Identification of immunoglobulin superfamily 11 (IGSF11) as a novel target for cancer immunotherapy of gastrointestinal and hepatocellular carcinomas

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We previously performed gene expression profile analyses of 20 intestinal-type gastric cancers, and identified a set of genes whose expression levels were elevated in cancer tissues compared to their corresponding non-cancerous tissues. In the present study we focused on the immunoglobulin superfamily 11 gene *(IGSF11)*. Its expression was also elevated in colorectal cancers and hepatocellular carcinomas as well as intestinal-type gastric cancers. Northern blot analysis showed that it was expressed abundantly in testis and ovary. These data suggest that IGSF11 is a good candidate of cancer-testis antigen. Furthermore, suppression of *IGSF11* by siRNA retarded the growth of gastric cancer cells. To investigate the possibility of clinical application of peptide vaccine to IGSF11, we synthesized candidate epitope peptides for IGSF11 and tested whether the peptides elicit IGSF11-specific CTL. As a result, we successfully established oligo-clonal CTL by stimulation with IGSF11-9-207 (ALSSGLYQC). In addition, we also established additional CTL using IGSF11-9V (ALSSGLYQV), anchor-modified peptides of IGSF11-9-207. These peptides showed IGSF11-specific cytotoxic activity in an HLA-A*0201-restricted fashion, suggesting that these peptides may be applicable for cancer immunotherapy. These findings have provided a novel insight into carcinogenesis of the stomach, colon and liver, and will be helpful for the development of novel therapeutic strategies to a wide range of human cancers. (*Cancer Sci* 2005; 96: 498–506)

Gastric cancer is one of the most frequent malignancies worldwide, accounting for 10.4% of cancer deaths in 2000.[1] Recent progress in the diagnosis of gastric cancer using endoscopy has enabled the detection of gastric tumors at relatively early stages, which improves the cure rate by surgical resection. However, the prognosis of patients with advanced gastric cancer still remains poor, because other therapeutic modalities, such as chemotherapy, remain ineffective; the overall 5-year survival rate of advanced tumors ranges from 5 to 15%.[2] Hence, the development of novel therapeutic strategies is urgently required for the treatment of patients with advanced cancer.

The effective induction of CTL by TAA has provided hope for the success of cancer immunotherapy.[3] Utilization of CTL elicited by TAA is an ideal therapeutic approach, if they specifically attack tumor cells expressing the antigen and reveal no or little adverse effect on normal cells. The identification of TAA immunodominant epitopes has highlighted the utilization of these epitopes as a promising therapeutic tool for immunotherapy. In addition, the understanding of mechanisms of antigen presentation to T lymphocytes in association with MHC has underscored the concept of cancer vaccines as a therapeutic option. More than 50 TAA have been identified to date, including MAGE-1, MAGE-2, MAGE-3, MAGE-12, BAGE, GAGE, PAGE, XAGE, NY-ESO-1, SSX, HER-2/neu, SPANX and TRAG-3.[4] Clinical trials using their epitope peptides alone or loaded on to DC have been carried out not only with malignant melanoma[5–8] but also with epithelial malignancies.[9–11] Recently, it was reported that four reactive peptide vaccines lead to prolonged survival in gastric cancer patients, and this was associated with cellular and humoral immunoresponses.[12] In addition, epitope peptides of immediate early response gene X-1 induced CTL toward gastric cancer in an HLA-A33-restricted manner.[13] However, a greater number of TAA are required to exert effective immunotherapy to cancer cells that are prone to escape from immunity.

Cancer-testis antigens are one category of TAA that are expressed in tumors as well as in germ cells within the testis, and in some cases also in the ovary.[14] The gonads

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Abbreviations: APC, antigen presenting cells; CTA, cancer-testis antigen; CTL, cytotoxic T lymphocyte; CXADR, coxsackie adenovirus receptor; DC, dendritic cell; ESAM, endothelial cell-selective adhesion molecule; EST, expressed sequence tag; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; IGSF11, immunoglobulin superfamily 11; IL, interleukin; MAGE, melanoma antigen; MHC, major histocompatibility complex; NK, natural killer; PBMC, peripheral blood mononuclear cell; PDZ, postsynaptic density protein 95/drosophila disks large/zona occludens-1; RP-HPLC, reverse phase high-performance liquid chromatography; RT-PCR, reverse transcription–polymerase chain reaction; SEREX, serological analysis of recombinant cDNA expression cloning; siRNA, short interfering RNA; TAA, tumor-associated antigen; TCR, T-cell receptor.

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themselves do not express molecules of MHC; therefore, when tumor-specific T-cell-mediated immune systems are activated, cytotoxic T cells elicited by CTA will attack CTA in cancer cells and have no effect on germ cells.

The immunoglobulin superfamily 11 gene (*IGSF11*) was identified as a gene expressed in the brain and testis.[15] The predicted protein comprises V-type and C2-type immunoglobulin domains, a C-terminal PDZ-binding domain and a transmembrane domain, and was classified as belonging to a novel immunoglobulin superfamily. A search for expressed sequence tags in nucleotide databases identified two forms of *IGSF11* transcripts that were composed of different exons in their 5′ terminal regions.[16] Homology searches revealed that both forms of the predicted *IGSF11* proteins share similarity in amino acid sequence with CXADR and ESAM.

To disclose mechanisms of gastric carcinogenesis and discover target molecules for their diagnosis and treatment, we had analyzed global expression profiles of 20 gastric cancers by means of a cDNA microarray representing 23 040 genes.[17] Among the genes commonly upregulated in cancer tissues, we focused, in this study, on *IGSF11*, a type-1 transmembrane protein. Its expression was also elevated in six of 11 colon cancers, and 12 of 20 hepatocellular carcinomas in our microarray data. In the present study, we demonstrate novel insights into carcinogenesis of the stomach, colon and liver, and document antigenic epitope peptides of IGSF11 for the treatment of human cancers as a vaccine strategy.

Materials and Methods

Cell lines

Human gastric cancer cell lines MKN1, MKN28 and MKN45 (expressing HLA-A*2402), MKN74 (HLA-A*3101/), Kato III (HLA-A*0201/0207), St-4 (HLA-A*0201/1101), a human colon cancer cell line SNU-C4 (HLA-A*0201/2402), a mouse fibroblast line NIH3T3, a monkey kidney cell line COS-7, and a human hybrid between B and T lymphoblastic cell line T2 (HLA-A*0201) were purchased from the American Type Culture Collection (ATCC). A human hepatocellular carcinoma cell line SNU475 (HLA-A*0201/1101) was obtained from Korea Cell-Line Bank (KCLB, Seoul National University, Seoul, Korea). An Epstein–Barr virus-transformed B-lymphoblastoid cell line A3LCL (HLA-A*0301/) was generously provided by Takara Shuzo Co. Although abundant HLA-A*0201 protein expression was observed in both SNU-C4 and SNU475 cells, low level and no expression of HLA-A*0201 protein was detected in St-4 and MKN74, respectively. All of these cells were cultured in appropriate media: Dulbecco's modified Eagle's medium (COS7, NIH3T3) and RPMI-1640 medium (T2, A3LCL, MKN1, MKN28, MKN45, MKN74, Kato III, St-4, SNU-C4 and SNU475), supplemented with 10% fetal bovine serum (Cansera International) and 1% antibiotic/antimycotic solution (Sigma-Aldrich). Cells were maintained at 37°C in an atmosphere of humidified air with 5% CO_2. Cancerous tissues and corresponding non-cancerous tissues were excised from patients during surgery, after informed consent had been obtained.

RNA preparation and RT-PCR analysis

Total RNA was extracted using Trizol reagent (Life Technologies) according to the manufacturer's instructions. Total RNA (10 μg) was reverse transcribed for single-stranded cDNA using the poly $dT_{12\text{-}18}$ primer (Amersham Pharmacia Biotech) with Superscript II reverse transcriptase (Life Technologies). Each single-stranded cDNA was then diluted for subsequent PCR amplification by monitoring *GAPDH* 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 *IGSF11*). Primer sequences were as follows: *GAPDH* forward, 5′-ACAACAGCCTCAAGATCATCAG-3′; *GAPDH* reverse, 5′-GGTCCACCACTGACACGTTG-3′; *IGSF11* forward, 5′-ACCTGTCTTCTGGATCTCCAG-3′; and *IGSF11* reverse, 5′-GTGTGAAATGCTTTGGCAGAAG-3′.

Multiple tissue northern blot analysis

Human multiple-tissue blots (Clontech) were hybridized with ^{32}P-labeled *IGSF11* cDNA as a probe. Prehybridization, hybridization and washing were carried out according to the manufacturer's recommendations. The blots were then autoradiographed with intensifying screens at −80°C for 120 h.

Colony formation assay of IGSF-11 in NIH3T3 cells

To prepare plasmids expressing *IGSF11-2* (pcDNA3.1myc/His-IGSF11), we amplified the coding region of *IGSF11-2* with a set of primers (5′-AGTTAAGCTTGCCGGGATGACTTCTCAGCGTTCCCCTCTGG-3′ and 5′-ATCTCGAGTACCAAGGACCCGGCCCGACTCTG-3′) by RT-PCR using cDNA from gastric cancer tissue as a template. The PCR products were cloned into an appropriate cloning site in the pcDNA3.1-Myc/His vector (Invitrogen). NIH3T3 cells were plated at a density of 1×10^5 cells/100 mm dish. After 24 h, the cells were transfected by plasmid vector using FuGENE6 reagent, and were cultured with an appropriate concentration of geneticin for 2 weeks. Cells were fixed with 100% methanol and stained using Giemsa solution.

Construction of IGSF-11 siRNA plasmids

We prepared psiH1BX3.0 vector plasmids that express siRNA under the control of the *H1RNA* promoter.[18] Control plasmid, psi-EGFP, was prepared by cloning double-stranded oligonucleotides of 5′-TCCCGAAGCAGCACGACTTCTTCTTCAAGAGAGAAGAAGTCGTGCTGCTTC-3′ and 5′-AAAAGAAGCAGCACGACTTCTTCTCTCTTGAAGAAGAAGTCGTGCTGCTTC-3′ into the *Bbs*I site in the psiH1BX3.0 vector. Plasmids expressing IGSF11 siRNA were prepared by cloning double-stranded oligonucleotides into the psiH1BX3.0 vector. The oligonucleotides used for IGSF11 siRNA were 5′-TCCCCCTTCCAGACATAGGGGGCTTCAAGAGAGCCCCCTATGTCTGGAAGG-3′ and 5′-AAAACCTTCCAGACATAGGGGGCTCTCTTGAAGCCCCCTATGTCTGGAAGG-3′ (psi-IGSF11-12). The plasmid psi-IGSF11-12 was transfected into St-4 cells using FuGENE6 reagent (Roche) or Nucleofector reagent (Alexa) according to the suppliers' recommendations. Total RNA was extracted from the cells 48 h after the transfection. Cells were cultured in the presence of 0.3 mg/mL geneticin for 14 days and stained with Giemsa solution (Merck) as described elsewhere.[18] To determine cell viability in response to siRNA to either IGSF11 or EGFP (a control gene), St-4 cells were plated in six-well plates at a density of

Table 1. Candidate peptides derived from *IGSF11* and their predicted binding affinities to HLA-A*0201

Position†	Sequence (9-mer)	Score‡	Position†	Sequence (10-mer)	Score‡
176	YLWEKLDNT	1314.6	176	YLWEKLDNTL	3344.0
11	LLLLSLHGV	1006.2	52	LINLNVIWMV	280.4
53	INLNVIWMV	49.2 (N.S.)§	207	ALSSGLYQCV	104.3
59	WMVTPLSNA	37.9	51	ALININVIWM	62.8 (N.S.)§
120	CLVNNLPDI	23.9	162	ILLCSSEEGI	32.1
15	SLHGVAASL	21.3	41	VLPCTFTTSA	32.0
252	VIIIFCIAL	18.9 (N.S.)§	12	LLLSIHGVAA	31.2
52	LINLNVIWM	14.6 (N.S.)§	356	SIYANGTHLV	30.6
40	AVLPCTFTT	13.9	111	QLSDTGTYQC	20.3
207	ALSSGLYQC	11.4	211	GLYQCVASNA	15.8
384	VMSRSNGSV	11.1	10	PLLLISLHGV	13.0
104	SIFINNTQL	10.8	32	IQVARGQPAV	11.9
327	KVHRNTDSV	10.4	106	FINNTQLSDT	10.8
413	RIGAVPVMV	9.5	364	LVPGQHKTLV	10.3
132	NIGVTGLTV	9.5	124	NLPDIGGRNI	8.5
356	SIYANGTHL	9.3	140	VLVPPSAPHC	8.4
163	LLCSSEEGI	8.6 (N.S.)§	251	AVIIIFCIAL	7.1
13	LLSLHGVAA	8.4	252	VIIIFCIALI	5.6 (N.S.)§
254	IIFCIALIL	7.5	261	ILGAFFYWRS	5.4
97	TMPATNVSI	7.5	137	GLTVIVPPSA	4.9

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-terminus of IGSF11. ‡The estimated binding affinity of each peptide to HLA-A*0201 molecules using Bioinformatics and Molecular Analysis Section software. §Not synthesized due to the hydrophobicity of amino acid sequences.

1×10^5 cells/well. On the next day, the cells were transfected in triplicate with psi-IGSF11 or psi-EGFP, and maintained in media containing 0.3 mg/mL geneticin. After 7 days of culture, cell viability was measured using the MTT assay with cell counting kit-8 (DOJINDO).

Peptide prediction and synthesis

Candidate peptide sequences of nine and 10 amino acids in length were predicted using the HLA Peptide Binding Predictions program,[19] available at http://bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform in the NIH Bioinformatics and Molecular Analysis Section (BIMAS). Among the predicted sequences, we synthesized 40 different peptides that were ranked according to highest binding affinity for the MHC class I molecule HLA-A*0201, the most common HLA-allele in the world (Table 1). The peptides synthesized revealed over 90% purity by RP-HPLC, and their structures were verified using mass spectrometry. $CMVpp65_{495–503}$ (NLVPMVATV), an HLA-A*0201-restricted epitope peptide derived from cytomegalovirus protein pp65, was used as a control for the cytotoxicity assay.[20]

CTL induction by candidate peptides *in vitro*

We prepared DC of HLA-A*0201 healthy volunteers for the use of APC as described previously.[21,22] Subsequently, the DC were pulsed with 20 μg/mL of the candidate peptides over 4 h at room temperature. The cells were then irradiated (50 Gy) and incubated with CD8-positive T lymphocytes in the presence of IL-7 at a concentration of 10 U/mL (Genzyme). We added IL-2 (BD Biosciences) into the culture medium at a final concentration of 20 U/mL at day 2. Two additional weekly stimulations with autologous peptide-loaded DC using the same procedure were carried out on days 7 and 14. The CTL activity was examined by cytotoxicity assay at day 21 as described elsewhere.[21]

Cytotoxicity assay

We examined the cytotoxic activity of the stimulated T cells by means of a 4-h ^{51}Cr-release assay. T2 cells (HLA-A*0201-positive) were pulsed with or without 20 μM peptide for 16 h, and tumor cells expressing *IGSF11* were removed with phosphate-buffered saline containing 0.53 mM ethylenediaminetetracetic acid. Each target was subsequently incubated with 100 μCi of ^{51}Cr-labeled sodium chromate for an additional hour at 37°C. The labeled cells (target) were then washed three times with phosphate-buffered saline and mixed with the pretreated T cells (effectors) at various effector-to-target (E/T) ratios in 200 μL of medium in 96-well plates. Half of the culture medium was collected from each well after 4 h of incubation, and its radioactivity was counted in a scintillation counter. Background activity was measured without effector cells, while maximum ^{51}Cr-releasing activity was examined by incubating target cells with 1 M hydrochloric acid. The percentage of cytotoxicity (%cytotoxicity) was calculated using the following formula:

$$[(^{51}\text{Cr-releasing activity of each experiment} - \text{background activity})/(\text{maximum } ^{51}\text{Cr-releasing activity} - \text{background activity})] \times 100(\%).$$

Establishment of oligo-clonal CTL

After testing the cytotoxicities, the peptide-pulsed CTL that responded with increased peptide-specific killing either were expanded to establish CTL lines or underwent limiting dilution and expansion to establish oligo-clonal CTL. To establish CTL lines, a total of 5×10^4 CTL were resuspended

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in 25 mL of AIM-V/2%AS, along with 2.5×10^7 irradiated (33 Gy) allogenic PBMC and 5×10^6 irradiated (80 Gy) A3LCL cells, in the presence of 30 ng/mL of anti-CD3 monoclonal antibody, as reported by Walter *et al*.[23] The following day, IL-2 was added to the cultures to a final concentration of 120 U/mL. The cultures were fed with fresh AIM-V/2% autologous serum containing 30 U/mL of IL-2 on days 5, 8, 11 and 14. To establish oligo-clonal CTL, we carried out limiting dilution of CTL that showed increased cytotoxicity to the peptide-pulsed target cells in our ^{51}Cr-release assay, before expansion.

Cold target-inhibition assay

For the cold target-inhibition assay, SNU475 cells expressing both HLA-A*0201 and IGSF11 were labeled with ^{51}Cr (hot target) and subsequently mixed with unlabeled T2 cells (cold target) pulsed with IGSF11-9-207 or control ($CMVpp65_{495–503}$) peptides at various cold target/hot target (C/H) ratios. After preparation of target cells, CTL (effectors) were added and reacted for 4 h. The cytotoxicity of each C/H ratio was measured using the ^{51}Cr-release assay. The percentage of specific inhibition (%inhibition) was calculated using the following formula:

$$\{[\%\text{cytotoxicity (C/H ratio 0)} - \%\text{cytotoxicity (each experiment)}]/\%\text{cytotoxicity (C/H ratio 0)}\} \times 100.$$

Antibody blocking assay

SNU475 cells pretreated with anti-HLA-A/B/C monoclonal antibody or anti-HLA-DR/DB monoclonal antibody for 1 h were co-cultured with oligo-clonal CTL that were pretreated with anti-CD4 monoclonal antibody or anti-CD8 monoclonal antibody for 1 h, at an E/T ratio of 20 (all monoclonal antibodies were purchased from Dako, Carpinteria, CA, USA). We used antimouse IgG2a monoclonal antibody for anti-HLA-A/B/C monoclonal antibody and antimouse IgG1 monoclonal antibody for the other, as isotype antibodies. The percentage of specific inhibition (%inhibition) was calculated using the following formula:

$$\{[\%\text{cytotoxicity (Isotype)} - \%\text{cytotoxicity (experimental)}]/\%\text{cytotoxicity (Isotype)}\} \times 100.$$

Results

Identification of *IGSF11*, a human gene commonly upregulated in gastric cancer

Using a genome-wide cDNA microarray containing 23 040 genes, we had previously examined the expression profiles of 20 gastric cancers and their corresponding normal gastric mucosae.[17] Among the genes commonly upregulated in the cancer cells, we focused on a gene corresponding to an EST, Hs.112873 of a UniGene cluster (http://www.ncbi.nlm.nih.gov/UniGene/), which later turned out to be *IGSF11* on chromosomal band 3q13 (GenBank accession no. AC068984). The signal intensity ratios of cancer-to-non-cancerous gastric tissues in the microarray data ranged between 4.09 and 48.6. Elevated *IGSF11* expression was subsequently confirmed in seven out of eight additional gastric cancer tissues (data not shown). Interestingly, its expression was also elevated in six of 11 colon cancers, and in 12 of 20 hepatocellular carcinomas in our microarray data. Enhanced expression was confirmed in five out of eight hepatocellular carcinomas by semiquantitative RT-PCR (data not shown). Because two forms of *IGSF11* transcripts had been reported,[16] we prepared PCR primers specific to each transcript and carried out RT-PCR experiments to examine the transcriptional levels of the two transcripts in gastric cancer. Although an isoform (IGSF11-2) containing exon 1b was successfully amplified using cDNA from gastric cancer tissues as a template, the other form (IGSF11-1) containing exons 1a, 2a and 3a could not be amplified with various sets of primers, suggesting that IGSF11-2 was the isoform upregulated in gastric cancer tissues. Hence, we further investigated this form of transcript (GenBank accession no. AB071618) in the present study.

Expression and characterization of *IGSF11*

We further analyzed *IGSF11* expression in normal adult organs by multiple-tissue northern-blot analysis, using two 3′ regions of *IGSF11* cDNA (which were conserved between the two isoforms) as probes, and revealed that the 3.5-kb transcript was abundantly expressed in testis and ovary, and expressed in brain, kidney and skeletal muscle at lower levels (Fig. 1a).

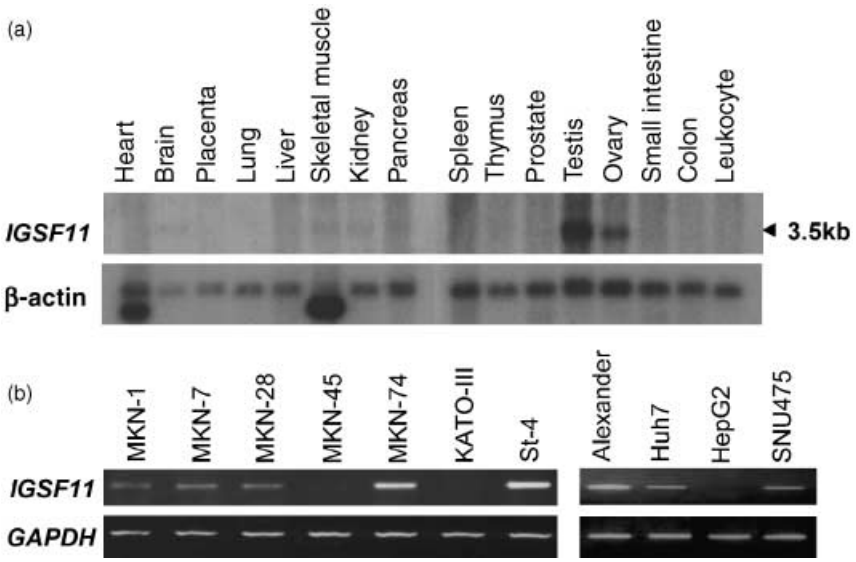

Fig. 1. (a) Multiple tissue northern-blot analyses of *IGSF11*. The transcript of *IGSF11* is approximately 3.5 kb in size. Expression of β-actin served as a control. (b) *IGSF11* expression in gastric cancer and hepatoma cell lines.

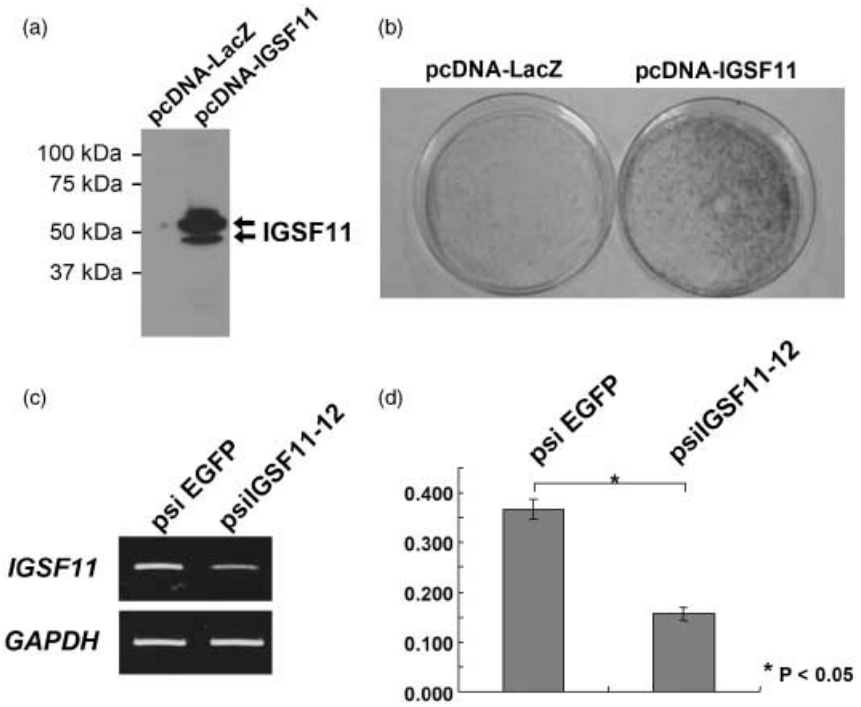

Fig. 2. (a) Expression of Myc-tagged IGSF11 in NIH3T3 cells transfected with pcDNA3.1myc/His-IGSF11 was examined by Western blot analysis using anti-Myc antibody. (b) Colony formation activity of exogenous *IGSF11* in NIH3T3 cells. (c) Gene-knockdown effect of IGSF11-specific siRNA. (d) Significant growth-inhibitory effect of the IGSF11-specific siRNA in St-4 cells ($P < 0.05$).

Semi-quantitative RT-PCR analysis revealed that MKN-1, MKN-7, MKN-28, MKN-74 and St-4 gastric cancer cells, and Alexander, Huh7 and SNU475 hepatoma cells expressed *IGSF11*, but not MKN-45 and Kato-III gastric cancer cells or HepG2 hepatoma cells (Fig. 1b).

Effect of IGSF11 on cell growth

To analyze the effect of IGSF11-2 on cell growth, we carried out a colony-formation assay by transfecting NIH3T3 cells with pcDNA3.1myc/His-IGSF11. Expression of exogenous Myc-tagged IGSF11 was confirmed by Western blot analysis using anti-Myc antibody (Fig. 2a). Compared with a control plasmid (pcDNA3.1myc/His-LacZ), pcDNA3.1myc/His-IGSF11 induced markedly more colonies in NIH3T3 cells (Fig. 2b). This result was confirmed in three independent experiments.

Effect of the siRNA to *IGSF11* on the growth of gastric cancer cells

To test whether the suppression of *IGSF11* may reduce the growth of cancer cells or not, we constructed plasmids expressing IGSF11 siRNA (psiIGSF11-12) and those expressing siRNA to a control gene (psiEGFP), and transfected them into St-4 gastric cancer cells that constitutively express *IGSF11*. As a result, transfection with psiIGSF11-12 reduced *IGSF11* expression (Fig. 2c) and decreased the number of transfected St-4 cells compared to psiEGFP plasmids (Fig. 2d), suggesting that the elevated expression of *IGSF11* may be essential for the growth of cancer cells.

Identification of peptides that induce CTL to cells expressing *IGSF11*

Using the HLA Peptide Binding Predictions in the NIH BIMAS, we synthesized a total of 40 different candidate 9- or 10 amino-acid peptides that were expected to have the highest binding affinity to HLA-A*0201, the most common HLA-allele (Table 1). Each of the 40 peptides was tested as to whether they could induce peptide-reactive CTL or not by means of the ^{51}Cr-release assay. As a result, IGSF11-9-207 (ALSSGLYQC) and IGSF11-10-207 (ALSSGLYQCV) were shown to induce peptide-reactive CTL (data not shown). Notably, these CTL did not show cytotoxic activity to T2 cells that were pulsed with the control peptides. We further carried out limiting dilutions to establish oligo-clonal CTL from both CTL lines.

Establishment of oligo-clonal CTL

Among the oligo-clonal CTL established from the CTL line against IGSF11-9-207, CTL207-11 cells showed higher cytotoxic activity against T2 cells (HLA-A*0201 positive) pulsed with the peptides compared to the parental cells, while the cells did not show cytotoxic activity against T2 cells without IGSF11-9-207-stimulation (Fig. 3a). Furthermore, SNU475 human hepatocellular carcinoma cells that express both HLA-A*0201 and *IGSF11* (Fig. 1b) were killed by CTL207-11 cells (Fig. 3b). On the other hand, CTL207-11 cells did not reveal any cytotoxity to SNU-C4 hepatocellular carcinoma cells that express HLA-A*0201 but do not express *IGSF11* (Fig. 3b). These data confirm the IGSF11-specific cytotoxic activity of CTL207-11 cells. To further examine HLA-dependence of the cytotoxity, we analyzed their cytotoxic activity using MKN74 human gastric cancer cells, which express *IGSF11* but do not express HLA-A*0201 (Fig. 3b). As expected, we observed no target killing, recapitulating IGSF11-specific and HLA-A*0201-restricted cytotoxity elicited by the peptide. Although we analyzed oligo-clonal CTL induced by IGSF11-10-207 (ALSSGLYQCV), the cells failed to display any (or displayed a low level of) cytotoxity against SNU475 cells (data not shown).

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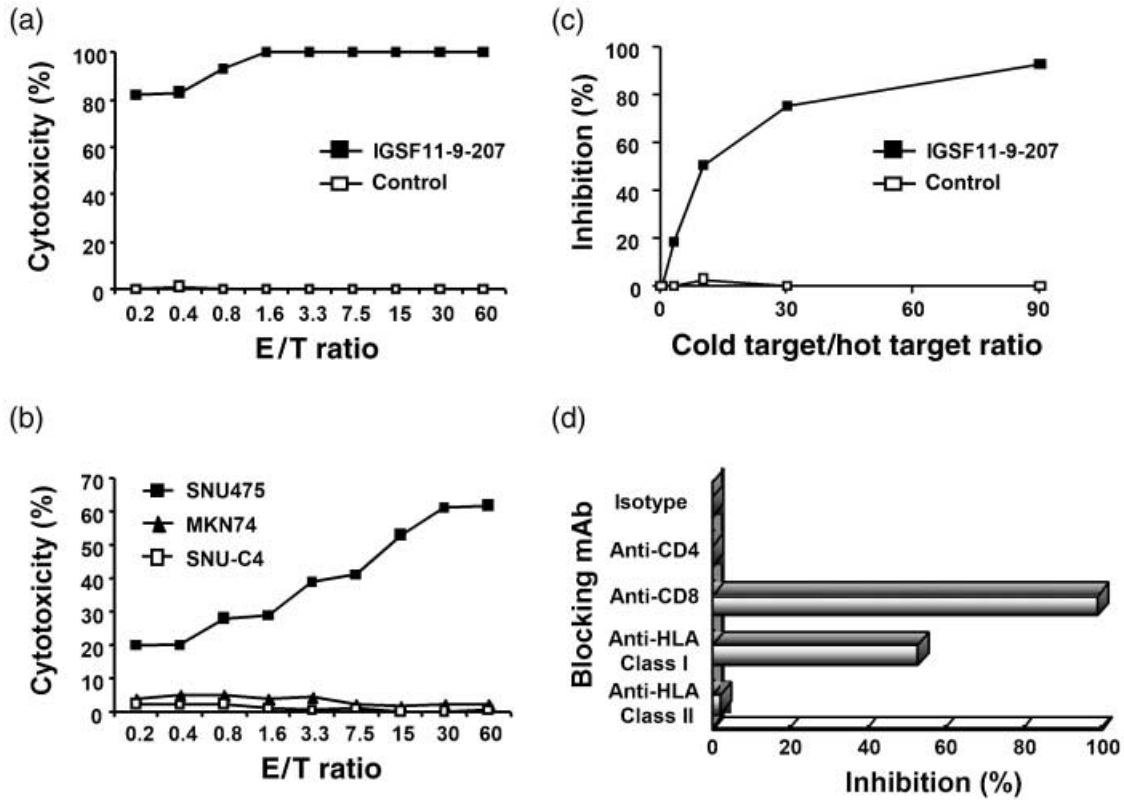

Fig. 3. Cytotoxic activities of CTL207-11 oligo-clonal CTL induced with IGSF11-9-207 peptide. Cytotoxic activities of CTL207-11 cells were examined using a 4-h ^{51}Cr-release assay. (a) Cytotoxic activities to T2 cells (HLA-A*0201) pulsed with IGSF11-9-207 (■) or control (□) peptides. (b) Cytotoxic activities to SNU475 (both IGSF11 and HLA-A*0201 positive), SNU-C4 (HLA-A*0201 positive and IGSF11 negative) and MKN74 (IGSF11 positive and HLA-A*0201 negative). CTL207-11 showed potent cytotoxic activities against not only T2 cells pulsed with IGSF11-9-207 but also SNU475 cells. There was no significant cytotoxic activity against SNU-C4 or MKN74, both of which do not express HLA-A*0201 and IGSF11 simultaneously. (c) A cold target inhibition assay. ^{51}Cr-labeled SNU475 cells were used as hot targets at an E/T ratio of 20. T2 cells pulsed with IGSF11-9-207 (■) or control (□) peptides were used as a cold target. 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 IGSF11-9-207 peptides. (d) An antibody-blocking assay. Cytotoxic activity against SNU475 cells was inhibited by anti-CD8 or anti-HLA-class I monoclonal antibody. *E/T ratio: 20.

Specificity of established oligo-clonal CTL

To assess the specificity of CTL207-11 cells, we carried out a cold target inhibition assay. We incubated SNU475 cells with various numbers of the cold target T2 cells that were pulsed with IGSF11-9-207 or control peptides. As a consequence, the killing of ^{51}Cr-labeled SNU475 cells was dramatically reduced when they were mixed with T2 cells pulsed with IGSF11-9-207 peptides, but not suppressed when they were incubated with T2 cells that were pulsed with control peptides (Fig. 3c). To confirm the HLA-dependent cytotoxity, we carried out ^{51}Cr-releasing assays with monoclonal antibody to MHC class I of the target cells, or cytotoxic T-cell-specific antibody to the effector cells. The cytotoxic activity was decreased significantly either when SNU475 cells were pre-treated with anti-HLA-ABC monoclonal antibody, or when the CTL207-11 cells were preincubated with anti-CD8 monoclonal antibody (Fig. 3d). However, the cytotoxic activity was decreased by approximately 50% in the presence of anti-HLA monoclonal antibody. Therefore, other effector cells, such as CD8-positive NK cells, may contribute to the cytotoxicity. Taken together, the CTL consist of CD8-positive cells that recognize IGSF11-9-207 in an MHC class I-restricted manner, and IGSF11-9-207 peptide is likely to be an epitope peptide restricted to HLA-A*0201.

CTL by anchor-modified peptides

According to the HLA-A*0201 antigen motif previously reported by Smith *et al.*,[24] leucine and isoleucine are the ideal anchor residues at position two that enhance the binding affinity of a peptide to HLA-A*0201, and valine and leucine are the preferred amino acids at position nine for nonamer peptides. The newly defined epitope IGSF11-9-207 was ranked at a relatively low binding score by the prediction, and it did not consist of valine or leucine at the ninth position. Hence, we synthesized two different anchor-modified peptides termed IGSF11-9V (ALSSGLYQV) and IGSF11-9L (ALSSGLYQL) and examined a possible enhancement of immunogenicity by these modifications (Table 2). The cysteine at position nine in IGSF11-9-207 was replaced by valine in IGSF11-9V and leucine in IGSF11-9L. Both of the two altered peptides were predicted to have higher HLA-A*0201-binding scores compared to the wild-type IGSF11-9-207 peptide by BIMAS's epitope prediction algorithm. We also prepared CTL pulsed with IGSF11-9V or IGSF11-9L peptides, and successfully established

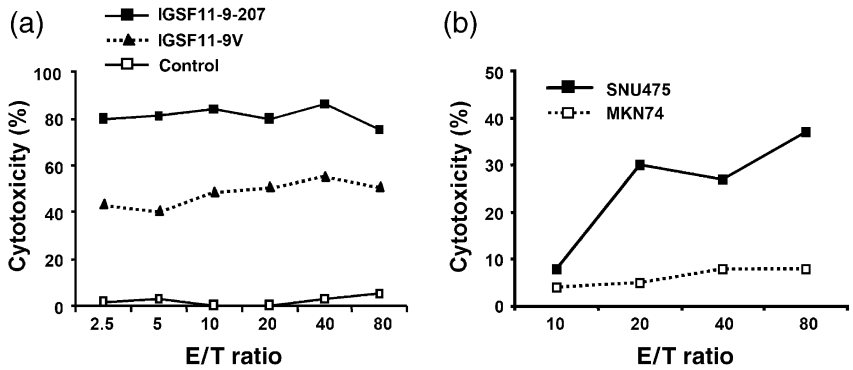

Fig. 4. Cytotoxic activity of CTL induced with the anchor-modified altered peptide IGSF11-9V. Examination of the cytotoxic activity of CTL9V-69 oligo-clonal CTL against (a) peptide-pulsed T2 cells (HLA-A*0201) and (b) tumor cell lines using 4-h ^{51}Cr-release assay. CTL9V-69 cells recognized not only IGSF11-9V (▲) but also the parental peptide IGSF11-9-207 (■). The cells killed SNU475 cells expressing both *IGSF11*and the HLA-A*0201 molecule.

Table 2. Anchor-modified peptides for IGSF11-9-207

Peptide	Type	Sequence	Score†
IGSF11-9-207	Wild type	ALSSGLYQC	11.4
IGSF11-9V	Anchor-modified-1	ALSSGLYQV	49.0
IGSF11-9L	Anchor-modified-2	ALSSGLYQL	159.8

†The estimated binding affinity of each peptide to HLA-A*0201 molecules using Bioinformatics and Molecular Analysis Section software.

oligo-clonal CTL with IGSF11-9V (CTL9V-69) (Fig. 4a). CTL9V-69 cells killed not only T2 cells pulsed with IGSF11-9-207 peptide but also SNU475 cells expressing IGSF11, indicating that IGSF11-9V is also capable of inducing CTL activity against IGSF11 (Fig. 4b). Notably, although we only managed to induce CTL in one individual out of four healthy HLA-A*0201-positive volunteers using IGSF11-9-207, this number was increased to three individuals out of the four by using IGSF11-9V peptides.

A homology search of protein databases revealed that IGSF11-9-207 and IGSF11-9V have no homologous peptide sequences derived from any other gene products, indicating that these peptides are likely to elicit IGSF11-specific cytotoxity.

Discussion

In the present study, we have shown that IGSF11 is a promising therapeutic target for human gastric and hepatocellular carcinomas, and that peptides corresponding to a part of IGSF11 [IGSF11-9-207 (ALSSGLYQC) or its anchor-modified form IGSF-9V (ALSSGLYQV)] elicit CTL towards human cancer cells. *IGSF11* was identified as an immunoglobulin superfamily gene that showed highest expression in testis and ovary, and lower expression levels in brain, kidney and adrenal gland.[15] *IGSF11* expression was barely detectable in other organs except for testis and ovary, brain, kidney and skeletal muscle in our study, suggesting that targeting *IGSF11* was highly unlikely to give rise to life-threatening adverse events. Our microarray data also showed that *IGSF11* expression was augmented in six of 11 colon cancers, 12 of 20 hepatocellular carcinomas and none of 20 diffuse-type gastric cancers. Hence, targeting *IGSF11* should be applicable for the treatment of colorectal cancer and hepatocellular carcinoma in addition to intestinal-type gastric cancer, but not for that of diffuse-type gastric cancer. Although we revealed frequent enhancement of *IGSF11* in a wide range of tumors, the mechanisms of its upregulation remain unresolved. Frequent gain of 3q was reported in diffuse-type gastric cancer,[25] but the amplification has not been reported so far in intestinal-type cancers, colorectal cancer or hepatocellular carcinomas. Thus, other mechanisms, such as transcriptional activation, may be involved in its upregulation. Notably, two forms of *IGSF11* transcripts were predicted, and our experiment revealed that gastric cancer cells abundantly express IGSF11-2 transcripts but not IGSF11-1 transcripts. Because the peptide sequence of IGSF11-9-207 (ALSSGLYQC) is conserved in both forms of *IGSF11* protein, immunotherapy targeting this peptide sequence may have an effect on cells expressing IGSF11-1 as well as those expressing IGSF11-2. Further studies of the function and physiological roles of IGSF11-1 and IGSF11-2 are necessary.

The IGSF11-2 protein shared 37% homology with human CXADR, 34% with Xenopus CTX homolog-like (CTXL), and 30% with ESAM.[16] CXADR serves as the cell-surface receptor for group B coxsackie viruses and most adenoviruses;[26] CTXL is a human homolog of Xenopus thymocyte receptor and is expressed in stomach, colon, prostate, trachea and thyroid gland;[27] ESAM plays a role in adhesion and vascular development.[28] These proteins comprise a signal peptide, an extracellular V-type immunoglobulin-like domain followed by a C2-type immunoglobulin-like domain, a transmembrane region and a cytoplasmic tail. The predicted IGSF11-2 protein also contains a hydrophobic signal sequence, two immunoglobulin domains, a transmembrane domain and a cytoplasmic region with a PZD-binding domain, suggesting that IGSF11-2 is a type-I transmembrane protein. In line with this prediction, we detected subcellular localization of exogenous IGSF11-2 protein in the cytoplasmic membrane when we transfected NIH-3T3 cells with plasmids expressing Flag-tagged IGSF11-2 (data not shown). Therefore, IGSF11 may mediate external signals as a receptor, play a role in cell–cell interaction, or enhance cellular proliferation and motility through the regulation of other receptors or adhesion molecules in a manner similar to Nectin-like molecule-5.[29] An antibody against the extracellular domain of IGSF11-2 is therefore an alternative approach for the treatment of gastric cancer. Because its elevated expression is essential for the growth of cancer cells, small compounds that inhibit IGSF11-2 or signals mediated by IGSF11-2 may also be a potential therapeutic option.

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A recent report summarized the objective response rate of immunotherapy in clinical trials to be as low as 2.6%.[30] This inefficacy may result from decreased expression of MHC class I antigen or co-stimulatory molecules, low levels of circulating immune cells, induction of immune suppressive mechanisms, insufficient observation periods or heterogeneous expression of TAA. Although cancer-testis antigens are expressed in a variety of cancers, the expression levels among cancer cells are very heterogeneous.[31,32] Moreover, peptides suitable for use in vaccination are different from patient to patient, mainly due to large diversity of TCR repertories.[33,34] Therefore, identification of further TAA is urgently needed to overcome the low efficacy. In particular, antigens that are homogenously expressed in all cancer cells are ideal target molecules. From this point of view, we are attempting to identify additional TAA and epitope peptides for cancer immunotherapy. As *IGSF11* expression is essential for the proliferation of cancer cells, all cancer cells should express *IGSF11* to some extent. Together with its low expression levels in important organs essential for life, IGSF11 should be an ideal therapeutic target.

The majority of TAA were identified using one of three approaches: (i) cDNA expression cloning of tumor infiltrating lymphocytes;[35,36] (ii) SEREX with antibodies from patients' sera;[14,37] and (iii) reverse immunology using previously defined oncogene products.[38,39] In addition to these conventional methods, advances in expression profile analysis have accelerated the identification of candidate TAA.[40–44] Although a number of TAA have been identified to date,[4,45] TAA of gastric cancer have not been intensively studied; only the expression of a few known TAA, such as MAGE-1, MAGE-2 and NY-ESO-1, have been investigated in gastric tumors.[46,47] To our knowledge, IGSF11 is the first TAA for immunotherapy to gastric cancer identified by expression profile analysis. Recent studies have identified an increasing number of antigenic peptides, some of which are under clinical trials.[3] However, data of clinically useful epitope peptides towards treatment of gastric cancer is limited. Several candidate epitope peptides were determined for peptide vaccines. For example, an-HLA-A31(A*31012)-restricted natural antigenic peptide of gastric signet ring cell carcinoma cells was identified by RP-HPLC.[48] Peptides of IEX-1 were recently identified as TAA for gastric cancer expressing HLA-A33 by cDNA expression cloning.[13] Another group tested a total of 30 antigenic peptides on HLA-A24 or HLA-A2 of 13 advanced gastric patients, which were identified by means of reverse immunology.[49] We added two epitope peptide sequences for HLA-A*0201-restricted immunotherapy to intestinal-type gastric cancer. Notably, HLA-A*0201 accounts for 30–40% of Caucasian and approximately 20% of Japanese populations.[50] Our data showed that IGSF11 is highly expressed in a wide range of human tumors. Taken together, the two IGSF11-derived peptides should be applicable for the treatment of a number of patients with cancer. Although cytotoxic activity was elicited by the anchor-modified peptides, IGSF11-9V was approximately half of that by IGSF11-9-207, binding affinity of IGSF11-9V peptides to HLA molecules was higher, and the frequency of induction of CTL with IGSF11-9V increased compared to IGSF11-9-207. Therefore, IGSF11-9V peptides may have an advantage for clinical use.

In summary, we have identified IGSF11 as a novel therapeutic target for gastric cancer, and further discovered two peptide sequences of IGSF11, both of which elicited CTL to gastric cancer cells expressing IGSF11 in an HLA-A*0201-restricted manner. These data may be useful not only for the gain of additional insights into human carcinogenesis but also for clinical applications of immunotherapy.

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