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Identification of Cerebral Infarction-Specific Antibody Markers from Autoantibodies Detected in Patients with Systemic Lupus Erythematosus

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Abstract

Background: Systemic lupus erythematosus (SLE) is an autoimmune disease which may be caused by development of the autoantibodies. On the other hand, SLE is a high-risk group of atherosclerosis, so it is possible that some of autoantibodies in SLE are the result of atherosclerosis-related diseases such as cerebral infarction (CI), cardiovascular disease (CVD) and diabetes mellitus (DM).

Methods: The initial screening of autoantibodies was performed using the protein array method. AlphaLISA was used to analyze the serum antibody levels using synthetic polypeptides as antigens.

Results: After the initial screening using protein array, we identified 67 antigens that were recognized by IgG antibodies in sera of patients with SLE. In the second screening, 170 peptides derived from amino acid sequences of 67 antigens were synthesized and used as antigens for analysis of serum antibody levels by AlphaLISA. The antibody levels for ten peptides were significantly higher in the sera of patients with SLE than in those of healthy donors. Further AlphaLISA analysis of sera of patients with CI, CVD or DM revealed that the serum antibody levels for four peptides derived from SOSTDC1, CTNND1, CLDND1 and CCNG2 were elevated in patients as compared to those of healthy donors.

Conclusions: Serum antibody levels against peptide antigens of SOSTDC1, CTNND1, CLDND1 and CCNG2 are useful markers for diagnosis of the progression of CI, CVD and/or DM.

Keywords: Systemic lupus erythematosus; Cerebral infarction; Cardiovascular disease; Diabetes mellitus; Antibody biomarker

Introduction

Systemic lupus erythematosus (SLE) is a chronic inflammatory disorder characterized by damage to multiple organ systems caused by the production of many autoantibodies, generation of immune complexes, and activation of the complement system [1-3]. Dysfunction of T cells and accelerated activation of B cells in SLE patients [4] enables the development of various autoantigens such as the anti-nuclear antibody [5]. SLE specific autoantibodies thus far reported were the anti-Sm antibody [6], anti-double-stranded DNA antibody [7], anti-U1RNP antibody [8], anti-SSA/Ro antibody [9,10] and the anti-P ribosomal protein antibody [11], yet the pathogenic role of these antibodies remains to be proven.

Accelerated atherosclerotic diseases have been recognized as major causes of mortality in SLE. In the study of large case series of patients with SLE, 6-20% and 4-15% of deaths were due to cardiovascular disease (CVD) and cerebrovascular disease, respectively [12-14]. To estimate the onset risk of accelerated atherosclerosis in SLE patients, several markers have been introduced including C-reactive protein [15], lipoprotein (a) [16], homocysteine [17], inflammatory cytokines [18,19], yet the satisfactory results have not been obtained.

On the other hand, recent studies have revealed that specific autoantibodies exist in the sera of patients with atherosclerosis, such as autoantibodies for phospholipid (Antiphospholipid syndrome) [20,21], apolipoprotein A-1 [22] and oxidized low-density lipoprotein [23]. We have also reported that the antibody levels against RPA2 were associated with the onset of ischemic stroke [24]. These antibody markers might be useful for evaluation of the onset of lethal atherosclerotic disease in patients with SLE.

In the present study, we have comprehensively screened autoantigens which were recognized by IgG antibodies in the sera of patients with SLE by the protein array method. We then selected and identified autoantigens specific for cerebral infarction (CI), CVD and/or diabetes mellitus (DM).

Materials and Methods

Patients and healthy donor sera

This study was approved by the Local Ethical Review Board of the Chiba University, Graduate School of Medicine as well as that of the National Hospital Organization, Shimoshizu Hospital and Chiba-East hospitals. Sera were collected from patients after they had given written informed consent. Each serum sample was centrifuged at 3,000 x g for 10 min, and then the supernatant was stored at -80°C until use.

The serum samples of SLE were obtained from Shimoshizu Hospital, and those of CI and transient ischemic attack (TIA) were obtained from Sawara Hospital, Rosai Hospital, Aoba Hospital and Chiba Medical Center. Samples of CVD and DM were obtained from Chiba University Hospital, and those of healthy donors were from Chiba University, Kashiwado Clinic and Fujikura Kasei Co.

Protein array screening

Initial screening was performed using ProtoArrays® Human Protein Microarrays v4.0 (Thermo Fisher Scientific, Waltham, MA), which were loaded with 9,480 species of proteins. A total of 11 sera, 6 from patients and 5 from healthy donors, were used to detect antigens recognized specifically by IgG antibodies in the sera of patients.

Peptide synthesis

Three epitope sites in the candidate antigen proteins were predicted using the program ProPred (http://www.imtech.res.in/raghava/propred/). N-terminal biotinylated 15mer peptides without purification were synthesized and used in the second screening. For the third screening, synthetic peptides were purified by HPLC. 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™ from Perkin Elmer) containing 2.5 µL of 1/100-diluted serum and 2.5 µL of biotinylated synthetic 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 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 plate was read on an EnSpire Alpha microplate reader (PerkinElmer).

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. 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

Initial screening of SLE-specific antigens by protein array

By using protein microarrays loading with 9,480 proteins, we examined 6 sera from SLE patients and 5 sera from healthy controls to identify SLE-associated antigens. Sixty-seven proteins such as SOSTDC1, CTNND1 were selected as antigens by reacting with more than 5 sera from SLE patients and not with any of the sera from healthy donors (Table 1). These proteins may include not only antigens specific for SLE but also those specific for the complication such as CI, CVD and DM.

Name	
ZIC4	C9orf32
SDHB	DKFZp762
MGC17553	SOSTDC1
RARS2	RNPC3
IPO11	CDC45L
SLC25A24	ZNF649
OTX1	ABAT
MKRN2	CLIC5
KCNS3	H2AFY

ATP6V0A1	TOP3B
SIAH1	KIAA0391
TAS2R13	SND1
FGF23	PCDHA7
TFAM	ZNF449
CLDND1	PPAN
MAFG	AGPAT6
CCNG2	CTRB1
ACTL6B	MAPK13
APEX1	ORC3L
PARP15	MAP4K4
CLK1	RAPGEF4
KIF12	SIRT1
CTNND1	VEGFD
TCF7L2	ZNF187
MYBBP1A	TUFM
ANK1	MIER3
C15orf15	ERp27
PRKCH	C3orf37
ENG	HAPLN1
NOLA1	RNF32
RPS15A	GLCE
C15orf15	UXS1
RBMS3	ETV3
CSNK1A1	

Table 1: List of Protein array-selected antigens recognized by serum antibodies of SLE patients.

Second screening using crude peptides

The amino acid sequences of the 170 peptides shown in Table 2 were predicted as epitope sites of 67 candidate antigen proteins selected in the first screening. In the second screening, these 170 peptides were synthesized and used as antigens for analysis of the serum antibody levels by AlphaLISA. The serum levels of eight peptides (No. 55, 57, 63, 79, 87, 88, 113 and 128) were significantly higher in SLE, CI and/or CVD patients than in healthy controls, and these peptides were selected as useful markers.

Third screening using purified peptides

We then obtained highly purified biotinylated polypeptides, SOSTDC1-156, CTNND1-211, CLDND1-69, CCNG2-231, TFAM-231, TOP3B-628, MYBBP1A-1134 and MYBBP1A-1306. The sera of HD and SLE patients used for AlphaLISA were obtained from Shimoshizu Hospital. Serum antibodies against SOSTDC1-156, CTNND1-211, TOP3B-628 and MYBBP1A-1306 showed significantly high levels in patients with SLE as compared to those in HD (Table 3). Other peptides showed no apparent difference, probably because the peptides were selected based on the difference between CI and HD in the second screening.

The antibody levels of most peptides were higher in patients with CI (Rosai Hospital and Aoba Hospital) or CVD (Chiba University Hospital and Kyoto University Hospital) than in HD (Table 4). In particular, the levels against SOSTDC1-156 and CLDND1-69 showed obvious differences between CI and HD. On the other hand, CTNND1-211 and CLDND1-69 showed large differences between CVD and HD. When the cut-off value was determined as the average + 2SD of healthy donors, the positivity of CTNND1-211 in CVD was 12.5%. We then examined another set of patients with CI (Narita Red Cross Hospital, Chiba Medical Center and Chiba University Hospital) together with DM (Chiba University Hospital). The differences between CI and HD were reproduced for SOSTDC1-156 and CLDND1-69, and CCNG2-231, and TOP3B-628 also showed clear differences (Table 5). By comparing between HD and patients with DM, CCNG2-231 showed the most obvious difference, although most of other peptides showed similar differences.

Validation test for acute CI and TIA

We further examined the serum antibody levels using another set of sera from patients with aCI (Sawara Hospital) as well as those who has experienced TIA. The serum antibody levels to SOSTDC1-156 were higher in TIA and aCI patients as compared with those of HD (Figure 1). The levels to CTNND1-211, CLDND1-69 and CCNG2-231 were elevated at the aCI stage but not at the TIA stage.

Correlation analysis

We then performed Spearman correlation analysis between the antibody levels and the information of the subject persons including gender, age, height, weight, BMI, maximum intima-media thickness (IMT), blood test data and lifestyle such as smoking, alcohol intake, and work and exercise habits. Data from more than 400 patients were analyzed. The levels of SOSTDC1-156 showed a positive correlation with age, max IMT, complication of hypertension and smoking habit but reverse correlation with working and Chinese tea drinking habits (Table 6). Correlation with IMT represents that this marker reflects atherosclerosis. The levels of CTNND1-211 showed reverse correlation with weight and BMI.

No.	Name	Sequence	No.	Name	Sequence	No.	Name	Sequence	No.	Name	Sequence
1	ZIC4-3	YKTSLVMRKRL RLYR	44	SIAH1-267	FAENGNLGINVT ISM	87	**MYBBP1A-113 4**	LYWQAMKTLGV QRPK	130	KIAA0391-1 80	KYLYLCVFHMQ TSEV

2	ZIC4-185	FKAKYKLVNHIRVHT	45	TAS2R13-30	INCIDWVSKRELSSV	88	**MYBBP1A-1306**	IRSPSLLQSGAKKKA	131	KIAA0391-478	DDPFLLYATLHSGNH
3	ZIC4-269	RGCDKCYTHPSSLRK	46	TAS2R13-110	KIASFSSPAFLYLKW	89	ANK1-34	CFVLKHIHQELDKEL	132	SND1-423	INIAEALVSKGLATV
4	SDHB-238	FSLYRCHTIMNCTRT	47	TAS2R13-179	VKFTMTMFSLTPFTV	90	ANK1-99	TKKIIRKVVRQIDLS	133	PCDHA7-172	LSPNEYFFLDVPTSN
5	MGC17553-17	PSKENWFRQLRSQAV	48	TAS2R13-284	GNAKLRQAFLLVAAK	91	C15orf15-22	VRNDCKVFRFCKSKC	134	PCDHA7-789	PNPDWRYSASLRAGM
6	MGC23985-18	LTCYADDKPDKPDDK	49	FGF23-6	LRLWVCALCSVCSMS	92	PRKCH-130	YDHFVANCTLQFQEL	135	ZNF449-69	ILELLVLEQFLTILP
7	RARS2-2	ACGFRRAIACQLSRV	50	FGF23-40	IHLYTATARNSYHLQ	93	PRKCH-171	TLTGSFTEATLQRDR	136	PPAN-69	RMTLQLIKVQEGVGE
8	RARS2-179	GLLGTGFQLFGYEEK	51	FGF23-85	ITGVMSRRYLCMDFR	94	PRKCH-360	SRSTLRRQGKESSKE	137	AGPAT6-45	LYMKSLLKIFAWATL
9	RARS2-359	QMLKIMGYDWAERCQ	52	FGF23-131	QYHFLVSLGRAKRAF	95	ENG-98	VLSVNSSVFLHLQAL	138	AGPAT6-171	RYCFLLPLRIALAFT
10	RARS2-402	LRMLQNMASIKTTKE	53	TFAM-5	RSMWGVLSALGRSGA	96	ENG-473	SFVQVRVSPSVSEFL	139	AGPAT6-368	MVTYLLRMMTSWAIV
11	RARS2-500	QHLLRFDEVLYKSSQ	54	TFAM-38	LPRWFSSVLASCPKK	97	ENG-583	TSKGLVLPAVLGITF	140	CTRB1-229	GIVSWGSDTCSTSSP
12	IPO11-52	HTLDINVRWLAVLYF	55	**TFAM-231**	LRRTIKKQRKYGAEE	98	NOLA1-123	FYFSVKLSENMKASS	141	MAPK13-189	RAPEVILSWMHYNQT
13	IPO11-143	RQHRALLTFYHVTKT	56	CLDND1-12	ACVLSLISTIYMAAS	99	RPS15A-2	VRMNVLADALKSINN	142	ORC3L-212	SPPVVVILKDMESFA
14	IPO11-215	LKVLRKLTVNGFVEP	57	**CLDND1-69**	FRYNGTVGLWRRCIT	100	RPS15A-31	SKVIVRFLTVMMKHG	143	ORC3L-297	QFPFKINEKVLQVLT
15	IPO11-320	CMNLIKMIVKNYAYK	58	CLDND1-177	HLLAGLCTLGSVSCY	101	RPS15A-99	FGFIVLTTSAGIMDH	144	ORC3L-410	MNYFLVLRCLHKFTS
16	IPO11-526	DQDLVVRIETATTLK	59	MAFG-34	VRELNQHLRGLSKEE	102	C15orf15-23	RNDCKVFRFCKSKCH	145	MAP4K4-40	VEVVGNGTYGQVYKG
17	IPO11-579	HVLHVLSCVIERVNM	60	CCNG2-84	LDRFLALMKVKPKHL	103	RBMS3-130	PTNLYISNLPISMDE	146	MAP4K4-152	GLAHLHIHHVIHRDI
18	IPO11-708	KIINGYIFLSSTEFL	61	CCNG2-130	QCKCTASDIKRMEKI	104	CSNK1A1-13	VGGKYKLVRKIGSGS	147	RAPGEF4-44	PLRPANTITKVPSEK
19	SLC25A24-113	QSLQTLGLTISEQQA	62	CCNG2-181	SLDKLEAQLKACNCR	105	CSNK1A1-109	TMKTVLMLADQMISR	148	RAPGEF4-391	MMHCVFMPNTQLCPA
20	SLC25A24-248	RSLWRGNGTNVIKIA	63	**CCNG2-231**	KKHSKINDTEFFYWR	106	CSNK1A1-277	LRQLFRILFRTLNHQ	149	RAPGEF4-583	DVSVFTTLTINGRLF
21	SLC25A24-389	LGCGALSSTCGQLAS	64	CCNG2-270	WIVSRRTAQNLHNSY	107	C9orf32-156	SLRPNGIIVIKDNMA	150	RAPGEF4-680	QFWVVTEICLCSQLS
22	SLC25A24-430	LFRRIISKEGIPGLY	65	ACTL6B-146	FFLCKTAVLTAFANG	108	C9orf32-184	CRDLDVVRRIICSAG	151	RAPGEF4-841	SYVRQLNVIDNQRTL
23	SLC25A24-444	YRGITPNFMKVLPAV	66	ACTL6B-376	KLIASNSTMERKFSP	109	DKFZp762-2	LHSMSRLLSTKPSSI	152	SIRT1-26	MTLWQIVINILSEPP
24	OTX1-68	REEVALKINLPESRV	67	APEX1-168	VTAYVPNAGRGLVRL	110	DKFZp762-142	KWLISPVKIVSRPTI	153	SIRT1-246	VIGSSLKVRPVALIP
25	MKRN2-109	LRDRNLSGMAERKTQ	68	APEX1-189	DEAFRKFLKGLASRK	111	DKFZp762-365	PHFQGFQKLPSSPLG	154	VEGFD-155	NTSTSYISKQLFEIS

26	MKRN2-300	PSVYWVEDQNKKNEL	69	APEX1-250	ADSFRHLYPNTPYAY	112	SOSTDC1-9	YLLPLACILMKSCLA	155	VEGFD-173	TSVPELVPVKVANHT
27	MKRN2-367	QGTVRFFNSVRLWDF	70	APEX1-271	MNARSKNVGWRLDYF	113	**SOSTDC1-156**	KITVVTACKCKRYTR	156	ZNF187-274	CQKAFRLNSHLAQHV
28	MKRN2-402	GDLFMHLSGVESSEP	71	PARP15-9	FLHNIVVVSNCFYFQ	114	RNPC3-234	VLFGKPMVVQFARSA	157	TUFM-25	FLLQGLLRLLKAPAL
29	KCNS3-76	FRYVLNFYYTGKLHV	72	PARP15-382	YVVRVLTGVFTKGRA	115	CDC45L-15	QSQRVLLFVASDVDA	158	TUFM-408	KFNLILRQPMILEKG
30	KCNS3-171	IWIRMENPAYCLSAK	73	CLK1-75	EYRNDYTQGCEPGHR	116	CDC45L-196	TSSAMVMFELAWMLS	159	MIER3-359	NILNSFTASDLTALT
31	KCNS3-193	SVVLASIVAMCVHSM	74	CLK1-115	SKHRIHHSTSHRRSH	117	CDC45L-459	LFSRPASLSLLSKHL	160	MIER3-507	FISAHALHQHAALHS
32	KCNS3-240	RLAAAPCQKKFWKNP	75	KIF12-102	LYISRQTAQQMPSVD	118	CDC45L-488	LLPLVMAAPLSMEHG	161	ERp27-74	ILHSMVQKFPGVSFG
33	ATP6V0A1-128	LKFILRKTQQFFDEM	76	KIF12-203	CVSPSAQCLPETLST	119	ZNF649-480	QGKSPVNMVTVAMVA	162	ERp27-159	VIQIHLLLIMNKASP
34	ATP6V0A1-214	YVHKSVFIIFFQGDQ	77	KIF12-218	LRYASRAQRVTTRPQ	120	ABAT-370	FRPNAPYRIFNTWLG	163	C3orf37-169	DNWRLLTMAGIFDCW
35	ATP6V0A1-269	QMVLNQTEDHRQRVL	78	CTNND1-134	VRLLRKARDMDLTEV	121	ABAT-387	SKNLLLAEVINIIKR	164	HAPLN1-261	FYYLIHPTKLTYDVA
36	ATP6V0A1-297	VRKMKAIYHTLNLCN	79	**CTNND1-211**	LRNVSSERSEARRKL	122	ABAT-446	DDSIRNKLILIARNK	165	RNF32-29	LQLRNLSVADHSKTQ
37	ATP6V0A1-426	RESRILSQKNEEMF	80	CTNND1-480	NKSGNRSEKEVRAAA	123	CLIC5-41	ILWLKGVVFNVTTVD	166	RNF32-187	IKCVTRIQAYWRGCV
38	ATP6V0A1-494	LRGNPVLQLNPALPG	81	CTNND1-525	NNASRSQSSHSYDDS	124	CLIC5-212	TGLWRYLKNAYARDE	167	GLCE-17	CALFTLVTVLLWNKC
39	ATP6V0A1-526	TNKLTFLNSFKMKMS	82	TCF7L2-162	SNKVPVVQHPHHVHP	125	H2AFY-42	LWLKGVVFNVTTVDL	168	GLCE-504	PSSFVLNGFMYSLIG
40	ATP6V0A1-774	LFFFFTAFATLTVAI	83	TCF7L2-334	KEMRAKVVAECTLKE	126	H2AFY-214	LWRYLKNAYARDEFT	169	UXS1-134	VSDLVNGLVALMNSN
41	SIAH1-43	VCFDYVLPPILQCQS	84	MYBBP1A-251	LKMAASSVKKDRKLP	127	TOP3B-301	LNTVEMLRVASSSLG	170	ETV3-124	NYPFINIRSSGKIQT
42	SIAH1-182	QSCFGFHFMLVLEKQ	85	MYBBP1A-395	VRFLSPPALQGYVAW	128	**TOP3B-628**	HRFMKYIQAKPSRLH			
43	SIAH1-211	LIGTRKQAENFAYRL	86	MYBBP1A-2036	KTLSMREVRSCFEDP	129	KIAA0391-4	YLFGIRSFPKLWKSP			

Table 2: List of amino acid sequences of synthetic peptides used for the second screening. A total of 170 peptides were predicted as epitopes derived from 67 antigen proteins. The selected useful antigen peptides are shown in bold. Numbers of peptide names represent the first amino acid number of the original proteins.

		SOSTDC1-156	**CTNND1-211**	**CLDND1-69**	**CCNG2-231**	**TFAM-231**	**TOP3B-628**	**MYBBP1A-1134**	**MYBBP1A-1306**
HD	Average	1,730	2,302	4,518	2,053	3,374	1,799	1,739	2,505
	SD	442	884	2,329	492	2,882	367	433	676
	Cut-off value	2,613	4,071	9,176	3,036	9,139	2,532	2,606	3,858
	Total No.	111	111	111	111	111	111	111	111
	Positive No.	5	6	5	5	1	5	6	3

	Positive (%)	4.50%	5.40%	4.50%	4.50%	0.90%	4.50%	5.40%	2.70%
SLE	Average	2,211	3,049	3,780	2,994	4,283	2,319	2,149	3,215
	SD	968	2,992	2,606	5,308	4,169	2,321	1,974	3,101
	Total No.	84	84	84	84	84	84	84	84
	Positive No.	13	8	4	10	1	11	10	11
	Positive (%)	**15.50%**	9.50%	4.80%	**11.90%**	1.20%	**13.10%**	**11.90%**	**13.10%**
	P (vs HD)	**0.000048**	**0.029**	-	0.109	0.089	**0.045**	0.065	**0.042**

Table 3: Comparison of serum antibody levels between HD and SLE patients examined by AlphaLISA. 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; 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 SLE patients; and P value of comparison between HD and SLE patients. P values lower than 0.05 and positive rates higher than 10% were marked in bold.

		SOSTDC1-156	CTNND1-211	CLDND1-69	CCNG2-231	TFAM-231	TOP3B-628	MYBBP1A-1134	MYBBP1A-1306
HD	Average	2,970	2,233	2,948	1,804	4,694	2,386	2,074	3,808
	SD	1,187	739	1,691	442	1,392	757	703	1,060
	Cut-off value	5,344	3,711	6,331	2,688	7,479	3,900	3,479	5,928
	Total No.	128	128	127	128	125	128	127	128
	Positive No.	6	6	3	7	5	6	6	7
	Positive (%)	4.70%	4.70%	2.40%	5.50%	4.00%	4.70%	4.70%	5.50%
CI	Average	3,549	2,529	3,587	1,936	5,133	2,619	2,292	4,672
	SD	1,241	1,227	1,941	413	1,508	941	902	5,267
	Total No.	128	128	128	128	125	127	128	128
	Positive No.	13	6	10	9	11	9	8	12
	Positive (%)	**10.20%**	4.70%	7.80%	7.00%	8.80%	7.10%	6.30%	9.40%
	P (vs. HD)	**0.00017**	**0.020**	**0.0055**	**0.015**	**0.018**	**0.030**	**0.033**	0.071
CVD	Average	3,260	2,815	3,624	1,948	5,084	2,358	2,278	4,136
	SD	1,076	876	1,568	454	1,514	901	740	1,114
	Total No.	128	128	128	128	124	128	128	128
	Positive No.	7	16	9	7	4	6	8	7
	Positive (%)	5.50%	**12.50%**	7.00%	5.50%	3.20%	4.70%	6.30%	5.50%
	P (vs. HD)	**0.042**	**2.70E-08**	**0.0011**	**0.011**	**0.036**	-	**0.025**	**0.016**

Table 4: Comparison of serum antibody levels among HD, CI patients and CVD patients examined by AlphaLISA.

Discussion

There are various types of autoantibodies in the sera of SLE patients due to the dysfunction of T cells and the accelerated activation of B cells. Available data suggest that young women with SLE are at a substantially increased risk of AMI, congestive heart failure, and cerebrovascular accidents [12-14]. If autoantibodies develop during the progress of CI and CVD, they can be amplified in patients with SLE due to their dysregulated immune systems. Thus, we performed the first screening using SLE sera and then the second and third screenings using CI and CVD samples. Through the first screening by protein array method followed by second screening using crude peptide antigens and validation tests using three sets of control HD and patients' sera, we identified SOSTDC1, CTNND1, CLDND1 and CCNG2 as novel useful markers for the diagnosis of atherosclerosis-related diseases such as CI, CVD and DM.

		SOSTDC1-156	CTNND1-211	CLDND1-69	CCNG2-231	TFAM-231	TOP3B-628	MYBBP1A-1134	MYBBP1A-1306
HD	Average	4,823	1,833	7,307	3,989	1,5203	7,546	3,680	5,356
	SD	1,793	179	3,501	1,226	5,761	1,690	2,469	1,039
	Cut-off value	8,409	2,190	14,308	6,442	26,725	10,926	8,618	7,435
	Total No.	137	137	137	137	136	136	136	136
	Positive No.	7	4	5	7	2	6	6	7
	Positive (%)	5.10%	2.90%	3.60%	5.10%	1.50%	4.40%	4.40%	5.10%
CI	Average	5,686	1,870	8,917	4,638	16,009	8,753	3,555	5,876
	SD	1,949	229	4,494	1,421	4,614	5,002	1,374	1,616
	Total No.	139	139	139	139	139	139	139	139
	Positive No.	11	8	14	12	1	12	3	16
	Positive (%)	7.90%	5.80%	**10.10%**	8.60%	0.70%	8.60%	2.20%	11.50%
	P (vs HD)	**0.00016**	0.137	**0.001**	**0.000063**	0.202	**0.0078**	-	**0.0017**
CVD	Average	5,565	1,938	9,274	4,580	17,035	9,013	4,117	5,845
	SD	2,033	397	5,780	1,418	3,718	5,739	2,160	1,451
	Total No.	108	108	108	108	108	108	108	108
	Positive No.	9	9	13	8	4	14	5	12
	Positive (%)	8.30%	8.30%	**12.00%**	7.40%	3.70%	**13.00%**	4.60%	**11.10%**
	P (vs HD)	**0.0032**	**0.012**	**0.0022**	**0.00071**	**0.003**	**0.011**	0.143	**0.0036**

Table 5: Comparison of serum antibody levels among HD, CI patients and DM patients examined by AlphaLISA.

	SOSTDC1-156		CTNND1-211		bCLDND1-69		bCCNG2-231	
	r value	P value	r value	P value	r value	P value	r value	P value
Gender	-0.079	**0.0408**	0.019	0.6341	-0.019	0.6226	0.057	0.1448
Age	0.182	**<0.0001**	0.157	**<0.0001**	0.102	**0.0089**	0.057	0.1420
Height	-0.062	0.1131	-0.054	0.1639	-0.009	0.8115	-0.028	0.4770
Weight	-0.008	0.8351	-0.125	**0.0013**	-0.065	0.0932	-0.044	0.2595
Body mass index	0.039	0.3227	-0.111	**0.0043**	-0.074	0.0586	-0.027	0.4849
Intima media thickness (IMT)	0.218	**<0.0001**	0.117	**0.0127**	0.040	0.3920	0.019	0.6819
Diabetes	0.110	**0.0045**	0.013	0.7397	-0.036	0.3614	0.017	0.6708
Hypertension	0.160	**<0.0001**	0.066	0.0919	0.038	0.3346	0.035	0.3678
Albumin/globulin ratio	0.011	0.7883	-0.005	0.9026	-0.001	0.9827	0.066	0.0962
Aspartate transaminase	0.004	0.9241	0.009	0.8197	0.016	0.6736	-0.011	0.7763
Alanine transaminase	-0.013	0.7353	0.015	0.7042	-0.051	0.1903	-0.006	0.8714
Alkaline phosphatase	0.046	0.2624	0.007	0.8733	-0.031	0.4473	-0.042	0.2991
Lactate dehydrogenase	-0.016	0.6972	0.061	0.1269	-0.015	0.7089	0.025	0.5356

Total billirubin	0.046	0.2502	-0.017	0.6647	0.026	0.5049	0.015	0.7068
Choline esterase	-0.039	0.3834	0.009	0.8342	0.018	0.6893	-0.001	0.9749
gamma-GTP	0.027	0.4988	-0.004	0.9311	-0.019	0.6432	0.003	0.9400
Total protein	-0.044	0.2729	-0.073	0.0656	-0.011	0.7861	0.002	0.9596
Albumin	-0.024	0.5439	-0.065	0.0994	-0.013	0.7397	0.054	0.1743
Blood urea nitrogen	-0.019	0.6331	-0.038	0.3306	0.000	0.9916	-0.040	0.3009
Creatinin	0.010	0.7904	-0.021	0.5848	0.023	0.5547	-0.007	0.8603
Estimated glomerular filtration rate	-0.004	0.9326	0.023	0.5866	-0.010	0.8060	-0.004	0.9214
Uric acid	-0.019	0.6690	0.030	0.5104	0.011	0.7992	0.025	0.5729
Amylase	-0.084	0.0875	-0.015	0.7540	0.017	0.7362	-0.074	0.1322
Total cholesterol	-0.067	0.1131	0.033	0.4346	-0.054	0.2030	-0.022	0.5983
HDL cholesterol	-0.002	0.9599	0.000	0.9931	0.038	0.4340	0.087	0.0694
Triglyceride	-0.031	0.5086	0.013	0.7773	-0.028	0.5419	-0.035	0.4594
Na	-0.001	0.9811	0.002	0.9500	0.003	0.9370	0.077	0.0507
K	-0.025	0.5245	0.043	0.2779	0.031	0.4397	0.058	0.1393
Cl	0.005	0.8985	0.061	0.1236	0.007	0.8680	0.036	0.3583
C-reactive protein	0.047	0.3018	-0.046	0.3182	0.056	0.2241	-0.050	0.2732
LDL cholesterol	-0.119	**0.0275**	0.043	0.4254	-0.070	0.1940	-0.091	0.0913
White blood cell	0.015	0.7028	-0.041	0.2992	0.030	0.4382	-0.036	0.3629
Red blood cell	-0.005	0.9062	-0.049	0.2110	0.030	0.4471	-0.009	0.8185
Hemoblobin	0.013	0.7468	-0.059	0.1360	0.034	0.3896	0.007	0.8621
Hematocrit	0.017	0.6567	-0.047	0.2325	0.039	0.3262	0.031	0.4225
Mean cell volume	0.072	0.0647	0.026	0.5057	-0.005	0.896	0.049	0.2145
Mean corpuscular hemoglobin	0.050	0.1988	-0.018	0.6498	0.009	0.8229	-0.002	0.9513
Mean corpuscular hemoglobin concentration	-0.021	0.6015	-0.070	0.0737	0.019	0.6314	-0.067	0.0891
Red cell dstribution width	0.021	0.5894	-0.011	0.7837	-0.002	0.9551	-0.030	0.4462
Platelet	-0.031	0.4254	-0.027	0.4944	0.027	0.4896	0.010	0.8020
Mean platelet volume	0.005	0.8969	0.025	0.5186	-0.019	0.6356	0.025	0.5217
Procalcitonin	-0.020	0.6023	-0.016	0.6912	0.035	0.3677	0.031	0.4303
Platelet distribution width	-0.002	0.9667	-0.002	0.9601	-0.037	0.3433	0.006	0.8785
Blood sugar	0.047	0.2467	0.011	0.7832	-0.069	0.0909	-0.063	0.1215
HbA1c	0.016	0.7264	0.015	0.7405	-0.067	0.1310	-0.042	0.3409
Smoking habit	0.152	**<0.0001**	-0.058	0.1368	-0.010	0.8047	-0.036	0.3532
Alcohol drinking habit	0.058	0.1386	-0.053	0.1762	0.029	0.4552	-0.033	0.3934
Green tea drinking habit	-0.017	0.6664	0.018	0.6377	-0.014	0.7178	0.054	0.1690
Coffee drinking habit	-0.064	0.1022	-0.005	0.8913	-0.008	0.8346	0.021	0.5904

Chinese tea drinking habit	-0.083	**0.0323**	-0.025	0.5245	-0.023	0.5616	0.003	0.9440
Working habit	-0.137	**0.0005**	-0.073	0.0659	-0.024	0.5457	-0.057	0.1472
Exercise habit	-0.029	0.484	-0.011	0.7888	-0.024	0.5572	-0.049	0.2309

Table 6: Correlation analysis between antibody marker levels and the subject's information. Shown are correlation coefficients (r) and P values calculated by Spearman's analysis. Significant correlations are marked in bold.

The following information is known for these selected markers: SOSTDC1/sclerostin domain containing 1 (Accession No.: NM_015464) is a member of bone morphogenetic protein (BMP) of TGF-β superfamily [25,26]. It works as a BMP antagonist and suppresses cell proliferation, differentiation or cell death induced by BMP. BMPs also play important parts in the development of atherosclerosis [27]. CTNND1/catenin (cadherin-associated protein), delta 1 (Accession No.: NM_001085458) is a member of the Armadillo protein family and mediates the signaling from the cell-adhesion molecule cadherin onto cells [28]. CLDND1/claudin domain containing 1 (Accession No.: NM_001040181) contains the domain of claudin which is involved in tight junction, but its function is not known [29]. CCNG2/cyclin G2 (Accession No.: NM_004354): It is a member of the cyclin family and induced by DNA damaging agents [30].

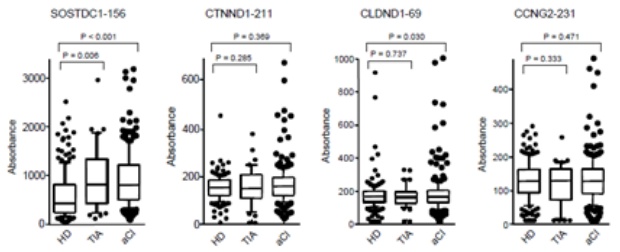

Figure 1: Serum antibody levels examined by AlphaLISA. The levels in the sera of 228 CI patients, 44 TIA patients and 137 HD were measured by AlphaLISA using synthetic antigen peptides, SOSTDC1-156, CTNND1-211, CLDND1-69 and CCNG2-231. The box-whisker plots display the 25th, 50th and 75th percentiles. The upper and lower cross bars of the box represent 90th and 10th percentiles, respectively. Values higher than 90th percentile or lower than 10th percentile are marked by dots. P values between HD and TIA or CI patients were calculated using the Mann-Whitney U test.

The positivity was approximately 10% and 13% at most. Multiple factors can affect the progress of CI, CVD and DM. Spearman correlation analysis between the antibody levels and the information of the patients revealed that the levels of SOSTDC1-156 but not of CTNND1-211, CLDND1-69 or CCNG2-231 are correlated with IMT, hypertension and smoking (Table 6). Thus, the SOSTDC1-156 marker can predict atherosclerotic CI caused by hypertension and/or smoking habit. There are many causes that affect the progress of CI, and each antibody marker may be associated with a respective cause of CI. Thus, the positivity of each maker cannot be expected to particularly high. The development of an increasing number of such antibody markers may make the prediction of the onset of CI at a strong possibility.

We used the sera of patients with CI within two weeks of onset. Various antigens 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 are known to have been 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 induce suddenly but mediated frequently by health issues such as TIA and asymptomatic CI. When small infarctions occur, it is possible for antigens to leak out from infarction lesions. Repeated exposure to such antigens may raise the antibodies to detectable levels. In fact, the antibody levels against SOSTDC1-156 were found to be higher in TIA patients than that of those in HD (Figure 1). The antibody levels of CCNG2-231 were highly associated with DM (Table 5), and therefore, it may be useful for the early diagnosis of DM. If the levels of both SOSTDC1-156 and CCNG2-231 were high, the patient might suffer from CI caused by DM. CTNND1-211 and CLDND1-69 may contribute to diagnose CVD. Application of these biomarkers for the clinical use is very important and the early development of the diagnosis kit is expected.

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