RESEARCH ARTICLE

Serum anti-DIDO1, anti-CPSF2, and anti-FOXJ2 antibodies as predictive risk markers for acute ischemic stroke

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Abstract

Background: Acute ischemic stroke (AIS) is a serious cause of mortality and disability. AIS is a serious cause of mortality and disability. Early diagnosis of atherosclerosis, which is the major cause of AIS, allows therapeutic intervention before the onset, leading to prevention of AIS.

Methods: Serological identification by cDNA expression cDNA libraries and the protein array method were used for the screening of antigens recognized by serum IgG antibodies in patients with atherosclerosis. Recombinant proteins or synthetic peptides derived from candidate antigens were used as antigens to compare serum IgG levels between healthy donors (HDs) and patients with atherosclerosis-related disease using the amplified luminescent proximity homogeneous assay-linked immunosorbent assay.

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Background
Atherosclerosis is a serious disease and a major cause of acute ischemic stroke (AIS) and acute myocardial infarction (AMI) [1]. Diabetes mellitus (DM) and chronic kidney disease (CKD) are closely related to and accompanied by atherosclerosis [2]. As atherosclerosis progresses, atherosclerotic plaques are formed on artery walls by foam cells, which are differentiated from smooth muscle cells or macrophages [3–5]. Diagnosing atherosclerosis is important to prevent the onset of AIS and AMI because the effectiveness of treatment and therapy is limited after their onset. Thus, to date, many risk factors and biomarkers including family history, age, obesity, smoking habit, dyslipidemia, hypertension, sleep, C-reactive protein level, interleukin-6 level, troponin level, and B-type natriuretic peptide level have been reported [6, 7]; however, they are still insufficient. Genome-wide association studies on stroke have identified many genes such as NOTCH3 [8], CSTA [9], and COL3A1 [10]. However, lifestyle diseases such as stroke and atherosclerosis can be prevented by improving individuals’ lifestyles.

Recent studies have discovered that the development of autoantibodies is not limited to autoimmune diseases but is also observed in other diseases. Some examples include autoantibody markers against proteins such as p53, NY-ESO-1, and RALA for cancer [11–14]; Hsp60 for stroke [15]; insulin [16], glutamic acid decarboxylase [17], and protein tyrosine phosphatase IA-2 [18, 19] for DM, as well as phospholipid [20], apolipoprotein A1 [21, 22], oxidized low-density lipoprotein [22, 23], and heat shock proteins [22, 24] for cardiovascular disease (CVD).

Previously, we searched for antibody markers using serological identification of antigens by cDNA expression cloning (SEREX) and the protein array method, and we reported on autoantibodies against Trop2/TACSTD2 [25], TRIM21 [26], Makorin 1 [27], and ECSA [28], for esophageal squamous cell carcinoma; FIR/PUF60 for colon cancer [29]; SH3GL1 [30] and filamin C [31] for glioma; EP300-interacting inhibitor of differentiation 3 for non-functional pancreatic neuroendocrine tumors [32]; proline-rich 13 for ulcerative colitis [33]; talin-1 for multiple sclerosis [34]; PSMA7 for amyotrophic lateral sclerosis [35]; NBL1/DAN [36] and SNX16 [37] for obstructive sleep apnea (OSA); and EXD2 for chronic thromboembolic pulmonary hypertension (CTEPH) [38]. We also reported on autoantibody markers for atherosclerosis-related diseases, e.g., RPA2 [39], PDCD11 [40], MMP1 [41], and DNAJC2 [42] for AIS; ASXL2 [43] for atherosclerosis; and nardilysin for acute coronary syndrome [44]. Here, we report on antibodies against death-inducer obliterator 1 (DIDO1), forkhead box J2 (FOXJ2), and cleavage and polyadenylation specificity factor (CPSF2) peptides, which are highly associated with AIS and could be useful as predictive markers.

Methods
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Patient and controls
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 the cooperating hospitals or institutes. Sera were collected from patients who had provided informed consent. Each serum sample was centrifuged at 3000g for 10 min, and supernatant was stored at −80°C until use. Repeated freezing and thawing of samples was avoided.

Serum samples from patients with DM, ulcerative colitis, CTEPH, pulmonary arterial hypertension (PAH), and OSA were obtained from Chiba University Hospital, and samples collected from patients with AIS, transient ischemic attack (TIA), asymptomatic cerebral infarction (asympt-Cl), chronic-phase CI (cCl), and deep and subcortical white matter hyperintensity (DSWMH) were obtained from Chiba Prefectural Sawara Hospital, Chiba Rosai Hospital, Chiba Aoba Municipal Hospital, and Chiba Medical Center. The stroke subtype of each patient was also determined according to the criteria of the Trial of Org 10172 in Acute Stroke Treatment classification system [45]. In this analysis, large-artery atherosclerosis or small-artery occlusion (lacune) were included as AIS or cerebral infarction.

Serum samples from patients with AIS used in the preliminary screening were provided by BioBank Japan. Serum samples from patients with AMI were obtained from Kyoto University Hospital [44]. Serum samples associated with AIS, TIA, and AMI were obtained within 2 weeks after disease onset. Samples collected from patients with CKD were obtained from the Kumamoto cohort [46, 47], whereas those collected from patients with colorectal carcinoma, esophageal squamous cell carcinoma, gastric cancer, breast cancer, and pancreatic cancer were obtained from the Department of Frontier Surgery, Chiba University Hospital. Serum samples from patients with Sjögren’s syndrome were obtained from Chiba Children’s Hospital. Serum samples from patients with rheumatoid arthritis and systemic lupus erythematosus (SLE) were obtained from the National Hospital Organization, Shimoshizu Hospital, and Chiba East Hospital [48]. Serum samples from healthy donors (HDs) were obtained from Chiba University, Port Square Kashiwado Clinic, Higashi Funabashi Hospital, and Chiba Prefectural Sawara Hospital. For comparisons with TIA and AIS, serum samples from HDs were selected from patients who exhibited no abnormalities on cranial magnetic resonance imaging.

**ProtoArray® screening**

The first screening was performed using ProtoArray® Human Protein Microarrays v. 4.0 (Thermo Fisher Scientific, Waltham, MA), which were loaded with 9480 proteins species as described previously [33, 38, 48]. In total, 30 serum samples (15 each from HDs and patients with atherosclerosis) were used to detect antigens specifically recognized by IgG antibodies in sera. Results were analyzed using the Prospector software (Thermo Fisher Scientific), which is based on M-statistics. When comparing the two groups, a cutoff for positivity was calculated for each protein using M-statistics. For both groups, the proportion of subjects with an immune response above the cutoff value was counted, and a P value representing the significance of the difference between both groups was calculated as described [49].

**Expression and purification of the DIDO1 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). The amino-terminal (amino acids 1–275) and carboxy-terminal half (amino acids 271–545) of the coding sequences of DIDO1 cDNA were amplified via PCR using Pyrobest DNA polymerase (Takara Bio Inc., Shiga, Japan) and cloned at the EcoRI/Sall site of pGEX-4 T-3 (GE Healthcare Life Sciences, Pittsburgh, PA), followed by confirmation by DNA sequencing. Expression of the cDNA product was induced by treating pGEX-4 T-3-DIDO1-transformed Escherichia coli (E. coli) with 0.1 mM isopropyl-β-D-thiogalactoside at 25°C for 4 h; the cells were subsequently lysed in BugBuster® Master Mix (Merck Millipore, Darmstadt, Germany). Then, glutathione S-transferase (GST)-tagged DIDO1 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 [34–37, 39–43].

**Western blotting**

GST-tagged amino-terminal (amino acids 1–275) and carboxy-terminal half (amino acids 271–545) DIDO1 proteins were designated as DIDO1-N and DIDO1-C, respectively, and purified as described above. GST–FOXJ2 and GST–CPSF2 were purchased from Abnova (Taipei, Taiwan). GST and GST fusion proteins (0.3 μg) were separated via sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrically transferred onto nitrocellulose membranes (Advantec, Tokyo, Japan). The membranes were blocked using a blocking solution [0.5% skim milk powder in a buffer comprising 20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.1% Tween 20], and the blotted proteins were probed with primary antibodies including anti-GST (goat) (Rockland, Gilbertsville, PA), anti-DIDO1 (rabbit) (Aviva Systems Biology, San Diego, CA), or anti-FOXJ2 (rabbit) (Thermo Fisher Scientific), anti-CPSF2 (rabbit) (GeneTex, Irvine, CA) or from sera from HDs (#30017) or patients with TIA.
Epitope prediction and peptide synthesis
Possible epitope sites in the CPSF2 and FOXJ2 proteins were predicted using the ProPred program (http://www.imtech.res.in/raghava/propred/) as described previously [38, 48]. The following amino acid sequences were designed:

- bCPSF3-165: biotin-FMIEIAGVKLLLYTGDbCPSF3-298: biotin-NPINPVFKHISSLKbCPSF3-545: biotin-KPALKVKFKNITVQEbCPSF2-607: biotin-QVRLLKDSLVSSLQFCbCPSF2-712: biotin-QSVFMNEPRLSDFKQbFOXJ2-426: biotin-KMVNRLNWSSIEQSQ

Peptide array method
The epitopes in the DIDO1 protein were screened comprehensively throughout the full-length DIDO1 protein using the peptide array method, in which we designed 83 peptides of 14mer derived from the DIDO1 protein. These peptides were synthesized onto cellulose membranes using Fmoc amino acids (Auto-Spot Robot ASP222; ABIMED Analysen-Technik GmbH, Langenfeld, Germany) as described previously [50]. The membranes were washed five times with PBS containing 1% (w/v) bovine serum albumin, 0.05% Tween 20, and 0.05% NaN3 (PBS-T-BSA) for 30 min each and then incubated with a 1:200 dilution of sera of HDs or patients with AIS 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 antihuman IgG (Jackson ImmunoResearch, West Grove, PA) for 1 h. After washing, the fluorescence levels of peptide spots were detected using the Typhoon 9400 Imager (GE Healthcare Life Sciences) with a 488-nm/520-nm filter, as described previously [30, 48, 51].

Peptide synthesis
N-terminal biotinylated 15-mer peptide of amino acids 426–440 derived from FOXJ2 (designated as bFOXJ2-426), N-terminal biotinylated 15-mer peptide of amino acids 607–621 derived from CPSF2 (designated as bCPSF2-607), and N-terminal biotinylated 18-mer peptide of amino acids 297–314 derived from DIDO1 (designated as bDIDO1-297) were purchased from Eurofins Genomics (Tokyo, Japan). Their amino acid sequences and purity were as follows:

- bFOXJ2-426: biotin-KMVNRLNWSSIEQSQ (94.9%)
- bCPSF2-607: biotin-QVRLLKDSLVSSLQFC (99.2%)
- bDIDO1-297: biotin-AMAASKKTAPPGSAVGKQ (98.4%)

Amplified luminescent proximity homogeneous assay-linked immunosorbent assay (AlphaLISA)
AlphaLISA was performed in 384-well microtiter plates (white opaque OptiPlate™; PerkinElmer, Waltham, MA) containing either 2.5 μL of 1:100 diluted serum with 2.5 μL of GST or GST–DIDO1 protein (10 μg/mL) or biotinylated peptides (bDIDO1-297, bFOXJ2-426, and bCPSF2-607; 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, after which antihuman 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, followed by 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 [32–38, 40–43, 48, 51]. Specific reactions were calculated by subtracting the Alpha counts of GST control and buffer control without antigenic peptides from the counts of GST-fusion proteins and biotinylated peptides, respectively.

Immunohistochemical staining
Tissue samples were obtained from surgically resected carotid atherosclerotic plaques. Paraffin-embedded vascular tissues were sectioned and then dewaxed using graded alcohol and xylene. After antigen retrieval at 98°C for 40 min in 10 mM citrate buffer (pH 6.0), endogenous peroxidase was blocked using 3% hydrogen peroxide in methanol for 30 min. Then, all sections were washed three times with a wash buffer (S3006; Agilent) for 1 h. After washing, the fluorescence levels of peptide spots were detected using the Envision Detection System (K5007; Agilent) at 37°C.
for 60 min. The bound antibodies were visualized with chromogen diaminobenzidine in 3% hydrogen peroxidase. Finally, the sections were counterstained with hematoxylin, dehydrated, and mounted on glass slides as described in the literature [25, 28, 39].

**Nested case–control study**

A nested case–control study was conducted using the abovementioned AlphaLISA detection antibody levels. This study was nested within the Japan Public Health Center (JPHC)-based Prospective Study [52, 53], which involved approximately 30,000 Japanese individuals aged 40–69 years at a baseline period of 1990–1994 whose plasma samples were stored. Serum DIDO1, bDIDO1-297, bFOXJ2-426, and bCPSF2-607 antibody levels were measured in 202 cases of incidental AIS in the cohort developed between the baseline and 2008 as well as in 202 controls whose sex, age (within 2 years), date of blood sampling (within 3 months), time since last meal (within 4 h), and study location (Public Health Center area) were matched with those of the cases. We used a conditional logistic regression model to estimate odds ratios and 95% confidence intervals (CIs) for AIS with respect to serum antibody levels of the DIDO1 protein and DIDO1, FOXJ2, and CPSF2 peptides.

**Statistical analysis**

Mann–Whitney U test, Student’s t test, and Kruskal–Wallis test were used to determine the significance of the differences between two groups or among multiple groups. Correlations were calculated using Spearman’s 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 using a receiver operating characteristic (ROC) curve analysis, and cutoff values were set to maximize the sums of sensitivity and specificity. All tests were two tailed, and P values of < 0.05 were considered statistically significant.

**Results**

**Recognition of DIDO1, CPSF2, and FOXJ2 via serum IgG antibodies of patients with atherosclerosis**

The first screening for AIS biomarkers was performed using SEREX and the protein array method. After the second screening using serum samples from HDs obtained from Chiba University Hospital and serum samples from patients with AIS obtained from BioBank Japan, we identified 74 antibody markers for AIS, some of which have been reported previously (Table 1). Preliminary validation tests using serum samples from patients with AIS obtained from BioBank Japan showed that the antibody levels against these three antigens, DIDO1, FOXJ2, and CPSF2, were reproducibly and significantly higher in AIS sera than control HD sera. Thus, we focused on these antibody markers highly associated with AIS.

The results of ProtoArray® loaded with 9480 protein species showed that DIDO1 (accession no. BC000770.1) antibodies were observed in 4 out of 5 serum samples from patients with atherosclerosis and 1 out of 5 serum samples from HDs. FOXJ2 (accession no. NM_018416.2) antibodies were found to react with antibodies in 7 out of 15 serum samples from patients with atherosclerosis and none of the 15 serum samples from HDs. CPSF2 (100 kDa; accession no. NM_017437.1) antibodies reacted with antibodies in 5 out of 10 serum samples from patients with atherosclerosis and 2 out of 10 serum samples from HDs. Subsequently, GST fusion proteins that contained DIDO1N or DIDO1C were expressed in E. coli and purified via affinity chromatography. In addition, 5 predicted epitopes of CPSF2 and 1 of FOXJ2 were prepared, and the following preliminary experiments showed that serum bFOXJ2-426 and bCPSF2-607 antibody levels more highly reacted with serum antibodies in patients with AIS than with those in HDs. To examine epitopes in the DIDO1 protein recognized by serum antibodies, we synthesized a peptide array [30, 48, 51] loaded with 83 species of 14-mer peptides derived from the DIDO1 protein. bDIDO1-297, which was most closely associated with AIS, was also used as an antigen to evaluate serum antibody levels.

**Presence of serum antibodies against purified proteins in patients with TIA or AIS**

We then confirmed the presence of antibodies against the GST fusion proteins of DIDO1N, DIDO1C, FOXJ2, and CPSF2 in serum samples from patients with TIA or AIS via Western blotting. GST, GST–DIDO1N, GST–DIDO1C, GST–FOXJ2, and GST–CPSF2 were recognized by the anti-GST antibody as reactions of 26-, 70-, 57-, 95-, and 110-kDa proteins, respectively (Fig. 1). GST–DIDO1N, GST–FOXJ2, and GST–CPSF2 were recognized by each specific commercial antibody. GST–DIDO1N and GST–DIDO1C (but not GST) reacted with antibodies in serum samples from patients with TIA #07207, AIS #07684, TIA #07175, and AIS #07115, whereas the serum antibodies of patients with AIS #07684 and TIA #07060 recognized GST–DIDO1N but not GST–DIDO1C. GST–CPSF2 reacted with antibodies in serum sample from a patient with TIA #07175, and GST–FOXJ2 reacted with antibodies in serum sample from patients with AIS #07115 and TIA #07060. None of these antigenic proteins were recognized by serum IgG in patients with HD #30017. As such, the reactivity of GST fusion antigenic proteins with serum antibodies may be primarily attributed to the antigenic protein regions but not to the GST domain. GST–DIDO1N was
| Abbreviated name | Accession number | Full name | Screening method | Reference |
|------------------|------------------|-----------|------------------|-----------|
| DIDO1            | BC00070.1        | Death inducer obliterator-1 | Protein array | This report |
| CPSF2            | NM_017437.1      | Cleavage and polyadenylation specific factor 2, 100 kDa | Protein array | This report |
| FOXJ2            | NM_018416.2      | Forkhead box J2 | Protein array | This report |
| ACTR3B           | NM_020445.6      | ARP3 actin-related protein 3 homolog B | SEREX | [40] |
| ADAMTS7          | NM_014272.3      | ADAM metallopeptidase with thrombospondin type 1 motif, 7 | SEREX | [40] |
| AR1413S2         | NM_133494        | NIMA (never in mitosis gene a)-related kinase 7 | SEREX | [40] |
| ASXL2            | NM_018263.6      | Additional sex combs-like 2 | SEREX | [43] |
| ATP2B4           | NM_001001396.2   | ATPase, Ca++ transporting, plasma membrane 4 | Protein array | [54] |
| BAZ1B            | NM_032408        | Bromodomain adjacent to zinc finger domain, 1B | SEREX | [54] |
| BMP1             | NM_006129.4      | Bone morphogenetic protein 1 | SEREX | [39, 54] |
| CBX1             | NM_001127228     | Chromobox homolog 1 | SEREX | [41] |
| CBX5             | NM_012117        | Chromobox homolog 5 | SEREX | [41] |
| CCNG2            | NM_004354.3      | Cyclin G2 | Protein array | [48] |
| CEP290           | NM_014684        | Centrosomal protein 290 kDa | SEREX | [48] |
| CLDN1D           | NM_001040181     | Claudin domain containing 1 | Protein array | [48] |
| COPE             | CR456886         | Coatamer protein complex subunit epsilon | SEREX | [36] |
| CRIM1            | NM_016441.2      | Cysteine-rich transmembrane BMP regulator 1 (chordin-like) | SEREX | [48] |
| CTNNA1           | NM_001903.5      | Catenin alpha 1 | SEREX | [40] |
| CTNNID1          | NM_001085458     | Catenin delta 1 | Protein array | [48] |
| DEF8             | NM_207514        | Differentially expressed in FDCP 8 homolog (mouse) | SEREX | [55] |
| DHP5             | NM_001930        | Deoxyhypusine synthase | Protein array | [42] |
| DNAJA1           | NM_001539        | DnaJ heat shock protein family (Hsp40) member A1 | SEREX | [42] |
| DNAJC2           | NM_014377        | DnaJ heat shock protein family (Hsp40) member C2 | SEREX | [42] |
| DST              | NM_015548        | Dystonin | SEREX | [48] |
| EEF1A1           | NM_001402.5      | Eukaryotic translation Elongation factor 1 alpha 1 | SEREX | [56] |
| EEF1G            | NM_001404.4      | Eukaryotic translation elongation factor 1 gamma | SEREX | [56] |
| EIF2A            | NM_032025.3      | Eukaryotic translation initiation factor 2A, 65 kDa | SEREX | [56] |
| FER1L3           | NM_133337        | Myoferlin | SEREX | [56] |
| GOPC             | NM_001017408     | Golgi associated PDZ and coiled-coil motif containing | SEREX | [39] |
| H3F3B            | NM_005324        | H3 histone, family 3B | SEREX | [57] |
| HM13             | AF483215         | Histocompatibility (minor) 13 | SEREX | [41] |
| HSPA8            | NM_006597        | Heat shock 70 kDa protein 8 | SEREX | [41] |
| HSPB1            | NM_001540.3      | Heat shock 27 kDa protein 1 | SEREX | [41] |
| KIAA0020         | NM_014878        | KIAA0020 | SEREX | [56] |
| LGALS9           | NM_009597        | Galectin 9 | SEREX | [39] |
| LRPA1            | NM_002337        | Low-density lipoprotein receptor–related protein–associated protein 1 | SEREX | [57] |
| MAGT1            | NM_032121.5      | Magnesium transporter 1 | SEREX | [41] |
| MMP1             | NM_002421        | Metalloproteinase 1 | SEREX | [41] |
| MYBBP1A          | NM_001105538     | MYB binding protein 1a | Protein array | [48] |
| NAV2             | NM_145117.4      | Neuron navigator 2 | SEREX | [48] |
| PARC             | NM_015089        | p53-associated parkin-like cytoplasmic protein | SEREX | [48] |
recognized by most, if not all, serum samples from patients with AIS and TIA. Thus, in the following experiments, GST–DIDO1N, not GST–DIDO1C, was used for the measurement of antibody levels.

Elevation of serum DIDO1-Ab, FOXJ2-Ab, and CPSF2-Ab levels in patients with TIA or AIS

We then examined the levels of anti-DIDO1_N protein, anti-FOXJ2 peptide (bFOXJ2-426), and anti-CPSF2 peptide (bCPSF2-607) antibodies (abbreviated as DIDO1-Ab, FOXJ2-Ab, and CPSF2-Ab, respectively) in serum samples from patients with TIA or AIS. Serum samples from HDs were obtained from the Port Square Kashiwado Clinic and compared with those from patients with TIA and AIS obtained from Chiba Prefectural Sawara Hospital, Chiba Rosai Hospital, Chiba Aoba Municipal Hospital, and Chiba Medical Center. AlphaLISA demonstrated that the serum levels of DIDO1-Ab, FOXJ2-Ab, and CPSF2-Ab were significantly higher in patients with AIS than in HDs (Fig. 2a, g and 3d). DIDO1-Abs and FOXJ2-Abs but not CPSF2-Abs were also elevated in patients with TIA as compared with those in HDs. At a cutoff value of the

| Abbreliated name | Accession number | Full name | Screening method | Reference |
|------------------|------------------|-----------|-----------------|-----------|
| PDCD11           | NM_014976.2      | Programmed cell death 11 | SEREX | [40, 58] |
| PFKFB3           | NM_004566        | 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase 3 | SEREX | |
| PHF20            | NM_016436        | PHD finger protein 20 | SEREX | |
| PPP1R15A         | NM_014330        | Protein phosphatase 1 regulatory subunit 1A | SEREX | [39, 59] |
| PRCP             | NM_005040.1      | Prolylcarboxypeptidase | Protein array | [60] |
| PSAP             | NM_002778        | Prosaposin | SEREX | |
| RANBP2L1         | NM_005054        | RAN binding protein 2-like 1 | SEREX | |
| RBCK1            | NM_031229        | RanBP-type and C3HC4-type zinc finger containing 1 | SEREX | |
| RBPJ             | NM_005349        | Recombination signal binding protein for immunoglobulin kappa J region | SEREX | |
| ROCK1            | NM_005406        | Rho-associated, coiled-coil containing protein kinase 1 | SEREX | |
| RPA1             | NM_002945        | Replication protein A1 | SEREX | |
| RPA2             | NM_002946        | Replication protein A2 | SEREX | [39] |
| RPL3 R           | NM_000967        | Ribosomal protein L3t | SEREX | |
| SC65             | BC007942         | Synaptosomal complex protein SC65 | SEREX | [39] |
| SH3BP5           | NM_004844        | SH3 domain-binding protein 5 | Protein array | [51] |
| SMARCA4          | NM_001128847     | SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4 | SEREX | |
| SNX16            | NM_022133.4      | Sorting Nexins 16 | SEREX | [37] |
| SOSTDC1          | NM_015464        | Sclerostin domain containing 1 | Protein array | [48] |
| SPARC            | NM_003118        | Secreted protein acidic and cysteine-rich | SEREX | |
| SPOCK1           | NM_004598        | SPARC (osteonectin), cwcw and kazal like domains proteoglycan 1 | SEREX | [56] |
| TBC1D2           | NM_001267571     | TBC1 domain family, member 2 | SEREX | |
| TBC1D4           | NM_014832        | TBC1 domain family, member 4 | SEREX | |
| TDX261           | NM_144582        | Testis expressed 261 | SEREX | |
| TFAM             | NM_003201        | Transcription factor A, mitochondrial | Protein array | [48] |
| THBS1            | NM_003246        | Thrombospondin 1 | SEREX | |
| TMEFF1           | NM_003692        | Transmembrane protein with EGF-like and two follistatin-like domains 1 | SEREX | |
| TOP3B            | NM_003935        | DNA topoisomerase III beta | Protein array | [48] |
| TUBB2C           | NM_006088        | Tubulin, beta 2C | SEREX | [56] |
| TYMS             | NM_001071        | Thymidylate synthetase | SEREX | |
| WDR36            | NM_139281.2      | T cell activation WD repeat protein | SEREX | [39] |
| XPO1             | NM_003400.3      | Exportin 1 | SEREX | |
| XRCC4            | NM_022406        | X-ray repair cross complementing 4 | SEREX | |
| ZFP36L1          | NM_004926        | ZFP36 ring finger protein like 1 | SEREX | |
mean HD value plus 2 standard deviation (SD), the DIDO1-Ab positive rate in HDs and patients with TIA, AIS, and cCl was 6.7%, 15.2%, 17.5%, and 15.4%, respectively (Table 2). Their FOXJ2-Ab and CPSF2-Ab positive rates were 5.6%, 14.1%, 19.6%, and 20.0%, respectively, and 4.2%, 16.3%, 14.9%, and 21.5%, respectively.

The serum levels of anti-bDIDO1-297 peptide antibodies (DIDO1pep-Abs) were also higher in patients with TIA and AIS than in HDs (Supplementary Figure S1).

**Elevation of serum DIDO1, FOXJ2, and CPSF2 antibody levels in patients with AMI or DM**

We then examined DIDO1-Ab, FOXJ2-Ab, and CPSF2-Ab in HDs and patients with AMI and DM. Serum samples from patients with AMI were obtained from Kyoto University Hospital, those from patients with DM were obtained from Chiba University Hospital, and those from HDs were obtained from the Port Square Kashiwado Clinic. The mean age (±SD) of HDs and patients with AMI and DM was 58.29 ± 5.63, 58.20 ± 8.50, and 58.37 ± 9.11 years, respectively. A total of 128 samples each of HDs and patients with AMI and type 2 DM were assayed simultaneously using AlphaLISA on a 384-well plate. Serum DIDO1-Ab levels were not visibly different between the serum samples from HDs and those from patients with AMI or DM (Fig. 3a). However, serum FOXJ2-Ab levels were significantly higher in patients with AMI or DM than in HDs (Fig. 3d). Using cutoff values as described in the previous section, positive rates were 3.1% in HDs, 34.4% in patients with AMI, and 22.7% in those with DM (Table 3). Serum CPSF2-Ab levels were significantly higher in patients with DM (although not in those with
AMI) than in HDs (Fig. 3g). The positive rate of CPSF2-Ab in patients with DM was 13.3% (Table 2).

Next, we examined antibody levels in serum samples from patients with CKD, which is also closely related to atherosclerosis. Patients with CKD were divided into three groups: type 1 (diabetic kidney disease), type 2 (nephrosclerosis), and type 3 (glomerulonephritis). Serum samples from patients with CKD were obtained from the Kumamoto cohort and those from HDs were obtained from Chiba University. All CKD groups had significantly higher serum DIDO1-Ab and FOXJ2-Ab levels than HDs (Fig. 4a and e). The positive rates of DIDO1-Ab in HDs and patients with type 1, type 2, and type 3 CKD were 13.3%, 18.6%, and 33.3%, respectively. ROC analysis was performed to assess the ability of DIDO1-Ab (b, c), FOXJ2-Ab (e, f), and CPSF2-Ab (h, i) to detect TIA and AIS. The numbers in the figures indicate the cutoff values for marker levels, and the numbers in parentheses indicate the sensitivity (left) and specificity (right). The areas under the curve (AUC), and 95% confidence intervals (CI) are also shown in Table 5.
type 3 CKD were 7.3%, 43.4%, 37.5%, and 26.8%, respectively, and those of FOXJ2-Ab were 3.7%, 28.3%, 34.4%, and 13.8%, respectively (Table 4). No apparent difference was found in CPSF2-Ab levels between HDs and patients with any type of CKD (Fig. 4i, Table 4).

ROC analysis
The results of the ROC analysis are shown in Figs. 2b, c, e, f, h, i, 3b, c, e, f, h, i, 4b, c, d, f, g, h, j, k, l, S1B, and S1C and summarized in Table 5, in which the area under the curve (AUC), 95% CI, cutoff value, sensitivity, specificity, and P value are shown. Serum anti-DIDO1 antibody levels showed markedly high AUC values against CKD. The AUCs of DIDO1-Ab versus type 1, type 2, and type 3 CKD were 0.8665, 0.8728, and 0.8227, respectively. Thus, irrespective of the CKD type, DIDO1-Ab may discriminate kidney failure. The AUCs of DIDO1-Ab versus TIA and AIS were 0.6819 and 0.6476, respectively, and similar values were observed for DIDO1pep-Ab (0.6503 and 0.6611, respectively). No significant increase above 0.6 was observed in AUCs of DIDO1-Ab versus AMI and DM.
AUCs of FOXJ2-Ab were > 0.65 versus TIA, AIS, AMI, DM, and CKD, among which AUC was the highest versus type 2 CKD (0.8769; Table 4). AUC versus DM was relatively low (0.6584). Thus, FOXJ2-Ab may be associated with kidney failure and atherosclerosis, but it does not primarily reflect DM. However, CPSF2-Ab was not associated with AMI or type 1/type 2/type 3 CKD. The lowest P values were observed versus AIS and DM, suggesting that CPSF2-Abs reflect diabetic AIS.

Table 2 Comparison of the serum antibody levels of HDs versus those of patients with transient ischemic attack (TIA) or acute ischemic stroke (AIS)

| Sample information | HD | TIA | AIS |
|--------------------|----|-----|-----|
| Total sample number | 285 | 92  | 464 |
| Male/female        | 188/97 | 55/37 | 271/193 |
| Age (average ± SD) | 52.3 ± 11.7 | 70.2 ± 11.6 | 75.5 ± 11.5 |

| Alpha analysis (antibody level) | HD | TIA | AIS |
|---------------------------------|----|-----|-----|
| DIDO1-Ab                        | 4736 | 6950 | 7309 |
| FOXJ2-Ab                        | 8568 | 12,390 | 13,255 |
| CPSF2-Ab                        | 2515 | 3792 | 3291 |
| SD                              | 3179 | 5251 | 5415 |
| Cutoff value                    | 11,095 | 12,390 | 7309 |
| Positive no.                    | 19 | 14 | 81 |
| Positive (%)                    | 6.7% | 15.2% | 17.5% |

The upper panel indicates the numbers of all samples and samples from males and females as well as the ages (average ± SD). The lower panel summarizes the serum antibody levels (alpha luminescent photon count) examined by AlphaLISA. Purified DIDO1 (amino acids 1-275)-glutathione S-transferase (GST) protein and synthetic peptides, bFOXJ2-426 and bCPSF2-607, were used as antigens. The cutoff values were determined as the average HD values plus two SDs, and positive samples for which the Alpha counts exceeded the cutoff value were scored. P values were calculated using the Kruskal-Wallis test. P values lower than 0.05 and positive rates higher than 10% are marked in bold. Box-whisker plots of the same results are shown in Fig. 2a, d, and g.

However, serum FOXJ2-Ab levels were significantly higher in patients with colorectal carcinoma but not in those with other types of cancer than in HDs.

Association of serum DIDO1-, FOXJ2-, and CPSF2-Ab levels with autoimmune diseases

Autoantibodies may have causal effects on autoimmune diseases such as Sjögren’s syndrome, rheumatoid arthritis, SLE, and ulcerative colitis. Some of these autoimmunity-related characteristics are known to be involved in the development of atherosclerosis [61–64]. We examined antibody levels in serum samples from patients with Sjögren’s syndrome, rheumatoid arthritis, SLE, and ulcerative colitis. Serum DIDO1-Ab and FOXJ2-Ab levels were significantly higher in patients with rheumatoid arthritis and SLE (but not in those with Sjögren’s syndrome or ulcerative colitis) than in HDs (Supplementary Table S2). Serum CPSF2-Ab levels were higher in patients with rheumatoid arthritis (but not in those with Sjögren’s syndrome, SLE, or ulcerative colitis) than in HDs.

Association of serum DIDO1-Ab, FOXJ2-Ab, and CPSF2-Ab levels with pulmonary diseases

OSA is frequently accompanied by hypertension. Serum anti-COPE was identified by SEREX screening using serum samples from patients with atherosclerosis, and
its level was elevated in patients with OSA compared with in HDs [65]. Pulmonary diseases including CTEPH and PAH are distinct from hypertension but could have an inflammatory condition similar to that in hypertension (e.g., elevation of Pentraxin 3 level) [66]. Serum FOXJ2-Ab levels were higher in patients with CTEPH and PAH (but not in those with OSA) than in HDs, whereas serum DIDO1-Ab and CPSF2-Ab levels did not show any apparent difference between HDs and patients with CTEPH, PAH, or OSA (Supplementary Table S3).

Correlation analysis
Comparative analysis of serum antibody levels and subject data was performed using 851 serum samples
obtained from Chiba Prefectural Sawara Hospital including 188 serum samples from HDs, 162 from patients with DSWMH, 18 from patients with asympt-CI, 66 from patients with TIA, 351 from patients with AIS, 66 from patients with cCI, and 66 from disease controls. Other subject information is shown in Supplementary Table S4. Comparison using Mann–Whitney U test revealed that serum DIDO1pep-Ab, FOXJ2-Ab, and CPSF2-Ab levels were significantly higher in patients with TIA, AIS, and cCI (but not in those with DSWMH) than in HDs (Table 6, uppermost panel). Then, antibody levels were compared between males and females; those with or without DM, hypertension, CVD, and dyslipidemia; and those with or without smoking and alcohol intake habits. Hypertension was defined as a history of systolic blood pressure of > 140 mmHg, diastolic blood pressure of > 90 mmHg, or use of antihypertensive agents. Significantly higher serum DIDO1pep-Ab levels were observed in patients with hypertension, CVD, dyslipidemia, or a smoking habit (but not in those with DM) than in their control groups (Table 6, lower panels). Serum FOXJ2-Ab levels showed similar results, except that they were not correlated with dyslipidemia. Meanwhile, serum CPSF2-Ab levels were associated with DM, hypertension, and smoking habit but not with CVD or dyslipidemia. Sex and alcohol intake displayed no association with any of these three antibody levels.

Spearman’s rank-order correlation analysis was performed to determine the correlation between serum antibody levels of DIDO, FOXJ2, and CPSF2 peptides and subject parameters including general information such as age, body height, weight, body mass index, and degree of artery stenosis (maximum intima media thickness, max IMT). The following blood test data were also included: albumin/globulin ratio, aspartate aminotransferase, alanine amino transferase, alkaline phosphatase, lactate dehydrogenase, total bilirubin, cholinesterase, γ-glutamyl transpeptidase, total protein, albumin, blood

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### Table 4 Comparison of serum antibody levels of HDs versus those of patients with chronic kidney disease (CKD)

| 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 ± SD) | 44.1 ± 11.2 | 66.0 ± 10.4 | 76.0 ± 9.8 | 62.0 ± 11.7 |

#### Alpha analysis (antibody level)

|        | DIDO1-Ab | FOXJ2-Ab | CPSF2-Ab |
|--------|----------|----------|----------|
| HD     | DIDO1-Ab | 3166     | 1300     | 914      |
|        | SD       | 1423     | 517      | 298      |
|        | Cutoff value | 6012     | 2334     | 1509     |
|        | Positive no. | 6        | 3        | 3        |
|        | Positive rate (%) | 7.3% | 3.7% | 3.7% |
| Type 1-CKD | Average | 6805     | 2141     | 939      |
|        | SD       | 4675     | 1330     | 382      |
|        | Positive no. | 63       | 41       | 7        |
|        | Positive rate (%) | 43.4% | 28.3% | 48.9% |
|        | P (vs HD) | < 0.0001 | < 0.0001 | 0.579 |
| Type 2-CKD | Average | 6693     | 2245     | 1020     |
|        | SD       | 3347     | 930      | 281      |
|        | Positive no. | 12       | 11       | 2        |
|        | Positive rate (%) | 37.5% | 34.4% | 6.3% |
|        | P (vs HD) | < 0.0001 | < 0.0001 | 0.081 |
| Type 3-CKD | Average | 5264     | 1770     | 936      |
|        | SD       | 2161     | 829      | 421      |
|        | Positive no. | 33       | 17       | 10       |
|        | Positive rate (%) | 26.8% | 13.8% | 8.1% |
|        | P (vs HD) | < 0.0001 | < 0.0001 | 0.656 |

CKD types 1, 2, and 3 correspond to diabetic kidney disease, nephrosclerosis, and glomerulonephritis, respectively. The upper panel indicates the numbers of all samples and samples from males and females as well as age (average ± SD). The lower panel summarizes the serum antibody levels examined by AlphaLISA using purified DIDO1-GST protein and synthetic bCPSF2 and bFOXJ2 peptides as antigens as described in the legend of Table 2. Box-whisker plots of the same results are shown in Fig. 4a, e, and i. P values lower than 0.05 and positive rates higher than 10% are marked in bold.
Table 5  Receiver operating characteristic (ROC) analysis

|                  | DIDO1-Ab vs TIA  | DIDO1-Ab vs AIS  |
|------------------|------------------|------------------|
| AUC              | 0.6767           | 0.6023           |
| 95% CI           | 0.6001–0.7533    | 0.5367–0.6680    |
| Cutoff value     | 14,184           | 19,924           |
| Sensitivity (%)  | 77.9%            | 27.9%            |
| Specificity (%)  | 49.6%            | 91.9%            |
| P value          | < 0.0001         | 0.0033           |

|                  | DIDO1-Ab vs AMI  | DIDO1-Ab vs DM  |
|------------------|------------------|------------------|
| AUC              | 0.5163           | 0.5347           |
| 95% CI           | 0.4454–0.5875    | 0.4638–0.6057    |
| Cutoff value     | 13,519           | 10,700           |
| Sensitivity (%)  | 22.7%            | 46.9%            |
| Specificity (%)  | 85.8%            | 63.8%            |
| P value          | 0.650            | 0.338            |

|                  | DIDO1-Ab vs type 1 CKD | DIDO1-Ab vs type 2 CKD | DIDO1-Ab vs type 3 CKD |
|------------------|------------------------|------------------------|------------------------|
| AUC              | 0.8665                 | 0.8728                 | 0.8227                 |
| 95% CI           | 0.8144 to 0.9186       | 0.8092 to 0.9364       | 0.7611 to 0.8843       |
| Cutoff value     | 3375                   | 3511                   | 3158                   |
| Sensitivity (%)  | 93.1%                  | 90.6%                  | 91.9%                  |
| Specificity (%)  | 69.1%                  | 70.2%                  | 63.1%                  |
| P value          | < 0.0001               | < 0.0001               | < 0.0001               |

|                  | DIDO1pep-Ab vs TIA    | DIDO1pep-Ab vs AIS    |
|------------------|-----------------------|-----------------------|
| AUC              | 0.6503                | 0.6611                |
| 95% CI           | 0.5751–0.7256         | 0.6138–0.7084         |
| Cutoff value     | 4662                  | 8413                  |
| Sensitivity (%)  | 87.9%                 | 43.9%                 |
| Specificity (%)  | 38.3%                 | 81.9%                 |
| P value          | 0.0003                | < 0.0001              |

|                  | FOXJ2-Ab vs TIA       | FOXJ2-Ab vs AIS       | CPSF2-Ab vs TIA       | CPSF2-Ab vs AIS       |
|------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| AUC              | 0.6696                | 0.7006                | 0.6314                | 0.6369                |
| 95% CI           | 0.6066 to 0.7326      | 0.6626 to 0.7386      | 0.5631–0.6997         | 0.5970–0.6768         |
| Cutoff value     | 8978                  | 8920                  | 2643                  | 2644                  |
| Sensitivity (%)  | 60.9%                 | 65.1%                 | 54.4%                 | 57.8%                 |
| Specificity (%)  | 66.0%                 | 66.0%                 | 67.7%                 | 67.7%                 |
| P value          | < 0.0001              | < 0.0001              | 0.0002                | < 0.0001              |

|                  | FOXJ2-Ab vs AMI       | FOXJ2-Ab vs DM        | CPSF2-Ab vs AMI       | CPSF2-Ab vs DM        |
|------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| AUC              | 0.7418                | 0.6584                | 0.5522                | 0.6464                |
| 95% CI           | 0.6813 to 0.8022      | 0.5922 to 0.7245      | 0.4817 to 0.6226      | 0.5792 to 0.7136      |
| Cutoff value     | 14,437                | 20,978                | 5356                  | 6145                  |
| Sensitivity (%)  | 68.0%                 | 34.4%                 | 63.3%                 | 55.5%                 |
| Specificity (%)  | 71.1%                 | 91.4%                 | 49.2%                 | 70.3%                 |
| P value          | < 0.0001              | < 0.0001              | 0.149                 | < 0.0001              |
urea nitrogen, creatinine, estimated glomerular filtration rate, uric acid, amylase, total cholesterol, high-density lipoprotein cholesterol, triglyceride, sodium, potassium, chloride, calcium, inorganic phosphate, iron, C-reactive protein, low-density lipoprotein cholesterol, white blood cells, red blood cells, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution width, platelets, mean platelet volume, procalcitonin, platelet distribution width, blood sugar, and glycated hemoglobin (HbA1c).

All three antibody levels were correlated with age and max IMT but inversely correlated with height and weight and cholinesterase, total protein, and albumin levels. Serum DIDO1 and FOXJ2 antibody levels, but not serum CPSF2 antibody level, were correlated with alkaline phosphatase, white blood cell count, and mean corpuscular volume (Table 7). Blood sugar and HbA1c, which reflect DM, were not correlated with these antibody levels, except for a slight correlation ($P = 0.195$) between serum DIDO1pep-Ab level and blood sugar.

**Immunohistochemical analysis of antigenic proteins**

Assuming that autoantibodies against DIDO1, FOXJ2, and CPSF2 peptides develop in patients with atherosclerotic diseases, these antigenic proteins should be expressed at high levels in atherosclerotic lesions. As such, we also examined the expressions of antigenic proteins in surgically resected carotid atherosclerotic plaques via immunohistochemistry. The DIDO1 and CPSF2 proteins were predominantly expressed in the intima of atherosclerotic plaques, similar to the localization of vimentin and smooth muscle actin, which are markers for smooth muscle cells (Fig. 5). DHPS, reported as an atherosclerosis marker [55], was also expressed in smooth muscle cells. The expression of FOXJ2 showed a similar pattern as that of CD31- and CD34-positive vascular endothelial cells. CD68 expression in macrophages was not similar to any of the other antigen expressions (Fig. 5).

**JPHC cohort analysis**

We conducted a case–control study nested within the JPHC-based Prospective Study, which involved approximately 30,000 plasma samples [52, 53]. The antibody level against the DIDO1 protein was positively and strongly associated with a risk of AIS: odds ratios (95% CIs) were 3.99 (1.93–8.23), 3.40 (1.62–7.13), and 4.02 (1.94–8.35) for those with the second, third, and highest quartiles of antibody levels, respectively, versus for those with the lowest quartile (Table 8). Likewise, the antibody levels of the DIDO1, FOXJ2, and CPSF2 peptides were positively correlated with a risk of cerebral infarction: odds ratios (95% CIs) of the highest quartile were 2.66 (1.43–4.95), 2.24 (1.27–3.95), and 2.41 (1.33–4.37), respectively. These results indicate that the antibody markers against the DIDO1 protein and DIDO1, FOXJ2, and CPSF2 peptides are useful in predicting the onset of AIS.

**Discussion**

Three novel antibody markers for atherosclerosis

We performed large-scale screening using SEREX and the protein microarray method and identified 69 candidate antigenic proteins related to atherosclerosis (Table 1). In the present study, we focused on three antigens—DIDO1, FOXJ2, and CPSF2—that appeared to be of

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**Table 5** Receiver operating characteristic (ROC) analysis (Continued)

|                      | FOXJ2-Ab vs type 1 CKD | FOXJ2-Ab vs type 2 CKD | FOXJ2-Ab vs type 3 CKD |
|----------------------|------------------------|------------------------|------------------------|
| AUC                  | 0.7812                 | 0.8769                 | 0.7151                 |
| 95% CI               | 0.7200 to 0.8424       | 0.8124 to 0.9413       | 0.6439 to 0.7862       |
| Cutoff value         | 1236                   | 1331                   | 1354                   |
| Sensitivity (%)      | 83.5%                  | 93.8%                  | 69.9%                  |
| Specificity (%)      | 59.5%                  | 71.4%                  | 69.1%                  |
| $P$ value            | < 0.0001               | < 0.0001               | < 0.0001               |

|                      | CPSF2-Ab vs Type-1 CKD | CPSF2-Ab vs Type-2 CKD | CPSF2-Ab vs Type-3 CKD |
|----------------------|------------------------|------------------------|------------------------|
| AUC                  | 0.5040                 | 0.6387                 | 0.5196                 |
| 95% CI               | 0.4262–0.5817          | 0.5274–0.7500          | 0.4395–0.5996          |
| Cutoff value         | 641.5                  | 901                    | 706                    |
| Sensitivity (%)      | 11.7%                  | 65.6%                  | 29.3%                  |
| Specificity (%)      | 93.9%                  | 62.2%                  | 80.5%                  |
| $P$ value            | 0.022                  | 0.022                  | 0.635                  |

Area under the curve (AUC), 95% CI, cutoff value, sensitivity (%), specificity (%), and $P$ value of the ROC analysis are shown. Purified GST-DIDO1 protein and synthetic peptides—bDIDO1-297 (DIDO1pep), bFOXJ2-426, and bCPSF2-607—were used as antigens. $P$ values lower than 0.05 and AUCs higher than 0.7 are marked in bold.
Table 6: Correlation analysis of antibody levels against synthetic bDIDO1, bCPSF2, and bFOXJ2 peptides with data of subjects in the Sawara Hospital cohort

| Present disease | HD | DSWMH | asympt-Cl | TIA | AIS | cCI |
|-----------------|----|-------|-----------|-----|-----|-----|
| Sample number   | 188| 162   | 18        | 66  | 351 | 66  |
| DIDO1pep-Ab level | Average | 3381 | 3523 | 3481 | 4443 | 4688 | 4347 |
|                 | SD  | 1660  | 1750      | 2099| 2576| 2740 | 3017 |
| P value (vs HD) | –  | ns    | ns        | < 0.01 | < 0.001 | < 0.05 |
| FOXJ2-Ab level | Average | 4627 | 4995 | 4902 | 5794 | 6298 | 7022 |
|                 | SD  | 1972  | 2232      | 1854| 2368| 3308 | 5646 |
| P value (vs HD) | –  | ns    | ns        | < 0.01 | < 0.001 | < 0.001 |
| CPSF2-Ab level | Average | 7322 | 7571 | 8312 | 11,778 | 8722 | 10,088 |
|                 | SD  | 3415  | 2942      | 2461| 16,843| 3970 | 4240 |
| P value (vs HD) | –  | ns    | < 0.05    | < 0.01 | < 0.001 | < 0.001 |

| Sex              | Male | Female |
|------------------|------|--------|
| Sample number    | 528  | 389    |
| DIDO1pep-Ab level | Average | 4081 | 4038 |
|                 | SD  | 2493  | 2244    |
| P value (vs Male)| 0.781|
| FOXJ2-Ab level | Average | 5772 | 5443 |
|                 | SD  | 3077  | 3084    |
| P value (vs male)| 0.111|
| CPSF2-Ab level | Average | 8633 | 8420 |
|                 | SD  | 5493  | 6553    |
| P value (vs male)| 0.155|

| Complication | DM– | DM+ |
|--------------|-----|-----|
| Sample number | 732 | 180 |
| DIDO1pep-Ab level | Average | 4059 | 4047 |
|                 | SD  | 2469  | 2027    |
| P value (vs DM–)| 0.949|
| FOXJ2-Ab level | Average | 5589 | 5763 |
|                 | SD  | 3104  | 2987    |
| P value (vs DM–)| 0.488|
| CPSF2-Ab level | Average | 8319 | 9437 |
|                 | SD  | 5373  | 7822    |
| P value (vs DM–)| 0.015|

| Complication | HT– | HT+ |
|--------------|-----|-----|
| Sample number | 347 | 565 |
| DIDO1pep-Ab level | Average | 3830 | 4196 |
|                 | SD  | 2217  | 2477    |
| P value (vs HT–)| 0.021|
| FOXJ2-Ab level | Average | 5093 | 5948 |
|                 | SD  | 2373  | 3405    |
| P value (vs HT–)| < 0.0001|
| CPSF2-Ab level | Average | 7699 | 9065 |
|                 | SD  | 6095  | 5804    |
| P value (vs HT–)| < 0.0001
Table 6 Correlation analysis of antibody levels against synthetic bDIDO1, bCPSF2, and bFOXJ2 peptides with data of subjects in the Sawara Hospital cohort (Continued)

| Complication | CVD− | CVD+ |
|--------------|------|------|
| Sample number | 861  | 51   |
| DIDO1pep-Ab level | Average | 4003 | 4966 |
|                | SD    | 2360 | 2673 |
| $P$ value (vs CVD−) | 0.015|
| FOXJ2-Ab level | Average | 5559 | 6712 |
|                | SD    | 3050 | 3408 |
| $P$ value (vs CVD−) | 0.022|
| CPSF2-Ab level | Average | 8499 | 9232 |
|                | SD    | 6037 | 4239 |
| $P$ value (vs CVD−) | 0.142|

| Complication | Lipidemia− | Lipidemia+ |
|--------------|------------|------------|
| Sample number | 649       | 263        |
| DIDO1pep-Ab level | Average | 4158 | 3806 |
|                | SD    | 2497 | 2073 |
| $P$ value (vs Lipidemia−) | 0.029|
| FOXJ2-Ab level | Average | 5702 | 5428 |
|                | SD    | 3171 | 2841 |
| $P$ value (vs Lipidemia−) | 0.203|
| CPSF2-Ab level | Average | 8146 | 9531 |
|                | SD    | 3583 | 9534 |
| $P$ value (vs Lipidemia−) | 0.145|

| Lifestyle | Non-smoker | Smoker |
|-----------|------------|--------|
| Sample number | 474       | 441    |
| DIDO1pep-Ab level | Average | 3732 | 4425 |
|                | SD    | 2037 | 2676 |
| $P$ value (vs non-smoker) | < 0.0001|
| FOXJ2-Ab level | Average | 5192 | 6111 |
|                | SD    | 2793 | 3309 |
| $P$ value (vs non-smoker) | < 0.0001|
| CPSF2-Ab level | Average | 8214 | 8901 |
|                | SD    | 6086 | 5801 |
| $P$ value (vs non-smoker) | 0.002|

| Lifestyle | Alcohol− | Alcohol+ |
|-----------|----------|----------|
| Sample number | 334 | 581 |
| DIDO1pep-Ab level | Average | 4001 | 4103 |
|                | SD    | 2236 | 2476 |
| $P$ value (vs Alcohol−) | 0.527|
| FOXJ2-Ab level | Average | 5691 | 5603 |
|                | SD    | 3542 | 2793 |
| $P$ value (vs Alcohol−) | 0.698|
| CPSF2-Ab level | Average | 8559 | 8591 |
|                | SD    | 6946 | 5341 |
| $P$ value (vs Alcohol−) | 0.361|

The subjects were divided as follows: sex (male and female); presence (+) or absence (−) of complication of DM, hypertension (HT), cardiovascular disease (CVD), or dyslipidemia, and lifestyle factors (smoking and alcohol intake habits). Antibody levels (Alpha counts) were compared using the Kruskal–Wallis test (upper panel) and the Mann–Whitney U test (lower panels). Sample numbers, averages, and SDs of counts as well as $P$ values are shown. Significant correlations ($P < 0.05$) are marked in bold.
much interest in relation to AIS. The presence of antibodies against these proteins was confirmed by Western blotting (Fig. 1). We then examined epitopes and selected bDIDO1-297, bFOXJ2-426, and bCPSF2-607 as useful antigenic peptides to measure serum antibody levels. The amino-terminal half of Dido1 was also used as an antigen. Serum antibody levels of these antigens were more elevated in patients with AIS and TIA than in HDs (Fig. 2, Supplementary Figure S1). All of bDIDO1-297, bFOXJ2-426, and bCPSF2-607 were closely correlated with max IMT (Table 7), which is a typical index of the development of atherosclerosis leading to AIS and CVD [67–70]. Thus, these serum antibodies can be markers for atherosclerosis. A case-control study nested within the JPHC-based Prospective Study showed that the three antibody markers are associated with the risk of cerebral infarction and indicated that these markers are useful in predicting the onset of cerebral infarction (Table 8). However, they had distinct characteristics.

The Dido1 protein was first identified as a regulator of apoptosis [71]. Serum Dido1 pep-Ab levels were elevated in patients with TIA, AIS, cCl, CKD, rheumatoid arthritis, and SLE but not in those with AMI, DM, any type of cancer, or ulcerative colitis (Figs. 2, 3, and 4; Tables 2, 3, and 4; Supplementary Tables S1 and S2). Serum CPSF2-Ab levels were correlated with aortic hypertension (Table 6) but not with pulmonary hypertension such as CTEPH and PAH (Supplementary Table S3). Moreover, the levels correlated most closely with max IMT (Table 7), indicating that CPSF2-Ab can mainly detect DM-caused atherosclerosis leading to AIS.

**Relationship between BMP/TGF-β and atherosclerosis**

Bone morphogenetic proteins (BMPs) are involved in the transforming growth factor-β (TGF-β) superfamily. It is well documented that BMP signals play important roles in the development of atherosclerosis [76, 77], BMP-2 and BMP-4 expressions were elevated in atherosclerotic endothelium [78, 79], and plasma BMP-2 levels are elevated in patients with type 2 DM [80]. Chronic infusion of BMP-4 induces endothelial dysfunction and hypertension [81], and treatment with the BMP antagonist, matrix Gla protein, and BMP inhibitors prevents the development of ATS [82, 83]. On the other hand, the knockdown of the BMP type II receptor BMPRII accelerates ATS [84]. Therefore, BMP family members may play a subtle regulatory role in the development of ATS. It should be noted that Dido1 is the target gene of BMP and promotes cell attachment, migration, invasion, and apoptosis resistance in melanoma [85].

CPSF proteins interact with Smad via Smicl and potentiate TGF-β/BMP-stimulated Smad-dependent transcriptional responses [86, 87]. We previously reported the elevation of autoantibodies against Sostdc1 and Nbl1/Dan, which are the antagonists of BMP, in patients with AIS [48] and Osa [36], respectively. As such, it is possible that some, if not all, autoantibodies against TGF-β/BMP-related proteins play causal or suppressive roles in the development of atherosclerosis-related diseases.

**Involvement of marker genes in development and differentiation**

Dido1 is the target gene of Oct4, Sox2, and Nanog; in reverse, Nanog and Oct4 are the target genes of Dido1 [88]. Thus, Dido1 plays a key role in the self-renewal of embryonic stem cells. Futterer suggested that Dido1 is a switchboard that regulates embryonic stem cell transition from pluripotency maintenance to differentiation [89]. During the development of atherosclerosis, smooth muscle cells differentiate into foam cells to form atheroma [90]. Highly expressed Dido1 in intimal smooth muscle cells (Fig. 5) may have an important role in their differentiation into foam cells.
| Parameter* | Number of XY pairs | DIDO1pep-Ab r value** | P value | FOXJ2pep-Ab r value | P value | CPSF2pep-Ab r value | P value |
|------------|--------------------|------------------------|--------|---------------------|--------|---------------------|--------|
| Age        | 851                | 0.2074                 | < 0.0001*** | 0.2688               | < 0.0001 | 0.1657               | < 0.0001 |
| Height     | 844                | −0.1227                | 0.0004  | −0.1229             | 0.0003  | −0.0799             | 0.0202  |
| Weight     | 848                | −0.1047                | 0.0023  | −0.1196             | 0.0005  | −0.0707             | 0.0396  |
| BMI        | 843                | −0.0311                | 0.3679  | −0.0552             | 0.1098  | −0.0343             | 0.3197  |
| max IMT    | 646                | 0.1908                 | < 0.0001 | 0.2717               | < 0.0001 | 0.2161               | < 0.0001 |
| A/G        | 820                | −0.0303                | 0.3858  | −0.0484             | 0.1662  | −0.0906             | 0.0094  |
| AST        | 848                | 0.0605                 | 0.0782  | 0.0205              | 0.5523  | −0.0496             | 0.1490  |
| ALT        | 847                | 0.0063                 | 0.8545  | −0.0079             | 0.8177  | −0.0800             | 0.0199  |
| ALP        | 786                | 0.0850                 | 0.0172  | 0.0743              | 0.0374  | 0.0319              | 0.3716  |
| LDH        | 822                | 0.0718                 | 0.0395  | 0.0291              | 0.4046  | −0.0134             | 0.7017  |
| tBil       | 830                | −0.0576                | 0.0972  | −0.0752             | 0.0304  | −0.1024             | 0.0031  |
| CHE        | 646                | −0.0895                | 0.0230  | −0.1671             | < 0.0001 | −0.0982             | 0.0125  |
| γ-GTP      | 795                | 0.0334                 | 0.3474  | 0.0240              | 0.4996  | −0.0028             | 0.9381  |
| TP         | 823                | −0.0971                | 0.0053  | −0.1443             | < 0.0001 | −0.1084             | 0.0018  |
| Albumin    | 832                | −0.0757                | 0.0289  | −0.1294             | 0.0002  | −0.1358             | < 0.0001 |
| BUN        | 846                | 0.0179                 | 0.6038  | 0.0431              | 0.2103  | −0.0381             | 0.2686  |
| CRE        | 842                | −0.0090                | 0.7946  | 0.0472              | 0.1714  | −0.0341             | 0.3233  |
| eGFR       | 758                | 0.0176                 | 0.6284  | −0.0255             | 0.4835  | 0.0230              | 0.5282  |
| UA         | 622                | 0.0336                 | 0.4023  | 0.0255              | 0.5261  | 0.0050              | 0.9006  |
| AMY        | 527                | −0.0780                | 0.0735  | −0.0422             | 0.3350  | −0.0391             | 0.3701  |
| T-CHO      | 744                | −0.0520                | 0.1568  | −0.0604             | 0.0994  | −0.1207             | 0.0010  |
| HDL-C      | 550                | −0.0458                | 0.2840  | −0.0521             | 0.2222  | 0.0553              | 0.1952  |
| TG         | 589                | 0.0199                 | 0.6303  | 0.0038              | 0.9274  | −0.0405             | 0.3261  |
| Na         | 833                | 0.0200                 | 0.5635  | 0.0233              | 0.5027  | 0.0005              | 0.9881  |
| K          | 832                | −0.0275                | 0.4280  | −0.0091             | 0.7928  | −0.0072             | 0.8359  |
| Cl         | 833                | 0.0056                 | 0.8708  | 0.0470              | 0.1752  | 0.0269              | 0.4376  |
| Ca         | 495                | −0.0210                | 0.6408  | −0.0815             | 0.0708  | −0.0405             | 0.3682  |
| IP         | 388                | −0.0023                | 0.9639  | −0.0465             | 0.3618  | 0.0546              | 0.2836  |
| Fe         | 400                | −0.0406                | 0.4185  | −0.0575             | 0.2526  | −0.0472             | 0.3465  |
| CRP        | 617                | 0.1172                 | 0.0035  | 0.0775              | 0.0552  | 0.1041              | 0.0096  |
| LDL-C      | 440                | −0.0513                | 0.2831  | −0.0771             | 0.1071  | −0.1180             | 0.0133  |
| WBC        | 846                | 0.1036                 | 0.0026  | 0.0848              | 0.0138  | 0.0417              | 0.2262  |
| RBC        | 846                | −0.0426                | 0.2155  | −0.0649             | 0.0596  | −0.0711             | 0.0386  |
| HGB        | 846                | −0.0113                | 0.7420  | −0.0329             | 0.3406  | −0.0672             | 0.0508  |
| HCT        | 846                | −0.0078                | 0.8214  | −0.0271             | 0.4317  | −0.0528             | 0.1249  |
| MCV        | 846                | 0.0683                 | 0.0472  | 0.0959              | 0.0053  | 0.0510              | 0.1387  |
| MCH        | 846                | 0.0474                 | 0.1681  | 0.0776              | 0.0242  | 0.0081              | 0.8136  |
| MCHC       | 846                | −0.0149                | 0.6659  | −0.0253             | 0.4635  | −0.0617             | 0.0728  |
| RDW        | 846                | 0.0489                 | 0.1551  | 0.0449              | 0.1928  | 0.0529              | 0.1245  |
| PLT        | 846                | −0.0047                | 0.8919  | −0.0443             | 0.1992  | 0.0128              | 0.7097  |
| MPV        | 846                | −0.0201                | 0.5589  | −0.0637             | 0.0646  | −0.0012             | 0.9716  |
| PCT        | 846                | −0.0030                | 0.9312  | −0.0568             | 0.0993  | 0.0188              | 0.5853  |
FOXJ2 expression is also regulated by Oct4 and involved in oocyte development [91]. Transient FOXJ2 transgenesis experiments have shown that FOXJ2 overexpression has a lethal effect on embryonic development from E10.5 [92]. FOXJ2 is also involved in differentiation and inhibits TGF-β1-induced epithelial–mesenchymal transition [93]. Thus, high FOXJ2 expression (Fig. 5) may affect otherwise normally functioning vascular endothelial cells.

### Relationship between atherosclerosis and cancer

BMP-induced DIDO1 promotes cell attachment, migration, invasion, and apoptosis resistance in melanoma [85]. Serum FOXJ2-Ab levels, which correlated well with hypertension, were elevated in patients with colorectal carcinoma (P < 0.001) but not in those with esophageal squamous cell carcinoma, gastric cancer, breast cancer, or pancreatic cancer (Supplementary Table S1). This is consistent with the report that hypertension is also a risk factor for cancer. (Continued)

### Table 7 Correlation analysis of serum antibody levels against synthetic bDIDO1, bCPSF2, and bFOXJ2 peptides with data on subjects in the Sawara Hospital cohort

| Parameter* | Number of XY pairs | DIDO1pep-Ab r value** | P value | FOXJ2pep-Ab r value | P value | CPSF2pep-Ab r value | P value |
|------------|-------------------|-----------------------|---------|---------------------|---------|---------------------|---------|
| PDW        | 846               | -0.0151               | 0.6611  | -0.0587             | 0.0886  | -0.0109             | 0.7512  |
| BS         | 783               | 0.0834                | 0.0195  | 0.0678              | 0.0581  | 0.0644              | 0.0718  |
| HbA1c      | 655               | -0.0204               | 0.6031  | 0.0170              | 0.6644  | -0.0277             | 0.4789  |

*Subjects’ data used were age, height, weight, body mass index (BMI), maximum intima–media thickness (max IMT), albumin/globulin ratio (A/G), aspartate aminotransferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), total bilirubin (TBIL), cholinesterase (CHE), γ-glutamyl transpeptidase (γ-GTP), total protein (TP), albumin, blood urea nitrogen (BUN), creatinine (CRE), estimated glomerular filtration rate (eGFR), uric acid (UA), amylase (AMY), total cholesterol (T-CHO), high-density lipoprotein cholesterol (HDL-C), triglyceride (TG), sodium (Na), potassium (K), calcium (Ca), inorganic phosphate (IP), iron (Fe), C-reactive protein (CRP), low-density lipoprotein cholesterol (LDL-C), white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), red cell distribution width (RDW), platelets (PLT), mean platelet volume (MPV), procalcitonin (PCT), platelet distribution width (PDW), blood sugar (BS), and glycated hemoglobin (HbA1c)

**Correlation coefficients (r values) and P values obtained through Spearman’s correlation analysis are shown

***Significant correlations (P < 0.05) are marked in bold

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**Fig. 5** Immunohistochemical staining of antigenic marker proteins in the atherosclerotic lesions. Surgically resected carotid atherosclerotic plaques were stained using immunohistochemistry. The antibodies used were anti-DIDO1 (Aviva Systems Biology), anti-FOXJ2 (Thermo Fisher Scientific), anti-CPSF2 (GeneTex), and anti-DHPS (Proteintech) antibodies for comparison. The tissue was also stained with antibodies against smooth muscle cell marker, vimentin (VIM) and smooth muscle actin (SMA), vascular endothelial cell marker, CD31 and CD34, and macrophage marker, CD68.
factor for colorectal carcinoma but not for esophageal squamous cell carcinoma or gastric cancer [73, 94]. FOXJ2 overexpression is associated with poor prognosis, progression, and metastasis in nasopharyngeal carcinoma [95]. FOXQ1, a member of the FOX family, is overexpressed in colorectal cancer, and it enhances tumorigenicity and tumor growth [96]. However, it has been reported that FOXJ2 suppresses migration and invasion in extrahepatic cholangiocarcinoma [97], hepatocellular carcinoma [98], glioma [99], and breast cancer [100]. Thus, FOXJ2 can promote or suppress malignancy depending on cancer type, which may account for the colorectal carcinoma-selective association of FOXJ2-Abs (Supplementary Table S1).

CPSF2 has a suppressive role in cell invasion in thyroid cancer and cancer stem cell population [101]. It is involved in the 6-gene prognostic signature for hepatocellular carcinoma overall survival prediction [102]. Our results showed only a slight association of CPSF2-Abs with esophageal squamous cell carcinoma ($P < 0.01$) but not with other types of cancer (Supplementary Table S1). CPSF2-Ab may reflect DM-caused atherosclerosis as described above, and the causes of cancer and atherosclerosis overlap with each other. Thus, CPSF2-Abs may be associated indirectly with some types of cancer.

**Characteristics of antibody biomarkers**

Atherosclerosis progresses slowly over many years, finally leading to the onset of AIS or AMI. The prodromal stages of AIS and AMI may be accompanied by tissue destruction in arteries. The development of autoantibodies may be caused by high expressions of antigenic proteins in arteries followed by tissue destruction-induced exposure of antigens to immune cells. Repeated destruction/exposure can considerably increase antibody levels while keeping the antigen level low. Thus, antibody markers are much more sensitive than antigen markers. In addition, serum IgG proteins are highly stable and not easily degraded. As such, antibody markers are highly suitable for detecting trivial alterations caused by early-stage lesions. This is consistent with results that in this study, serum DIDO1-Ab, FOXJ2-Ab, and CPSF2-Ab were elevated not only in patients with AIS but also in those with TIA, a prodromal lesion of AIS (Fig. 2).

AIS is a severe disease that often leads to death. Once it occurs, even without death, affected patients require a long rehabilitation period, with this disease also being the first cause of being bedridden. However, if the onset of AIS is predicted, most patients can avoid it via an appropriate treatment. Therefore, the development of highly sensitive and predictive biomarkers is eagerly expected. We discovered that serum DIDO1-Ab, FOXJ2-Ab, and CPSF2-Ab are useful in predicting the onset of AIS (Table 8), although these three markers may not be sufficient to diagnose all AIS types. AIS is a multifactorial disease, and each marker may associated with a different cause. The more biomarkers are identified, the more precise predictions can be achieved. Further investigations may be necessary for practical use.

**Limitation**

Although the present study suggests kidney failure-associated DIDO1-Ab, hypertension-related FOXJ2-Ab, and DM-related CPSF2-Ab markers as risk factors of AIS, further study using the increasing number of specimens is needed to verify the suggestion. Because our present study was carried out using specimens obtained from hospitals and universities in Japan, it is obscure whether our conclusion is generalized in other populations. Further international collaborative research using the specimens from many countries is necessary for the practical use in the world.

**Conclusions**

Serum DIDO1-Ab, FOXJ2-Ab, and CPSF2-Ab appear to be useful for diagnosing AIS and may originate from kidney disease, hypertension, and DM, respectively.

**Abbreviations**

AIS: Acute ischemic stroke; AlphaLISA: Amplified luminescent proximity homogeneous assay-linked immunosorbent assay; AMI: Acute myocardial infarction; asympt-CI: Asymptomatic cerebral infarction; AUC: Area under the curve; bCPSF2-607: Biotinylated peptide of CPSF2 amino acids 607-621, biotin-QVRLKDSLVSSLQFC; bDIDO1-297: Biotinylated peptide of DIDO1 amino acids 297-314, biotin-AMAASKKTAPPGSAVGKQ; bFOXJ2-426: Biotinylated peptide of FOXJ2 amino acids 426-440, biotin-KMVNRLNWSSIEQSQ; bCPSF2-607: Biotinylated peptide of CPSF2 amino acids 607-621, biotin-QVRLKDSLVSSLQFC; bDIDO1-297: Biotinylated peptide of DIDO1 amino acids 297-314, biotin-AMAASKKTAPPGSAVGKQ; bFOXJ2-426: Biotinylated peptide of FOXJ2 amino acids 426-440, biotin-KMVNRLNWSSIEQSQ;
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Authors’ contributions
TH, TMac, Y, MMo, EN, and HKu created the concept and design of the study. HW, KG, KS, Mmu, AH, MY, SKo, TA, AK, KI, SYL, GT, NSh, and RN performed experiments and acquired data. SM, MKun, K, AU, MO, YM, KK, HKa, RI, HT, KM, TMar, MT, Ym, TN, JT, TMatum, Yka, AN, MI, FS, Msu, MSh, and SYo contributed to the preparation of reagents, materials, analysis tools, and data. YY, AA, TK, YYo, MT, NK, NT, SS, TKu, HD, and HA contributed to the analysis and interpretation of the data. HW, KG, Msu, KYa, and NS performed the statistical analyses. TH, TMac, YY, TMatum, MKu, SYa, and Yw contributed to the drafting of the manuscript. HI, ST, SKh, KYo, FN, HM, KT, HS, and Yw supervised the study. All authors gave final approval for the article to be published.

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Availability of data and materials
The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations
Ethics approval and consent to participate
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 the cooperating hospitals or institutes. Sera were collected from participants who had provided informed consent. The study was conducted in accordance with the principles of the Declaration of Helsinki (2013).

Consent for publication
Not applicable.

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

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70. Mannami T, Konishi M, Baba S, Nishi N, Terao A. Prevalence of asymptomatic carotid atherosclerotic lesions detected by high-resolution ultrasonography and its relation to cardiovascular risk factors in the general population of a Japanese city: the Saita study. Stroke. 1997;28(3):518–25. https://doi.org/10.1161/01.STR.28.3.518. PMID: 9056605.

71. Garcia-Domingo D, Leonardo E, Grandien A, Martinez P, Alcar JP, Izpisu-Belmonte JC, et al. DIO-1 is a gene involved in onset of apoptosis in vitro, whose misexpression disrupts limb development. Proc Natl Acad Sci USA. 1999;96:7992–7. https://doi.org/10.1073/pnas.96.14.7992. PMID: 10393935.

72. Pérez-Sánchez C, Gómez-Ferreria MA, de La Fuente CA, Granadino B, Velasco G, Esteban-Gambao A, et al. FKh, a novel fork head factor with a dual DNA binding specificity. J Biol Chem. 2002;275:12909–16. https://doi.org/10.1074/jbc.M202430200. PMID: 12077590.

73. Soriano LC, Soriano-Gabarró M, García Rodríguez LA. Trends in the contemporary incidence of colorectal cancer and patient characteristics in the United Kingdom: a population-based cohort study using The Health Improvement Network. BMC Cancer. 2018;18:402. https://doi.org/10.1186/s12885-018-4265-1. PMID: 29636012.

74. Qian J, Li M, Zhang X, Wang Q, Zhao J, Tian Z, et al. Long-term prognosis of patients with systemic lupus erythematosus-associated arterial pulmonary hypertension: CSTAR-PAH cohort study. Eur Respir J. 2019;53:1800081. https://doi.org/10.1183/13993003.00081818. PMID: 30635295.

75. Murthy KKG, Manley JL. The 160-Id subunit of human cleavage-polyadenylation specificity factor coordinates pre-miRNA 3-prime-end formation. Genes Dev. 1995;9:2627–83. https://doi.org/10.1101/gad.9.21.2627. PMID: 7590244.

76. Cai J, Pardali E, Sánchez-Duffhues G, ten Dijke P. BMP signaling in vascular diseases. FEBS Lett. 2012;586:1993–2002. https://doi.org/10.1016/j.febslet.2012.04.030. PMID: 22710160.

77. Dyer LA, Pi X, Patterson C. The role of BMPs in endothelial cell function and dysfunction. Trends Endocrinol Metab. 2014;25:472–80. https://doi.org/10.1016/j.tem.2014.05.003. PMID: 24908616.

78. Dhore CR, Cleutjens JP, Lutgens E, Cleutjens KB, Geusens PP, Kitslaar PJ, et al. Differential expression of bone matrix regulatory proteins in human atherosclerotic plaques. Arterioscler Thromb Vasc Biol. 2001;21:1998–2003. https://doi.org/10.1161/01.HT.101.000229. PMID: 11742876.

79. Siorescu GP, Sykes M, Weiss D, Platt MD, Saha A, Hwang J, et al. Bone morphogenetic protein 4 produced in endothelial cells by oscillatory shear stress stimulates an inflammatory response. J Biol Chem. 2003;278:1128–35. https://doi.org/10.1074/jbc.M300703200. PMID: 12766166.

80. Zhang M, Sara JD, Wang FL, Liu LP, Su LX, Zhe J, et al. BMP-2 levels are associated with atherosclerosis burden and coronary artery disease. Circ Res. 2002;90:879–86. https://doi.org/10.1161/01.RES.0000024771.00008.AE. PMID: 11742876.

81. Miriyala S, Gongora Nieto MC, Mingone C, Smith D, Dikalov S, Harrison DG, et al. Smiclr is a novel Smad interacting protein and cleavage and dual DNA binding specificity. J Biol Chem. 2000;275:12909–16. https://doi.org/10.1074/jbc.M300703200. PMID: 12766166.

82. Yao Y, Bennett BJ, Wang X, Rosenfeld ME, Giachelli C, Lusis AJ, et al. Bone morphogenic protein-4 induces hypertension in mice: role of Notch signaling pathway in non-small lung cancer. Cell Biol Int. 2017;41:79–83. https://doi.org/10.1002/cbin.10680. PMID: 27611107.

83. Radulavskas R, Kuzmickiene I, Milanaviciene E, Everatt R. Hyperpertension, serum lipids and cancer risk: a review of epidemiological evidence. Medicina [Kaunas]. 2015;52:89–28. https://doi.org/10.1016/j.medicina.2016.10.002. PMID: 27170481.

84. Shy Q, Tang C, Shi S, Tang M, Bao L, Li L, et al. Foxj2 overexpression is associated with poor prognosis, progression, and metastasis in nasopharyngeal carcinoma. Onco Targets Ther. 2017;10:3733–41. https://doi.org/10.2147/OTT.S134915. PMID: 28769576.

85. Kaneda H, Aso T, Tanaka K, Tamura D, Komatani K, Kudo K, et al. FOXJ1 is overexpressed in colorectal cancer and enhances tumorigenicity and tumor growth. Cancer Res. 2010;70:2053–63. https://doi.org/10.1158/0008-5472.CAN-09-2161. PMID: 20415541.

86. Qiang Y, Wang F, Yan S, Zhang H, Zhu L, Chen Z, et al. Abnormal expression of Forkhead Box J2 (FOXJ2) suppresses migration and invasion in extrahepatic cholangiocarcinoma and is associated with prognosis. Int J Oncol. 2015;46:249–59. https://doi.org/10.3892/ijo.2015.2957. PMID: 25873280.

87. Zhang H, Tang QF, Sun MY, Zhang CY, Zhu YJ, Shen YL, et al. ARHGAP9 suppresses the migration and invasion of hepatocellular carcinoma cells through up-regulating FOXJ2/E-cadherin. Cell Death Dis. 2018;9:201. https://doi.org/10.1038/s41419-018-0076-0. PMID: 29126221.

88. Ou X, Ji B, Yang L, Huang Q, Shi W, Ding Z, et al. The role of FoxJ2 in the migration of human glioma cells. Pathol Res Pract. 2015;211:89–97. https://doi.org/10.1016/j.prp.2015.01.005. PMID: 25661068.

89. Wang Y, Yang S, Ni Q, He S, Zhao Y, Yuan Q, et al. Overexpression of forkhead box J2 can decrease the migration of breast cancer cells. J Cell Biochem. 2012;113:2729–37. https://doi.org/10.1002/jcb.24146. PMID: 2241887.

90. Nilubol N, Boufaqech M, Zhang L, Kebebew E. Loss of CPSF2 expression is associated with increased thyroid cancer cellular invasion and cancer stem cell population, and more aggressive disease. J Clin Endocrinol Metab. 2014;99:E1173–82. https://doi.org/10.1210/jc.2013-4140. PMID: 24654752.

91. Wang Z, Teng D, Li Y, Hu Z, Liu L, Zheng H. A six-gene-based prognostic signature for hepatocellular carcinoma overall survival prediction. Life Sci. 2018;203:83–91. https://doi.org/10.1016/j.lfs.2018.04.025. PMID: 2978742.

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