Identification of *secernin 1* as a novel immunotherapy target for gastric cancer using the expression profiles of cDNA microarray

Takako Suda,[1,2] Takuya Tsunoda,[1] Naotaka Uchida,[1] Takeshi Watanabe,[3,4] Suguru Hasegawa,[4] Seiji Satoh,[4] Shigetsugu Ohgi,[2] Yoichi Furukawa,[3] Yusuke Nakamura[3] and Hideaki Tahara[1,5]

[1]Department of Surgery and Bioengineering, Institute of Medical Science, The University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108-8639; [2]Division of Organ Regeneration Surgery, Department of Surgery, Tottori University Faculty of Medicine, 36-1 Nishi-machi, Yonago, Tottori 683-8504; [3]Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108-8639; and [4]Department of Gastroenterological Surgery, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan

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Despite the discovery of multiple TAAs, only a limited number is available for clinical application, particularly against epithelial malignancies. In this study we searched for novel TAAs using expression profiles of gastric cancer examined with cDNA microarray, and identified the *SCRN1* gene as a candidate. *SCRN1* was confirmed to be expressed in five out of seven gastric cancers with semiquantitative RT-PCR. With Northern blot analysis, it was detected abundantly in the testis and ovary, but it was barely detectable in 14 other normal human adult organs. Colony formation assay revealed that its augmented expression is associated with promoted cell growth. As these expression profiles and functional features of *SCRN1* appeared to be compatible with the characteristics of the hypothesized ideal TAAs, we examined whether SCRN1 protein contains antigenic epitope peptides restricted to HLA-A*0201. We synthesized the candidate peptides derived from *SCRN1*, and tried to induce CTLs with each peptide. The CTL clones were successfully induced with a peptide SCRN1-196 (KMDAEHPEL), and they lyzed not only the peptide-pulsed targets but also the tumor cells expressing both SCRN1 and HLA-A*0201 endogenously. These results strongly suggest that SCRN1-196 is an epitope peptide restricted to HLA-A*0201. Furthermore, we synthesized an anchor-modified peptide SCRN1–9 V (KMDAEHPEV), in which leucine at position 9 was substituted for valine to increase the binding affinity to the HLA-A*0201 molecules. The CTL clones induced by SCRN1–9 V also recognized tumor cells expressing its natural SCRN1 protein endogenously. These results strongly suggest that SCRN1 is a novel TAA and these peptides, both native and modified, may be applicable for cancer vaccines to treat gastric cancer. (*Cancer Sci* 2006; *97*: 411–419)

Gastric cancer is one of the most common malignancies in the Asian population, especially in Japan, accounting for a large percentage of cancer-related deaths.[1] Advances in diagnostic and therapeutic modalities have improved long-term survival for early stage disease, but those patients with advanced tumors still have uniformly poor prognoses[2] and are in need of newer therapeutic modalities.[3] With the discovery of TAAs,[4,5] cancer vaccine strategy has become a promising therapeutic approach. Advances in molecular biology and tumor immunology have led to the isolation of more than 50 TAAs, including MAGE-1, MAGE-2, MAGE-3, MAGE-12,[6,7] BAGE, GAGE, PAGE, XAGE, NY-ESO-1,[8] SSX, HER-2/neu,[9] SPANX and TRAG-3. They were identified with cDNA expression cloning,[10,11] serological analysis of recombinant cDNA expression libraries,[12,13] proteomic analysis, and expression profiling with microarray.[14–16] Clinical trials using their epitope peptides conjugated with incomplete Freund's adjuvant or pulsed on DCs have been carried out for patients with malignant melanoma[7,17–21] and other epithelial malignancies.[9,22–24] However, further exploration of new TAAs appears to be necessary, as the number of clinically applicable peptides is still limited, considering the diversity of the phenotypic characteristics of tumors. In this study, we focused on cDNA microarray containing 23 040 human genes[25] that the *SCRN1* gene was upregulated in the majority of gastric cancers. We also noted that its expression is associated with augmented cell proliferation. As *SCRN1* appeared to be a promising immunological target, judging from its tumor specificity and functional features, we hypothesized SCRN1 as a novel TAA. To test this hypothesis, we examined whether the SCRN1 protein contains antigenic peptides. We also tried to identify epitope peptides restricted to HLA-A*0201 to clinically apply it to gastric cancer immunotherapy.

Materials and Methods

Cell lines

Human gastric cancer cell lines (MKN1, MKN28, and MKN45) expressing HLA-A*2402, Kato III, another gastric cancer line expressing HLA-A*0201/0207, a mouse fibroblast cell line (NIH3T3), a monkey kidney cell line (COS-7), and a human hybrid between B and T lymphoblastic cell lines (T2)

[5]To whom correspondence should be addressed. E-mail: tahara@ims.u-tokyo.ac.jp
Abbreviations: AS, autologous serum; BIMAS, Bioinformatics and Molecular Analysis Section; C/H, cold target/hot target; CTL, cytotoxic T lymphocyte; DC, dendritic cell; E/T, effector/target; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; mAb, monoclonal antibody; MAGE, melanoma antigen; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; SCRN1, secernin 1; TAA, tumor-associated antigen.

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expressing HLA-A*0201 were purchased from the American Type Culture Collection (Rockville, MD). A human hepatocellular carcinoma cell line, SNU475 (HLA-A*0201/1101) was obtained from the Korean Cell Line Bank (Seoul National University, Seoul, Korea). A3LCL, an Epstein–Barr virus-transformed B-lymphoblastoid cell line (HLA-A*0301/), was generously provided by Takara Shuzo (Otsu, Japan). All of these cells were cultured as monolayers in appropriate media: Dulbecco's modified Eagle's medium (COS7, NIH3T3), and RPMI-1640 medium (T2, A3LCL, MKN1, MKN28, MKN45, Kato III and SNU475), supplemented with 10% fetal bovine serum (Cansera International, Ontario, Canada) and 1% antibiotic/antimycotic solution (Sigma-Aldrich, St Louis, MO, USA). Cells were maintained at 37°C in an atmosphere of humidified air with 5% CO_2. Cancerous tissues and corresponding non-cancerous mucosae were excised from 20 patients during surgery, after informed consent had been obtained.

RNA preparation and RT-PCR analysis

Total RNA was extracted using either the RNeasy kit (Qiagen, Dusseldorf, Germany) or the Trizol reagent (Life Technologies, Rockville, MD, USA) according to the manufacturers' instructions. Ten μg of total RNA was reverse-transcribed for single-stranded cDNAs using the poly $dT_{12–18}$ primer (Amersham Pharmacia Biotech, Buckinghamshire, UK) with Superscript II reverse transcriptase (Life Technologies). Each single-stranded cDNA was then diluted for subsequent PCR amplification for target genes along with the *GAPDH* gene as a quantitative control. All of the reactions involved initial denaturation at 94°C for 4 min followed by 20 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s (for GAPDH); or 30 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s (for SCRN1). Primer sequences were as follows: for GAPDH forward 5′-ACAACAGCCTCAAGATCATCAG-3′; for GAPDH reverse 5′-GGTCCACCACTGACACGTTG-3′; for SCRN1 forward 5′-TGCATAGACTCTGAGTTTTTCC-3′; and for SCRN1 reverse 5′-CTTTCTCATTTTACTCAAACAGG-3′.

Northern blot analysis on multiple tissues

Human multiple-tissue blots (Clontech, Palo Alto, CA) were hybridized with ^{32}P-labeled SCRN1 cDNA as a probe. Pre-hybridization, hybridization and washing were performed according to the manufacturer's recommendations. The blots were then autoradiographed with intensifying screens at −80°C for 24 h.

Immunocytochemical staining

The entire coding region of SCRN1 was amplified by RT-PCR using a set of SCRN1-specific primers, 5′-GCGGATCCAGGATGGCTGCAGCTCCTCCAAG-3′ and 5′-TAGAATTCTTAAAGAACTTAATCTCCGTGTCAACAC-3′. The PCR products were then cloned into the appropriate cloning site of pcDNA3.1myc/His (Invitrogen, Carlsbad, CA, USA) vector. COS7 cells transfected with pcDNA3.1myc/His-SCRN1 were fixed with PBS containing 4% paraformaldehyde for 15 min, and were then rendered permeable with PBS containing 0.1% Triton X-100 for 2.5 min at room temperature. Subsequently, the cells were covered with 2% bovine serum albumin in PBS for 24 h at 4°C to block any non-specific hybridization. Mouse antimyc monoclonal antibody (Sigma) at 1 : 1000 dilution was used as the first antibody, and the reaction was visualized after incubation with rhodamine-conjugated antimouse antibody (Leinco, St Louis, MO, USA; ICN, Costa Mesa, CA, USA). Nuclei were counter-stained with 4′,6′-diamidine-2′-phenylindole dihydrochloride. Fluorescent images were obtained under an Eclipse E800 microscope (Nikon, Tokyo, Japan).

Colony formation assay

The entire coding region of SCRN1 was amplified by RT-PCR using a set of primers, 5′-GCGGATCCAGGATGGCTGCAGCTCCTCCAAG-3′ and 5′-TAGAATTCTTAAAGAACTTAATCTCCGTGTCAACAC-3′, and the PCR product was subsequently cloned into an appropriate cloning site of pcDNA3.1myc/His vector (Invitrogen). NIH3T3 cells were transfected transiently with pcDNA3.1myc/His-SCRN1 or pcDNA3.1myc/His (mock) using FuGENE6 transfection reagent (Roche, Grenzacherstrasse, Switzerland), and incubated with 0.9 mg/mL of geneticin for 14 days. The surviving cells were fixed with 100% methanol and stained using Giemsa solution. All experiments were carried out in duplicate.

Selection and synthesis of candidate peptides derived from SCRN1

From the 9-mer and 10-mer peptides derived from SCRN1, candidates for the antigenic epitope were selected based on the predicted binding affinities to HLA-A*0201 molecules, the most frequently observed HLA-allele, using the HLA Peptide Binding Predictions program,[26,27] available at the BIMAS (National Institutes of Health, Bethesda, MD) website http://bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform. These peptides were synthesized by Mimotopes (San Diego, CA) using the standard solid-phase synthesis method and were purified by reverse phase high-performance liquid chromatography. The purity (>90%) and the identity of the peptides were determined by analytical reverse phase high-performance liquid chromatography and mass spectrometry analysis, respectively. These peptides were dissolved in dimethylsulfoxide at 20 mg/mL and stored at −80°C. $CMVpp65_{495–503}$ (NLVPMVATV),[28] an HLA-A*0201-restricted epitope peptide derived from cytomegalovirus protein pp65, was used for cytotoxicity assay as a control.

CTL induction by candidate peptides *in vitro*

DCs were prepared from PBMC donated by HLA-A*0201 healthy volunteers for the use of antigen presenting cells as described previously.[14,29] Briefly, the adherent monocyte-enriched population isolated from PBMC in a plastic tissue culture flask was cultured in the presence of 1000 IU/mL of granulocyte macrophage colony-stimulating factor (generous gift from Kirin Brewery, Tokyo, Japan) and 1000 IU/mL of IL-4 (Techne, Minneapolis, MN) in AIM-V (Life Technologies) containing 2% heat-inactivated AS. After 5 days in the culture, OK-432 (generously provided by Chugai Pharmaceutical, Tokyo, Japan) was added to the culture at the concentration of 10 μg/mL to induce the maturation of DCs. After 7 days in the culture, the mature DCs were harvested and pulsed with 20 μM of the candidate peptides for 4 h at 20°C, and these peptide-pulsed DCs were irradiated (55 Gy) and incubated with autologous $CD8^+$ T lymphocytes, obtained by positive selection

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with Dynabeads M-450 CD8 and Detachabead (both from Dynal, Lake Success, NY) at a 1 : 20 ratio. These cultures were set up in 48-well plates (Corning, Corning, NY); each well contained 1.5×10^4 peptide-pulsed DCs, 3×10^5 $CD8^+$ T cells and 10 ng/mL IL-7 (Techne) in 0.5 mL of AIM-V/2%AS. Three days later, IL-2 (Chiron, Emeryville, CA) was added to these cultures at a final concentration of 20 IU/mL. On days 7 and 14, the T cells were restimulated with the autologous peptide-pulsed DCs as described above. Cytotoxicity was tested on day 21 against peptide-pulsed T2 cells at day 21 as described elsewhere.[29]

Cytotoxicity assay

Cytotoxicity of the stimulated T cells was measured with ^{51}Cr release assay. The T2 cells (HLA-A*0201-positive) and tumor cells were used as target cells. The T2 cells were pulsed with 20 μM peptide for 16 h before the labeling. The target cells were subsequently labeled with 100 μCi of $Na_2{}^{51}CrO_4$ (Perkin-Elmer Life Sciences, Boston, MA) for an additional 1 h at 37°C. The labeled cells (target) were then washed with PBS and mixed with the pretreated T cells (effector) at various E/T ratios in 96-well plates. Half of the supernatant was collected from each well after 4 h of incubation, and its radioactivity was measured in a gamma scintillation counter. The percentage of specific cytotoxicity was determined by calculating the percentage of specific ^{51}Cr-release by the following formula: [(^{51}Cr-releasing activity of each experiment-background activity)/(maximum ^{51}Cr-releasing activity-background activity)] × 100 (%).

Background release was determined by incubating the target cells alone, in the absence of effectors, and the maximum release was obtained by incubating the targets with 1 mol/L HCl.

Establishment of CTL clones

The lymphoid cells induced on 48-well plates and tested for positive peptide-specific lysis as described above were expanded to establish CTL lines, and subsequently subjected for limiting dilution to establish CTL clones. To establish CTL lines, a total of 5×10^4 CTLs were re-suspended and expanded in 25 mL of AIM-V/2%AS, along with 2.5×10^7 irradiated (3300 rads) allogenic PBMCs and 5×10^6 irradiated (8000 rads) A3LCL cells, in the presence of 30 ng/mL of anti-CD3 mAb (BD Bioscience-PharMingen, San Diego, CA) and 120 IU/mL of IL-2 as reported by Walter *et al*.[30] One day after initiating the cultures, 120 IU/mL IL-2 was added to the cultures, and the cultures were fed with fresh AIM-V/5%AS containing 30 IU/mL IL-2 on days 5, 8, 11 and 14. The cytotoxicity of the expanded CTLs was tested on day 14, and we carried out limiting dilution of the CTL lines showing significant cytotoxicities against their corresponding peptides were carried out to establish CTL clones. Limiting dilution[14] was performed with 7×10^4 cells of allogenic PBMC, 1×10^4 cells of A3LCL cells, 30 ng/mL of anti-CD3 mAb and 125 IU/mL of IL-2 per well in 96 round-bottomed microtiter plates and each microculture was tested for cytotoxicity. Cells from CTL lines were added 0.3, 0.5, 1, 3, and 5 cells per 96 well (0.3 = 1 cell per 3 wells). Three plates were prepared for each cell number. We picked up the cells that grew well. The CTLs in a positive well were expanded once again as 'CTL clones' for further examination.

Cold target inhibition assay

Antigen specificity was confirmed by the cold target inhibition assay. SNU475 cells expressing both HLA-A*0201 and SCRN1 were labeled with $Na_2{}^{51}CrO_4$ (hot target) and subsequently mixed at various cold target : hot target (C/H) ratios with unlabeled T2 cells (cold target) pulsed with the peptide (20 μM for 16 h at 37°C) to compete for the recognition of ^{51}Cr-labeled SNU475 cells. After preparation of target cells, CTLs (effector) were added and incubated for 4 h. The number of target cells was fixed to 1×10^4/well. The cytotoxicity of each C/H ratio was measured using ^{51}Cr release assay. The percentage of specific inhibition (% inhibition) was calculated using the following formula: [(%Lysis (C/H ratio 0) – %Lysis (each experiment))/%Lysis (C/H ratio 0)] × 100 (%).

Antibody blocking assay

SNU475 cells pretreated with anti-HLA-A/B/C mAb or anti-HLA-DR/DB mAb for 1 h were co-cultured with CTL clones that were pretreated with anti-CD4 mAb or anti-CD8 mAb (all mAbs from Dako, Carpinteria, CA) for 1 h, at an E/T of 20. Isotype antibodies, antimouse IgG2a mAb for anti-HLA-A/B/C mAb and antimouse IgG1 mAb for anti-HLA-DR/DB mAb, anti-CD4 mAb, and anti-CD8 mAb were used as the controls. The percentage of specific inhibition (% inhibition) was calculated with the following formula: [(%lysis (isotype) – %lysis (experimental))/%lysis (isotype)] × 100 (%).

Results

Identification of *SCRN1*, a human gene commonly upregulated in gastric cancer

Using a genome-wide cDNA microarray containing 23 040 genes, we previously examined expression profiles of 20 gastric cancers and their corresponding normal gastric mucosae.[31] Among the commonly upregulated genes in the cancers, we identified a gene with an in-house accession number of C8121. Its signal intensity ratios of cancer to non-cancerous gastric tissues were greater than two. Microarray examination was carried out in nine of 11 gastric cancers that passed our cut-off filter (Fig. 1a). A homology search with the nucleotide sequence of C8121 revealed that it corresponded with the *SCRN1* gene. Semiquantitative RT-PCR experiments confirmed its increased expression in five of the seven gastric cancers compared with their corresponding non-cancerous tissues (Fig. 1b). Additional examination of other types of malignancies showed that *SCRN1* was upregulated in 22 of 24 colon cancers examined and one of six hepatocellular carcinomas examined (data not shown).

Expression of SCRN1 in normal vital organs

Multi-tissue Northern blot analyses using *SCRN1* cDNA as a probe showed a 4.9-kb transcript was expressed abundantly in the testis and ovary and marginally in brain, prostate, thymus and small intestine (Fig. 2a). Additional semiquantitative RT-PCR analysis of *SCRN1* in gastric cancer cell lines revealed that it was expressed abundantly in MKN-1, -7, -28, -45, and -74 cells but not in Kato III (Fig. 2b).

Effect of *SCRN1* overexpression on cell growth

To analyze the effect of *SCRN1* overexpression on cell growth, we carried out a colony-formation assay on NIH3T3

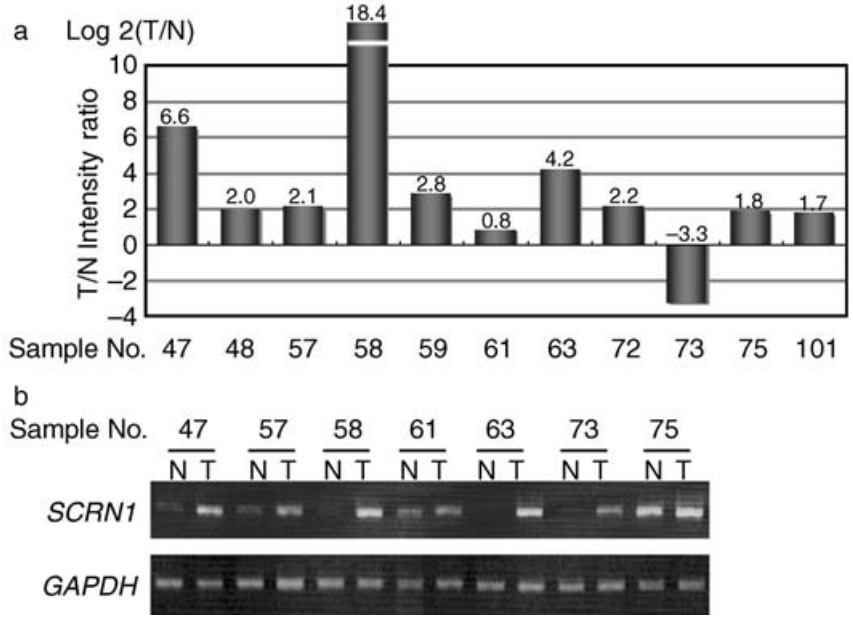

Fig. 1. Expression of *SCRN1* in gastric cancers. (a) Expression of *SCRN1* in gastric cancers measured by cDNA microarray. Relative expression ratio (cancer/non-cancer) of *SCRN1* was shown in the 11 primary gastric cancers examined by cDNA microarray. Upregulated expression (Cy3/Cy5 intensity ratio >2.0) was observed in nine of the 11 gastric cancers that passed through the cut-off filter. (b) Expression of *SCRN1* in seven gastric cancer tissues (T) and the corresponding normal tissues (N), as measured by semiquantitative RT-PCR. Expression of *GAPDH* serves as an internal control.

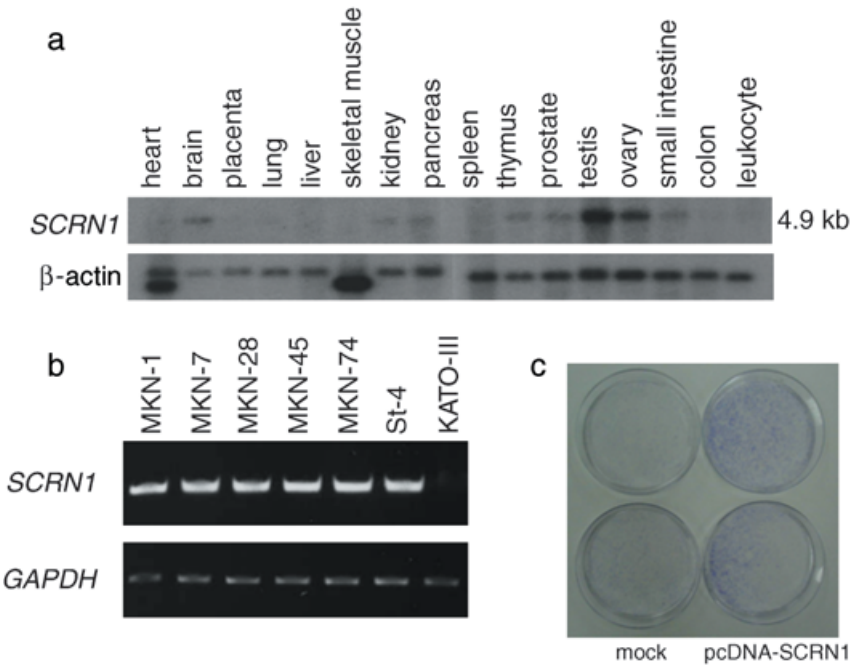

Fig. 2. Expression of *SCRN1* in normal human tissues and gastric cancer cell lines, and its effect on cell growth. (a) Northern blot analysis of *SCRN1* on multiple tissue. The transcript of *SCRN1* is approximately 4.9 kb in size. Expression of β-actin served as a control. (b) Expression of *SCRN1* in gastric cancer cell lines by semi-quantitative RT-PCR. Expression of *GAPDH* serves as a control. (c) Colony formation assays showed that *SCRN1* promoted cell growth in NIH3T3 cells. Cells transfected with mock or plasmids expressing *SCRN1* were fixed, and subsequently stained with Giemsa solution. Similar results were obtained in three experiments.

cells transfected with pcDNA3.1myc/His-SCRN1 that express myc-tagged SCRN1 protein. Expression of myc-tagged SCRN1 was confirmed by Western blot analysis with antimyc antibody (data not shown). Compared to the NIH3T3 cells transfected with a control plasmid (pcDNA3.1myc/His-LacZ), those with pcDNA3.1myc/His-SCRN1 showed markedly more colonies in NIH3T3 cells (Fig. 2c). This result was repeated and confirmed in three independent experiments. We did not observe morphological changes in cells using normal microscopy.

Successful induction of CTLs with a candidate peptide derived from *SCRN1*

Table 1 shows the predicted peptide sequences binding to HLA-A*0201 in the order of predicted binding affinities, indicated as the binding score. The 40 peptides were tested as candidates whether they could induce peptide-reactive CTLs or not. SCRN1-196 (KMDAEHPEL) induced the proliferation of lymphoid cells. The lymphoid cells showed significantly higher cytotoxicity against T2 cells pulsed with SCRN1-196 peptide compared to those pulsed with the control peptide

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Table 1. Candidate peptides derived from *SCRN1* and their predicted binding affinities to HLA-A*0201

Position†	Sequence‡ (9-mer)	Score§	Position†	Sequence‡ (10-mer)	Score§
303	SIFKPFIFV	369.7	308	FIFVDDVKLV	374.3
75	WLWGAEMGA	189.6	158	LIVDRDEAWV	366.6
68	IMISRPAWL	144.2	272	FLTTASGVSV	319.9
107	LLGMDLVRL	83.5	371	TMLEIEKQGL	234.0
308	FIFVDDVKL	49.9	106	ALLGMDLVRL	181.7
265	VCIDSEFFL	31.0 (N.S.)	67	AIMISRPAWL	59.7
306	KPFIFVDDV	25.1 (N.S.)	264	GVCIDSEFFL	59.6
159	IVDRDEAWV	22.7	315	KLVPKTQSPC	17.3
257	TLRDKASGV	21.6	220	FNFSEVFSPV	14.6
196	KMDAEHPEL	21.6	56	YISIDQVPRT	10.8
126	ALDVIVSLL	19.6	216	GEGEFNFSEV	10.5
207	YAQSQGWWT	19.6 (N.S.)	175	WAAEKVTEGV	8.9
367	KLRSTMLEL	13.0	281	VLPQNRSSPC	8.4
157	YLIVDRDEA	11.1	2	AAAPPSYCFV	7.9
3	AAPPSYCFV	7.9	253	TMMNTLRDKA	6.5
109	GMDLVRLGL	6.1	397	EVGDIFYDCV	5.2
179	KVTEGVRCI	6.0	79	AEMGANEHGV	5.0
266	CIDSEFFLT	4.5	20	GLVVFGKNSA	4.9
250	TVQTMMNTL	4.2	190	QLSLTTKMDA	4.9 (N.S.)
80	EMGANEHGV	3.7	302	RSIFKPFIFV	4.7

The amino acid sequences of candidate peptides are listed in the order of binding affinities to HLA-A*0201 molecules.
†The amino acid sequence number for position 1 in the candidate peptide, counting from the N-terminal of SCRN1; ‡The standard single letter amino acid code is used. §The estimated half-life of dissociation score for each peptide was calculated using BIMAS software. Higher scores indicate higher affinity to MHC class I molecules. N.S., not synthesized due to the high hydrophobicity of amino acid sequences.

$CMVpp65_{495–503}$ (data not shown). We carried out further limiting dilutions in order to establish CTL clones from the CTL lines.

Of the CTL clones established from the CTL lines against SCRN1-196, CTL Clone-23 showed higher cytotoxicity against T2 cells pulsed with SCRN1-196 compared to those pulsed with the control peptide (Fig. 3a). Additionally, CTL Clone-23 was examined for its ability to recognize and lyze the tumor cells endogenously expressing SCRN1. CTL Clone-23 showed specific cytotoxicity against SNU475, human hepatocellular carcinoma cells that express both HLA-A*0201 and SCRN1, but it did not show cytotoxicity against HLA-A*0201-negative MKN-45, which expressed SCRN1, nor against SCRN1-negative Kato III, which expressed HLA-A*0201 (Fig. 3b). These data suggest that CTL Clone-23 can recognize SCRN1-196 peptide processed and presented by SCRN1-positive tumor cells with HLA-A*0201 restriction.

Specificity of established CTL clones against SCRN1 peptide

Cold target inhibition assay was performed to confirm the specificity of CTL Clone-23 against SCRN1-196 peptide. The SNU475 cells labeled with ^{51}Cr were used as hot targets, and T2 cells pulsed with peptides without ^{51}Cr labeling were used as cold targets. In this assay, the specific lysis of ^{51}Cr-labeled SNU475 cells was dramatically reduced when they were mixed with unlabeled T2 cells pulsed with the SCRN1-196 peptide, but not when they were mixed with unlabeled T2 cells that were pulsed with the control peptide (Fig. 3c). To confirm the HLA-restriction, we examined the cytotoxic activity after the incubation with blocking mAbs. Significantly decreased cytotoxicities were observed when SNU475 cells were pretreated with anti-HLA-A/B/C mAb, or when the CTL Clone-23 cells were preincubated with anti-CD8 mAb (Fig. 3d). Taken together, the CD8-positive cells in CTL Clone-23 recognize and lyze SNU475 cells in the MHC-class I restricted manner. These results indicate that SCRN1-196 peptide is a HLA-A*0201 restricted epitope derived from SCRN1.

CTLs induced with peptides modified at anchoring positions

According to the HLA-A*0201 antigen motif previously reported by Smith *et al.*[32] the binding affinities of the peptides to the HLA are determined by amino acid residues at position 2 and 9. The newly identified epitope peptide SCRN1-196 has a relatively low binding score. We synthesized the peptides SCRN1–9 V (KMDAEHPEV) and SCRN1–2 L (KLDAEHPEL), which have modified amino acids only at anchoring positions and are predicted to have much higher binding affinity to HLA-A*0201 (Table 2). The leucine at position 9 in SCRN1-196 was changed to valine in SCRN1–9 V, and the methionine at position 2 in SCRN1-196 was changed to leucine in SCRN1–2 L. These two peptides were predicted to have the capability of binding to the T cell receptor, recognizing native SCRN1-196 peptide. We again prepared CTLs pulsed with SCRN1–9 V or SCRN1–2 L peptides, and successfully established CTL clones with SCRN1–9 V (Fig. 4a). CTL Clone-16 killed not only T2 cells pulsed with SCRN1-196 peptide but also SNU475 cells expressing SCRN1, implicating that SCRN1–9 V can also induce CTLs specific to SCRN1 (Fig. 4b). However, CTL Clone-16 had only modest cytotoxicity against tumor cells expressing SCRN1. We further compared the efficiency of CTL induction of SCRN1–9 V with that of SCRN1-196. When we used SCRN1–9 V, CTL lines showing significant cytotoxicity against the native SCRN1-196 peptide were successfully

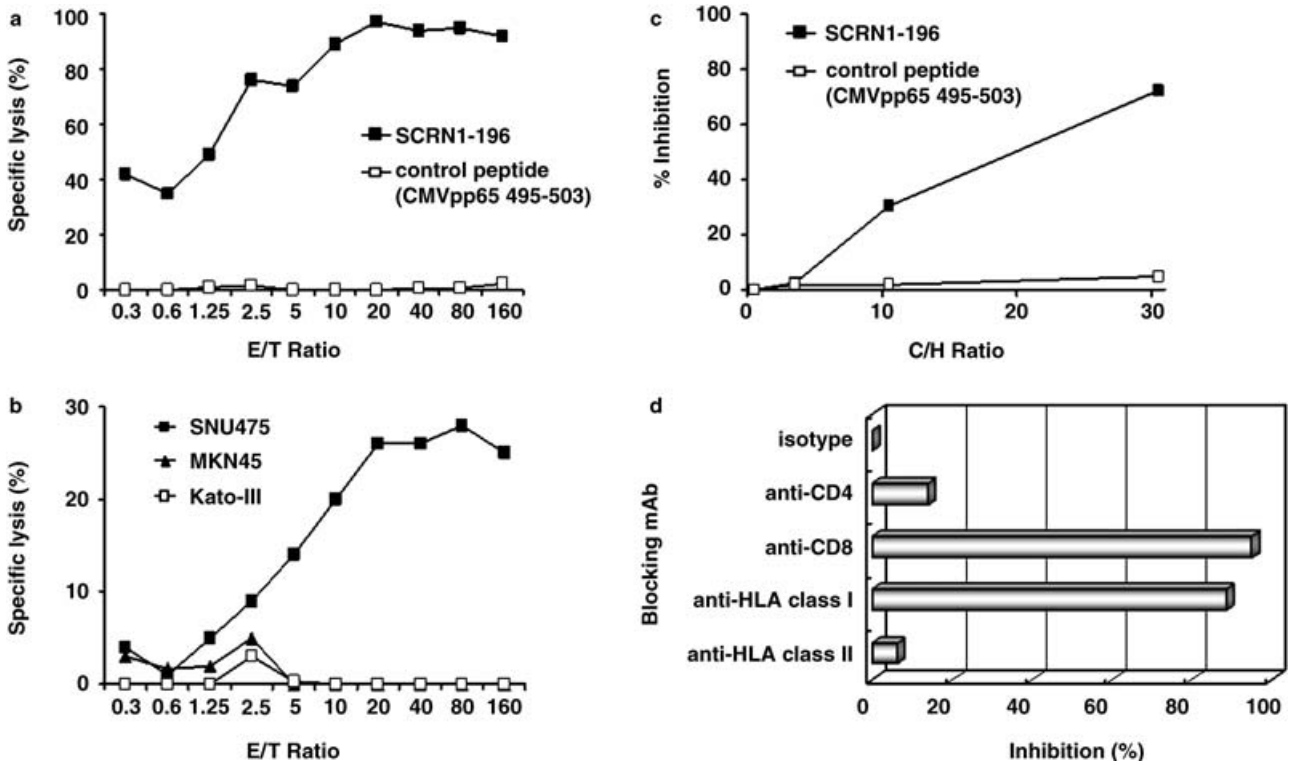

Fig. 3. Cytotoxicities of cytotoxic T lymphocyte (CTL) clones induced with SCRN1-196 peptide. Cytotoxicities of CTLs were examined using a 4 h ^{51}Cr release assay. (a) Cytotoxicities to T2 cells (HLA-A*0201) pulsed with SCRN1-196 (closed square) or control (open square) peptides. (b) Cytotoxicities to SNU475 (both SCRN1 and HLA-A*0201 positive), Kato III cells (SCRN1 negative, HLA-A*0201 positive), and MKN-45 cells (SCRN1 positive, HLA-A*0201 negative). CTL Clone-23 induced with SCRN1-196 showed potent cytotoxicities against not only T2 cells pulsed with SCRN1-196 but also SNU475 cells. There was no significant cytotoxicity against Kato III cells or MKN45 cells, neither of which express HLA-A*0201 and SCRN1 simultaneously. (c) A cold target inhibition assay. ^{51}Cr-labeled SNU475 cells were used as hot targets at an effector/target (E/T) ratio of 20. T2 cells pulsed with SCRN1-196 (closed square) or control (open square) peptides were used as a cold target. The number of target cells is fixed to 1×10^4/well. We added T2 cells to SNU475 cells in each microculture at various cold target/hot target ratios, and examined cytotoxic activity. T2 cells inhibited cytotoxic activity against SNU475 cells only when pulsed with SCRN1-196 peptides. (d) Antibody blocking assay. The cytotoxicity against SNU475 cells was inhibited by anti-CD8 monoclonal antibody or anti-HLA-class I monoclonal antibody. Specific lysis of SNU475 by CTLs in the presence of an isotype-matched control Ig was 23%. E/T ratio: 20.

Table 2. Peptides modified from SCRN1-196 at anchoring positions

Peptide	Sequence	Score†
SCRN1-196 (wild-type)	KMDAEHPEL	21.6
SCRN1–2 L (anchor-modified-1)	KLDAEHPEL	29.9
SCRN1–9 V (anchor-modified-2)	KMDAEHPEV	70.3

Modification of SCRN1-196 peptide was performed to create artificial peptides with increased binding affinities for the HLA-A*0201 molecule. †The estimated binding score of each peptide as calculated by BIMAS software.

established in three out of four individuals, whereas the CTL lines were established in only one out of four individuals using SCRN1-196 peptide. Moreover, positive wells were more frequently observed in the initial cultures when SCRN1–9 V was used (data not shown).

A homology search in protein databases revealed that neither SCRN1-196 nor SCRN1–9 V has homologous peptide sequences derived from any other gene products (data not shown), indicating that these peptides are likely to induce SCRN1-specific cytotoxicity.

Discussion

TAAs have been identified using multiple methods, including cDNA expression cloning with tumor-infiltrating lymphocytes,[10,11] serological analysis of recombinant cDNA expression librarieswith antibodies from patients' sera,[12,13] and reverse immunology methodusing previously defined tumor-related products.[24,33] However, the number of identified TAAs remains limited, especially for common cancer types including gastrointestinal cancers.

Recently, the number of newly identified genes has increased dramatically with the development of novel bioinformatics approaches and the growing use of cDNA microarray. We applied comprehensive gene expression analyses between cancer and normal tissues using cDNA microarray to identify novel TAA candidates in an efficient manner.

We previously reported the successful identification of RNF43 as a novel immunotherapy target for colorectal cancer using this strategy.[14] As RNF43 is restrictedly expressed in cancer tissues, we focused on RNF43 as a candidate for a novel TAA, and successfully identified the epitope peptides inducing potent RNF43-specific CTLs. We have started a phase I clinical trial for patients with RNF43-expressing

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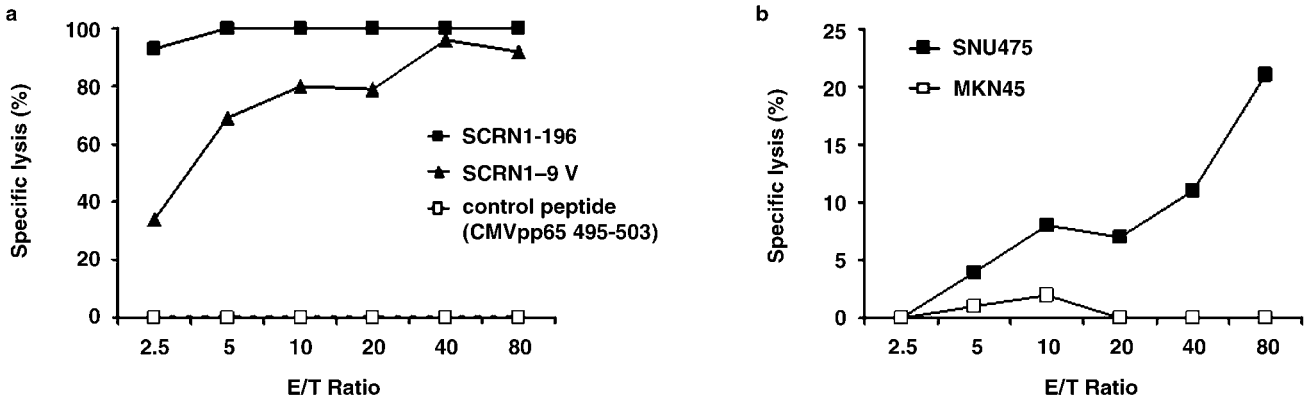

Fig. 4. Cytotoxicities of cytotoxic T lymphocyte (CTL) clones induced with SCRN1–9 V, modified from native SCRN1-196 peptide. Cytotoxicities of the CTL Clone-16, which were induced with SCRN1–9 V, were tested against (a) peptide-pulsed T2 cells (HLA-A*0201) and (b) tumor cell lines using 4 h ^{51}Cr release assay. CTL Clone-16 recognized not only SCRN1–9 V (closed triangle) but also the parental peptide SCRN1-196 (closed square). This CTL clone killed SNU475 cells expressing both the *SCRN1* gene and the HLA-A*0201 molecule. E/T, effector/target.

advanced or metastatic colon cancers using these epitope peptides. We have also shown that immunoglobulin superfamily 11 is a novel TAA that is expressed in gastric, colorectal and liver cancers, and reported antigenic epitope peptides for immunotherapy.[34]

In this study, we have shown that SCRN1 is frequently expressed in gastric cancer and colon cancer tissues. Northern blot analysis also showed that expression of this gene is abundant in testis and ovary but barely detectable in other normal adult tissues examined. These expression data suggest that SCRN1 could be a novel TAA. With regard to the function of SCRN1, it is a cytosolic protein that stimulates exocytosis in mast cells.[35] However, the mechanisms of its involvement in exocytosis remain unresolved. SCRN1 might cause the recruitment of secretory granules to the site of exocytosis or increase granule swelling, core expulsion, or breakdown. Notably, a number of GTP binding proteins such as G alpha i3,[36] beta gamma subunits,[37] rac,[38] rho,[39] and cdc42[40] play a role in secretion. These molecules are involved in other physiological functions, so SCRN1 might also play different roles other than exocytosis in the cells. From the result of colony formation assay, it is involved in cell growth as well.

To evaluate the immunogenicity of SCRN1, we attempted to identify the epitope peptides. Among the 40 candidate peptides predicted by the BIMAS algorithm, a 9-mer peptide SCRN1-196 (KMDAEHPEL) induced CTLs that killed not only T2 cells pulsed with its corresponding peptide but also SNU475 hepatocellular carcinoma cells, which endogenously express SCRN1. These data suggest that SCRN1-196 peptide is certainly processed in cytoplasm and presented on to the cell surface with HLA-A*0201 molecules. Specificity of CTLs to the SCRN1-196 peptide was confirmed by cold target inhibition and antibody blocking assay. Therefore, we defined SCRN1-196 to be an epitope peptide, and we also certified here that SCRN1 could serve as a TAA which could be recognized and killed by CTLs. These data suggest that SCRN1 itself could be used as the antigen in the form of DNA, mRNA, or protein.

It has been reported by multiple investigators that epitope peptides derived from TAAs frequently have mediocre bindings. The binding score of SCRN1-196 peptide with HLA molecule was 21.6, which was ranked at relatively low affinity compared to the binding scores of viral antigens or other TAAs.[41] Peptides containing a higher affinity for the MHC class I molecule are presented on cell surfaces for longer periods than those peptides containing low affinities,[42] and thus considered to have a greater potential to induce T cell-mediated immune responses.[43–46] It has been shown that the utility of anchor residue modifications at position 2 or 9 increases the immunogenicity of native epitope peptides conserving the binding affinity to T cell receptors.[47,48] To achieve this situation, we made a modified peptide, SCRN1–9 V, which has a higher binding score than that of SCRN1-196, analyzed by BIMAS, and we examined whether SCRN1–9 V could induce CTLs reactive to the naturally processed wild-type epitope peptide presented by tumor cells. The CTL clone was successfully established with the stimulation with the mutated peptide, SCRN1–9 V, and this CTL clone (Clone-16) had a potent cytotoxic capability on T2 cells pulsed with the SCRN1-196 peptide, a native peptide of SCRN1–9 V. However, the potency of the CTL clone induced with SCRN1–9 V was not signficantly different from that of SCRN1-196. Furthermore, the CTL Clone-16 generated by stimulation with the SCRN1–9 V peptide had less potent cytotoxicity on SCRN1–9 V-pulsed T2 cells when compared with SCRN1-196-pulsed T2 cells, especially at the lower E/T ratio. Thus, these results cannot support the advantage of using SCRN1–9 V instead of SCRN1-196. These confusing results could have been caused by the dissociation between the BIMAS scores and the actual binding affinities of peptides to HLAs. Although the difference of SCRN1-196 and SCRN1–9 V in actual binding affinity was not examined in this study, it could be smaller than the one predicted by BIMAS. We also examined the binding affinities of the peptides with another prediction system (SYFPEITHI), but the scores were not different between the two peptides. Further experiments, including the measurement of binding affinities, need to be carried out to clarify the advantage of this mutated peptide. The usefulness of anchor-modified peptides is still controversial[49–52] and there are a certain differences in clinical responses among individual anchor-modified peptides.

Analysis on immune responses *in vivo* in clinical trials could give us useful information to solve these questions.

In summary, using a genome-wide exploration for TAAs with cDNA microarray profiling, we identified a human gene, *SCRN1*, that was upregulated in human cancers. *SCRN1* is expressed in the testis and ovary, but is barely detectable in other normal adult tissues. We also discovered a sequence of SCRN1-196 and its modified form, SCRN1–9 V, both of which induce CTLs reactive to cancer cells expressing SCRN1 in an HLA-A*0201 restricted manner. These data might be useful not only to gain insights into human carcinogenesis but also for clinical applications of immunotherapy. We are currently preparing clinical trials using SCRN1-196 and/or SCRN1–9 V peptide.

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