS: amyotrophic lateral sclerosis, PD: Parkinson disease and CBD: corticobasal degeneration; Group A: total cases in Group A.

**: Significantly high incidence compared with background, Fisher exact probability test, $p < 0.01$. +: Mann-Whitney *U* test, $p < 0.05$, ++: Mann-Whitney *U* test, $p < 0.01$.

The average AG stage of 1,241 cases was 0.53. The incidence of AG in the background was 36%. Both the frequency and stage of argyrophilic grains were higher in PSP, NFTD, and DLB.

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