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Research Article

Association of Serum Antibody Levels against TUBB2C with Diabetes and Cerebral Infarction

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Abstract

Cerebral infarction (CI), cardiovascular disease (CVD), diabetes mellitus (DM) and chronic kidney disease (CKD) are atherosclerosis-related diseases, which are major causes of health damage. For early and sensitive diagnosis, development of novel biomarkers is expected and of significant practical importance. First screening was carried out by phage expression cloning to identify antigen proteins recognized by serum IgG antibodies in patients with atherosclerosis. RPA2, LRPAP1, EEF1A1, SPOCK1, LOC729260, tubulin beta 2C (TUBB2C) and KIAA0020 markers were identified. We then compared the serum antibody levels against the candidate proteins between healthy donors (HD) and patients with CI, CVD, DM, or CKD by Alpha (amplified luminescent proximity homogeneous assay)-LISA method. The results showed that the serum TUBB2C antibody levels were significantly higher in patients with CI, DM, or CKD than those in HD. Using the average + 2SD of HD as the cut-off value, the positive thresholds of TUBB2C antibody markers were 14.8% in CI, 25.8% in DM, and 18.3% in CKD. TUBB2C antibody levels were well correlated with artery stenosis degrees such as plaque score, maximum intima-media thickness and cardio ankle vascular index. Consequently, TUBB2C antibody markers are useful to diagnose atherosclerosis, DM, and CKD, and can be applied to the prediction of the onset of CI. The serum anti-TUBB2C antibody markers are useful for the diagnosis of DM and CKD.

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

Abbreviations: ABI: Ankle Brachial Pressure Index; CAVI: Cardio Ankle Vascular Index; CI: Cerebral Infarction; CKD: Chronic Kidney Disease; CVD: Cardiovascular Disease; DM: Diabetes Mellitus; *E. coli: Escherichia coli*; GST: Glutathione-S-Transferase; HD: Healthy Donor; HDL: High Density Lipoprotein; IMT: Intima-Media Thickness; IPTG: Isopropyl-β-D-Thiogalactoside; LDL: Low Density Lipoprotein; SLE: Systemic Lupus Erythematosus; SEREX: Serological Identification of Antigens by Recombinant cDNA Expression Cloning; s-bTUBB2Cpep-Abs: Serum Anti-biotinylated TUBB2C-375 Peptide Antibodies; s-TUBB2C-Abs: Serum Anti-TUBB2C Antibodies

Introduction

Cerebrovascular disease occupies approximately 10% of the cause of mortality in Japan. After the onset of a stroke, patients have suffered from prognostic symptoms for a long time, and the disease is a major cause of being bedridden. Despite of the development of treatment with urokinase and anticoagulants, the effect is limited [1-3]. Thus, it is absolutely important to treat before the onset of a cerebral infarction (CI) such as at the stage of transient ischemic attack. For the diagnosis of CI, various risk factors have been developed including body mass index (BMI), low density lipoprotein (LDL) cholesterol, uric acid, smoking habits and family history [4-6].

On the other hand, diabetes mellitus (DM) is a major risk factors of CI and cardiovascular diseases (CVD) [7]. DM frequently induces atherosclerosis, which is accompanied by complications such as CI and CVD. Therefore, prevention of CI and CVD has been attempted by using DM markers, which are blood glucose, glycoalbumin and glycohemoglobin (HbA1c) [8]. However, despite frequent application for diagnosis, prevention of the onset remains unsuccessful. Thus, the development of more sensitive markers is awaited.

We have performed a large scale screening of esophageal squamous cell carcinoma by serological identification of antigens by recombinant cDNA expression cloning (SEREX), and identified many SEREX antigens [9-11]. We also reported that the antibody levels against SEREX antigens were excellent and sensitive for diagnosis of tumors. Although many autoantibodies have been reported for cancer [12,13] and autoimmune diseases [14], only few have been identified for atherosclerosis-related diseases. Among the ones reported were phospholipid [15], apolipoprotein A-1 [16] and oxidized low-density

lipoprotein [17] and heat shock proteins (Hsps), for CVD [18], Hsp60 for stroke [19], and GAD, insulin and protein tyrosine phosphatase for DM [20].

We introduced the SEREX screening method to identify autoantigens responsible to atherosclerosis and reported anti-RPA2 antibody as a CI-related marker [21]. In this report, further screening identified novel TUBB2C as an antigen, against which the serum antibody levels were higher in patients with DM or CKD than in HD.

Materials and Methods

Patients and healthy donor sera

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

The serum samples of CI were obtained from Chiba Prefectural Sawara Hospital, Chiba Rosai Hospital and Chiba Aoba Municipal Hospital. Samples of CVD and DM were obtained from Chiba University Hospital, and those of CKD were from Kumamoto University. Serum samples of CVD were obtained from the Kita-Nagoya Genomic Epidemiology (KING) Study, which is a prospective population-based observational survey of aged Japanese [22]. The sera of healthy donors were obtained from Chiba University, Shimoshizu National Hospital and Port Square Kashiwado Clinic.

Screening by expression cloning

Recombinant DNA studies were performed with the official permission of the Chiba University Graduate School of Medicine and were carried out in accordance with the rules of the Japanese government. We used a commercially available human microvascular endothelial cell cDNA library (Uni-ZAP XR Premade Library, Stratagene, La Jolla, CA) to screen for clones that were immunoreactive against serum IgG from patients with severe carotid stenosis as described previously [9-11,21]. *Escherichia coli* (*E. coli*) XL1-Blue MRF' was infected with Uni-ZAP XR phage and the expression of resident cDNA clones was induced after blotting the infected bacteria onto NitroBind nitrocellulose membranes (Osmonics, Minnetonka, MN) that had been treated with 10 mM isopropyl-β-D-thiogalactoside (IPTG, Wako Pure Chemicals, Osaka, Japan) for 30 min. The membranes with bacterial proteins were rinses 3 times with TBS-T [20 mM Tris -HCl (pH 7.5), 0.15 M NaCl and 0.05% Tween-20], and non-specific binding was blocked by incubation with 1% protease-free BSA (Wako Pure Chemicals) in TBS-T for 1 h. The membranes were exposed to 1:2000-diluted plasma for 1 h. After 3 washes with TBS-T, the membranes were incubated for 1 h with 1:5000-diluted alkaline phosphatase-conjugated goat anti-human IgG (Jackson ImmunResearch Laboratories, West Grove, PA). Positive reactions were developed using 100 mM Tris-HCl (pH 9.5) containing 100 mM NaCl, 5 mM $MgCl_2$, 0.15 mg/mL of 5-bromo-4-chloro-3-indolylphosphate and 0.3 mg/mL of nitro blue tetrazolium (Wako Pure Chemicals). Positive clones were re-cloned twice until obtaining monoclonality.

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

Expression and purification of GST-fusion proteins

Recombinant proteins tagged with glutathione-S-transferase (GST) were constructed by recombining the insertion sequences of pBluescript into pGEX-4T (GE Healthcare Life Sciences, Pittsburgh, PA) vector plasmids. The pBluescript plasmids were digested using a combination of EcoRI and XhoI or SmaI and XhoI. The inserted DNA fragments were ligated in frame to SmaI/XhoI-digested pGEX-4T-1 or EcoRI/XhoI-digested pGEX-4T-3 using Ligation Convenience Kits (Nippon

Gene, Toyama, Japan). The ligation mixtures were used to transform ECOS™ competent *E. coli* BL-21 (Nippon Gene) and appropriate recombinants were confirmed by DNA sequencing as well as protein expressions. Treating the transformed *E. coli* with 0.1 mM IPTG for 3 h induced the expression of the GST-fusion proteins. The GST recombinant proteins were purified by glutathione-Sepharose column chromatography according to the manufacturer's instructions (GE Healthcare Life Sciences) and dialyzed against PBS as described [9,10,21].

Peptide array

The possible epitope sites in the candidate antigen proteins can be predicted using the program ProPred (http://www.imtech.res.in/raghava/propred/) as described [23]. We designed 100 peptides derived from seven candidate antigens. These peptides together with positive or negative controls were synthesized onto the cellulose membranes using F-moc amino acids (Auto spot robot ASP222; ABIMED Analysen-Technik GmbH, Langenfeld, Germany) as previously described [24]. Membranes loaded with 150 peptides were washed with PBS-T-BSA [phosphate-buffered saline containing 1% (w/v) bovine serum albumin and 0.05% Tween-20 and 0.05% NaN_3] for 30 min five times, and then incubated with the sera of patients at 1:200 dilutions for 18 h. The membranes were washed with PBS-T-BSA five times, and then treated with FITC-conjugated goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA) at 1:10000 dilutions for 1-2 h. After washing, the fluorescence of the peptide spots were detected using Typhoon 9400 Imager (Amersham Biosciences, Stockholm, Sweden) with a 488 nm/520 nm filter as described previously [25].

Peptide synthesis

N-terminal biotinylated 14mer peptides corresponding to the amino acid sequences of the positive spots of peptide arrays were synthesized and used for AlphaLISA. The purity of each peptide was determined to be higher than 90%.

AlphaLISA (Amplified Luminescence Proximity Homogeneous Assay)

To evaluate the serum antibody levels, AlphaLISA was used. AlphaLISA was performed in 384-well microtiter plates (white opaque OptiPlate™, Perkin Elmer) containing 2.5 μL of 1/100-diluted sera and 2.5 μL of GST or GST-fusion proteins (10 μg/mL) or biotinylated peptides (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, then anti-human IgG-conjugated acceptor beads (2.5 μL at 40 μg/mL) and glutathione- or streptavidin-conjugated donor beads (2.5 μL at 40 μg/mL) were added and incubated at room temperature in the dark for another 1-14 days. The chemical emission was read on an EnSpire Alpha microplate reader (PerkinElmer) as described [23]. Specific reaction was calculated by subtracting Alpha counts of GST control and those of buffer control without antigenic peptides from the counts of GST-fusion proteins and biotinylated peptides, respectively.

Statistical analyses

Fisher's exact (two-sided) probability test and the Mann-Whitney U test were used to determine the significance of the differences between the two groups. Correlation was examined by Spearman's correlation analysis. All statistical analyses were carried out using the GraphPad Prism 5 (GraphPad Software, La Jolla, CA). P values lower than 0.05 were considered statistically significant.

Results

Identification of antigens recognized by sera of patients with atherosclerosis

Expression cloning using sera of patients with atherosclerosis has identified seven antigens; replication protein A2 (RPA2), low density lipoprotein-related protein-associated protein 1 (LRPAP1), eukaryotic translation elongation factor 1 alpha 1 (EEF1A1), sparc/osteonectin, cwcv and kazal-like domains proteoglycan 1 (SPOCK1), LOC729260, tubulin beta 2C (TUBB2C) and KIAA0020. RPA2 has also been identified and reported previously [21]. LRPAP1, EEF1A1, LOC729260 and TUBB2C proteins were expressed as GST-fusion proteins in *E. coli*

and purified by affinity-chromatography using GST-Sepharose as described previously [9-11,21]. It was unable to purify SPARC and KIAA0020 proteins.

Identification of epitope peptides by peptide array

A total of 100 peptides were designed as possible epitope sites based on the amino acid sequences of seven candidate antigen proteins (Table 1). These peptides as well as positive or negative controls were synthesized directly onto the cellulose membranes, which were then treated with sera followed by treatment with FITC-labeled second antibodies and detection of fluorescence. Figure 1 shows the representative results after treatment with sera of patients #1 (a) and #2 (b) or with that of HD (c).

Based on the results using sera of 22 patients and 10 HD, we selected three spots, numbers E6 (LOC729260-337), F6 (TUBB2C-375) and F19 (KIAA0020-477), of which the fluorescence was more potent after treatment with patients' sera as compared with that with HD sera. No peptide spot was selected from peptides derived from the sequence of RPA2, LRPAP1, EEF1A1 and SPARC. N-terminal biotinylated peptides, bLOC729260-337, bTUBB2C-375 and bKIAA0020-477, were synthesized with the purity higher than 90%, and used as antigens in the following study.

The levels of s-TUBB2C-Abs increased in patients with CI

We examined the serum antibody levels against selected antigens using specimens of CI and CVD, which have been obtained from Chiba Rosai Hospital, Chiba Aoba Municipal Hospital, Chiba Prefectural Sawara Hospital, Kita-Nagoya Genomic Epidemiology (KING) Study and Chiba University Hospital. HD sera were obtained from Shimoshizu National Hospital and Chiba University. AlphaLISA was introduced to measure the serum antibody levels because of the low background levels and small variation. When control GST was used as an antigen, serum antibody levels were not apparently different between HD and patients with CI or CVD (Figure 2a). On the other hand, the levels of s-TUBB2C-Abs were significantly higher in patients with CI than in HD (Figure 2b). When the cut-off value was determined as the average +2SD of HD, the positive rates of s-TUBB2C-Abs in HD and CI patients were

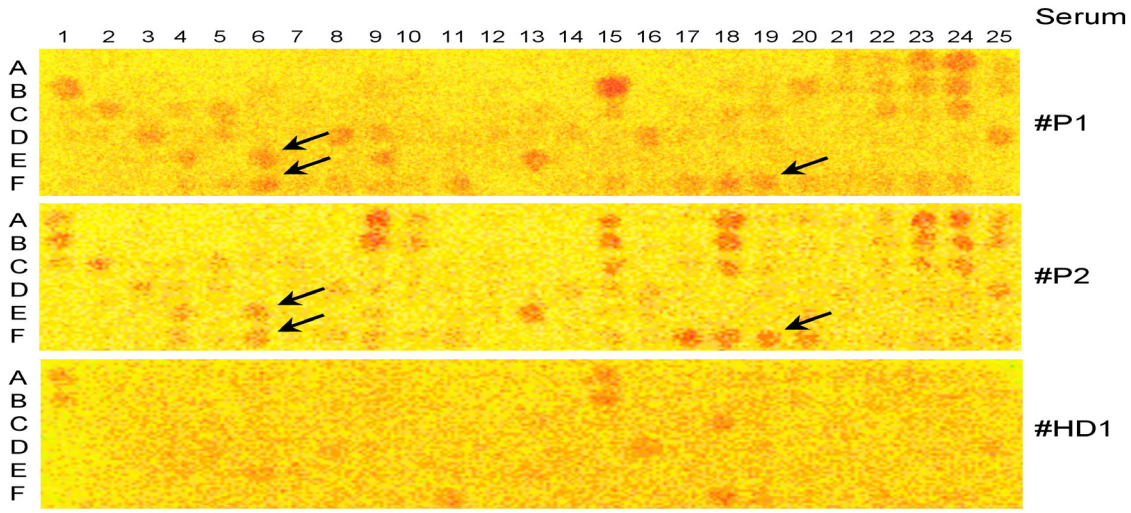

Figure 1: Results of peptide arrays. Cellulose membranes loaded with 150 peptides described in Table 1 were treated with sera from patients with atherosclerosis (#P1 and #P2) or from HD (#HD1). Antibody binding to peptide spots was detected by treatment with FITC-conjugated anti-human IgG antibody followed by imaging by a fluorescence laser scanner. Representative results are shown. Arrows indicate the position of selected peptides, numbers E6, F6 and F19.

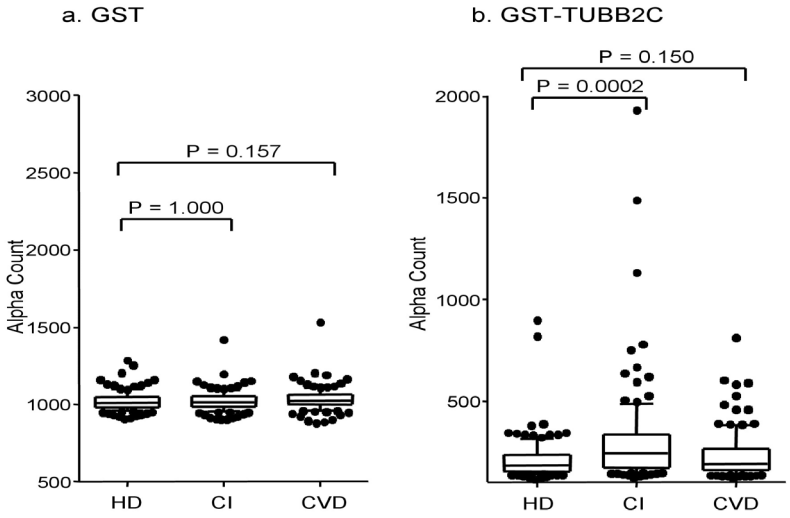

Figure 2: Comparison of serum antibody levels of HD and patients with CI or CVD. Antigens used were control GST (a) and GST-TUBB2C proteins (b). Serum antibody levels examined by AlphaLISA are shown by box-whisker plot. The box plots display the 10th, 20th, 50th, 80th and 90th percentiles. P values versus HD specimens are shown.

1.6% and 14.8%, respectively (Table 2). s-TUBB2C-Abs levels were somewhat higher in patients with CVD as compared with that in HD, yet the difference was not significant. The levels of anti-LOC729260 antibodies were not altered between patients with CI or CVD and HD, and anti-LRPAP1 antibody levels were slightly higher in patients with CI but not in patients with CVD as compared to those in HD (Table 2). Thus, these antigens were not used in the following study.

The levels of serum anti-biotinylated TUBB2C-375 peptide antibodies (s-bTUBB2Cpep-Abs) and anti-biotinylated LOC729260-337 peptide antibodies were not apparently different between HD and patients with CI (Table 2).

The levels of s-TUBB2C-Abs were responsible to DM

Atherosclerosis is closely related to DM, and thus, we then examined specimens of DM as well as another set of CI and HD which were obtained from Chiba University Hospital and Port Square Kashiwado Clinic. Both of s-TUBB2C-Abs and s-bTUBB2Cpep-Abs increased significantly and markedly in patients with DM as compared with those in HD (Figure 3 and Table 3). The positive rates of s-TUBB2C-Abs in HD and DM patients were 2.3% and 25.6%, respectively (Table 3). The levels of s-TUBB2C-Abs and s-bTUBB2Cpep-Abs were significantly higher in patients with CVD than in HD with the positive rates of 7.5% for both antigens. Anti-KIAA0020-477 peptide antibodies showed a marginal increase in DM but not in CVD.

The levels of s-TUBB2C-Abs were elevated in patients with chronic kidney disease

Chronic kidney disease (CKD) was divided into three groups; type 1: diabetic kidney disease, type 2: nephrosclerosis, and type 3: glomerulonephritis. s-TUBB2C-Ab levels in CKD patients were higher than those in HD (Figure 4). The positive rates of CKD type 1, type 2 and type 3 were 22.1%, 28.1% and 11.4%, respectively (Table 4). The average positive rate of CKD was 18.3% (55 positive specimens out of 300). Similar results were obtained when bTUBB2C-375 peptide was used as an antigen.

The levels of s-TUBB2C-Abs were not associated with cancer

Autologous antibodies developed frequently in cancer patients [13]. We then examined the specimens

Table 1: List of peptides loaded onto a peptide array

No.	Name	Sequence	No.	Name	Sequence	No.	Name	Sequence
A1	Control	KKKKKKKKKKKKKK	B1	Control	KKKKKKKKKKKKKK	C1	RPA2-44	QHIVPCTISQLLSA
A2	Control	AAAAAAAAAAAAA	B2	Control	AAAAAAAAAAAAA	C2	RPA2-63	VFRIGNVEISQVTI
A3	Control	CCCCCCCCCCCCCC	B3	Control	CCCCCCCCCCCCCC	C3	RPA2-75	TIVGIIRHAEKAPT
A4	Control	DDDDDDDDDDDDDD	B4	Control	DDDDDDDDDDDDDD	C4	RPA2-91	VYKIDDMTAAPMDV
A5	Control	EEEEEEEEEEEEEE	B5	Control	EEEEEEEEEEEEEE	C5	RPA2-125	YVKVAGHLRSFQNK
A6	Control	FFFFFFFFFFFFFF	B6	Control	FFFFFFFFFFFFFF	C6	RPA2-139	KSLVAFKIMPLEDM
A7	Control	GGGGGGGGGGGGGG	B7	Control	GGGGGGGGGGGGGG	C7	RPA2-162	VINAHMVLSKANSQ
A8	Control	HHHHHHHHHHHHHH	B8	Control	HHHHHHHHHHHHHH	C8	RPA2-198	SFMPANGLTVAQNQ
A9	Control	IIIIIIIIIIIII	B9	Control	IIIIIIIIIIIII	C9	RPA2-211	QVLNLIKACPRPEG
A10	Control	LLLLLLLLLLLLL	B10	Control	LLLLLLLLLLLLL	C10	RPA2-229	DLKNQLKHMSVSSI
A11	Control	MMMMMMMMMMMMM	B11	Control	MMMMMMMMMMMMM	C11	RPA2-257	STVDDDHFKSTDAE
A12	Control	NNNNNNNNNNNNN	B12	Control	NNNNNNNNNNNNN	C12	LRPAP1-7	RSFLRGLPALLLLL
A13	Control	PPPPPPPPPPPPP	B13	Control	PPPPPPPPPPPPP	C13	LRPAP1-22	FLGPWPAASHGGKY
A14	Control	QQQQQQQQQQQQQ	B14	Control	QQQQQQQQQQQQQ	C14	LRPAP1-52	EEFRMEKLNQLWEK
A15	Control	RRRRRRRRRRRRR	B15	Control	RRRRRRRRRRRRR	C15	LRPAP1-67	QRLHLPPVRLAELH
A16	Control	SSSSSSSSSSSSS	B16	Control	SSSSSSSSSSSSS	C16	LRPAP1-77	AELHADLKIQERDE
A17	Control	TTTTTTTTTTTTT	B17	Control	TTTTTTTTTTTTT	C17	LRPAP1-91	LAWKKLKLDGLDED
A18	Control	VVVVVVVVVVVVV	B18	Control	VVVVVVVVVVVVV	C18	LRPAP1-109	ARLIRNLNVILAKY
A19	Control	WWWWWWWWWWWWW	B19	Control	WWWWWWWWWWWWW	C19	LRPAP1-120	AKYGLDGKKDARQV
A20	Control	YYYYYYYYYYYYY	B20	Control	YYYYYYYYYYYYY	C20	LRPAP1-155	WHKAKTSGKFSGEE
A21	Control	LTPALTSYKLVIKG	B21	Control	LTPALTSYKLVIKG	C21	LRPAP1-178	HHKEKVHEYNVLLE
A22	Control	NVLYYRNGKAFKFF	B22	Control	NVLYYRNGKAFKFF	C22	LRPAP1-218	HSRHTELKEKLRSI
A23	Control	TTYKLVINGKTLKG	B23	Control	TTYKLVINGKTLKG	C23	EEF1A1-8	INIVVIGHVDSGKS
A24	Control	VTYYKLVINGKTLK	B24	Control	VTYYKLVINGKTLK	C24	EEF1A1-53	SFKYAWVLDKLKAE
A25	Control	VTYYKLVINFKTLK	B25	Control	VTYYKLVINFKTLK	C25	EEF1A1-72	TIDISLWKFETSKY

No.	Name	Sequence	No.	Name	Sequence	No.	Name	Sequence
D1	EEF1A1-97	DFIKNMITGTSQAD	E1	LOC729260-226	FYAKFNMANALASA	F1	TUBB2C-266	FMPGFAPLTSRGSQ
D2	EEF1A1-147	QLIVGVNKMDSTEP	E2	LOC729260-262	YHEQCFVCAQCFQQ	F2	TUBB2C-279	QQYRALTVPELTQQ
D3	EEF1A1-172	KEVSTYIKKIGYNP	E3	LOC729260-291	EHDFQMLFAPCCHQ	F3	TUBB2C-329	QMLNVQNKNSSYFV
D4	EEF1A1-183	YNPDTVAFVPISGW	E4	LOC729260-307	EFIIGRVIKAMNNS	F4	TUBB2C-340	YFVEWIPNNVKTAV
D5	EEF1A1-209	PWFKGWKVTRKDGN	E5	LOC729260-325	CFRCDLCQEVLADI	F5	TUBB2C-362	KMSATFIGNSTAIQ
D6	EEF1A1-252	DVYKIGGIGTVPVG	E6	LOC729260-337	DIGFVKNAGRHLCR	F6	TUBB2C-375	QELFKRISEQFTAM
D7	EEF1A1-274	PGMVVTFAPVNVTT	E7	LOC729260-363	KYICQKCHAIIDEQ	F7	TUBB2C-388	MFRRKAFLHWYTGE
D8	EEF1A1-308	VGFNVKNVSVKDVR	E8	LOC729260-431	IEGRVVNAMGKQWH	F8	KIAA0020-218	IVKKFLMYGSKPQI
D9	EEF1A1-321	RRGNVAGDSKNDPP	E9	LOC729260-457	FLGHRHYERKGLAY	F9	KIAA0020-229	PQIAEIIRSFKGHV
D10	EEF1A1-343	QVIILNHPGQISAG	E10	LOC729260-472	ETHYNQLFGDVCFH	F10	KIAA0020-241	HVRKMLRHAEASAI
D11	EEF1A1-398	DAAIVDMVPGKPMC	E11	LOC729260-487	NRVIEGDVVSALNK	F11	KIAA0020-280	FQLYKSADHRTLDK
D12	EEF1A1-421	LGRFAVRDMRQTVA	E12	LOC729260-500	KAWCVNCFACSTCN	F12	KIAA0020-307	DEMKQILTPMAQKE
D13	EEF1A1-433	VAVGVIKAVDKKAA	E13	LOC729260-517	TLKDKFVEIDLKPV	F13	KIAA0020-324	KHSLVHKVFLDFFT
D14	SPOCK1-4	WIFFLLCLAGRALA	E14	TUBB2C-2	REIVHLQAGQCGNQ	F14	KIAA0020-357	YLAHTHDGARVAMH
D15	SPOCK1-165	LRMRDWLKNVLVTL	E15	TUBB2C-42	LQLERINVYYNEAT	F15	KIAA0020-384	VKTMKTYVEKVANG
D16	SPOCK1-192	QKLRVKKIHENEKR	E16	TUBB2C-55	TGGKYVPRAVLVDL	F16	KIAA0020-399	YSHLVLLAAFDCID
D17	SPOCK1-224	YNMYIFPVHWQFGQ	E17	TUBB2C-85	FRPDNFVFGQSGAG	F17	KIAA0020-416	LVKQIIISEIISSL
D18	LOC729260-1	MESFLLDDISSVIQ	E18	TUBB2C-121	RKEAESCDCLQGFQ	F18	KIAA0020-439	RKVLLYLLSPRDPA
D19	LOC729260-26	IVQLCHLLISMERK	E19	TUBB2C-131	QGFQLTHSLGGGTG	F19	KIAA0020-477	VRRRELLESISPAL
D20	LOC729260-61	EDFVQVVKSSGNTE	E20	TUBB2C-153	SKIREEYPDRIMNT	F20	KIAA0020-505	KSACVLVSDILGSA
D21	LOC729260-80	PVIVNAALVFQKAV	E21	TUBB2C-164	MNTFSVVPSPKVSD	F21	KIAA0020-523	QPTMNAIASLAATG
D22	LOC729260-92	AVVVWVFKSEKHGI	E22	TUBB2C-178	TVVEPYNATLSVHQ	F22	KIAA0020-559	KWLIEQDKKMKENG
D23	LOC729260-111	WQICKARVDISFSW	E23	TUBB2C-221	TYGDLNHLVSATMS	F23	KIAA0020-577	FAKTLVEHVGMKNL
D24	LOC729260-122	FSWRLTRLTCVGTV	E24	TUBB2C-246	LNADLRKLAVNMVP	F24	KIAA0020-587	MKNLKSWASVNRGA
D25	LOC729260-208	SKLQRRHSDVKVYK	E25	TUBB2C-256	NMVPFPRLHFFMPG	F25	KIAA0020-597	NRGAIILSSLLQSC

Columns A and B are positive or negative controls, and columns C - F are synthetic peptides corresponding to the amino acid sequences predicted as epitopes of seven candidate proteins. The selected three peptides are marked in color.

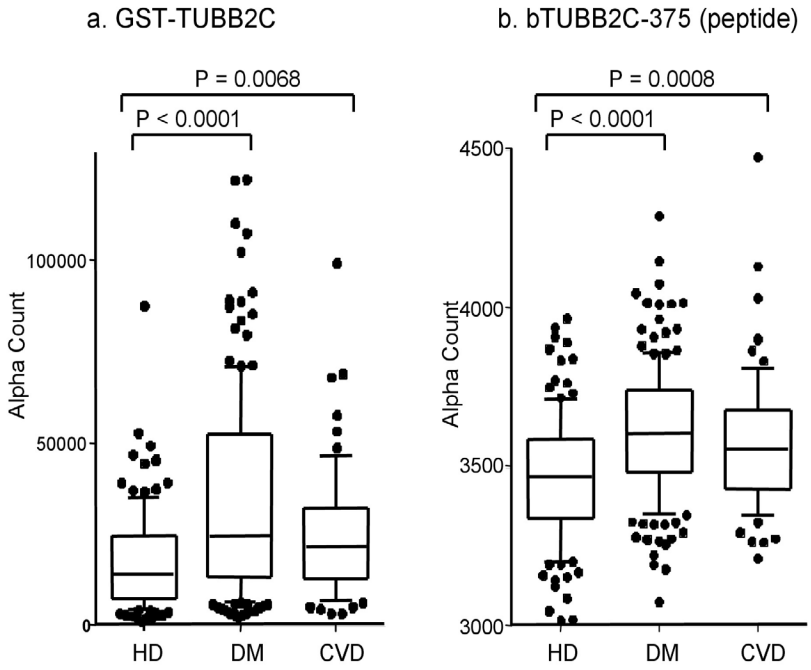

Figure 3: Comparison of serum antibody levels of HD and patients with DM or CVD. Antigens used were GST-TUBB2C protein (a) and bTUBB2C-375 peptide (b). Serum antibody levels examined by AlphalLISA are shown as described in the legends of Figure 2. In Table 3, averages, SDs, cut-off values, P values, total numbers, positive numbers and positive rates (%) are shown.

of patients with benign glioma, malignant glioma or esophageal squamous cell carcinoma obtained from Toho University Hospital and Chiba University Hospital. The levels of s-TUBB2C-Abs were not significantly altered between HD and cancer patients (Figure 5).

Correlation between s-TUBB2C-Abs and atherosclerosis indices

We then performed Spearman correlation analysis between the antibody marker levels and the information of the subject persons including age, gender, height, weight, BMI, smoking habit, artery stenosis degrees such as plaque score, maximum intima-media thickness (maxIMT), cardio ankle vascular index (CAVI) and ankle brachial pressure index (ABI), blood test data such red blood cell, white blood cell, platelet, hemoglobin, hematocrit, total protein, albumin, uric nitrogen, uric acid, creatinin, Na, K, Cl, Ca, P, Fe, Mg, AST, ALT, LD, gamma-GTP, alkaline phosphatase, total billirubin, amylase, creatin kinase, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglyceride, and C-reactive protein. Total of 384 specimens were analyzed. The levels of s-TUBB2C-Abs showed a positive correlation with plaque score, max IMT and CAVI (Table 5), suggesting the close association between s-TUBB2C-Ab levels and atherosclerosis. s-TUBB2C-Ab levels were also correlated with AST but not with ALT. This implicates that the high s-TUBB2C-Abs levels are responsible to some disorder in cardiac muscle, skeletal muscle or erythrocytes but not of liver. s-TUBB2C-Abs also showed positive correlation with C-reactive protein, age and triglyceride but inverse correlation with the levels of albumin and HDL-cholesterol.

Discussion

Through the phage expression cloning, we have identified six candidate antigens which can be recognized by serum IgG antibodies in patients with atherosclerosis. Replication protein A2 (RPA2) which plays a role in the repair of DNA double-strand breaks [26] has been identified in our previous study

Table 2: Comparison of serum antibody levels between HD and patients with CI or CVD examined by AlphaLISA

		EEF1A1	LOC729260	TUBB2C	LRPAP1	bTUBB2C-375	bLOC729260-337	bKIAA0020-477
HD	Average	839	91	2,138	1,530	1,402	1,508	2,525
	SD	848	141	1,034	828	148	205	185
	Cut-off value	2,534	374	4,205	3,185	1,698	1,917	2,896
	Total No.	118	118	128	96	128	128	189
	Positive No.	5	0	2	4	3	3	9
	Positive rate (%)	4.2%	0.0%	1.6%	4.2%	2.3%	2.3%	4.8%
CI	Average	1,033	81	3,008	1,931	1,411	1,541	2,601
	SD	1,107	156	2,363	1,498	155	184	524
	Total No.	132	132	128	96	128	128	190
	Positive No.	7	0	19	9	5	5	18
	Positive rate (%)	5.3%	0.0%	14.8%	9.4%	3.9%	3.9%	9.5%
	P (vs HD)	0.118	0.582	0.00019	0.023	0.639	0.165	0.061
CVD	Average	1,141	125	2,332	1,744	1,415	1,494	ND
	SD	911	143	1,118	1,124	99	162	ND
	Total No.	93	93	128	96	128	128	ND
	Positive No.	8	2	8	7	3	4	ND
	Positive rate (%)	8.6%	2.2%	6.3%	7.3%	2.3%	3.1%	ND
	P (vs HD)	0.015	0.092	0.15	0.134	0.396	0.567	ND

Shown are average, SD, cut-off values (average + 2SD), total sample numbers, the number of positive sera of which the antibody levels were higher than the cut-off value, and the positive rate (%) of HD; and average, SD, total sample number, number of positive sera of which the antibody levels were higher than the cut-off value, and the positive rate (%) of patients; and P value of comparison between HD and patients. Antigens used were purified GST-fusion proteins such as EEF1A1, LOC729260, TUBB2C and LRPAP1 and synthetic peptides such as bLOC729260-337 and bKIAA0020-477. P values lower than 0.05 and positive rates higher than 10% were marked in color.

and reported [21]. Low density lipoprotein-related protein-associated protein 1 (LRPAP1) functions as a chaperone protein in the intracellular transport of low-density lipoprotein-related receptor and in involved in anti-aging [27]. Because low-density lipoprotein is closely related to atherosclerosis [28], LRPAP1 may also be involved in the related disease. In fact, the serum levels of anti-LRPAP1 antibodies were higher in patients with CI but not CVD than in HD (Table 2). Eukaryotic translation elongation factor 1 alpha 1 (EEF1A1) is one of elongation factors and inhibits p53-dependent apoptosis [29]. Autoantibody against EEF1A1 was elevated in patients with Felty's syndrome [30], which shows similar symptoms as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). It should be noted that RA and SLE are high risk groups of CVD [31]. We observed higher levels of anti-EEF1A1 antibodies in patients with CVD as compared with those in HD (Table 2). Sparc/osteonectin, cwcv and kazal-like domains proteoglycan 1 (SPOCK1) is a Ca^{2+}-binding proteoglycan and contains Kazal-like protease-inhibitory domains, with no further information on the function [32]. KIAA0020 protein contains a sequence of a minor histocompatibility antigen, HA-8 [33]. The function of LOC729260 is unknown.

Tubulin beta 2C (TUBB2C) is highly expressed in heart and testis [34]. TUBB2C is of much interest because s-TUBB2C-Abs levels were higher in patients with CI, DM, CVD or CKD (Figures 2-4 and Tables 2-4). The most prominent difference of the levels was observed between HD and patients with DM or CKD. Similar results were obtained by using bTUBB2C-375 peptide as an antigen. The positivity of s-TUBB2C-Abs for CI was 14.8% (Table 2) whereas those for DM and type 2-CKD were 25.6% and 28.1%, respectively (Tables

3 and 4). Because CI is caused by atherosclerosis or cardiogenic thrombus, it is possible that s-TUBB2C-Ab marker can discriminate CI caused by atherosclerosis but not by thrombus.

Both levels of s-TUBB2C-Abs and s-bTUBB2Cpep-Abs were higher in CVD specimens than in those of HD (Figure 3a and b and Table 3), whereas their levels showed no significant difference between CVD and HD specimens (Figure 2 and Table 2). Most specimens (92 out of 128 specimens) were old myocardial infarction in Figure 2 and Table 2, and therefore, the sera were

Table 3: Comparison of serum antibody levels between HD and patients with DM or CVD examined by AlphaLISA

		TUBB2C-GST	bTUBB2C-375	bKIAA0020-477
HD	Average	18,122	3,463	4,831
	SD	16,967	197	1,331
	Cut-off value	52,055	3,856	7,494
	Total No.	128	128	128
	Positive No.	3	5	4
	Positive rate (%)	2.3%	3.9%	3.1%
DM	Average	34,372	3,616	5,095
	SD	30,278	201	822
	Total No.	172	172	172
	Positive No.	44	17	1
	Positive rate (%)	25.6%	9.9%	0.6%
	P (vs HD)	0.00000001	0.0000000002	0.049
CVD	Average	25,329	3,570	5,096
	SD	17,612	214	963
	Total No.	67	67	67
	Positive No.	5	5	4
	Positive rate (%)	7.5%	7.5%	6.0%
	P (vs HD)	0.0068	0.00082	0.112

Antigens used were purified GST-fused TUBB2C protein and bTUBB2C-375 and bKIAA0020-477 peptides. Shown numbers are as described in Table 2.

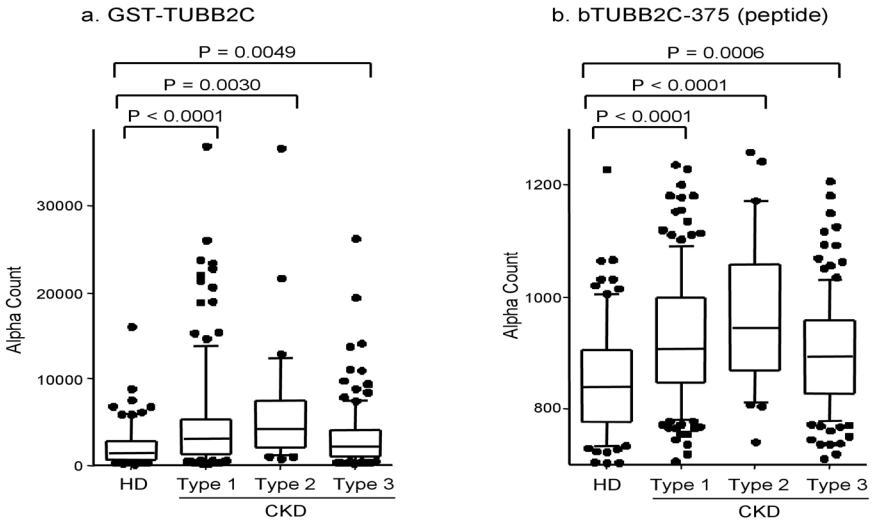

Figure 4: Comparison of serum antibody levels of HD and patients with CKD types 1, 2 and 3. Antigens used were GST-TUBB2C protein (a) and bTUBB2C-375 peptide (b). Serum antibody levels examined by AlphaLISA are shown as described in the legends of Figure 2. In Table 4, averages, SDs, cut-off values, P values, total numbers, positive numbers and positive rates (%) are shown.

Table 4: Comparison of serum antibody levels between HD and patients with CKD examined by AlphaLISA

		TUBB2C	bTUBB2C-375
HD	Average	2,041	850
	SD	2,403	99
	Cut-off value	6,848	1,049
	Total No.	82	82
	Positive No.	3	3
	Positive rate (%)	3.7%	3.7%
CKD Type 1	Average	5,425	925
	SD	7,829	116
	Total No.	145	145
	Positive No.	32	24
	Positive rate (%)	22.1%	16.6%
	P (vs HD)	0.0000030	0.00000069
CKD Type 2	Average	6,146	968
	SD	7,090	132
	Total No.	32	32
	Positive No.	9	9
	Positive rate (%)	28.1%	28.1%
	P (vs HD)	0.0030	0.000040
CKD Type 3	Average	3,276	900
	SD	3,812	99
	Total No.	123	123
	Positive No.	14	11
	Positive rate (%)	11.4%	8.9%
	P (vs HD)	0.0049	0.00056

CKD types 1, 2 and 3 were diabetic kidney disease, nephrosclerosis and glomerulonephritis, respectively. Antigens used were purified GST-TUBB2C protein and bTUBB2C-375 peptide. Shown numbers are as described in Table 2.

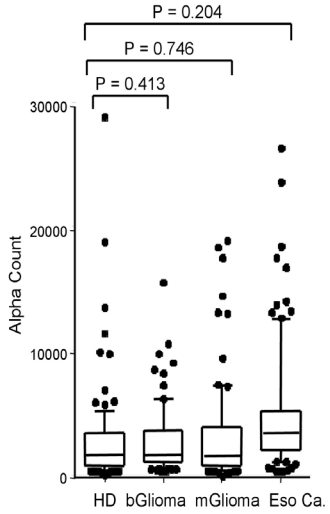

Figure 5: Comparison of serum antibody levels of HD and patients with cancer. Antigens used were control GST and GST-TUBB2C proteins. Serum antibody levels examined by AlphaLISA are shown as described in the legends of Figure 2. Numbers of specimens used were 111 of HD, 83 of benign glioma (bGlioma), 90 of malignant glioma (mGlioma) and 100 of esophageal squamous cell carcinoma (Eso Ca).

Table 5: Correlation analysis between TUBB2C antibody marker levels and the subjects' information

	TUBB2C	
	r value	P value
Age	0.1167	0.0222
Gender	0.0156	0.7610
Height	-0.0136	0.7908
Weight	-0.0145	0.7768
Body Mass Index	-0.0031	0.9518
Smoking	0.0656	0.2003
Plaque score	0.1089	0.033
MaxIMT	0.1069	0.0375
CAVI (right)	0.1333	0.0096
CAVI (left)	0.1265	0.0137
ABI (right)	-0.0814	0.1120
ABI (left)	-0.0778	0.1282
RBC	-0.0807	0.1144
WBC	0.0264	0.6059
Platelet	-0.0896	0.0796
Hemoglobin	-0.0666	0.1928
Hematocrit	-0.0544	0.2879
Total protein	0.0090	0.8608
Albumin	-0.1088	0.0331
Urea nitrogen	-0.0769	0.1326
Uric acid	-0.0841	0.0999
Creatinin	-0.0979	0.0553
Na	0.0317	0.5352
K	-0.0417	0.4148
Cl	0.0083	0.8706
Ca	-0.0235	0.6461
P	0.0128	0.8021
Fe	-0.0706	0.1676
Mg	0.0148	0.7721
AST (GOT)	0.1613	0.0015
ALT (GPT)	0.0672	0.1885
LD (LDH)	0.0837	0.1016
gamma-GTP	0.0840	0.1004
Alkaline phosphatase	0.0566	0.2688
Total billirubin	0.0039	0.9400
Amylase	-0.0615	0.2294
Creatin kinase	-0.0849	0.0965
Total cholesterol	-0.0990	0.0525
HDL-cholesterol	-0.1921	0.0002
LDL-cholesterol	-0.0669	0.1911
Triglyceride	0.1149	0.0244
C-reactive protein	0.1539	0.0025

Shown are correlation coefficients (r) and P values calculated by Spearman's correlation analysis. Significant correlations are marked in color.

obtained more than one month after the onset of myocardial infarction. On the other hand, all of sera used in Figure 3 and Table 3 were obtained within two weeks after the onset of acute myocardial infarction or unstable angina. Thus, some of serum antibodies may disappear in one month after the onset. Alternatively, because substantial populations of patients die within one month after the onset of myocardial infarction, patients in Figure 2 and Table 2 were selected as those with mild symptoms, resulting in lower marker levels than patients in Figure 3 and Table 3 with occasional severe symptoms.

Both s-TUBB2C-Abs and s-TUBB2Cpep-Abs levels were highly responsible to CKD almost irrespective of the types (type 1: diabetic kidney disease, type 2: nephrosclerosis, and type 3: glomerulonephritis). Thus, the marker levels may not necessarily reflect

DM but renal failure itself. The highest responsibility of TUBB2C marker to this renal failure (Figure 4 and Table 4) suggests that renal failure is one of the initial events to cause the progression of atherosclerosis followed by the onset of CI or CVD. Consistently, the association of reduced glomerular filtration rate and stroke outcome was reported [35]. The correlation between c-TUBB2C-Abs and blood concentrations of triglyceride and HDL-cholesterol (Table 5) suggests that these lipid metabolism may lead to the development of not only DM but also CKD.

In this study, we introduced peptide arrays in marker screening. Automated peptide synthesis onto cellulose membranes has been developed by a co-author, Mitoshi Kunimatsu [24]. Successful identification of a useful marker, bTUBB2C-375, indicates that this peptide array method is effective in marker screening.

There are many factors that affect the progress of CI, and each antibody marker may be associated with the causes such as body mass index, smoking habit, atherosclerosis, uric acid, HDL- or LDL-cholesterol and triglyceride. It is probable that each marker is associated with one or few of those factors. If so, the positivity of each maker cannot be expected to be particularly high. Therefore, examination by using as more markers may provide as more precise diagnosis.

Our antibody markers were selected by using sera collected within two weeks after the onset of CI. Various antigens may appear immediately after the onset of CI whereas the antibodies are not produced until two weeks later. Thus, the antibodies specifically detected in sera immediately after the onset were present prior to the onset. By measuring the levels of these antibodies, it is possible to predict the onset, i.e., serum antibody markers can be prediction markers for the onset of CI. In most cases, CI is not induced suddenly but mediated frequently by health issues such as TIA and asymptomatic CI. It is possible for TUBB2C to leak out from infarct lesions repeatedly, which may evoke the immune system leading to development of the autoantibodies to detectable levels. Thus, diagnosis of autoantibody levels such as s-TUBB2C-Abs may enable to predict the onset of CI.

Conclusion

The levels of s-TUBB2C-Abs were associated with DM and CKD. s-TUBB2C-Abs may be useful to predict the onset of CI caused by atherosclerosis, DM and CKD.

Competing interests

This work was performed in collaboration with Fujikura Kasei Co., Ltd. and Celish Fd Inc.

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