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Potential Utility of Soluble p3-Alcadeinα Plasma Levels as a Biomarker for Sporadic Alzheimer's Disease

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Abstract. Alcadeins (Alcs) constitute a family of neuronal type I membrane proteins (α, β, γ) that share identical localization and function to the amyloid-β protein precursor (AβPP) in the brain. Alcs are proteolyzed in neurons through successive cleavages via secretases, resulting in non-aggregative p3-Alc, where p3 corresponds to the AβPP-fragment. We found p3-Alcα detected in human plasma reflected the pathological process of amyloid-β accumulation in Alzheimer's disease (AD) patients and therefore investigated the utility of p3-Alcα as a plasma biomarker in AD. We measured p3-Alcα plasma levels in 83 sporadic-AD, 18 mild cognitive impaired (MCI), and 24 control subjects using the sandwich-ELISA system. Pooled samples with previously published data (171 AD and 45 controls) were also analyzed. The plasma p3-Alcα concentrations in patients with AD and MCI were significantly higher compared with control subjects (224.7 ± 40.4, 223.3 ± 53.9, and 189.1 ± 32.9 pg/ml, respectively; $p = 0.0012$). In AD patients, the plasma p3-Alcα concentration significantly correlated with age ($r = 0.23$, $p = 0.037$) and serum creatinine levels ($r = 0.23$, $p = 0.0012$). Even after adjusting for confounding factors of age, gender, renal function, and ApoE-ε4, high plasma p3-Alcα levels were correlated with significant AD risk, with an odds ratio 1.47 (95% confidence interval: 1.18–1.93, $p = 0.0019$) for every 10 pg/ml increase. Pooled analysis further confirmed these findings. Increased plasma p3-Alcα, evident in the early stages of cognitive impairment, suggests that Alc metabolites are useful plasma biomarkers of AD.

Keywords: Alcadein, Alzheimer's disease, amyloid-β, blood biomarker, mild cognitive impairment

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INTRODUCTION

Alzheimer's disease (AD) is a common neurodegenerative disorder characterized by progressive cognitive and behavioral deficits. The hallmarks of AD are the presence of senile plaques and neurofibrillary tangles, together with neuronal loss. The major component of senile plaques is the amyloid β-peptide (Aβ), generated by consecutive cleavages of the amyloid-β protein precursor (AβPP) [1]. The production and aggregation of Aβ in the brain is believed to be the primary cause of AD pathogenesis [2], a property that hampers assessment of Aβ generation in the brain. While the presence of Aβ in the cerebrospinal fluid (CSF) is a potentially useful biomarker for AD [3, 4], the invasive

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nature of CSF sampling procedures limits its utility in routine clinical practice. Furthermore, despite extensive research, useful blood biomarkers for estimating Aβ generation in the brain have not yielded consistent results [3, 5, 6].

Alcadeins (Alc) constitute a family of three neuronal type I membrane proteins found in mammals (Alcα, Alcβ, and Alcγ). They colocalize in the brain with AβPP and share identical functional properties with the cargo-receptors of the kinesin-1 motor protein, which mediates anterograde axonal transport [7–9]. Alcs, also known as calsyntenins, have been identified as postsynaptic Ca^{2+}-binding membrane proteins and play an important role in associative learning [10]. Both Alc and AβPP mainly colocalize in dystrophic neurites within the senile plaques of an AD brain [7]. Alcs are successively cleaved by α- and γ-secretases, leading to the release of soluble Alc ectodomains (corresponding to the soluble AβPP ectodomain) and p3-Alc (corresponding to the AβPP fragment, p3) (supplementary Figure 1; available online: http://www.j-alz.com/issues/31/vol31-2.html#supplementarydata03). Detecting changes in non-aggregative p3-Alc species, possible in both human CSF and blood, could reflect the pathological process of Aβ accumulation, including γ-secretase dysfunction. Indeed, several recent studies have suggested that monitoring p3-Alcα C-terminal alterations in CSF may be useful for detecting γ-secretase dysfunction in sporadic AD patients [11, 12].

Recently, we developed a p3-Alcα-specific ELISA combined with an extraction method. The ELISA is very sensitive to the major p3-Alcα species p3-Alcα35, a product of γ-secretase cleavage, along with other p3-Alcα species (supplementary Figure 2), indicating its reliability to quantify total plasma p3-Alcα. A recent preliminary study using this ELISA showed a significant increase in plasma p3-Alcα levels in AD patients, in association with $Aβ_{40}$ [13], suggesting that detection of p3-Alcα in plasma could be a useful biomarker for sporadic AD.

A number of factors may influence the levels of p3-Alcα detected. In our previous study, we observed that plasma p3-Alcα levels in female AD patients were significantly increased but not in male AD patients, compared with controls subjects [13]. However, underlying mechanisms responsible for this gender difference, if any, need to be further evaluated. Age and duration of disease in AD patients could also influence plasma p3-Alcα levels. In addition, renal function may present changes in the metabolism of p3-Alcα, thereby influencing the concentration of plasma p3-Alcα. We further speculate that an apolipoprotein E (APOE) phenotype may influence plasma p3-Alcα levels by affecting the clearance rate of p3-Alcα produced by neurons, which is observed in the clearance rate of Aβ [14].

To assess the clinical usefulness of plasma p3-Alcα as biomarker for AD, we conducted a replication study to confirm the quantitative variation of plasma p3-Alcα in AD subjects and analyzed pooled data from previously reported cases. In addition, we investigated the possible association between plasma p3-Alcα and confounding factors such as age, gender, renal function, and ApoE phenotype. Finally, we evaluated plasma p3-Alcα levels in patients with mild cognitive impairment (MCI), proposed as the clinical stage preceding AD dementia.

METHODS

Study subjects

This study enrolled patients with AD ($n = 83$), MCI ($n = 18$), and control subjects ($n = 24$), all living in Ehime, Japan. The clinical diagnosis of AD was made in the Ehime University Hospital by a neurologist certified for dementia (T.M.) using the NINCDS-ADRDA criteria [15]. A brain computed tomography (CT) and/or magnetic resonance imaging (MRI) were performed to exclude the presence of other diseases. All patients underwent medical, neurological, and psychiatric examinations, as well as appropriate diagnostic studies to exclude other disorders related to dementia. The diagnosis of MCI was based on Petersen's criteria [16]. Control subjects were healthy, independent, community-dwelling subjects from the Ehime Prefecture with normal cognitive function. The aim of the study was fully explained and written informed consent was obtained from each participant. The Ethics Committee of the Ehime University Graduate School of Medicine approved this series of studies.

The severity of cognitive impairment was evaluated in patients with AD and MCI at the time of blood sampling using the Revised Hasegawa Dementia Scale (HDS-R). This brief and reliable system for measuring global cognitive function consists of a series of items that assess orientation, memory, attention/calculation, and verbal fluency. It has been suggested that the HDS-R is more useful than the Mini-Mental State Examination (MMSE) for cognitive screening in early AD [17].

For the pooled analysis, we included previously published p3-Alcα measurements from AD patients

($n = 49$) in Japanese cohort 1 and AD patients ($n = 39$) and controls ($n = 21$) in Japanese cohort 2 from our previous study [13]. Detailed information on these subjects can be found in the original publication [13].

Measurement of plasma p3-Alcα35

To obtain plasma fractions, blood was collected into tubes containing EDTA and separated immediately by centrifugation at 3000 rpm for 15 min and stored at −80°C.

Details determining the plasma concentration of p3-Alcα peptides have been reported elsewhere [13]. In brief, p3-Alcα peptides were extracted by adding 4 volumes (800 μl) of organic reagent (chloroform : methanol [2 : 1]) to 200 μl of plasma in conical tubes (1.5 ml). Tubes were mixed for 10 s with a vortex mixer and then left to stand for 1 h at room temperature. After adding 160 μl of distilled water, samples were mixed again with a vortex mixer and centrifuged at 15,000 rpm for 15 min, after which the aqueous phases were recovered and dried using a SpeedVac system (Sakuma, Tokyo, Japan). Dried samples were dissolved in 250 μl PBS containing 1% (w/v) BSA and 0.05% (v/v) Tween-20 (buffer A). The samples and samples further diluted with buffer A (2- and 4-fold) were used for ELISA. Aliquots of 100 μl were analyzed in duplicate.

The p3-Alcα peptide concentrations were measured using a specific ELISA system [13]. A polyclonal rabbit antibody 839 raised against the peptide between positions 839 and 852 of p3-Alcα 35 was used to capture p3-Alcα. A horseradish peroxidase-conjugated pan p3-Alcα antibody 817 raised against the peptide between positions 817 and 822 of p3-Alcα 35 was used with tetramethyl benzidine to colorimetrically detect (OD_{450}) the captured p3-Alcα. A serial dilution of the synthetic p3-Alcα 35 peptide was used as the standard. ELISA specificity for p3-Alcα was confirmed using the synthetic p3-Alcα 35 (major species of CSF p3-Alcα), p3-Alcα 39 and p3-Alcβ 37 peptides [13].

Renal parameters

Confounding parameters such as serum creatinine (sCre) concentration and estimated-glomerular filtration rate (eGFR) were assessed. A conventional method was used for sCre and the Modification of Diet in Renal Disease (MDRD) Study equation modified for Japanese subjects [18] was used to determine eGFR. All Ehime subjects were free from chronic renal failure (as defined by sCre ≥2.0 mg/dL).

Genotyping of ApoE

The ApoE isotype-related genotypes are combinations of the ApoE ε2, ε3, and ε4 alleles derived from the two genotypes of the rs429358 (T334C) and rs7412 (C472T): ε2, 334T/472T; ε3, 334T/472C; and ε4, 334C/472C. Risk genotype for AD (ε4) was detected by analyzing the SNP rs429358 (T334C) [19].

Genomic DNA was extracted from peripheral blood using a QIAamp DNA blood kit (Qiagen, Hilden, Germany). A single-nucleotide polymorphism (SNP; T334C [Cys112Arg], rs429358) on the ApoE gene was analyzed using the TaqMan probe assay (Applied Biosystems, Foster City, CA, USA) with commercially available primers and probes purchased from the Assay-on-Demand system (ref: C_3084793_20). The fluorescence level of PCR products was measured using an ABI PRISM 7900HT sequence detector (Applied Biosystems).

Statistical analysis

Values are expressed as mean ± standard deviation (SD), unless otherwise specified. A student's t test was used to evaluate the means between the two groups and Pearson's correlation coefficients were used to analyze associations with two variables. The χ^2 test was used to assess frequency differences between groups. Multiple regression analyses of plasma were performed to evaluate the gender differences associated with p3-Alcα and AD and to evaluate the parameters independently related to plasma p3-Alcα levels. Comparisons among the three groups were assessed using ANOVA followed by Tukey-Kramer. Odds ratios (ORs) for the presence of AD were obtained by logistic regression analysis after correcting for the confounding parameters. Receiver operating characteristic (ROC) analysis was performed to evaluate the diagnostic potential of plasma p3-Alcα. All analyses were conducted using commercially available statistical software (JMP ver. 9.0, SAS Institute, Cary, NC, USA), with $p < 0.05$ considered as statistically significant.

RESULTS

The clinical characteristics of the subjects in the Ehime and pooled populations are summarized in Table 1. AD and MCI subjects were significantly older than normal subjects ($p < 0.05$). Renal function (based on sCre and eGFR levels) was similar among the three groups.

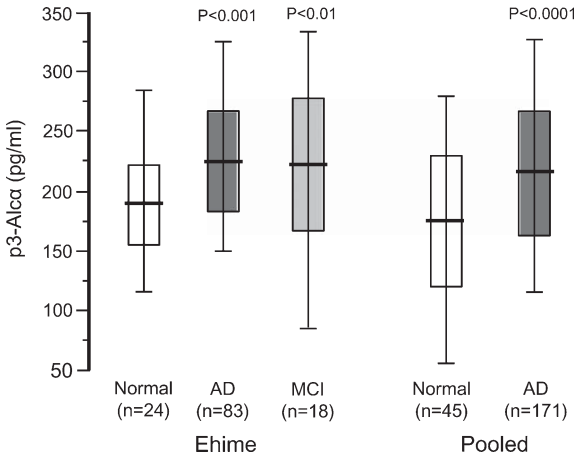

Fig. 1. Plasma p3-alcadeineα (p3-Alcα) levels in normal subjects and patients with mild cognitive impairment (MCI) and Alzheimer's disease (AD) in Ehime and pooled populations. Horizontal bold bar indicates mean. Box represents standard deviation. Vertical bar shows 95% value range. p values indicate difference from normal subjects.

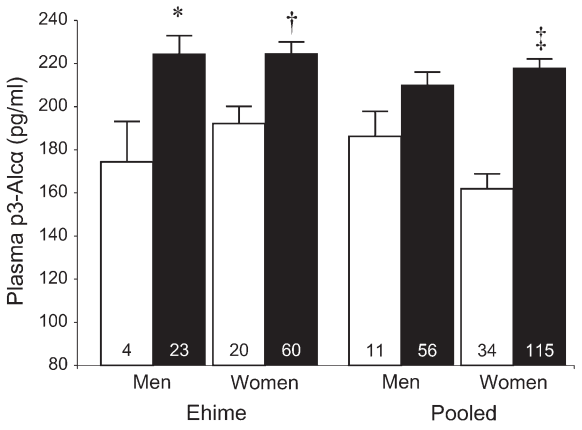

Fig. 2. Plasma levels of p3-alcadelinα (p3-Alcα) in normal subjects (white column) and AD patients (black column) in men and women in Ehime and pooled populations. Number in the column indicates number of subjects. Effect of gender, presence of AD and their interaction was assessed in a multiple regression analysis for plasma p3-Alcα. In the Ehime population; gender, F = 0.59, $p = 0.44$; presence of AD, $F = 12.5$, $p = 0.0006$; gender*AD interaction, F = 0.57, $p = 0.45$. In pooled population, gender, F = 0.44, $p = 0.51$, presence of AD, F = 11.1, $p = 0.001$; gender*AD interaction, F = 2.7, $p = 0.10$. *$p < 0.05$, †$p < 0.01$, ‡$p < 0.0001$ versus normal subjects.

Plasma p3-Alcα levels in AD patients

Plasma p3-Alcα levels were significantly increased in AD patients compared with both the control subjects in the Ehime (224.7 ± 40.4 versus 189.1 ± 32.9 pg/ml, $p = 0.0001$) and pooled populations (224.7 ± 50.9 versus 176.9 ± 54.7 pg/ml, $p < 0.0001$) (Fig. 1). Furthermore, we noted no significant differences between plasma p3-Alcα levels in MCI and AD patients in the Ehime population (Fig. 1).

Confounding parameters for plasma p3-Alcα levels

We noted no gender-dependent differences between plasma p3-Alcα levels and AD status in either the Ehime or pooled populations (Fig. 2). However, no significant difference in male plasma p3-Alcα levels was found between normal subjects and AD patients from the pooled population (Fig. 2). Similarly, no significant differences in plasma p3-Alcα levels were found between male or female AD patients in both the Ehime (224.6 ± 48.8 versus 224.7 ± 37.1 pg/ml, $p = 0.99$) and pooled populations (220.6 ± 49.4 versus 210.9 ± 53.7 pg/ml, $p = 0.24$). Taken together, these findings suggest no significant relationship between plasma p3-Alcα levels and AD status based on gender.

We found a significant positive correlation between age and plasma p3-Alcα levels in AD patients in both the Ehime ($r = 0.23$, $p = 0.037$) (Fig. 3A) and pooled populations ($r = 0.28$, $p = 0.0002$), compared with normal subjects ($r = 0.09$, $p = 0.57$). Plasma p3-Alcα levels were significantly and positively associated with sCre and negatively correlated with eGFR in AD patients (Fig. 3B, C).

In AD patients, plasma p3-Alcα levels did not significantly differ between ApoEε4 carriers and non-carriers. However, after correction for age, gender, and sCre levels, plasma p3-Alcα levels tended to be lower in ApoEε4 carriers compared with non-carriers (193.9 ± 48.1 versus 206.6 ± 43.5 pg/ml, $p = 0.096$) (Table 2).

No significant relationship was noted between plasma p3-Alcα levels and either HDS-R score ($r = 0.12$, $p = 0.28$) or duration of disease in Ehime AD patients ($r = -0.16$, $p = 0.13$).

Multiple regression analysis of plasma p3-Alcα levels

Multiple regression analyses were performed to further evaluate whether or not p3-Alcα was independently associated with AD status. Our results demonstrate that AD was indeed significantly and independently associated with high plasma p3-Alcα levels, even after adjustment for age, gender, renal function, and ApoEε4 (Table 2). Logistic regression analysis further showed that every 10 pg/ml increase in

Table 1
Patient characteristics at baseline

	Ehime			Pooled samples	
	Normal ($n=24$)	AD ($n=83$)	MCI ($n=18$)	Normal ($n=45$)	AD ($n=171$)
Age, year	70.2 ± 6.6	79.1 ± 7.0*	78.9 ± 6.4*	71.6 ± 6.6	77.3 ± 7.2*
Male, *n* (%)	4 (17)	23 (28)	4 (22)	11 (24)	56 (33)
HDS-R, score	–	15.0 ± 6.2	24.9 ± 2.9	–	–
Serum creatinine, mg/dl	0.71 ± 0.16	0.71 ± 0.23	0.69 ± 0.20	–	–
Estimated GFR, ml/min/1.73 m^2	67.8 ± 11.6	70.9 ± 21.5	70.1 ± 15.2	–	–
ApoEε4, *n* (%)	3 (12.5)	34 (41)*	4 (22)	–	–

Values are mean ± SD. AD, Alzheimer's disease; MCI, mild cognitive impairment; HDS-R, Hasegawa dementia score-revised; GFR, glomerular filtration ratio; ApoE ε4, apolipoprotein E ε4. Pooled samples are the combined samples of the Ehime population and Japanese cohorts 1 and 2.
*$p < 0.05$ versus normal subjects.

Table 2
Multiple regression analysis of plasma p3-Alcadeinα concentration in control subjects and Alzheimer's disease patients in the Ehime population

	Model 1		Model 2		Model 3	
	β	*p*	B	*p*	B	*p*
Gender, female	0.04	0.67	0.26	0.008	0.05	0.55
Age, year	0.27	0.01	0.21	0.035	0.16	0.12
AD, presence = 1	0.24	0.022	0.31	0.002	0.33	0.002
ApoEε4, carrier = 1	–	–	–0.15	0.096	0.13	0.16
Serum creatinine, mg/dl	–	–	0.42	<0.0001	–	–
Estimated GFR, ml/min/1.72 m^2	–	–	–	–	–0.31	0.0005
r^2	0.185		0.318		0.282	

AD, Alzheimer's disease; ApoE ε4, apolipoprotein E ε4; GFR, glomerular filtration ratio. – : not included in the model.

Table 3
Odds ratio of a 10 pg/ml increase in plasma p3-Alcadeinα concentration for the presence of Alzheimer's disease

P3-A1cα35, (10 pg/ml)	OR	95% CI	*p*
Model 1	1.31	1.14–1.54	<0.0001
Model 2	1.28	1.07–1.56	0.005
Model 3	1.45	1.17–1.88	0.0003
Model 4	1.51	1.20–2.00	0.0001
Model 1*	1.20	1.11–1.31	<0.0001
Model 2*	1.17	1.07–1.28	0.0002

OR, odds ratio; CI, confidence interval. Model 1, no adjustment; model 2, adjusted for age and gender; model 3, model 2 + adjustment for ApoEε4 carrier and serum creatinine; model 4, model 2 + adjustment for ApoE ε4 carrier and estimated glomerular filtration rate. Models 1 to 4: Logistic regression analyses were performed for the presence of Alzheimer's disease in 24 normal subjects and 83 Alzheimer's disease patients in Ehime. Model 1*, adjusted for cohort in pooled population; model 2*, adjusted for age, gender, and cohort in pooled population. Models 1* and 2*: Logistic regression analyses were performed for the presence of Alzheimer's disease in 45 normal subjects and 171 Alzheimer's patients in pooled samples.

plasma p3-Alcα concentration was significantly associated with an increased risk of AD, with an OR of 1.45–1.51 after adjustment for all possible confounding parameters examined (Table 3).

ROC analysis

To further evaluate the diagnostic and screening potential of plasma p3-Alcα levels as a marker for AD, ROC analysis was performed in three models (Fig. 4). In the Ehime population, area under the curve (AUC) of plasma p3-Alcα ROC for the presence of AD was 0.77. Inclusion of age and gender to the model increased the AUC to 0.87, and additional inclusion of sCre and ApoEε4 genotype further increased it to 0.93. In the pooled population, AUC of ROC for the presence of AD was 0.88, inclusive of plasma p3-Alcα, age, gender, and cohort parameters.

DISCUSSION

In agreement with previous findings, our present study showed that in the Ehime population, plasma p3-Alcα levels in AD patients were significantly increased compared with normal subjects [13]. In addition, we found plasma p3-Alcα levels were significantly associated with age and renal function in AD patients (sCre and/or eGFR). Our findings show an increased plasma p3-Alcα concentration to be an independent predictor for the presence of AD, even after adjustment for

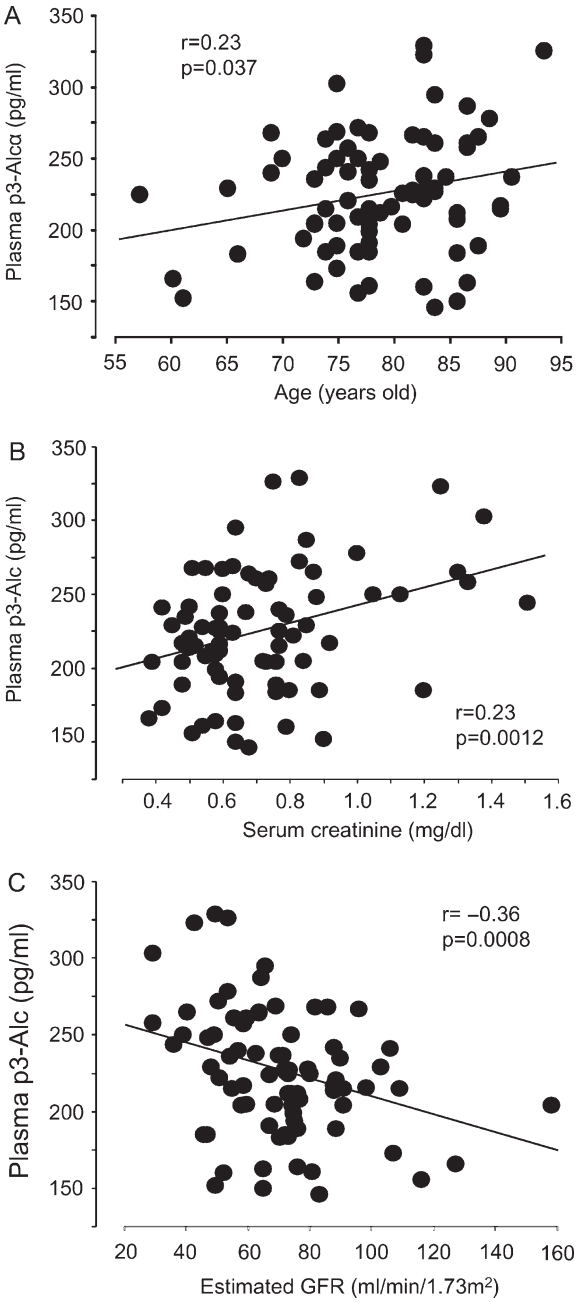

Fig. 3. Relationship between plasma levels of p3-alcadelinα (p3-Alcα) and age (A), serum creatinine level (B), and estimated glomerular filtration rate (GFR) (C) in Alzheimer's disease patients ($n = 83$) in Ehime.

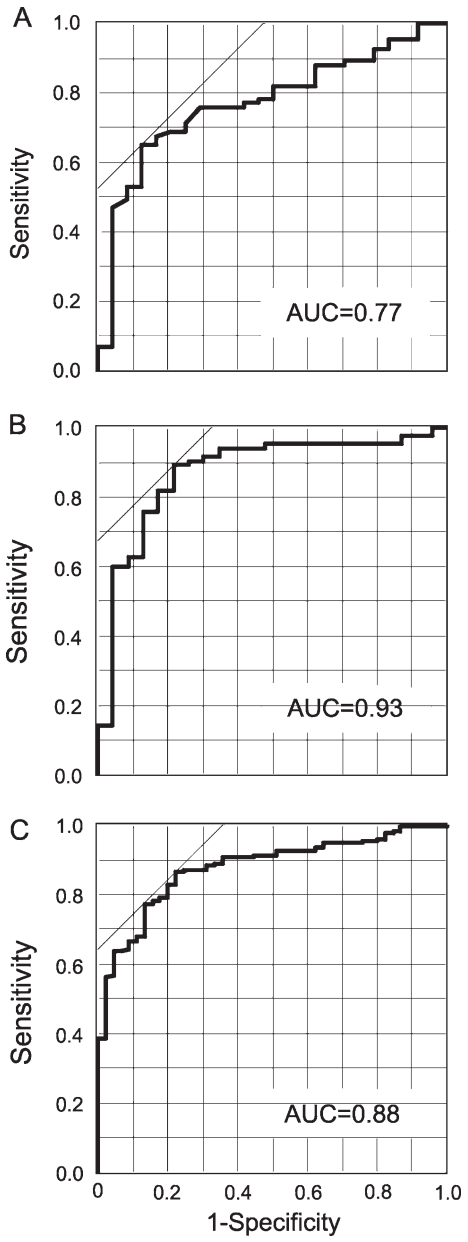

Fig. 4. Receiver operating characteristic (ROC) analysis. ROC analysis of plasma levels of p3-alcadelinα (p3-Alcα) for the presence of Alzheimer's disease (AD) in the Ehime population with 24 normal subjects and 83 AD patients [top]. Area under the curve (AUC) of ROC of plasma p3-Alcα for the presence of AD was 0.77. Inclusion of age, gender, and serum ApoEε4 genotype to the model increased AUC to 0.93 [middle]. In the pooled population, AUC of ROC for the presence of AD was 0.88 with plasma p3-Alcα, age, gender, and cohort.

confounding factors of age, gender, renal function, and ApoE genotype. Our findings suggest the clinical utility of plasma p3-Alcα levels as a novel biomarker for the presence of AD.

A previous study reported an association between plasma p3-Alcα levels and AD in female subjects [13]. In contrast, our study found no significant difference in the association between AD and plasma p3-Alcα levels in either gender in the Ehime and pooled populations, suggesting the association between plasma

p3-Alcα levels and AD are not gender-specific. The discrepancy between the influence of gender on the association between AD and p3-Alcα level may have been due to differences in the control subjects used in these two studies. One more independent cohort also showed no significant differences in plasma p3-Alcα levels between male and female AD subjects (Hata S, Matsubara E, Suzuki T, unpublished observation).

In our present study, we observed a significant correlation between plasma p3-Alcα levels and age in AD patients compared with the control subjects, with similar results observed in the pooled analysis. Given the inclusion of estimated GFR, which is an age- and gender-dependent index of renal function, eliminated the effect of age and gender on p3-Alcα in a multiple regression analysis (Table 1), the association between age and p3-Alcα may reflect an age-dependent decline in renal function.

We found that both positive sCre and negative eGFR levels were associated with increased plasma p3-Alcα levels, indicating that plasma p3-Alcα concentration increased with renal impairment. These findings suggest that circulating p3-Alcα may be excreted from or metabolized in the kidney, a concept supported by the fact that Aβ co-metabolites of p3-Alcα were found in urine of AD patients [20, 21].

Previous studies have shown that ApoE plays a role in the clearance of Aβ from the brain [14] and therefore could influence the clearance of other transmembrane-bound peptides, including Aβ. Furthermore, compared with ApoEε3, ApoEε4 showed reduced activity for receptor-mediated Aβ clearance and perivascular drainage [14]. Our findings that plasma p3-Alcα levels were decreased in ApoEε4 carriers compared with non-carriers, although not significant, may reflect the possibility of reduced p3-Alcα clearance from the brain, similar to Aβ. These findings, together with the possible kidney metabolism of p3-Alcα, suggest that plasma p3-Alcα levels indicate the balance between p3-Alcα neuronal synthesis by neurons, p3-Alcα clearance from brain into the circulation, and the metabolic removal of p3-Alcα by the kidney.

Our findings have demonstrated that p3-Alcα in plasma is a useful biomarker of AD, even in the presence of confounding factors. In the Ehime cohort, ROC analysis revealed an AUC value of 0.87 inclusive of plasma p3-Alcα, age, and gender, with a sensitivity of 0.89 at the highest specificity of 0.68. Although the inclusion of sCre and ApoE genotype further increased AUC to 0.93, these findings indicate plasma p3-Alcα, along with age and gender parameters, has a strong potential to detect AD in patients, as confirmed in the pooled samples (Fig. 4).

Interestingly, plasma p3-Alcα levels in patients with MCI were also significantly higher compared with controls. Although MCI patients have an increased risk of converting to AD, MCI is heterogeneous with several possible outcomes including returning to normal cognition. Accordingly, it is essential to identify high-risk subjects with MCI who will develop AD [22]. Showing an elevation in plasma p3-Alcα levels in MCI patients leads to the question of whether p3-Alcα might be of substantial benefit even at very early clinical, even preclinical, stages of AD. These potential benefits must be further examined using serial measurements with a long-term follow-up to determine the potential of plasma p3-Alcα levels in predicting AD at the preclinical stage.

Several limitations to our study warrant mention. Given its cross-sectional nature, we were unable to specify the causality of increased plasma p3-Alcα levels in AD. In addition, our sample size was relatively small, even in the pooled analysis, and therefore statistical power is limited. We also did not determine the p3-Alcα species that corresponded to $A\beta_{42}$ or $A\beta_{40}$, which could provide further information about the pathological alteration of γ-secretase in sporadic AD patients. Further studies in a larger population on the potential influence of specific p3-Alcα species levels in AD will be needed to confirm our results.

In conclusion, we confirmed that plasma p3-Alcα levels were significantly increased in AD patients compared with cognitively normal subjects. These levels were significantly correlated to renal function and moderately correlated to ApoE genotype. Plasma p3-Alcα levels is a potent marker for the detection of AD, even after correcting for possible confounding parameters, indicating that plasma p3-Alcα is indeed a novel circulating biomarker for sporadic AD.

DISCLOSURE STATEMENT

Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=1274).

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