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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].

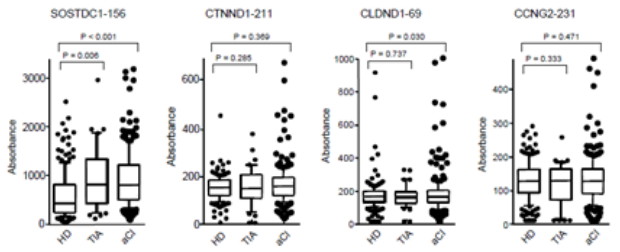


**Figure 1:** Serum antibody levels examined by AlphaLISA. The levels in the sera of 228 CI patients, 44 TIA patients and 137 HD were measured by AlphaLISA using synthetic antigen peptides, SOSTDC1-156, CTNND1-211, CLDND1-69 and CCNG2-231. The box-whisker plots display the 25th, 50th and 75th percentiles. 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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