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Plasma protein profiling for potential biomarkers in the early diagnosis of Alzheimer's disease

Yuki Kitamura[a], Ryoko Usami[a], Sahoko Ichihara[b], Hirotaka Kida[c], Masayuki Satoh[c], Hidekazu Tomimoto[c,d], Mariko Murata[a] and Shinji Oikawa[a]

[a]Department of Environmental and Molecular Medicine, Mie University Graduate School of Medicine, Tsu, Japan; [b]Graduate School of Regional Innovation Studies, Mie University, Tsu, Japan; [c]Department of Dementia Prevention and Therapeutics, Mie University Graduate School of Medicine, Tsu, Japan; [d]Department of Neurology, Mie University Graduate School of Medicine, Tsu, Japan

ABSTRACT

Objectives: Alzheimer's disease (AD) is the most common cause of dementia in elderly persons. Since the pathology of AD develops slowly from a preclinical or early phase into a fully expressed clinical syndrome, at the time of diagnosis the disease has been progressing for many years. To facilitate the early diagnosis of AD, we performed protein profiling of blood in patients with mild AD as defined by the Functional Assessment Staging (FAST) scale.

Methods: Plasma samples from mild AD patients and healthy controls were analyzed using two-dimensional differential gel electrophoresis (2D-DIGE) combined with matrix-assisted laser desorption ionization time-of-flight tandem mass spectrometry (MALDI-TOF/TOF/MS) followed by peptide mass fingerprinting.

Results: Three downregulated proteins were identified: apolipoprotein A-1, alpha-2-HS-glycoprotein, and afamin. Two proteins, including apolipoprotein A-4 and fibrinogen gamma chain, were upregulated in mild AD patients.

Discussion: Our results suggest that altered expression levels of these proteins in plasma may yield candidate biomarkers for the early diagnosis of AD.

Abbreviations: AD, Alzheimer's disease; FAST, Functional Assessment Staging; 2D-DIGE, two-dimensional differential gel electrophoresis; MALDI-TOF/TOF/MS, matrix-assisted laser desorption ionization time-of-flight tandem mass spectrometry; CSF, cerebrospinal fluid; Aβ, amyloid beta; MMSE, Mini Mental State Examination; MRI, magnetic resonance imaging; NINCDS-ADRDA, National Institute for Neurological Diseases and Stroke/Alzheimer's Disease and Related Disorders Association; CHAPS, 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate; DTT, dithiothreitol; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; DIA, differential in-gel analysis; BVA, biological variation analysis; CBB, Coomassie brilliant blue; 2DE, two-dimensional gel electrophoresis; TFA, trifluoroacetic acid; ACTH, adrenocorticotropic hormone; Apo A-1, apolipoprotein A-1; AHSG, alpha-2-HS-glycoprotein; Apo A-4, apolipoprotein A-4; MCI, mild cognitive impairment.

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Alzheimer's disease; plasma; 2D-DIGE; biomarkers; proteomics

Introduction

Alzheimer's disease (AD) is the most common cause of dementia in elderly persons [1], with a rapidly increasing prevalence worldwide. The development of AD has been closely associated with oxidative damage, but its molecular mechanism still remains unclarified [2–4]. AD slowly develops from a preclinical or early phase into a fully expressed clinical syndrome [5]. By the time it is recognized, the disease has been progressing for many years, and thus, early diagnosis of AD is important. The clinical diagnosis of AD is based on the patient's medical history, brain imaging scans, and neuropsychological assessments, as well as testing of cerebrospinal fluid (CSF) [6,7].

Proteins such as amyloid-beta (Aβ), total tau, and phosphorylated tau in CSF can contribute to the diagnosis of AD. However, obtaining CSF requires that patients undergo lumbar puncture, a relatively painful and invasive procedure. In addition, it is unclear to what extent blood Aβ and tau levels accurately reflect the presence or state of AD [5]. Blood is a complex tissue-containing proteins originating in many organs, and it is thus a useful potential source of screening biomarkers [1,8]. Blood is easily collected via noninvasive and safe procedures [9]. Dysfunction of blood–brain barrier permeability has been reported in AD [10], which implies that protein exchange may occur between the brain and peripheral

CONTACT Shinji Oikawa s-oikawa@doc.medic.mie-u.ac.jp
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bloodstream. Therefore, the peripheral circulation may be a promising source of AD-related biomarkers [11,12].

In this study, we performed protein profiling of blood in patients with mild AD to identify biomarkers for the early diagnosis of AD. Mild AD patients were defined using the Functional Assessment Staging (FAST) scale. The FAST scale is designed to evaluate the progression of AD based on patients' daily functioning [13] and has been used often in both research and clinical settings [14,15]. In addition, cognitive function was tested by the Mini Mental State Examination (MMSE) [16], and the scores supported the diagnosis of mild AD (Table 1). In discovery-phase investigations, the two-dimensional differential gel electrophoresis (2D-DIGE) technique has been validated as a powerful tool to analyze differences in protein expression profiles [17,18]. 2D-DIGE is able to minimize gel-to-gel variation and allows for the comparison of protein expression across different gels due to the use of an internal fluorescent standard. Thus, to identify candidate biomarkers for the early diagnosis of AD, the expression levels of plasma proteins were analyzed and compared between mild AD patients and healthy controls using 2D-DIGE, and the proteins were identified by matrix-assisted laser desorption ionization time-of-flight tandem mass spectrometry (MALDI-TOF/TOF/MS) followed by peptide mass fingerprinting.

Materials and methods

Clinical samples

Patients underwent a comprehensive clinical neurological examination, routine blood analysis, structural magnetic resonance imaging (MRI), and a detailed neuropsychological assessment that included the MMSE. The MRI scan revealed atrophy of the frontal and/or temporal lobes. Patients were diagnosed according to the National Institute for Neurological Diseases and Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria for probable AD [19]. The FAST scale is a reliable scale and has been validated for evaluating general functional capabilities, with patients assigned a stage from 1 (normal adults) to 7 (severe AD) [13]. We selected AD patients with stage 4, which is defined as mild AD. The patients in this stage may have difficulty with finances, counting money, and traveling to new locations [13]. Blood samples from mild AD patients with FAST stage 4 were collected at the University Hospital of Mie, Japan.

Control blood samples were collected from healthy subjects who were medically examined during health check-ups at Inabe General Hospital, Japan, and confirmed to have no history, symptoms or signs of psychiatric or neuronal disease. Demographic details of patients are listed in Table 1. The mean age of the control group was significantly lower than that of the AD patient group. To eliminate the influence of gender on protein expression [20], we used AD and control groups with matched gender ratios that were similar to those reported in the AD population as a whole (male:female, 1:1.5~3) [21].

Blood samples were collected in EDTA-2Na tubes and centrifuged at 1,000 × *g* for 10 min, and the supernatants were stored at −80 °C. The study was approved by the Ethical Committees of Mie University (No.2092) and Inabe General Hospital (No. 2015–12). All participants or their relatives gave informed consent for participation in the study, which was performed in accordance with the Declaration of Helsinki.

Table 1. Characteristics of healthy subjects and patients with AD.

Demographic variables	Control	AD
Number of subjects (M/F)	10 (2/8)	9 (2/7)
Age (years)	63.8 ± 5.3	72.7 ± 8.5*
FAST scale	N/A	4
MMSE scores	N/A	21.7 ± 3.4
Total cholesterol (mg/dl)	215 ± 30	222 ± 43
Triglycerides (mg/dl)	93 ± 30	127 ± 57

Data are presented as mean ± SD. M/F = male/female. N/A = not applicable. FAST = Functional Assessment Staging. MMSE = Mini Mental State Examination.
*$p < 0.05$.

Preparation of plasma proteins

The high-abundance proteins in the collected plasma samples, such as albumin, alpha-1-antitrypsin, IgA, IgG, transferrin, and haptoglobin, were depleted using the Multiple Affinity Removal Spin Cartridge for Human Serum (Agilent Technologies, Santa Clara, CA, USA). The depleted plasma samples were precipitated using the 2D Clean-Up Kit (GE Healthcare, England, UK). The samples were resuspended in lysis buffer (30 mM Tris-HCl, 7 M urea, 2 M thiourea, 4% (w/v) 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS), a protease inhibitor cocktail, pH 8.5). Protein concentrations of the samples were measured in triplicate by the Bradford assay (Thermo Fisher Scientific, Waltham, MA, USA), using bovine serum albumin as a standard [22].

2D-DIGE

Each protein sample was labeled with CyDyes developed for the 2D-DIGE technology (GE Healthcare) [23,24]. Twenty-five micrograms of total protein per sample were labeled with 200 pmol of CyDyes and incubated on ice for 30 min in the dark. Five samples from healthy subjects were initially labeled with Cy3 and five samples from AD patients were labeled with Cy5. The CyDyes were then switched to avoid preferential binding of a CyDye to a group-specific protein: five samples from healthy subjects were labeled with Cy5, while four samples from AD patients were labeled with Cy3. An internal standard was generated by combining equal amounts of all samples and then labeling with Cy2. The pooled sample with Cy2

was run on all gels, allowing spot matching and normalization of signals from different gels. Equal protein amounts of Cy2-, Cy3-, and Cy5-labeled samples were mixed and added to an equal volume of 2 × sample buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 130 mM dithiothreitol (DTT), 2% IPG buffer (pI 3–10; GE Healthcare), a protease inhibitor cocktail). After incubation on ice for 10 min in the dark, the samples were added to rehydration buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 13 mM DTT, 1% IPG buffer (pI 3–10), a protease inhibitor cocktail) and applied to IPG strips (pI 3–10 NL strips, 24 cm; GE Healthcare) for rehydration for 16 h. Isoelectric focusing was carried out on an Ettan IPGphor 3 (GE Healthcare). The strips were treated by reduction and alkylation of disulfide bonds with 10 mg/ml DTT and 25 mg/ml iodoacetamide, respectively. Then, standard SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 10 vertical 12.5% gels (Ettan DALTsix Large Format Vertical System, GE Healthcare). Ten gel images (from 9 mild AD patients and 10 controls) were scanned using a Typhoon FLA 9500 scanner (GE Healthcare). Intragel spot detection and intergel matching were performed using the differential in-gel analysis (DIA) and biological variation analysis (BVA) modules in DeCyder 2D software version 7.2 (GE Healthcare). Spot integration was carried out with the following parameters: spot max slope, spot area, spot volume, and spot peak height. Protein expression values were statistically analyzed using Student's *t*-test with the DeCyder 2D software.

Protein identification by in-gel digestion and MALDI-TOF/TOF/MS

For peptide mass fingerprinting, the Coomassie brilliant blue (CBB)–stained portions of the two-dimensional gel electrophoresis (2DE) gel were excised and digested with trypsin [24]. The differential protein spots on the gel were cut out, decolorized, dehydrated, added to trypsin solution (Promega Corporation, Madison, WI, USA) and digested overnight at 37 °C. After digestion, tryptic peptides were extracted with 45% acetonitrile/0.1% trifluoroacetic acid (TFA) and concentrated. The solutions were spotted 1:1 with saturated α-cyanohydroxycinnamic acid (Wako Pure Chemical, Osaka, Japan) matrix solution, and mixed on a stainless-steel target plate.

Mass analysis was performed using MALDI-TOF/TOF/MS (4800 Plus MALDI-TOF/TOF™ Analyzer System, Sciex, Toronto, Canada) with 4000 Series Explorer version 3.5 software. The mass scale was calibrated using mixtures of des-arg1-bradykinin ($[M + H]^+$ 904.468 m/z), angiotensin 1 ($[M + H]^+$ 1296.685 m/z), glu1-fibrinopeptide B ($[M + H]^+$ 1570.677 m/z), adrenocorticotropic hormone (ACTH)-(1–17)-clip ($[M + H]^+$ 2093.087 m/z), ACTH-(18–39)-clip ($[M + H]^+$ 2465.199 m/z), and ACTH-(7–38)-clip ($[M + H]^+$ 3657.929 m/z). For MS mode, the MS reflector positive-ion mode was set up as follows: automatic acquisition; mass range: 800–4000 m/z; total laser shots per spectrum: 1250. The MS/MS 1-kV positive mode was set up as follows: relative precursor mass window: 200 (full width, half mass); total laser shots per spectrum: 2500. The peak detection criteria used were a minimum S/N of 3, a local noise window width mass/charge (m/z) of 250 and a minimum full-width half-maximum (bins) of 2.9. A maximum of the 10 strongest precursor ions per protein spot were chosen for MS/MS analysis. The following monoisotopic precursor selection criteria were used for MS/MS: minimum S/N filter of 20 and excluding the precursors within 200 resolutions.

Protein identification was performed using the MS/MS ion search tool in ProteinPilot version 4.0 software (Sciex) with the Uniprot database as the search engine. The following sample parameters were used: cysteine alkylation: iodoacetamide; digestion: trypsin; species: *Homo sapiens*; ID focus: biological modifications and amino acid substitutions; result quality: detected protein threshold > 2.0 (99.0% confidence). Protein identification was based on the criterion of ProtScore > 2 (corresponding to 99% confidence) to minimize false positives.

Statistical analysis

A stepwise multiple regression analysis was performed in SPSS Statistics 23 (IBM Corporation, NY, USA) to determine the influence of age on expression levels of proteins between the AD and control groups.

Results

To examine the profiles of plasma proteins between mild AD patients and healthy controls, we performed 2D-DIGE and MALDI-TOF/TOF/MS. Figure 1a shows a representative image of a 2D-DIGE gel containing two samples, one labeled with Cy3 (control) and the other with Cy5 (AD). The green spots on the 2D-DIGE gel depict plasma proteins and show downregulated proteins in AD compared to control, whereas the red spots show upregulated proteins. The yellow spots indicate proteins with similar expression in AD patient and control. A comparison of nine mild AD patients and 10 controls showed significant differences between the two groups in the expression levels of 31 spots. Since 13 of these spots were invisible or hardly visible on CBB-stained gels, the remaining 18 spots were excised, in-gel digested and identified by MALDI-TOF/TOF/MS analysis. Seven of these spots contained several proteins in each spot. Therefore, 11 spots contained a single differentially expressed protein (Table 2). The positions of these protein spots are shown on CBB-stained gel in Figure 1b. The graphical expression profiles of each protein spot and the mass spectra obtained by MALDI-TOF/TOF/MS are shown in the Supplementary Figures. We found five proteins that were significantly decreased

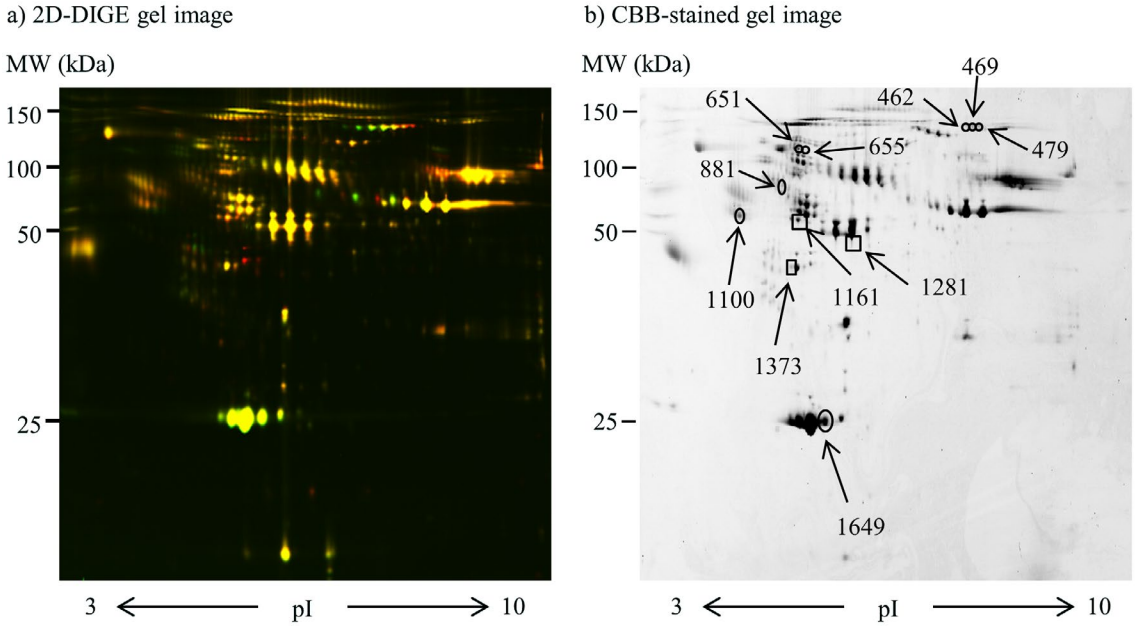

Figure 1. Image of plasma proteins detected by 2D-DIGE (a) and CBB staining (b).
(a) 2D-DIGE image of AD patient (Cy5) and control (Cy3). The proteins (25 µg) were labeled with Cy3 and Cy5 dyes, mixed and subjected to 2D-DIGE analysis. Green spots indicate downregulated proteins in AD compare to control, while red spots indicate upregulated proteins. Yellow spots represent proteins that are unchanged. (b) Typical image of a CBB-stained 2DE map of plasma samples. Various protein spots showed either significant increases (squares) or decreases (circles) in intensity in AD patients compared to controls; numbers indicate spot ID number.

Table 2. MALDI-TOF/TOF/MS identification of plasma proteins from AD patients and controls.

Spot No.	Protein	Average ratio	Uniprot ID	No. of peptide matches	Sequence coverage (%)	Theoretical MW (kDa)/ pI
1649	Apolipoprotein A-1	−1.30	P02647	10	30.0	30.8/5.6
1373	Apolipoprotein A-4	1.38	P06727	7	21.7	45.4/5.3
1100	Alpha-2-HS-glycoprotein	−1.32	P02765	4	12.0	39.3/5.4
1281	Fibrinogen gamma chain	1.23	P02679	6	26.3	51.5/5.4
1161	Fibrinogen gamma chain	1.24	P02679	10	37.8	51.5/5.4
651	Afamin	−1.29	P43652	6	10.9	69.1/5.6
655	Afamin	−1.32	P43652	2	6.3	69.1/5.6
881	Kininogen-1	−1.15	P01042	7	8.4	72.0/6.3
462	Plasminogen	−1.35	P00747	7	12.7	90.7/7.0
469	Plasminogen	−1.27	P00747	9	17.0	90.7/7.0
479	Plasminogen	−1.22	P00747	10	21.1	90.7/7.0

in the plasma of AD patients compared to controls. These were identified as apolipoprotein A-1 (Apo A-1; spot 1649), kininogen-1 (spot 881), alpha-2-HS-glycoprotein (AHSG; spot 1100), afamin (spots 651 and 655), and plasminogen (spots 462, 469 and 479). In contrast, apolipoprotein A-4 (Apo A-4; spot 1373) and fibrinogen gamma chain (spots 1161 and 1281) were significantly increased in the plasma of AD patients. Afamin, plasminogen, and fibrinogen gamma chain were identified in multiple spots, likely reflecting the existence of different protein isoforms.

There was a difference in mean age between AD patients and healthy controls in this study (Table 1, $p < 0.05$). We investigated the influence of age on expression levels of identified proteins in AD patients and controls using multiple regression analysis. Differences between these two groups in the expression levels of Apo A-1 (spot 1649), Apo A-4 (spot 1373), AHSG (spot 1100), fibrinogen gamma chain (spot 1281), and afamin (spot 651) remained statistically significant after adjusting for age. On the other hand, differences in the expression levels of kininogen-1 (spot 881), fibrinogen gamma chain (spot 1161), afamin (spot 655), and plasminogen (spots 462, 469 and 479) were influenced by age. The graphical expression profiles of Apo A-1 (spot 1649), Apo A-4 (spot 1373), AHSG (spot 1100), fibrinogen gamma chain (spot 1281), and afamin (spot 651) are represented in Figure 2.

Discussion

In this study, we compared protein profiles of blood samples from patients with mild AD, defined by a classification of stage 4 on the FAST scale, and healthy controls without symptoms of dementia to discover biomarkers for early diagnosis of AD. After adjusting for age by

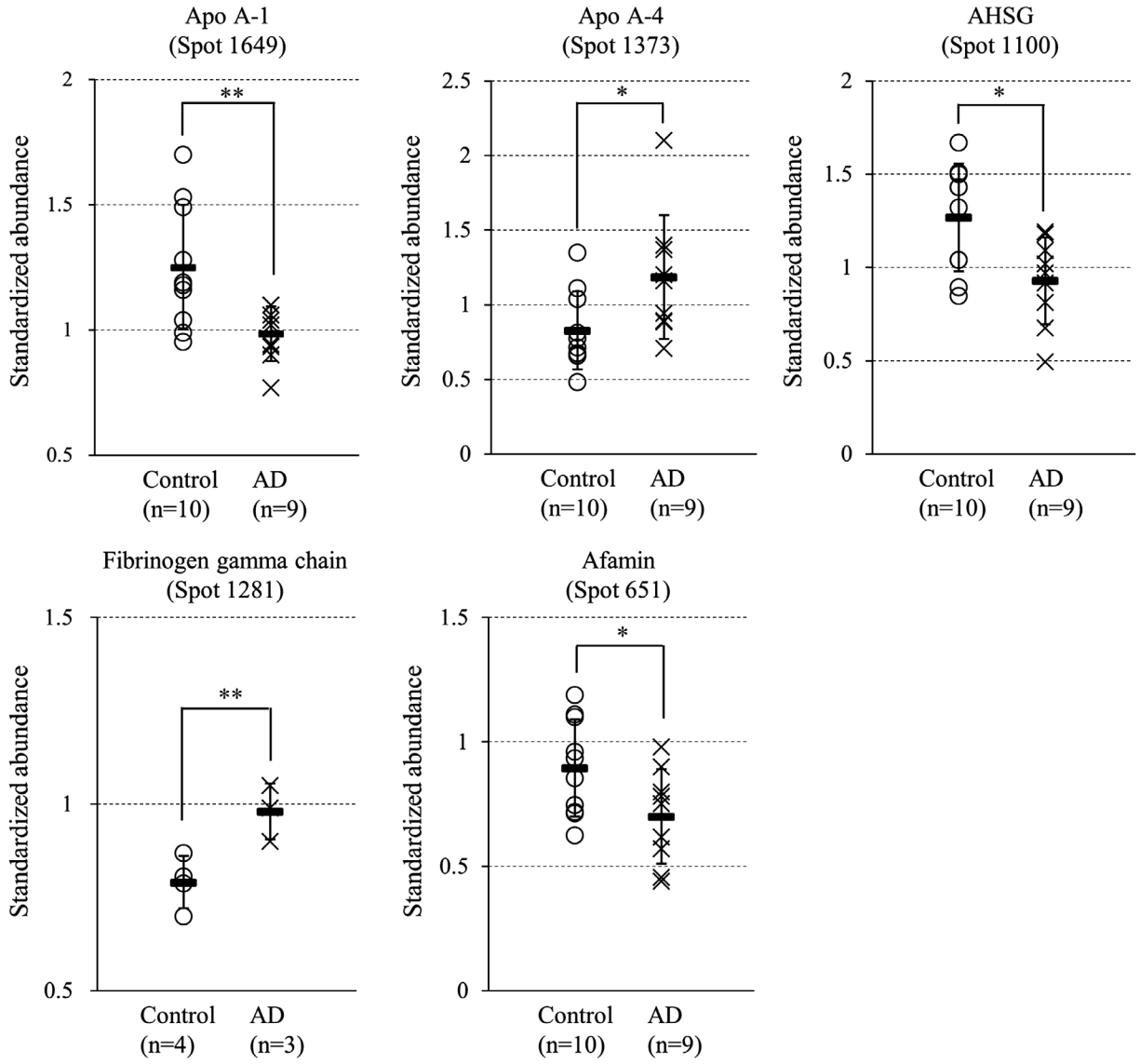

Figure 2. Graphical expression profile of the identified proteins.
The levels of identified proteins were visualized by graph views of the standardized abundance of spot intensity. Each data point represents an individual subject. The bars indicate mean ± SD. Statistical analysis was performed using multiple regression analysis (*$p < 0.05$, **$p < 0.01$). Apo A-1: apolipoprotein A-1; Apo A-4: apolipoprotein A-4; AHSG: alpha-2-HS-glycoprotein.

multiple regression analysis, we found that the expression levels of 3 proteins (Apo A-1 (spot 1649), AHSG (spot 1100), and afamin (spot 651)) were significantly decreased in the plasma of AD patients compared to controls, and those of 2 proteins (Apo A-4 (spot 1373) and fibrinogen gamma chain (spot 1281)) were significantly increased in the plasma of AD patients. The functions of the identified proteins and their potential as biomarkers for AD are discussed below.

Apolipoproteins

Apolipoproteins are a group of proteins related to cholesterol and lipid metabolism. Recent findings indicate that apolipoproteins might also be involved in neurodegenerative processes [25]. Apo A-1 was shown to inhibit the aggregation of Aβ oligomers and to diminish their accumulation *in vitro* [26]. It was reported that decreased Apo A-1 was associated with an increased risk of dementia [27]. Thus, decreased ApoA-1 may be related to the progression of AD [28]. We showed that expression levels of Apo A-1 were significantly decreased in the plasma of mild AD patients as compared to controls. Our results are consistent with a previous report of decreased concentrations of ApoA-1 in the CSF of neuropathologically confirmed AD [29]. In addition, Liu et al. showed significantly decreased Apo A-1 in the serum of patients with moderate-to-severe AD [30]. Taken together, decreased plasma ApoA-1 may serve as useful biomarker for the early diagnosis of AD.

Although several functions of Apo A-4 correlate with AD pathogenesis, the pathologic role of Apo A-4 in AD is still unknown. Our results showed that the expression levels of Apo A-4 were significantly increased in plasma samples of mild AD patients as compared to controls. The expression levels of Apo A-4 in the serum

of AD patients were previously found to be significantly increased [7]. It was reported that ablation of Apo A-4 promoted amyloid formation and facilitated Aβ clearance in an AD mouse model [31]. It is suggested that Apo A-4 may play an important role in the progression of AD and could thus be a susceptibility biomarker for the early diagnosis of AD.

Alpha-2-HS-glycoprotein (AHSG)

AHSG, also known as fetuin-A, plays a role in anti-inflammatory and neuroprotective effects [32]. Our results revealed that AHSG levels in the plasma of mild AD patients were significantly lower than in controls. A recent study showed that plasma concentrations of AHSG in patients with mild-to-moderate AD were decreased in direct proportion to the degree of cognitive impairment [33]. In addition, plasma AHSG levels were found to significantly correlate with the MMSE score, which may be related to the severity or progression of AD [33]. Further, it was reported that the concentrations of AHSG were decreased in the CSF of AD patients [6]. Our results and those of these previous studies suggest that decreased plasma levels of AHSG could be a possible biomarker for the early diagnosis of AD.

Fibrinogen gamma chain

Fibrinogen is the primary protein component of blood clots. It is composed of three pairs of polypeptide chains, designated alpha, beta, and gamma, which are connected by disulfide bonds. *In vitro* studies suggested that the interaction between Aβ and fibrinogen modifies fibrinogen's structure, which may then lead to abnormal fibrin clot formation and vascular abnormalities in AD [9,34]. Increased plasma fibrinogen levels were found to be associated with an increased risk of AD [35]. Our results showed that the expression levels of fibrinogen gamma chain were significantly increased in the plasma of mild AD patients as compared to controls. In addition, it was reported that fibrinogen gamma chain levels in the plasma of patients with mild cognitive impairment (MCI) who subsequently progressed to AD were also increased [36]. Therefore, elevated fibrinogen gamma chain may be a potential biomarker of early AD.

Afamin

Afamin is a specific binding protein for vitamin E and facilitates vitamin E transport across the blood–brain barrier [37]. It was reported that afamin and vitamin E display neuroprotective activity against oxidative damage by hydrogen peroxide or Aβ *in vitro* [38]. Several recent studies suggested that afamin is a possible marker in various neurological pathologies, including AD [9,39,40]. We showed that plasma afamin levels were significantly lower in mild AD patients. Therefore, we suggested that decreased plasma afamin levels might be used as biomarker for early AD.

Our experimental results showed that Apo A-1, Apo A-4, AHSG, fibrinogen gamma chain, and afamin were differentially expressed in plasma of patients with mild AD compared to healthy controls. Interestingly, low expression levels of Apo A-1 and AHSG have been reported in the CSF of AD patients [6,30]. In addition, Simonsen et al. reported that some candidate biomarkers, including Apo A-1 in CSF, may be useful to distinguish between AD and vascular dementia, the latter of which is the second most common form of dementia [41]. Certainly, CSF directly interacts with the central nervous system and reflects biochemical changes that occur in the brain, but lumbar puncture is difficult and invasive. Therefore, we consider that altered plasma expression levels of Apo A-1, Apo A-4, AHSG, fibrinogen gamma chain, and afamin could serve as candidate biomarkers for the early diagnosis of AD. Furthermore, a recent review identified blood-based candidate biomarkers of vascular dementia that differed from the proteins described in this study, suggesting variations in biomarkers between AD and other types of dementia [42]. However, one of the limitations of this study was its relatively small sample size. Future validation studies of identified proteins using larger samples and a targeted quantitative approach (e.g. ELISA) are needed to prove biomarker effectiveness.

Authors contribution

Conceived and designed the study: Shinji Oikawa and Hidekazu Tomimoto. Blood collection: Sahoko Ichihara, Hirotaka Kida and Masayuki Satoh. Analyzed the data: Yuki Kitamura and Ryoko Usami. Wrote the paper: Yuki Kitamura. Paper modification: Shinji Oikawa and Mariko Murata.

Ethics approval

The study was approved by the Ethical Committees of Mie University (No. 2092) and Inabe General Hospital (No. 2015-12).

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Disclosure statement

No potential conflict of interest was reported by the authors.

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