

# Serum SH3BP5-specific Antibody Level is a Biomarker of Atherosclerosis

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## Abstract

**Background:** The discovery and development of novel biomarkers that could facilitate early diagnosis and thus prevent the progression of atherosclerosis-related diabetes mellitus (DM), cerebral infarction (CI), and cardiovascular disease (CVD) has garnered much research interest. Notably, recent reports have described a number of highly sensitive antibody markers. In this study, we aimed to identify additional antibody markers that would facilitate screening.

**Methods:** The amplified luminescent proximity homogeneous assay (AlphaLISA) method, which incorporates glutathione- or streptavidin-donor beads and anti-human-IgG-acceptor beads, was used to evaluate serum antibody levels in serum samples. The protein array method was used for the initial screening, and peptide arrays were used to identify epitope sites.

**Results:** The protein array identified SH3 domain-binding protein 5 (SH3BP5) as a target antigen of serum IgG antibodies in the sera of patients with atherosclerosis. We prepared recombinant glutathione S-transferase (GST)-fused SH3BP5 protein. Peptide arrays revealed that the epitope site recognized by serum antibodies is located within amino acids 161–174 of SH3BP5. AlphaLISA revealed significantly higher serum antibody levels against both the SH3BP5 protein and peptide in patients with DM, acute-phase CI, transient ischemic attack, CVD or chronic kidney disease (CKD), than in healthy donors. Furthermore, areas under the receiver operating characteristic curves of these antibodies were higher in patients with CKD and DM than in other patients. Spearman correlation analysis revealed associations between the serum antibody levels against SH3BP5 peptide and artery stenosis, hypertension, and smoking.

**Conclusions:** The serum anti-SH3BP5 antibody marker appears to be useful for estimating the progress of atherosclerosis and may discriminate atherosclerosis associated with hypertension and/or habitual smoking.

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

## Introduction

Current clinical practices include biochemical blood analyses as part of a typical health checkup. These analyses are useful because organ dysfunction may lead to the leakage of an intracellular protein, such as an enzyme, into circulation. Notably, enzymes can be simply quantitated by measuring the enzymatic activity; for example, alanine aminotransferase (ALT) and  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GTP) are

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Received April 04, 2016; Accepted April 25, 2017; Published May 01, 2017

**Citation:** Hiwasa T, Tomiyoshi G, Nakamura R, Shinmen N, Kuroda H, et al. (2017) Serum SH3BP5-specific Antibody Level is a Biomarker of Atherosclerosis. Immunome Res 13: 132. doi: [10.4172/17457580.1000132](https://doi.org/10.4172/17457580.1000132)

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abundantly expressed in the liver, and an increase in their enzymatic activities in the blood indicates liver failure. However, the quantitative measurement of non-enzymatic proteins is less straightforward.

At present, blood levels of non-enzymatic proteins can be measured using enzyme-linked immunosorbent assays (ELISAs) that incorporate specific antibodies. However, this method is less sensitive than enzymatic assays, although considerable recent progress has been made. Therefore, ELISAs are limited to analyze abundant proteins, such as C-reactive protein (CRP) [1], glycated hemoglobin (HbA1c) [2], and adiponectin [3]. Notably, recent remarkable advances in biotechnology, particularly in the polymerase chain reaction methodology, have enabled the highly sensitive detection of nucleic acids, such as cell-free DNA and RNA, in the blood [4]. These assays measure the extracellular or “leaked” levels of otherwise intracellular DNA and RNA, a phenomenon that occurs very frequently in response to tissue destruction. In some organs, even small lesions may cause sufficient cell membrane destruction and consequent DNA, RNA, and protein leakage; these leaked components will then enter systematic circulation [5]. However, the highly sensitive nature of these assays does not necessarily mean that DNA and RNA are more suitable biomarkers than proteins. DNA is found in most cells of the body; therefore its presence alone does not indicate organ dysfunctional, unless particular mutations have occurred in a certain tumor tissue. Although some RNAs, unlike DNA, are expressed in a tissue-specific manner, RNA is highly labile and difficult to handle. In contrast, proteins are relatively stable and suitable as biomarkers.

Although immunoassays are known to be insensitive for determining protein concentrations, as mentioned above, whether circulating antibody levels would provide more information remains unclear. Potentially, autoantibodies may develop against leaked self-proteins (antigens). A recently developed assay technology has led to the finding that most self-proteins can serve as antigens against which autoantibodies are elicited [6]. Because of the fact that both antigens and antibodies may remain at very low concentrations and are rapidly degraded after a single round of tissue destruction, the levels of these factors may be difficult to detect. However, repeated cycles of tissue destruction during the course of disease progression could lead to a tremendous increase in autoantibody levels while keeping low levels of antigens. Therefore, antibody measurement is thought to be much more sensitive than antigen measurement.

Recent studies have discovered and implemented antibody markers against proteins such as phospholipid [7], apolipoprotein A-1 [8,9], oxidized low-density lipoprotein [9,10] and heat shock proteins (Hsps) [9,11] for cardiovascular disease (CVD); Hsp60 for stroke [12]; insulin [13], glutamic acid decarboxylase (GAD) [14] and protein tyrosine phosphatase IA-2 for diabetes mellitus (DM) [15,16] and p53 for cancer [17]. Similarly, our group has searched for antibody markers using the serological identification of antigens by recombinant cDNA expression cloning (SEREX) method, and has previously reported the discovery of antibodies against Trop2, TRIM21, makorin 1, and ECSA for esophageal squamous cell carcinoma (SCC) [18-21]; SH3GL1 and filamin C for glioma [22,23]; talin-1 for multiple sclerosis [24]; RPA2 and SOSTDC1 for ischemic stroke [25,26]; ATP2B4 and BMP-1 for atherosclerosis [27]; and TUBB2C and adiponectin for DM [28,29]. Here, we describe our recent discovery of a SH3 domain-binding protein 5 (SH3BP5)-specific antibody as a common marker of atherosclerosis-related diseases.

## Materials and Methods

### Patient and healthy donor (HD) sera

This study was approved by the Local Ethical Review Board of the Chiba University Graduate School of Medicine (Chiba, Japan) as well as

the review boards of co-operating hospitals. Serum was collected from patients who had provided written informed consent. Each serum sample was centrifuged at  $3,000 \times g$  for 10 min, and the supernatant was stored at  $-80^{\circ}\text{C}$  until use. Repeated thawing and freezing of samples were avoided.

Serum samples from patients with DM, CVD, and obstructive sleep apnea (OSA) were obtained from Chiba University Hospital, and samples from patients with acute-phase cerebral infarction (aCI) and transient ischemic attack (TIA) were obtained from Chiba Prefectural Sawara Hospital, Chiba Rosai Hospital, Chiba Aoba Municipal Hospital, and Chiba Medical Center. Serum samples associated with aCI, TIA, and CVD were obtained within 2 weeks after disease onset. Samples from patients with chronic kidney disease (CKD) were obtained from the Kumamoto cohort [30,31], and samples from patients with esophageal or colon carcinomas were obtained from Toho University Hospital. Sera of HDs were obtained from Chiba University, Port Square Kashiwado Clinic, and Chiba Prefectural Sawara Hospital. For comparisons associated with TIA and aCI, sera of HDs from Port Square Kashiwado Clinic were selected from among patients who exhibited no abnormalities on cranial magnetic resonance imaging.

### ProtoArray® screening

First The initial screening was performed using ProtoArray® Human Protein Microarrays v4.0 (Thermo Fisher Scientific, Waltham, MA), which were loaded with 9,480 species of proteins as described previously [26]. In total, 10 serum samples (five each from patients and HDs) were used to detect antigens specifically recognized by IgG antibodies in patient sera.

### Expression and purification of SH3BP5 protein

Total RNA was isolated from human U2OS osteosarcoma cells using the High Pure RNA Isolation Kit (Roche, Basel, Switzerland), and cDNA was synthesized using the Superscript III First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific). Full-length SH3BP5 cDNA was amplified by PCR using Pyrobest DNA polymerase (Takara Bio Inc., Shiga, Japan) and cloned into the EcoRI/SalI site of pGEX-4T-1 (GE Healthcare Life Sciences, Pittsburgh, PA), followed by confirmation by DNA sequencing. Expression of the cDNA product was induced by treating pGEX-4T-1-SH3BP5-transformed *Escherichia coli* (*E. coli*) with 0.1 mM isopropyl- $\beta$ -D-thiogalactoside at  $25^{\circ}\text{C}$  for 4 h; the cells were subsequently lysed in BugBuster Master Mix (Merck Millipore, Darmstadt, Germany). GST-tagged SH3BP5 protein was purified by glutathione-Sepharose (GE Healthcare Life Sciences) column chromatography according to the manufacturer's instructions, and dialyzed against phosphate-buffered saline (PBS) as described previously [18,20,25].

### Peptide array

Possible epitope sites in the SH3BP5 protein were predicted using the program ProPred (<http://www.imtech.res.in/raghava/propred/>) as described previously [26]. We designed five peptides derived from SH3BP5 and one control peptide derived from C9orf156. These peptides were synthesized onto cellulose membranes using F-moc amino acids (Auto spot robot ASP222; ABIMED Analysen-Technik GmbH, Langenfeld, Germany) as described previously [32]. The following amino acid sequences were used:

SH3BP5-43: FRSVLVEATVKLDE  
SH3BP5-149: VHKETAARYNAAMG  
SH3BP5-161: MGRMRQLEKCLKRA  
SH3BP5-185: KAKYYVQLEQLKKT

SH3BP5-327: EFGMMFVVLGPRSE  
C9orf156-428: HMTGPVGLVSLGS

After loading the six peptides, the membranes were washed five times with PBS containing 1% (w/v) bovine serum albumin, 0.05% Tween-20, and 0.05% NaN<sub>3</sub> (PBS-T-BSA) for 30 min each and then incubated with 1:200 dilutions of patient serum for 18 h. The membranes were subsequently washed five times with PBS-T-BSA and treated with a 1:10,000 dilution of FITC-conjugated goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA) for 1 h. After washing, the fluorescence levels of the peptide spots were detected using a Typhoon 9400 Imager (GE Healthcare Life Sciences) with a 488-nm/520-nm filter, as described previously [22,28].

### Peptide synthesis

N-terminal biotinylated 14-mer peptides (amino acid positions 161-174) derived from SH3BP5 (designated as bSH3BP5-161) were purchased from Eurofins Genomics (Tokyo, Japan). The purity of the HPLC-purified peptide was 94.92%.

### Amplified luminescence proximity homogeneous assay (AlphaLISA)

The AlphaLISA was performed in 384-well microtiter plates (white opaque OptiPlate™, Perkin Elmer, Waltham, MA) containing either 2.5 µL of 1:100-diluted serum with 2.5 µL of GST or GST-SH3BP5 protein (10 µg/mL), or a biotinylated peptide (bSH3BP5-161; 400 ng/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, following which anti-human IgG-conjugated acceptor beads (2.5 µL at 40 µg/mL) and glutathione- or streptavidin-conjugated donor beads (2.5 µL at 40 µg/mL) were added and incubated prior to another incubation at room temperature in the dark for 1-14 days. Chemical emissions were read on an EnSpire Alpha microplate reader (PerkinElmer) as described previously [26-29]. Specific reactions were calculated by subtracting the alpha counts of the GST control and of the buffer control without antigenic peptides, from the counts of GST-fusion proteins and biotinylated peptides, respectively.

### Statistical analyses

The Mann-Whitney U test were used to determine the significance of differences between the two groups. Correlations were calculated using Spearman correlation analysis. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). The predictive values of putative disease markers were assessed via a receiver operating characteristic (ROC) curve analysis, and the cutoff values were set to maximize the sums of sensitivity and specificity. All the tests were two-tailed, and *P* values of <0.05 were considered statistically significant.

## Results

### Recognition of SH3BP5 by serum components of patients with atherosclerosis

ProtoArray loaded with 9,480 protein species was used to identify the antigens recognized by antibodies in the sera of patients with atherosclerosis. SH3BP5 (Accession Number: NM\_004844) was found to react with three of the five serum samples from patients with atherosclerosis, and none of the five samples from HDs. Subsequently, GST-fused SH3BP5 proteins were expressed in *E. coli* and purified by affinity-chromatography. In addition, a peptide array containing

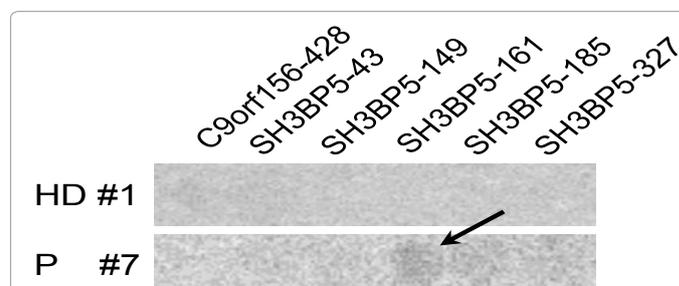
five predicted epitope sites of SH3BP5 was prepared, and it was found that one peptide, bSH3BP5-161, reacted with seven of the 17 serum samples from patients with aCI, but none of the nine samples from HDs. Although bSH3BP5-185 reacted with five of the 17 patient samples, the remaining three peptides and control C9orf156-428 were not recognized by any of the 17 patient sera, or nine HD sera. A representative result is shown in Figure 1.

### Elevation of serum antibody levels against SH3BP5 in patients with DMFirst

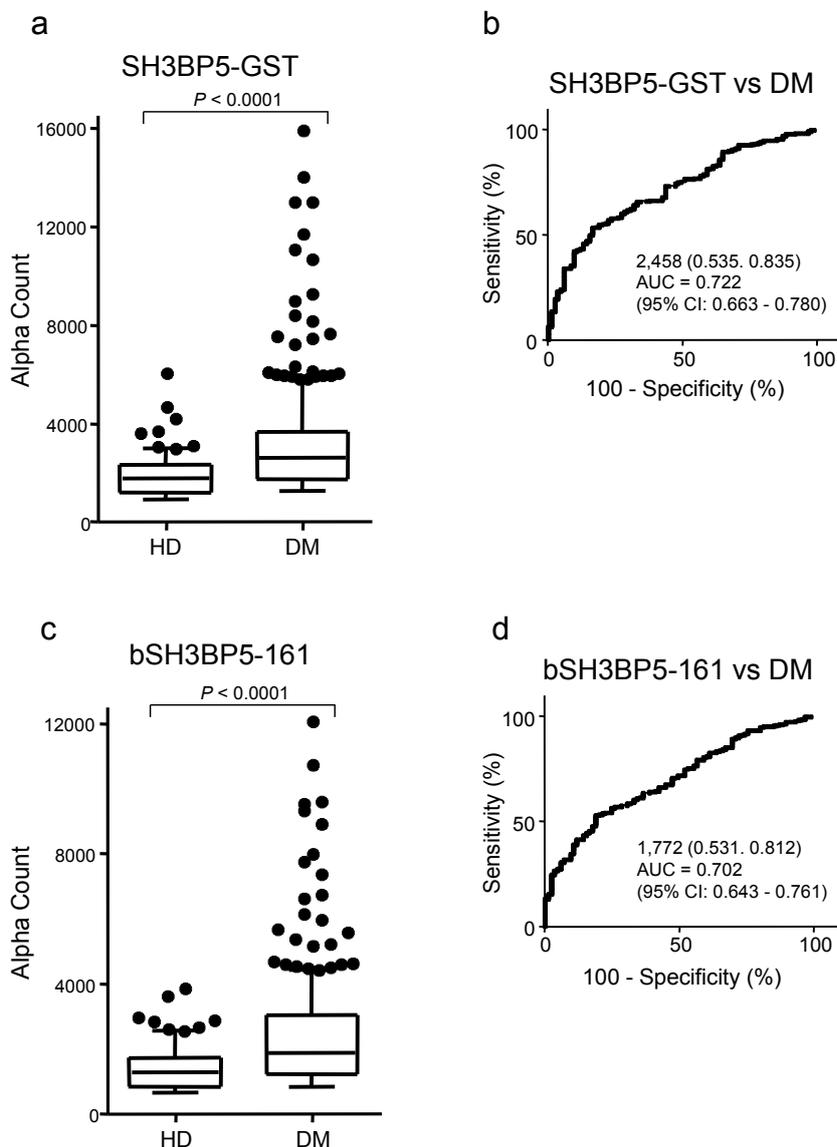
We next examined the levels of antibodies against the GST-fusion SH3BP5 protein and bSH3BP5-161 peptide in sera of HDs and patients with DM, using the highly sensitive and stable AlphaLISA method [26-29]. Serum samples of HDs and patients with DM were obtained from Chiba University Hospital (Figures 2-5). The levels of serum antibodies against GST-SH3BP5 protein (s-SH3BP5-Abs) and bSH3BP5-161 peptide (s-bSH3BP5-161-Abs) were significantly higher in samples from patients with DM than in those from HDs (Figures 2a and 2c). At a cutoff value equivalent to the average plus two standard deviations (SDs) of the HD specimen values, the s-SH3BP5-Ab positive rates in HDs and patients with DM were 3.7% and 22.2%, respectively, and the s-bSH3BP5-161-Ab positive rates were 6.2% and 27.6%, respectively (Table 1). ROC analysis was performed to evaluate the abilities of these antibody markers to indicate the presence of DM. The areas under the ROC curves (AUCs) for s-SH3BP5-Abs and s-bSH3BP5-161-Abs were 0.722 and 0.702, respectively, yielding sensitivity and specificity values of 53.5% and 83.5%, respectively, for s-SH3BP5-Abs (Figure 2b), and 53.1% and 81.2%, respectively, for s-bSH3BP5-161-Abs for the diagnosis of DM (Figure 2d). The similar values obtained for these markers suggest that the epitope domain between amino acid positions 161 and 174 of SH3BP5 represents the overall reactivity of the whole protein against serum antibodies.

### Elevation of levels of s-SH3BP5-Abs and s-bSH3BP5-161-Abs in patients with TIA and CI

We then examined the levels of s-SH3BP5-Abs and s-bSH3BP5-161-Abs in the sera of patients with TIA or aCI. Sera of HD were obtained from Port Square Kashiwado Clinic, and compared with those of patients with TIA and aCI obtained from Chiba Prefectural Sawara Hospital, Chiba Rosai Hospital, Chiba Aoba Municipal Hospital, and Chiba Medical Center. The AlphaLISA results demonstrated that levels of both s-SH3BP5-Ab and s-bSH3BP5-161-Ab were significantly



**Figure 1:** Representative peptide array. Cellulose membranes were loaded with six peptides derived from C9orf156 or SH3BP5 proteins and treated with sera from a healthy donor (HD, #1) or a patient with atherosclerosis (P, #7); Antibodies binding to peptide spots were detected by treatment with FITC-conjugated anti-human IgG antibody, followed by imaging with a fluorescence laser scanner. The arrow indicates the position of a positive signal observed only using the patient's serum.

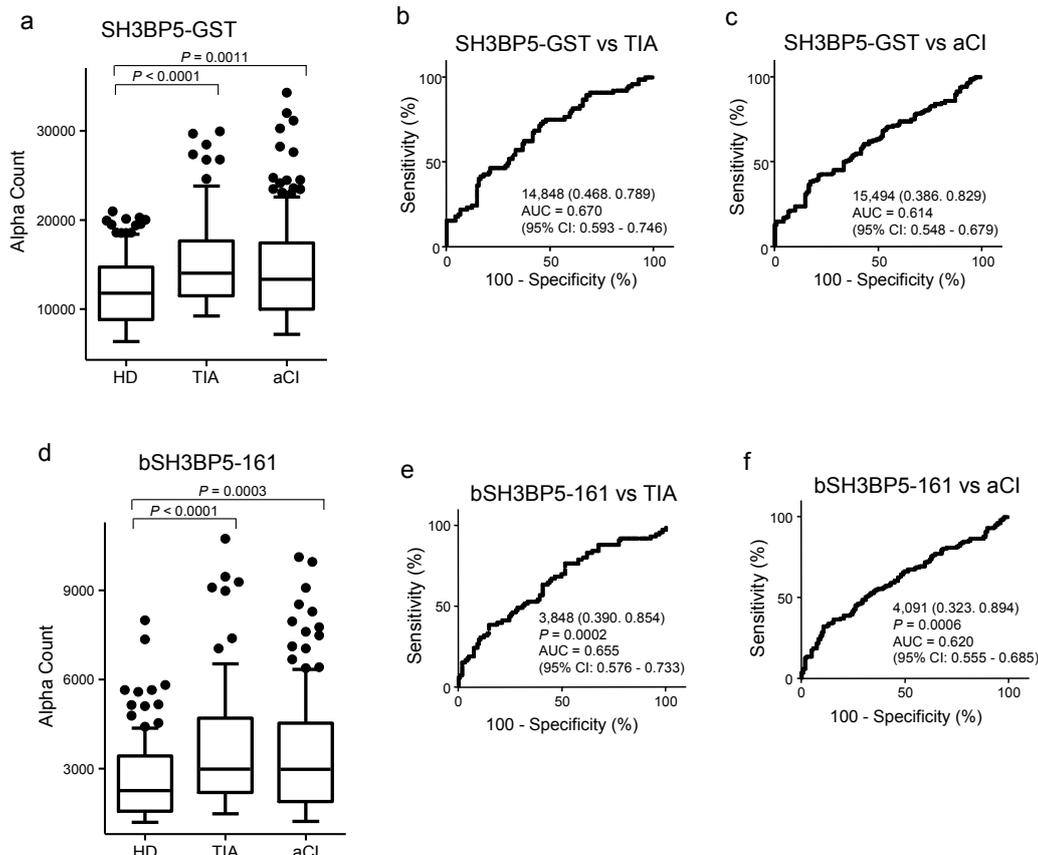


**Figure 2:** Comparison of serum SH3BP5-Ab levels between HDs and patients with diabetes mellitus (DM); GST-SH3BP5 protein (a) and bSH3BP5-161 peptides (c) were used as the antigens; AlphaLISA-determined serum antibody levels after subtraction of the levels against those of control GST are shown as box-whisker plots displaying the 10th, 20th, 50th, 80th, and 90th percentiles; The total (male/female) numbers, average values, standard deviations, cutoff values, positive numbers, positive rates (%), and *P* values obtained using the Mann-Whitney U test are summarized and shown in Table 1; Receiver operating characteristic curve (ROC) analysis was performed to assess the abilities of s-SH3BP5-Abs (b) and s-bSH3BP5-161-Abs (d) to detect DM; Numbers in the figures indicate the cutoff values for marker levels, and numbers in parentheses indicate sensitivity (left) and specificity (right); areas under the curve (AUC), and 95% confidence intervals (CI) are also shown. *P* values calculated using ROC analysis are shown in a and c.

higher in patients with TIA or aCI than in HDs (Figures 3a and 3d). Using cut-off values determined as described in the previous section, the s-SH3BP5-Ab positivity rates in HDs, patients with TIA and those with aCI were found to be 0.8%, 15.6%, and 15.2%, respectively, and the s-bSH3BP5-161-Ab positivity rates were found to be 4.9%, 18.2%, and 15.8%, respectively (Table 2). ROC analysis revealed that AUCs of s-SH3BP5-Abs and s-bSH3BP5-161-Abs were 0.670 [95% confidence interval (CI)=0.593–0.746] and 0.655 (95% CI=0.576–0.733), respectively, for TIA (Figures 3b and 3e), and 0.614 (95% CI=0.548–0.679) and 0.620 (95% CI=0.555–0.685), respectively, for aCI (Figures 3c and 3f). In other words, SH3BP5 antibodies were useful as diagnostic markers not only of aCI, but also of TIA.

### Association of s-SH3BP5-Abs and s-bSH3BP5-161-Abs levels with CVD and OSA

Next, we examined the antibody levels in samples from patients with CVD and OSA. CVD included acute myocardial infarction and unstable angina, obtained from Chiba University Hospital and Kyoto University Hospital. Because OSA is related to atherosclerosis and is associated with high risks of CI and CVD [33–36], we also examined the sera of patients with OSA, obtained from Chiba University Hospital. Compared with HDx, the levels of s-SH3BP5-Abs were higher in patients with CVD but not in those with OSA (Figure 4a), on the other hand, the levels of s-bSH3BP5-161-Abs were significantly higher in



**Figure 3:** Comparison of serum SH3BP5-Ab levels between HDs and patients with transient ischemic attack (TIA) or acute cerebral infarction (aCI). GST-SH3BP5 protein (a) and bSH3BP5-161 peptides (d) were used as antigens. Serum antibody levels were determined by AlphaLISA and are shown as box-whisker plots, as described in the legend of Figure 2. The same results are summarized in Table 2. Responses to s-SH3BP5-Abs (b and c) and s-bSH3BP5-161-Abs (e and f) were also evaluated using ROC analysis, and the numbers in figures are as described in the legend of Figure 2.

Sample information	HD	DM
Total sample number	81	275
Male/Female	46/35	158/117
Type 1 DM/Type 2 DM	0/0	26/216
Age (Average ± SD)	45.20 ± 10.95	63.12 ± 12.04

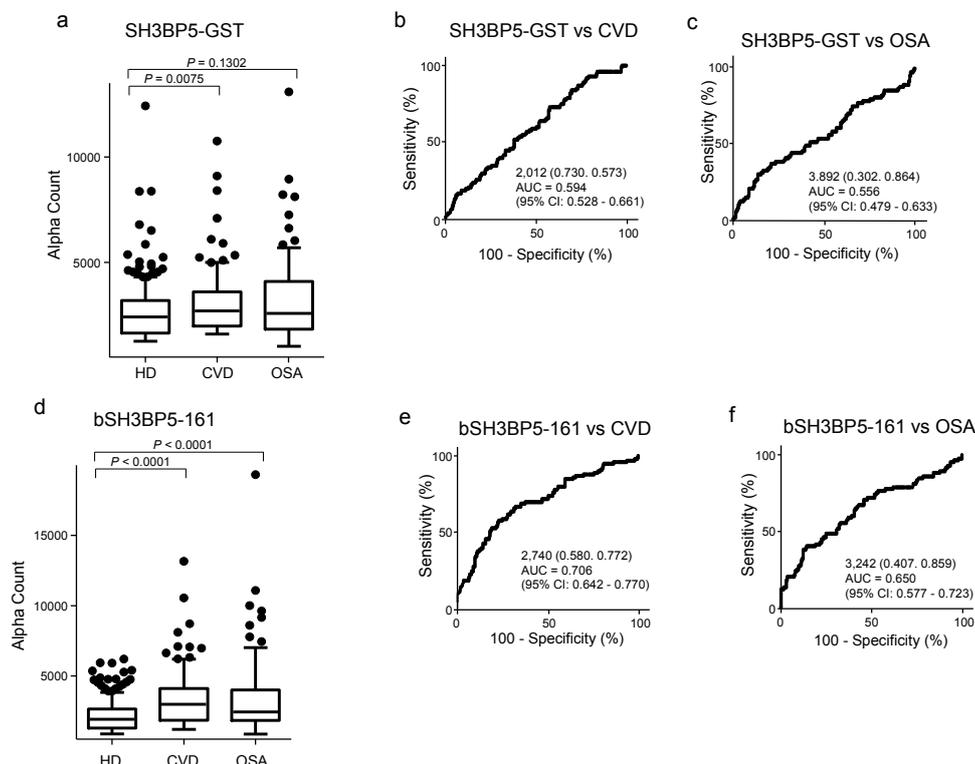
Alpha analysis (antibody level)	SH3BP5-GST	bSH3BP5-161
HD Average	1,850	1,387
SD	955	737
Cutoff value	3,760	2,861
Positive No.	3	5
Positive rate (%)	3.7%	6.2%
DM Average	3,087	2,428
SD	2,244	2,202
Positive No.	61	76
Positive rate (%)	<b>22.2%</b>	<b>27.6%</b>
<i>P</i> value (vs HD)	<b>&lt; 0.0001</b>	<b>&lt; 0.0001</b>

**Table 1:** Comparison of the serum antibody levels of healthy donors (HDs) vs those of patients with diabetes mellitus (DM). The upper panel indicates the numbers of total samples, samples from male and female subjects, and samples from patients with type 1 and type 2 DM as well as ages (average ± standard deviation (SD)). The lower panel summarizes the serum antibody levels (Alpha count) examined by AlphaLISA. Purified SH3BP5-GST protein and synthetic bSH3BP5-161 peptide were used as antigens; cutoff values were determined as the average HD values plus two SD, and positive samples for which the Alpha counts exceeded the cutoff value were scored. *P* values were calculated using the Mann-Whitney U test; *P* values <0.05 and positive rates >10% are marked in bold. Box-whisker plots of the same results are shown in Figure 2.

patients with CVD or OSA than in HDs (Figure 4d). The s-SH3BP5-Ab positivity rates in HDs, patients with CVD and those with OSA were 3.9%, 6.0% and 10.5%, respectively, and the s-bSH3BP5-161-Ab positivity rates were 1.5%, 19.0% and 20.9%, respectively (Table 3). ROC analysis revealed that AUCs of s-SH3BP5-Abs and s-bSH3BP5-161-Abs for CVD were 0.594 (95% CI: 0.528–0.661) (Figure 4b) and 0.706 (95% CI: 0.642–0.770), respectively (Figure 4e), and those for OSA were 0.556 (95% CI: 0.479–0.633) (Figure 4c) and 0.650 (95% CI: 0.577–0.723), respectively (Figure 4f). Despite the high AUC value of s-SH3BP5-Ab for OSA, the *P* values (calculated using the Mann-Whitney U test) of s-SH3BP5-Abs and s-bSH3BP5-161-Abs for OSA were 0.043, and 0.054, respectively, suggesting weaker association of these antibody markers with OSA than with CVD (Table 3).

### Elevation of levels of s-SH3BP5-Abs and s-bSH3BP5-161-Abs in patients with CKD

We examined antibody levels in the sera of patients with CKD, which is also closely related to atherosclerosis. CKD was divided into three groups as follows: type 1, diabetic kidney disease; type 2, nephrosclerosis; and type 3, glomerulonephritis. Samples from patients with CKD were obtained from the Kumamoto cohort, and those from HDs from Chiba University Hospital. Patients from all three groups of CKD had significantly higher serum levels of s-SH3BP5-Abs and s-bSH3BP5-161-Abs than HDs (Figures 5a and 5e). The s-SH3BP5-Ab positivity rates in HDs and patients with CKD type 1, type 2



**Figure 4:** Comparison of serum SH3BP5-Ab levels between HDs and patients with cardiovascular disease (CVD) or obstructive sleep apnea (OSA). GST-SH3BP5 proteins (a) and bSH3BP5-161 peptides (d) were used as antigens. Serum antibody levels were examined by AlphaLISA and are shown as box-whisker plots, as described in the legend of Figure 2. The same results are summarized in Table 3. Responses to s-SH3BP5-Abs (b and c) and s-bSH3BP5-161-Abs (e and f) were also evaluated using ROC analysis as described in the legend of Figure 2.

Sample information	HD	TIA	aCI
Total sample number	123	77	158
Male/Female	85/38	49/28	120/38
Age (Average ± SD)	51.85 ± 8.74	69.60 ± 11.74	57.67 ± 7.61

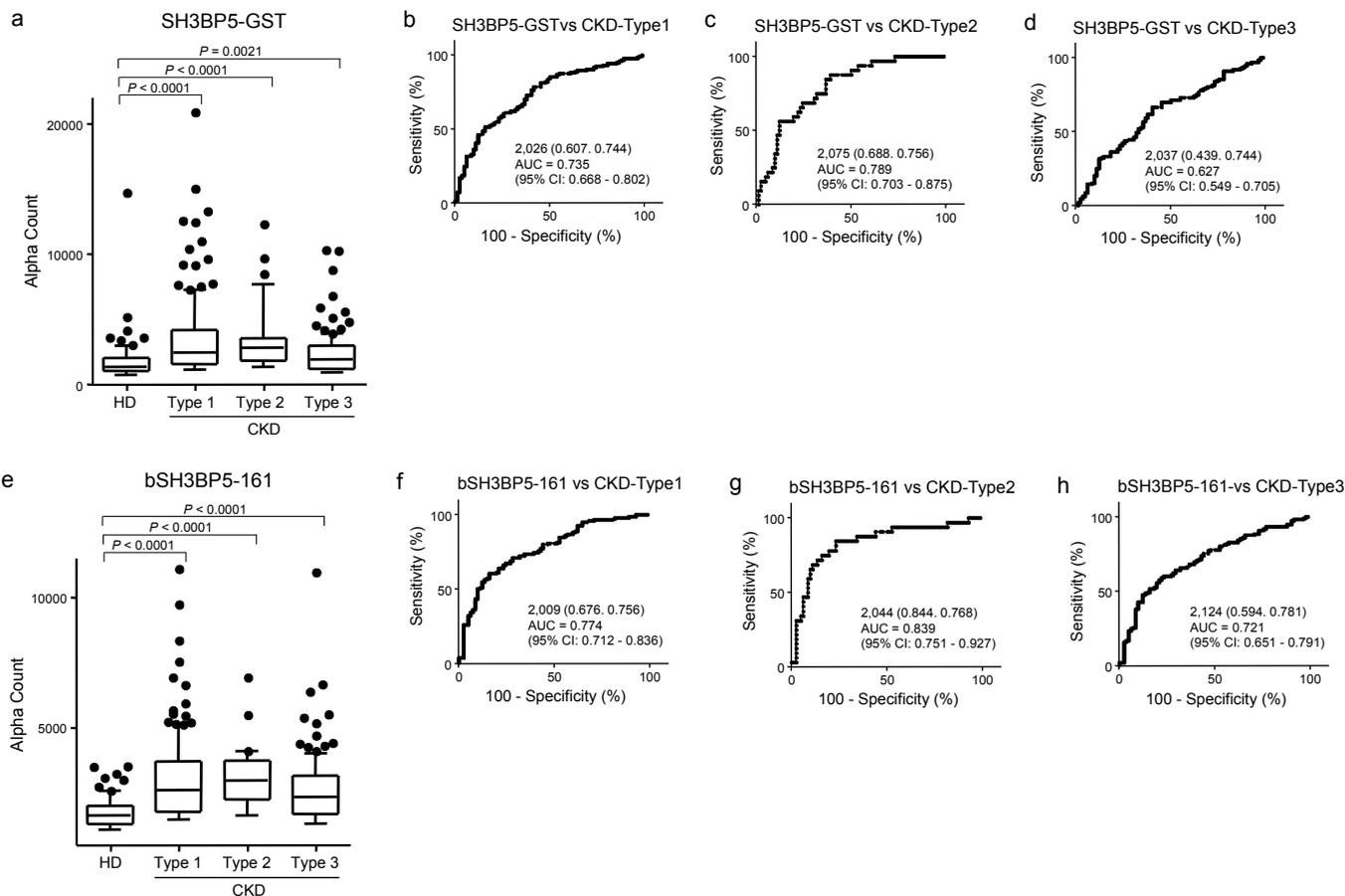
Sample information	HD	CVD	OSA
Total sample number	206	100	86
Male/Female	125/81	85/15	60/26
Age (Average ± SD)	57.61 ± 5.96	66.01 ± 11.48	57.78 ± 12.49

Alpha analysis (antibody level)	SH3BP5-GST	bSH3BP5-161
HD Average	11,820	2,575
SD	4,336	1,374
Cutoff value	20,492	5,323
Positive No.	1	6
Positive (%)	0.8%	4.9%
TIA Average	15,188	3,641
SD	5,693	2,217
Positive No.	12	14
Positive (%)	<b>15.6%</b>	<b>18.2%</b>
P value (vs HD)	<b>&lt; 0.0001</b>	<b>0.0002</b>
aCI Average	14,285	3,485
SD	5,901	2,270
Positive No.	24	25
Positive (%)	<b>15.2%</b>	<b>15.8%</b>
P value (vs HD)	<b>0.0011</b>	<b>0.0006</b>

Alpha analysis (antibody level)	SH3BP5-GST	bSH3BP5-161
HD Average	2,628	2,135
SD	1,435	1,173
Cutoff value	5,497	4,481
Positive No.	8	3
Positive (%)	3.9%	1.5%
CVD Average	3,092	3,390
SD	1,666	2,124
Positive No.	6	19
Positive (%)	6.0%	<b>19.0%</b>
P value (vs HD)	<b>0.0019</b>	<b>0.0009</b>
OSA Average	3,124	3,403
SD	2,074	2,873
Positive No.	9	18
Positive (%)	<b>10.5%</b>	<b>20.9%</b>
P value (vs HD)	<b>0.043</b>	<b>0.054</b>

**Table 2:** Comparison of the serum antibody levels of HDs vs those of patients with transient ischemic attack (TIA) or acute-phase cerebral infarction (aCI). Upper panel indicates the numbers of total samples and samples from male and female samples as well as ages (average ± SD). The lower panel summarizes the serum antibody levels examined by AlphaLISA using purified SH3BP5-GST protein and synthetic bSH3BP5-161 peptide as antigens as described in the legend of Table 1. Box-whisker plots of the same results are shown in Figure 3.

**Table 3:** Comparison of the serum antibody levels of HDs vs those of patients with cardiovascular disease (CVD) or obstructive sleep apnea (OSA). The upper panel indicates the numbers of total samples and samples from male and female samples as well as ages (average ± SD). The lower panel summarizes the serum antibody levels examined by AlphaLISA using purified SH3BP5-GST protein and synthetic bSH3BP5-161 peptide as antigens as described in the legend of Table 1. Box-whisker plots of the same results are shown in Figure 4.



**Figure 5:** Comparison of serum SH3BP5-1-Ab levels between HDs and patients with chronic kidney disease (CKD). Serum antibody levels against GST-SH3BP5 protein (a) and bSH3BP5-161 peptides (e) were compared between HDs and patients with CKD types 1, 2, and 3. *P* values of CKD types 1, 2, and 3 versus HD controls are shown. Results are presented as described in the legend of Figure 2. *P* values vs. HD specimens are shown. Averages, SDs, cutoff values, total numbers, positive numbers, positive rates (%), and *P* values are shown in Table 4. The abilities of s-SH3BP5-Abs (b–d) and s-bSH3BP5-161-Abs (f–h) to detect CKD were also evaluated using receiver operating characteristic curve analysis.

and type 3 were 2.4%, 15.2%, 12.5%, and 4.1%, respectively, and the s-bSH3BP5-161-Abs positivity rates were 2.4%, 25.5%, 28.1%, and 13.0%, respectively (Table 4). In other words, strong positive rates were observed in patients with type 1 and type 2 CKD. ROC analysis revealed AUCs of s-SH3BP5-Abs and s-bSH3BP5-161-Abs to be 0.789 (95% CI: 0.703–0.875) (Figure 5c) and 0.839 (95% CI: 0.751–0.927) (Figure 5g), respectively, for CKD type 2, which are the highest values observed to date. CKD type 3 exhibited much weaker associations with s-SH3BP5-Abs and s-bSH3BP5-161-Abs.

### No close correlation between levels of s-bSH3BP5-161-Abs and cancer

Because autologous antibodies frequently develop in patients with cancer [17], we examined the samples from patients with esophageal SCC or colon carcinoma, from Toho University Hospital. Notably, the levels of s-SH3BP5-Abs were significantly higher in samples from patients with esophageal SCC and colon carcinoma than in those from HDs, who had positive rates of <10% (Supplementary Table S1). Although the s-bSH3BP5-161-Ab levels were slightly elevated in samples from patients with esophageal SCC with a positive rate of 10.9% (*P*=0.011), no significance was observed between HDs and patients with colon carcinoma (*P*=0.081).

### Correlation analysis

Correlation analysis of s-bSH3BP5-161-Ab levels and subject data was performed using 665 specimens from Chiba Prefectural Sawara Hospital, including 139 specimens from HDs, 122 from patients with deep and subcortical white matter hyperintensity (DSWMH), 17 from patients with asymptomatic CI, 44 from patients with TIA, 225 from patients with aCI, 59 from patients with chronic-phase CI (cCI), and 41 from disease controls. In this analysis, using the Mann–Whitney U test, s-bSH3BP5-161-Ab levels were compared between male and female subjects; with or without present aCI, TIA, cCI, and DSWMH; with or without diseases of DM, hypertension (HT), CVD, and dyslipidemia; and with or without smoking and alcohol intake habits. Notably, significant differences were observed in the following comparisons: aCI vs. HD, cCI vs. HD, with vs. without HT, and smoker vs. nonsmokers (Table 5). Although the levels of s-SH3BP5-Abs were significantly higher in samples from patients with TIA than in those from HDs (*P*=0.0002) (Table 2), the difference of those between HDs and patients with TIA was not significant (*P*=0.1155). This was probably because to the smaller number of samples than those in the analyses in Table 2.

Spearman correlation analysis was performed to determine the correlation between the s-bSH3BP5-161-Ab levels and subject

Sample information		HD	Type 1 CKD	Type 2 CKD	Type 3 CKD
Total sample number		82	145	32	123
Male/Female		44/38	106/39	21/11	70/53
Age (Average $\pm$ SD)		44.10 $\pm$ 11.19	66.04 $\pm$ 10.38	76.03 $\pm$ 9.78	61.98 $\pm$ 11.69

Alpha analysis (antibody level)		SH3BP5-GST	bSH3BP5-161
HD	Average	1,839	1,839
	SD	1,887	902
	Cutoff value	5,614	3,644
	Positive No.	2	2
	Positive rate (%)	2.4%	2.4%
Type 1 CKD	Average	3,467	3,011
	SD	3,094	1,660
	Positive No.	22	37
	Positive (%)	<b>15.2%</b>	<b>25.5%</b>
	<i>P</i> value (vs HD)	<b>&lt; 0.0001</b>	<b>&lt; 0.0001</b>
Type 2 CKD	Average	3,392	3,052
	SD	2,530	1,183
	Positive No.	4	9
	Positive (%)	<b>12.5%</b>	<b>28.1%</b>
	<i>P</i> value (vs HD)	<b>&lt; 0.0001</b>	<b>&lt; 0.0001</b>
Type 3 CKD	Average	2,335	2,602
	SD	1,692	1,347
	Positive No.	5	16
	Positive (%)	4.1%	<b>13.0%</b>
	<i>P</i> value (vs HD)	<b>0.0021</b>	<b>&lt; 0.0001</b>

**Table 4:** Comparison of serum antibody levels of HDs vs those of patients with chronic kidney disease (CKD). CKD types 1, 2, and 3 correspond to diabetic kidney disease, nephrosclerosis, and glomerulonephritis, respectively. The upper panel indicates the numbers of total samples and samples from male and female samples as well as age (average  $\pm$  SD). The lower panel summarizes the serum antibody levels examined by AlphaLISA using purified SH3BP5-GST protein and synthetic bSH3BP5-161 peptide as antigens as described in the legend of Table 1. Box-whisker plots of the same results are shown in Figure 5.

parameters including general information such as age, body height, weight, body mass index (BMI), and blood pressure; degree of artery stenosis, including the maximum intima-media thickness (max IMT); and life style factors such as smoking duration (year) and alcohol intake frequency (times/week). The following previously described blood test data were also included; low-density lipoprotein cholesterol (LDL-C), alkaline phosphatase (ALP), total cholesterol, chlorine, high-density lipoprotein cholesterol, potassium, creatinine,  $\gamma$ -GTP, uric acid, HbA1c, albumin, total protein, sodium, ALT, triglyceride, aspartate aminotransferase, cholinesterase, blood urea nitrogen, total bilirubin, blood sugar (BS), and lactate dehydrogenase levels; estimated glomerular filtration ratio; and albumin/globulin ratio [26-28]. The average values and SDs of these parameters are shown in Supplementary Table S2. The s-bSH3BP5-161-Ab level was found to significantly correlate with HT (blood pressure), artery stenosis (max IMT), smoking duration and age (Table 6). The results also indicated a weak association of antibody levels with ALP but an inverse correlation with LDL-C, suggesting that s-bSH3BP5-161-Ab distinguishes atherosclerotic CI accompanied by hypertension and/or smoking.

## Discussion

Our initial ProtoArray screening identified SH3BP5 as an antigen,

as recognized by serum IgG in patients with atherosclerosis, and subsequent peptide array analysis identified a possible epitope site between the amino acid positions of 161 and 175 in SH3BP5 (Figure 1). Subsequent analyses demonstrated higher levels of serum antibodies against the SH3BP5 protein and bSH3BP5-161 peptide in patients with DM, aCI, cCI, TIA, CVD, and CKD than in HDs (Figures 2-5 and Tables 1-5). Although the antibody levels against the SH3BP5 protein were very similar to those against the SH3BP5 peptide, it cannot be ruled out that serum antibodies against other protein(s) cross-reacted with the SH3BP5 peptide.

SH3BP5 was first identified as a protein that could bind the SH3 domain of, and thus inhibit, Bruton tyrosine kinase (BTK) [37,38]. SH3BP5 is necessary for JNK-mediated induction of lipotoxicity and nonalcoholic steatohepatitis (NASH) [39], both of which are thought to closely correlate with atherosclerosis, as suggested by the involvement of common mediators such as macrophages, nuclear factor (erythroid-derived 2)-like 2, and oxidative stress [40-42]. Notably, the detected BTK-binding region of SH3BP5 was located within amino acids 163-193 [37], which overlaps with bSH3BP5-161, a peptide of SH3BP5 consisting of amino acids 161-174. This finding suggests that the SH3 domain-binding biological function of SH3BP5 could be affected by autoantibody binding.

Present disease		HD	aCI	cCI	TIA	DSWMH
Sample number		139	228	59	44	122
Alpha count	Average	348.1	461.5	510.9	467.5	341.2
(Antibody level)	SD	268.3	333.3	375.1	472.5	274.1
<i>P</i> value (vs HD)		-	<b>0.0006</b>	<b>0.0012</b>	0.1155	0.7548

Other disease		DM-	DM+
Sample number		525	135
Alpha count	Average	409.1	418.2
(Antibody level)	SD	329.6	312.9
<i>P</i> value (vs DM-)			0.4860

Other disease		HT-	HT+
Sample number		239	421
Alpha count	Average	370.2	434.1
(Antibody level)	SD	305.3	335.4
<i>P</i> value (vs HT-)			<b>0.0007</b>

Other disease		CVD-	CVD+
Sample number		623	37
Alpha count	Average	405.1	509.8
(Antibody level)	SD	319.5	414.1
<i>P</i> value (vs CVD-)			0.0938

Other disease		Dyslipidemia-	Dyslipidemia+
Sample number		475	185
Alpha count	Average	414.5	401.9
(Antibody level)	SD	337.7	294.9
<i>P</i> value (vs Lipidemia-)			0.8324

Sex		Male	Female
Sample number		395	270
Alpha count	Average	410.2	414.1
(Antibody level)	SD	315.1	343.4
<i>P</i> value (vs Male)			0.6773

Life style		Nonsmoker	Smoker
Sample number		344	319
Alpha count	Average	386.6	438.7
(Antibody level)	SD	323.4	329.1
<i>P</i> value (vs nonsmoker)			<b>0.0175</b>

Life style		Alcohol-	Alcohol+
Sample number		238	419
Alpha count	Average	406.0	418.8
(Antibody level)	SD	330.3	325.8
<i>P</i> value (vs Alcohol-)			0.5304

**Table 5:** Correlation analysis of s-bSH3BP5-161-Ab levels with data of subjects in the Sawara Hospital cohort. The subjects were divided as follows: sex (male and female); presence (+) or absence (-) of complication of DM, hypertension (HT), CVD, or dyslipidemia, and life style factors (smoking and alcohol intake habits). Antibody levels (Alpha counts) were compared using the Mann-Whitney U test; Sample numbers, averages and SD of counts as well as *P* values are shown. Significant correlations (*P*<0.05) are marked in bold text.

Given the strong associations of the levels of s-SH3BP5-Abs and s-bSH3BP5-161-Abs with DM (Figure 2), we further speculated a potential association with CKD type 1 (diabetic kidney disease). However, CKD type 2 (nephrosclerosis) was found to associate even more strongly with these markers than CKD type 1 (Figure 5). Spearman correlation analysis revealed a significant association between s-bSH3BP5-161-Ab levels and max IMT, which reflects arterial

stenosis (Table 6). Therefore, the SH3BP5 antibody marker could be used to identify atherosclerosis, rather than DM. Consistent with this finding; we did not observe significant correlations between BS and HbA1c levels (Table 6) or complications of DM and s-bSH3BP5-161-Ab levels (Table 5). However, the antibody levels significantly correlated with HT and habitual smoking (Tables 5 and 6), which are well-known risk factors for atherosclerosis [43]. According to ROC analysis, the

	Subjects' information	Abbreviation	Spearman	
			r value	P value
General	Age		0.123	<b>0.0016</b>
	Body height	Height	-0.070	0.0710
	Body weight	Weight	-0.037	0.3361
	Body mass index	BMI	0.008	0.8336
	Blood pressure	BP	0.121	<b>0.0023</b>
Artery stenosis	Maximum intima-media thickness	max IMT	0.159	<b>0.0007</b>
Life style	Smoking habit period		0.135	<b>0.0005</b>
	Alcohol intake frequency		0.070	0.0737
Blood test	LDL-cholesterol	LDL-C	-0.108	<b>0.0449</b>
	Alkaline phosphatase	ALP	0.082	<b>0.0458</b>
	Total cholesterol	T-CHO	-0.068	0.1047
	Chlorine	Cl	0.056	0.1536
	HDL-cholesterol	HDL-c	-0.053	0.2696
	Potassium	K	0.030	0.4415
	Creatinin	CRE	0.030	0.4447
	Gamma-glutamyl transpeptidase	-GTP	0.030	0.4660
	Uric acid	UA	0.024	0.5948
	Hemoglobin A1c	HbA1c	0.023	0.6079
	Albumin	ALB	-0.018	0.6444
	Total Protein	TP	-0.016	0.6827
	Sodium	Na	0.014	0.7159
	Alanine aminotransferase	ALT (GPT)	0.012	0.7503
	Triglyceride	TG	0.013	0.7872
	Estimated glomerular filtrating ratio	eGFR	-0.011	0.8026
	Aspartate aminotransferase	AST (GOT)	0.008	0.8330
	Cholinesterase	CHE	-0.005	0.9054
	Blood urea nitrogen	BUN	-0.003	0.9349
	Total bilirubin	tBil	-0.002	0.9609
Blood sugar	BS	-0.001	0.9761	
Lactate dehydrogenase	LDH	0.000	0.9932	
Albumin/globulin ratio	A/G	0.000	0.9967	

**Table 6:** Correlation analysis of s-bSH3BP5-161-Ab levels with data on subjects in the Sawara Hospital cohort. Correlation coefficients (*r* values) and *P* values obtained through Spearman's correlation analysis are shown. Significant correlations (*P*<0.05) are marked in bold text.

sensitivity of s-bSH3BP5-161-Abs varied from 40.7% (OSA, Figure 4f) to 84.4% (type 2 CKD, Figure 5g). Therefore, this antibody marker may identify a certain type of atherosclerosis caused by HT and/or smoking habit.

The levels of s-SH3BP5-Abs and s-bSH3BP5-161-Abs were higher in the sera of patients with esophageal SCC or colon carcinoma than in those of HDs, although the positivity rates (<11%; Supplementary Table S1) were lower than those of patients with DM and CKD (>20%; Tables 1 and 4). This result may be explained by the fact that many types of cancers are attributable to obesity, the complications of which include DM, CVD, CKD, and stroke [44,45]. Furthermore, adiponectin which are negatively correlated with obesity and atherosclerosis [46] was negatively associated with the onset of KRAS-mutant colorectal cancer atherosclerosis [47]. Thus, atherosclerosis can be one of the causes of a certain part of cancers, which may be responsible to SH3BP5 antibody marker. Patients with OSA also exhibited higher SH3BP5 antibody levels than HDs (Table 3). OSA is accompanied by the high risk of aCI and CVD [33-36,48] which may be mediated by atherosclerosis discriminated by SH3BP5 antibody marker.

In most cases, aCI is not induced suddenly but is frequently mediated by premonitory phenomena such as TIA and asymptomatic

CI. Each premonitory phenomenon may induce low-level tissue destruction and subsequent leakage of intracellular proteins, leading to amplified antibody expression and very low antigen levels after each event. The resulting highly sensitive antibody markers could therefore be used for early, and even pre-onset, detection. We observed higher levels of both s-SH3BP5-Abs and s-bSH3BP5-161-Abs in patients with TIA than in HDs (Figure 3). Occasionally, TIA is not recognized by the affected patients; yet treatment at an early stage of TIA can prevent the onset of otherwise fatal aCI. Therefore, the detection of some, if not all, cases of TIA using this SH3BP5 antibody marker may represent a significant contribution to preventive healthcare.

## Conclusion

Serum anti-SH3BP5 antibody markers appear to be useful for the diagnosis of DM, aCI, TIA, CVD, and CKD, and may reflect the progress of atherosclerosis.

## 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.

## Acknowledgments

The authors thank Prof. Masaki Takiguchi (Department of Biochemistry and Genetics, Graduate School of Medicine, Chiba University) for the valuable discussion and Dr. Sho Takahashi (Clinical Research Center, Chiba University Hospital) for the support on statistical analysis.

This work was supported, in part, by research grants from the Japan Agency for Medical Research and Development (AMED) (Practical Research Project for Life-Style related Diseases including Cardiovascular Diseases and Diabetes Mellitus), Japan Science and Technology Agency (JST), and the Ministry of Education, Culture, Sports, Science and Technology (MEXT) in Japan.

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