Ring Finger Protein 43 as a New Target for Cancer Immunotherapy

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ABSTRACT

We have performed genome-wide exploration by using cDNA microarray profiling, and successfully identified a new tumor-associated antigen (TAA) that can induce potent cytotoxic T lymphocytes (CTLs) specific to tumor cells. In our preceding study, we identified multiple new genes by using gene expression profiling with a genome-wide cDNA microarray containing 23,040 genes. Among them, we selected *RNF43* (*ring finger protein 43*) as a promising candidate for a TAA expressed by colon cancer cells. In this study, we examined whether the RNF43 protein contains antigenic epitope peptides restricted to *HLA-A*0201* or *HLA-A*2402*. The CTL clones were successfully induced with stimulation by using the peptides binding to *HLA-A*0201* (ALWPWLLMA and ALWPWLLMAT) and *HLA-A*2402* (NSQPVWLCL), and these CTL clones showed the cytotoxic activity specific to not only the peptide-pulsed targets but also the tumor cells expressing RNF43 and respective HLAs. Lytic activities mediated by two HLA-A2-restricted epitopes were marginal, whereas tumor lysis mediated by the HLA-A24 epitope was clearly better. These findings might be caused by the poor natural presentation of RNF43-11(IX) and RNF43-11(X) by tumors or poor T-cell receptor avidity for these specific epitopes. These results strongly suggest that RNF43 is a new TAA of colon cancer. Furthermore, these results also suggest that our strategy might be a promising one to efficiently discover clinically useful TAAs.

INTRODUCTION

It has been demonstrated that $CD8^+$ cytotoxic T lymphocytes (CTLs) recognize epitope peptides derived from tumor-associated antigens (TAAs) presented on MHC Class I molecules and then lyse the tumor cells. After the discovery of melanoma antigen gene (MAGE) family as the first example of TAAs, other TAAs have been discovered by using similar immunologic approaches (1–5). These TAAs include gp100 (5), SART (6), and NY-ESO-1 (7). Furthermore, several gene products, which had been already been known to be preferentially overexpressed by the tumor cells, have recently been shown to be recognized by CTLs as TAAs. These gene products include p53 (8), HER2/neu (9), and carcinoembryonic antigen (10). With antigenic epitope peptides derived from these TAAs, clinical trials have been initiated by multiple groups to treat cancer patients (11–13).

Although significant progress has been made in the development of cancer vaccine with specific epitope peptides as described above, usefulness of this strategy is still greatly hampered by the fact that limited numbers of TAAs are currently available for the treatment of cancer patients. To identify a greater number of useful TAAs, multiple identification strategies including serologic identification of antigens by recombinant expression cloning (SEREX; ref. 7) have been performed. Although new TAAs, NY-ESO1 (7) for an example, have been successfully identified after the extensive efforts with these measures, one might need to admit that useful new TAAs are still needed for clinical application. Thus, development of a new and efficient method to discover new TAAs would drastically change this situation.

Development of cDNA microarray technologies, coupled with genome information, has enabled us to obtain comprehensive profiles of gene expressions of malignant cells and to compare them with those of normal cells (14–18). This approach discloses the complex nature of cancer cells and leads to identification of genes of which expression patterns are different in tumors when compared with the patterns in nontransformed cells (19). Because TAAs should theoretically be expressed excessively and preferentially by the tumor cells but not by the normal tissues, gene expression profiling with cDNA microarray technologies is useful to identify TAAs (20, 21). We analyzed the expression profiles of the newly identified genes with a genome-wide cDNA microarray technology, selected TAA candidates from these genes with the information, and examined whether they contain antigenic T-cell epitope peptides to prove that they are indeed TAAs.

We report herein that we identified a new TAA, RNF43 (Ring Finger Protein 43), as the successful example of the genome-wide exploration of tumor-associated antigens with cDNA microarray profiling.

MATERIALS AND METHODS

Cell Lines. The T2 (*HLA-A*0201*-positive cell line generously provided as a gift by Dr. Shiku of the University of Mie, Mie, Japan) and human B-lymphoblastoid cell lines (*HLA-A*2402*-positive A24LCL and *HLA-A*0301*-positive A3LCL generously provided as a gift by Takara Shuzo Co, Ltd. Otsu, Japan) were used for peptide-mediated cytotoxicity assays. HT29 (colon carcinoma cell line, HLA-A24/01), WiDR (colon

Received 1/21/04; revised 9/17/04; accepted 9/23/04.
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carcinoma cell line, HLA-A24/01), DLD-1 (colon carcinoma cell line, HLA-A24/02), HCT15 (colon carcinoma cell line, HLA-A24/02) and HCT116 (colon carcinoma cell line, HLA-A02/01), all purchased from American Type Culture Collection, were also used in cytotoxicity assays as target cells. Examinations with cDNA microarray and reverse transcription-PCR showed strong *RNF43* expression in HT29, WiDR, DLD-1, HCT15n and HCT116 (data not shown).

Selection of *RNF43* as a TAA Candidate. Among the transcripts that we recently identified as up-regulated ones in cancer cells with a genome-wide cDNA microarray containing 23,040 genes, we selected an annotated gene as *FLJ20315* (GenBank accession no. NM 017763) as a TAA candidate. Because the deduced 784-amino-acid sequence contained a ring finger domain, the nomenclature committee in the Human Genome Organization (HUGO) termed the gene as *RNF43* (ring finger protein; refs. 15, 17, 18). Although *RNF43* was up-regulated in more than 80% of colorectal cancer tissues compared with the corresponding noncancerous mucosa, *RNF43* expression cannot be detected in 29 normal organs of the adult human with Northern blotting. The expression of *RNF43* in the normal organs was detectable only in the lung and kidney of the fetus. Furthermore, it was suggested that the function of *RNF43* was associate with the proliferation of tumor cells.

Selection of Candidate Peptides Derived from *RNF43*. Among the 9mer-and 10mer-peptides derived from *RNF43*, candidates for antigenic epitope were selected based on the predicted binding affinities to *HLA-A*0201* or *HLA-A*2402* molecules BIMAS binding prediction software[3] and SYFPEITHI prediction software.[4] These peptides were synthesized by Mimotopes (San Diego, CA) according to the standard solid-phase synthesis method and were purified by reverse phase high-performance liquid chromatography (HPLC). The purity (>90%) and the identity of the peptides were determined by analytical HPLC and mass spectrometry analysis, respectively. These peptides were dissolved in DMSO at 20 mg/mL and stored at −80°C.

***In vitro* CTL Induction and Expansion.** The CTLs were induced with monocyte-derived dendritic cells (DCs) pulsed with candidate-peptides. DCs were generated *in vitro* as described elsewhere (10, 22, 23). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from normal volunteer (*HLA-A*0201* or *HLA-A*2402*) with Ficoll-Plaque PLUS (Amersham Biosciences, Uppsala, Sweden) solution and placed onto a plastic tissue culture flask (Becton Dickinson, Franklin Lakes, NJ) to obtain adherent monocyte fraction. The monocyte-enriched population was cultured in the presence of 1,000 units/mL of granulocyte macrophage colony-stimulating factor (generous gift from Kirin Brewery Company, Tokyo, Japan) and 1,000 units/mL of IL-4 (TECHNE Co., Minneapolis, MN) in AIM-V (Life Technologies, Inc., Invitrogen Co., Grand Island, NY) containing 2% heat-inactivated autologous serum. After 5 days in the culture, OK-432 (generously provided by Chugai Pharmaceutical Co., 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, mature DCs were harvested and pulsed with 20 μg/mL concentration of candidate peptides in the presence of 3 μg/mL β2-microglobulin for 4 hours at 20°C in AIM-V. These peptide-pulsed DCs were irradiated (5,500 rads) and mixed at a 1:20 ratio with autologous $CD8^+$ T cells, obtained by positive selection with Dynabeads M-450 CD8 and Detachabead (both from Dynal, Lake Success, NY). These cultures were set up in 48-well plates (Corning Inc., 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% autologous serum. Three days later, IL-2 (CHIRON Co., 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 DCs pulsed with the peptide. The DCs were prepared each time with the same method described above. Cytotoxicity was tested on day 21 against peptide-pulsed A24LCLs after the third round of peptide stimulation.

When T cells with detectable cytotoxic activities were identified, these T cells were expanded as CTLs in culture with the method similar to the one described by Riddell *et al.* (24) and Walter et al. (25). A total 5×10^4 of CTLs were resuspended in 25 mL of AIM-V/5% autologous serum with 25×10^6 irradiated (3300 rads) PBMCs and 5×10^6 irradiated (8,000 rads) A3LCLs in the presence of 40 ng/mL anti-CD3 monoclonal antibody (BD Bioscience-PharMingen, San Diego, CA). One day after initiating the cultures, 120 IU/mL IL-2 was added to the cultures. The cultures were fed with fresh AIM-V/5% autologous serum containing 30 IU/mL IL-2 on days 5, 8, and 11.

Establishment of CTL Clones. The CTL suspension was diluted to have 0.3, 1, and 3 CTLs per well in 96 round-bottomed microtiter plates (Nalge Nunc International; Rochester, NY). The CTLs were cultured with 7×10^4 cells of allogenic PBMCs per well, 1×10^4 cells of A3LCLs per well, 30 ng/mL anti-CD3 antibody, and 125 unit/mL IL-2 in a concentration of 150 μL of AIM-V containing 5% autologous serum per well. Then, 50 μL IL-2 per well was added to the medium 10 days later so that IL-2 became 125 unit/mL in the final concentration. Cytotoxic activity of the expanded CTLs was tested on the 14th day, and the CTLs showing significant cytotoxicity against the target cells pulsed with the candidate peptides were expanded once again as "CTL clones" with the same method as above.

Cytotoxicity Assay. Target cells were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ (Perkin-Elmer Life Sciences, Boston, MA) for 1 hour at 37°C in the CO_2 incubator. When peptide-pulsed targets were needed, they were prepared by incubating the cells with a 20-μg/mL concentration of the peptide for 16 hours at 37°C. Target cells were rinsed and mixed with effectors in a final volume of 0.2 mL in round-bottomed microtiter plates. The plates were centrifuged (4 minutes at 800 × *g*) to increase cell-to-cell contact and were placed in a CO_2 incubator at 37°C. After 4 hours of incubation, 0.1 mL of the supernatant was collected from each well, and the radioactivity was determined with a gamma counter. The percentage of specific cytotoxicity

[3] BIMAS binding prediction software, Internet address: http://bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform.
[4] SYFPEITHI prediction software, Internet address: http://syfpeithi.bmi-heidelberg.com/.

Table 1 Candidate selection from peptides derived from *RNF43* based on predicted binding affinities to *HLA-A*0201*

		Binding affinity				Binding affinity	
Start position	AA sequence (9mers)	BIMAS	SYFPEITHI	Start position	AA sequence (10mers)	BIMAS	SYFPEITHI
60	KLNLTLEGV	274	27	81	KLMQSHPLYL	1521	23
8	QLAALWPWL	199	24	357	YLLGPSRSAV	1183	27
82	LMQSHPLYL	144	21	202	LMTVVGTIFV	469	19
358	LLGPSRSAV	118	25	290	CLHEFHRNCV	285	23
11	ALWPWLLMA	94	24	500	SLSSDFDPLV	264	23
15	WLLMATLQA	84	19	8	QLAALWPWLL	160	22
200	WILMTVVGT	40	21	11	ALWPWLLMAT	142	24
171	KLMEFVYKN	34	22	7	LQLAALWPWL	127	15
62	NLTLEGVFA	27	15	726	WLCLTPRQPL	98	20
156	GLTWPVVLI	23	25	302	WLHQHRTCPL	98	20

Note. Table 1 (*HLA-A*0201*) shows the predicted peptides binding to *HLA-A*0201* in the order of predicted binding affinities shown as binding scores. Predicted 9mer-peptides are shown in the left half, and predicted 10mer-peptides are shown in the right half of the table. The prediction of the binding affinities was performed with the software described in Materials and Methods.

was determined by calculating the percentage of specific ^{51}Cr-release by the following formula:

$$\frac{\text{cpm of the test sample release} - \text{cpm of the spontaneous release}}{\text{cpm of the maximum release} - \text{cpm of the spontaneous release}} \times 100$$

Spontaneous 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.

Antigen specificity was confirmed by the cold target inhibition assay, which used unlabeled T2 or A24LCLs that were pulsed with the peptide (20 μg/mL for 16 hours at 37°C) to compete for the recognition of ^{51}Cr-labeled DLD-1 or HT29 tumor cells. The MHC restriction of the induced CTLs was examined by measuring the inhibition of the cytotoxicity with anti-HLA-class I (W6/32) antibody and anti-HLA-class II antibody, anti-CD4 antibody and anti-CD8 antibody (DAKO, Carpinteria, CA).

CTL Induction from Patients with Colorectal Cancer. The PBMCs (1×10^5 cells per well) were incubated with 10 μmol/L peptide in wells of the U-bottomed-type 96-well microculture plates in 200 μL of culture medium. The culture medium contained 45% RPMI 1640 , 45% AIM-V medium, 10% fetal bovine serum, 100 units/ml IL-2, and 0.1 μmol/L nonessential amino acid solution. One-half of the medium was removed and was replaced with new medium containing the corresponding peptide every 3 days. After incubation for 13 days, these cells were harvested and then tested for their ability to produce IFN-γ in response to T2 cells (*HLA-A*0201*) or A24LCLs (*HLA-A*2402*) loaded with either the candidate peptide or control HIV peptide (*HLA-A*0201*:SLYNTYATL, *HLA-A*2402*:RYLRQQLLGI) with ELISA specific to IFN-γ (Pierce, Endogen, Rockford, IL). The lymphoid cells, which showed IFN-γ production on the stimulation with the candidate peptide at the levels higher than that with control HIV peptide, were expanded with the expansion procedure described above to generate CTL lines.

RESULTS

Identification of Gene Up-Regulated in Colorectal Cancers and Expression Analysis of *RNF43*. We compared expression of genes at colon cancer tissues with their corresponding noncancer mucosa of the colon normal tissues. With genome-wide cDNA microarray with 23,040 genes, Yagyu *et al.* (18) detected the *RNF43* gene, which was up-regulated two times higher than tumor-to-normal intensity ratios in 10 of the 11 cancer tissues examined. Lin *et al.* (15) or Takemasa *et al.*

Table 2 Candidate selection from peptides derived from *RNF43* based on predicted binding affinities to *HLA-A*2402*

		Binding affinity				Binding affinity	
Start position	AA sequence (9mers)	BIMAS	SYFPEITHI	Start position	AA sequence (10mers)	BIMAS	SYFPEITHI
331	SYQEPGRRL	360	22	449	SYCTERSGYL	200	20
350	HYHLPAAYL	200	19	350	HYHLPAAYLL	200	21
639	LFNLQKSSL	30	16	718	CYSNSQPVWL	200	21
24	GFGRTGLVL	20	16	209	IFVIILASVL	36	16
247	RYQASCRQA	15	10	313	VFNITEGDSF	15	18
397	RAPGEQQRL	14	12	496	TFCSSLSSDF	12	17
114	RAPRPCLSL	12	12	81	KLMQSHPLYL	12	12
368	RPPRPGPFL	12	12	54	KMDPTGKLNL	9	12
45	KAVIRVIPL	12	13	683	HYTPSVAYPW	8	11
721	NSQPVWLCL	10	12	282	GQELRVISCL	4	11

Note. Table 2 (*HLA-A*2402*) shows the predicted peptides binding to *HLA-A*2402* in the order of predicted binding affinities shown as binding scores. Predicted 9mer-peptides are shown in the left half, and predicted 10mer-peptides are shown in the right half of the table. The prediction of the binding affinities was performed with the software described in Materials and Methods.

Table 3 Cytotoxicity of CTL lines (*HLA-A*0201*)

Start position	AA sequences	Cytotoxicity (%) Pep(+)	Cytotoxicity (%) Pep(−)	Start position	AA sequences	Cytotoxicity (%) Pep(+)	Cytotoxicity (%) Pep(−)
60	KLNLTLEGV	−2.1	0.2	81	KLMQSHPLYL	18.0	27.6
8	QLAALWPWL	3.5	0.0	357	YLLGPSRSAV	18.2	15.4
82	LMQSHPLYL	1.7	1.2	202	LMTVVGTIFV *		
358	LLGPSRSAV	−0.4	−0.7	290	CLHEFHRNCV	9.6	9.7
11	ALWPWLLMA	90.2	1.5	500	SLSSDFDPLV †		
15	WLLMATLQA	−0.2	0.0	8	QLAALWPWLL	6.7	9.0
200	WILMTVVGT *			11	ALWPWLLMAT	91.5	27.1
171	KLMEFVYKN	2.6	0.0	7	LQLAALWPWL †		
62	NLTLEGVFA *			726	WLCLTPRQPL †		
156	GLTWPVVLI	−0.4	0.7	302	WLHQHRTCPL	7.4	6.1

Note. Table 3 shows cytotoxicities of CTL lines with *HLA-A*0201* binding peptides derived from *RNF43*. Pep(+) shows cytotoxicity against the targets pulsed with corresponding peptides, and Pep(−) shows cytotoxicity against targets without peptide pulse at 20 as Effector-to-Targets ratio.
Abbreviations: AA, amino acid(s); Pep, peptides.
* Peptide was not synthesized because of the technical difficulties caused by highly hydrophobic nature of the peptides.
† CTL line was not established.

(17) also showed *RNF43* as one of the up-regulated genes in colorectal cancer tissues on cDNA microarray. Subsequent semiquantitative reverse transcription-PCR confirmed the elevated expression in 15 of 18 additional colon cancer tissues analyzed (18). We carried out Northern blot analysis on human adult and fetal multiple-tissue with a PCR product of *RNF43* as a probe. Although human adult tissue blots did not show a detectable band, the transcripts were detected in the fetal lung and fetal kidney (18). Furthermore, we confirmed that elevated expression of *RNF43* is associated with better proliferation of the cancer cells with colony formation assay (18). These results suggest that *RNF43* has the expression profile and functions hypothesized for the ideal TAA candidates. Therefore, we tried to demonstrate its immunogenicity through identification of epitope peptides with reverse immunology approach.

Candidate Selection from Peptides Derived from *RNF43* on the Basis of Predicted Binding Affinities to *HLA-A*0201* or *A*2402*. Tables 1and 2 show the predicted peptides restricted to *HLA-A*0201* and *HLA-A*2402,* respectively, in the order of predicted binding affinity that is exhibited as binding score. Forty peptides in total were selected and examined as described below. These tables also show binding score predicted by SYFPEITHI prediction software program for reference.[4]

Successful Induction of CTLs with the Candidate Peptides. The CTLs were induced with these peptides derived from *RNF43* as described in Materials and Methods. CTL inductions were set up from, at most, five healthy donors for each peptide. Furthermore, CTL inductions were tried by using 6 to 48 wells in 48-well plates for one peptide from each donor. Resulting CTLs showing detectable cytotoxic activity were expanded to establish CTL lines.

The cytotoxic activities of CTL lines induced by the *HLA-A*0201* binding peptides are shown in Table 3. The CTL line stimulated with RNF43-11(IX) (ALWPWLLMA) and RNF43-11(X) (ALWPWLLMAT) showed higher cytotoxic activities against targets pulsed with each corresponding peptide than against those targets not pulsed with any peptides. The cytotoxic activities of CTL lines induced by the *HLA-A*2402* binding peptides are shown in Table 4CTL lines stimulated by RNF43-

Table 4 Cytotoxicity of CTL lines (*HLA-A*2402*)

Start position	AA sequences	Cytotoxicity (%) Pep(+)	Cytotoxicity (%) Pep(−)	Start position	AA sequences	Cytotoxicity (%) Pep(+)	Cytotoxicity (%) Pep(−)
331	SYQEPGRRL	2	1	449	SYCTERSGYL	1	1
350	HYHLPAAYL	26	17	350	HYHLPAAYLL †		
639	LFNLQKSSL	42	33	718	CYSNSQPVWL †		
24	GFGRTGLVL	8	9	209	IFVIILASVL *		
247	RYQASCRQA	71	82	313	VFNITEGDSF *		
397	RAPGEQQRL	41	32	496	TFCSSLSSDF	8	9
114	RAPRPCLSL	23	26	81	KLMQSHPLYL	10	5
368	RPPRPGPFL	1	0	54	KMDPTGKLNL	5	2
45	KAVIRVIPL †			683	HYTPSVAYPW	0	0
721	NSQPVWLCL	68	0	282	GQELRVISCL †		

Note. Table 4 shows cytotoxicities of CTL lines with *HLA-A*2402* binding peptides derived from *RNF43*. Pep(+) shows cytotoxicity against the targets pulsed with corresponding peptides, and Pep(−) does cytotoxicity against targets without peptide pulse at 20 as Effectors to Targets ratio.
Abbreviations: AA, amino acid(s); Pep, peptides.
* Peptide was not synthesized because of the technical difficulties caused by highly hydrophobic nature of the peptides.
† CTL line was not established.

350 (HYHLPAAYL), RNF43-639 (LFNLQKSSL), and RNF43-721 (NSQPVWLCL) showed higher cytotoxic activities against the targets pulsed with peptides than against those targets not pulsed with any peptides. The CTL lines stimulated with RNF43-81 (KLMQSHPLYL) and RNF43-54 (KMDPTGKLNL) showed marginally higher cytotoxic activity against the peptide-pulsed target than against those targets not pulsed with any peptides.

These CTL lines stimulated with RNF43-11(IX) (Fig. 1*A*), RNF43-11(X) (Fig. 1*B*) or RNF43-721 (Fig. 1*C*) showed potent cytotoxic activity against the peptide-pulsed target without showing any significant cytotoxic activity against targets not pulsed with any peptides.

Establishment of CTL Clones with Potent and Specific Cytotoxicity Against the Targets Pulsed with the Candidate Peptides. CTL clones were propagated from the CTL lines with limiting dilution methods as described in Materials and Methods. With *HLA-A*0201* binding peptides, 14 and 6 CTL clones were established against RNF43-11(IX) and RNF43-11(X), respectively. With *HLA-A*2402* binding peptides, 13 CTL clones were established against RNF43-721. Cytotoxic activities of RNF43-11(IX), RNF43-11(X), and RNF43-721 CTL clones are shown in Fig 1*D, E,* and *F*, respectively. Every CTL clone tested had potent cytotoxic activity against the peptide-pulsed target without showing any cytotoxic activity against the target not pulsed with any peptides.

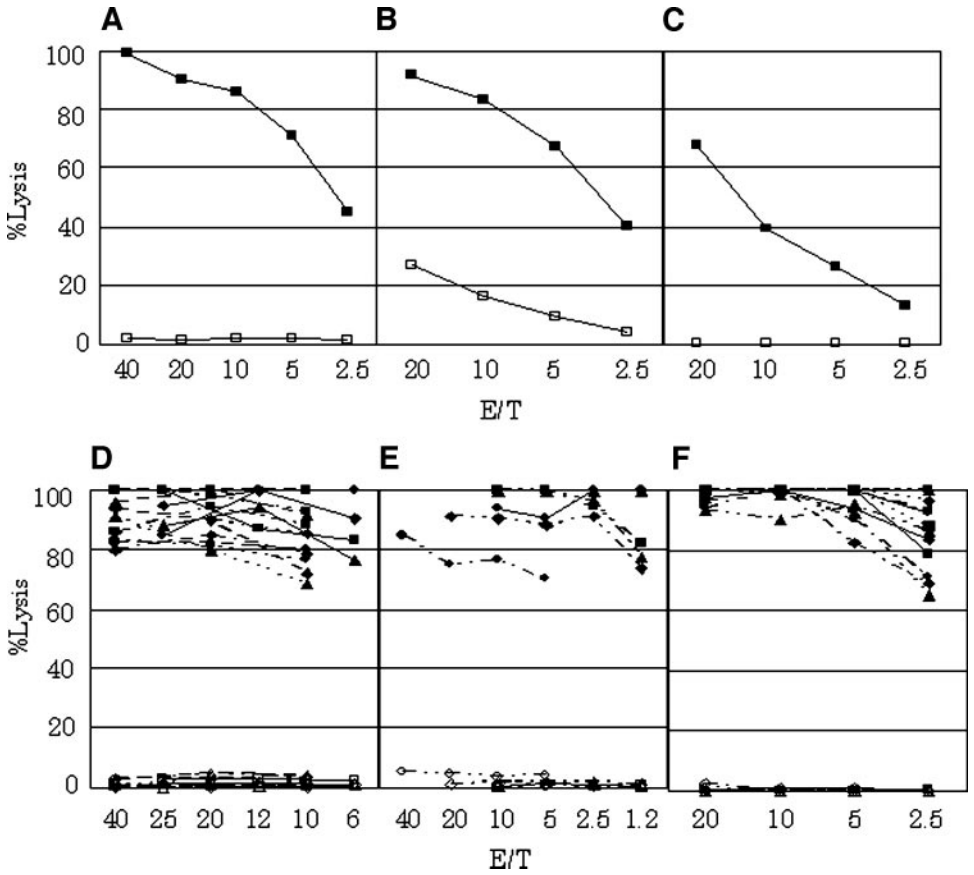

Fig. 1 The CTL lines and clones raised by RNF43-11(IX), RNF43-11(X) and RNF43-721 have peptide specific cytotoxicity. The CTL lines induced with RNF43-11(IX) (*A*), RNF43-11(X) (*B*) or RNF43-721 (*C*) (*filled square line*) showed high cytotoxic activity against target cells pulsed with respective peptides. However, they did not show significant cytotoxic activity against the same target cells without peptide pulse (*empty square line*). Target cells used on *A* and *B* were T2; whereas target cells used on *C* was A24LCL. These demonstrated that CTL lines had peptide-specific cytotoxicity. Cytotoxic activities of CTL clones induced with RNF43-11(IX) (*D*), RNF43-11(X) (*E*), and RNF43-721 (*F*) were tested against the targets pulsed with corresponding peptides as described in Materials and Methods. The 14, 6, and 13 CTL clones established with RNF43-11(IX), RNF43-11(X), and RNF43-721, respectively, had potent cytotoxic activities against target cells pulsed with the peptides without showing any significant cytotoxic activity against the same target cells not pulsed with any peptides. The targets used for the clone for RNF43-11(IX) or RNF43-11(X) were T2 cells with or without pulsing the corresponding peptides. The target used for the clone for RNF43-721 was A24LCLs with or without pulsing the corresponding peptide. *D*: clone8+/−:-■-/-□-, clone17+/−:–■–/–□–; clone31+/−:–◆–/–◇–, clone34+/−:-◆-/-◇-; clone61+/−:–▲–/–△–, clone64+/−:–●–/–○–; clone69+/−:-◆-/-◇-, clone71+/−:-▲-/-△-; clone78+/−:-■-/-□-, clone83+/−:-▲-/-△-; clone90+/−:-●-/-○-, clone93+/−:-●-/-○-; clone112+/−:–◆–/–◇–, clone119+/−:–■–/–□–. *E*: clone2+/−:-▲-/-△-, clone3+/−:-●-/-○-; clone15+/−:–■–/–□–, clone16+/−:–●–/–○–; clone17+/−:–◆–/–◇–, clone25+/−:–▲–/–△–. *F*: clone16+/−:-■-/-□, clone37+/−:-◆-/−◇-; clone45+/−:-▲-/−△-, clone80+/−:-●-/-○-; clone83+/−:-■-/-□-, clone87+/−:-◆-/−◇-; clone104+/−:-▲-/-△-, clone112+/−:-●-/-○-; clone113+/−:–■–/–□–, clone115+/−:–◆–/–◇–; clone122+/−:–▲–/–△–, clone133+/−:–●–/–○–; clone136+/−:–■–/–□–.

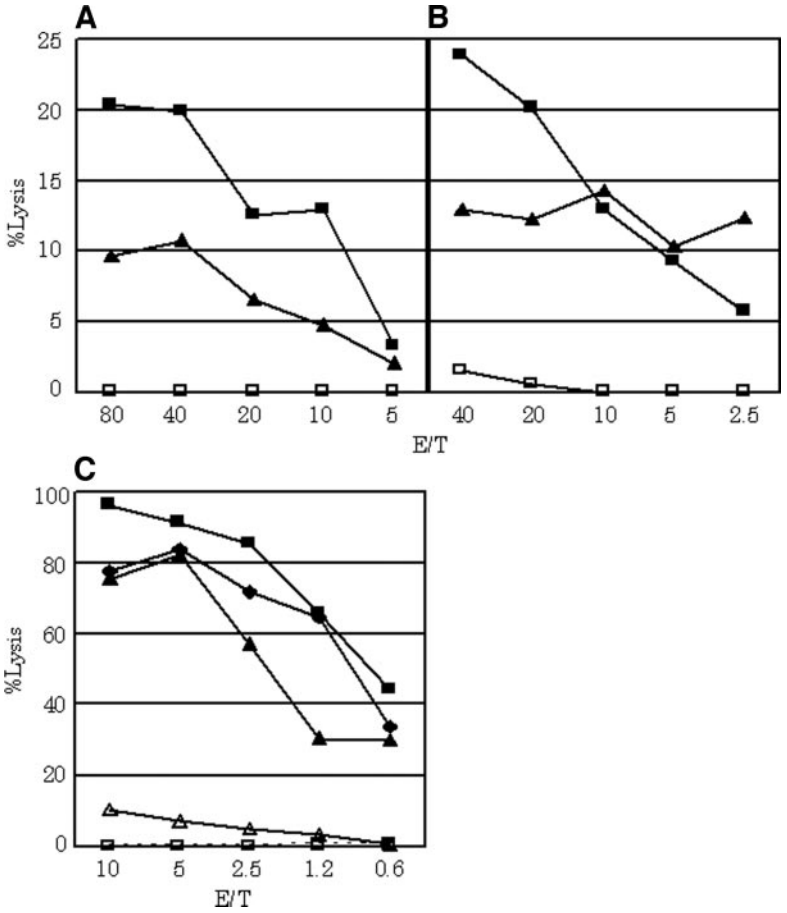

Fig. 2 The CTL clones induced with RNF43-11(IX), RNF43-11(X), and RNF43-721 recognize and lyse the tumor cells endogenously expressing *RNF43* in the HLA-restricted fashion. *A, B*, cytotoxic activities against DLD-1, HCT15, and HT29, all of which endogenously express *RNF43,* were tested, as described in Materials and Methods, with RNF43-11(IX) CTL clone 90 (*A*) or RNF43-11(X) (*B*) CTL clone 25 as effector cells. These CTL clones showed high cytotoxic activity against DLD-1 (*filled square line*) and HCT15 (*filled triangle line*) that have *RNF43* and *HLA-A*0201* genotype. On the other hand, they did not show significant cytotoxic activity against HT29 (*empty square line*), which expresses *RNF43* but not *HLA-A*0201*. *C*, cytotoxic activities against HT29, WiDR, and HCT116, all of which endogenously express *RNF43,* were tested, as described in Materials and Methods, with RNF43-721 CTL clone 45 as effector cells. A24LCL was used as the target that does not express *RNF43*. RNF43-721 CTL clone 45 showed high cytotoxic activity against HT29 (*filled triangle solid line*) and WiDR (*filled diamond solid line*), which express both *RNF43* and HLA-A24. On the other hand, it did not show significant cytotoxic activity against HCT116 (*empty triangle solid line*), which expresses *RNF43* but not HLA-A24, and A24LCL (*empty square dotted line*), which expresses HLA-A24 but not *RNF43*. Moreover, RNF43-721 CTL clone 45 did not show the cytotoxic activity against A24LCL pulsed with irrelevant peptide (*empty square solid line*).

Specific and HLA-Restricted Cytotoxic Activities of Established CTL Clones Against Colorectal Cancer Cell Lines Endogenously Expressing *RNF43*. The CTL clones raised against candidate peptides were examined for their ability to recognize and lyse the tumor cells endogenously expressing *RNF43*. Figure 2*A* shows the cytolytic activities of CTL clone 90 raised against RNF43-11(IX). Although CTL clone 90 showed cytotoxic activity against DLD-1 and HCT15, which express *RNF43* and *HLA-A*0201*, it showed no cytotoxic activity against *HLA-A*0201*-negative HT29, which expresses *RNF43*. Figure 2*B* shows the cytolytic activities of CTL clone 25 raised against RNF43-11(X). The CTL clone showed cytotoxic activity against DLD1 and HCT15, which express both *RNF43* and *HLA-A*0201*. However, it showed no cytotoxic activity against *HLA-A*0201*-negative HT29, which expresses *RNF43*. We established CTL lines against RNF43-11(IX) from three of three healthy donors and CTL lines against RNF43-11(X) from three of four healthy donors. Figure 2*C* shows the cytolytic activities of CTL clone 45 raised against RNF43-721. The CTL clone showed potent cytotoxic activity not only against A24LCLs pulsed with RNF43-721 peptide but also against HT29 and WiDR, which express *RNF43* and *HLA-A*2402*. However, it showed no cytotoxic activity against *HLA-A*2402*-negative HCT116, which expresses *RNF43*, A24LCLs pulsed with irrelevant peptide, or A24LCLs pulsed with no peptide. We established CTL lines against RNF43-721 from two of five healthy donors.

A cold-target inhibition assay was performed to confirm the specificity of RNF43-11(IX) CTL clone and RNF43-11(X) CTL clone. The DLD-1 cells labeled with ^{51}Cr were used as hot targets, and T2 cells pulsed with RNF43-11(IX) or RNF43-11(X) without ^{51}Cr labeling were used as cold targets. Specific cell lysis against DLD-1 cell target was significantly inhibited, when T2 pulsed with RNF43-11(IX) or RNF43-11(X) was added in the assay at various ratios (Fig. 3*A* and *B*). A similar result was observed with *RNF43-7*21. HT29 cells labeled with ^{51}Cr were used as hot targets, and A24LCLs pulsed with RNF43-721 without ^{51}Cr labeling were used as cold targets. Specific cell lysis against the HT29 cell target was significantly inhibited, when A24LCLs, pulsed with RNF43-721, were added in the assay at various ratios (Fig. 3*C*). All of these results were indicated as a percentage of specific lysis at and E/T ratio of 40.

The CTL clones against RNF43-11(IX), RNF43-11(X), and RNF43-721 were incubated with antibodies against HLA-Class I, HLA-Class II, CD4, and CD8, and were tested for their capacity to lyse the target cells that was DLD-1 in the case of RNF43-11(IX) (Fig. 3*D*) and RNF43-11(X) (Fig. 3*E*), or WiDR in the case of RNF43-721 (Fig. 3*F*). The cytotoxicity of CTL

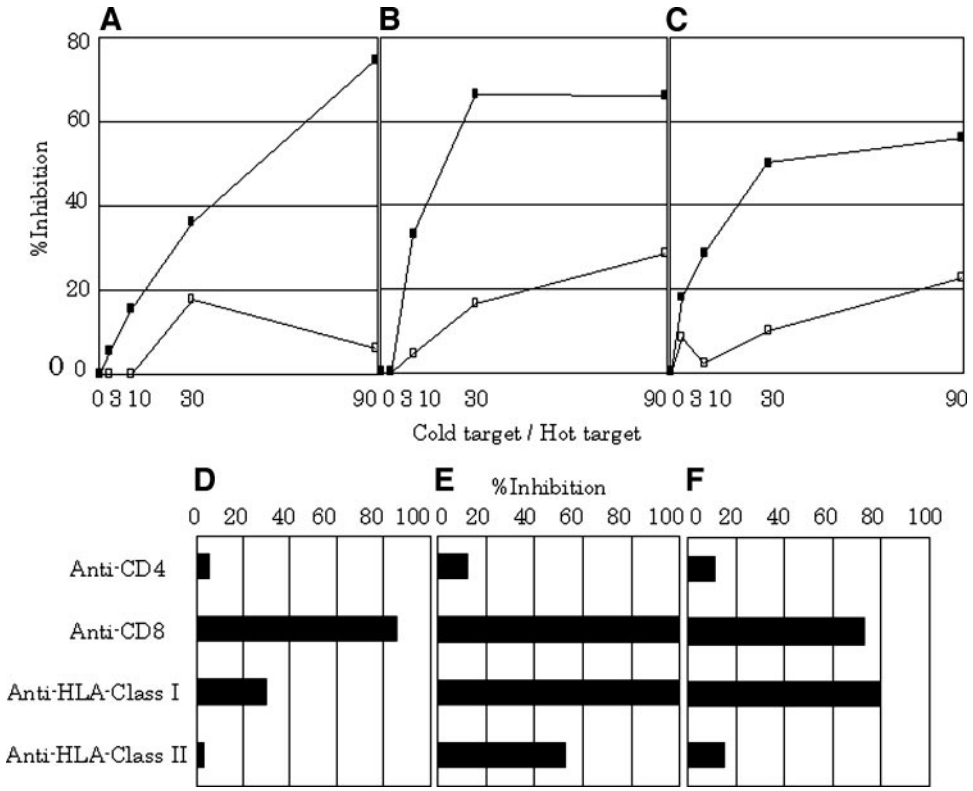

Fig. 3 RNF43-11(IX), RNF43-11(X), and RNF43-721 CTL clones specifically recognize corresponding peptides in the *HLA-A*0201* or *A*2402* restricted manner. *A, B,* the cold target inhibition assay was performed as described in Materials and Methods. DLD-1 labeled by $Na_2{}^{51}Cr\ O_4$ was prepared as a hot target, whereas RNF43-11(IX) (*A*) or RNF43-11(X) (*B*) peptide-pulsed T2 (Peptide +) was used as a cold target (*Inhibitors*). E/T ratio was fixed as a 40. The cytotoxic activity against DLD-1 was inhibited by the addition of T2 pulsed with the identical peptide (*filled quadrilateral line*), whereas it was almost uninhibited by the addition of T2 without peptide pulse (*empty quadrilateral line*). *C* the cold target inhibition assay was performed as described in Materials and Methods. HT29 labeled by $Na_2{}^{51}Cr\ O_4$ was prepared as a hot target, and RNF43-721 peptide-pulsed A24LCL (*Peptide* +) was used as a cold target (*Inhibitors*). E/T ratio was fixed as a 20. The cytotoxic activity against HT29 was inhibited by the addition of A24LCL pulsed with the identical peptide (*filled quadrilateral line*), whereas it was almost uninhibited by the addition of A24LCL without peptide pulse (*empty quadrilateral line*). In *D, E,* and *F*, to examine the characteristics of CTL clones raised against RNF43 –11(IX) (*D*), RNF43-11(X) (*E*), or RNF43-721(*F*), antibodies against HLA-Class I, HLA-Class II, CD4, and CD8 were tested for their capacity to inhibit the cytotoxic activity. *The horizontal axis* reveals percentage (%) inhibition of the cytotoxicity. The cytotoxicity of CTL clones against DLD-1 (*D, E*) or WiDR (*F*) targets was significantly reduced when anti-class I- and -CD8 antibodies were used.

clones against target cells was significantly inhibited when anti-HLA-Class I antibody and anti-CD8 antibody were used (Fig. *3D, E,* and *F*), indicating that the CTL clones, consisting mainly of CD8+ T cells, recognize the *RNF43*-derived peptide in a HLA-Class I-restricted manner.

Given that the sequences of RNF43-11(IX) and RNF43-11(X) peptides are largely overlapping, we examined whether CTL clones against them may have identical specificities or not. The CTL clone for RNF43-11(IX) showed cytotoxic activity against T2 cells pulsed with the corresponding peptide but showed no cytotoxic activity against T2 cells pulsed with RNF43-11(X) peptide (Fig. 4*A*). On the other hand, the CTL clone for RNF43-11(X) showed potent cytotoxic activity against T2 cells pulsed with RNF43-11(X) but showed significantly less cytotoxic activity against T2 cells pulsed with the RNF43-11 (IX) peptide (Fig. 4*B*). These results suggest that the CTL clone against the RNF43-11 (IX) CTL clone and the one for the RNF43-11(X) CTL clone appear to have different specificities.

These results strongly suggest that the CTL clones established against the candidate peptides have specific and HLA-restricted cytotoxic activity against colorectal cancer cell lines expressing endogenous *RNF43*. Lytic activities mediated by two HLA-A2-restricted epitopes were marginal, whereas tumor lysis mediated by the HLA-A24 epitope was clearly better. These findings might be caused by the poor natural presentation of RNF43-11(IX) and RNF43-11(X) by tumors or poor T-cell receptor avidity for these specific epitopes.

Identification of No Other Known Gene Products Containing the Peptides with Completely Homologous to RNF43-11(IX), RNF43-11(X), or RNF43-721 Peptide. The CTL clones established against RNF43-11(IX), RNF43-11(X), and RNF43-721 showed cytotoxic activity against targets pulsed with corresponding peptides as well as targets endogenously expressing *RNF43*. To confirm the sequences of RNF43-11(IX), RNF43-11(X), and RNF43-721 are unique to *RNF43*, we performed a homology search of these peptides in public databases

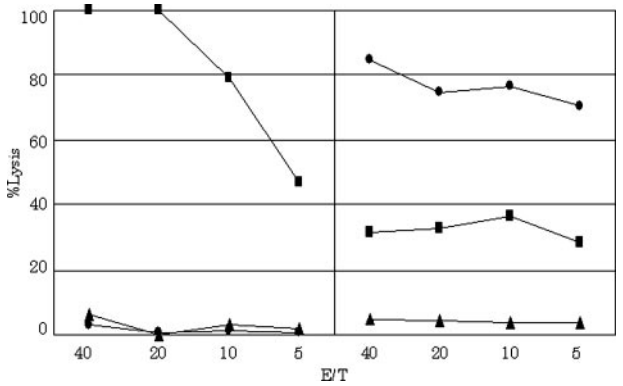

Fig. 4 The CTL clones for RNF43-11(IX) and RNF43-11(X) show different specificities. Given that the sequences of RNF43-11(IX) and RNF43-11(X) peptides are largely overlapping, we examined whether CTL clones against them may have identical specificities or not. The CTL clone for RNF43-11(IX) showed cytotoxic activity against T2 cells pulsed with the corresponding peptide (*filled square line*) but showed no cytotoxic activity against T2 cells pulsed with RNF43-11(X) peptide (*filled circle line*) and T2 cells without peptide pulse (*filled triangle line*; *A*). On the other hand, the CTL clone for RNF43-11(X) showed potent cytotoxic activity against T2 cells pulsed with RNF43-11(X) (*filled circle line*) but showed significantly less cytotoxic activity against T2 cells pulsed with the RNF43-11(IX) peptide (*filled square line*) and no cytotoxic activity against T2 cells without peptide pulse (*filled triangle line*; *B*). These results suggest that the CTL clone against RNF43-11(IX) CTL clone and the one for RNF43-11(X) CTL clone have different specificities.

with BLAST.[5] There was no sequence completely or highly homologous to RNF43-11(IX), RNF43-11(X), or RNF43-721 in the databases. These results strongly suggest that the sequences of RNF43-11(IX), RNF43-11(X), and RNF43-721 are unique to the RNF43 to the best of our knowledge.

Existence of CTL-Precursors in PBMCs of Colorectal Cancer Patients. We then examined whether RNF43-specific CTLs could be induced also in cancer patients. PBMCs from two *HLA-A*0201*-positive patients with colorectal cancer expressing RNF43 were stimulated with RNF43-11(IX). The RNF43-11(IX) peptide successfully induced the CTL lines, which showed potent cytotoxicity against target cells pulsed with the corresponding peptide (Fig. 5*A*) from one patient. These CTL lines against RNF43-11(IX) also showed potent cytotoxic activity against DLD-1, which expresses both *RNF43* and *HLA-A*0201*. However, they did not show detectable cytotoxic activity against HT29, which expresses *RNF43* but not *HLA-A*0201* (Fig. 5*B*). PBMCs from three *HLA-A*2402*-positive patients with colorectal cancer expressing RNF43 were stimulated with RNF43-721. The stimulation with RNF43-721 peptide successfully induced the CTL lines that showed moderate cytotoxicity against target cells pulsed with the corresponding peptide (Fig. 5C) from one patient. The CTL line against RNF43-721 also showed potent cytotoxic activity against HT29, which expresses both *RNF43* and *HLA-A*2402*. However, they did not show detectable cytotoxic activity against HCT116, which expresses *RNF43* but not *HLA-A*2402* (Fig. 5D).

These results strongly suggest that CTLs specific to *RNF43* protein can also be induced in colorectal cancer patients.

DISCUSSION

Identification of novel TAAs that induce potent and specific antitumor immune responses, warrants further development of clinical application of peptide vaccination strategy in various types of cancers (2–7, 26–33). However, at the moment, not many promising TAAs have been found in common type cancers, including colon cancer. New TAAs have been explored with multiple measures. The initial TAA discoveries have been achieved mostly by the immunologic screening of the products expressed in tumor cell lines with cDNA library (1). More recent studies performed by the multiple groups have shown that novel TAAs can also be identified as well with new strategies, including SEREX (7). Although these methods are useful and reliable, they are time-consuming and laborious and need specific techniques. These difficulties might result in the limited number of TAAs identified by these methods.

To overcome the situation described above, we have invented a novel method that uses information from gene expression profiles. Because cDNA microarray technologies can disclose comprehensive profiles of gene expression of malignant cells (14–16), this approach should help to identify candidates of TAA (20, 21). In previous studies with these technologies, we have identified multiple gene transcripts that are up- or down-regulated in colorectal cancers (15, 17, 18, 34). From among these, we selected a novel human gene, *RNF43* (15, 17, 18), as the prime candidate for a TAA. The *RNF43* gene is enhanced in colorectal cancer cells. Additionally, Northern blot analysis detected its expression in the lung and kidney of the human fetus but not in human adult tissues that we examined. Significant expression of *RNF43* was detectable only in the lung and kidney of the human fetus. In addition, we also revealed that *RNF43* was involved in the proliferation of cancer cells, which is one of the essential properties of malignant tumor cells. Because the ideal TAA to be used in cancer vaccination should be abundantly and specifically expressed in the proliferating tumor cells, we hypothesized that *RNF43* might serve as a good immunologic target.

To test this hypothesis, we examined, in this study, whether the *RNF43* protein contains antigenic epitope peptides or not. The *RNF43*-derived peptide-candidates were predicted based on the theoretical binding affinities to *HLA-A*0201* or *HLA-A*2402*, both of which are known to have higher frequencies in certain clusters of human populations (35–37) and have been synthesized for evaluation. With *in vitro* stimulation on PBMCs of healthy volunteers by DCs pulsed with these peptides, CTL clones were successfully established with RNF43-11(IX) (ALWPWLLMA) and RNF43-11(X) (ALWPWLLMAT), and they showed potent cytotoxic activities against T2 (*HLA-A*0201*) pulsed with the corresponding peptides. CTL clones established with RNF43-721 (NSQPVWLCL) also showed potent cytotoxic activity against the A24LCL (*HLA-A*2402*) pulsed with corresponding peptide. Furthermore, CTL clones established from RNF43-11(IX) and RNF43-11(X) showed specific cytotoxicity

[5] Internet address: http://www.ncbi.nlm.nih.gov/BLAST/.

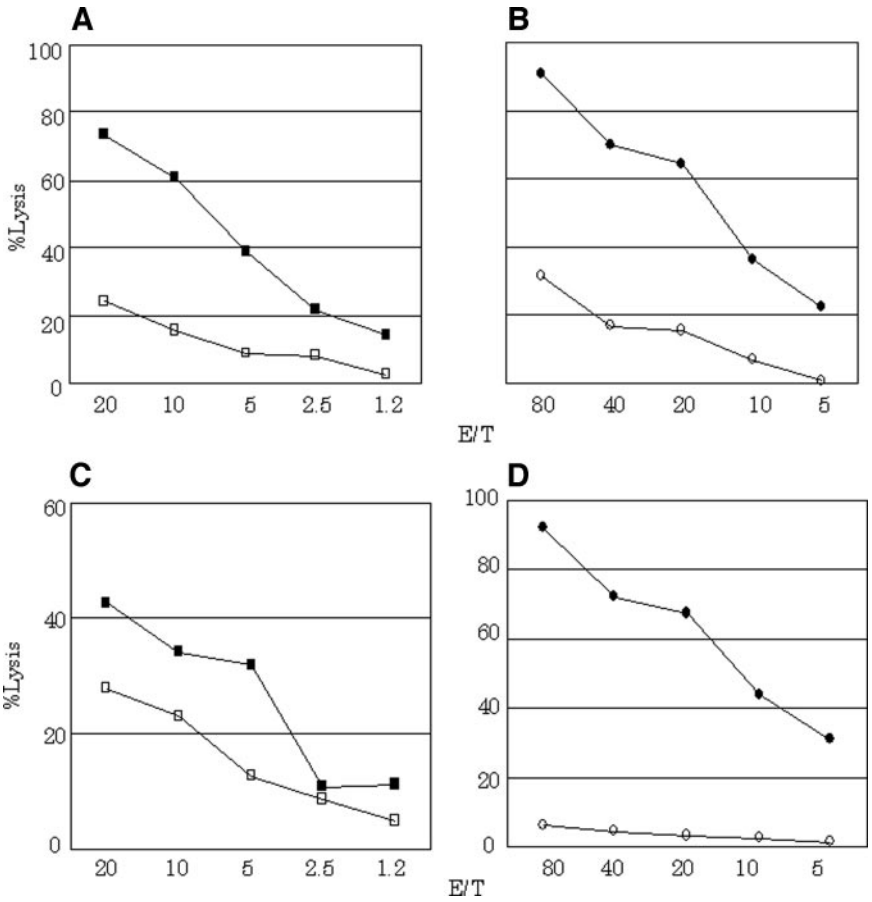

Fig. 5 Existence of CTL precursors in PBMCs from colorectal cancer patients. The CTL line against RNF43-11(IX) peptide was established. The CTL line against RNF43-11(IX) showed high cytotoxic activity not only against the target cells pulsed with the corresponding peptide (*A*) but also against the target cells (*DLD-1*) that express *RNF43* and *HLA-A2* (*B*). ■, cytotoxic activity against target with the peptide pulse; □, cytotoxic activity against target without any peptide pulse; ●, cytotoxic activity against DLD-1; ○, cytotoxic activity against HT29. The CTL lines against RNF43-721 peptide were established. The CTL line against RNF43-721 showed potent cytotoxic activity not only against the target cells pulsed with the corresponding peptide (*C*) but also against the target cells (HT29) that expresses *RNF43* and *HLA-A24* (*D*). ■, cytotoxic activity against target with the peptide pulse; □, cytotoxic activity against target without any peptide pulse, ●, cytotoxic activity against HT29; ○, cytotoxic activity against HCT116.

against *HLA-A*0201*-positive colorectal carcinoma cell lines that endogenously express *RNF43*. The CTL clones induced with RNF43-721 also showed specific cytotoxicity against HLA-A24-positive colorectal carcinoma cell lines that endogenously express *RNF43*. These CTL clones, mostly CD8 positive, showed significant cytotoxic activities specific to the peptide in HLA-Class I–restricted manner. These results strongly suggest that RNF43-11(IX), RNF43-11(X), and RNF43-721 peptides are among the native peptides that are cleaved from the *RNF43* protein, processed, and presented on HLA molecules of the cell surface, and that induce potent CTL responses against themselves. In other words, *RNF43* is immunogenic and could serve as a TAA of colorectal cancers. Because we now know that *RNF43* is also expressed in lung, gastric, and liver cancers (data not shown), *RNF43* could be a TAA to these cancers as well.

Homology analysis of RNF43-11(IX), RNF43-11(X), and RNF43-721 peptides showed that there are no highly homologous peptides in the databases. These results support the proposal that identified peptides are *RNF43* specific and that they are unlikely to possess cross-reactivity against other known molecules. This might also suggest that these peptides could be clinically applied without adverse effects, because CTLs induced with these peptides would not react to the epitope peptides of the gene products expressed by noncancerous tissues. Furthermore, we have shown that CTLs specific to the identified peptide could be successfully induced also from the PBMCs obtained from the patients with colon cancer expressing *RNF43*.

The results of this study strongly suggest that *RNF43* is a new TAA of which epitope peptides may induce potent immune responses. The epitope peptides derived from *RNF43* are now in the process of clinical application as a phase I study. Furthermore, our results also suggest that many more new TAAs can be discovered in various types of cancers.

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Clin Cancer Res 2004;10:8577-8586.