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Elevated Levels of Autoantibodies against ATP2B4 and BMP-1 in Sera of Patients with Atherosclerosis-related Diseases

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Abstract

Background: Atherosclerosis-related life-style diseases such as cerebral infarction (CI), cardiovascular disease (CVD), diabetes mellitus (DM), and chronic kidney disease (CKD) are a serious problem in the recently aging society. The development of novel and sensitive diagnostic markers is necessary and expected for the early treatment. **Methods and Results:** Through the first screening by phage expression cloning, we identified ATPase, Ca^{++} transporting, plasma membrane 4 (ATP2B4) and bone morphogenetic protein 1 (BMP-1) as antigens recognized by IgG antibodies in the sera of patients with atherosclerosis. The presence of autoantibodies against these antigens in serum specimens was confirmed by Western blotting. We then compared serum antibody levels against recombinant ATP2B4 and BMP-1 proteins between healthy donors (HD) and patients with atherosclerotic diseases, such as CI, transient ischemic attack (TIA), CVD, DM, or CKD, by the Alpha (amplified luminescent proximity homogeneous assay)-LISA method. The results revealed that both antibody levels were significantly higher in patients with these diseases than in HD and exhibited most prominent differences in CKD vs. HD. Correlation analysis showed that both antibody levels were well correlated with the degree of artery stenosis, such as maximum intima-media thickness with some different patterns, i.e., anti-ATP2B4 antibody levels were related to hypertension, whereas anti-BMP-1 antibodies were related to smoking habits.

Conclusions: The serum antibody levels against ATP2B4 and BMP-1 can be useful diagnostic markers for atherosclerosis and its related diseases caused by hypertension and smoking habits, respectively.

Keywords: Atherosclerosis; Diabetes mellitus; Chronic kidney disease; Cerebral infarction; Cardiovascular disease; Antibody biomarker

Abbreviations: ABI: Ankle-Brachial Pressure Index; ATP2B4-Abs: Anti-ATP2B4 Antibodies; ATS: Atherosclerosis; AUC: Areas Under the Curve; BMP-1-Abs: Anti-BMP-1 Antibodies: CAVI: Cardio-Ankle Vascular Index: CI: Cerebral Infarction: CKD: Chronic Kidney Disease; CVD: Cardiovascular Disease; DM: Diabetes Mellitus; *E. coli*: *Escherichia coli*; GST: Glutathione-S-Transferase; HD: Healthy Donor; HDL: High Density Lipoprotein; IMT: Intima-media Thickness; IPTG: Isopropyl-β-D-Thiogalactoside; LDL: Low Density Lipoprotein;

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ROC: Receiver Operating Curve; SEREX: Serological Identification of Antigens by Recombinant cDNA Expression Cloning.

Introduction

The development of atherosclerosis (ATS) leads to the onset of cerebral infarction (CI), cardiovascular disease (CVD), and chronic kidney disease (CKD), which are also caused by diabetes mellitus (DM) [1]. Thus, these ATS-related diseases are regarded as typical life-style diseases and are major causes of mortality worldwide [2,3]. Thus far, many risk factors have been identified for these diseases, including hypertension, hyperlipidemia, body mass index (BMI)/obesity, smoking habits, and family history [4-6]. Blood test parameters, such as high-density lipoprotein (HDL)-cholesterol, low-density lipoprotein (LDL)-cholesterol, total cholesterol (TC), glycohemoglobin (HbA1c) [7], and uric acid [8], were also introduced for evaluating the development of ATS-related diseases. However, these parameters are still insufficient for predicting the onset of CI and CVD. It is possible to prevent the onset of these diseases by early treatment and/or changing the life-style. The development of an early diagnosis and prediction system using novel and sensitive biomarkers is indispensable.

Till date, only few antibody biomarkers have also been identified, for example, oxidized low density lipoprotein and β2-glycoprotein I for ATS [9,10], and heat shock proteins (Hsps) for CVD [11], Hsp60 for stroke [12], and GAD for DM [13,14]. The SEREX (serological identification of antigen by recombinant cDNA expression cloning) [15] method is effective and convenient for comprehensive screening of tumor antigens [16-18]. We also introduced SEREX into the screening of autoantigens and autoantibodies responsible for ATS-related diseases and identified RPA2 for stroke [19] and TUBB2C for DM and CI [20]. Here, we report anti-ATP2B4 and anti-BMP-1 antibodies as general ATS markers related to CI, CVD, DM, and CKD.

Materials and Methods

Patients and healthy donor (HD) sera

The Local Ethical Review Board of the Chiba University, Graduate School of Medicine as well as those of co-operating hospitals approved the study. Sera were collected from patients after they had given written informed consent. Each serum sample was centrifuged at 3,000 ×g for 10 min, and then the supernatant was stored at -80°C until use. Repeated thawing and freezing of samples were avoided.

The serum samples of CI were obtained from Chiba Prefectural Sawara Hospital, Chiba Rosai Hospital and Chiba Aoba Municipal Hospital. The samples of CVD and DM were obtained from Kyoto University Hospital and Chiba University Hospital, and those of CKD were from the Kumamoto cohort and Sanai Memorial Soga Hospital. Sera from HDs were obtained from Chiba University, Chiba Prefectural Sawara Hospital, Shimoshizu National Hospital and Port Square Kashiwado Clinic.

Screening by expression cloning

Recombinant DNA studies were performed with the official permission of the Chiba University Graduate School of Medicine and were carried out in accordance with the rules of the Japanese government. We used a commercially available human microvascular endothelial cell cDNA library (Uni-ZAP XR Premade Library, Stratagene, La Jolla, CA) to screen for clones that were immunoreactive against serum IgG from patients with severe carotid stenosis as previously described [19]. *Escherichia coli* (*E. coli*) XL1-Blue MRF' was infected with Uni-ZAP XR phage and the expression of resident cDNA clones was induced after blotting the infected bacteria onto NitroBind nitrocellulose membranes (Osmonics, Minnetonka, MN) that had been treated with 10 mM isopropyl-β-D-thiogalactoside (IPTG, Wako Pure Chemicals, Osaka, Japan) for 30 min. The membranes with bacterial proteins were rinsed 3 times with TBS-T [20 mM Tris-HCl (pH 7.5), 0.15M NaCl and 0.05% Tween-20], and non-specific binding was blocked by incubation with 1% protease-free bovine serum albumin (Nacalai Tesque, Inc., Kyoto, Japan) in TBS-T for 1 h. The membranes were exposed to 1:2000-diluted sera of patients for 1 h. After three washes with TBS-T, the membranes were incubated for 1 h with 1:5000-diluted alkaline phosphatase-conjugated goat anti-human IgG (Jackson ImmunResearch Laboratories, West Grove, PA). Positive reactions were developed using 100 mM Tris-HCl (pH 9.5) containing 100 mM NaCl, 5 mM $MgCl_2$, 0.15 mg/ml of 5-bromo-4-chloro-3-indolylphosphate, and 0.3 mg/ml of nitro blue tetrazolium (Wako Pure Chemicals). Positive clones were re-cloned twice until obtaining monoclonality as previously described [17,19,20].

Monoclonal phage cDNA clones were converted to pBluescript phagemids by excision *in vivo* using the ExAssist helper phage (Stratagene). Plasmid pBluescript containing cDNA was obtained from the *E. coli* SOLR strain after transformation by the phagemid. The sequences of cDNA inserts were evaluated for homology with identified genes or proteins within the public sequence database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Expression and purification of antigen proteins

The expression plasmids of glutathione-S-transferase (GST)-fused proteins were constructed by recombining the cDNA sequences into pGEX-4T-3 (GE Healthcare Life Sciences, Pittsburgh, PA). The inserted DNA fragments were ligated in frame to pGEX-4T-3 using the Ligation Convenience Kits (Nippon Gene, Toyama, Japan). Ligation mixtures were used to transform ECOS™-competent *E. coli* BL-21 (Nippon Gene), and appropriate recombinants were confirmed by DNA sequencing as well as protein expressions. Treating the transformed *E. coli* with 0.1 mM IPTG for 3 h induced the expression of the GST-fusion proteins. The GST-fused recombinant proteins were purified by glutathione-Sepharose column chromatography according to the manufacturer's instructions (GE Healthcare Life Sciences) and dialyzed against phosphate-buffered saline as previously described [16,21].

Western blotting

GST, GST-ATP2B4, and GST-BMP-1 proteins (0.3 μg) were electrophoresed through SDS-polyacrylamide gel followed by Western blotting using anti-GST (Rockland, Gilbertsville, PA) or sera from patients with CI (#350, #462, and #692). After incubation with horseradish peroxidase-conjugated secondary antibody, immunoreactivity was detected with the Immobilon (Merck Millipore, Darmstadt, Germany) as previously described [18,22].

AlphaLISA (Amplified Luminescence Proximity Homogeneous Assay)

AlphaLISA was performed using 384-well microtiter plates (white opaque OptiPlate™, Perkin Elmer) containing 2.5 μl of 1/100-diluted sera and 2.5 μl of GST or GST-fusion proteins (10 μg/ml) in AlphaLISA buffer (25 mM HEPES, pH 7.4, 0.1% casein, 0.5% Triton X-100, 1 mg/ml dextran-500, and 0.05% Proclin-300). The reaction mixture was incubated at room temperature for 6-8 h. Next, anti-human IgG-conjugated acceptor beads (2.5 μl of 40 μg/ml) and glutathione-conjugated donor beads (2.5 μl of 40 μg/ml) were added and incubated further for 7-14 days at room temperature in the dark. The chemical emission was read on an EnSpire Alpha microplate reader (PerkinElmer) as previously described [20,23]. Specific reactions were calculated by subtracting Alpha values of GST control from the values of GST-fusion proteins.

Statistical analyses

Student's *t* test and the Mann-Whitney U test were used to determine the significance of the differences between two groups. Correlation was examined by Spearman's correlation analysis. All statistical analyses were carried out using the GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Multivariate logistic regression analysis was used to find a set of variables classifying the subjects into those with and without a history of stroke. The predictive values of markers for diseases were assessed by receiver operating curve (ROC) analysis and the cut-off values were set at the values that maximize the sums of the sensitivity and specificity. All tests were two-tailed and a *P* value below 0.05 was considered significant.

Results

Identification of BMP-1 recognized by sera of patients with ATS

Expression cloning using the sera of patients with both ATS has identified two clones, TS22I and A1J3, which showed a sequence homology with ATPase, Ca^{++} transporting, plasma membrane 4 (ATP2B4) (Accession number: NM_001001396.2), and bone morphogenetic protein 1 (BMP-1) (Accession number: NM_006129.4), respectively. Recombinant ATP2B4 and BMP-1 proteins were expressed in *E. coli* as GST-fusion proteins and purified by affinity-chromatography using glutathione-Sepharose.

Confirmation of the presence of serum antibodies by Western blotting

The presence of anti-ATP2B4 antibodies (ATP2B4-Abs) and anti-BMP-1 antibodies (BMP-1-Abs) in the sera of patients was confirmed by Western blotting analysis. GST-ATP2B4 and GST-BMP-1 as well as GST proteins were recognized by anti-GST antibody as reactions of 55-kDa, 85-kDa and 28-kDa proteins, respectively (Figure 1). On the other hand, only GST-ATP2B4 or GST-BMP-1 was reacted with the serum antibodies of patients # 350 and #462, respectively. Serum #692 showed the reactivity to GST, which eventually enabled the recognition of both GST-ATP2B4 and GST-BMP-1. Therefore, in the following study, specific reactions against ATP2B4 or BMP-1 proteins were estimated by antibody levels toward GST-tagged antigen proteins subtracted by the levels toward GST.

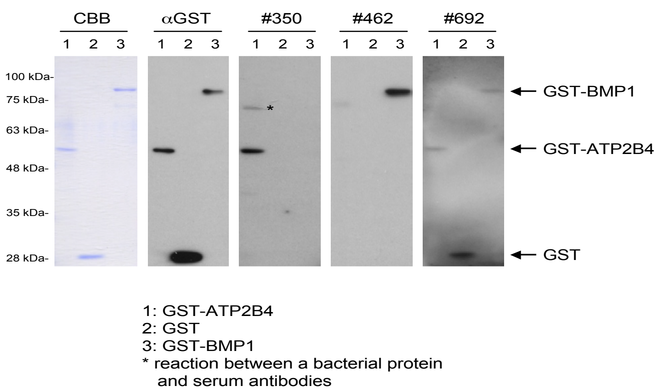

Figure 1: Serum antibodies recognized GST-ATP2B4 or GST-BMP-1. Representative results of Western blotting are shown. The original SDS-polyacrylamide gel was stained with Coomassie Brilliant Blue (CBB), or the membranes blotted with antigen proteins were probed with anti-GST (αGST), or patient sera, numbers #350, #462 and #692. Arrows indicate specific reactions, and the asterisk represents a reaction between a bacterial protein and serum antibodies, which does not affect the AlphaLISA results because the reactivity to only GST-fusion proteins is evaluated by trapping by glutathione-conjugated donor beads. Molecular weights are shown to the left.

Levels of ATP2B4-Abs and BMP-1-Abs are increased in patients with CI

We examined the levels of serum ATP2B4-Abs and BMP-1-Abs using the sera of HD and patients with TIA or in the acute phase of CI (aCI) obtained from Chiba Prefectural Sawara Hospital, Chiba Rosai Hospital and Chiba Aoba Municipal Hospital. HD subjects from Kashiwado Clinic and Chiba Prefectural Sawara Hospital were selected as those exhibiting no abnormalities on MRI examination. The results of AlphaLISA showed that both levels of ATP2B4-Abs and BMP-1-Abs were significantly higher in patients with TIA or aCI than in HD (Figures 2a and 2b). When the cut-off value was determined as the average + 2SD of HD, the positive rates of ATP2B4-Abs in HD and patients with TIA and aCI patients were 3.9%, 13.8%, and 17.3%,

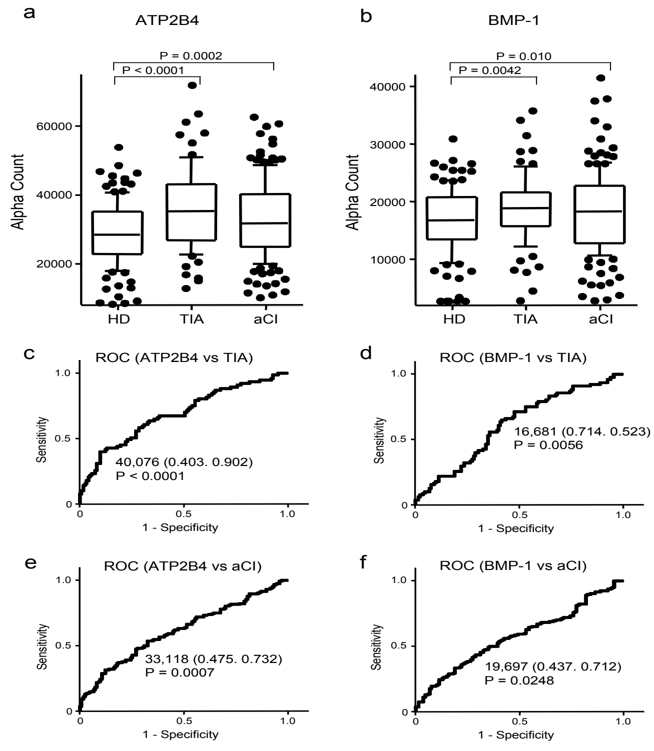

Figure 2: Comparison of serum ATP2B4-Ab and BMP-1-Ab levels between HD and patients with TIA or aCI. Antigens used were GST-ATP2B4 (a) and GST-BMP-1 proteins (b). Serum antibody levels subtracted by the levels against control GST examined by AlphalLISA are shown by a box-whisker plot. The box plots display the 10th, 20th, 50th, 80th, and 90th percentiles. P values vs. HD specimens are shown. In Table 1, averages, SDs, cut-off values, total numbers, positive numbers, positive rates (%), and P values are shown. Receiver operating curve (ROC) analysis was carried out for assessing the ability of ATP2B4-Abs (c and e) and BMP-1-Abs (d and f) to detect TIA (c and d) or aCI (e and f). Areas under the curve (AUC) were 0.690 (95% CI: 0.614-0.766) (c), 0.615 (95% CI: 0.537-0.693) (d), 0.619 (95% CI: 0.553-0.684) (e), and 0.577 (95% CI: 0.511-0.642) (f). Numbers in the curves indicate cut-off values of marker levels and those in the parentheses indicate sensitivity (left) and specificity (right). P values are also shown.

respectively, whereas the positive rates of BMP-1-Abs were 0.8%, 3.9%, and 3.9%, respectively (Table 1). Thus, positivity of BMP-1-Abs in TIA and aCI was less prominent as compared with that of ATP2B4-Abs. Receiver operating curve (ROC) analysis was carried out to evaluate the ability of these markers to detect TIA and aCI. The areas under the curve (AUC) of ATP2B4-Abs and BMP-1-Abs for TIA were 0.690 (95% CI: 0.614-0.766) (Figure 2c) and 0.615 (95% CI: 0.537-0.693) (Figure 2d), respectively, whereas those for aCI were 0.619 (95% CI: 0.553-0.684) (Figure 2e) and 0.577 (95% CI: 0.511-0.642) (Figure 2f), respectively. When the cut-off value of the ATP2B4-Ab level was determined to be 40,076, the sensitivity and specificity of the antibody level for the diagnosis of TIA were 40.3% and 90.2%, respectively (Figure 2c).

Levels of BMP-1-Abs are associated with CVD

We next examined the antibody levels of CVD, including acute myocardial infarction and unstable angina, in samples obtained from Chiba University Hospital and Kyoto University Hospital. The levels of both ATP2B4-Abs and BMP-1-Abs were significantly higher in patients with CVD than those in HD; yet, BMP-1-Ab levels differed more than ATP2B4-Ab levels (Figures 3a and 3b), which was reflected in the positive rates. The positive rates of ATP2B4-Abs in HD and patients with CVD were 4.4% and 17.2%, respectively, whereas the positive rates of BMP-1-Abs were 5.7% and 23.4%, respectively (Table 2). ROC analysis revealed that AUCs of ATP2B4-Abs and BMP-1-Abs for CVD were 0.653 (95% CI: 0.567-0.738) (Figure 3c) and 0.680 (95% CI: 0.636-0.725) (Figure 3d), respectively.

Levels of ATP2B4-Abs and BMP-1-Abs are related to DM

Because ATS is closely related to DM, we then compared the specimens of HD and DM obtained from Shimoshizu National Hospital and Chiba University Hospital, respectively. Both ATP2B4-Abs and BMP-1-Abs markedly increased in patients with DM as compared with those in HD (Figure 4). The positive rates of ATP2B4-Abs in HD and patients with CVD were 4.9% and 28.0%, respectively, whereas the positive rates of BMP-1-Abs were 2.5% and 17.8%, respectively (Table 3). ROC analysis revealed that AUCs of ATP2B4-Abs and BMP-1-Abs for DM were 0.714 (95% CI: 0.655-0.772) (Figure 4c) and 0.670 (95% CI: 0.606-0.733) (Figure 4d), respectively.

		GST-ATP2B4	GST-BMP-1
HD	Average	51,248	16,161
	SD	30,365	6,858
	Cut-off value	111,979	29,878
	Total No.	128	123
	Positive No.	5	1
	Positive rate (%)	3.9%	0.8%
TIA	Average	72,672	18,806
	SD	36,128	5,871
	Total No.	29	77
	Positive No.	4	3
	Positive rate (%)	**13.8%**	3.9%
	P (vs HD)	**0.0052**	**0.0042**
aCI	Average	74,380	18,806
	SD	35,931	5,871
	Total No.	127	77
	Positive No.	22	3
	Positive rate (%)	**17.3%**	3.9%
	P (vs HD)	**7.38E-08**	**0.0042**

Table 1: Comparison of serum antibody levels between HD and patients with TIA or acute CI (aCI) examined by AlphaLISA. Shown are average, SD, cut-off values (average+2SD), total sample number, the number of positive sera of which antibody levels were higher than the cut-off value, and the positive rate (%) of HD; and average, SD, total sample number, number of positive sera of which the antibody levels were higher than the cut-off value, and the positive rate (%) of patients; and P values of statistical comparison between HD and patients. Antigens used were purified GST-ATP2B4 and GST-BMP-1 proteins. P values lower than 0.05 and positive rates higher than 10% are marked in bold.

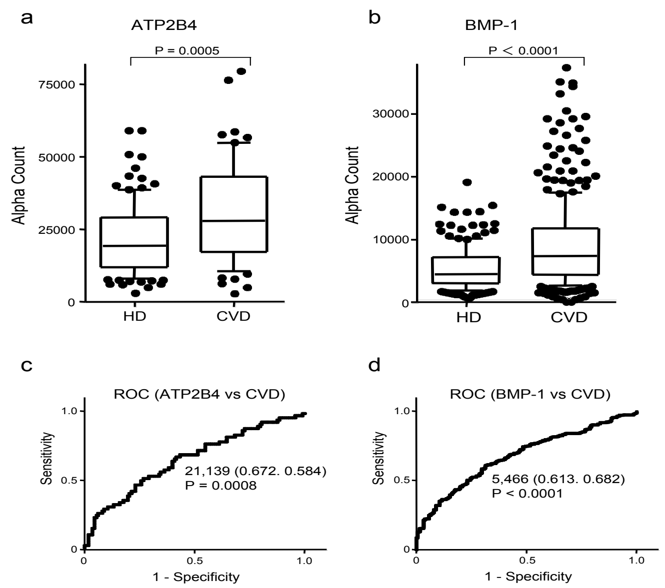

Figure 3: Comparison of serum ATP2B4-Ab and BMP-1-Ab levels between HD and patients with CVD. Antigens used were GST-ATP2B4 (a) and GST-BMP-1 proteins (b). Serum antibody levels examined by AlphaLISA are shown by a box-whisker plot as described in the legends of Figure 2. In Table 2, averages, SDs, cut-off values, total numbers, positive numbers, positive rates (%), and P values are shown. The results were also evaluated by ROC analysis (c and d). AUCs of ATP2B4-Abs and BMP-1-Abs were 0.653 (95% CI: 0.567-0.738) (c) and 0.680 (95% CI: 0.636-0.725) (d), respectively.

		ATP2B4	BMP-1
HD	Average	21,325	4,859
	SD	12,132	3,386
	Cut-off value	45,588	11,630
	Total No.	113	192
	Positive No.	5	11
	Positive rate (%)	4.4%	5.7%
CVD	Average	29,923	8,667
	SD	16,926	7,258
	Total No.	64	380
	Positive No.	11	89
	Positive rate (%)	**17.2%**	**23.4%**
	P (vs HD)	**0.00054**	**1.14E-16**

Table 2: Comparison of serum antibody levels between HD and patients with CVD examined by AlphaLISA. Antigens used were purified GST-ATP2B4 and GST-BMP-1 proteins. See Table 1 for further details.

Levels of ATP2B4-Abs and BMP-1-Abs are elevated in patients with CKD

CKD is also closely related to ATS and was divided into three groups as follows: type 1, diabetic kidney disease; type 2, nephrosclerosis; and type 3, glomerulonephritis; samples were obtained from the Kumamoto cohort. ATP2B4-Ab and BMP-1-Ab levels in patients with CKD were higher in all three types than those in HD (Figures 5a and

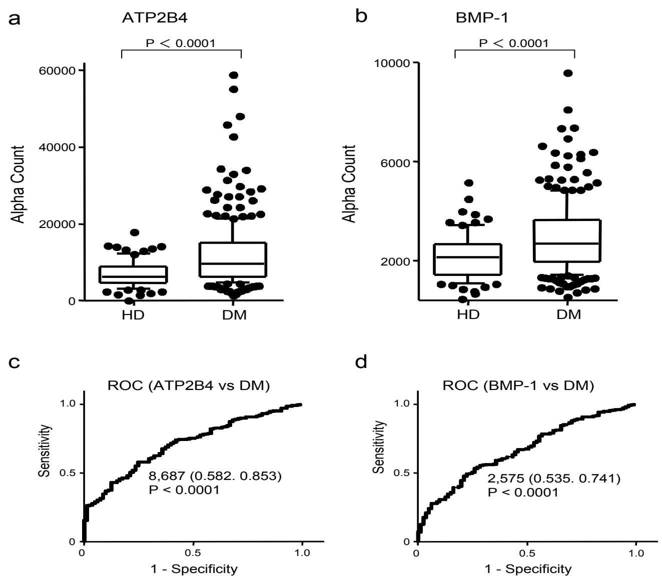

Figure 4 : Comparison of serum ATP2B4-Ab and BMP-1-Ab levels between HD and patients with DM. Antigens used were GST-ATP2B4 (a) and GST-BMP-1 proteins (b). Serum antibody levels examined by AlphalLISA are shown by a box-whisker plot as described in the legends of Figure 2. In Table 3, averages, SDs, cut-off values, total numbers, positive numbers, positive rates (%), and P values are shown. The results were also evaluated by ROC analysis (c and d). AUCs of ATP2B4-Abs and BMP-1-Abs were 0.714 (95% CI: 0.655-0.772) (c), and 0.670 (95% CI: 0.606-0.733) (d), respectively.

		GST-ATP2B4	GST-BMP-1
HD	Average	6,865	2,150
	SD	3,507	928
	Cut-off value	13,880	4,006
	Total No.	81	81
	Positive No.	4	2
	Positive rate (%)	4.9%	2.5%
DM	Average	11,833	3,013
	SD	8,388	1,859
	Total No.	275	275
	Positive No.	77	49
	Positive rate (%)	**28.0%**	**17.8%**
	P (vs HD)	**1.05E-13**	**3.71E-08**

Table 3: Comparison of serum antibody levels between HD and patients with DM examined by AlphaLISA. Antigens used were purified GST-ATP2B4 and GST-BMP-1 proteins. See Table 1 for further details.

5b). The positive rates of ATP2B4-Abs in HD, CKD type 1, type 2 and type 3 were 5.0%, 37.2%, 43.8%, and 19.5%, respectively, whereas the positive rates of BMP-1-Abs were 3.8%, 35.9%, 37.5%, and 20.3% respectively (Table 4). Little difference was found among CDK types. The total positive rates of ATP2B4-Abs and BMP-1-Abs were 30.7% and 29.7%, respectively. BMP-1-Ab levels were also examined in serum samples obtained from patients on dialysis in Sanai Memorial Soga Hospital, and similar differences between HD and patients with CKD were observed (Figure 5c and Table 5). ROC analysis revealed that AUCs of ATP2B4-Abs and BMP-1-Abs for CKD type 1 were 0.789 (95% CI: 0.730-0.848) (Figure 5d) and 0.770 (95% CI: 0.708-0.831) (Figure 5e), respectively. Thus, CKD was most associated with both ATP2B4-Abs and BMP-1-Abs with such high sensitivity and specificity.

Levels of BMP-1-Abs are related to cancer

Autologous antibodies frequently developed in patients with cancer [24]. We therefore examined the samples from patients with benign glioma, malignant glioma or esophageal squamous cell carcinoma (SCC) obtained from Chiba University Hospital and Toho University Hospital. The levels of BMP-1-Abs were significantly elevated in esophageal SCC as compared with HD (Table 6). ATP2B4-Ab levels were also elevated in esophageal SCC but less prominently. No apparent difference in both ATP2B4-Abs and BMP-1-Abs was observed between HD and patients with benign or malignant gliomas.

Correlation analysis between ATP2B4-Abs and BMP-1-Abs and atherosclerosis indices

We then carried out Spearman correlation analysis and multivariate logistic regression analysis between antibody marker levels and data on study individuals, including gender, age, height, weight, BMI, blood pressure, smoking habit, and the degree of artery stenosis such as maximum intima-media thickness (max IMT), as well as complication of DM, hypertension, CVD, lipidemia, and CI. Blood test data, such as data on total protein, albumin/globulin ratios, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, lactate dehydrogenase, total bilirubin, γ-glutamyl transpeptidase, albumin, blood urea nitrogen, creatinin, estimated glomerular filtration rates, uric acid, amylase, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglyceride, Na, K, Cl, Ca, P, Fe, C-reactive protein, blood

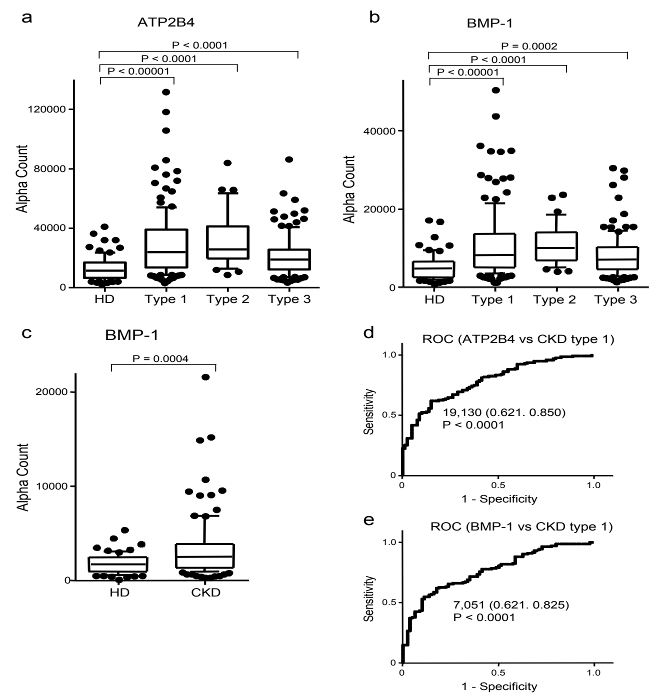

Figure 5: Comparison of serum ATP2B4-Ab and BMP-1-Ab levels between HD and patients with CKD. Serum antibody levels against GST-ATP2B4 (a) and GST-BMP-1 proteins (b) were compared between HD and patients with CKD types 1, 2, and 3. (c) Serum antibody levels against GST-BMP-1 proteins were also compared between HD and dialysis patients in the Sanai Memorial Soga Hospital. Results are shown as described in the legends of Figure 2. In Tables 4 and 5, averages, SDs, cut-off values, total numbers, positive numbers, positive rates (%), and P values are shown. The results were also evaluated by ROC analysis to detect CKD type 1 (d and e). AUCs of ATP2B4-Abs and BMP-1-Abs were 0.789 (95% CI: 0.730-0.848) (d), and 0.770 (95% CI: 0.708-0.831) (e), respectively.

		GST-ATP2B4	GST-BMP-1
HD	Average	12,825	5,023
	SD	8,074	3,163
	Cut-off value	28,973	11,349
	Total No.	80	80
	Positive No.	4	3
	Positive rate (%)	5.0%	3.8%
Type 1 CKD	Average	29,204	10,881
	SD	21,835	8,530
	Total No.	145	145
	Positive No.	54	52
	Positive rate (%)	**37.2%**	**35.9%**
	P (vs HD)	**5.62E-14**	**3.63E-12**
Type 2 CKD	Average	31,991	10,642
	SD	17,866	5,083
	Total No.	32	32
	Positive No.	14	12
	Positive rate (%)	**43.8%**	**37.5%**
	P (vs HD)	**1.14E-06**	**7.85E-07**
Type 3 CKD	Average	21,662	8,056
	SD	13,537	5,521
	Total No.	123	123
	Positive No.	24	25
	Positive rate (%)	**19.5%**	**20.3%**
	P (vs HD)	**2.30E-08**	**1.46E-06**
CKD total	Total No.	300	300
	Positive No.	92	89
	Positive rate (%)	**30.7%**	**29.7%**

Table 4: Comparison of serum antibody levels between HD and patients with CKD examined by AlphaLISA. CKD types 1, 2 and 3 were diabetic kidney disease, nephrosclerosis and glomerulonephritis, respectively. Antigens used were purified GST-ATP2B4 and GST-BMP-1 proteins. See Table 1 for further details.

		GST-BMP-1
HD	Average	2,021
	SD	1,542
	Cut-off value	5,104
	Total No.	78
	Positive No.	5
	Positive rate (%)	6.4%
CKD	Average	3,292
	SD	3,221
	Total No.	111
	Positive No.	17
	Positive rate (%)	**15.3%**
	P (vs HD)	**0.00040**

Table 5: Comparison of serum antibody levels between HD and patients with CKD examined by AlphaLISA. Antigens used were purified GST-ATP2B4 and GST-BMP-1 proteins. See Table 1 for further details.

sugar, and HbA1c were also included as described [20,23]. A total of 741 specimens from the Sawara Hospital including 139 specimens from HD, 79 from patients with deep and subcortical white matter hyperintensity, 15 patients with asymptomatic CI, 29 patients with TIA, 227 with aCI, 58 with chronic CI, and 194 from disease controls. Both Spearman and multivariate analyses showed similar results. The degree of artery stenosis, such as max IMT and IMT values, smoking habit and smoking period were well correlated with both ATP2B4-Ab and BMP-1-Ab levels (Table 7). In addition, ATP2B4-Abs were associated well with blood pressure and complication of hypertension.

A similar correlation analysis was also performed for 384 specimens obtained from patients with CKD of the Kumamoto cohort were analyzed. The levels of ATP2B4-Abs showed a positive correlation with max IMT and cardio-ankle vascular index (CAVI) (Table 8), suggesting a close relation between ATP2B4-Ab levels and ATS. ATP2B4-Ab levels also correlated with ferritin, Fe and K levels. On the other hand, the levels of BMP-1-Abs were correlated with ferritin, cigarette smoking habit, AST and triglyceride but showed inverse correlation with HDL-cholesterol. This implicates that the high BMP-1-Abs levels may reflect liver malfunction caused by smoking habit. No other patient data significantly correlated with the levels of ATP2B4-Abs or BMP-1-Abs.

Discussion

Phage expression cloning, also called SEREX, is effective for the screening of tumor antigens [15-18] and biomarkers of stroke [19] and DM [20]. Through the expression cloning method, we have identified two antigens that were recognized by serum IgG antibodies in patients with ATS. The presence of serum antibodies against ATP2B4 and BMP-1 in patient sera was confirmed by Western blotting (Figure 1). Furthermore, AlphaLISA enabled us to evaluate those antibody levels, and thereby, to compare the levels between HD and patients.

ATP2B4 is a member of the plasma membrane Ca^{2+}-ATPase family and involved in calcium homeostasis [25]. Several mutations in the ATP2B4 gene have been found in familial spastic paraplegia [26]. The positive rates of ATP2B4-Abs were higher in patients with DM (28.7%) and CKD (30.7%) than those in patients with aCI (17.3%), CVD (17.2%), and TIA (13.8%) (Tables 1-4). Similar high positive rates of ATP2B4-Abs in patients with DM and CKD may suggest that these

		GST-ATP2B4	GST-BMP-1
HD	HD Av	14,221	4,645
	HD SD	13,869	4,367
	HD Av+2SD	41,959	13,380
	HD Total No.	111	111
	HD Positive No.	4	5
	HD Positive (%)	3.6%	4.5%
Glioma-B	P Av	14,882	5,078
	P SD	9,280	4,078
	P Total No.	83	83
	P Positive No.	1	6
	P Positive (%)	1.2%	7.2%
	P (P vs HD)	0.692	0.479
Glioma-M	P Av	14,469	5,125
	P SD	10,366	4,151
	P Total No.	90	90
	P Positive No.	2	3
	P Positive (%)	2.2%	3.3%
	P (P vs HD)	0.885	0.427
Esophag-SCC	P Av	20,144	6,895
	P SD	12,377	4,918
	P Total No.	100	100
	P Positive No.	5	11
	P Positive (%)	5.0%	**11.0%**
	P (P vs HD)	**0.0012**	**0.00058**

Table 6: Comparison of serum antibody levels between HD and patients with cancer examined by AlphaLISA. Cancer specimens included benign glioma (Glioma-B), malignant glioma (Glioma-M) and esophageal squamous cell carcinoma (Esophag-SCC). Antigens used were purified GST-ATP2B4 and GST-BMP-1 proteins. See Table 1 for further details.

		ATP2B4			**BMP-1**	
	Spearman		**Multivariate**	**Spearman**		**Multivariate**
	r value	**P value**	**r value**	**r value**	**P value**	**r value**
Gender	0.012	0.762	0.025	-0.006	0.868	-0.005
Age	**0.124**	**0.001**	**0.140**	0.066	0.091	0.077
Height	-0.072	0.065	**-0.100**	-0.031	0.425	-0.046
Weight	-0.058	0.136	**-0.081**	-0.034	0.385	-0.033
BMI	-0.019	0.621	-0.044	-0.016	0.687	-0.018
IMT (right)	**0.150**	**0.001**	**0.115**	**0.096**	**0.040**	**0.088**
IMT (left)	**0.104**	**0.027**	**0.119**	**0.120**	**0.011**	**0.130**
max IMT	**0.152**	**0.001**	**0.129**	**0.124**	**0.008**	**0.119**
Blood pressure	**0.128**	**0.001**	**0.125**	0.070	0.080	0.067
Smoking	**0.102**	**0.009**	**0.109**	**0.139**	**0.000**	**0.150**
Smoking period	**0.151**	**0.000**	**0.188**	**0.181**	**< 0.0001**	**0.201**
DM (Complication)	0.050	0.202	0.016	0.038	0.323	0.030
Hypertension (Complication)	**0.097**	**0.012**	**0.103**	0.030	0.442	0.034
CVD (Complication)	0.033	0.398	0.034	0.010	0.803	0.004
Lipidemia (Complication)	-0.033	0.395	-0.064	-0.023	0.550	-0.030
CI (Complication)	-0.018	0.649	-0.012	0.013	0.732	0.008
Total protein	-0.047	0.239	-0.031	**-0.099**	**0.013**	-0.075
Albumin/globulin ratio	-0.062	0.119	-0.087	0.049	0.225	0.046
Aspartate aminotransferase	0.056	0.149	0.004	0.020	0.611	0.004
Alanine aminotransferase	-0.021	0.587	-0.015	0.017	0.666	0.022
Alkaline phosphatase	**0.100**	**0.015**	0.069	0.067	0.100	0.060
Lactate dehydrogenase	0.049	0.219	0.098	0.026	0.512	0.069
Total bilirubin	0.023	0.560	0.019	0.014	0.732	0.010
γ-glutamyl transpeptidase	-0.026	0.522	0.023	0.042	0.297	0.062
Albumin	-0.066	0.094	**-0.092**	-0.021	0.599	-0.015
Blood urea nitrogen	-0.042	0.286	0.037	-0.055	0.159	0.011
Creatinin	-0.018	0.640	0.015	-0.021	0.589	-0.002
Estimated glomerular filtration rate	-0.002	0.965	-0.008	0.039	0.365	0.033
Uric acid	-0.056	0.216	0.037	-0.022	0.626	0.040
Amylase	-0.081	0.100	**-0.086**	-0.092	0.060	**-0.090**
Total cholesterol	**-0.093**	**0.028**	**-0.087**	-0.065	0.122	-0.060
HDL-cholesterol	-0.003	0.954	0.022	-0.013	0.792	0.016
LDL-C	-0.097	0.073	**-0.111**	-0.066	0.220	-0.078
Triglyceride	-0.058	0.212	-0.039	0.007	0.874	0.054
Na	-0.044	0.263	-0.077	-0.030	0.455	-0.028
K	-0.054	0.174	-0.047	-0.077	0.052	**-0.099**
Cl	-0.028	0.479	-0.065	-0.008	0.838	-0.020
Ca	-0.063	0.218	-0.077	-0.088	0.086	**-0.109**
Inorganic phosphate	-0.019	0.741	-0.016	-0.002	0.973	-0.012
Fe	-0.053	0.352	0.001	-0.015	0.792	0.022
C-reactive protein	0.069	0.133	0.026	0.012	0.800	0.005
Blood sugar	0.076	0.065	0.046	0.036	0.381	0.062
HbA1c	-0.039	0.383	0.047	-0.063	0.161	0.039

Table 7: Correlation analysis between ATP2B4 and BMP-1 antibody marker levels and data on study individuals from Sawara Hospital cohort. Correlation coefficient (r) values and P values were calculated by Spearman's correlation analysis. r values obtained by multivariate logistic regression analysis are also shown. Significant correlations are marked in bold.

diseases are interrelated; i.e., one may be the cause of the other and vice versa. However, multivariate logistic regression analysis revealed no significant correlation between ATP2B4-Abs and blood sugar, DM marker HbA1c and complication of DM (Table 7). Thus, the primary cause detected by ATP2B4-Abs may be kidney failure. The close association of ATP2B4-Abs with blood pressure and complication of hypertension (Tables 7 and 8) may be well consistent with the high expression of ATP2B4 in the aorta [27], because the aorta is most susceptible to hypertension produced by heart beating. Therefore, it is possible that the simple leaking out of ATP2B4 protein by hypertension in the aorta as well as by ATS in the neighboring artery caused the development of the antibodies. Further, continuous high expression of ATP2B4 may disrupt calcium homeostasis leading to tissue calcification, which is the final stage of ATS.

It has been well documented that BMP signals are involved in the development of ATS [28,29]. The expression of BMP-2 and BMP-4 is elevated in atherosclerotic endothelium [30,31]. Plasma BMP-2 levels are elevated in patients with type 2 DM [32]. The chronic infusion

	ATP2B4	BMP-1
Ferritin	**<0.0001**	**0.0035**
C-reactive protein	**0.0030**	0.0578
max IMT	**0.0041**	0.1836
Fe	**0.0042**	0.9711
CAVI (right)	**0.0111**	0.1203
CAVI (left)	**0.0167**	0.2364
K	**0.0215**	0.4760
Smoking	0.2445	**0.0068**
Aspartate aminotransferase	0.0607	**0.0114**
Triglyceride	0.0563	**0.0260**

Table 8: Correlation analysis between ATP2B4 and BMP-1 antibody marker levels and data on study individuals from Kumamoto cohort. P values were calculated using Spearman's correlation analysis. Significant correlations are marked in bold.

of BMP-4 induced endothelial dysfunction and hypertension [33], and treatment with the BMP antagonist, matrix gla protein, or BMP inhibitors prevent from the development of ATS [34,35]. On the other hand, the knockdown of BMP type II receptor, BMPRRII, accelerates ATS [36]. Therefore, BMP family members may have a subtle regulatory role in the development of ATS.

Unlike other members of the BMP family including BMP-2 - 15, BMP-1 is a metalloproteinase containing an astacin-like domain and is also known as procollagen C-proteinase, [37]. BMP-1 activates BMP signaling by degrading chordin which can inhibit the action of BMPs [38]. The positive rates of BMP-1-Abs were higher in patients with CKD (29.7%) and CVD (23.4%) than those in patients with DM (17.8%) and aCI (3.9%) (Tables 1-4), suggesting that BMP-1-Abs are associated with most, if not all, ATS-related diseases. It should be noted that we have found elevated antibody levels in patients with CI against SOSTDC1 [23], which is an antagonist of BMPs [39,40]. Therefore, these autologous antibodies may not be generated due to the high expression of antigen proteins; however, they may play a role in the development of ATS. This notion further implies that the antigenic cytokines can be therapeutic targets by treating patients with agonists or antagonists as appropriate.

Correlation analysis using specimens from the Kumamoto cohort and Sawara hospital cohort revealed that BMP-1-Abs correlated with smoking habit (Tables 7 and 8). Consequently, this marker can detect CVD accompanied by CKD or DM, of which the primary cause may be the smoking habit. This type of correlation analysis of biomarkers is of utmost importance because the high risk of the disease onset might be reduced by changing only marker-related life-style behaviors of the examinee.

BMP-1-Ab levels, which reflect smoking, were also higher in patients with esophageal SCC than those in HD (Table 6), probably because smoking habit is one of the major causes of esophageal SCC. However, it should be taken into account that DM is also a risk factor for cancers such as colorectal cancer and esophageal carcinoma [41-43]. Consistently, ATP2B4-Ab levels were also higher in patients with esophageal SCC than those in HD but less prominently compared with BMP-Ab levels (Table 6).

Serum samples from patients with CI were collected within two weeks after disease onset. The onset may induce spreading of various antigens, however, the antibodies against these antigens are not immediately produced. Therefore, the antibodies that were specifically detected in patient sera immediately after the onset, such as ATP2B4-Abs and BMP-1-Abs, were probably present prior to disease onset. Consistently, these autoantibody levels were markedly elevated not only in a CI but also in TIA, a harbinger of CI, compared with HD samples (Figure 2). Thus, these antibody markers appear to be prediction markers but not simple risk markers.

ATP2B4-Abs and BMP-1-Abs are promising biomarkers for ATS-related diseases. The positive rates of each marker may not be high enough, this may be because they were associated with different causes, such as hypertension and smoking habit. Further diagnosis using combination of as many markers as possible may improve the sensitivity.

Conclusion

The levels of ATP2B4-Abs and BMP-1-Abs were higher in patients with TIA, aCI, CVD, DM and CKD, and may therefore prove valuable for the early diagnosis of these ATS-related diseases.

Competing interests

This work was performed in collaboration with Fujikura Kasei Co., Ltd. and Celish Fd Inc. RN, GT, NS and HK are employees of Fujikura Kasei Co., Ltd., and TK and HD are employees of Celish Fd Inc.

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