impairment, particularly verbal recall and executive function deficits [2] in mild cognitive impairment (MCI) patients, is regarded as a critical factor for predicting the conversion of MCI to AD. According to the criteria for MCI set out by Petersen et al. [3], such patients should be classified as amnestic MCI cases [4]. The authors recommended that a standard deviation of approximately 1.5–2.0 below the education-adjusted values for healthy subjects on the delayed recall portion of the Logical Memory subtest (Logical Memory II: LM-II) be used as the diagnostic threshold for amnestic MCI [5]. They also found evidence of some impairment in other cognitive domains in MCI patients, including visual memory. These impairments were assessed using delayed recall in the Visual Reproduction (VR) subtests of the WMS-R for evaluating the nonverbal domain [3]. These findings suggest that the assessment of a combination of LM [6, 7] and other cognitive domains, such as VR, may prove useful for predicting conversion from MCI to AD [8–10].

In the Japanese version of the WMS-R (WMS-R-J) [11], scores on most of the 11 subtests were equivalent to the scores on the WMS-R across 6 age-matched groups of healthy subjects in the USA. However, scores on 2 visual memory tests, immediate Visual Reproduction (VR-I) and delayed Visual Reproduction (VR-II), which are designed to evaluate delayed memory at least 30 min after the visual presentation of figures, were significantly higher in healthy Japanese subjects compared to healthy American subjects for each age-matched group ($p < 0.00001$ for all) [12]. The stimuli in the VR tests of the WMS-R-J are the same as those in the US version except for the Japanese language instructions. The authors hypothesized that the characteristic high scores of Japanese subjects on the VR subtest might be attributable to the fact that Japanese subjects have learned Chinese characters, 'Kanji', from an early age – an activity that requires and may enhance visual reproduction skills.

No studies to date have evaluated visual memory and its specificity for predicting conversion to AD in Japanese MCI patients. In this study, we aimed to clarify whether Japanese MCI patients maintain good visual memory capabilities, and to evaluate the usefulness of VR scores for predicting the conversion of MCI to AD in the near future.

Participants and Methods

Research Participants

This was a retrospective, single-center, longitudinal cohort study. Participants were recruited from memory clinic outpatients of the Tokyo Medical and Dental University Hospital, between April 2007 and April 2011. In total, 247 participants took the WMS-R-J and Mini Mental State Examination (MMSE) tests, and 144 of the 157 participants who obtained ≥24 points on the MMSE were enrolled in our study (table 1; mean age, 68.8 years; male:female ratio, 75:69). The remaining 13 patients were diagnosed with other dementias. A cutoff point for the MMSE of <24 is the most commonly used threshold for determining cognitive impairment [13], and all patients classified as MCI scored ≥24 points on the MMSE. We only analyzed MCI and AD patients who received ≥24 points on the MMSE, in order to compare differently classified participants who were experiencing similar levels of cognitive impairment. Twenty-three of the 27 MCI patients were observed for at least 2 years for classification into 2 groups – converted within 2 years and nonconverted – until October 2012 for analysis of conversion prediction from MCI to AD.

A medical interview including present illness and assessment of the activity of daily function (activities of daily living/instrumental activities of daily living) were performed circumstantially to confirm the participant's social or occupational functioning and whether a participant had experienced a significant decline from a previous level of function. Physical examination, MMSE, complete blood count, electrolytes, liver and kidney functions, thyroid function, vitamin B_{12}, syphilis, magnetic resonance imaging (MRI), and/or brain single-photon emission computed tomography (SPECT) were all utilized for diagnostic classification. When required, the Self-Rating Depression Scale was also used. The WMS-R-J was administered by trained neuropsychologists. The first examinations were performed within 2 months from the first visit for all patients. Baseline clinical groupings were determined according to the criteria described below, based on the results of all subtest scores and index scores of MMSE, including verbal memory, visual memory, and delayed recall,

Table 1. Comparisons of baseline clinical and neuropsychological data of participants

Clinical data	ND (n = 67)	MCI (n = 27)	AD (n = 50)	ND vs. MCI p value		MCI vs. AD p value	F (d.f. = 2)
Age, years	62.3±13.9	72.4±5.8	75.5±7.8	*<0.001*		0.243	23.3
Sex, male	33 (49.3)	18 (66.7)	24 (48)	0.129		0.120	1.42
Educational years	14.2±3.1	13.1±3.8	12.9±3.3	0.168		0.774	2.35
Neuropsychological tests	**ND**	**MCI**	**AD**	**ND vs. MCI p value**	**F (d.f. = 1)**	**MCI vs. AD p value**	**F (d.f. = 1)**
MMSE	28.27±1.67	27.59±1.76	25.98±1.55	*0.036*	4.55	*<0.001*	18.0
WMS-R-J							
LM-I	19.7±7.1	11.9±5.2	9.8±5.7	0.084	3.06	0.092	2.92
LM-II	15.2±7.3	5.7±5.7	2.9±4.1	0.454	0.56	0.137	6.37
LM-II story A	8.3±4.2	3.2±3.3	1.7±2.3	0.343	0.91	*0.017*	5.94
LM-II/I	75.4±20.7	44.3±32.9	20.9±24.9	0.942	0.01	*<0.001*	12.1
VR-I	36.7±4.4	33.3±5.6	25.7±7.9	0.053	3.84	*<0.001*	19.7
VR-II	29.2±9.9	20.5±14.0	7.2±9.6	0.051	3.91	*<0.001*	24.1
VR-II/I	79.2±25.3	58.7±37.4	24.3±29.5	0.070	3.37	*<0.001*	19.5
rIRI	111.2±22.9	87.1±18.7	67.9±24.6	0.121	2.45	*<0.001*	12.6
rDRI	67.0±17.4	44.2±21.4	23.8±16.3	0.791	0.07	*<0.001*	22.0
rDRI/rIRI	60.3±10.9	48.6±18.9	34.0±16.5	*0.046*	4.08	*<0.001*	12.4

Values are presented as means ± SD or numbers with figures in parentheses indicating percentages. Comparisons of clinical data and comparisons of neuropsychological tests for MCI and AD are performed using a one-way analysis of variance, followed by a two-tailed multiple t test, with a Bonferroni/Dunn correction. Because the ND group is significantly younger than the MCI group, analysis of covariance which added age as covariate performed neuropsychological tests comparisons of ND and MCI.

F = F value; d.f. = degrees of freedom; SD = standard deviation; rIRI = raw score of Immediate Recall Index; rDRI = raw score of Delayed Recall Index; rIRI and rDRI scores were calculated from subtests (rIRI is the sum total of LM-I, VR-I, doubled VIPA-I, and doubled VEPA-I; rDRI is calculated in the same way, but for II); I = immediate recall of that subtest; II = delayed recall of that subtest; II/I or rDRI/rIRI = percentage retention score (percentage retention from immediate to delayed recall). The significance level was determined by a 3-group multiple-comparison t test with Bonferroni/Dunn correction ($p < 0.0167$) or by a 2-group comparison t test with Bonferroni/Dunn correction ($p < 0.05$); significant p values are italicized.

as well as MRI and SPECT findings. The index scores were changed to reflect age-adjusted deviation values. WMS-R-J data from subjects >75 years old have not been standardized [11].

Baseline Diagnostic Criteria

Diagnosis of AD was based on the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association criteria for diagnosing 'probable' AD [14]. All participants enrolled in this study scored ≥24 points on the MMSE, and were therefore all classified as 'very early-stage AD'. MCI was diagnosed on the basis of objective memory complaints reported by the participant's family, or the occurrence of episodes related to memory disturbances, and a clinical dementia rating (CDR) of 0.5 [15, 16], in which the Memory Box score of CDR must be 0.5, but the patient does not meet other criteria for dementia. These MCI criteria were intended to signify 'amnestic MCI', similar to that in the Alzheimer's Disease Neuroimaging Initiative (ADNI) study [17]. However, our MCI criteria did not require a logical memory decline in the LM-II story A, on the basis of the participant's educational status. Because of this difference in the criteria, 8 of 27 MCI patients in this study were compatible with no-dementia (ND) participants in the ADNI. The previous study by Saxton et al. [18] indicated that the CDR was sensitive to subtle changes in cognition that were not identified by the neuropsychological-based algorithm, which was leading to a greater number of false-positive diagnoses of MCI. For this reason, we used not only CDR, but also a clinically confirmed objective memory impairment for the diagnosis of MCI in this study, resulting in

Table 2. Baseline findings and WMS-R-J scores in the 2y-MCI-C and 2y-MCI-NC groups

More than 2-years-followed baseline MCI patients (n = 23)	2y-MCI-C (n = 10)	2y-MCI-NC (n = 13)	p value	F (d.f. = 1)
Age, years	74.2±6.0	72.2±4.1	0.341	0.95
Sex, male	5 (50)	8 (61.5)	0.600	0.28
Educational years	12.6±3.7	13.8±4.1	0.489	0.50
MMSE	27.0±1.7	28.2±1.7	0.119	2.65
WMS-R-J				
LM-I	8.7±4.0	13.9±5.6	*0.022*	6.17
LM-II	2.5±3.2	7.5±6.2	*0.032*	5.30
LM-II story A	1.3±1.1	4.5±3.9	*0.021*	6.25
LM-II/I	27.0±28.2	53.2±31.4	0.050	4.32
VR-I	30.5±4.8	35.5±6.0	*0.043*	4.64
VR-II	7.2±8.7	29.8±9.3	*<0.001*	35.2
VR-II/I	21.9±26.0	84.0±21.1	*<0.001*	40.1

Values are presented as means ± SD or numbers with figures in parentheses indicating percentages. F = F value; d.f. = degrees of freedom; SD = standard deviation; 2y-MCI-C = MCI patients who progressed to AD within 2 years (MCI converters); 2y-MCI-NC = MCI patients who did not progress to dementia within 2 years (MCI nonconverters); I = immediate recall of subtests; II = delayed recall of subtests; II/I = percentage retention score (percentage retention from immediate to delayed recall). Significant p values are italicized.

our criteria being more similar to MCI criteria in the ADNI than to CDR-based criteria. All participating patients were classified into 1 of 3 groups: ND, MCI, or AD, in accordance with the criteria. Data from other patients who could not be classified into 1 of the 3 groups were not analyzed.

Most of the enrolled MCI patients were followed continuously after diagnosis in the outpatient clinic. Twenty-three MCI patients were followed for over 2 years, and the remaining 4 MCI patients were not followed past 4 months because of either a caregiver's illness (1 patient) or because of unwillingness (3 patients). The 23 patients for whom long-term follow-up was possible were classified into 2 subgroups, according to their diagnosis of converting from MCI to AD within 2 years or not. The 2-year converters were classified as 2y-MCI-C (10 patients), and 2-year nonconverters as 2y-MCI-NC (13 patients; table 2).

The baseline clinical features of these groups were investigated. Medical histories of high blood pressure (≥140 mm Hg), diabetes mellitus, hypercholesterolemia, smoking, alcoholism, donepezil usage, previous stroke, MRI findings (Fazekas grade of periventricular hyperintensity and deep white matter hyperintensity, number of lacunar infarctions), and familial dementia were all examined.

Calculation of WMS-R Index Scores and Percentage Retention Scores

All participants took all 13 subtests of the WMS-R-J, and our analysis focused especially on 8 subtests, including the immediate memory tests (class I) and delayed (30 min or more) memory tests (class II) of Logical Memory (LM-I and LM-II), Visual Paired Associates (VIPA-I and VIPA-II), Verbal Paired Associates (VEPA-I and VEPA-II), and Visual Reproduction (VR-I and VR-II). We used raw scores for the analysis of the participants because the WMS-R-J has not been standardized for subjects over 75 years old [11]. We also examined the raw scores of the Delayed Recall Index (rDRI), and the raw scores of the Immediate Recall Index (rIRI) calculated from these 8 subtests. The original WMS-R defines the DRI as an adjusted deviation score, which is calculated from delayed recall subtests (LM-II + VR-II + doubled VIPA-II + doubled VEPA-II), and standardized to age-matched controls [11]. Because the DRI has not been standardized for participants >75 years old, too, we used rDRI to evaluate comprehensive delayed memory in these participants. The rIRI is our original index score, which is calculated from the immediate recall subtests (LM-I + VR-I + doubled VIPA-I + doubled VEPA-I) to evaluate the comprehensive immediate memory of participants, and for a direct comparison of immediate memory with delayed memory. Furthermore, the percentage retention scores (= percent savings from immediate to delayed recall, II/I), defined as 100 × II divided by I, were calculated (LM-II/I, VR-II/I, and rDRI/rIRI) in order to provide an estimate of memory retention. We finally analyzed for LM(-I, -II, -II/I), VR(-I, -II, -II/I), and rIRI, rDRI and rDRI/rIRI.

Statistical Analyses

All statistical analyses, except for the receiver-operating characteristic (ROC) curve analyses, were performed using StatView J software, version 5.0 (SAS Institute Inc., Cary, N.C., USA). ROC curve analyses were performed using IBM SPSS (Armonk, New York, N.Y., USA).

Comparisons of neuropsychological tests between ND and MCI groups, and MCI and AD groups were performed using a one-way analysis of variance, followed by a two-tailed multiple t test, with a Bonferroni/Dunn correction. Because of a significant difference in mean ages between ND and MCI groups, neuropsychological tests were analyzed using a covariance analysis which added age as a covariate. A comparison between the 2y-MCI-C and 2y-MCI-NC groups was performed in order to confirm the usefulness of each candidate item of the WMS-R and other factors for predicting the conversion from MCI to AD, using a one-way analysis of variance, followed by a two-tailed multiple t test. The threshold for significance was set at $p < 0.05$. The ROC curve analysis was performed to evaluate the utility of candidate items in predicting conversion to AD. The area under the ROC curve was used to compare subtest performance for accurate prediction of converters. Multiple logistic regression model analysis was performed to distinguish significantly different items on the WMS-R-J and other candidates between the 2y-MCI-N and 2y-MCI-NC groups, and to investigate the utility of the WMS-R-J subtests, index scores, and percentage retention scores for predicting conversion from MCI to AD. Pearson product-moment correlation coefficients among MMSE, WMS-R subtest scores, index scores, and percentage retention scores were examined in order to investigate the associations.

Results

Baseline Neuropsychological Features of Participants

Participants' demographic characteristics, MMSE scores, and main scores on the WMS-R are shown in table 1. Sixty-seven, 27, and 50 participants were diagnosed as having ND, MCI, and AD with ≥24 points on the MMSE, respectively. Participants in the ND group were significantly younger than those in either of the other 2 groups ($p < 0.001$). No significant differences were observed in gender or educational background among the 3 groups. The mean MMSE scores in the ND and in the MCI groups were significantly higher than those in the MCI and in the AD groups, respectively ($p = 0.036$ and $p < 0.001$). For the comparisons of subtests and indices in the WMS-R-J between ND and MCI, rDRI/rIRI was significantly lower in the MCI group. In contrast, the mean scores of LM-II story A, LM-II/I, VR-I, VR-II, VR-II/I, rIRI, rDRI, and rDRI/rIRI in the AD group were significantly lower than those in the MCI group.

Comparison of Baseline Data between the 2y-MCI-C and 2y-MCI-NC Groups

Because VR-II and VR-II/I are useful predictors for non-Japanese MCI patients' conversion to AD [8–10], we evaluated the usefulness of VR-II and VR-II/I for the conversion in Japanese MCI patients, and compared the scores of other WMS-R subtests. The mean follow-up period for MCI patients was 40.4 ± 20.5 months, and 11 patients converted to AD (40.7%) within this follow-up period. Thus, the annual rate of conversion from MCI to AD was 18.8%. Among 23 patients who were followed up for more than 2 years, 5 (21.7%) converted to AD within 1 year, and 10 patients (43.5%) converted within 2 years.

MMSE scores and mean scores in LM- and VR-related indices on WMS-R-J in the 2y-MCI-C and 2y-MCI-NC groups are shown in table 2. There were no significant differences between the groups in terms of medical histories of high blood pressure (≥140 mm Hg), diabetes mellitus, hypercholesterolemia, smoking experience, alcohol experience (>22 g/day), donepezil usage, previous stroke, white matter hyperintensity on brain MRI (Fazekas grade), multiple lacunar infarctions on brain MRI, or family history of dementias. Although MMSE scores were not different between the 2 groups, most mean scores in the LM- and VR-related indices were significantly lower in the 2y-MCI-C group than in the 2y-MCI-NC group, indicating that those scores could be useful in predicting conversion to AD (the p value of LM-II/I was 0.0502). The difference in the mean VR-II score between 2y-MCI-C and 2y-MCI-NC

Table 3. Comparison of the present Japanese study with previous American studies

	VR-II			LM-II		
	MCI-C	MCI-NC	p value	MCI-C	MCI-NC	p value
This study (WMS-R-J)	7.2±8.7 (−2.22)	29.8±13.9 (0.06)	*<0.001*	2.5±3.2 (−1.73)	7.5±6.2 (−1.05)	*0.010*
Tabert et al. [8], 2006 (WMS)	3.0±2.7 (−1.41)	6.9±3.8 (−0.26)	*<0.001*			
Griffith et al. [10], 2006 (WMS-III)	12.9±16.3 (−1.91)	27.7±16.0 (−1.03)	*0.006*	11.4±7.3 (−1.59)	16.6±8.7 (−1.00)	0.060

Values are presented as means ± SD. Z-scores for these test measures – given in parentheses – were generated from each study's normal control data. SD = Standard deviation; VR-II = visual reproduction delayed recall. The follow-up period for the MCI patients (MCI-C and MCI-NC) varied among studies: for this study, 2 years (n = 23), for Tabert et al. [8], 3 years (n = 115), for Griffith et al. [10], 1 or 2 years (n = 49). Significant p values are italicized.

(Z scores generated from normal control data) was 2.28, which was higher than that in the mean LM-II score (0.68; table 3). Additionally, unilateral or bilateral hypoperfusion of the precuneus area in SPECT analyzed by statistical methods, 3-dimensional stereotactic surface projection [19] or the easy Z score Imaging System [20], was significantly different between the MCI-C and the MCI-NC groups (online suppl. table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000346738).

Utility of VR-II and VR-II/I for Predicting Conversion from MCI to AD

We visualized the VR-II and -II/I scores (fig. 1a) for the MCI patients in a scattergram and evaluated these factors as predictors for the conversion to AD. Patients with lower scores on these indices were more likely to convert within 2 years, and a linear correlation between VR-II and -II/I is clearly shown. Comprehensive memory markers, rDRI and rDRI/rIRI scores were also useful for prediction and linear correlation (fig. 1b). When we set the cutoff score for the 2-year conversion prediction of VR-II at 18 points, the sensitivity, specificity, positive prediction value, and negative prediction value were 90, 84.6, 81.8, and 91.7%, respectively. When we set the cutoff score of VR-II/I at 50.6%, those values were 90, 92.3, 90, and 92.3%, respectively. We focused on LM-I, LM-II, LM-II/I, VR-I, VR-II and VR-II/I of the WMS-R-J, which were contradistinction items and most of them were significantly lower in the 2y-MCI-C group than in the 2y-MCI-NC group. In the ROC analyses of those scores for conversion within 2 years, the area under the curve values for those scores were 0.77, 0.82, 078, 0.79, 0.95, and 0.95, respectively (fig. 2a, b). The VR-related values were higher than the LM-related values, especially scores in delayed memory and percentage retention (fig. 2).

The cutoff scores for VR-I, VR-II, and VR-II/I assessed by univariate logistic regression were 31.7, 17.2, and 51.8%, respectively (p = 0.058, 0.017 and 0.011), which was compatible with the optimal cutoff point on the ROC curve closest to (0, 1) (fig. 2).

We analyzed MCI groups with multiple logistic regression using age, sex, and educational years as covariates, and found that delayed recall in MMSE, LM-I, VR-II, VR-II/I, rDRI/rIRI, and hypoperfusion of the precuneus in SPECT were statistically significant for the prediction conversion (online suppl. table 2).

Correlation Coefficients for Linear Regression between Cognitive Scores in the MCI and ND Groups

In order to clarify the correlations between MMSE scores and all cognitive subtests in our cohort, we calculated their correlation coefficients for the MCI and ND groups (online suppl.

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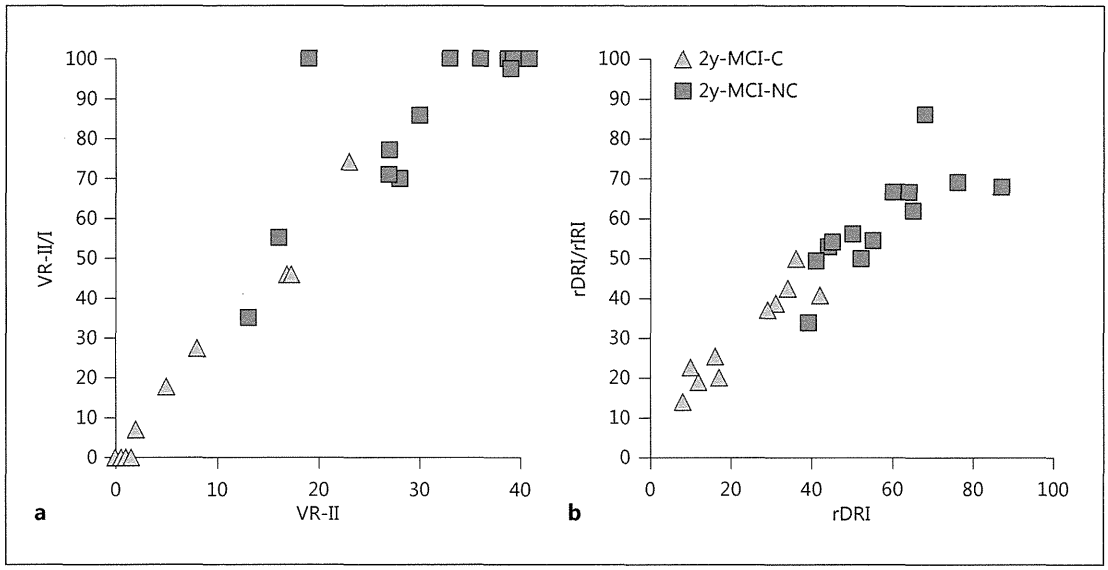

Fig. 1. Scattergrams of VR-II and VR-II/I on the WMS-R-J (**a**), and the rDRI and rDRI/rIRI (**b**) for each 2-year-followed baseline MCI patient (n = 23). **a** Correlation coefficient of VR-II and VR-II/I, correlation coefficient 0.956, $p < 0.001$, 95% confidence interval 0.898–0.981. **b** Correlation coefficient of rDRI and rDRI/rIRI, correlation coefficient 0.931, $p < 0.001$, 95% confidence interval 0.842–0.971.

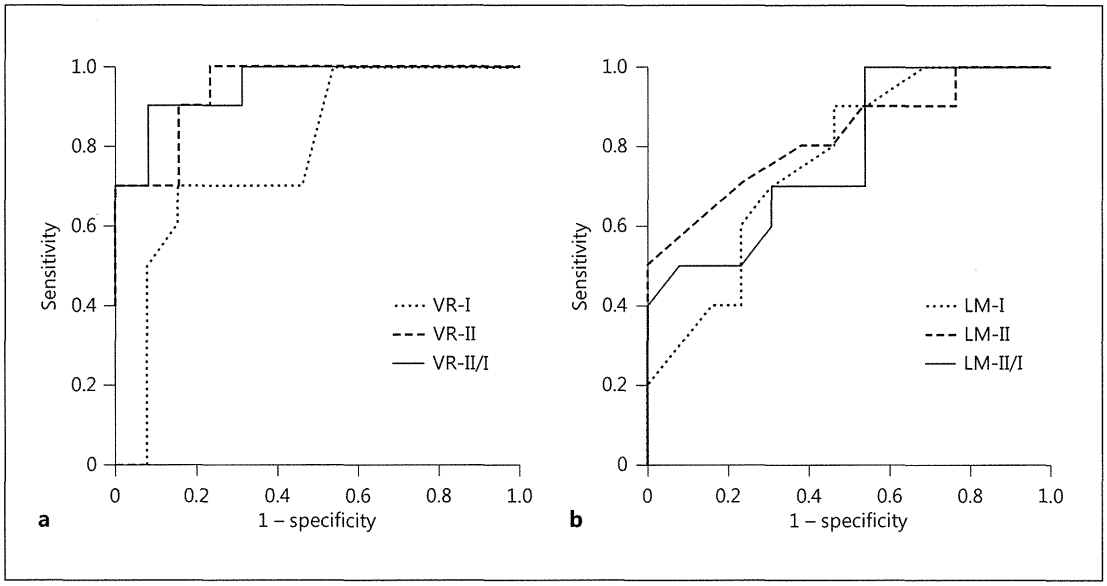

Fig. 2. The ROC curves, discrimination between subjects in the 2y-MCI-C and 2y-MCI-NC groups. **a** ROC curves of VR-I, VR-II and VR-II/I. **b** ROC curves of LM-I, LM-II and LM-II/I. The true rate (sensitivity) is plotted as a function of the false-positive rate (1 – specificity). Area under the ROC curve, p value, and 95% confidence interval of those items are as follows: VR-I: 0.79, 0.022, 0.59–0.98; VR-II: 0.95, <0.001, 0.00–1.00; VR-II/I: 0.95, <0.001, 0.00–1.00; LM-I: 0.77, 0.032, 0.57–0.96; LM-II: 0.82, 0.010, 0.64–1.00, and LM-II/I: 0.78, 0.024, 0.59–0.97. Optimal cutoff points on the ROC curve closest to (0, 1), sensitivity and specificity of those items are as follows: LM-I: 10.5 points, 70 and 69.2%; LM-II: 2.5 points, 70 and 76.9%; LM-II/I: 29.8, 70 and 69.2%; VR-I: 32 points, 70 and 84.6%; VR-II: 18 points, 90 and 84.6%; VR-II/I: 50.6, 90 and 92.3%.

table 3). The correlation coefficient between VR-II and rDRI in MCI patients was 0.940 points (online suppl. table 3a), which was much higher than any of the other correlation coefficients. In contrast, the correlation coefficients of VR-II and rDRI in the ND group were not so high (online suppl. table 3b). The ND group correlations were calculated using data from 66 participants, because of a partial deficit of data from 1 participant. Although a high correlation between VR-II and VR-II/I (fig. 1a), and between rDRI and rDRI/rIRI (fig. 1b) was observed in all MCI patients (correlation coefficients were 0.959 and 0.936, respectively), a high correlation of delayed memory and percentage retention scores was not confirmed between other subtests, such as LM-II/I and LM-II, VIPA-II/I and VIPA-II, and VEPA-II/I and VEPA-II (correlation coefficients were 0.802, 0.415, and 0.252, respectively).

Discussion

A Comparison of VR Scores between Participants from this Study with Healthy Subjects from Previous Reports

We compared the mean VR scores of the ND, MCI, and AD groups in our study with the VR scores of healthy adults reported both in Japan and in the USA in each age group [12] (online suppl. fig. 1). The mean VR-I and VR-II scores of each age classification in our MCI group were higher than those of normal, healthy American adults, and the mean VR-I scores were almost the same as those of healthy Japanese adults. The mean VR-I score in the MCI group was almost the same as that in the ND group, but the mean VR-II scores in the MCI group were lower than those in the ND group, except for patients in the 65- to 69-year age group. This result suggested that the decline in visual memory occurs first in VR-II, followed by a decline in immediate recall.

We speculate that relatively higher scores of VR-I and VR-II in MCI patients than those in healthy American adults might be due to one, or a combination of several, ethnic, racial, educational, or linguistic factors. Sugishita and Omura [12] suggested that studying Chinese characters as 'Kanji' from early childhood in the Japanese educational system, in which Japanese children have to memorize and exercise reading and writing about 2,000 Chinese characters during the 12 years from elementary school to senior high school, may have an effect on visual cognitive domain performance, including visual reproduction. Chinese characters are composed of the meaningful figure units, and each unit is composed of some vertical, horizontal and diagonal lines. Cognitive processes in between Chinese characters and English alphabets were suggested to be distinct [21]. Kanji reading and writing performance has been successfully predicted by visual memory in Japanese children and adults [22, 23]. Those reports support the high scores of VR-related indices in the ND group. As for high scores of VR-related indices in the MCI group, it has also been suggested that a person with extensive educational experience is less likely to develop dementia [24, 25].

Comparison of VR-II between the MCI Subgroups in this Study, and in Studies Performed in the USA

We compared VR scores between MCI patients who converted to AD during the research period (MCI-C), and MCI patients who did not convert to AD during the same period (MCI-NC; table 3). The follow-up periods were different among the studies: in the present study, 2 years; in Tabert et al. [8], 3 years, and in Griffith et al. [10], 1 or 2 years. In our study, the difference in the mean VR-II score between MCI-C and MCI-NC was much higher than the difference reported previously in MCI patients in the USA (table 3) [8, 10]. The difference in Z scores of VR-II was higher than that of LM-II in this study (table 3).

This result indicated that the declined VR-II and LM-II scores are comparable to those observed in the USA, where patients typically do not have experience studying Chinese char-

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acters. The decline of LM-II scores in MCI patients in this study preceded the VR-II score, followed by the conversion to AD. Previous reports have suggested that dementia patients with more educational years experienced memory decline more rapidly following the initial onset of decline [26]. We speculate that the remarkable decline of VR-II scores in MCI-C patients in this study is related to learning geometrical logographical letters like 'Kanji' from their childhood. This raises the possibility that the VR-II score may largely decline in Japanese patients just before patients are converting from MCI to AD.

Predicting the Conversion from MCI to AD

Although we analyzed a relatively small sample size, we found a significant difference in baseline VR-II and VR-II/I data between the 2y-MCI-C and 2y-MCI-NC groups ($p < 0.001$; table 2). Further investigation is required to confirm the usefulness of those scores for the prediction of the conversion from MCI to AD in Japanese MCI patients. The compatibility of our diagnostic criteria for MCI with those used in other studies should be confirmed by pathologically confirmed markers, such as the decline of β-amyloid levels in cerebrospinal fluid, or elevation of brain β-amyloid deposition, which recently has come to be detectable by positron emission tomography [27].

The conversion rate within 2 years for patients of our study was 43.5%, which was almost the same as that reported for the US-ADNI cohort (39.2%) [28]. In 2 other studies investigating AD conversion, one reported a lower conversion rate than was observed in this study (26.4% within 3.88 years [8]), while the other reported conversion rates very similar to ours (26.5% within 1.1 years [10]). The adjusted annual conversion rate to AD by Mayo MCI criteria (Petersen's MCI criteria) was 8.1%, according to a meta-analysis [29]. This rate is much lower than those of our study and the US-ADNI, but in that meta-analysis, they did not take into consideration the effect of the length of study duration. All of their studies were performed for 3 years or more; thus, their calculated conversion rate within 2 years might be higher than 16.2% (2 × 8.1).

The emergence of discrepancies in cognitive functions is useful for predicting the conversion from MCI to AD, and these cognitive functions tend to decline several years before being diagnosed as AD [30, 31]. Previous studies [30, 31] reported discrepancies in verbal and visual cognitive functions as a useful predictor; however, verbal memory impairment preceded visual memory impairment in most 2y-MCI-C patients in our study. Our finding that the LM-II score of Japanese MCI patients had declined in both the 2y-MCI-C and 2y-MCI-NC groups indicated that it was difficult to divide into 2y-MCI-C and 2y-MCI-NC groups by LM-II or LM-II/I. The sensitivity and specificity of LM-II and LM-II/I were all lower than those of VR-II and VR-II/I (fig. 2). VR-II is a useful predictive marker when a raw score is used as a predictor, which is independent of age and educational level of patients.

In this study, baseline scores of VR-II in the 2y-MCI-C group (7.2 ± 8.7) and in the AD group (7.2 ± 9.6) were similar. We speculate that this effect was caused by the AD and MCI classifications of this study, in which AD criteria included a requirement of amnestic features, and a decline from previous activities in daily living. Many patients coming to the memory clinic had attained a high educational level, and some of the AD patients obtained comparatively high scores on memory tests but showed decline in other aspects of cognitive functions. Moreover, AD patients in this study were limited to those who scored 24 points or more on the MMSE, and 5 of 50 AD patients who had high VR-II scores (more than 18 points) showed scores below AD criteria of the ADNI in the LM-II story, a task of WMS-R-J. In contrast, MCI criteria of this study required memory dysfunction more specifically (the Memory Box score of CDR must be 0.5) than the AD criteria, so the proportion of low VR-II scoring patients was similar to the level of AD. Although 4 of 23 MCI patients already scored 0 points in the VR-II test, their daily living abilities were not disturbed. When we use ADNI criteria, a classification of all MCI-C patients was not changed, but only 7 of 13 MCI-NC patients were classified into

MCI, indicating that the decline of LM-II precedes VR-II, and all converted patients showed the decline of LM-II. MCI-C patients might correspond to a 'late' or 'advanced' stage of MCI, but it is unclear whether the decline of VR-II occurred only at the progressive stage of MCI or not. It is also difficult to determine whether patients showing decline of LM-II and VR-II should in fact be classified as AD, because each score indicates one aspect of cognitive function, and is not necessarily correlated with activities of daily living/instrumental activities of daily living. This confound represents a limitation of studies evaluating AD conversion.

The usefulness of VR findings for predicting the conversion to AD agreed with the findings of previous studies, confirming VR-II and VR-II/I as reliable predictors of conversion of MCI to AD within 3 years [8]. A combination of VR-II/I and the Dementia Rating Scale has a 76.9% sensitivity and 91.7% specificity as a predictor [10]. The sensitivity and specificity of these predictors (VR-II and VR-II/I) in our study were compatible with, and may be higher than, that found in studies of American patients.

The usefulness of VR tests for predicting conversion to AD is supported by the finding that precuneus function declines during the MCI stage. The precuneus area is involved in both visuospatial abilities and episodic memory [32], and includes memory function distribution networks [33]. Precuneus deterioration in MCI, or early stages of AD, has been reported morphologically in a number of volumetric MRI studies [34, 35]. It has also been observed pathologically [36] and functionally using regional cerebral blood flow decreases on SPECT [37], with further activation during the retrieval process shown on functional MRI [38]. Choline acetyltransferase activity is generally low in precuneus tissue [39]. Although only 2 of 12 MCI-NC patients were positive in precuneus hypoperfusion on SPECT, 6 of 9 MCI-C patients were positive in this study, which was a significantly higher rate, as revealed by multiple logistic regression. However, hypoperfusion of the posterior cingulate and medial frontal gyrus were not significant (data not shown). It is possible that the functional decline in the precuneus partially underlies the progression from MCI to early-stage AD.

Although rIRI, rDRI, and rDRI/rIRI were not standardized by age, these items also had a very high utility for predicting the conversion to AD (online suppl. table 1). This result might be due to the homogeneity of patient ages (mean, 73.0; SD, 5.0; range, 63–80), and the fact that rDRI scores reflected comprehensive delayed memory functions.

High Correlations among VR-II, VR-II/I, rDRI, and rDRI/rIRI in MCI Patients

Many abilities are reflected by VR-I, such as vision, attentiveness, and the acquisition of immediate memory output. VR-II includes the VR-I abilities, plus the retention ability from immediate to recent memory. VR-II/I is believed to reflect retention abilities [40]. We compared the usefulness of VR-II/I and VR-II, for predicting conversion to AD, and found that they were equally useful. The correlation between rDRI/rIRI and rDRI was also very high. It is reasonable that rDRI and rDRI/IRI are good predictable markers from MCI to AD, because these scores indicate comprehensive delayed memory ability, a disturbance of which is the most important core symptom of AD. The reasons why VR-II and VR-II/I scores are well correlated with rDRI are unclear, but it is important to know that there are high correlations between VR-II, VR-II/I, rDRI, and rDRI/rIRI mutually in MCI patients, because comprehensive rDRI in MCI patients could be detected only by a VR-II or VR-II/I examination.

Conclusions

Our results show that VR tests (VR-II and VR-II/I) of the WMS-R-J are beneficial for Japanese MCI patients as a predictive marker for AD, while their predictive value is more limited for North American MCI patients. Predicting conversion to AD is extremely useful for

MCI patients and their families, and assists them in determining their plans and treatment strategy in the early stages of AD. This constitutes the first report on the usefulness of VR-II and VR-II/I for predicting conversion to AD in Japanese MCI patients. Although large sample sizes are quite difficult to obtain for longitudinal studies like this one, particularly when studying elderly populations, further verification with a larger sample size will be required. The J-ADNI is under way in Japan, and in other countries, but VR tests were not included as either a diagnostic criterion or an evaluation item. The results of our study indicate specific features of visual memory in Japanese MCI patients that require further investigation, and comparison with the corresponding features of MCI patients in other countries.

Our results also show the impact of differences in nationality, potentially attributable to cultural learning differences, and suggest that care should be taken when comparing nonverbal cognitive functions between countries and cultures.

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Disclosure Statement

The authors report no conflicts of interest.

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Relationships between Clinicopathological Features and Cerebrospinal Fluid Biomarkers in Japanese Patients with Genetic Prion Diseases

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Abstract

A national system for surveillance of prion diseases (PrDs) was established in Japan in April 1999. Here, we analyzed the relationships among prion protein gene (*PRNP*) mutations and the clinical features, cerebrospinal fluid (CSF) markers, and pathological characteristics of the major genotypes of genetic PrDs (gPrDs). We retrospectively analyzed age at onset and disease duration; the concentrations and incidences of 14-3-3 protein, tau protein, and abnormal prion protein (PrP^{Sc}) in the CSF of 309 gPrD patients with P102L, P105L, E200K, V180I, or M232R mutations; and brain pathology in 32 autopsied patients. Three clinical phenotypes were seen: rapidly progressive Creutzfeldt-Jakob disease (CJD), which included 100% of E200K cases, 70% of M232R, and 21% of P102L; slowly progressive CJD, which included 100% of V180I and 30% of M232R; and Gerstmann-Sträussler-Scheinker disease, which included 100% of P105L and 79% of P102L. PrP^{Sc} was detected in the CSF of more than 80% of patients with E200K, M232R, or P102L mutations but in only 39% of patients with V180I. V180I was accompanied by weak PrP immunoreactivity in the brain. Patients negative for PrP^{Sc} in the CSF were older at disease onset than positive patients. Patients with mutations associated with high 14-3-3 protein levels in the CSF typically had synaptic deposition of PrP in the brain and a rapid course of disease. The presence of small PrP protein fragments in brain homogenates was not correlated with other clinicopathological features. Positivity for PrP^{Sc} in the CSF may reflect the pathological process before or at disease onset, or abnormality in the secretion or metabolism of PrP^{Sc}. The amount of 14-3-3 protein in the CSF likely indicates the severity of the pathological process and accompanying neuronal damage. These characteristic features of the CSF in cases of gPrD will likely facilitate accurate diagnosis and clinicopathological study of the various disease subtypes.

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Introduction

Genetic prion diseases (gPrDs) are classified into three major phenotypes: fatal familial insomnia (FFI), Gerstmann-Sträussler-Scheinker disease (GSS), and genetic Creutzfeldt-Jakob disease (gCJD). These diseases are characterized by disease-specific mutations in the prion protein gene (*PRNP*), some of which are inherited in an autosomal dominant fashion. More than 30 point mutations and repeated octapeptide insertions have been reported [1]. The frequency of gPrDs and the proportion of each *PRNP* mutation differ from country to country. The E200K mutation is the most common in European countries and the only mutation observed in Slovakia, whereas V210I is the most frequent mutation observed in Italy [2]. In Japan, where a nationwide surveillance system for PrDs was established in April 1999, a 10-year review of PrDs was published in 2010 [3]. Several of the mutations detected, namely V180I, P105L, and M232R, are seen almost exclusively in patients from Japan.

Here, we analyzed in detail the clinical, laboratory, and pathological characteristics of Japanese patients with gPrD. We also investigated biomarkers of prion disease in the cerebrospinal fluid (CSF) of patients with gPrD. Biomarkers including 14-3-3 protein, tau protein, neuron-specific enolase, and S100 protein, are helpful tools for the diagnosis of sporadic CJD [4]. Although these markers previously were investigated in 174 cases of gPrDs, the data were limited to the major mutations found in European countries [4]. In addition, development of the real-time quaking-

induced conversion (RT-QUIC) method [5,6] has enabled us to detect abnormal prion protein (PrP^{Sc}) in the CSF. In the current study, we analyzed PrP^{Sc}, 14-3-3, and tau proteins in the CSF of patients with gPrD characterized by major mutations found in Japan.

Methods

Patients

The CJD Surveillance Committee of Japan diagnosed gPrDs in accordance with the WHO Case Definition Criteria for epidemiological surveillance of these diseases (Table S1). Pathologically confirmed cases were classified as "definite" gPrD and cases diagnosed clinically without pathological analysis were classified as "probable." From April 1999 through August 2011, 336 patients throughout Japan were categorized as having a gPrD, and we analyzed data from 309 patients with definite or probable gPrD associated with five major mutations: P102L, P105L, V180I, E200K, and M232R. Other mutations, such as D178N-129MM (the causative mutation of FFI) were not included, because few patients had these mutations. Whether M232R actually causes gPrD has not yet been determined definitively [3]; however, we provisionally classified patients with the M232R mutation as having a gPrD, according to the classification convention of the Surveillance Committee of Japan [3].

Clinical Analyses and Laboratory Examinations

We collected information regarding patients' age at onset, sex, family history, clinical duration (duration from onset to death, or to the point when we confirmed the condition of the patient if he or she was alive), and clinical signs (dementia, psychological disturbance, cerebellar disturbance, visual disturbance, pyramidal or extrapyramidal signs, myoclonus, and akinetic mutism). Disease onset was defined as the time when the patient started to show one of the aforementioned clinical signs. Patients with the M232R mutation were categorized into two phenotypes: rapid (M232R-rapid) and slow (M232R-slow) [7]. Here, we defined patients with M232R-rapid as those in whom akinetic mutism developed within 7 months of disease onset or those who died less than 8 months after disease onset with or without akinetic mutism. Because divergent clinical and neuropathological phenotypes have been associated with the P102L mutation [8,9], we categorized patients with P102L into those with the GSS phenotype, who showed cerebellar symptoms first, and those with the CJD phenotype, who showed rapidly progressive dementia.

We evaluated electroencephalograms (EEG) for the appearance of periodic sharp wave complexes (PSWC) and assessed the cerebral cortices, basal ganglia, and thalamus for hyperintensities on diffusion-weighted imaging (DWI), fluid-attenuated inversion recovery (FLAIR) imaging, and T2-weighted magnetic resonance imaging (MRI) [3]. The schedules for the EEG and MRI examinations were determined by each patient's physician.

Genomic DNA extracted from patients' blood was used to analyze the open reading frame and polymorphisms of codons 129 and 219 of the *PRNP* gene [10]. We classified the family history as "definite" when any member of the patient's family had the same mutation as the patient and as "possible" when any member of the patient's family had a prion disease with an unknown mutation or dementia due to neurodegenerative disease (see Table S2).

CSF Examinations

Analysis was performed on available CSF samples stored at Nagasaki University [3,5,10,11], obtained from patients with the P102L, P105L, V180I, E200K, or M232R mutations. Although an assay for 14-3-3 protein in the CSF has been described previously, the method was not standardized until April 2009 [3]. Here we reevaluated 14-3-3 protein levels in the CSF with Western blotting by using polyclonal antibodies specific for the γ isoform of 14-3-3 (dilution, 1:500; catalog no. 18647, IBL, Gunma, Japan) and with semiquantitative analysis by using chemiluminescence (model no. LAS-3000, FufiFilm, Tokyo, Japan) [11]. Total tau protein was measured by means of an ELISA, as previously described [12]. PrP^{Sc} in the CSF was detected by using RT-QUIC, as previously described [5]; negative results were confirmed by performing a second QUIC reaction. For RT-QUIC analysis of the CSF of patients with gPrDs, we also used a recombinant prion protein with or without the aforementioned mutations. The time between disease onset and acquisition of the CSF sample was noted. We used CSF samples from patients with definite MM1-type sporadic CJD as positive controls and those from healthy adults as negative controls.

Neuropathological Examinations and Western Blot Analysis of PrP^{Sc}

Of the 184 patients who had died, 32 were autopsied and their brains examined histopathologically. Brain tissues obtained from the autopsies were sectioned by using routine neuropathological techniques and stained with hematoxylin and eosin. Immunohistochemistry for PrP^{Sc} was performed by using mouse monoclonal antibodies specific for the PrP^{Sc} protein (3F4) [13]. Frozen samples of brain tissue were homogenized and evaluated in Western blot analyses by using the 3F4 antibody to determine PrP^{Sc} levels [14].

Statistical Analysis

The Mann–Whitney *U* test was used to compare patient groups in regard to age at onset, disease duration, and level of tau protein in the CSF. Fisher's exact probability test was used for comparisons of sex, rate of occurrence of each clinical sign, presence of PSWC on EEG, presence of hyperintensity on MRI sequences, and rates of positive detection of 14-3-3 and PrP^{Sc} proteins. For analysis of the correlation between CSF markers and each clinical parameter, Student's *t*-test or analysis of variance (ANOVA) was used. Significance was defined as $P<0.05$. All analyses were performed by using Prism 5 software (GraphPad Software, La Jolla, CA) and IBM SPSS Statistics (IBM, New York, NY).

Ethical Issues

The family of each patient gave informed consent for patient inclusion in the study. The study protocol was approved by the Institutional Ethics Committees of Kanazawa University and Tokyo Medical and Dental University.

Results

Clinical Features

The clinical characteristics of all patients are summarized in Table S2, categorized by *PRNP* mutation. All of the patients with the P105L mutation exhibited clinical features of GSS. Of the 57 patients with P102L, 45 manifested the GSS phenotype (P102L-GSS), and 12 had the slowly progressive CJD phenotype (P102L-CJD). All patients with E200K and 33 patients with M232R manifested the rapidly progressive CJD phenotype. The slowly progressive CJD phenotype was seen in 14 patients with M232R (M232R-slow) and all patients with V180I.

There were no significant differences in the male-to-female ratios among the mutations ($P>0.05$; Fisher's exact probability test). The range in median age at onset was wider for our patients

than was that of gPrD patients in European countries [2]; our youngest group of patients was 44.3 years at an average disease onset (patients with P105L), and our oldest group of patients (who had V180I) was 76.5 years at an average (Figure 1A and Table S2). Relative to patients with the CJD phenotype (gCJD), those with the GSS phenotype (P102L-GSS, P105L) had significantly longer disease duration and less frequent myoclonus (Figure 1B and Table S2; $P<0.005$; Mann–Whitney U test). Among patients with gCJD, those with E200K or M232R-rapid showed clinical courses typical of classical CJD, but patients with P102L-CJD, V180I, or M232R-slow showed atypical clinical features, including prolonged duration (Figure 1B and Table S2). Myoclonus was less frequent in patients with V180I or M232R-slow than in those with E200K or M232R-rapid ($P<0.005$; Fisher's exact probability test). In addition, patients with the V180I mutation had a lower incidence of cerebellar signs than did those with the E200K or M232R-rapid mutation ($P<0.005$; Fisher's exact probability test).

Relative to patients with any other mutation, patients with E200K or M232R-rapid showed a higher incidence of PSWCs on EEG ($P<0.005$; Fisher's exact probability test). Hyperintensity on MRI was frequently observed in gCJD patients, especially those with V180I, but was rare in GSS patients (P102L-GSS, P105L vs. all other mutations; $P<0.05$; Fisher's exact probability test, Table S2).

Unlike European patients [15], most (92%) normal Japanese adults are methionine homozygous at codon 129 (129 MM), with only 8% who are methionine-valine heterozygous (129 MV). Patients with the P102L or M232R mutation showed the same distribution of codon 129 polymorphisms as that of the normal population. All patients with the E200K mutation had the 129 MM variant, and all patients with the P105L mutation had 129 MV heterozygosity, with the 129 V codon on the same allele as the 105 L codon. Although 24% of patients with V180I had 129 MV heterozygosity, clinical features did not differ between patients with the 129 MM and 129 MV variants (data not shown). Four patients (1 with P102L, 2 with E200K, and 1 with M232R) had 219 EK heterozygosity, and the rest had 219 EE homozygosity.

Patients with the P102L, P105L, or E200K mutation showed a high frequency of familial PrDs, although none of the patients with M232R and only 1 patient with V180I had a "definite" family history of PrD (Table S2).

Clinical Features and Biomarkers in CSF

CSF was positive for the 14-3-3 protein in 85% of patients with E200K and in 50% to 75% of the remaining patients with gCJD; however, this protein was rarely present in the CSF of patients with GSS (P102L-GSS, 14.2%; P105L, 0%; Figure 2A). The concentration of tau protein in CSF was highest (14,215±15,058 pg/mL) in patients with M232R-rapid and lowest (711±214 pg/mL) in patients with P105L (Figure 2B). The rate of tau protein positivity (that is, exceeding the cut-off level of 1260 pg/mL [12]) was 78.5% or greater in patients with P102L-CJD, V180I, E200K, or M232R-rapid but less than 15% in patients with GSS (shaded in Figure 2B). PrP^{Sc} was detected in the CSF of most patients with P102L (-GSS; 80% and -CJD; 100%), E200K (84.6%), or M232R-rapid (77.8%; Figure 2C); these rates are comparable to those of patients with sporadic CJD (sCJD) [5]. The rate of PrP^{Sc} positivity was much lower in patients with V180I (39%) than in patients with sCJD [5,16], E200K, or M232R-rapid ($P<0.05$; Fisher's exact probability test).

To investigate associations between CSF biomarkers and clinical features, we analyzed correlations between age at onset, disease duration, and each CSF biomarker. We also investigated whether the positivity of CSF biomarkers varied with the time that the sample was acquired relative to disease onset. The date of CSF analysis was obtained from the records of 124 gPrD patients (P102L-GSS, 11 cases; P102L-CJD, 4 cases; P105L, 2 cases; V180I, 71 cases; E200K, 16 cases; M232R-rapid, 13 cases; and M232R-slow, 7 cases). Age at onset was significantly greater in PrP^{Sc}-negative patients than in PrP^{Sc}-positive patients (Figure 3A; $P=0.001$) and in 14-3-3-positive patients than in 14-3-3-negative patients (Figure 3B; $P<0.05$) but was not associated with the concentration of tau protein (Figure 3C). Disease duration was not correlated with PrP^{Sc} positivity but was shorter in 14-3-3-positive patients than 14-3-3-negative patients (Figure 3E; $P<0.001$) and

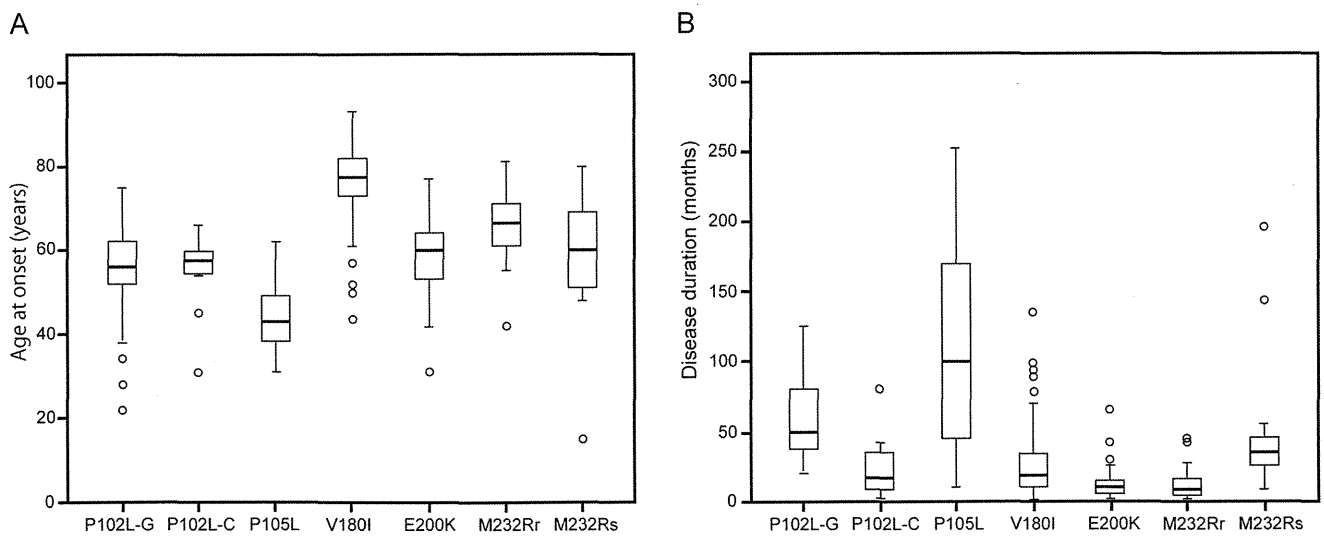

Figure 1. Age of onset and disease duration. Box-and-whisker plots show (A) age at onset and (B) disease duration for each type of gPrD (P102L-GSS, P102L-CJD, P105L, V180I, E200K, M232R-rapid, and M232R-slow). The horizontal line inside each box indicates the median value, and the length of the box is the interquartile range (from 25th to 75th percentile). The extremes of the whiskers contain 95% of values. Open circles indicate outliers. P102L-G, P102L GSS type; P102L-C, P102L CJD type; M232Rr, M232R-rapid; M232Rs, M232R-slow.
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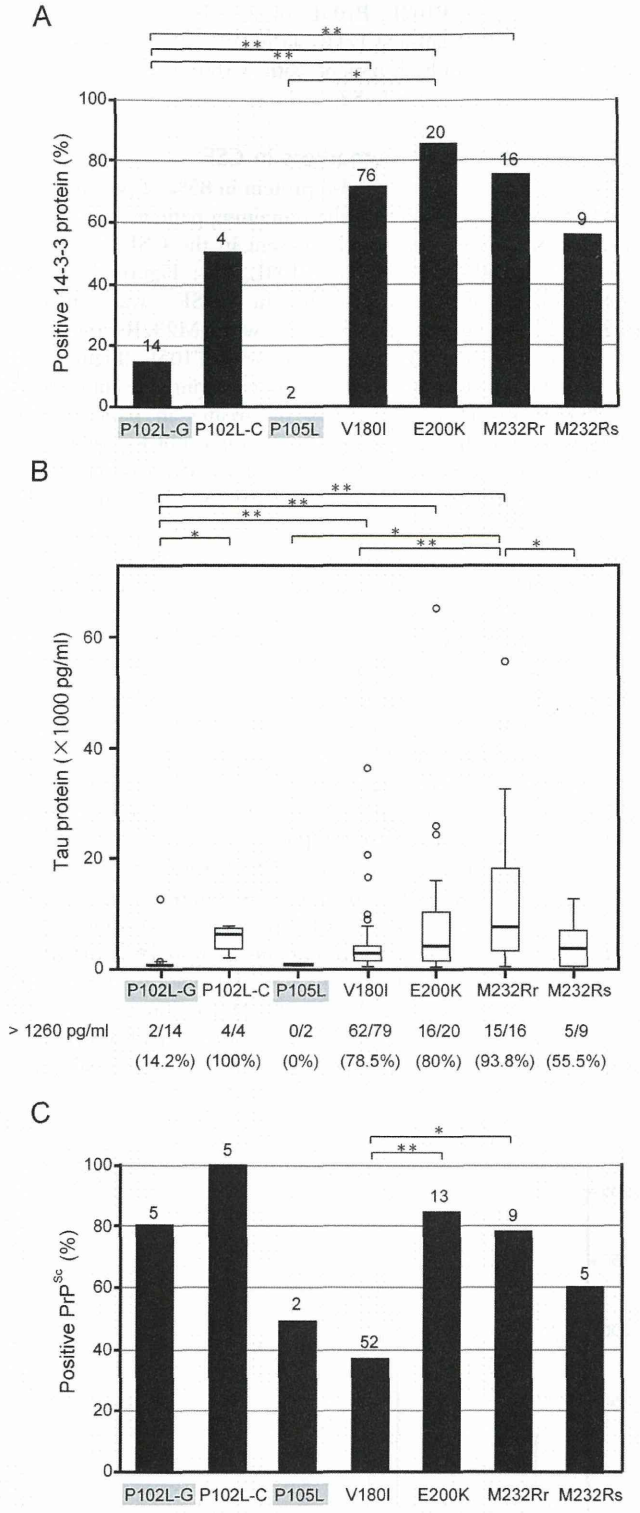

Figure 2. Relationships between CSF biomarkers and genotypes. (A) Rates of positivity of 14-3-3 protein in the CSF of 141 gPrD patients. The number of patients for whom data were available is shown at the top of each column. (B) Box-and-whisker plots of the concentrations of tau proteins in the CSF of 144 gPrD patients. The number of patients with a tau concentration of 1260 pg/mL or greater and the detection rate for each mutation are shown below the graph. (C) Positivity rates of PrP^{Sc} protein in the CSF of 91 gPrD patients. *$P<0.05$; **$P<0.005$ (Fisher's exact probability test).
doi:10.1371/journal.pone.0060003.g002

patients with high tau concentration (Figure 3F; $r=-0.226$). The interval from disease onset to the date of CSF examination was shorter in 14-3-3-positive patients than in 14-3-3-negative patients (Figure S1B; $P<0.001$) and was not correlated with the concentration of tau protein or PrP^{Sc} positivity in CSF samples (Figure S1A, C). The positive correlation between the interval from disease onset to the date of CSF examination and disease duration was significant ($r=0.835$; data not shown).

Disease duration decreased with increasing 14-3-3 or tau protein concentration, depending on the mutation present (Figure 4A, B), and there was a trend toward a positive correlation between these biomarkers and age at onset, as analyzed by mutation (Figure 4C, D).

Western Blot Analysis of Brain Homogenates and Biomarkers in CSF

Western blot analyses of proteinase K (PK)-treated PrP^{Sc} typically show three bands that correspond to the di-, mono-, and unglycosylated PrP^{Sc} fragments [17]. The two subtypes of PrP^{Sc} differ in the molecular weight of the unglycosylated PrP^{Sc}: subtype 1 (21 kDa) is cleaved by PK primarily at residue 82 (the range of residues 74–97 is possible PK digestion sites) of PrP^{Sc} (Figure 5, MM1 type), whereas subtype 2 (19 kDa) is cleaved primarily at residue 97 (residues 82–102; Figure 5, MM2 type). Subtype 1 PrP^{Sc} bands were detected in brain homogenates from our patients with E200K, M232R-rapid, or P102L-GSS (Figure 5). Subtype 2A PrP^{Sc} bands were detected in brain homogenates from patients with V180I or M232R-slow, although V180I cases showed only weak bands of the mono- and unglycosylated fragments (Figure 5), as reported previously [18]. An additional low-molecular-weight (<7 kDa) band was detected in brain homogenates from patients with V180I or P102L-GSS. Whereas a faint band that corresponds to a <6-kDa PK-resistant fragment has been reported previously to occur in cases with P105L [19], we detected a prominent band at ~7 kDa in brain homogenates from our patients with P105L.

Bands associated with the typical PK-resistant PrP^{Sc} fragments were weak or undetectable in patients with P105L or V180I; these patients also were characterized by low rates of PrP^{Sc} positivity in the CSF. We did not find any relationship between the band pattern on Western blots and 14-3-3 positivity or tau protein concentration in CSF.

To exclude the possibility that the RT-QUIC procedure is not well-suited for amplifying the PrP^{Sc} conformer associated with the V180I mutation, we performed RT-QUIC by using a recombinant prion protein with or without the V180I mutation and CSF from patients with V180I CJD. The PrP^{Sc} positivity rate in the CSF of these patients, measured with the RT-QUIC method by using recombinant prion protein, was the same with or without the mutation (data not shown).

Neuropathology, Immunohistochemistry, and Biomarkers in CSF

Only 32 (17.3%) of the 184 patients who died were autopsied; several of these cases have been described previously [7,20–22]. Usually, the cerebral cortices of patients with the V180I mutation showed typical spongiform changes, neuronal loss, and astrocytosis (Figure S2A); destructive changes (status spongiosus) were rarely observed, however, even in cases of relatively short clinical duration. Immunohistochemistry of patients with V180I showed fairly weak synaptic-type PrP deposition (Figure S2B) [20]. The brain tissues of patients with E200K (Figure S2C, D) or M232R-rapid (Figures S2E, F) showed typical spongiform changes and synaptic-type PrP deposition, resembling those of patients with

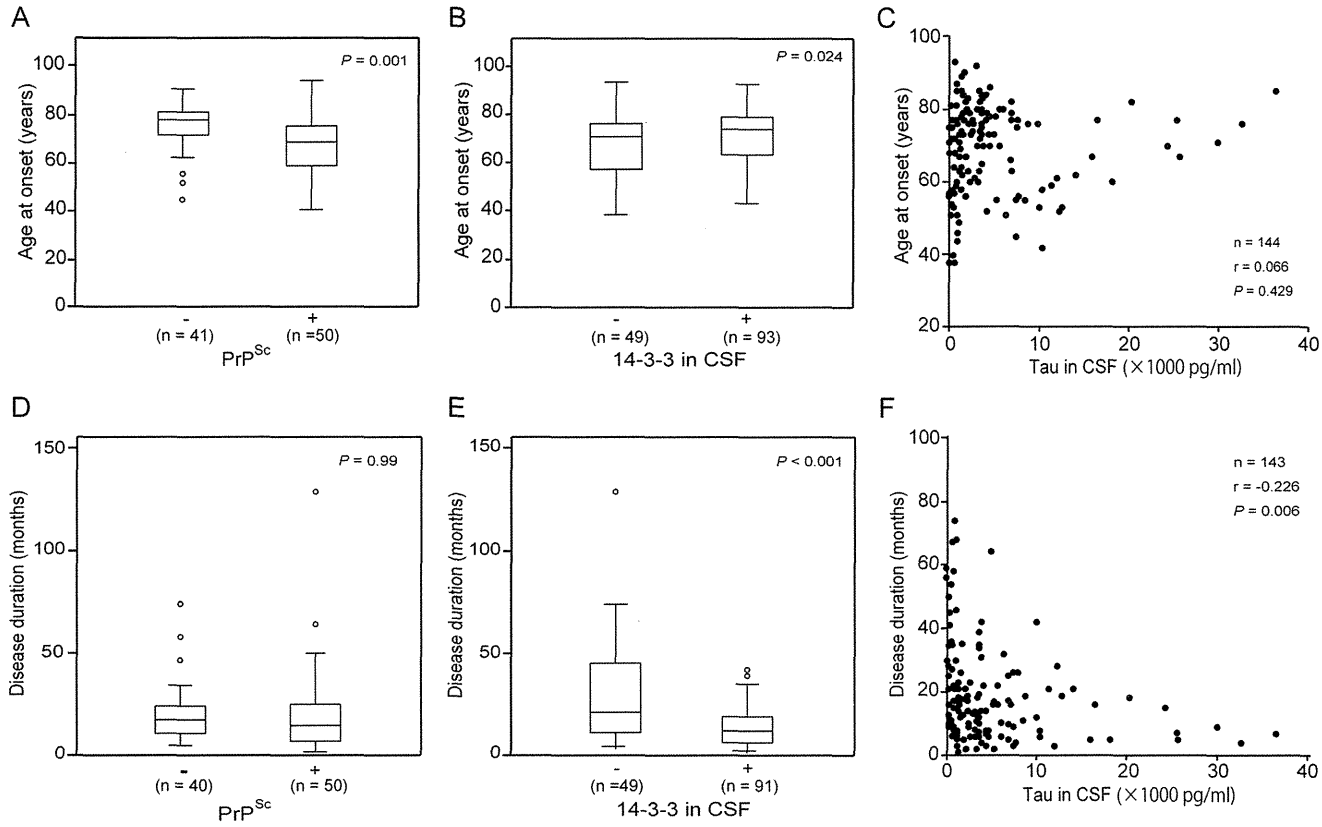

Figure 3. Relationships between CSF biomarkers and clinical features. (A–C) Age at onset and (D–F) disease duration (time from disease onset to death or to when the condition was confirmed) were compared between patients with and without (A, D) PrP^{Sc} protein or (B, E) 14-3-3 protein. In addition, (C, F) the correlation between these clinical features and the concentration of tau protein in CSF was examined for patients with the P102L, P105L, V180I, E200K, or M232R mutation.
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MM1-type sCJD [6,19]. Neuropathological findings in patients with M232R-slow showed large, confluent, vacuole-type spongiform changes and perivacuolar-type PrP deposition in the cortex (Figures S2G, H). These features differed from those in MM1-type sCJD and resembled those in MM2 cortical-type sCJD [6,18]. In patients with P105L, we found multiple PrP-positive amyloid plaques and diffuse PrP deposition in the deep layers of the cerebral cortex without spongiform changes, consistent with a previous report [19]. Cases of P102L have been associated with PrP-positive amyloid plaques in the cerebral cortex with or without spongiform changes or synaptic-type deposits [23,24].

The low PrP^{Sc} positivity in the brains of patients with the V180I mutation was consistent with the weak immunoreactivity observed in Western blotting and immunohistochemistry (Figure 5 and Figure S2B). The cerebral cortices of patients with the V180I, E200K, or M232R mutation showed typical spongiform changes (Figure S2), which also were associated with a high concentration of 14-3-3 or tau protein in CSF. Patients with synaptic-type of PrP deposition in the cerebral cortex (V180I, E200K, or M232R-rapid) were older at disease onset (53.7±13.6 years vs. 71.1±10.9 years; $P<0.001$) and had shorter disease duration (59.4±44.6 months vs. 17.9±15.9 months; $P<0.001$) than did patients with plaque-type PrP deposition (P102L, P105L, or M232R-slow).

Discussion

The mutations and clinical characteristics associated with gPrDs in Japan differ from those in European countries [2,3]. Although clinical variation is observed, more than 60% of the gPrD cases we examined had mutations of V180I, P105L, and M232R; these mutations were mostly reported from Japan [3]. Patients with P105L were characterized by onset at a young age, prolonged clinical duration, spastic paraparesis, and dementia; however, clinical variation is sometimes observed [25,26]. PSWC on EEG and hyperintensity on MRI studies are usually negative in patients with P105L. Relative to patients with E200K, those with P102L-CJD or V180I showed slower disease progression, with less myoclonus and fewer PSWC on EEG (Table S2; $P=0.033$ and $P<0.0001$, respectively). Among all patients with gPrDs, those with V180I typically are the oldest at disease onset, have the fewest cerebellar symptoms, and show a characteristic pattern of lesions on MRI [27]. Although a few patients with V180I [28] or M232R [29,30] mutations have been reported from Korea [26,27] and China [28], long-term nationwide surveillance in Asia has been attempted only in Japan and Taiwan; therefore, further accumulation of cases is needed before ethnic variability can be discussed. Controversy regarding whether the M232R mutation actually causes gPrD remains, not only because patients with this mutation have no family history of the disease, but also because this amino acid substitution has been discovered in a few patients with dementia with Lewy bodies as well as several healthy individuals [31]. Here, we provisionally classified the disease associated with the M232R mutation as gPrD and analyzed its clinical and pathological features.

Few reports have correlated clinical or pathological features of gPrDs and CSF biomarkers of the disease [4,32]. Here, we

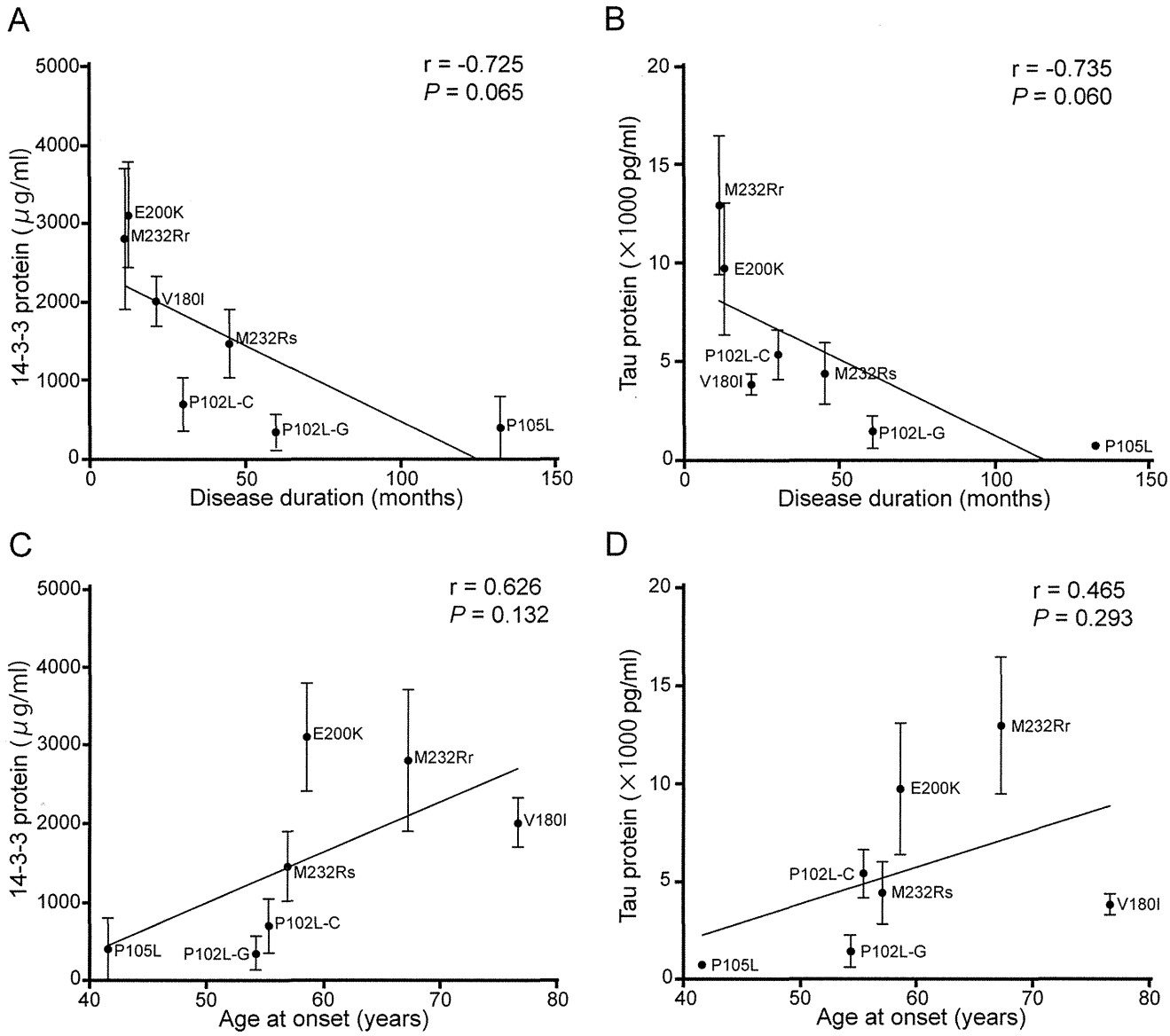

Figure 4. Linear regression analysis of *PRNP* mutations. The correlation between average concentration of 14-3-3 (A and C) or tau (B and D) protein in the CSF with disease duration (mean ± standard error; A and B) or age at onset (C and D) for each mutation was analyzed by using linear regression. P102L-G, P102L GSS type; P102L-C, P102L CJD-type; M232Rr, M232R-rapid; M232Rs, M232R-slow.
doi:10.1371/journal.pone.0060003.g004

investigated the presence of 14-3-3, tau, and PrP^{Sc} proteins in the CSF of patients with gPrD. To our knowledge, the patient population in the current study is the largest in which CSF levels of PrP^{Sc} have been examined in gPrDs. The low number of patients with V180I for whom PrP^{Sc} in the CSF was positive suggests that either little PrP^{Sc} is produced with this mutation or that the mutant protein is unstable; each possibility is consistent with the weak immunoreactivity seen in the Western blots of brain homogenates and immunohistochemistry. However, we need to perform RT-QUIC by using various concentrations of brain homogenate from patients with V180I to confirm the relationship between the quantity of PrP^{Sc} and the sensitivity of RT-QUIC for detecting PrP^{Sc} in the CSF. In this regard, we already have used recombinant prion proteins with or without the target mutation to confirm that brain PrP^{Sc} from patients with P102L, V180I, or M232R is amplified efficiently by RT-QUIC.

Like those with V180I, few of our patients with P105L were positive for PrP^{Sc} in their CSF (Figure 2C). Nonetheless, we will need to analyze more patients with this mutation to confirm this finding. Given that the Western blots of brain homogenates from patients with P105L showed only low-molecular-weight PK-resistant bands, the physiology of PrP^{Sc} in patients with the P105L mutation likely differs from that in patients with other mutations, in terms of the behavior of the mutant protein as a prion [33].

Disease duration was shorter in 14-3-3-positive patients than in 14-3-3-negative ones, regardless of whether the data were analyzed by patient (Figure 3E) or by mutation (Figure 4A). In addition, tau protein concentration was negatively correlated with disease duration (Figures 3F, 4B). The presence of 14-3-3 and tau protein likely reflects the severity or speed of progression of the disease; in pathological analysis, increased 14-3-3 protein levels in

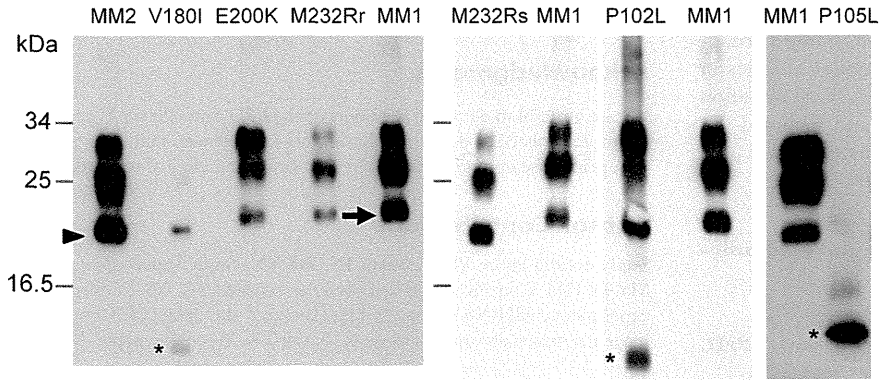

Figure 5. Western blot analysis of PrP^{Sc} in PK-treated brain homogenates. MM2-type sCJD, V180I, E200K (1:30 dilution of homogenate), M232R-rapid (1:10 dilution), M232R-slow, P102L-GSS, and P105L (a different case from that in Figure 2). The 3 different molecular-weight bands from large to small correspond to di-, mono-, and unglycosylated fragments. Bands with a shorter migration distance indicate PrP^{Sc} subtype 1 (arrow), which is processed at residues 74–97, whereas bands with a longer migration distance indicate subtype 2 (arrowhead), which is processed at residues 82–102. There were additional bands of smaller molecular weight in the V180I, P102L-GSS, and P105L samples (asterisks).
doi:10.1371/journal.pone.0060003.g005

patients and increased tau concentrations were seen with mutations associated with synaptic-type PrP deposition, such as V180I, E200K, and M232R-rapid (Figure S2B, D, F). Our results are consistent with those of a previous analysis of patients with the E200K mutation and sCJD in Israel [32]; both indicate that these markers leak into the CSF in the presence of acute brain damage.

To examine the relationship between biomarker positivity and disease stage, we measured the interval from disease onset to acquisition of the CSF sample (Figure S1). The presence of 14-3-3 or tau is reportedly unrelated to the timing of CSF sampling during the clinical course of disease progression (that is, early, middle, and advanced stages) [4]. However, in the current study, CSF samples were obtained during an earlier stage in 14-3-3-positive patients compared with 14-3-3-negative patients. This difference likely reflects that 14-3-3-positive patients experienced more rapid disease progression and came to the hospital sooner than did 14-3-3-negative patients; this explanation is supported by our finding that disease duration was strongly correlated with the length of the interval from disease onset to CSF examination ($r = 0.835$). We were able to obtain data on disease duration and the timing of CSF sampling but not on disease stage at the time of sampling; therefore, whether measuring CSF levels of 14-3-3 protein during the early phase of gPrDs is useful is difficult to determine.

In the current study population, age at disease onset was greater in 14-3-3-positive patients than in 14-3-3-negative ones (Figure 3B), consistent with the data in one previous study [32] but not another [4]. However, whereas age at onset was lower in PrP^{Sc}-positive patients than in PrP^{Sc}-negative patients, disease duration was not correlated with PrP^{Sc} positivity (Figure 3D). This result suggests that the detection of PrP^{Sc} does not reflect rapid disease progression after the onset of clinical disease. Although PrP^{Sc} is a marker of a pathological process evaluated after disease onset, perhaps a low PrP^{Sc} level in the CSF is associated with slow disease progression during the preclinical stage. Alternatively, the amount of PrP^{Sc} in the CSF may reflect abnormalities in the secretion or metabolism of the mutant proteins. This hypothesis is consistent with the very weak immunoreactivity for PrP^{Sc} in the brains of patients with V180I, which was associated with the oldest age at onset among all mutations. In a clinicopathological study of P102L patients, destructive (e.g., spongiform) changes in the brain were not always consistent with the degree of PrP^{Sc} deposition [9,23], indicating that cofactors other than the amount or aggregation of PrP^{Sc} may play an important role in the process of disease onset and progression. Other possibilities are that the central nervous system excretes abnormal PrP into the CSF (as a self-protective mechanism) or produces protective factors more strongly in younger patients. Further investigation is needed to clarify whether additional factors that promote pathological changes or that protect the brain affect disease onset and progression in patients with gPrD.

In conclusion, the presence or amount of CSF biomarkers in patients with genetic prion disease varies with the *PRNP* mutation. Levels of the 14-3-3 and tau proteins are likely to reflect the severity of brain damage, and measurement of these markers may therefore be useful in the diagnosis of gCJD. The absence of PrP^{Sc} in the CSF may be related to older age at onset. RT-QUIC for the detection of PrP^{Sc} in the CSF is a helpful tool for diagnosing gPrDs as well as sCJD, but we note that its sensitivity regarding the V180I mutation is not yet determined. These CSF data, together with characteristic differences in clinical and pathological phenotypes, will help in determining the pathologic mechanisms of these intractable neurodegenerative diseases.

Supporting Information

Figure S1 Relationship between CSF biomarkers and the interval from disease onset to the date of CSF examination. The interval between disease onset and date of CSF examination was compared between patients with or without (A) PrP^{Sc} or (B) 14-3-3 protein. (C) The correlation between this interval and tau protein concentration was examined.
(PDF)

Figure S2 Hematoxylin and eosin staining, and immunohistochemistry with anti-PrP antibody in the cerebral cortex. (A, C, E, G) Hematoxylin and eosin staining, as well as (B, D, F, H) immunohistochemistry with an anti-PrP antibody of brain sections from the temporal lobe of patients with *PRNP* mutations. (A) Samples from patients with the V180I mutation showed typical spongiform changes, mild neuronal loss, and astrocytosis. (B) PrP immunostaining of samples from patient with V180I showed very weak synaptic-type PrP immunoreactivity. Samples from patients with the E200K mutation showed (C) typical spongiform changes and (D) synaptic-type PrP deposition. (E) Samples from patients with the M232R-rapid (M232R-R) mutation also showed spongiform changes, as well as neuronal loss

and proliferation of hypertrophic astrocytes in the cortex. (F) Immunohistochemistry of patients with M232R-R showed synaptic-type PrP accumulation in the cortex. Pathological changes in samples from patients with the M232R-slow (M232R-S) mutation were different from those of other mutations and included (G) large, confluent, vacuole-type spongiform changes and (H) perivacuolar-type PrP deposits. Scale bar, 300 μm.
(PDF)

Table S1 WHO Case Definition Criteria for epidemiological surveillance of gPrDs
(DOC)

Table S2 Clinical characteristics of patients with gPrD.
(DOC)

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Author Contributions

Study coordination: YS K. Sakai IN TH YN. Study supervision: SS SM MY JT HM. Conceived and designed the experiments: NS. Performed the experiments: MH NS K. Satoh TK. Analyzed the data: NS. Contributed reagents/materials/analysis tools: K. Satoh TK. Wrote the paper: MH NS.

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Early Detection of Abnormal Prion Protein in Genetic Human Prion Diseases Now Possible Using Real-Time QUIC Assay

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Abstract

Introduction: The definitive diagnosis of genetic prion diseases (gPrD) requires pathological confirmation. To date, diagnosis has relied upon the finding of the biomarkers 14-3-3 protein and total tau (t-tau) protein in the cerebrospinal fluid (CSF), but many researchers have reported that these markers are not sufficiently elevated in gPrD, especially in Gerstmann-Sträussler-Scheinker syndrome (GSS). We recently developed a new *in vitro* amplification technology, designated "real-time quaking-induced conversion (RT-QUIC)", to detect the abnormal form of prion protein in CSF from sporadic Creutzfeldt-Jakob disease (sCJD) patients. In the present study, we aimed to investigate the presence of biomarkers and evaluate RT-QUIC assay in patients with gPrD, as the utility of RT-QUIC as a diagnostic tool in gPrD has yet to be determined.

Method/Principal Findings: 56 CSF samples were obtained from gPrD patients, including 20 cases of GSS with P102L mutation, 12 cases of fatal familial insomnia (FFI; D178N), and 24 cases of genetic CJD (gCJD), comprising 22 cases with E200K mutation and 2 with V203I mutation. We subjected all CSF samples to RT-QUIC assay, analyzed 14-3-3 protein by Western blotting, and measured t-tau protein using an ELISA kit. The detection sensitivities of RT-QUIC were as follows: GSS (78%), FFI (100%), gCJD E200K (87%), and gCJD V203I (100%). On the other hand the detection sensitivities of biomarkers were considerably lower: GSS (11%), FFI (0%), gCJD E200K (73%), and gCJD V203I (67%). Thus, RT-QUIC had a much higher detection sensitivity compared with testing for biomarkers, especially in patients with GSS and FFI.

Conclusion/Significance: RT-QUIC assay is more sensitive than testing for biomarkers in gPrD patients. RT-QUIC method would thus be useful as a diagnostic tool when the patient or the patient's family does not agree to genetic testing, or to confirm the diagnosis in the presence of a positive result for genetic testing.

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Introduction

Prion diseases (PrD) are fatal neurodegenerative disorders characterized by the accumulation of abnormal prion protein (PrP^{Sc}) in the CNS. The genetic form of human PrD (gPrD) is caused by mutations in the *prion protein gene* (*PRNP*), and is classified into genetic CJD (gCJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI). Patients with GSS and FFI have symptoms such as dementia, dyskinesia and sleep disorders, but show no specific signal in diffusion-weighed MR imaging, and therefore the clinical discrimination of GSS and FFI from non-prion diseases such as spinocerebellar degeneration (SCA) [1] and chronic refractory sleep disorders, respectively, is problematic.