Efficient Identification of a Novel Cancer/Testis Antigen for Immunotherapy Using Three-Step Microarray Analysis

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Abstract

Advanced technology in molecular biology has provided us powerful tools for the diagnosis and treatment for cancer. We herein adopted a new methodology to identify a novel cancer/testis (CT) antigen with high frequency of expression in colorectal cancer as follows: (*a*) combining laser microdissection and cDNA microarray was used to analyze the gene expression profile of colorectal cancer cells; (*b*) genes overexpressed in testis and underexpressed in normal colon epithelium were analyzed using cDNA microarray; and (*c*) the gene expression profile of colorectal cancer cells was compared with that of normal testis. Using this methodology, we selected 38 candidates for CT antigen. Among these genes, we identified a novel CT antigen, serine/threonine kinase 31 (STK31), which was previously reported as a gene expressed in spermatogonia. Reverse transcription–PCR analysis showed that STK31 gene expression levels in cancer samples were significantly higher ($P < 0.0001$) than those in normal samples. The STK31 gene was frequently expressed not only in colorectal cancer but also in gastric and esophageal cancer. Moreover, STK31 peptide was able to elicit specific CTLs and induced CTLs lysed either peptide-loading or endogenously STK31-expressing target cells. These results showed that the new methodology in this study facilitated identification of CT antigens and that STK31 may be a candidate for cancer immunotherapy against gastrointestinal cancer. [Cancer Res 2008;68(4):1074–82]

Introduction

Over 55,000 patients with colorectal cancer, the second leading cause of cancer death, died in the United States in 1 year, and the total number of gastrointestinal cancer deaths was ~80,000 (1). In Japan, total of gastrointestinal cancer death rate has been increased, although gastric cancer death has decreased. Survival has been little improved over the last half century regardless of development of surgery, chemotherapy, and radiotherapy. Therefore, novel cancer therapy that is completely different from conventional modality is required to improve survival rate. One of the expected modalities is cancer immunotherapy. We have been conducting cancer immunotherapy clinical trials (2).

Recently, cancer immunotherapy using T cells, which recognize cancer antigens, has been carried out for melanoma patients (3–6). Many tumor antigens have been discovered to date, and a large number of tumor antigen specific T-cell epitopes were identified. However, tumor antigens and T-cell epitopes, which are available for immunotherapy for gastrointestinal cancers, are not sufficient (7). Thus, identification of novel tumor antigens and tumor-specific T-cell epitopes are required.

Tumor antigens are divided into five classes: cancer/testis (CT) antigen, mutated antigen, tumor virus, differentiation antigen, and overexpressed protein (7). Cancer immunotherapy using specific T cells that recognized CT antigens does not pose significant risk of adverse events, because expression of CT antigen is normally limited to testis. This is an immune privileged organ because spermatogonic cells do not express human leukocyte antigen (HLA) class I and II molecules at the cell surface (8). CT antigens are ideal targets for cancer immunotherapy, as CT antigens tend to be expressed in multiple kinds of cancer (9–13). Currently, the representative methods to identify CT antigens are cDNA expression cloning, e.g., MAGEA1 (14), BAGE (15), GAGE (16), and SEREX, e.g., NY-ESO-1 (9), SCP-1 (17), and SSX (18). Although these are useful, these methods require abundant experiments, time, and researcher skills.

Microarray technology, high-throughput mRNA expression analysis (19, 20), has been used to identify tumor antigens including cancer testis antigens (21–23). We previously identified genes that were expressed in colorectal cancer cells using laser microdissection and cDNA microarray (24, 25). In the current study, to identify CT antigens more efficiently, we analyzed genes that were overexpressed in both colorectal cancer cells and normal testis using three-step microarray analysis. Using this methodology, we identified a novel CT antigen, serine/threonine kinase 31 (STK31), which was frequently expressed in colorectal cancer. There was no evidence that STK31 was expressed in colorectal cancer previously. Furthermore, we verified that STK31 is a candidate for cancer immunotherapy using cancer-specific T cells in colorectal cancer and other gastrointestinal cancers.

Materials and Methods

Tissue sampling, laser microdissection, and cDNA microarray. The samples of cancer tissues and noncancerous tissues were obtained from 16 patients with colorectal cancer who underwent surgical resection in Kyushu University Hospital. Written informed consent was obtained from all patients. Tumors and adjacent normal tissues were immediately embedded in Tissue-Tek OCT compound medium (Sakura) and were kept frozen at −80°C until laser microdissection was done. Serial 8-μm frozen sections were generated by a cryostat. Sections were mounted onto a foil-coated glass slide 90 FOIL-SL25 (Leica Microsystems) for laser microdissection.

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Slides were stained with H&E at room temperature and dehydrated with ethanol. The Application Solutions Laser Microdissection System (Leica Microsystems) was introduced for laser microdissection to obtain the cancer cells and normal epithelial cells and to discard the mesenchymal tissues. Laser microdissection was done for several sequential sections. The target cells dissected from a section were dropped immediately into a microcentrifuge tube cap filled with 30 μL of RLT lysis buffer (Qiagen). At least 600 cancer cells were collected into 0.5-mL tube, and then the total RNA was extracted with an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. As the extracted total RNA was insufficient for hybridization to the cDNA microarray, the RNA was subjected to T7-based RNA amplification (26). The purity and concentration of the amplified RNA were determined by an Agilent 2100 Bioanalyzer (Agilent Technologies) as described previously (27). In brief, high-quality amplified RNA run on a bioanalyzer typically has the shape of a hump peak and one marker peak, indicating no contamination of rRNA. Of 16 cancer and 16 normal microdissected and T7-based amplified RNA samples, 8 samples from the cancer sections and 10 samples from the normal sections were determined to be of sufficient quality. Each of the eight RNA samples from cancer sections and the mixture of 10 RNA samples from normal epithelium were hybridized competitively to Agilent Human 1 cDNA Microarray (Agilent Technologies) containing 12,814 genes. To identify the gene expression profile in normal testis, not including normal colon epithelium, normal testis mRNA (Human RNA Master Panel II; Clontech Laboratories, Inc.) was subjected to T7-based RNA amplification (26). Normal testis RNA and a mixture of 10 RNA samples from normal epithelium were hybridized competitively to Agilent Human 1 cDNA Microarray (Agilent Technologies). The fluorescent intensities were analyzed by G2567AA Feature Extraction Software version A.7.5.1 (Agilent), which used the LOWESS (locally weight linear regression curve fit) normalization method (28). This microarray study followed MIAME (minimum information about a microarray experiment) guidelines issued by the Microarray Gene Expression Data group (29). All microarray data are available from Center for Information Biology Gene Expression database (http://cibex.nig.ac.jp/cibex2/index.jsp).

Microarray analysis. After subtracting the local and global background signals, expression values were calculated as intensity of the dye-normalized red (Cy5) and green (Cy3) channel signals. Data flagged as poor quality according to the Agilent data extraction software were removed from the analysis. All data calculated by data extraction software were imported to the Rosetta Luminator System version 2.0 (Rosetta Biosoftware). Candidate genes were selected with three-step analysis: (*a*) on expression data obtained from cDNA microarray of colorectal cancer, overexpressed genes were selected according to the criteria that the control intensity was <500, the fold changes were >2, and the *P* value was <0.01; (*b*) on expression data obtained from cDNA microarray of normal testis, overexpressed genes were selected according the criteria that the control intensity was <500, the fold changes were >2, and the *P* value was <0.01; (*c*) within the selected genes that satisfied the criteria *b*, the genes that satisfied the criteria *a* and overexpressed in two or more RNA samples in colorectal cancer cells (eight total samples) were further analyzed.

Quantitative real-time PCR. Quantitative real-time reverse transcription–PCR (RT-PCR) used 100 operatively resected cancer and paired normal samples that were not previously used for microarray analysis. Total RNA was extracted from each bulk sample, and cDNA was synthesized from 8.0 μg of total RNA as described previously (30). The purity and concentration of total RNA were determined using an Agilent 2100 Bioanalyzer. The following primers were used to amplify the STK31 gene: sense primer 5′-GGTCTCCTTACAATGAGCTTGG-3′ and antisense primer 5′-TGTCAACATCCACAGAATAGCC-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sense primer 5′-TTGGTATCGTGGAAGGACTCA-3′ and antisense primer 5′-TGTCATCATATTTGGCAGGTTT-3′) was used as an internal control. Reactions were performed in a LightCycler System (Roche Applied Science) using the LightCycler Fast Start DNA Master SYBR Green I kit (Roche Diagnostics). Details of each reaction are described elsewhere (31). Briefly, thermal cycling for all genes was initiated with a denaturation step of 95°C for 10 min, followed by 40 cycles at 95°C for 10 s, 65°C (60°C for GAPDH) for 10 s, and 72°C for each optimal length (1 s/25 bp). All calculated concentrations of target genes were divided by the amount of the endogenous reference (GAPDH) to obtain normalized STK31 expression values. Each assay was performed in triplicate.

Reverse transcription-PCR analysis. STK31 cDNA was detected by PCR amplification using oligonucleotide primers specific for the different exons of the STK31 gene. STK31 PCR primer sequences were sense 5′-GGTCAACTTGTTCTCAGGAACC-3′ and antisense 5′-GTGGTTAGTCAAATCCACAGCA-3′. PCR was performed as follows: 30 s at 94°C, 30 s at 65°C, and 30 s at 72°C for 29 cycles. To ensure that RNA was not degraded, a PCR assay using primers specific for GAPDH cDNA was carried out for each sample. The PCR products were size-fractionated on 2% agarose gel.

Treatment of cells with 5-aza-2′-deoxycytidine. We initially determined the optimal concentrations of 5-aza-2′-deoxycytidine (Sigma Chemical Co.) for each cell line. Cells were plated at a concentration of 1×10^6 per 100-mm dish and treated the next day with 1 to 5 μmol/L 5-aza-2′-deoxycytidine. Cells were harvested, and RNA was extracted before and after (72 h) the 5-azacytidine treatment. The cDNA was synthesized as mentioned above.

Immunohistochemistry. Immunohistochemical studies of STK31 were examined on formalin-fixed, paraffin-embedded surgical sections. A formalin-fixed, paraffin-embedded section of normal human testis was purchased from US Biomax (HuFPT151). After deparaffinization and blocking, the antibodies were incubated overnight at 4°C. Primary rabbit polyclonal antibody against the human STK31 (Abgent) was used at dilutions of 1:75. The LSAB2 kit (DAKO) was used to detect the signal of the STK31 antigen-antibody reaction. All sections were counterstained with hematoxylin.

Cell lines. The .221 (A2.1) cells, produced by transferring the HLA-A*0201 gene into the HLA-A, HLA-B, HLA-C null mutant human B lymphoblastoid cell line .221, were supplied by Takara Shuzo Co. Ltd. (32). The gastric cancer cell line AZ521 was obtained from Cell Resource Center for Biomedical Research at Tohoku University. The colon cancer cell line colo205 and the gastric cancer cell line NUGC3 were obtained from the Japanese Collection of Research Bioresources Cell Bank. T2, a TAP-deficient lymphoblastoid line of HLA-A2 genotype, was obtained from Cell Bank, Riken Bioresource Center. All of these cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 μg/mL streptomycin. Other cell lines used in this study were as follows: colorectal cancers (SW480, HCT15, CaR1, RCM1, colo201, colo320DM, DLD1, HT29, LoVo, LS174T, and WiDr), gastric cancers (KATOIII, MKN1, MKN28, and MKN94), and esophageal cancers (KY30, KY110, KY170, KY200, KSE2, TE2, TE5, TE8, TE11, and TE14). CaR1 was maintained in Eagle's MEM with 10% FBS. RCM1 was maintained in 45% RPMI 1640 with 45% Ham's F12 and 10% FBS. HT29 was maintained in McCoy's 5a medium with 10% FBS. LoVo and LS174T were maintained in DMEM with 10% FBS. Other cell lines were maintained in RPMI 1640 supplemented with 10% FBS. Antibiotics (100 IU/mL penicillin, 100 μg/mL streptomycin) were added to all media.

Synthetic peptide. To screen for HLA-A*0201–restricted peptide sequences of STK31, we used two epitope prediction algorithms (BIMAS[5] and SYFPEITHI[6]). Five peptides of nine residues were found to contain the binding motif for HLA-A*0201 with a highest predicted binding in both algorithms. Peptides used for CTL induction were purchased from Sigma Aldrich and were purified by repeated ether precipitations. Purity was determined by analytic reverse-phase high-performance liquid chromatography and proved to be ≥95% pure. Peptides were dissolved in DMSO and stored at −20°C before use.

Peptide binding assay. Peptide binding affinity to HLA-A2 was assessed by a HLA-A2 stabilization assay as described previously (33). In brief, TAP-deficient T2 cells were pulsed with 50 mg/mL of peptide and 5 μg/mL of β2-microglobulin (Becton Dickinson) for 18 h at 37°C. HLA-A*0201 expression was then measured by flow cytometry using monoclonal antibody BB7.2 (Serotech) followed by incubation with FITC-conjugated $F(ab')_2$ rabbit anti-mouse immunoglobulin (Serotech).

[5] http://bimas.dcrt.nih.gov/molbio/hla_bind
[6] http://syfpeithi.bmi-heidelberg.com

Table 1. Expression of STK31 mRNA in colorectal cancer

A. Overexpressed 38 genes in both colorectal cancer cells and normal testis relative to normal colon epithelium

Gene name	Symbol	Accession no.	Locus	Fold change A*	Fold change B†
Integral membrane glycoprotein-like	—	U21556	5p13.3	3.55	16.94
Chromosome 12 open reading frame 24	*C12orf24*	AW958081	12q24.11	2.63	10.63
Solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	*SLC7A5*	M80244	16q24.3	4.82	10.08
Serine/threonine kinase 31	*STK31*	AF332194	7p15.3	3.11	9.76
Membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3)	*MPP3*	U37707	17q12-q21	3.78	9.13
Low density lipoprotein receptor-related protein 8, apolipoprotein e receptor	*LRP8*	D86407	1p34	3.93	8.91
Expressed sequence tags	*TDRD9*	AA844124	14q32.33	2.38	8.48
Dipeptidase 1 (renal)	*DPEP1*	D13138	16q24.3	17.70	8.12
Oviductal glycoprotein 1, 120 kDa (mucin 9, oviductin)	*OVGP1*	NM_002557	1p13	3.32	8.06
Centromere protein F, 350/400 ka (mitosin)	*CENPF*	U19769	1q32-q41	4.17	7.24
Mevalonate kinase (mevalonic aciduria)	*MVK*	BG474232	12q24	2.98	7.04
Interleukin 8 receptor, β	*IL8RB*	AW969370	2q35	3.46	6.52
GREB1 protein	*GREB1*	AB011147	2p25.1	3.28	6.15
Expressed sequence tags	*AK126318*	BE672109	17q22	3.61	5.76
Fatty acid binding protein 6, ileal (gastrotropin)	*FABP6*	X90908	5q33.3-q34	4.81	5.69
Zinc finger protein 200	*ZNF200*	NM_003454	16p13.3	2.83	4.77
Calcium channel, voltage-dependent, L type, α 1S subunit	*CACNA1S*	AI417964	1q32	2.45	4.49
Zinc finger protein 783	*ZNF783*	AF035281	7q36.1	2.85	4.08
Thyroid hormone receptor interactor 13	*TRIP13*	AL562757	5p15.33	4.23	3.78
Matrix metalloproteinase 11 (stromelysin 3)	*MMP11*	AW301093	22q11.23	6.12	3.54
Nucleoporin 43 kDa	*NUP43*	AU155156	6q25.1	2.25	3.54
F-box and leucine-rich repeat protein 2	*FBXL2*	AAF03128	3p22.3	2.69	3.40
Calpain 3, (p94)	*CAPN3*	AI978885	15q15.1-q21.1	3.01	3.24
Tubulin, β4	*TUBB4*	X00734	19p13.3	2.70	3.10
Neuronal pentraxin II	*NPTX2*	BC009924	7q21.3-q22.1	3.77	3.06
Ubiquitination factor E4A (UFD2 homologue, yeast)	*UBE4A*	BF691464	11q23.3	2.36	2.87
Transcription factor Dp-1	*TFDP1*	BC011685	13q34	2.57	2.86
Renal tumor antigen	*RAGE*	BF037188	14q32	2.85	2.65
Ras-related associated with diabetes	*RRAD*	AI186786	16q22	3.82	2.62
Prostaglandin E receptor 4 (subtype EP4)	*PTGER4*	NM_000958	5p13.1	3.76	2.48
KIAA0251 protein	*KIAA0251*	AA318390	16p13.11	4.13	2.48
Melanoma antigen family A, 11	*MAGEA11*	NM_005366	Xq28	2.69	2.43
PHD finger protein 16	*PHF16*	D86969	Xp11.3	2.60	2.31
Wolfram syndrome 1 (wolframin)	*WFS1*	Y18064	4p16	2.95	2.22
Isovaleryl coenzyme A dehydrogenase	*IVD*	M34192	15q14-q15	3.75	2.18
Methionine adenosyltransferase II, α	*MAT2A*	F07456	2p11.2	3.44	2.07
γ-Aminobutyric acid B receptor, 1	*GABBR1*	NM_021905	6p21.31	3.10	2.01
Lactamase, β 2	*LACTB2*	BC000878	8q22-q22.3	2.78	2.01

(*Continued on the following page*)

CTL induction. CTL induction *in vitro* was performed according to the procedure described previously (34, 35). In brief, peripheral blood mononuclear cells (PBMC) of healthy donors (HLA-A*0201) were collected by centrifugation on a Ficoll-Paque density gradient. PBMCs were prepulsed by purified peptides at a final concentration of 20 μg/mL for 2 h at 37°C. Cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated human antibody serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 50 μmol/L 2-mercaptoethanol (Wako Pure Chemical Industries, Ltd.), with the addition on keyhole limpet hemocyanin (5 μg/mL; Calbiochem-Novabiochem Co.) and interleukin 7 (IL-7; 25 ng/mL; Peprotech EC Ltd.). On day 3, recombinant IL-2 (Peprotech) was then added to the culture at 50 IU/mL. Responder cells were restimulated every 7 days with freshly isolated autologous PBMCs that had been prepulsed with peptide and treated with mytomicin C (Kyowa Hakko Co., Ltd.). Cultures were fed with fresh medium containing IL-2, 1 day after stimulation. CTL activity was assessed on day 21.

Cytotoxicity assay. Target cells were labeled with 100 μCi of ^{51}Cr for 1 h at 37°C, and labeled cells were then washed and resuspended. Peptide-pulsed targets, .221 cells, were prepared by incubating the cells with peptides for 2 h at 37°C and then labeling them with ^{51}Cr. Effector cells were placed in each well of round-bottomed microtiter plates. Labeled

Table 1. Expression of STK31 mRNA in colorectal cancer (Cont'd)

B. Clinicopathologic variables and STK31 mRNA expression in 100 colorectal cancers

Variables	Expression		*P*
	High (*n* = 32)	Low (*n* = 68)	
Age	61.7 ± 11.6	69.3 ± 9.4	0.002
Gender			
Male	24	38	0.061
Female	8	30	
Histologic grade			
Well	17	18	0.010
Moderately and poorly	15	50	
Tumor site ‡			
Right colon	7	23	0.216
Left colon	25	45	
Serosal invasion			
Absent	22	46	0.912
Present	10	22	
Lymph node metastasis			
Absent	20	41	0.833
Present	12	27	
Lymphatic permeation			
Absent	19	47	0.341
Present	13	21	
Venous permeation			
Absent	28	45	0.019
Present	4	23	
Liver metastasis			
Absent	26	59	0.478
Present	6	9	
Duke's classification			
A and B	17	39	0.691
C and D	15	29	
Cancer-related death			
Alive	25	50	0.618
Death	7	18	

*At the ratio of colorectal cancer to colon normal epithelium.
†At the ratio of testis to colon normal epithelium.
‡Relative to splenic flexure.

target cells (1×10^4 cells per well) were incubated with various numbers of effector cells for 4 h at 37°C. Radioactivity of the culture supernatant was measured by an automated Gamma counter, and the percentage of specific lysis was calculated according to the following formula: (experimental ^{51}Cr release − spontaneous ^{51}Cr release) / (maximum ^{51}Cr release − spontaneous ^{51}Cr release) × 100.

Inhibition of cytotoxicity with monoclonal antibody. Appropriate target cells were incubated with monoclonal antibodies at a final concentration of 20 μg/mL for 1 h at 4°C before the assay for cytotoxicity. The monoclonal antibody used was anti-HLA class I antibody (Abcam).

Statistical analysis. Differences between groups were estimated using Student's *t* test and χ^2 test, as well as a repeated measure ANOVA analysis. Survival curves were estimated by the Kaplan-Meier method, and a comparison between the curves was made by the log-rank test. A probability level of 0.05 was chosen for statistical significance. Statistical analyses were performed using the JMP 5 for Windows software package (SAS Institute, Inc.).

Results

Identification of the gene expressed in both colorectal cancer cells and normal testis and mRNA expression of STK31 in colorectal cancer. We selected the candidate 38 genes that were overexpressed in both colorectal cancer cells and normal testis relative to normal colon epithelium with the three-step microarray analysis (Table 1A). To verify that the selected genes were overexpressed in colorectal cancer, we carried out quantitative real-time RT-PCR on colorectal cancer samples. Moreover, to validate that selected genes were expressed only in testis, we performed RT-PCR on cDNA templates synthesized from 8.0 μg of commercially available mRNA (Human RNA Master Panel II, Clontech) encompassing 20 normal tissues, obtained from multiple disease-free individuals. Among the selected genes, expression of the STK31 gene was significantly higher in colorectal cancer tissues and was restricted to testis and fetal brain in normal tissues

(Fig. 1*A*); thus, STK31 gene was further analyzed. In this study, the mean expression level of STK31 mRNA in cancer tissues, 0.105 ± 0.117 (mean ± SD), was significantly higher than 0.027 ± 0.016 of corresponding normal tissues ($P < 0.0001$; Fig. 1*B*). In the current study, patients with values less than the mean expression level of 0.100 in tumor tissues were assigned to a low expression group ($n = 68$), whereas those with values of ≥0.100 were assigned to a high expression group ($n = 32$). Table 1B shows the clinicopathologic data and STK31 mRNA expression in tumor specimens from 100 colorectal cancer patients. Patients with colorectal cancer were significantly younger ($P = 0.002$) in the high expression group (61.7 ± 11.6) than in the low expression group (69.3 ± 9.4). Histologic grade was significantly more differentiated ($P = 0.010$) in the high expression group (17 of 32, 53%) than those in the low

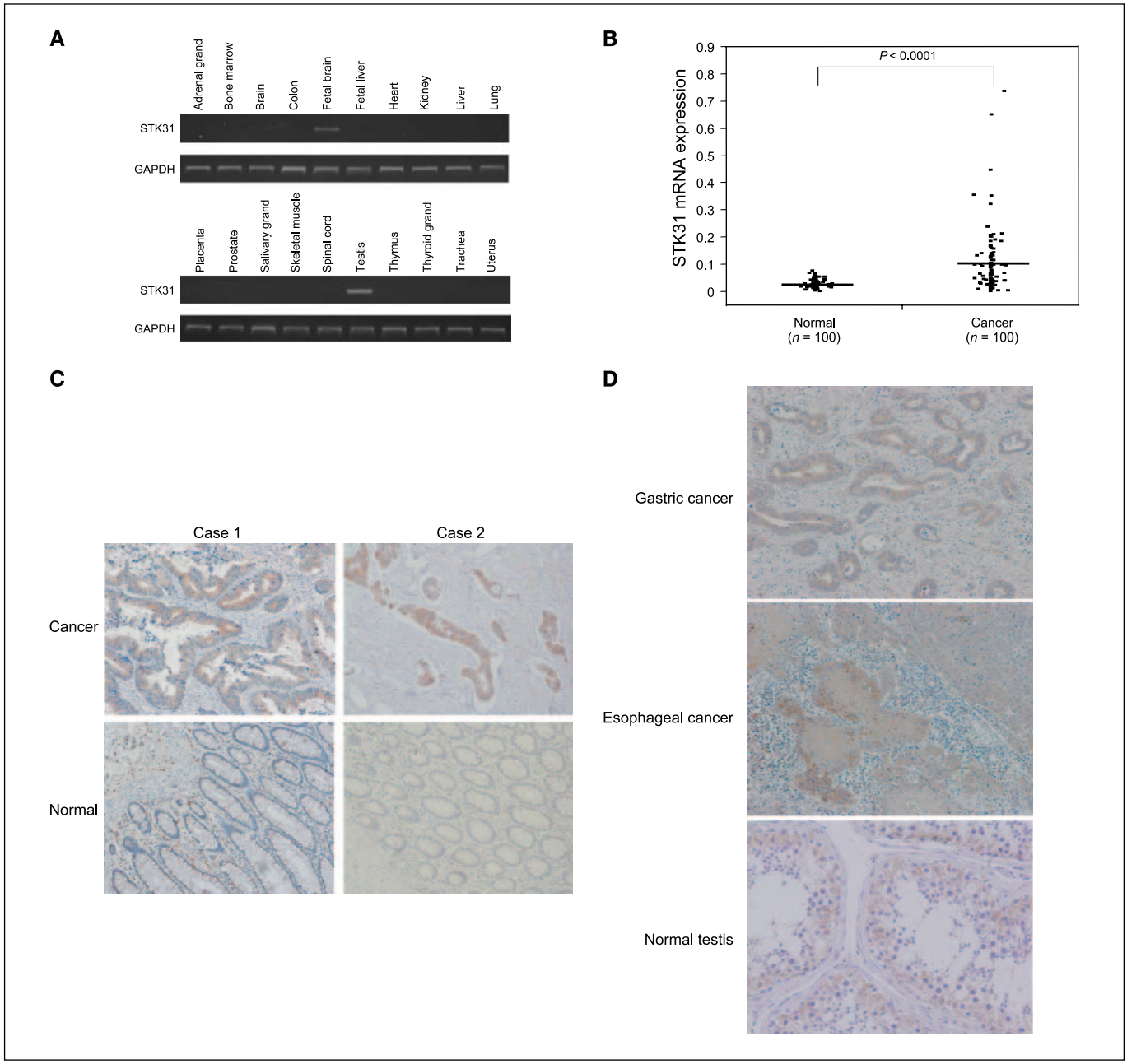

Figure 1. Expression of STK31 in gastrointestinal cancer and normal tissues. *A,* expression of the STK31 gene analyzed by RT-PCR in normal tissues. Each cDNA template was synthesized from 8.0 μg commercially available mRNA (Human RNA Master Panel II, Clontech) encompassing 20 normal tissues, obtained from multiple disease–free individuals. *Top,* STK31 gene expression; *bottom,* GAPDH. Expression of the STK31 gene was restricted to testis and fetal brain. *B,* quantitative real-time RT-PCR was carried out on 100 colorectal cancer and paired normal samples. The expression value of STK31 in cancer samples was significantly higher than that in normal samples ($P < 0.0001$, Student's *t* test). The *y*-axis represents normalized expression values of STK31 divided by the amount of glyceraldehyde-3-phosphate. *Horizontal lines,* mean. *C* and *D,* STK31 protein expression in gastrointestinal cancer and normal testis. Formalin-fixed, paraffin-embedded surgical specimens obtained from gastrointestinal cancer patients were stained with anti-STK31 polyclonal antibody at the dilution of 1:75. A section of normal human testis was purchased from US Biomax, obtained from a disease-free individual. *C,* colorectal cancer and paired normal epithelium. Original magnification, 100×. *D,* gastric, esophageal cancer (original magnification, 100×) and normal human testis (original magnification, 200×).

Table 2. Induction of STK31 gene expression by 5-aza-2′-deoxycytidine in human gastrointestinal cancer cell lines

Cell line	STK31 gene expression	
	Before 5-aza-2′-deoxycytidine treatment	After 5-aza-2′-deoxycytidine treatment
HCT15	−	+
colo205	−	+
MKN45	−	+
NUGC3	−	+
TE8	−	+
KYSE30	−	+

Abbreviations: −, no expression; +, positive expression of STK31 gene determined by RT-PCR.

expression group (18 of 68, 26.5%). Survival rate between these two groups was not statistically significant (data not shown).

Expression of STK31 in cancer originated from other gastrointestinal organs and in normal testis. Expression of the STK31 gene in cancer originated from gastrointestinal organs was determined by RT-PCR analysis on cancer cell lines and by immunohistochemistry on formalin-fixed, paraffin-embedded surgical sections from gastrointestinal cancer patients. On RT-PCR analysis, STK31 gene was expressed in 4 (38%) of 13 colorectal cancer cell lines, 4 of 6 gastric cancer cell lines (67%), and 6 of 10 esophageal cancer cell lines (60%). Immunohistochemical studies of clinical samples of colorectal cancer revealed that STK31 was expressed in the cytoplasm of colorectal cancer cells (Fig. 1*C*). No staining was observed in the same sections with control antibody. Expression rate of STK31 protein in colorectal cancer is 10 of 27 (37%). In addition to colorectal cancer, STK31 protein expression in gastric cancer and esophageal cancer was also revealed in this study (Fig. 1*D*). Furthermore, we studied the expression of STK31 protein in normal testicular tissue by immunohistochemistry. The STK31 protein was expressed predominantly in cytoplasm of spermatogonia (Fig. 1*D*).

Relative expression of the STK31 gene with MAGE genes. We have been treating cancer patients with MAGEA1 or MAGEA3 and DC vaccine (2). Therefore, we analyzed the synchronous expression of the STK31 gene with MAGEA1 or MAGEA3 gene in colorectal cancer. A total of 8 of 76 colorectal cancer samples (10.5%) expressed STK31 with synchronous expression of MAGEA1 or MAGEA3 genes. We also analyzed expression of the STK31 gene without MAGEA1 or MAGEA3 genes to determine whether STK31 is useful target for cancer immunotherapy for the patients with no expression of MAGE genes. A total of 20 of 76 colorectal cancer samples (26.3%) expressed the STK31 gene without expression of either MAGEA1 or MAGEA3. A total of 43 of 76 colorectal cancer samples (56.6%) expressed at least one CT antigen, that is, STK31, MAGEA1, or MAGEA3.

Mechanism of STK31 gene expression on cancer cell. Demethylation has been shown as one of the mechanisms of CT gene expression (36). To assess the kinetics of STK31 expression, we performed 5-aza-2′-deoxycytidine treatment for colorectal (HCT15 and colo205), gastric (MKN45 and NUGC3), and esophageal (TE8 and KYSE30) cancer cell lines that did not express STK31 gene. STK31 gene expression was induced after 5-aza-2′-deoxycytidine treatment on all of the tested cell lines (Table 2).

Screening for HLA-A*0201–restricted peptide. To assess peptide binding, five peptides were tested for binding affinity by pulsing with β2-microglobulin onto TAP-deficient T2 cells. Results from the binding assay showed two peptides bound to HLA-A*0201 with high affinity and one peptide was an intermediate HLA-A*0201 binder. This binding affinity was not correlated with the predicted binding scores (Table 3). Furthermore, to determine the epitope, which is able to elicit STK31 specific CTL, we pulsed PBMCs obtained from healthy donors using the three peptides (FLMPKEQSV, LLPLIFLFL, KLIEENEKL) of high or intermediate binding affinity to HLA-A*0201 by simplified methods (34, 35). After two stimulations, we performed a cytotoxicity assay against the cancer cell line AZ521, which expressed both STK31 gene endogenously and HLA-A*0201. The cytotoxicity assay against AZ521 revealed that the two peptides with high binding affinity, FLMPKEQSV and LLPLIFLFL, were able to induce the effectors exhibiting high cytotoxicity. However, the effectors pulsed with peptide KLIEENEKL exhibited exceedingly low cytotoxicity. We determined that FLMPKEQSV, which exhibited the highest cytotoxicity against AZ521, was the most putative peptide to elicit specific CTL among the five candidate peptides (Fig. 2).

Specific cytotoxic activity of effector cells induced by STK31-derived peptide. With simplified methods of CTL induction, after 21 days of culture, the effector cells, induced by using the FLMPKEQSV peptide, exhibited higher cytotoxicity against the peptide-pulsed .221 cells than nonpulsed .221 cells (Fig. 3*A*). Next,

Table 3. Binding affinity of STK31-derived peptides to HLA-A*0201 molecule

Peptide	Sequence	BIMAS score	SYFPEITHI score	Mean fluorescence index*
STK31 972–980	FLMPKEQSV	1,184	27	1.21
STK31 319–327	ALLESYKAL	486	26	0.01
STK31 790–798	LLPLIFLFL	380	23	1.14
STK31 449–457	YMSVEDFIL	169	20	0.26
STK31 305–313	KLIEENEKL	150	27	0.76
Influenza matrix (positive control)	GILGFVFTL	551	30	1.34

*(Mean fluorescence with peptide − mean fluorescence without peptide) / (mean fluorescence without peptide).

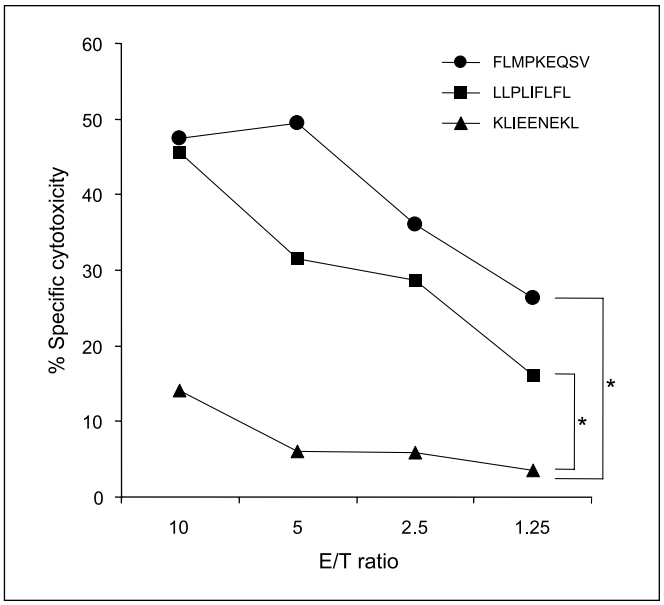

Figure 2. Cytotoxicity of effector cells against the gastric cancer cell line AZ521 (HLA-A*0201), which expresses STK31 endogenously. Each group of effector cells was generated from PBMCs by pulsing with each of three peptides (FLMPKEQSV, LLPLIFLFL, and KLIEENEKL) shown to have high or intermediate affinity to HLA-A*0201 by a peptide binding assay and restimulated with peptide-pulsed autologous PBMC twice. After 21 d, the effector cells were cocultured with ^{51}Cr-labeled AZ521 cells for 4 h and radioactivity of the culture supernatant was counted by a Gamma counter. The effector cells pulsed with peptides FLMPKEQSV or LLPLIFLFL exhibited significantly higher cytotoxicity than the ones pulsed with KLIEENEKL. *, $P < 0.01$.

we assessed the antitumor activity of induced effector cells against various cancer cell lines. Effector cells induced by FLMPKEQSV showed high toxicity against the AZ521 cell line (STK31$^+$, HLA-A*0201$^+$). However, cytolysis was diminished against colo205 (HLA-A*0201$^+$) and NUGC3 (HLA-A*0201$^-$), which do not express STK31 mRNA (Fig. 3*B*). To determine whether the FLMPKEQSV-induced effector cells recognized STK31-expressing targets in an HLA-restricted manner, a monoclonal antibody generated against HLA class I molecules was used to block recognition by effectors. Cytotoxic activity of the effector cells against AZ521 was eliminated by the anti-HLA class I antibody (Fig. 3*C*). The results suggested that induced effectors mainly lysed target cells expressing STK31 in an HLA class I–restricted manner.

Discussion

To identify a novel CT antigen of which expression rate was high in colorectal cancer, we adopted a new methodology using laser microdissection and cDNA microarray. This methodology consisted of three steps. First, to reveal the gene profile of colorectal cancer cells, we combined laser microdissection and cDNA microarray. Laser microdissection enabled us to extract cancer cells or normal epithelium selectively under a microscope. cDNA microarray analysis using laser microdissected samples revealed a precise expression profile of cancer cells (30). Second, to analyze the profile of genes overexpressed in testis and underexpressed in normal colon epithelium, we performed another cDNA microarray using commercially available normal testis mRNA and the mixture of clinical RNA samples from normal colon epithelium. Third, we compared the expression profile of colorectal cancer cells to that

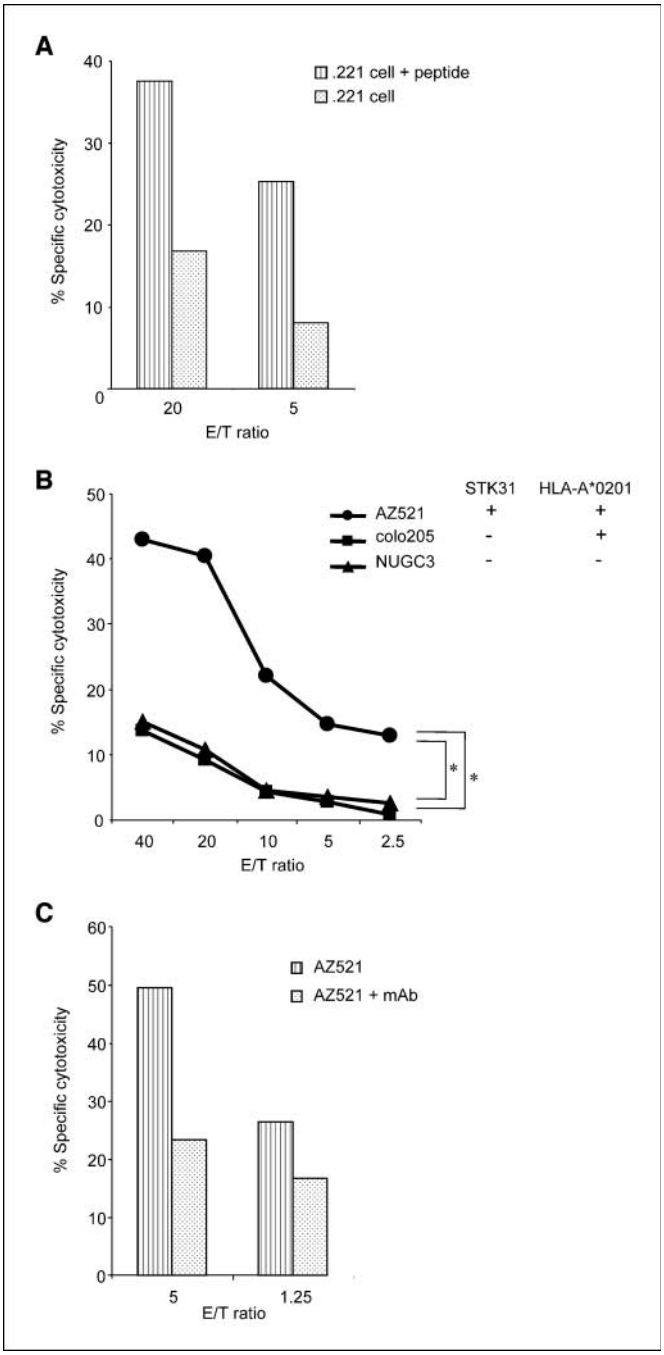

Figure 3. Specific cytotoxicity of the effector cells stimulated with STK31/HLA-A*0201 peptide FLMPKEQSV. PBMCs from a healthy donor were peptide-pulsed and stimulated with mytomicin-treated autologous PBMCs every 7 d. After 21 d of incubation, the effector cells were cocultured with ^{51}Cr-labeled target cells in a 4-h cytotoxicity assay at various E/T ratios. *A,* the cytotoxic activity of effector cells against HLA-A*0201 presented .221 cells with peptide loaded or not at an E/T ratio of 5 and 20. *B,* the cytotoxic activity of effector cells against AZ521 (STK31$^+$, HLA-A*0201$^+$), colo205 (STK31$^-$, HLA-A*0201$^+$), and NUGC3 (STK31$^-$, HLA-A*0201$^-$). There is a statistically significant difference between AZ521 and other targets. *, $P < 0.01$. *C,* the activity of the effector cells against AZ521 (STK31$^+$, HLA-A*0201$^+$) was diminished by anti-HLA class I monoclonal antibodies. Target cells, AZ521, were incubated with anti-HLA class I monoclonal antibody (*mAb*) at a final concentration of 20 μg/mL for 1 h at 4°C before coculture with effector cells stimulated with the STK31/HLA-A*0201 peptide FLMPKEQSV. The cytotoxic activity of the effector cells was estimated after coculture with anti-HLA class I antibody–treated cells or nontreated cells at an E/T ratio of 5 and 1.25.

of normal testis and investigated 38 overexpressed genes of candidate CT antigens. To our knowledge, this is the first report of this methodology. In the selected genes, we searched for genes which expressions were both frequently on patients with colorectal carcinoma and restricted in testis among normal organs. STK31 satisfied this condition. Certainly, selected genes with this methodology contained the ones that were expressed in other normal organs. The reason why other CT antigens were not identified is that the microarray analysis of this study used normal colon epithelium, but not all of the normal organs. However, this methodology is useful for investigation of new CT antigen, because it does not require abundant experiments, time, and researcher skills.

STK31 is one of the genes that Wang et al. identified through cDNA subtraction that is specific to mouse spermatogonia. Its human homologue indicated that it was a testis-specific gene (37). In agreement with these data, we verified that STK31 mRNA expression was restricted to testis and fetal brain in normal tissues. We validated cDNA microarray results by real-time RT-PCR, and the results also showed that expression levels of the STK31 gene in colorectal cancer was significantly higher than normal colon mucosa (Fig. 1*B*). In this study, immunohistochemical staining was carried out with anti-STK31 polyclonal antibody because reliable STK31 monoclonal antibody could not be available, to our knowledge. A few epithelial cells were marginally stained on normal colon section (Fig. 1*C*). This result is considered to be the nonspecific reaction of this polyclonal antibody. However, expression level of STK31 protein between colon cancer cells and normal colon epithelium was strongly different in the immunohistochemical analysis with this antibody as shown in Fig. 1*C*. The expression of STK31 in cancer tissue was heterogeneous. In the previous issues, expression of CT antigens is shown to be heterogeneous in cancer tissue, and this seems to be a characteristic feature of CT antigens (38–40). The heterogeneous expression of STK31 and other CT antigens in cancer tissue, which is not well known, is considered to be the same mechanism. Overexpression of the STK31 gene had no correlation with progression of colorectal cancer, although we previously showed that STK31 was one of the gene overexpressed in esophageal cancer cells with lymph node metastasis by microarray analysis (41). Moreover, STK31 did not contribute to proliferation of cancer cells, whereas we carried out STK31 small interfering RNA transfection to elucidate the function of STK31 gene (data not shown). The appearance of CT antigens in cancer represents the induction or activation of a gametogenic program in cancer, and programmatic acquisition is one of driving forces of tumorigenesis (42). Therefore, the STK31 gene may be concerned in tumorigenesis.

The analysis of expression rate of the STK31 gene in various cell lines, the STK31 gene, was frequently expressed in colorectal cancer, gastric cancer, and esophageal cancer. On the other hand, the expression rate of the STK31 gene in breast and hepatocellular carcinoma cell lines was rarely observed (data not shown). Generally, the incident of CT antigen expression in various tumor types has low frequency in colon and gastric cancers and moderate frequency in esophageal cancer (43). In immunologic analysis, effector cells generated with STK31-derived peptide FLMPKEQSV showed high cytotoxicity limited to AZ521, which expressed both STK31 and HLA-A*0201 (Fig. 3*B*). Furthermore, we studied the cytotoxic activity of effector cells stimulated with this peptide FLMPKEQSV against SW480 ($STK31^+$, HLA-A*0201^+) and MKN1 ($STK31^+$, HLA-A*0201^-). Specific cytotoxicities of these effector cells at E/T ratio of 10, 5, and 2.5 were 30.7%, 24.9%, and 19.2% (when cultured with SW480) and 14.8%, 11.2%, and 7.6% (when cultured with MKN1), respectively. There is statistically significant difference between two groups ($P < 0.01$; a repeated measure ANOVA analysis). This study indicated that the STK31-derived peptide was able to elicit CTL in a HLA-A*0201–restricted manner. HLA-A*0201 is the most commonly expressed allele in Caucasian and Asian individuals, and its expression rate is 40% to 50% (44, 45). We have conducted dendritic cell vaccinations with MAGEA3 peptide for gastrointestinal cancer (2). However, the expression rate of MAGEA3 gene in colorectal cancer was only 20% (46). Overlapping gene expression of STK31 and MAGEA3 in patients with colorectal cancer was rarely observed. If cancer immunotherapy by specific CTL recognizing STK31 is possible in clinical settings, we would expect the extension of indication of immunotherapy for colorectal cancer. We hypothesized that the reason for nonsynchronous expression of STK31 and MAGEA3 is because STK31 is a non-X CT antigen, located in 7p15.3. Therefore, the synchronous expression by global DNA hypomethylation among CT-X antigens, such as MAGE, NY-ESO-1, and LAGE-1 (47), is not observed (40). It is very significant that the expression rate of STK31 in esophageal and gastric cancers, which infrequently express CT antigens, was high.

As a methodology for identification of a T-cell epitope, "reverse immunology" has recently been performed (32). Here, we examined T-cell responses to STK31 in healthy donors, which were only affected in normal mechanisms of tolerance according to the following strategy: (*a*) a computer-based epitope prediction from amino acid sequence of a candidate antigen, (*b*) a peptide-binding assay to determine the affinity of the predicted peptide with MHC molecule, (*c*) the stimulation of primary T-cell response against the predicted peptide *in vitro*, and (*d*) testing of the resulting CTLs toward target cells endogenously expressing the antigen (48). In the current study, we were able to elicit specific CTLs using peptide FLMPKEQSV in four of five experiment (success rate was 80%) in healthy donor. We are planning clinical studies to elucidate whether CTLs elicited from cancer patients (44) by the STK31-derived peptide lyse cancer cells.

In this study, we efficiently identified a novel CT antigen with new methodology using laser microdissection and three steps of cDNA microarray. Expression of the STK31 gene was not only in colorectal cancer, but also in gastric and esophageal cancer. Moreover, the STK31-derived peptide FLMPKEQSV was able to elicit specific CTLs that lysed cancer cells in a HLA-A*0201–restricted manner. Our results provide evidence that this methodology to identify CT antigens is useful and that STK31 may be a candidate for cancer immunotherapy for gastrointestinal cancer.

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