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Research Article

An Anti-Deoxyhypusine Synthase Antibody as a Marker of Atherosclerosis-Related Cerebral Infarction, Myocardial Infarction, Diabetes Mellitus, and Chronic Kidney Disease

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

Abstract

Background: Atherosclerosis increases the risk of acute-phase CI (aCI) and acute myocardial infarction (AMI), which can be life threatening. Atherosclerosis is also closely related to diabetes mellitus (DM) and chronic kidney disease (CKD). Novel biomarkers are needed to follow the progress of atherosclerosis.

Methods and Results: Screening by protein arrays identified deoxyhypusine synthase (DHPS) as an antigen recognized by IgG antibodies in sera of patients with both atherosclerosis and aCI and the antibody was detected in Western blots. The serum antibody levels in healthy donors (HDs) and patients with aCI, transient ischemic attack (TIA), DM, AMI, or CKD were determined by enzyme-linked immunosorbent assay (ELISA) and amplified luminescent proximity homogeneous assay (Alpha) LISA using recombinant DHPS protein. Serum DHPS antibody levels were significantly higher in patients with any of these diseases than in HDs. The difference was greatest in patients with DM and CKD. DHPS antibody levels were well correlated with artery stenosis and the presence of hypertension. The serum level of this DHPS antibody may reflect the extent of atherosclerosis caused by DM, CKD and/or hypertension.

Conclusions: Serum antibody levels against DHPS may be useful in diagnosing atherosclerosis and associated aCI, TIA, DM, AMI, and CVD.

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Abbreviations aCI: acute-phase cerebral infarction; ALB: albumin; ALP: alkaline phosphatase; AlphaLISA: amplified luminescence proximity homogeneous assay-linked immunosorbent assay; ALT: alanine aminotransferase; AMI: acute myocardial infarction; AMY: amylase; AST: aspartate aminotransferase; asympt-CI: asymptomatic cerebral infarction; BMI: body mass index; BS: blood sugar; BUN: blood urea nitrogen; cCI: chronic cerebral infarction; CHE: cholinesterase; CI: cerebral infarction; CKD: chronic kidney disease; CRE: creatinine; CRP: C-reactive protein; CVD: cardiovascular disease; DHPS: deoxyhypusine synthase; DM: diabetes mellitus; eGFR: estimated glomerular filtration ratio; ELISA: enzyme-linked immunosorbent assay; eso-SCC: esophageal squamous cell carcinoma; *E. coli: Escherichia coli*; gamma-GTP: gamma-glutamyltranspeptidase; GST: glutathione-s-transferase; HbA1c: glycated hemoglobin; HD: healthy donor; HDL-c: high-density lipoprotein cholesterol; HGB: hemoglobin; HT: hypertension; IMT: intima-media thickness; IP: inorganic phosphate; IPTG: isopropyl-β-D-thiogalactoside; LDH: lactate dehydrogenase; LDL-c: low-density lipoprotein cholesterol; ; max IMT: maximum intima-media thickness; OSA: obstructive sleep apnea; PBS: phosphate-buffered saline; PLT: platelet; RBC: red blood cell; ROC: receiver operating curve; SEREX: serological identification of antigens by recombinant cDNA expression cloning; s-DHPS-Abs: serum anti-DHPS antibodies; tBil: total bilirubin; T-CHO: total cholesterol; TG: triglyceride; TIA: transient ischemic attack; TP: total protein; UA: uric acid; UAP: unstable angina pectoris; WBC: white blood cell

Introduction

The effects of arteriosclerosis on arterial function leads to serious disorders such as cerebral infarction (CI), cardiovascular disease (CVD), and chronic kidney disease (CKD). These disorders are also associated with diabetes mellitus (DM), which can accelerate atherosclerosis. The clinical significance of atherosclerosis is the possibility of sudden death from acute-phase CI (aCI) or acute myocardial infarction (AMI) [1]. Prevention and control of atherosclerosis is important. The risk of atherosclerosis is increased by hypertension (HT), high body mass index (BMI), uric acid level, smoking, and family history, which may also indicate the presence of atherosclerosis [2,3]. Blood chemistry assays such as triglyceride (TG), low-density lipoprotein cholesterol (LDL-c), homocysteine [4], osteoprotegerin [5], and osteopontin [6] are better indicators of atherosclerosis. Serum autoantibodies, including phospholipid [7], apolipoprotein A-1 [8], oxidized low-density lipoprotein [9], and heat shock proteins (HSPs) [10] have been associated with CVD, HSP60 with stroke [11], and insulin [12], glutamic acid decarboxylase [13], and protein tyrosine phosphatase IA-2 with DM [14,15].

We previously performed a large scale screening of atherosclerosis markers by recombinant cDNA expression cloning (SEREX) of serum antigens and identified some serum antibody markers including RPA2 and SOSTDC1 for ischemic stroke [16,17], ATP2B4 and BMP-1 for atherosclerosis [18], and TUBB2C and adiponectin for DM [19,20].

In this study, we used a human protein microarray to identify autoantigens responsive to atherosclerosis and identified an anti-deoxyhypusine synthase (DHPS) antibody as a common atherosclerosis marker.

Materials and Methods

Patient and healthy donor sera

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

Sera of CI patients were obtained from Chiba Prefectural Sawara Hospital, Chiba Rosai Hospital and Chiba Aoba Municipal Hospital. Sera of patients with CVD, DM, obstructive sleep apnea (OSA), and glioma were obtained from Chiba University Hospital, and CKD sera were obtained from Kumamoto [21,22]. Sera of esophageal squamous cell carcinoma (eso-SCC) were obtained from Toho University Hospital; Healthy donor (HD) sera were obtained from Chiba University Hospital, Chiba Prefectural Sawara Hospital, and Port Square Kashiwado Clinic. HDs were participants recruited at Chiba Prefectural Sawara Hospital and Port Square Kashiwado Clinic during regular health checkups, including cranial magnetic resonance imaging, and were found to have no health problems.

Protein microarray screening

Screening was performed using the ProtoArray v-4.0 human protein microarray system (Thermo Fisher Scientific, Waltham, MA), which was loaded with 9,480 protein species. Ten serum samples, five from patients and five from HDs, were used as previously described to detect antigens recognized by specific IgG antibodies in the sera of patients with atherosclerosis [17].

Expression and purification of DHPS protein

Total RNA was isolated from human U2OS osteosarcoma cell using High Pure RNA IsolatioKits (Roche, Basel, Switzerland). Superscript III First-Strand Synthesis System for reverse transcription-polymerase chain reaction (RT-PCR) (Thermo Fisher Scientific) was used to synthesize cDNA. DHPS cDNA amplified by PCR using Pyrobest DNA polymerase (Takara Bio Inc., Shiga, Japan) was cloned into the EcoRI/XhoI site of pGEX-4T-1 (GE Healthcare Life Sciences, Pittsburgh, PA) and confirmed by DNA sequencing. Expression of the cDNA product was induced by treating transformed Escherichia coli containing pGEX-4T-1–DHPS with 0.1 mM isopropyl-β-D-thiogalactoside(IPTG) at 25°C for 4 hrs. The *E. coli* cells were then lysed in BugBuster Master Mix (Merck Millipore, Darmstadt, Germany). glutathione-S-transferase (GST)-tagged DHPS protein was purified by glutathione-Sepharose column chromatography following the manufacturer's instructions (GE Healthcare Life Sciences), and dialyzed against phosphate-buffered saline (PBS) as previously described [16,23,24].

DHPS cDNA was recombined into the prokaryotic expression vector, pET28, and the expression of His-tagged DHPS was induced by 0.1 mM IPTG at 37°C for 2 hrs followed by lysis in BugBuster Master Mix. The His-tagged protein was purified on ProBond Resin (Thermo Fisher Scientific) after solubilization of precipitated inclusion bodies in 8 M Urea.

Western blotting

Purified recombinant proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were then incubated with sera from patients, HDs, or goat anti-GST antibodies (Abcam, Cambridge, MA). After incubation with horseradish peroxidase (HRP)-conjugated secondary antibody, immunoreactivity was visualized as described previously using Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore) [16,23-25].

Enzyme-linked immunosorbent assay

For the enzyme-linked immunosorbent assay (ELISA), purified recombinant proteins diluted to 5 μg/ml in PBS were added to each well of a 96-well MaxiSorp plates (Thermo Fisher Scientific) and incubated at 4°C overnight. Wells were washed four times with 0.1% Tween-20 in PBS (PBS-T) and then blocked with 10% fetal bovine serum (FBS) in PBS (PBS-FBS) for 1 hrs. Wells were washed four times with PBS-T and then sera diluted in PBS-FBS (1:100) were added to the wells and incubated for 1 hrs. Wells were washed four times with PBS-T and incubated with HRP-conjugated anti-human IgG antibody (1:20,000 dilution in PBS-FBS, Abcam, Cambridge, England) for 45 min. After washing, peroxidase substrate (3,3′,5,5′-tetramethylbenzidine[TMB One], Promega, Madison, WI) was added and incubated for 5 min. The reaction was stopped by adding 2M sulfuric acid. Absorbance was measured at 450 nm using a microplate reader (Emax, Molecular Devices, Sunnyvale, CA). Each blood sample was tested in duplicate against GST-fusion proteins, as well as a GST control. After normalization against standards, the serum antibody level was calculated as previously described by subtracting the absorbance of serum against GST from that against GST-tagged antigens [16,23,24].

AlphaLISA

Amplified luminescent proximity homogeneous assay (Alpha)-LISA was performed in 384-well microtiter plates (white opaque ProxiPlate™, PerkinElmer, Waltham, MA) containing 2.5 μL of 1/100 diluted sera and 2.5 μL of GST or GST-DHPS proteins (10 μg/mL) in AlphaLISAImmunoAssay Buffer (25 mM HEPES, pH 7.4, 0.1% casein, 0.5% Triton X-100, 1mg/mL dextran-500, and 0.05% Proclin-300). The reaction mixture was incubated at room temperature for 6-8 hrs, after which anti-human IgG-conjugated acceptor beads (2.5 μL at 40 μg/mL) and glutathione-conjugated donor beads (2.5 μL at 40 μg/mL) were added and incubated at room temperature in the dark for 1-14 days. The chemical emission was read on an EnSpire Alpha microplate reader (PerkinElmer) as previously described [17-20]. Specific reactions were quantified by subtracting the alpha counts of the GST control from the counts of the GST-DHPS protein.

Statistical analysis

Student's t-test and the Mann-Whitney U test were used to determine the significance of the significance of between-group differences. Correlations were determined by chi-square tests, multivariate logistic regression analysis, and Spearman's correlation analysis. The predictive values of markers for diseases were assessed by receiver operating curve (ROC) analysis, and the cutoff values were chosen to maximize the sum of sensitivity and specificity. All statistical analyses were carried out using GraphPad Prism 5 (GraphPad Software, La Jolla, CA); P values < 0.05 were considered statistically significant.

Results

Identification of DHPS in sera of patients with atherosclerosis

A Proto array loaded with 9,480 protein species was used to select antigens recognized by serum antibodies. DHPS reacted with three of the five serum samples from patients with atherosclerosis, and none of five samples from HDs. GST-fusion DHPS and His-tag-conjugated DHPS proteins were expressed in *E. coli* and purified by affinity chromatography.

Anti-DHPS antibodies in sera of patients with atherosclerosis

Preliminary experiments suggested that the major epitope was located in the carboxy-terminal half of the full-length DHPS 369-amino-acid sequence. We therefore constructed an expression plasmid consisting of GST and the amino acids from positions 183 to 369. GST-DHPS and control GST proteins were electrophoresed and assayed by Western blotting. Both the GST and GST-DHPS (aa.186-369) proteins were recognized by the anti-GST antibody (Figure 1). Weak reactivity was observed at 35 kDa, which may be a degradation product produced during protein purification. Sera from two HDs (#193 and #256) reacted weakly with GST and GST-DHPS proteins, whereas GST-DHPS, but not GST, reacted strongly with sera of two patients with atherosclerosis, (#545 and #573). The 35-kDa protein was also recognized by patients' sera, suggesting that the main epitope was located in the amino-terminal region of DHPS (183-369). We designated the serum anti-DHPS antibodies as s-DHPS-Abs.

Elevated levels of s-DHPS-Abs in patients with acute-phase CI or TIA

We compared s-DHPS-Ab levels in sera of HDs and in patients with TIA or aCI, all of which were obtained from Chiba Prefectural Sawara Hospital. aCI sera were collected within 2 weeks of the onset of aCI. The levels of s-DHPS-Abs were significantly higher in patients with TIA or aCI than in the HDs (Figures 2a and 2c). ROC analysis revealed that the AUC values of TIA and aCI were 0.694 and 0.674, respectively (Figures 2b and 2d). At a cutoff value of 0.055, the

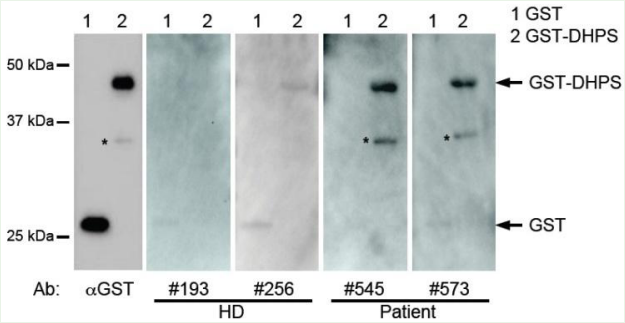

Figure 1: The presence of serum antibodies against DHPS antigenic protein. Representative results of Western blotting are shown. GST (lane 1) and GST-fusion DHPS protein (lane 2) were electrophoresed through SDS-polyacrylamide gels followed by Western blotting using goat anti-GST (αGST) and sera of healthy donors (HD) (#193 and #256) or patients with atherosclerosis (#545 and #573). Arrows indicate specific reactions to GST and GST-DHPS, and the asterisks represent degradation products after electrophoresis. Molecular weights are shown to the left.

sensitivity and specificity of s-DHPS-Abs for TIA were 61.4% and 73.5%, respectively, whereas the corresponding values for aCI were 56.9% and 77.8%, respectively, with cutoff value of 0.090. Similar sensitivity for TIA and aCI suggest that s-DHPS-Abs may become elevated at an early stage of CI.

We determined the serum antibody levels by AlphaLISA using a greater number of specimens. The average ages (± SDs) of HDs, TIA, and aCI patients were 51.80 ± 12.95, 68.45 ± 12.14, and 77.09 ± 11.02 years, respectively. The levels of s-DHPS-Abs were significantly higher in patients with aCI than in HDs (Figure 3a, Table 1). At a cutoff value calculated as the average HD value + 2SDs, the seropositivity rate of TIA was 18.2% and that of aCI was 21.5%, with *P* values of 0.0024 for both (Table 1). ROC analysis indicated AUC values of 0.666 for TIA and 0.659 for aCI (Figures 3b and 3c). At a cutoff value that maximized the sum of sensitivity and specificity, the sensitivity of s-DHPS-Abs for TIA was 52.3% and the specificity was 76.3%. The corresponding sensitivity and specificity for aCI were 52.2% and 74.8%. These results were similar to those obtained with ELISA.

Elevated s-DHPS-Abs levels in patients with AMI or DM

AMI and DM are also associated with atherosclerosis. Therefore, we assayed s-DHPS-Abs in patients with AMI, DM, and HDs. Sera of AMI patients were obtained at Kyoto University Hospital, DM sera were obtained at from Chiba University Hospital, and HD sera were

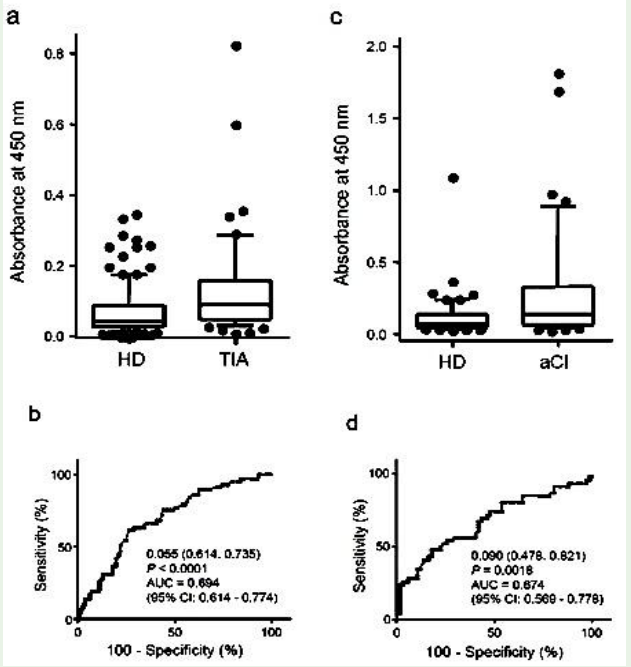

Figure 2: The levels of s-DHPS-Abs in HDs and patients with transient ischemic attack (TIA) or acute-phase cerebral infarction (aCI) determined by ELISA. HD versus TIA (a) and HD versus aCI(c). Serum antibody levels calculated by subtraction of the level against the GST control are shown in absorbance at 450 nm using a box-whisker plot. The box plots show the 10th, 20th, 50th, 80th and 90th percentiles of the antibody level. P values calculated by the Mann-Whitney U test are shown. Receiver operating characteristic curve (ROC) analysis assessed the ability of s-DHPS-Abs to detect TIA (b) and aCI (d). Numbers to the right of the curve give the marker cutoff value; sensitivity (left) and specificity (right) in parentheses, *P* value, AUC, area under the curve (AUC), and 95% confidence intervals (CI).

Table 1: Serum antibody levels in healthy donors (HDs) and patients with transient ischemic attack (TIA) or acute-phase cerebral infarction (aCI) determined by AlphaLISA. Data are average, SD, cutoff values (average + 2SD), total sample numbers, the number of positive sera in which the antibody levels were higher than the cutoff value and the positive rate (%) of HD; and average, SD, total sample number, number of positive sera in which the antibody levels were higher than the cutoff value and the positive rate (%) of patients; and *P* value of comparison between HDs and patients were calculated by the student's *t*-test. The antigen used was a purified GST-fusion DHPS protein. The *P* values lower than 0.05 and positive rates higher than 10% were marked in bold. Box-whisker plots of the same results are shown in (Figure 3).

		s-DHPS-Abs
HD	Total No.	138
	Average	1,263
	SD	1,120
	Cutoff value	3,503
	Positive No.	4
	Positive rate (%)	2.90%
TIA	Total No.	44
	Average	2,121
	SD	1,672
	Positive No.	8
	Positive rate (%)	**18.20%**
	P (*vs* HD)	**0.0024**
aCI	Total No.	228
	Average	2,072
	SD	3,723
	Positive No.	49
	Positive rate (%)	**21.50%**
	P (*vs* HD)	**0.0024**

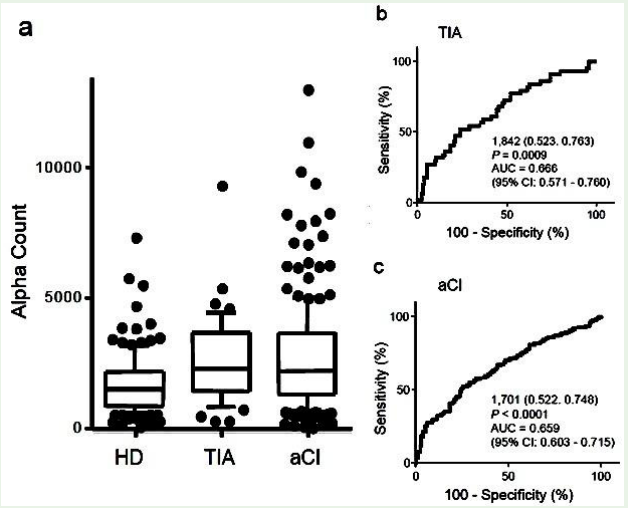

Figure 3: The levels of s-DHPS-Ab in HDs and in patients with TIA or aCI determined by AlphaLISA (a). The box plots display the 10th, 20th, 50th, 80th, and 90th percentiles of antibody level. In Table1, averages, SDs, a cutoff value, total numbers, positivity numbers, positivity rates (%), and *P* valuesare shown. Responses of TIA (b) and aCI (c) to s-DHPS-Abs were also evaluated using ROC analysis, and the numbers in figures are as described in the legend to Figure 2.

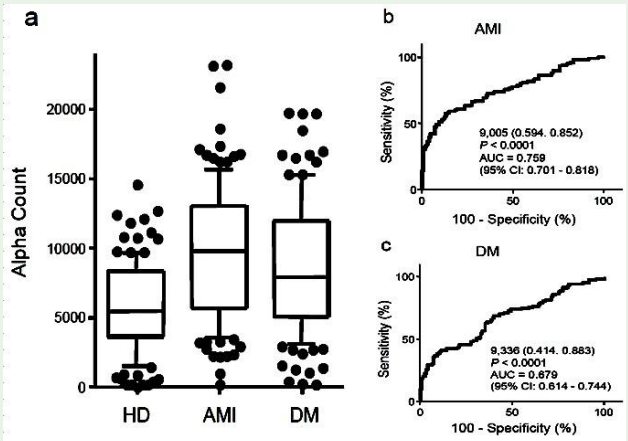

Figure 4: The levels of s-DHPS-Ab in HDs and in patients with acute myocardial infarction (AMI) or diabetes mellitus (DM) determined by AlphaLISA (a). The box plots show the 10th, 20th, 50th, 80th, and 90th percentiles of antibody level. The average values, standard deviations, cut-off values, total numbers, positive numbers, positive rates (%), and P values are summarized and shown in Table 2. The results of ROC analysis of s-DHPS-Ab levels for predicting AMI (b) or DM (c) are also shown. Numbers in the figures are the same as those shown in Figure 2.

Table 2: Comparison of serum antibody levels between HDs and patients with acute myocardial infarction (AMI) or diabetes mellitus (DM) examined by AlphaLISA. Shown are average, SD, cutoff values (average + 2SDs), total sample numbers, the number of positive sera of which the antibody levels were higher than the cutoff value and the positive rate (%) of HD; and average, SD, total sample number, number of positive sera in which the antibody levels were higher than the cutoff value and the positive rate (%) of patients; and *P* value of comparison between HD and patients. The antigen used was a purified GST-fusion DHPS protein. Shown numbers are as described in Table 1;
P values lower than 0.05 and positive rates higher than 10% were marked in bold. Box-whisker plots of the same results are shown in (Figure 4).

		s-DHPS-Abs
HD	Total No.	128
	Average	5,655
	SD	3,246
	Cutoff value	12,147
	Positive No.	3
	Positive rate (%)	2.30%
AMI	Total No.	128
	Average	9,812
	SD	4,696
	Positive No.	44
	Positive rate (%)	**34.40%**
	P (*vs* HD)	**1.40E-14**
DM	Total No.	128
	Average	8,605
	SD	5,013
	Positive No.	28
	Positive rate (%)	**21.90%**
	P (*vs* HD)	**6.80E-08**

obtained at Port Square Kashiwado Clinic. The average ages (± SDs) of HDs, AMI, and DM patients were 58.29 ± 5.63, 58.2 ± 8.50, and 58.37 ± 9.11 years, respectively. A total of 128 specimens each of HDs, AMI, and type II DM patients were assayed simultaneously by AlphaLISA on a 384-well plate. The levels of s-DHPS-Ab were significantly higher in patients with AMI or DM than in HDs (Figure 4a, Table 2). At a cutoff value of the average HD value + 2SDs, the seropositivity rates were 2.3% in HDs, 34.4% in AMI patients, and 21.9% in DM patients (Table 2). ROC analysis indicated that the sensitivity and specificity for AMI were 59.4% and 85.2%, respectively, and 41.4% and 88.3% for DM (Figures 4b and 4c), respectively. The AUC values for AMI and DM were 0.759 and 0.679, respectively. AMI patients had even higher s-DHPS-Ab levels than DM patients.

Serum specimens collected from HD, CVD, and OSA patients were also collected at Chiba University Hospital and assayed. CVD sera were collected not only from AMI patients but also from those with unstable angina pectoris (UAP). OSA is frequently accompanied by atherosclerosis and patients are also considered at high-risk of CVD and CI [26-28]. The levels of s-DHPS-Abs were significantly higher in patients with CVD than in HDs (Supplementary Table S1), whereas the levels in patients with OSA were not significantly different from those in HDs. ROC analysis revealed that the sensitivity of s-DHPS-Abs for CVD was 33.0% and the specificity was 90.3%. The AUC was 0.658 (data not shown). At a cutoff value of the average HD value + 2SDs, the seropositivity rates were 3.8% for HDs, 8.0% for CVD patients, and 7.0% for OSA patients (Supplementary Table S1).

Elevated s-DHPS-Abs levels of in patients with CKD

CKD patients in the Kumamoto cohort were divided into three groups: type-1, diabetic kidney disease; type-2, nephrosclerosis;

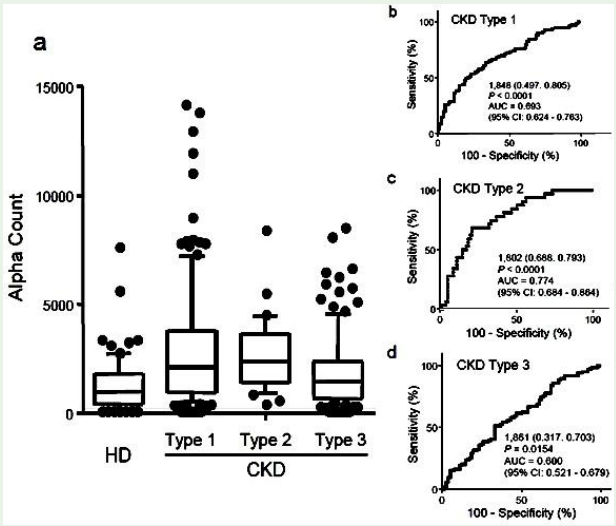

Figure 5: The levels of s-DHPS-Ab in HDs and in patients with chronic kidney disease (CKD) (type-1: diabetic kidney disease, type-2: nephrosclerosis, and type-3: glomerulonephritis) (a). Serum antibody levels against DHPS in HDs and CKD patients were determined by AlphaLISA. The box plots show the 10th, 20th, 50th, 80th and 90th percentiles of antibody level as described in the legend to Figure 2. In Table 3, averages, SDs, cutoff values, total numbers, positive numbers and positive rates (%), and *P* values are shown. The results were also evaluated by ROC analysis for predicting CKD type 1 (b), type 2 (d) or type 3 (e) are also shown. Numbers in the figures are the same as those shown in Figure 2.

Table 3: Comparison of serum antibody levels between HDs and patients with chronic kidney disease (CKD) examined by AlphaLISA. CKD types -1, -2 and -3 were diabetic kidney disease, nephrosclerosis, and glomerulonephritis, respectively. The antigen used was a purified GST-DHPS protein. Shown numbers are as described in Table 1. Box-whisker plots of the same results are shown in (Figure 5).

		s-DHPS-Abs
HD	Total No.	82
	Average	1,161
	SD	1,602
	Cutoff value	4,365
	Positive No.	4
	Positive rate (%)	4.90%
Type-1 CKD	Total No.	145
	Average	2,790
	SD	3,559
	Positive No.	29
	Positive rate (%)	**20.00%**
	P (*vs* HD)	**4.10E-06**
Type-2 CKD	Total No.	32
	Average	2,376
	SD	1,623
	Positive No.	2
	Positive rate (%)	6.30%
	P (*vs* HD)	**0.00066**
Type-3 CKD	Total No.	123
	Average	1,623
	SD	1,725
	Positive No.	12
	Positive rate (%)	9.80%
	P (*vs* HD)	0.051

Table 4: Comparison of serum antibody levels between HDs and patients with cancer examined by AlphaLISA. Sera of HD and patients with benign glioma (b-Glioma), malignant glioma (m-Glioma), and esophageal squamous cell carcinoma (eso-SCC) were examined. The antigen used was a purified GST-DHPS protein. Shown numbers are as described in Table 1.

		DHPS-tv1-GST
HD	Total No.	111
	Average	10516
	SD	10,434
	Cutoff value	31,383
	Positive No.	4
	Positive rate (%)	3.60%
b-Glioma	Total No.	83
	Average	10,628
	SD	8,581
	Positive No.	3
	Positive rate (%)	3.60%
	P (*vs* HD)	0.935
m-Glioma	Total No.	90
	Average	11,822
	SD	8,762
	Positive No.	4
	Positive rate (%)	4.40%
	P (*vs* HD)	0.336
eso-SCC	Total No.	100
	Average	16,622
	SD	11,929
	Positive No.	11
	Positive rate (%)	**11.00%**
	P (*vs* HD)	**0.00011**

and type-3, glomerulonephritis. HD sera were collected at Chiba University Hospital. Eighty-two HD, 145 type-1, 32 type-2, and 123 type-3 specimens were assayed. The average ages (±SDs) of HDs, type -1, -2 and -3 CKD patients were 45.82 ± 11.66, 65.78 ± 10.28, 75.97 ± 9.94 and 66.05 ± 14.60 years. The levels of s-DHPS-Ab were higher in CKD patients than in HDs, especially in patients with types-1 and -2 CKD (Figure 5a, Table 3). The AUC values obtained in the ROC analysis were 0.693 for type-1 CKD, 0.774 for type-2 (Figures 5b and 5c), and 0.600 for type-3 (Figure 5d). At a cutoff value of the average HD value plus two SDs, the positive rate for type-1 (i.e., diabetic CKD) was 20.0%.

The levels of s-DHPS-Abs were not closely associated with cancer Autologous antibodies frequently develop in cancer patients [23,24,29-31]. We evaluated sera of patients with benign or malignant glioma or eso-SCC obtained at Chiba University Hospital and Toho University Hospital. The levels of s-DHPS-Abs were not significantly different in HD and benign or malignant glioma, but were significantly different in HDs and in patients with eso-SCC (Table 4).

Correlation of s-DHPS-Abs and atherosclerosis indices

The significance of correlations between the antibody marker level and patient characteristics including sex, age, BMI, maximum intima-media thickness (max IMT), smoking, presence of aCI, TIA, chronic phase CI (cCI), asymptomatic CI (asympt-CI), complications of DM, HT, and hyperlipidemia were tested by chi-square and multivariate logistic regression analysis. A total of 665 specimens, 227 aCI, 58 cCI, 45 TIA, 19 asympt-CI, 122 white matter softening, 139 HD, and 55 others, were assayed. Both chi-square tests and multivariate logistic regression analysis showed significant correlations of s-DHPS-Ab level, age and presence of aCI and TIA. The correlation was strongest with aCI (Table 5). Chi-square testing also found positive correlations with max IMT and complication of HT. Multivariate analysis revealed positive correlations with gender and smoking, but a negative correlation with BMI. Because max IMT is an index specific to atherosclerosis, s-DHPS-Abs can distinguish aCI and TIA caused by atherosclerosis that is associated with aging, HT or smoking. The negative correlation with BMI suggests that s-DHPS-Abs may not be a marker of aCI induced by obesity.

Spearman's correlation coefficients were also calculated for s-DHPS-Ab and a large number of blood chemistry values. These included red blood cell (RBC), white blood cell (WBC), and platelet (PLT) counts, total protein (TP), hemoglobin (HGB), albumin (ALB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), gamma-

Table 5: Correlation of s-DHPS-Ab marker level and patient clinical characteristics. Shown are results of chi-squared test and multivariate logistic regression analysis. Significant correlations are marked in bold.

			Chi-square					Multivariate	
			Low	%	High	%	*P* value	r value	*P* value
General	Gender	male	295	74.90%	99	25.10%	0.2152	0.119	**0.0024**
		female	189	70.50%	79	29.50%			
	Age	<69	261	79.10%	69	20.90%	**0.0005**	0.093	**0.0176**
		≥70	222	67.10%	109	32.90%			
	BMI	<22.5	230	70.10%	98	29.90%	0.0994	-0.098	**0.0125**
		≥22.6	248	75.80%	79	24.20%			
	max IMT	<2.1	182	77.80%	52	22.20%	**0.0415**	0.028	0.4708
		≥2.2	154	69.40%	68	30.60%			
	Smoking habit	No	259	75.30%	85	24.70%	0.1885	0.119	**0.0023**
		Yes	225	70.80%	93	29.20%			
Present disease	aCI	No*	114	82.00%	25	18.00%	**<0.0001**	0.131	**0.0008**
		Yes	142	62.60%	85	37.40%			
	TIA	No*	114	82.00%	25	18.00%	**0.0045**	0.088	**0.0249**
		Yes	27	61.40%	17	38.60%			
	cCI	No*	114	82.00%	25	18.00%	0.1469	0.068	0.0833
		Yes	43	72.90%	16	27.10%			
	asympt-CI	No*	114	82.00%	25	18.00%	0.5222	-0.019	0.6241
		Yes	15	88.20%	2	11.80%			
Complication	DM	No	388	73.90%	137	26.10%	0.3813	-0.023	0.5608
		Yes	94	70.10%	40	29.90%			
	HT	No	187	78.20%	52	21.80%	**0.0258**	0.007	0.8506
		Yes	295	70.20%	125	29.80%			
	Hyperlipidemia	No	340	71.70%	134	28.30%	0.1908	-0.068	0.0850
		Yes	142	76.80%	43	23.20%			

Table 6: Correlation of s-DHPS-Ab marker level and patient laboratory test values. Shown are results of Spearman's correlation analysis. Significant correlations are marked in bold.

	r value	*P* value
RBC	-0.060	0.1247
WBC	0.071	0.0693
PLT	-0.078	**0.0467**
TP	-0.033	0.4130
HGB	-0.053	0.1761
ALB	-0.058	0.1451
AST	-0.021	0.5919
ALT	-0.063	0.1067
ALP	0.071	0.0842
LDH	0.081	**0.0430**
gamma-GTP	-0.054	0.1824
CHE	-0.089	**0.0442**
AMY	-0.062	0.2061
CRE	-0.033	0.4055
tBil	0.015	0.7072
BUN	-0.025	0.5162
UA	-0.050	0.2695
eGFR	-0.013	0.7677
Na	-0.034	0.3898
K	-0.087	**0.0277**
Cl	-0.010	0.8066
Ca	-0.104	**0.0420**
IP	0.057	0.3205
Fe	-0.051	0.3747
TG	-0.070	0.1315
T-CHO	-0.085	**0.0445**
LDL-c	0.001	0.9950
HDL-c	-0.014	0.7753
BS	0.068	0.0969
HbA1c	-0.031	0.4906
CRP	0.088	0.0560

glutamyltranspeptidase (gamma-GTP), cholinesterase (CHE), amylase (AMY), creatinine(CRE), total bilirubin (tBil), blood urea nitrogen (BUN), uric acid (UA), estimated glomerular filtration ratio (eGFR), Na, K, Cl, Ca, inorganic phosphate (IP), Fe, Mg, TG, total cholesterol (T-CHO), LDL-c, high-density lipoprotein cholesterol (HDL-c), blood sugar (BS), glycated hemoglobin (HbA1c), and C-reactive protein (CRP). A weak positive correlation of s-DHPS-Abs and LDH, and negative correlation with PLT and CHE, K, Ca, and T-CHO were found (Table 6). No significant correlations of s-DHPS-Abs and TG, LDL-c, HDL-c, or DM markers such as BS and HbA1c, which had been expected to be associated with atherosclerosis, were found.

Discussion

The ProtoArray assay identified DHPS antigens that were recognized by serum IgG antibodies in patients with atherosclerosis. ELISA and AlphaLISA determination of s-DHPS-Ab levels showed that the antibody level was elevated in patients with aCI, TIA, AMI, DM, and CKD, all of which are related to atherosclerosis (Figures 2-5).

Deoxyhypusination is a post-translational modification; DHPS catalyzes the conjugation of the Lys-50 ε-amino residue of the eukaryotic translation initiation factor 5A (eIF5A) and the 4-aminobutyl residue of spermidine. The deoxyhypusinated eIF5A is then converted to hypusinated EIF5A by deoxyhypusine hydrolase, which can stimulate the nuclear export of mRNA and translation of polyproline motifs [32]. The target genes stimulated by hypusinated eIF5A include iNOS, which can suppress ATP generation and induce impaired insulin release and cell death [33]. The DHPS inhibitor, N1-guanyl-1,7-diamino heptane, has been shown to enhance pancreatic islet β cell function and survival in mice [34]. DHPS thus plays a key role in the development of DM.

If elevated s-DHPS-Abs levels result from increased expression of the antigenic DHPS protein, they might be associated with DM.

However, higher AUC values for s-DHPS-Abs were observed with type-2 CKD (0.774) and AMI (0.759) than for type-1 CKD (0.693), DM (0.679), TIA (0.666), aCI (0.659), and type-3 CKD (0.600), (Figures 3-5). Although type-2 CKD is diabetes-related, nephrosclerotic type-2 CKD, which is not diabetes-related, was even more closely associated with s-DHPS-Ab levels. Furthermore, the correlation analyses revealed that s-DHPS-Ab levels were not correlated with DM markers such as BS and HbA1c, but were correlated with max IMT (Tables 5 and 6). Antibody levels were associated with age and complications of HT, but not DM complications. TG, LDL-c, and HDL-c levels were not associated with s-DHPS-Ab levels, and negative correlations were seen between antibody levels, BMI, and T-CHO. Consequently, s-DHPS-Abs may not directly reflect the progression of DM, obesity, or cholesterol metabolism, but might be a common marker of atherosclerosis caused by aging, HT, and/or smoking. The levels of s-DHPS-Abs were associated with aCI, TIA, AMI, DM, and CKD probably as a result of atherosclerosis progression.

DHPS and hypusination of EIF5A are known to be involved in proliferation of cervical cancer cells [35] and metastasis of eso-SCC [36] via c-Abl, RHOA, HSP27, NM23, and DJ-1 [37-40]. Evaluation of the cancer specimens in this study showed that s-DHPS-Ab levels were weakly associated with eso-SCC but not with benign or malignant glioma (Table 4). With a cutoff value calculated as the average HD value plus two SDs, the seropositivity rate of eso-SCC was 11.0%, which contrasted with the high seropositivity rates for AMI (34.4%) and DM (21.9%) (Table 2). It has been reported that atherosclerosis and cancer share common molecular pathogenic and progression pathways [41,42]. The s-DHPS-Ab marker might thus allow to distinguish cancers that originate from atherosclerotic lesions.

The s-DHPS-Abseropositivity rate in AMI was as high as 34.4%, whereas in CVD, which includes both AMI and UAP, it was relatively low (8.0%, Table 2, Supplementary Table S1). The sensitivity and specificity for diagnosis of AMI were 59.4% and 85.2%, respectively (Figure 4b). These values imply that AMI was highly associated with s-DHPS-Abs. Such autoantibodies do not appear immediately after AMI onset but are present prior to its onset. Thus, it is possible that s-DHPS-Ab might be predictive of the onset of AMI but not UAP. Similarly, the onset of aCI might also be predicted by s-DHPS-Ab. In this study, TIA, which is a predictor of aCI [43,44], was associated with higher s-DHPS-Abs levels than observed in HDs (Figures 2a, 2b, 3a and 3b, Table 1). Further analysis of cohort specimens might confirm s-DHPS-Ab as a predictive marker for AMI and aCI.

Conclusion

The levels of s-DHPS-Abs were associated with atherosclerotic conditions including AMI, DM, CKD, TIA, and aCI. This antibody may be useful to evaluate the progress of atherosclerosis leading to aCI and AMI.

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References

1. Carandang R, Seshadri S, Beiser A, Kelly-Hayes M, Kase CS, et al. Trends in incidence, lifetime risk, severity, and 30-day mortality of stroke over the past 50 years. JAMA 2006; 296: 2939-2946.
2. Kubo M, Hata J, Doi Y, TanizakiY, Iida M, et al. Secular trends in the incidence of and risk factors for ischemic stroke and its subtypes in Japanese population. Circulation 2008; 118: 2672-2678.
3. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature 2011; 473: 317-325.
4. Hankey GJ, Eikelboom JW. Homocysteine and stroke. CurrOpinNeurol 2001; 14: 95-102.
5. Kiechl S, Schett G, Wenning G, Redlich K, Oberhollenzer M, et al. Osteoprotegerin is a risk factor for progressive atherosclerosis and cardiovascular disease. Circulation 2004; 109: 2175-2180.
6. Mendioroz M, Fernandez-Cadenas I, Rosell A, Delgado P, Domingues-Montanari S, et al. Osteopontin predicts longterm functional outcome among ischemic stroke patients. J Neurol 2011; 258: 486-493.
7. Liang KP, Kremers HM, Crowson CS, Snyder MR, Therneau TM, et al. Autoantibodies and the risk of cardiovascular events. JRheumatol 2009; 36: 2462-2469.
8. Montecucco F, Vuilleumier N, Pagano S, Lenglet S, Bertolotto M, et al. Anti-apolipoprotein A-1 auto-antibodies are active mediators of atherosclerotic plaque vulnerability. Eur Heart J 2011; 32: 412-421.
9. Fesmire J, Wolfson-Reichlin M, Reichlin M. Effects of autoimmune antibodies anti-lipoprotein lipase, anti-low density lipoprotein, and anti-oxidized low density lipoprotein on lipid metabolism and atherosclerosis in systemic lupus erythematosus. Rev BrasReumatol 2010; 50: 539-551.
10. Carbone F, Nencioni A, Mach F, Vuilleumier N, Montecucco F. Evidence on the pathogenic role of auto-antibodies in acute cardiovascular diseases. ThrombHaemost 2013; 109: 854-868.
11. Kramer J, Harcos P, Prohászka Z, Horvath L, Karadi I, et al. Frequencies of certain complement protein alleles and serum levels of anti-heat-shock protein antibodies in cerebrovascular diseases. Stroke 2000; 31: 2648-2652.
12. Palmer JP, Asplin CM, Clemons P, Lyen K, Tatpati O, et al. Insulin antibodies in insulin-dependent diabetics before insulin treatment. Science 1983; 222: 1337-1339.
13. Baekkeskov S, Aanstoot H, Christgau S, Reetz A, Solimena MS, et al. Identification of the 64K autoantigen in insulin dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature 1990; 347; 151-156.
14. Payton MA, Hawkes CJ, Christie MR. Relationship of the 37,000- and 40,000-M(r) tryptic fragments of islet antigens in insulin-dependent diabetes to the protein tyrosine phosphatase-like molecule IA-2 (ICA512). J Clin Invest 1995; 96: 1506-1511.
15. Taplin CE, Barker JM. Auto-antibodies in type 1 diabetes. Autoimmunity 2008; 41: 11-18.
16. Machida T, Kubota M, Kobayashi E, Iwadate Y, Saeki N, et al. Identification of stroke-associated-antigens via screening of recombinant proteins from the human expression cDNA library (SEREX). JTranslat Med 2015; 13: 71.
17. Goto K, Sugiyama T, Matsumura R, Zhang XM, Kimura R, et al. Identification of Cerebral Infarction-Specific Antibody Markers from Auto-antibodies Detected in Patients with Systemic Lupus Erythematosus. JMolBiomarkDiagnos 2015; 6: 219.

18. Hiwasa T, Machida T, Zhang XM, Kimura R, Wang H, et al. Elevated levels of auto-antibodies against ATP2B4 and BMP-1 in sera of patients with atherosclerosis-related diseases. Immunome Res 2015; 11: 097.

19. Hiwasa T, Zhang XM, Kimura R, Machida T, Kitamura K, et al. Association of serum antibody levels against TUBB2C with diabetes and cerebral infarction. Integ Biomed Sci 2015; 1: 49-63.

20. Hiwasa T, Zhang XM, Kimura R, Ohno M, Chen PM, et al. Elevated adiponectin antibody levels in sera of patients with atherosclerosis-related coronary artery disease, cerebral infarction, and diabetes mellitus. J CircBiomark 2016; 5: 8.

21. Nishiura R, Fujimoto S, Sato Y, Yamada K, Hisanaga S, et al. Elevated osteoprotegerin levels predict cardiovascular events in new hemodialysis patients. Am JNephrol 2009; 29: 257-263.

22. Komatsu H, Fujimoto S, Hara S, Fukuda, A, Fukudome K, et al. Recent therapeutic strategies improve renal outcome in patients with IgA nephropathy. Am JNephrol 2009; 30: 19-25.

23. Nakashima K, Shimada H, Ochiai T, Kuboshima M, Kuroiwa N, et al. Serological identification of TROP2 by recombinant cDNA expression cloning using sera of patients with esophageal squamous cell carcinoma. Int J Cancer 2004; 112: 1029-1035.

24. Shimada H, Shiratori T, Yasuraoka M, Kagaya A, Kuboshima M, et al. Identification of Makorin 1 as a novel SEREX antigen of esophageal squamous cell carcinoma. BMC Cancer 2009; 9: 232.

25. Hiwasa T, Arase Y, Kikuno K, Hasegawa R, Sugaya S, et al. Increase in ultraviolet sensitivity by overexpression of calpastatin in ultraviolet-resistant UVr-1 cells derived from ultraviolet-sensitive human RSa cells. Cell Death Diff 2000; 7: 531-537.

26. Dewan NA, Nieto FJ, Somers VK. Intermittent hypoxemia and OSA: implications for comorbidities. Chest 2015; 147: 266-274.

27. Arnardottir ES, Mackiewicz M, Gislason T, Teff KL, Pack AI. Molecular signatures of obstructive sleep apnea in adults: a review and perspective. Sleep 2009; 32: 447-470.

28. Lavie L. Oxidative stress in obstructive sleep apnea and intermittent hypoxia--revisited--the bad ugly and good: implications to the heart and brain. Sleep Med Rev 2015; 20: 27-45.

29. Sahin U, Tureci O, Schmitt H, Cochlovius B, Johannes T, et al. Human neoplasms elicit multiple specific immune responses in the autologous host. ProcNatlAcadSci USA 1995; 92: 11810-11813.

30. Kagaya A, Shimada H, Shiratori T, Kuboshima M, Nakashima-Fujita K, et al. Identification of a novel SEREX antigen family, ECSA, in esophageal squamous cell carcinoma. Proteome Sci 2011; 9: 31.

31. Matsutani T, Hiwasa T, Takiguchi M, Oide T, Kunimatsu M, et al. Autologous antibody to src-homology 3-domain GRB2-like 1 specifically increases in the sera of patients with low-grade gliomas. J Experiment Clin Cancer Res 1012; 31: 85.

32. Gutierrez E, Shin BS, Woolstenhulme CJ, Kim JR, Saini P, et al. eIF5A promotes translation of polyproline motifs. Mol Cell 2013; 51, 35-45.

33. Maier B, Ogihara T, Trace AP, Tersey SA, Robbins RD, et al. The unique hypusine modification of eIF5A promotes islet beta cell inflammation and dysfunction in mice. J Clin Invest 2010; 120: 2156-2170.

34. Robbins RD, Tersey SA, Ogihara T, Gupta D, Farb TB, et al. Inhibition of deoxyhypusine synthase enhances islet β cell function and survival in the setting of endoplasmic reticulum stress and type 2 diabetes. J BiolChem 2010; 285: 39943-39952.

35. Memin E, Hoque M, Jain MR, Heller DS, Li H, et al. Blocking eIF5A modification in cervical cancer cells alters the expression of cancer-related genes and suppresses cell proliferation. Cancer Res 2014; 74: 552-562.

36. Muramatsu T, Kozaki KI, Imoto S, Yamaguchi R, Tsuda H, et al. The hypusine cascade promotes cancer progression and metastasis through the regulation of RhoA in squamous cell carcinoma. Oncogene 2016; 35: 5304-5316.

37. Pallmann N, Braig M, Sievert H, Preukschas M, Hermans-Borgmeyer I, et al. Biological Relevance and Therapeutic Potential of the Hypusine Modification System. JBiolChem 2015; 290: 18343-18360

38. Straume O, Shimamura T, Lampa MJ, Carretero J, Oyan AM, et al. Suppression of heat shock protein 27 induces long-term dormancy in human breast cancer. ProcNatlAcadSci USA 2012; 109: 8699-8704.

39. Cipollini G, Berti A, Fiore L, Rainaldi G, Basolo F, et al. Down-regulation of the nm23.h1 gene inhibits cell proliferation. Int J Cancer 1997; 73: 297-302.

40. He X, Zheng Z, Li J, Ben Q, Liu J, et al. DJ-1 promotes invasion and metastasis of pancreatic cancer cells by activating SRC/ERK/uPA. Carcinogenesis 2012; 33: 555-562.

41. Ross JS, Stagliano NE, Donovan MJ, Breitbart RE, Ginsburg GS. Atherosclerosis and cancer: common molecular pathways of disease development and progression. Ann NYAcadSci 2001; 947: 271-292.

42. Jara-Palomares L, Otero R, Jimenez D, Carrier M, Tzoran I, et al. Development of a risk prediction score for occult cancer in patients with venous thromboembolism. Chest. 2017; 151: 564-571.

43. Johnston SC, Rothwell PM, Nguyen-Huynh MN, Giles MF, Elkins JS, et al. Validation and refinement of scores to predict very early stroke risk after transient ischaemic attack. Lancet 2007; 369: 283-292.

44. Amarenco P, Lavallée PC, Labreuche J, Albers GW, Bornstein NM, et al. One-year risk of stroke after transient ischemic attack or minor stroke. NEngl J Med 2016; 374: 1533-1542.