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ORIGINAL ARTICLE

Imaging discrepancies between magnetic resonance imaging and brain perfusion single-photon emission computed tomography in the diagnosis of Alzheimer's disease, and verification with amyloid positron emission tomography

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Abstract

Background: In the diagnosis of Alzheimer's disease (AD), discrepancies are often observed between magnetic resonance imaging (MRI) and brain perfusion single-photon emission computed tomography (SPECT) findings. MRI, brain perfusion SPECT, and amyloid positron emission tomography (PET) findings were compared in patients with mild cognitive impairment or early AD to clarify the discrepancies between imaging modalities.

Methods: Several imaging markers were investigated, including the cortical average standardized uptake value ratio on amyloid PET, the Z-score of a voxel-based specific regional analysis system for AD on MRI, periventricular hyperintensity grade, deep white matter hyperintense signal grade, number of microbleeds, and three indicators of the easy Z-score imaging system for a specific SPECT volume-of-interest analysis. Based on the results of the regional analysis and the three indicators, we classified patients into four groups and then compared the results of amyloid PET, periventricular hyperintensity grade, deep white matter hyperintense signal grade, and the numbers of microbleeds among the groups.

Results: The amyloid deposition was the highest in the group that presented typical AD findings on both the regional analysis and the three indicators. The two groups that showed an imaging discrepancy between the regional analysis and the three indicators demonstrated intermediate amyloid deposition findings compared with the typical and atypical groups. The patients who showed hippocampal atrophy on the regional analysis and atypical AD findings using the three indicators were approximately 60% amyloid-negative. The mean periventricular hyperintensity grade was highest in the typical group.

Conclusions: Patients showing discrepancies between MRI and SPECT demonstrated intermediate amyloid deposition findings compared with patients who showed typical or atypical findings. Strong white matter signal abnormalities on MRI in patients who presented typical AD findings provided further evidence for the involvement of vascular factors in AD.

Key words: *Alzheimer's disease, amyloid, dementia, magnetic resonance imaging, positron-emission tomography.*

INTRODUCTION

Currently, various neuroimaging modalities contribute to progress in the diagnosis of Alzheimer's disease (AD). In particular, image statistical analysis techniques, such as the voxel-based specific regional analysis system for Alzheimer's disease (VSRAD) in magnetic resonance imaging (MRI) and the easy Z-score Imaging System (eZIS) in single-photon emission computed tomography (SPECT),[1,2] are becoming increasingly prevalent.

In practical imaging diagnosis, even if MRI demonstrates hippocampal atrophy indicating AD, there are

several cases that do not show typical findings on brain perfusion SPECT, and cases to the contrary are also seen. Such imaging discrepancies between MRI and SPECT are often observed, and they are puzzling in the diagnosis of dementia. In contrast, amyloid positron emission tomography (PET) is one of the most useful diagnostic markers of AD.[3–5] Negative amyloid PET findings are a key factor in ruling out AD.[5]

The findings of MRI, SPECT, and amyloid PET were compared in patients with mild cognitive impairment (MCI) or early AD to clarify the discrepancies between imaging modalities.[6]

METHODS

Consecutive patients visiting our outpatient clinic with MCI (n = 29) and early AD (n = 15) with Mini-Mental State Examination (MMSE) scores in the 20–30 range were enrolled. The MCI diagnosis was performed according to the report by Petersen *et al.*[6] AD was diagnosed based on the Diagnostic and Statistical Manual of Mental Disorders, fourth edition.[7] The research protocol was explained to patients and/or their relatives, and informed consent for their participation was obtained. The study design was approved by the ethics committee of Nanpuh Hospital (Kagoshima, Japan).

The level of the depression was evaluated with the Geriatric Depression Scale (GDS).[8]

A superconductive 1.5-T MRI scanner (Achieva HP; Philips Healthcare, Best, Netherlands) was used to acquire 3-D T_1-weighted imaging of the individual subjects, and the imaging data were analyzed by VSRAD advance. A Z-score map from a subject's grey matter imaging was obtained by comparing it with the mean ± SD of controls' grey matter images for each voxel after anatomical standardization and voxel normalization to a global mean using the following equation:

$$\text{Z-score} = (\text{[Control mean]} - \text{[Individual value]})/(\text{Control SD}).[1]$$

T_2*-weighted images were also obtained for all patients. Microbleeds, observed as small areas of signal loss on T_2*-weighted images, were counted. The findings were assessed as positive if at least one microbleed was exhibited. In order to evaluate vascular factors on MRI, the Fazekas scale for cerebral white matter lesions was used.[9,10] Periventricular hyperintensity (PVH) was graded as follows: 0 = an absence; 1 = 'caps' or a pencil-thin lining; 2 = a smooth halo; and 3 = an irregular PVH extending into the deep white matter. Separate deep white matter hyperintense signals (DWMH) were rated as follows: 0 = an absence; 1 = punctate foci; 2 = a beginning confluence of foci; and 3 = large confluent areas.

Each patient received a 600-MBq intravenous injection of ^{99m}Tc-ethyl cysteinate dimer just prior to SPECT (Infinia Hawkeye-4; General Electric, Milwaukee, WI, USA). Ten minutes after the injection, brain SPECT images were recorded. SPECT images for all patients were automatically standardized with an original ^{99m}Tc-ethyl cysteinate dimer template using the easy Z-score imaging system.[2] Three indicators for characterizing regional cerebral blood flow (rCBF) decreases in patients with very early AD were determined. First, the severity of rCBF decrease in a specific region showing rCBF reduction in very early AD was obtained from the averaged positive Z-scores in the volume of interest. Second, the extent of a region showing a significant rCBF reduction in the volume of interest was obtained; that is, the percentage rate of coordinates with a Z-value exceeding the threshold value of 2 was determined. Third, the ratio of the extent of a region showing significant rCBF reduction in the volume of interest to the extent of a region showing significant rCBF reduction in the whole brain was obtained; that is, the percentage rate of coordinates with a Z-value exceeding the threshold value of 2 was determined.[2]

PET was acquired on a Discovery ST Elite PET scanner (General Electric). The academic use of [C-11] Pittsburgh compound B (PiB) is permitted by Dr Chester A. Mathis, Department of Radiology, University of Pittsburgh Medical Center PET Facility, University of Pittsburgh School of Medicine (Pittsburgh, PA, USA).

The dose of PiB was 555 ± 185 MBq. The PET and magnetic resonance image volumes were co-registered. The regions of interest (ROI) were defined according to previously reported methods.[11] The ROI used in this study were as follows: anterior ventral striatum; dorsal frontal cortex; lateral temporal cortex; pregenual anterior cingulate; parietal cortex (PAR); and upper precuneus. The cerebellum was used as the reference region. The standardized uptake value ratio (SUVR) of each target ROI was calculated for each 20-min and 30-min time window by determining

the ratio of the integrated activities between the target ROI and the reference cerebellar ROI. The average values of the dorsal frontal cortex, lateral temporal cortex, pregenual anterior cingulate, PAR, and upper precuneus were used to determine the cortical SUVR average.

We also evaluated the whole brain visually with amyloid PET, based on accumulations in the precuneus, posterior cingulate gyrus, frontal cortex, lateral temporal cortex, lateral PAR, and striatum.[12] Stronger cortical retention than white matter in at least one cortex was assessed as positive; suspicious stronger cortical retention was assessed as equivocal; and weaker cortical retention than white matter was assessed as negative. The assessments were performed by radiologists who were blinded to the clinical data.

The cortical average SUVR and each cortical regional SUVR were compared with the age, MMSE score, GDS score, VSRAD Z-score, PVH grade, DWMH grade, the number of microbleeds, and the three eZIS indicators to examine the correlations between the variables.

Based on the results of VSRAD and the three eZIS indicators, we classified the patients into four groups. The threshold for the average Z-score VSRAD value was 2.0. The thresholds of the three eZIS indicators were 1.19 (sensitivity), 14.2% (extent), and 2.22 times (ratio). Values above these three thresholds were assessed as abnormal. The groups were classified as follows. In the typical group, the VSRAD Z-score was 2.0 or more, and all three eZIS indicators were abnormal. In discrepancy group 1, the VSRAD Z-score was 2.0 or more, and no more than two eZIS indicators were abnormal. In discrepancy group 2, the VSRAD Z-score was less than 2.0, and all three eZIS indicators were abnormal. In the atypical group, the VSRAD Z-score was less than 2.0, and no more than two eZIS indicators were abnormal (Fig. 1).

We compared the amyloid-positive rate, the cortical average SUVR, the SUVR in each cortex, PVH grade, DWMH grade, the numbers of microbleeds, and GDS score among the four groups. MRI, brain perfusion SPECT, and amyloid PET were performed within 3 months.

In the present study, the correlation between the SUVR from amyloid PET and other variables were analyzed using Pearson's correlation analysis. Additionally, χ^2 tests, a one-way ANOVA, and Mann–Whitney *U*-test were used for comparisons among the four groups, which were classified based on the VSRAD and eZIS results. *P*-values less than 0.05 were considered significant.

Discrepancy group 1 **(n = 7)** **VSRAD Z-score ≧ 2.0** **0–2 eZIS indicators are abnormal**	**Typical group** **(n = 5)** **VSRAD Z-score ≧ 2.0** **All 3 eZIS indicators are abnormal**
Atypical group **(n = 14)** **VSRAD Z-score < 2.0** **0–2 eZIS indicators are abnormal**	**Discrepancy group 2** **(n = 18)** **VSRAD Z-score < 2.0** **All 3 eZIS indicators are abnormal**

Figure 1 Group assignments based on the results of voxel-based specific regional analysis system for Alzheimer's disease (VSRAD) and easy Z-score Imaging System (eZIS). Typical group: VSRAD Z-score was 2.0 or more, and all three eZIS indicators were abnormal. Discrepancy group 1: VSRAD Z-score was 2.0 or more, and no more than two eZIS indicators were abnormal. Discrepancy group 2: VSRAD Z-score was less than 2.0, and all three eZIS indicators were abnormal. Atypical group: VSRAD Z-score was less than 2.0, and no more than two eZIS indicators were abnormal.

RESULTS

The 44 patients (16 men and 28 women) included 29 with MCI and 15 with AD. The average age was 75.0 ± 1.2 years (mean ± standard error [SE]), and the average MMSE and GDS scores were 25.1 ± 0.5 (mean ± SE) and 3.5 ± 0.4 (mean ± SE), respectively (Table 1).

Table 2 shows the correlations of the cortical average SUVR with other data. A negative correlation was found between the cortical average SUVR and MMSE score ($r = -0.550$, $P < 0.001$). Furthermore, when we examined the SUVR in each cortex, a negative correlation was found in all cortices. In particular, the right precuneus ($r = -0.567$, $P < 0.001$) and the left precuneus ($r = -0.606$, $P < 0.001$) showed stronger negative correlations with MMSE score. The cortical average SUVR showed no correlation with the VSRAD Z-score ($r = 0.235$, $P = 0.125$) or with the three eZIS indicators: severity ($r = 0.294$, $P = 0.053$); extent ($r = 0.294$, $P = 0.053$); and ratio ($r = 0.242$, $P = 0.114$). However, the right precuneus ($r = 0.352$, $P = 0.019$)

Table 1 Patient profiles

	n	Sex (men/women)	Age (mean ± SE)	Diagnosis (MCI/AD)	MMSE (mean ± SE)	GDS (mean ± SE)
Typical group	5	2/3	78.4 ± 2.5	1/4	23.2 ± 1.6	3.4 ± 0.9
Discrepancy group 1	7	1/6	79.1 ± 2.1	4/3	22.9 ± 1.2	2.9 ± 0.5
Discrepancy group 2	18	11/7	73.2 ± 1.8	12/6	25.3 ± 0.7	2.6 ± 0.7
Atypical group	14	2/12	74.0 ± 2.7	12/2	26.5 ± 0.7	5.1 ± 0.7
Total	44	16/28	75.0 ± 1.2	29/15	25.1 ± 0.5	3.5 ± 0.4

AD, Alzheimer's disease; GDS, Geriatric Depression Scale; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; SE, standard error.

Table 2 Correlations between the cortical average standardized uptake value ratio and different variables

	Pearson's correlation coefficient	*P*-value
Age	0.111	0.475
MMSE	−0.550	<0.001
GDS	−0.006	0.969
VSRAD	0.235	0.125
PVH	0.055	0.722
DWMH	0.200	0.194
Micobleeds	0.170	0.270
Severity	0.294	0.053
Extent	0.294	0.053
Ratio	0.242	0.114

DWMH, deep white matter hyperintense signals; GDS, Geriatric Depression Scale; MMSE, Mini-Mental State Examination; PVH, periventricular hyperintensity; VSRAD, voxel-based specific regional analysis system for Alzheimer's disease.

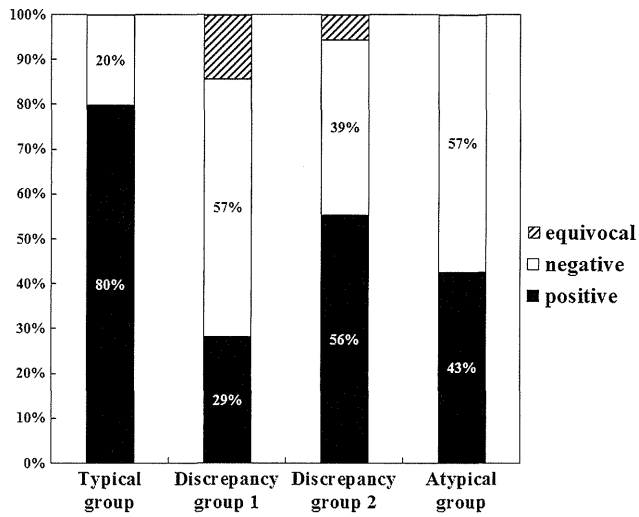

Figure 2 A graph showing the results of amyloid positron emission tomography by visual assessment. The ratio of amyloid-positive patients was highest in the typical group, but the difference was not significant (χ^2 test).

and the left precuneus ($r = 0.321$, $P = 0.034$) showed correlations with VSRAD Z-scores.

Based on the results of VSRAD and the three eZIS indicators, we classified the patients into four groups (Fig. 1). Five patients were assigned to the typical group, 7 patients to discrepancy group 1, 18 patients to discrepancy group 2, and 14 patients to the atypical group (Table 1).

According to the visual assessment of amyloid PET, 22 cases were positive, 20 cases were negative, and 2 cases were equivocal. The ratio of amyloid-positive patients was the highest in the typical group, but there was no significant difference among the groups (Fig. 2). The ratio of negative patients in each group was as follows: typical group, 20%; discrepancy group 1, 57%; discrepancy group 2, 39%; and atypical group, 57%. Amyloid PET ruled out AD in almost 60% of the patients in discrepancy group 1 (Fig. 2).

The cortical average SUVR was the highest in the group that presented typical AD findings on both VSRAD and eZIS. A significant difference was found between the typical group and the atypical group (one-way ANOVA, $P = 0.040$). The two groups that showed imaging discrepancies between VSRAD and eZIS (discrepancy groups 1 and 2) demonstrated intermediate findings of amyloid deposition when compared with the typical and atypical groups (Fig. 3). A difference in the amyloid deposition was not found between discrepancy group 1 and 2.

Assessments of SUVR in each cortex demonstrated that the amyloid depositions in the anterior ventral striatum (right), dorsal frontal cortex (right), pregenual anterior cingulate (right), PAR (left), and upper precuneus (right and left) were significantly higher in the typical group than in the atypical group. Discrepancy groups 1 and 2 demonstrated intermediate findings on each cortical regional SUVR study compared with the typical and atypical groups (Table 3).

The PVH grade results are shown in Figure 4. The average PVH grade (mean ± SE) was highest in the typical group (2.60 ± 0.25), and significant differences

Table 3 Cortical average SUVR and each cortical regional SUVR in the four groups

	n	AVS R (mean ± SE)	DFC R (mean ± SE)	LTC R (mean ± SE)	PAC R (mean ± SE)	PAR R (mean ± SE)	PCU R (mean ± SE)	Cortical average SUVR (mean ± SE)
Typical group	5	2.31 ± 0.20*	2.33 ± 0.25*	2.10 ± 0.20	2.28 ± 0.17*	2.14 ± 0.18	2.56 ± 0.29*	2.26 ± 0.20*
Discrepancy group 1	7	1.74 ± 0.20	1.73 ± 0.17	1.69 ± 0.12	1.70 ± 0.12	1.79 ± 0.15	1.91 ± 0.22	1.75 ± 0.14
Discrepancy group 2	18	1.95 ± 0.14	1.92 ± 0.14	1.80 ± 0.12	1.89 ± 0.13	1.87 ± 0.13	1.85 ± 0.15	1.84 ± 0.13
Atypical group	14	1.56 ± 0.10	1.56 ± 0.10	1.57 ± 0.10	1.54 ± 0.11	1.62 ± 0.09	1.62 ± 0.13	1.57 ± 0.10
Total	44	1.83 ± 0.08	1.82 ± 0.08	1.74 ± 0.07	1.79 ± 0.08	1.81 ± 0.07	1.87 ± 0.09	1.79 ± 0.07

	n	AVS L (mean ± SE)	DFC L (mean ± SE)	LTC L (mean ± SE)	PAC L (mean ± SE)	PAR L (mean ± SE)	PCU L (mean ± SE)
Typical group	5	2.22 ± 0.22	2.25 ± 0.24	2.04 ± 0.20	2.23 ± 0.15	2.21 ± 0.21*	2.43 ± 0.26*
Discrepancy group 1	7	1.72 ± 0.19	1.65 ± 0.15	1.70 ± 0.11	1.77 ± 0.10	1.69 ± 0.16	1.84 ± 0.22
Discrepancy group 2	18	1.91 ± 0.14	1.85 ± 0.15	1.68 ± 0.12	1.92 ± 0.14	1.78 ± 0.13	1.87 ± 0.16
Atypical group	14	1.56 ± 0.09	1.56 ± 0.10	1.48 ± 0.08	1.62 ± 0.09	1.53 ± 0.08	1.60 ± 0.13
Total	44	1.80 ± 0.08	1.77 ± 0.08	1.66 ± 0.07	1.84 ± 0.07	1.73 ± 0.07	1.84 ± 0.09

*$P < 0.05$, Comparison of typical group and atypical group. AVS, anterior ventral striatum; DFC, dorsal frontal cortex; L, left; LTC, lateral temporal cortex; PAC, pregenual anterior cingulate; PAR, parietal cortex; PCU, upper precuneus; R, right; SE, standard error; SUVR, standardized uptake value ratio.

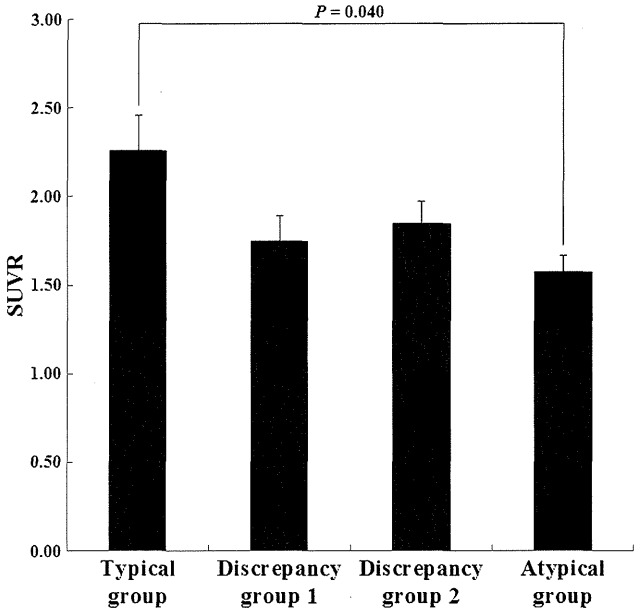

Figure 3 The cortical average standardized uptake value ratio (SUVR) was highest in the group that presented typical AD findings on both voxel-based specific regional analysis system for Alzheimer's disease (VSRAD) and easy Z-score Imaging System (eZIS). A significant difference (one-way ANOVA, $P = 0.040$) was found between the typical and atypical groups. The two groups that showed imaging discrepancies between VSRAD and eZIS (discrepancy groups 1 and 2), demonstrated intermediate amyloid retention findings compared with the typical and atypical groups.

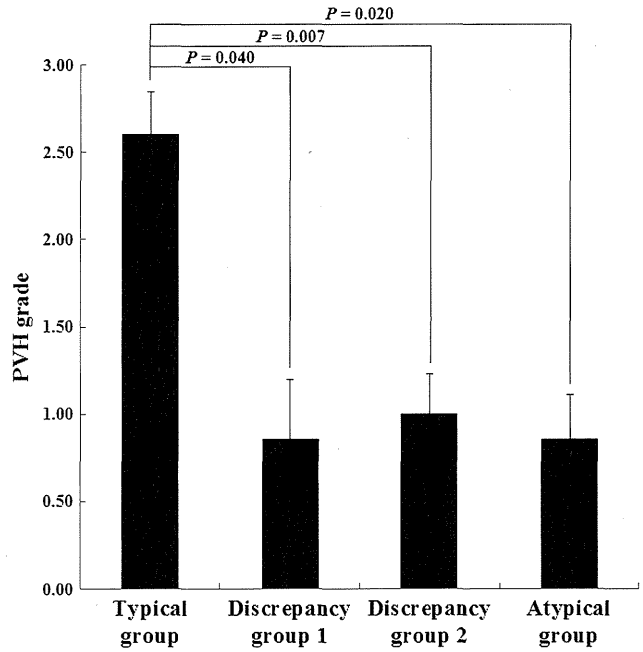

Figure 4 The average periventricular hyperintensity (PVH) grade (mean ± standard error) was highest in the typical group (2.60 ± 0.25), and a significant difference was found between the other three groups (Mann–Whitney *U*-test: discrepancy group 1, $P = 0.040$; discrepancy group 2, $P = 0.007$; atypical group, $P = 0.020$).

were found between the typical group and the other three groups (Mann–Whitney *U*-test, discrepancy group 1: 0.86 ± 0.34, $P = 0.040$, discrepancy group 2: 1.00 ± 0.23, $P = 0.007$, atypical group 0.86 ± 0.25, $P = 0.020$). Although the mean DWMH grade was highest in the typical group, a significant difference was not demonstrated.

The positive rate of the microbleeds was 60% in the typical group, 21% in the atypical group, 14% in discrepancy group 1, and 6% in discrepancy group 2.

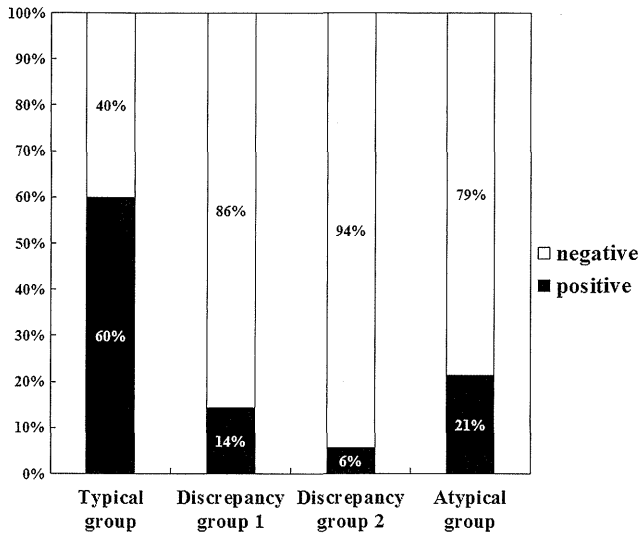

Figure 5 A graph showing the rate of positive microbleeds in each group. The typical group showed a higher tendency, but a significant difference was not found (χ^2 test).

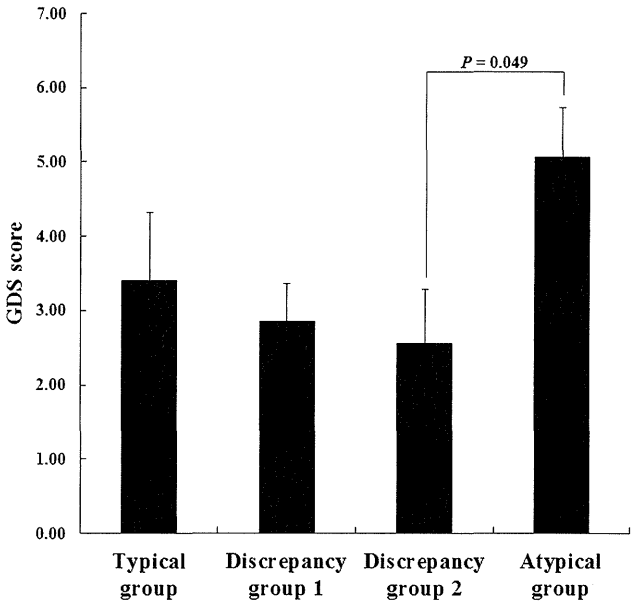

Figure 6 The average Geriatric Depression Scale (GDS) score (mean ± standard error) was highest in the atypical group (5.07 ± 0.67), followed by the typical group (3.40 ± 0.93), discrepancy group 1 (2.86 ± 0.51), and discrepancy group 2 (2.56 ± 0.74). A significant difference was shown between the atypical group and discrepancy group 2 (Mann–Whitney *U*-test, $P = 0.049$).

The typical group showed a higher tendency, but a significant difference was not demonstrated (Fig. 5).

The average GDS score (mean ± SE) was the highest in the atypical group (5.07 ± 0.67), followed by the typical group (3.40 ± 0.93), discrepancy group 1 (2.86 ± 0.51), and discrepancy group 2 (2.56 ± 0.74). A significant difference was shown between the atypical group and discrepancy group 2 (Mann–Whitney *U*-test, $P = 0.049$) (Fig. 6).

DISCUSSION

It is widely accepted that PET imaging with tracers can detect the presence of amyloid-beta in the brain. Brain imaging demonstrating a lack of amyloid-beta depositions is a key factor in ruling out AD.[5] It has been reported that PiB binding did not correlate with dementia severity in AD.[13] Amyloid deposition in the brain reaches a plateau in the early clinical stages of AD and, therefore, did not correlate with a decline in cognition.[14] There was a strong relationship between impaired episodic memory performance and PiB binding in both MCI and healthy ageing, but the relationship was weaker in AD.[15] Global cortical amyloid deposition demonstrated a significant correlation with cognitive test performance in AD and amnestic MCI patients.[16] Our results demonstrated a negative correlation between cortical average SUVR and MMSE score. This is probably because the subjects included only MCI or early AD patients. In each of the cortical regional SUVR assessments in the present study, amyloid deposition of the precuneus showed a correlation with the VSRAD Z-score. A previous report demonstrated that precuneal SUVR deposition showed the strongest associations with hippocampal atrophy.[17] It is suggested that amyloid accumulation in the precuneus plays an important role in the progression of AD. However, the cortical average SUVR showed no correlation with the VSRAD Z-score or the three eZIS indicators. In addition to amyloid retention, complicated processes may exist in the progression of hippocampal atrophy as well as hypoperfusion in the posterior cingulate gyrus and/or precuneus.

According to the visual assessments of amyloid PET, AD was ruled out in almost 60% of discrepancy group 1 patients who showed hippocampal atrophy on MRI but did not demonstrate the typical hypoperfusion pattern of AD on brain perfusion SPECT. It is suggested that there is a risk of misdiagnosis if the diagnosis is only based on MRI findings of hippocampal atrophy. A brain perfusion SPECT study is recommended for the assessment of patients with MCI and early AD.

The cortical average SUVR was the highest in patients who presented typical AD findings on both VSRAD and eZIS (typical group). A significant difference was found between the typical group and the atypical group. Assessments of SUVR in each cortex demonstrated that the amyloid depositions in several cortices were significantly higher in the typical group than in the atypical group. The two groups that showed imaging discrepancies between VSRAD and eZIS (discrepancy groups 1 and 2) demonstrated intermediate findings of amyloid deposition compared with the typical group and the atypical group in the SUVR assessments. When a discrepancy is seen between the results of VSRAD and eZIS, it is necessary to be aware that an AD diagnosis is less likely than in typical cases. We could not show a difference in amyloid deposition between discrepancy groups 1 and 2.

Cerebral white matter lesions demonstrated on MRI have been identified as vascular disease.[18,19] Hypertension-caused arteriosclerotic changes of the long penetrating medullary arteries may cause misery perfusion and later ischemic damage in the periventricular white matter.[19] Patients with silent brain infarcts and cerebral white matter lesions are at an increased risk of stroke and dementia.[9,18] In this study, we evaluated cerebral white matter lesions using PVH and DWMH to investigate the causes of discrepancy between VSRAD and eZIS. However, the severity of cerebral white matter lesions was rather mild in discrepancy groups 1 and 2. Interestingly, cerebral white matter lesions were more remarkable in the group with typical AD findings on VSRAD and eZIS. Small areas of signal loss on T_2*-weighted images have been histologically correlated with a previous extravasation of blood related to various microangiopathies.[20] Fazekas *et al.* reported that magnetic resonance signal loss foci showed moderate to severe fibrohyalinosis, and there was additional evidence of amyloid angiopathy.[21] Microbleeds, PVH grades, and lacunes have been reported as markers of cerebral microangiopathy.[22] Although a statistically significant difference was not revealed, this study found a high incidence of microbleeds as well as cerebral white matter lesions in the typical group. These results suggest the participation of microangiopathy in the progress of AD. In the Nun study by Snowdon *et al.*, participants who met the neuropathologic criteria for AD and those with brain infarcts had poorer cognitive function and a higher prevalence of dementia than those without infarcts.[23] These findings indicated that cerebrovascular disease, including microangiopathy, plays an important role in not only the cause of vascular dementia, but also the clinical symptoms of AD. Although vascular dementia has been regarded as exclusively related to AD, it has been reported that there is a spectrum ranging from patients with pure vascular dementia to patients with pure AD, and within that spectrum, a large majority of patients have contributions from both Alzheimer's and vascular pathologies.[24] Our study showed remarkable cerebral white matter lesions in patients with typical findings of AD on VSRAD and eZIS. Even if patients have typical MRI and SPECT findings of AD, the clinician should be aware of the potential coexistence of vascular dementia.

The GDS is commonly used to measure depression in the elderly.[8] A depressed state as evaluated by GDS did not correlate with discrepancies between VSRAD and eZIS. However, it is necessary to be conscious of depression in patients presenting atypical AD findings both on VSRAD and eZIS, because such patients have higher GDS scores.

In conclusion, based on the results of VSRAD and the three eZIS indicators, we classified patients into four groups. The cortical average SUVR was highest in the group that presented typical AD findings on both VSRAD and eZIS. A significant difference was found between the typical and atypical groups. The two groups that showed imaging discrepancies between VSRAD and eZIS demonstrated intermediate amyloid deposition findings compared with the typical and atypical groups.

According to the amyloid PET investigations, AD was ruled out in almost 60% of patients who showed hippocampal atrophy on MRI but did not demonstrate a typical AD hypoperfusion pattern on SPECT. It is suggested that there is a risk of misdiagnosis if the diagnosis is only based on MRI findings of hippocampal atrophy. Patients who presented typical AD findings demonstrated strong white matter signal abnormalities on MRI and provided further evidence for the involvement of vascular factors in AD.

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Milk and Dairy Consumption and Risk of Dementia in an Elderly Japanese Population: The Hisayama Study

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OBJECTIVES: To determine the effect of milk and dairy intake on the development of all-cause dementia and its subtypes in an elderly Japanese population.

DESIGN: Prospective cohort study.

SETTING: The Hisayama Study, Japan.

PARTICIPANTS: Individuals aged 60 and older without dementia (N = 1,081).

MEASUREMENTS: Milk and dairy intake was estimated using a 70-item semiquantitative food frequency questionnaire grouped into quartiles. The risk estimates of milk and dairy intake on the development of all-cause dementia, Alzheimer's disease (AD), and vascular dementia (VaD) were computed using a Cox proportional hazards model.

RESULTS: Over 17 years of follow-up, 303 subjects developed all-cause dementia; 166 had AD, and 98 had VaD. The age- and sex-adjusted incidence of all-cause dementia, AD, and VaD significantly decreased as milk and dairy intake level increased (P for trend = .03 for all-cause dementia, .04 for AD, .01 for VaD). After adjusting for potential confounders, the linear relationship between milk and dairy intake and development of AD remained significant (P for trend = .03), whereas the relationships with all-cause dementia and VaD were not significant. The risk of AD was significantly lower in the second, third, and fourth quartiles of milk and dairy intake than in the first quartile.

CONCLUSION: Greater milk and dairy intake reduced the risk of dementia, especially AD, in the general Japanese population. **J Am Geriatr Soc 62:1224–1230, 2014.**

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The increasing prevalence of dementia worldwide is a major public health concern. According to the World Health Organization and Alzheimer's Disease International, the number of people living with dementia will double by 2030 and more than triple by 2050,[1] but the causes of dementia, especially Alzheimer's disease (AD), remain unclear, and there are no disease-modifying therapies. Thus, there is an urgent need to identify factors that can prevent development of dementia to decrease the burden of this disease. Diet is one of the factors that can be modified, and it may have a protective influence against dementia. Milk and dairy intake has been reported to decrease cerebrovascular risk factors, such as hypertension,[2] diabetes mellitus,[3] and obesity,[4] which are associated with the development of dementia,[5] but a limited number of epidemiological studies have assessed the relationship between milk and dairy intake and cognitive impairment or dementia.[6–12] To this end, a community-based prospective cohort study was established to evaluate risk factors for or protective factors against dementia in the Japanese population. A feature of this study is that the subtypes of dementia have been verified using detailed neurological and morphological examination, including neuroimaging and autopsy. The purpose of this study was to elucidate the relationship between milk and dairy intake and the development of dementia and its subtypes in an elderly Japanese population.

METHODS

Study Populations

The Hisayama Study is an ongoing population-based prospective cohort study in the town of Hisayama, a suburb

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of the Fukuoka metropolitan area in the southern part of Japan.[13] This study was begun in 1961 to determine the prevalence and incidence of cerebro- and cardiovascular diseases and their risk factors in Japanese. Data from the national census and nutrition survey indicate that the age and occupational distributions and nutrient intake of the population of Hisayama are similar to those of Japan as a whole for each year from 1961 to the present.[14] Full community surveys of the health status and neurological condition of residents aged 40 and older have been repeated every 1 to 2 years since 1961. Comprehensive surveys of cognitive impairment have also been performed every 6 or 7 years in the elderly adults of the town since 1985.[15,16] In 1988, 1,228 residents aged 60 and older (participation rate 91.1%) underwent a screening examination for the present study. After excluding 35 subjects who already had dementia at baseline, 111 subjects whose dietary questionnaires were not available, and one subject with no blood sample, 1,081 subjects (457 men, 624 women) were enrolled in this study.

Follow-Up Survey

The subjects were followed prospectively for 17 years, from December 1988 to November 2005, during which time health examinations were repeated every 1 to 2 years.[13] Letters or telephone calls were used to collect the health information of subjects who did not have examinations or who had moved out of town. A daily monitoring system was also established with the study team and local physicians or members of the town's Health and Welfare Office to collect information about new events, including stroke, cognitive impairment, and dementia. Follow-up screening surveys of cognitive function, including neuropsychological tests (the Hasegawa Dementia Scale,[17] the Hasegawa Dementia Scale—Revised,[18] or the Mini-Mental State Examination[19]), were conducted in 1992, 1998, and 2005. The study physician and psychiatrist carefully evaluated any subject suspected of having new neurological symptoms, including cognitive impairment, by conducting a comprehensive investigation including interviews of the family or attending physician, physical and neurological examinations, and a review of the clinical records. Furthermore, when a subject died, all the available clinical information was reviewed, the attending physician and family of the deceased were interviewed, and an attempt was made to obtain permission for an autopsy from the family. During follow-up, 518 subjects died, 387 (74.7%) of whom underwent brain examination at autopsy. No subjects were lost to follow-up.

Diagnosis of Dementia

The guidelines of the *Diagnostic and Statistical Manual of Mental Disorders, Revised Third Edition*, were used to define the diagnosis of dementia,[20] the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association were used to define subjects with AD,[21] and the criteria of the National Institute of Neurological Disorders and Stroke-Association Internationale pour la Recherche et l'Enseignement en Neurosciences were used to determine the diagnoses of vascular dementia (VaD).[22] Clinical information, including neuroimaging, was used to diagnose possible and probable dementia subtypes. Definite dementia subtypes were also determined on the basis of clinical and neuropathological information in subjects with dementia who underwent autopsy. The diagnostic procedure for autopsy cases has been previously reported.[23] A neuropathological diagnosis of AD was made following the National Institute on Aging—Reagan Institute criteria;[24] the frequency of neuritic plaques and neurofibrillary tangles was evaluated using the Consortium to Establish a Registry for Alzheimer's Disease criteria[25] and Braak stage.[26] Definite VaD cases were confirmed with causative stroke or cerebrovascular change and no neuropathological evidence of other forms of dementia. Expert stroke physicians and psychiatrists adjudicated each case of dementia.

During the 17 years of follow-up, 303 subjects (103 men, 200 women) developed dementia; 261 (86.1%) were evaluated using brain imaging, 155 (51.2%) underwent autopsy, and both were performed in 143. Thus, 273 subjects (90.0%) had some kind of morphological examination. Of subjects with dementia cases, 25 with AD and 18 with VaD had other, coexisting subtypes of dementia, 14 of which were a mixed type of AD and VaD. These cases were counted as events in the analyses for each subtype. Finally, 166 subjects had AD (77 definite AD, 68 probable AD, 21 possible AD), and 98 had VaD (63 definite VaD, 35 probable VaD).

Nutritional Survey

The dietary survey was conducted using a 70-item semiquantitative food frequency questionnaire (SFFQ) concerning food intake.[27] Average food intake per day was calculated from the weekly frequency of various foods and the amount (quantity) of each food portion. The validity of this questionnaire has been reported previously.[28] Briefly, 65 subjects were randomly selected from 981 individuals aged 40 and older who underwent a health examination in 1987. Information regarding food intake was collected for 7 successive days using a weighted food record. Similarly, information regarding food intake was collected from the same subjects using the SFFQ. As a result, the 1-day average intake of milk and dairy products based on SFFQ was 84.6 g, and that based on the weighted food record was 103.9 g. The correlation coefficient in the amount of milk and dairy intake between the SFFQ and weighted food record was 0.53 ($P < .001$); this correlation was considered moderate.

The questionnaire was administered before initiation of this study; a trained dietician or nutritionist questioned each participant in the screening examination. Nutritional intake was calculated using the *Standard Tables of Food Composition in Japan, Fourth Revision*.[29] Each food group was adjusted for energy intake using the residual method.[30]

Risk Factor Measurements

At the baseline survey, each subject was asked to complete a self-administered questionnaire covering medical history, antidiabetes and antihypertensive treatments, educational status, smoking habits, alcohol consumption, and physical

activity. History of stroke was defined as a preexisting sudden onset of nonconvulsive and focal neurological deficit persisting for longer than 24 hours on the basis of all available clinical data. Low educational level was defined as less than 7 years of formal education. Smoking habits and alcohol consumption were categorized as current use or no current use. Regular exercise was defined as engaging in sports more than three times a week during leisure time. Blood pressure was measured three times using a standard mercury sphygmomanometer in the sitting position after at least 5 minutes rest. The mean of three measurements was used for the analysis. Hypertension was defined as blood pressure of 140/90 mmHg or greater or current use of antihypertensive drugs. Body height and weight were measured in light clothing without shoes, and body mass index (kg/m^2) was calculated. Diabetes mellitus was defined as fasting plasma glucose of 7.0 mmol/L or more, 2-hour postload glucose concentrations or postprandial glucose concentrations of 11.1 mmol/L or more, or current use of insulin or oral medication for diabetes mellitus. Serum total cholesterol levels were measured enzymatically.

Statistical Analysis

Subjects were grouped into quartiles based on amount of milk and dairy intake per day, according to sex. The quartiles for milk and dairy intake were less than 45, 45 to 96, 97 to 197, and 198 g/d or more for women and less than 20, 20 to 75, 76 to 173, and 174 g/d or more for men. The trends in the mean values of risk factors for the milk and dairy intake levels were tested using linear regression and the frequencies using logistic regression analysis. Participants were censored at date of death or end of follow-up for survival analyses. The incidence of dementia was calculated using a person-year method and adjusted for age and sex using the direct method using 10-year age groups of the overall study population. The age- and sex-adjusted or multivariable-adjusted hazard ratios (HRs) with their 95% confidence intervals (CIs) were estimated using the Cox proportional hazards model. The assumption of proportional hazards was checked graphically using log cumulative hazard plots for outcomes according to milk and dairy intake levels. In the multivariable-adjusted model, 15 covariates known to be potential risk or protective factors for dementia were selected: age; sex; low education; history of stroke; hypertension; diabetes mellitus; total cholesterol; body mass index; smoking habits; regular exercise; and energy, vegetable, fruit, fish, and meat intake.[31] Heterogeneity in the relationship between subgroups was tested by adding multiplicative interaction terms to the relevant Cox model. Two-sided $P < .05$ was considered statistically significant in all analyses. SAS version 9.3 (SAS Institute, Inc., Cary, NC) was used to perform all statistical analyses.

Ethical Considerations

This study was conducted with the approval of the Kyushu University institutional review board for clinical research. Written informed consent was obtained from participants.

RESULTS

The baseline characteristics of subjects according to milk and dairy intake levels are summarized in Table 1. Mean age and total cholesterol levels and frequencies of diabetes mellitus and regular exercise were higher with higher milk and dairy intake levels, whereas mean systolic blood pressure and frequencies of hypertension, smoking habits, and alcohol consumption were lower with higher milk and dairy intake levels. In relation to dietary factors, subjects in the fourth quartile of milk and dairy intake ate more fruit and had lower intake of fish and meat than those in the first quartile.

Figure 1 shows the age- and sex-adjusted incidence of all-cause dementia, AD, and VaD according to quartiles of milk and dairy intake levels. The age- and sex-adjusted incidence of all-cause dementia, AD, and VaD was significantly lower with higher milk and dairy intake levels (P for trend = .03 for all-cause dementia, = .04 for AD, and = .01 for VaD).

Table 2 shows the estimated HRs and 95% CIs for the development of dementia and its subtypes according to milk and dairy intake level. There was a significant inverse relationship between milk and dairy intake level and age- and sex-adjusted HR of all-cause dementia (P for trend = .03). This linear relationship did not remain significant after adjustment for age; sex; low education; diabetes mellitus; hypertension; total cholesterol; history of stroke; body mass index; smoking habits; regular exercise; and total energy, vegetable, fruit, fish, and meat intake (P for trend = .09), but the risk of all-cause dementia remained significantly lower in the third quartile than in the first quartile (adjusted HR = 0.69, 95% CI = 0.50–0.96).

With regard to dementia subtypes, multivariable-adjusted HRs of AD were significantly lower with higher milk and dairy intake, but no such relationship was observed for VaD (P for trend = .03 for AD; P for trend = .14 for VaD). The multivariable-adjusted HR of AD was significantly lower in subjects in the second, third, and fourth quartile of milk and dairy intake than in those in the first quartile (adjusted HR = 0.64, 95% CI = 0.41–0.99 for the second quartile; adjusted HR = 0.57, 95% CI = 0.37–0.87 for the third quartile; adjusted HR = 0.63, 95% CI = 0.41–0.98 for the fourth quartile). Although the age- and sex-adjusted HR of VaD was significantly lower in subjects in the fourth quartile of milk and dairy intake than in those in the first quartile, this relationship was not significant after multivariable adjustment (adjusted HR = 0.69, 95% CI = 0.37–1.29 for the fourth quartile). There was no evidence of heterogeneity between men and women in the risk of dementia and its subtypes.

DISCUSSION

This long-term prospective study of an elderly Japanese population demonstrated a significant inverse relationship between milk and dairy intake and risk of development of all-cause dementia, AD, and probably VaD. This is, to the best of the authors' knowledge, the first prospective cohort study to investigate the protective relationship between milk and dairy intake and risk of dementia and its subtypes.

Table 1. Baseline Characteristics of Subjects According to Quartile of Milk and Dairy Consumption: The Hisayama Study, 1988

Characteristic	Q1 (Low), n = 270	Q2, n = 270	Q3, n = 271	Q4 (High), n = 270	*P* for Trend
Female, %	57.8	57.8	57.6	57.8	.99
Age, mean ± SD	68.6 ± 6.4	69.8 ± 6.4	68.9 ± 6.1	70.4 ± 6.8	.008
Education ≤6 years, %	12.0	16.8	11.2	12.0	.56
History of stroke, %	4.1	4.4	4.4	4.4	.84
Systolic blood pressure, mmHg, mean ± SD	142 ± 24	138 ± 23	139 ± 21	137 ± 21	.02
Diastolic blood pressure, mmHg, mean ± SD	77 ± 11	76 ± 11	77 ± 10	75 ± 10	.10
Hypertension, %	57.8	53.7	53.5	48.5	.04
Diabetes mellitus, %	11.5	13.7	14.4	20.0	.007
Total cholesterol, mg/dL, mean ± SD	200 ± 42	204 ± 45	213 ± 43	220 ± 43	<.001
Body mass index, kg/m^2, mean ± SD	22.3 ± 3.1	22.1 ± 3.2	22.5 ± 3.2	22.4 ± 2.7	.40
Smoking habits, %	27.4	23.7	22.9	19.3	.03
Alcohol consumption, %	30.0	26.7	27.7	20.4	.02
Regular exercise, %	13.7	10.0	13.7	21.5	.005
Dietary intake, mean ± SD					
Energy, kcal/d[a]	1,703 ± 402	1,509 ± 395	1,721 ± 400	1,605 ± 372	.44
Vegetable, g/d[a]	251 ± 118	242 ± 102	257 ± 124	256 ± 128	.30
Fruit, g/d[a]	69 ± 69	74 ± 69	91 ± 93	84 ± 64	.002
Fish, g/d[a]	43 ± 43	41 ± 28	36 ± 28	37 ± 22	.006
Meat, g/d[a]	22 ± 24	20 ± 14	19 ± 15	19 ± 15	.03

Quartiles for milk and dairy intake were <45, 45–96, 97–197, ≥198 g/d for women and <20, 20–75, 76–173, ≥174 g/d for men.
SD = standard deviation.
[a]All food groups were adjusted for energy intake using the residual method.

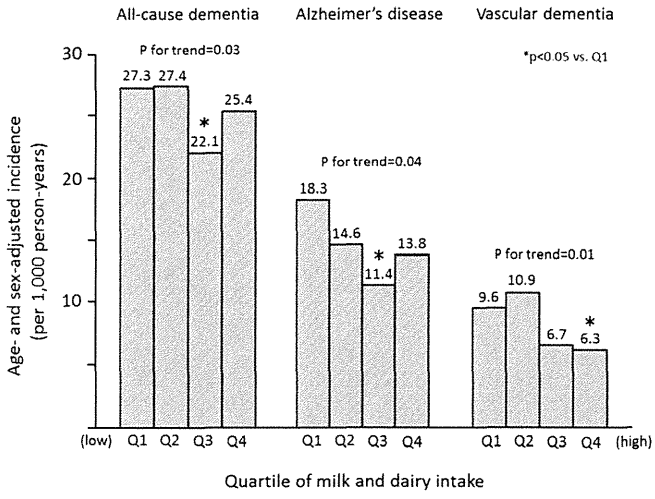

Figure 1. Age- and sex-adjusted incidence of all-cause dementia, Alzheimer's disease, and vascular dementia according to quartile of milk and dairy intake at baseline, 1988–2005.

Several epidemiological studies have investigated the relationship between milk and dairy intake and cognitive impairment or dementia.[6–12] Some cross-sectional studies have evaluated this relationship and found that higher milk and dairy intake is likely to have a protective effect against cognitive impairment.[6–8] A study in Australia demonstrated that low-fat milk and dairy consumption was associated with significantly lower likelihood of poor cognitive function but found the opposite to be true for whole-fat cream and ice cream rich in fat.[9] Similarly, a few prospective studies conducted in Western countries have reported that higher consumption of full-cream milk, milk and dairy desserts, and ice cream increased the risk of cognitive decline.[10,11] These results suggest that low-fat milk and dairy intake might have a more-favorable influence on cognitive function, especially in Western populations, although only one study has evaluated the relationship between milk intake and the risk of dementia longitudinally; the Adult Health Study with atomic bomb survivors in Japan retrospectively evaluated the relationship between milk intake, assessed 25 to 30 years earlier, and the prevalence of AD and VaD. The study concluded that subjects who consumed milk every day had significantly lower prevalence of VaD, but not of AD, than those who consumed milk twice a week or less.[12] This finding is inconsistent with that of the current study, but because the current study and the Adult Health Study had different designs (prospective vs retrospective), the heterogeneity of the methods may explain the discrepancy.

A few cohort studies in Western countries have found that it is possible that the Mediterranean dietary pattern provides protection against dementia, especially AD.[32,33] This diet recommends low to moderate consumption of milk and dairy products. Again, this is a finding that is inconsistent with that of the present study, although in a previous study of the present cohort, the greater adherence to the dietary pattern derived using a reduced rank regression analysis, which was characterized by high intake of milk and dairy products, was associated with a lower risk of dementia.[34] According to data from the Food and Agriculture Organization of the United Nations, there has consistently been a clear difference in the amount of milk and

Table 2. Likelihood of Development of All-Cause Dementia, Alzheimer's Disease, and Vascular Dementia According to Quartile of Milk and Dairy Consumption, 1988–2005

Outcome	Q1 (Low), n = 270	Q2, n = 270	Q3, n = 271	Q4 (High), n = 270	*P* for Trend
All-cause dementia					
Events, n	82	77	67	77	
HR (95% CI)[a]	1.0	0.90 (0.66–1.22)	0.66 (0.48–0.91)	0.76 (0.56–1.04)	.03
HR (95% CI)[b]	1.0	0.85 (0.62–1.18)	0.69 (0.50–0.96)	0.80 (0.57–1.11)	.09
Alzheimer's disease					
Events, n	49	38	37	42	
HR (95% CI)[a]	1.0	0.72 (0.47–1.10)	0.58 (0.38–0.89)	0.68 (0.45–1.03)	.04
HR (95% CI)[b]	1.0	0.64 (0.41–0.99)	0.57 (0.37–0.87)	0.63 (0.41–0.98)	.03
Vascular dementia					
Events, n	28	30	21	19	
HR (95% CI)[a]	1.0	1.04 (0.62–1.74)	0.65 (0.37–1.15)	0.54 (0.30–0.98)	.01
HR (95% CI)[b]	1.0	1.02 (0.59–1.77)	0.74 (0.42–1.33)	0.69 (0.37–1.29)	.14

HR = hazard ratio; CI = confidence interval.

[a]Adjusted for age and sex.

[b]Adjusted for age; sex; low education; history of stroke; hypertension; diabetes mellitus; total cholesterol; body mass index; smoking habits; regular exercise; and energy, vegetable, fruit, fish, and meat intake.

dairy consumption in Japan and Western countries; consumption in the Japanese population is historically approximately half that of Western populations.[35] This evidence, together with the findings of the present study, suggest that the difference in the amount of milk and dairy consumed in Japan and in Western countries could be the reason for the discrepancy in the influence of these foods on the risk of dementia between the populations. In populations with low intake of milk and dairy, such as the Japanese, a "high" intake of these foods is considered to reduce the risk of dementia. Further investigation is needed to clarify this in other ethnic populations.

In the present study, the age- and sex-adjusted HR of VaD was significantly lower in subjects in the fourth quartile of milk and dairy intake than in the first quartile, but this relationship was attenuated after adjustment for other covariates. This finding may have been due to the small number of VaD cases. In addition, because the frequencies of other known cerebrovascular risk factors, such as hypertension and smoking habits, were low in the fourth quartile of milk and dairy intake (Table 1), the risk of VaD may have appeared to decrease in this quartile through mediation of these risk factors.

There are presumably mechanisms for the protective influence of dairy intake against the risk of dementia. In several prospective studies, higher intake of milk and dairy was associated with lower risk of developing stroke and its risk factors, such as hypertension,[2] diabetes mellitus,[3] and obesity,[4] and these same factors were also recognized as risk factors for dementia.[5] Therefore, it is possible that milk and dairy intake decreases the risk of dementia, especially VaD, through mediating these risk factors. Another possible mechanism could be the benefits from some of the nutritional components of milk and dairy. It was previously reported that calcium and magnesium, which are components of milk and dairy, reduced the risk of development of dementia.[36] Milk and dairy consumption is also an important source of vitamin B_{12}, which is known to reduce plasma homocysteine levels. Because low serum vitamin B_{12} levels and high plasma homocysteine levels are reported risk factors for the development of dementia, especially AD,[37,38] milk and dairy consumption could decrease risk because of the influence of these nutrients.[39] Whey protein, another component of milk and dairy products, may also have favorable influence against dementia by reducing fat and improving insulin resistance.[40,41]

The strengths of the current study include its longitudinal, population-based, prospective design; the long follow-up period; perfect follow-up of subjects; and the ability to perform a morphological examination of the brains of most dementia cases using autopsy and neuroimaging, although some potential limitations should be noted. Information regarding the intake of dietary nutrients derived from a semiquantitative food frequency questionnaire may not be fully valid. In addition, the dietary assessment was performed only once, at baseline. These limitations are likely to have introduced some misclassification of food intake, and such misclassifications would weaken the relationship found in the study, biasing the results toward the null hypothesis. Finally, because dairy products are not part of traditional Japanese diets and represent a degree of westernization of lifestyle, the possibility of bias introduced by unmeasurable confounding factors cannot be eliminated.

In conclusion, these findings emphasize the need to consider higher intake of milk and dairy as a potentially protective factor against all-cause dementia, AD, and probably VaD in an elderly Japanese population. Further research will be necessary to clarify the relationship between milk and dairy intake and the risk of developing all-cause dementia and its subtypes in other prospective cohort studies and intervention trials.

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Conflict of Interest: The editor in chief has reviewed the conflict of interest checklist provided by

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Altered Expression of Diabetes-Related Genes in Alzheimer's Disease Brains: The Hisayama Study

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Diabetes mellitus (DM) is considered to be a risk factor for dementia including Alzheimer's disease (AD). However, the molecular mechanism underlying this risk is not well understood. We examined gene expression profiles in postmortem human brains donated for the Hisayama study. Three-way analysis of variance of microarray data from frontal cortex, temporal cortex, and hippocampus was performed with the presence/absence of AD and vascular dementia, and sex, as factors. Comparative analyses of expression changes in the brains of AD patients and a mouse model of AD were also performed. Relevant changes in gene expression identified by microarray analysis were validated by quantitative real-time reverse-transcription polymerase chain reaction and western blotting. The hippocampi of AD brains showed the most significant alteration in gene expression profile. Genes involved in noninsulin-dependent DM and obesity were significantly altered in both AD brains and the AD mouse model, as were genes related to psychiatric disorders and AD. The alterations in the expression profiles of DM-related genes in AD brains were independent of peripheral DM-related abnormalities. These results indicate that altered expression of genes related to DM in AD brains is a result of AD pathology, which may thereby be exacerbated by peripheral insulin resistance or DM.

Keywords: animal model, hippocampus, insulin, microarray, postmortem brains

Introduction

More than 20 million people worldwide suffer from dementia, and this number is expected to exceed 80 million by 2040 because of the rapid increase in the numbers of elderly (Ferri et al. 2005). The prevalences of all-cause dementia and Alzheimer's disease (AD) in the general population of Japanese elderly have increased significantly over the past 20 years, especially among subjects aged ≧75 years (Sekita et al. 2010). Thus, it is important to establish effective prevention strategies for dementia, and particularly for AD. To reach this goal, it is essential to understand the risk factors for developing dementia, including AD, in the elderly population.

Several recent studies have indicated effects of insulin and glucose metabolism on the risk of developing dementia, especially AD (Kuusisto et al. 1997; de la Monte and Wands 2008; Schrijvers et al. 2010). The results of the Hisayama study suggested that hyperinsulinemia and hyperglycemia caused by insulin resistance accelerate the formation of neuritic plaques (NPs) in combination with the effect of the *APOE* ε4 allele, a major risk factor for AD (Matsuzaki et al. 2010).

To identify molecular pathological alterations in AD brains, we performed interspecies comparative microarray analyses using RNA prepared from postmortem human brain tissues donated for the Hisayama study (Katsuki 1966; Matsuzaki et al. 2010; Sekita et al. 2010), and hippocampal RNAs from the triple-transgenic mouse model of AD (3xTg-AD) (Oddo et al. 2003). We found altered expression profiles of diabetes mellitus (DM)-related genes in AD brains, which were independent of peripheral DM-related abnormalities.

Materials and Methods

Postmortem Brain Tissues

We examined 88 autopsy samples from Hisayama residents obtained between 15 December 2008 and 24 February 2011. Clinical data related to DM or prediabetes were collected as described (Ohara et al. 2011). The study was approved by the Ethics Committee of the Faculty of Medicine, Kyushu University. Written informed consent for all subjects was obtained from their families. Neuropathologic changes were examined as described previously (Matsuzaki et al. 2010). Sections were routinely stained using hematoxylin–eosin, Klüver-Barrera stain, and a modified Bielschowsky method. Specimens from each subject were immunostained using antibodies against phosphorylated microtubule-associated protein tau (MAPT) (AT8, mouse monoclonal, 1:500; Innogenetics, Belgium) and the assessment of AD pathology was conducted according to the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) guidelines (Mirra et al. 1991) and the Braak stage (Braak and Braak 1991). During autopsy dissection, parts of the frontal cortex, temporal cortex, and hippocampus were cut out from each brain and preserved at −80 °C until RNA preparation.

Animals

3xTg-AD-H mice harboring a homozygous $Psen1_{M146V}$ mutation and homozygous mutant transgenes for APP_{Swe} and $MAPT_{P301L}$, 3xTg-AD-h mice harboring a homozygous $Psen1_{M146V}$ mutation and hemizygous APP_{Swe} and $MAPT_{P301L}$ transgenes, and nontransgenic control mice (non-Tg) (Oddo et al. 2003) were used in this study. At age 14 months, brains were removed ($N = 3$ male mice of each type) under pentobarbital anesthesia (i.p.), with perfusion of 40 mL of saline via the left ventricle. Hippocampi were isolated and preserved at −80 °C until RNA preparation. The handling and killing of all animals was performed in accordance with the national prescribed guidelines, and ethical approval for the study was granted by the Animal Experiment Committee of Kyushu University.

Gene Expression Profiling with Microarray Analyses

Total RNA was isolated using a combination of Isogen (Nippon Gene, Tokyo, Japan) and the RNeasy Mini Kit (Qiagen, Tokyo, Japan),

according to the manufacturers' instructions. RNA concentration was determined by the measurement of UV absorbance spectra, and the total RNA profile was analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies Japan, Tokyo, Japan) to determine RNA integrity number (RIN). Expression profiles of the RNA samples with an RIN ≧6.9 were determined using Affymetrix Human or Mouse Gene 1.0ST arrays (Affymetrix Japan, Tokyo, Japan) according to the manufacturer's instructions. The Ambion WT Expression Kit (Life Technologies Japan, Tokyo, Japan) and the GeneChip WT Terminal Labeling and Controls Kit (Affymetrix Japan) were used to generate amplified and biotinylated sense-strand DNA targets from expressed transcripts (100 ng of total RNA). Manufacturer's instructions were followed for hybridization, washing, and scanning steps, and CEL files were generated. CEL files were imported into Partek Genomics Suite software (Partek, St Louis, MO, USA), and both exon-level and gene-level estimates were obtained for all transcript clusters. The gene-level estimates were subjected to statistical analysis and hierarchical and partitioning clustering by the software. Transcript clusters with differential expression were investigated for functional inter-relatedness and networks using the Ingenuity Pathway Analysis (IPA) program (Ingenuity Systems, Redwood, CA, USA).

Reverse-Transcription and Quantitative Polymerase Chain Reaction

To validate the microarray data, we performed quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) on individual genes. We selected 17 genes of interest as potential targets for real-time RT-PCR; the corresponding gene-specific primer pairs are listed in Supplementary Table S1. Three genes, *RN18S1*, *ACTB*, and *GAPDH*, were used as internal controls. Each sample was reverse-transcribed to first-strand cDNA using 1 μg of total RNA, random primers and the High-Capacity cDNA Reverse-Transcription Kit (Life Technologies Japan). For each quantitative PCR reaction, 0.5% of the total complementary DNA yield was used. Transcript quantifications were carried out on a Thermal Cycler Dice® Real-Time System Single (Takara, Kyoto, Japan). Each reaction was performed using the appropriate amount of complementary DNA, optimized amounts of forward and reverse primers, and 12.5 mL of 2 × SYBR Green Ready Reaction Mix with Rox (Life Technologies Japan) in a total volume of 25 mL. Dissociation curves were generated for all wells. No primer dimers were observed.

Tissue Processing and Immunofluorescence Microscopy

Animals deeply anesthetized with pentobarbital were perfused intracardially with saline followed by cold 4% paraformaldehyde (PFA) in PBS. The brains were removed, immersed for 12 h in the same 4% PFA fixative, then in 20% followed by 30% sucrose in PBS for 24 h at 4 °C. The brains were stored as paraffin-embedded blocks. Tissue sections (4-μm thick) were cut from the blocks on a microtome and mounted from warm water (42 °C) onto slides. Sections were allowed to dry overnight at room temperature, deparaffinized in xylene, and rehydrated through a graded ethanol series. Antigen retrieval was performed by boiling sections in plastic Coplin jars containing sodium citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0) using a water bath (100 °C) for 10 min followed by cooling for 30 min to room temperature. Sections were blocked with a solution containing 1 × Block Ace (Dainippon Pharmaceutical, Osaka, Japan) for 30 min at room temperature, incubated with anti-PCSK1 (sc-100578, 1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and antineuron-specific nuclear protein (NeuN) (ABN78, 1:1000, Merck Japan, Tokyo, Japan) antibodies in 10% Block Ace at 4 °C overnight, and then incubated with an Alexa Fluor-labeled second antibody (Invitrogen Japan, Tokyo, Japan) for 45 min at room temperature. Confocal images were acquired using an LSM510 META Confocal Microscope System (Carl Zeiss MicroImaging, Tokyo, Japan).

Western Blot Analysis

Frozen hippocampus samples were homogenized in buffer containing 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl, pH 8.0) with 2% protease inhibitor, and a 5% phosphatase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) using a tissue homogenizer at 1500 rpm for 30 s. The homogenates were mixed with 2 × SDS sample buffer and subjected to 10% SDS polyacrylamide gel electrophoresis followed by western blotting using anti-PCSK1 (sc-100578), anti-PCSK2 (MAB6018, R&D Systems, Minneapolis, MN, USA), and anti-GAPDH (14C10, Cell Signaling Technology, Beverly, MA, USA) antibodies with appropriate fluorophore-conjugated secondary antibodies (LI-COR, Lincoln, NE, USA). Quantitative detection of fluorescent bands was performed using the Odyssey infrared imaging system (LI-COR). The blots were stained with Ponceau S (Sigma-Aldrich Japan, Tokyo, Japan), and digital images generated by a document scanner were used to quantitate total proteins on the blots in Image Gauge software (Fujifilm, Tokyo, Japan).

Statistical Analysis

Gene-level estimates from human microarray data were subjected to 3-way analysis of variance (ANOVA); then, the results of a specific comparison (AD vs. non-AD) were obtained using false discovery rate (FDR, $q < 0.05$) controlling procedures (Benjamini and Hochberg 1995). Gene-level estimates from mouse microarray data were subjected to ANOVA, and the obtained list of transcript clusters with $P < 0.05$ was subjected to a specific comparison (3x-TG-AD-H vs. non-Tg) with FDR ($q < 0.05$) control and a fold-change >1.3 as a threshold for the comparison. Statistical analysis of western blot data was performed by unpaired *t*-test using JMP 8.0 software (SAS Institute, Raleigh, NC, USA). A *P*-value <0.05 was considered statistically significant.

Results

Clinical and Pathological Features of Subjects

High-quality RNA samples with an RIN ≧6.9 from the gray matter of frontal and temporal cortices and hippocampi of 88 postmortem brains were subjected to microarray analysis. Among these, 26 subjects were pathologically diagnosed as having AD or an AD-like disorder. Only those results that

Table 1

Mean F ratios in 3-way ANOVAs of the microarray data

Comparison	Mean *F* ratios								
	Frontal cortex			Temporal cortex			Hippocampus		
	A	B	C	A	B	C	A	B	C
AD vs. non-AD	1.77	3.97	5.28	2.15	5.85	10.24	3.50	22.14	23.49
VD vs. non-VD	0.83	0.64	0.66	1.06	1.01	0.98	1.37	2.37	2.49
Sex	2.30	1.91	2.91	2.57	1.33	1.27	2.73	3.33	2.71

Note: A: mean *F* ratios for all transcript clusters (33 297) are shown. *B*: mean F ratios for transcript clusters (1387) exhibiting significantly altered expression between AD and non-AD hippocampal RNAs. *C*: mean F ratios for the top 200 transcription clusters in *B*. Statistical power analysis revealed that the smallest sample size that could be set for the 3-way ANOVAs was 5 with a significance (*P*-value) level of 0.01 and power of 0.8.

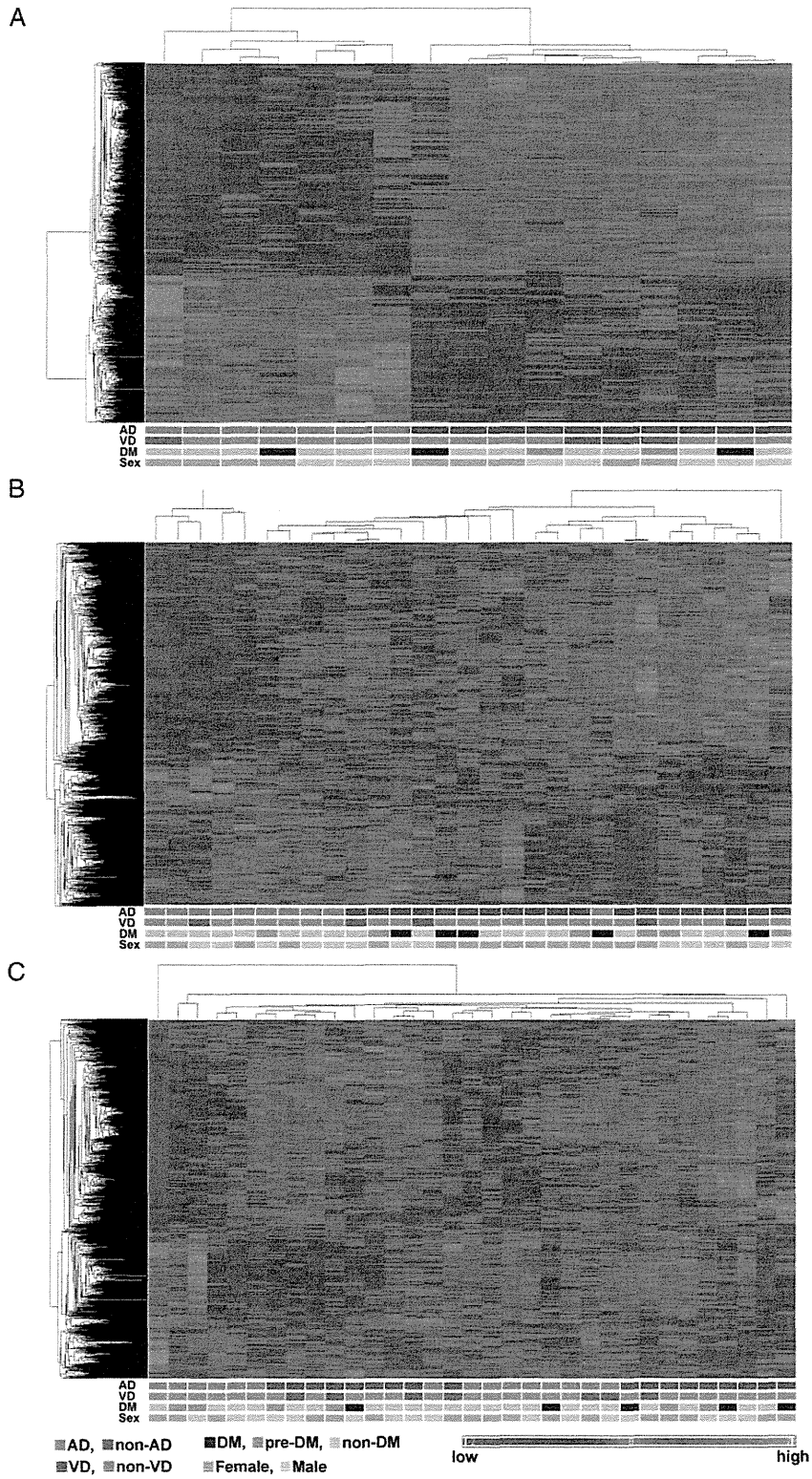

Figure 1. Hierarchical and partitioning clustering of the 1387 transcript clusters in the 3 regions of brain. (*A*) Cluster heat map of the 1387 transcript clusters based on expression data in the hippocampus. (*B*) Cluster heat map of the 1387 transcript clusters based on expression data in the temporal cortex. (*C*) Cluster heat map of the 1387 transcript clusters based on expression data in the frontal cortex. AD (red), non-AD (nAD, dark blue); vascular dementia (VD, dark green), non-VD (nVD, purple); diabetes mellitus (DM, black), prediabetes (pre-DM, gray), non-DM (light green); female (F, orange), male (M, light blue), In the heat map, blue represents a lower expression level and red indicates a higher expression level.

passed examinations for quality assurance and quality control of the GeneChip Human Gene 1.0 ST arrays were retrieved. In total, we obtained gene expression profiles from the following samples: 33 frontal cortex samples, among which 15 were from AD patients; 29 temporal cortex samples, among which 10 were from AD patients; 17 hippocampus samples, among

which 7 were from AD patients (see Supplementary Tables S2 and S3). NPs, assessed according to the CERAD guidelines (Mirra et al. 1991), were frequent (score of 3) in all 16 AD brains that provided RNA samples of suitable quality. The distribution patterns of neurofibrillary tangles (NFTs), assessed according to Braak stage (Braak and Braak 1991), were all stage V–VI. Two subjects given a pathological diagnosis of AD had been clinically diagnosed as having vascular dementia (VD), while another 3 subjects with a pathological diagnosis of AD had been clinically diagnosed with DM or prediabetes (defined as a blood glucose level of 140–199 mg/dL 2 h after a 75-g oral glucose tolerance test, or a blood glucose level of 110–125 mg/dL in the fasting condition) (see Supplementary Tables S2 and S3).

Altered Gene Expression Profiles in the Hippocampus, Temporal Cortex, and Frontal Cortex with AD Pathology

Three-way ANOVA of the microarray data with AD versus non-AD, VD versus non-VD, and female versus male as factors revealed that the comparison of AD versus non-AD exhibited the highest mean F ratio (3.50) based on expression data for all 33 297 transcript clusters obtained from hippocampal RNAs (Table 1). In total, 348 transcript clusters in the temporal cortex (98 up and 250 down) and 1387 transcript clusters in the hippocampus (569 up and 818 down), but none in the frontal cortex, showed significantly altered expression levels in AD versus non-AD brains (see Supplementary Tables S4 and S5). Of the 348 transcript clusters in the temporal cortex, 125 were also among the 1387 transcript clusters in the hippocampus. The mean F ratios for the 1387 transcript clusters identified in the hippocampus (Table 1) confirmed that the gene expression profile in the hippocampus is the most significantly altered in AD brain. No genes in any cluster showed a significant difference in expression levels between patients with DM or prediabetes (data not shown).

Hierarchical and partitioning clustering of the 1387 hippocampal transcript clusters (Fig. 1*A*) based on data from hippocampal samples revealed clustering of the 7 AD cases separately from the 10 non-AD cases, with statistical significance. Using data from temporal cortex samples, 9 of 10 AD cases were clustered together (Fig. 1*B*). Using data from frontal cortex samples, 6 and 8 AD cases were separately clustered out of 15 AD cases, and 8 and 9 non-AD cases were separately clustered out of 18 non-AD cases (Fig. 1*C*). Thus, the expression profiles of the 1387 transcript clusters identified as being altered in the hippocampus are similarly changed in the temporal and frontal cortices, but to lesser extents.

Genes Whose Expression Levels are Significantly Altered in AD Hippocampus

To retrieve genes whose expression levels were significantly altered in AD brains in comparison with non-AD brains, it is essential to consider the changes in the population of brain cells in AD brains. Therefore, we compared the expression levels of genes encoding specific markers for 4 major types of brain cells, namely, neurons, astrocytes, oligodendrocytes, and microglia (Table 2). The expression levels of 10 neuronal markers, including *RBFOX3* encoding NeuN (Dredge and Jensen 2011), which is expressed in about 68% of cells in the gray matter of the adult cerebral cortex (Azevedo et al. 2009), were consistently decreased in AD brains relative to the levels in non-AD brains, most significantly in the hippocampus. Conversely, the expression levels of *GFAP*, *S100B*, and *AQP4* transcripts, representing the astrocyte population, and to a lesser extent those for *AIF1*, *LGALS3*, *CD68*, and *EMR1* representing the microglial population, were increased, especially in the temporal cortex and hippocampus. The expression levels of *MBP*, *SOX10*, *MOG*, and *MAG*, representing the oligodendrocyte population, were largely unchanged. These data are likely to reflect neuronal loss and gliosis in AD brains; neuronal loss is most evident in the hippocampus, and gliosis is most evident in the temporal cortex and hippocampus.

Taking the mean relative expression levels of these markers in different brain regions (Table 2) into account, we selected the top 200 transcript clusters that exhibited a fold-change >1.563 from among the 1387 transcript clusters identified as being altered in the hippocampus (see Supplementary Table S6). In AD brains, 143 of the 200 transcript clusters were markedly downregulated in the hippocampus beyond the level expected based on the cell population change. Because of the population change in AD brains, the number of upregulated genes in AD brains was likely to have been underestimated (57 transcript clusters in see Supplementary Table S6).

We next individually analyzed the raw expression levels of 12 genes showing significant alterations (downregulated: *MET*, *PCSK1*, *RGS4*, *HS3ST2*, *NPTX2*, *NEUROD6*, *RAB27B*, *HCN1*, *HOMER1*; upregulated: *GJA1*, *AEBP1*, *GALNTL2*) in 3 brain regions from each subject (see Supplementary Fig. 1, left panels), confirming that AD hippocampi exhibited the most significant alterations of gene expression (Fig. 2*A*). The expression levels of individual exons within the 12 genes were also most significantly altered in the hippocampi of AD

Table 2
Altered expression levels of marker genes for various brain cell types in AD brains

Cell type	Relative expression (% non-AD)			
	Marker gene	Frontal cortex	Temporal cortex	Hippocampus
Astrocytes	*GFAP*	126.95	162.93	136.25
	S100B	101.32	128.95	125.96
	AQP4	107.40	132.57	146.39
	Mean	111.89	141.48	136.20
	SD	13.39	18.66	10.22
Oligodendrocytes	*MBP*	101.57	102.15	96.83
	SOX10	98.41	100.62	103.07
	MOG	104.55	130.64	116.20
	MAG	108.60	116.36	102.50
	Mean	103.28	112.44	104.65
	SD	4.34	14.05	8.20
Microglia	*CD68*	103.66	134.19	110.55
	AIF1	96.81	114.67	107.54
	LGALS3	103.20	114.04	112.30
	EMR1	101.13	115.21	102.74
	Mean	101.20	119.53	108.28
	SD	3.12	9.78	4.19
Neurons	*RBFOX3*	84.26	79.43	63.82
	ENO2	98.75	94.25	78.10
	CHGA	94.11	89.88	53.31
	TUBB	96.40	96.03	86.51
	SYP	92.00	86.73	65.49
	NEFH	90.60	96.27	54.13
	NEFM	97.17	90.80	64.43
	NEFL	89.76	83.80	58.93
	SNAP25	86.35	79.55	60.83
	SYT1	87.70	82.85	63.15
	Mean	91.71	87.96	64.87
	SD	4.86	6.45	10.27