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Identification of ribosomal protein L19 as a novel tumor antigen recognized by autologous cytotoxic T lymphocytes in lung adenocarcinoma

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The purpose of the present study was to identify a novel tumor-specific antigen capable of inducing a specific cellular immune response in lung cancer patients. The co-culture of regional lymph node lymphocytes and the CD80-transfected autologous lung adenocarcinoma cell line H1224L resulted in a successful induction of bulk cytotoxic T lymphocytes (CTL). CTL clone L7/8 was established by the limiting dilution method from these bulk CTLs and lysed H1224L but not autologous Epstein–Barr virus-transformed B cells or K562. The CTL clone also recognized allogeneic lung cancer cell lines in an HLA-A*31012-restricted manner. Using the CTL clone, an antigen-coding gene was identified using the cDNA expression cloning technique, which encodes ribosomal protein L19 (RPL19). Finally, a 9 mer antigenic peptide was identified by means of construction of mini-genes. RPL19 was overexpressed in the lung cancer tissue from patient H1224. All of the normal tissues examined expressed lower levels of RPL19 mRNA than that of the lung cancer tissue. RPL19 was also found to be overexpressed in 12 of 30 (40%) non-small-cell lung cancer tissues by immunohistochemical staining. The expression level of RPL19 in tumor cell lines correlated positively with the production of interferon (IFN)-γ by CTL clone L7/8 in response to such cell lines. In addition, the suppression of RPL19 expression by transfection with small interfering RNA resulted in the suppression of cyclinD1, D3 synthesis, and the growth inhibition of lung cancer cell lines overexpressing RPL19. Therefore, this growth suppression could be ascribed to the inhibition of the cell cycle. These results may indicate that RPL19 is a novel overexpressed antigen which may therefore be a useful candidate as a target for specific immunotherapy. (*Cancer Sci* 2010; 101: 46–53)

Lung cancer is one of the most common malignant tumors in the world and has the highest age-adjusted death rate among malignancies in 45 countries.[1,2] The most effective therapy is a surgical resection in early stage non-small-cell lung cancer (NSCLC), because advanced NSCLC tends to show a poor response to radiotherapy and chemotherapy. It is important to concentrate our efforts on developing more effective cancer therapies.

Specific immunotherapies have been utilized since the identification of the tumor-specific antigen derived from malignant melanoma.[3] Tumor-associated antigens are classified into cancer-testis antigen, overexpressed antigen, mutated antigen, and differentiation antigen, which have been identified with CTL clones derived from regional lymph node lymphocytes (RLNL), peripheral blood lymphocytes (PBL), and tumor-infiltrating lymphocytes (TIL).[3–6] These antigens have been employed for cancer vaccines.[7–9] In 2004, however, the results of clinical vaccine studies in patients with metastatic cancer were reviewed and the clinical response was not satisfactory.[10] The identification of a novel tumor antigen for more effective immunotherapy to induce clinical response is further needed to develop more effective immunotherapy, in addition to establishing a strategy against immunological escape mechanisms.

Twenty-nine lung cancer cell lines have been established from 1109 lung cancer specimens in our laboratory from 1994 to 2007 in order to analyze autologous tumor-specific immune response.[11] Successful induction of tumor-specific CTL clones from patients with NSCLC[12–14] and the identification of several types of tumor antigens recognized by such autologous CTL clones[15–20] have been reported. The present study identified a novel overexpressed antigen, RPL19, recognized by autologous CTL clones using cDNA expression cloning methods in patient H1224 with lung adenocarcinoma. The expression levels of RPL19 in lung cancer tissues and normal tissues were evaluated, and moreover, a functional analysis of RPL19 was performed using siRNA.

Materials and Methods

The study protocol was approved by the Human and Animal Ethics Review Committee of the University of Occupational and Environmental Health and a signed consent form was obtained from each patient before taking the tissue samples used in this study.

Patient H1224 and cell line H1224L. Patient H1224 was a 55-year-old female with adenocarcinoma of the lung showing T2N2M0, stage IIIA, according to the International Union Against Cancer (UICC) TNM classification. H1224L, a lung adenocarcinoma cell line, was established from the surgical specimen obtained from patient H1224, which had the genotype of HLA-A*2402/31012, B*4801/5601, Cw*0401/1201. To augment its capacity to stimulate autologous CTL, H1224L was transfected with CD80 cDNA cloned into a pMXs retrovirus plasmid vector (H1224L-B7).[21,22]

The regional mediastinal and hilar lymph nodes from patient H1224 were obtained at the time of surgery. Regional lymph node lymphocytes were prepared using mononuclear cells from RLN and preserved in a deep freezer at −80°C until use. Epstein–Barr virus transformed B cells of H1224 (H1224 EBV-B) were derived from PBL of H1224 treated with 1 μg/mL cyclosporine A (Sandoz, Basel, Switzerland) and 20% of the supernatant of the Epstein–Barr virus transformed marmoset monkey lymphocyte B95-8.

Allogeneic lung cancer cell lines and HLA genotypes. Lung adenocarcinoma cell lines A110L (HLA-A*2402, B*5201, Cw*1202), B203L (HLA-A*2402/31012, B*1501/5401, Cw*0102/0304), F1121L (HLA-A*2402/0201, B*4006/1507, Cw*0303/0801), G821L (HLA-A*2602, B*5101, Cw*1402),

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K420PL (HLA-A*2402, B*5201, Cw*1202); squamous cell carcinoma cell lines B1203L (HLA-A*2402/2402, B*5201/5401, Cw*0102/1201), QG56 (HLA-A*2601, B*4601, Cw*0102); large cell carcinoma cell line A904L (HLA-A*2402, B*0702, Cw*0702); and pleomorphic carcinoma cell line G603L (HLA-A*2602/31012, B*1501/4002, Cw*0303/0304) were established in the laboratory.[12–14,18,19] PC-9 (HLA-A*0602/2402, B*0702/5502, Cw*0302/0702) was kindly donated by Dr K. Itoh, Kurume University, Kurume. K562 is an erythroleukemia cell line, which is sensitive to natural-killer cell cytotoxicity. Rosi-EBV-B (HLA-A*2402/3201, B*3503/44031, Cw*0401/0401), tumor necrosis factor (TNF)-sensitive WEHI 164c13 cells, and 293-EBNA cells were kindly donated by Dr P.G. Coulie (Cellular Genetics Unit, Université catholique de Louvain, Brussels, Belgium). WEHI-164c13 cells were maintained in culture medium with 5% FCS and 293-EBNA cells were maintained in DMEM (Life Technologies, Gaithersburg, MD, USA) with 5% FCS, while the other cell lines were maintained in CM with 10% FCS.

Induction of the CTL clone. Frozen preserved RLNL obtained at the time of surgery were rapidly thawed and were stimulated with irradiated (100 Gy) CD80-transfected H1224L weekly at a tumor cell-to-lymphocyte ratio of 1:10 in CM with 10% FCS in the presence of 25 U/mL recombinant interleukin (rIL)-2 (kindly donated by Takeda Chemical Industries, Osaka, Japan) and 5 ng/mL IL-7 (Genzyme Techne, Cambridge, MA, USA) for 4 weeks. To establish T cell clones, a limiting dilution was performed from bulk CTL 7 days after the last stimulation as described previously.[14]

mAb (monoclonal antibodies). Hybridomas (HB-145, HB-95) were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). C7709.A2.6 (anti-HLA-A24) and B1.23.2 (anti-HLA-B, C) were kindly donated by Dr P.G. Coulie. The culture supernatants of ATCC HB–145 (IVA12; anti-HLA-DR, DP, DQ), HB-95 (W6/32; anti-HLA-A, B, C) were used for analyzing the HLA restriction of T-cell clones.

Cytotoxicity assay and cytokine production of CTL. The cytotoxicity of CTL was assessed by a standard 4-h ^{51}Cr release assay as described previously.[13] Cytokine production of CTL was measured as described previously.[23,24] In the blocking assay using mAb, a 1/4-diluted culture supernatant of hybridomas such as HB-95, C7709.A2.6, B1.23.2, and HB–145 was added to the co-culture of CTL and H1224L.

Cloning of HLA-A*31012. The total RNA was extracted from H1224L using the RNeasy Mini kit (Qiagen, Hilden, Germany) and it was converted to cDNA using an oligo (dT) primer. The cDNA served as a template for PCR amplification using each HLA-A specific forward primer (5′-ACTGGGCGGATCCGGACTCAGAATCTCCCCAGACGCCGAG-3′) and reverse primer (5′-ACTGCCCGAATTCTCTCAGTCCCTCACAAGGCAGCTGTC-3′). The PCR product was cloned into pcDNA3 by using the TOPO cloning Kit (Invitrogen, Tokyo, Japan), and then sequenced.

Construction and screening of the cDNA library. The CTL clone L7/8 also recognized B203L; therefore, the cDNA library was constructed from the mRNA of B203L, an allogenic lung adenocarcinoma cell line. The cDNA library was constructed and screened as described previously.[18]

Quantitative RT–polymerase chain reaction (RT-PCR). Quantitative RT-PCR was carried out in an ABI Prism 7000 (Applied Biosystems, Foster, CA). The relative amount of RPL19 mRNA was measured by means of detection of intercalated TaqMan. PCR was performed with the 10-μL TaqMan Universal Master Mix (Applied Biosystems), with either 1 μL of cDNA or 1 μL of water and each primer set as described below in a total volume of 20 μL. The PCR conditions were 10 min at 95°C for denaturing, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. The primer sequences of RPL19 for quantitative RT-PCR were OKY 988: 5′-CTCAGGCTTCAGAAGAGGCT-3′ and OKY 989: 5′-ATTGGCGATTTCATTGGTCT-3′, and the TaqMan probe sequences were 5′-TGTCCTCCGCTGTGGCAAGAA-3′. The quantitative PCR primers of β-actin were TaqMan Beta-actin Control Reagents (Applied Biosystems).

The threshold cycle number (C_T) was defined as a fractional cycle number at which the amount of amplified target product reached a fixed threshold. ΔC_T was obtained by comparing C_T of RPL19 with C_T of β-actin in same amount of templates. Relative quantification was achieved by comparison with the ΔC_T of H1224L or H1224 normal lung tissue or H1224 EBV-B. The relative expression was calculated using the following formula:

$$\text{relative expression} = 2^{-(\Delta C_T \text{sample} - \Delta C_T \text{control})}.$$

Immunohistochemical staining of RPL19. Four-μm-thick sections sliced from formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene, and after washing in a graded series of ethanol, the sections were placed in PBS for 10 min. The solution was boiled for 10 min and then cooled for 20 min. Endogenous peroxidase activity was blocked for 10 min in methanol containing 0.3% H_2O_2 and then the slides were placed in PBS. The sections were incubated with PBS including 1% concentration of bovine serum albumin for 10 min to block nonspecific binding of the immunoreagents. After washing in PBS, the tissue sections were incubated with 1:200 diluted mouse monoclonal antihuman RPL19 antibody (Abnova, Taipei, Taiwan) at 4°C for overnight and washed in PBS. Then an immunoperoxidase staining was done using the histofine simple stain MAX-PO (MULTI; Nichirei Bioscience, Tokyo, Japan). The localization of RPL19 was visualized with diaminobenzidine tetrahydrochloride. Cancer cells demonstrating RPL19 staining in more than 30% of all cells were regarded as tissue specimens showing positive staining in tumor tissues. The immunohistochemical staining slides were independently reviewed by two investigators (K.K. and T.B.).

Western blot analysis. After the lung cancer cell lines were transfected with siRNA of RPL19 and control siRNA, whole protein (50 μg) was loaded onto polyacrylamide gel in Tris-Glycine SDS Running buffer (Invitrogen). PAGE-separated protein was electroblotted to polyvinylidene difluoride (ATTO, Tokyo, Japan). The blots were probed with monoclonal antibodies against RPL19 (Abnova), cyclinD1, cyclinD3, p27kip1, and p16INK4A (Cell Signaling Technology, Danvers, MA, USA). The secondary antimouse or rabbit IgG antibody was conjugated with horseradish peroxidase. Imuunoblots were enhanced by lumi-lightplus Western blotting substrate (Roche, Indianapolis, IN, USA).

RNA interfering assay. Three RNAi of RPL19 were synthesized by Invitrogen. The siRNA sequences used were as follows: siRNA1-UUCCGCUUACCUAUGCCCAUGUGCC and GGCACAUGGGCAUAGGUAAGCGGAA; siRNA2-ACAUGUGGCGAUCGA UCUUCUUAGA and UCUAAGAAGAUCGA UCGCCACAUGU; and siRNA3-CUU GGAUAAAGUCUUGAUGAUCUCC and GGAGAUCAUCAAGACUUUAUCCAA G. siRNA was transfected into cancer cell lines using Lipofectamine 2000 (Invitrogen). The control siRNA was commercially available stealth RNAi negative control medium GC duplex (Invitrogen). H1224L cells transfected with siRNA using Lipofectamine 2000 were seeded in flat-bottomed plates in triplicate and viable cells were counted at the indicated time.

Cell cycle analysis. H1224L cells (2×10^5) transfected with siRNA of RPL19 and control siRNA were seeded into six flat-bottomed plates and cultured. The cells were recovered 24-h later and suspended in 70% ethanol with vortexing. The cells were then resuspended in 100 μg/mL RNase for 1 h at 37°C and 50 μg/mL propidium iodide (Sigma, St. Louis, MO, USA) in PBS. A flow cytometric analysis was performed with a

Coulter Epics analyzer, and the data were analyzed using the Multicycle program (Phoenix Flow System, San Diego, CA, USA) to determine the relative fractions of cells with G1-, S-, and G2/M-amounts of DNA.

Results

Generation of a CTL clone recognizing autologous adenocarcinoma cells. RLNLs from patient H1224 were stimulated weekly with irradiated H1224L-B7 in the presence of IL-2 and IL-7. The bulk of the CTLs were obtained after four such stimulations and lysed H1224L-B7 as well as H1224L, but did not lyse autologous EBV-B or K562 (data not shown). Then, the CTL clone L7/8 was established from the bulk of the CTLs by a limiting dilution. CTL clone L7/8 lysed H1224L, but did not lyse autologous EBV-B or K562 (Fig. 1A). CTL clone L7/8 produced TNF in response to H1224L which was inhibited in the presence of anti-HLA class I mAb, but not anti-HLA-A24 mAb or anti-HLA-B/C mAb (Fig. 1B). The genotype of HLA of H1224L was HLA-A*2402/31012, B*4801/5601, Cw*0401/1201. Therefore, the restriction element of HLA class I of CTL clone L7/8 was suggested to be HLA-A*31012.

The cross reactivity of CTL clone L7/8 against an HLA-A31-positive tumor cell line (B203L) and an HLA-A31-negative tumor cell line (G821L) were tested. CTL L7/8 produced TNF in response to B203L as well as in response to G821L when transfected with HLA- A31 but not with HLA-A24 (Fig. 1C). This suggested that the tumor antigen recognized by CTL clone L7/8 is shared among autologous H1224L and some allogeneic tumor cell lines.

Identification of gene encoding antigen recognized by CTL L7/8. A cDNA library (1.4×10^5) prepared from the mRNA of B203L to which CTL L7/8 could respond was cloned into expression vector pCEP4. The screening of the cDNA library was performed by measuring the TNF production of CTL L7/8 in response to 293 EBNA cells co-transfected with HLA-A*31012 and each cDNA fraction (100 clones/one pool). One positive pool of cDNA was obtained and was subcloned from 100 to 12 clones, then a single cDNA clone (cDNA clone 2-275) was finally isolated. The sequence of cDNA 2-275 was 694 bp long with a poly A sequence at the 3′ end. CTL L7/8 showed TNF production in response to 293 EBNA cells co-transfected with cDNA clone 2-275 and HLA-A*31012, but not with any other combination of cDNA (Fig. 2A). The sequence of cDNA 2-275 was identical to ribosomal protein L19 according to the NCBI data bank (BLAST accession no. NM 000981; Fig. 2B).

Identification of the antigenic peptide. To identify the antigenic peptide, cDNA fragments (mini-genes) which were shortened from the 3′ end of RPL19 were amplified from the cDNA clone 2-275 with PCR. The PCR products were cloned into expression plasmid pcDNA3.1 and the constructs were co-transfected with the HLA-A31 cDNA clone into 293EBNA cells. Transfections of truncated forms of cDNA clone 2-275 indicated that sequence coding for the C-terminal end of the antigenic peptide was located around 140–142 amino acid (Fig. 2C). Three nonapeptides covering this region were synthesized and tested for reactivity of CTL L7/8 by pulsing each peptide on T2 cells transfected with HLA-A*31012. The peptide KNKRILMEH: $RPL19_{133\text{-}141}$ was recognized in a dose-dependent manner by the CTL (Fig. 2D). The interferon (IFN)-γ production of the CTL clone in response to these peptides was also evaluated. The peptide, $RPL19_{133\text{-}141}$, was recognized by the CTL in a dose-dependent manner, in which the sensitivity was higher in the lysis assay than in the IFN-γ assay (Fig. 2E). In addition, T2 cells transfected with HLA- A*31012 and pulsed with the peptide KNKRILMEH: $RPL19_{133\text{-}141}$ were recognized by the CTL clone (Fig. 2F).

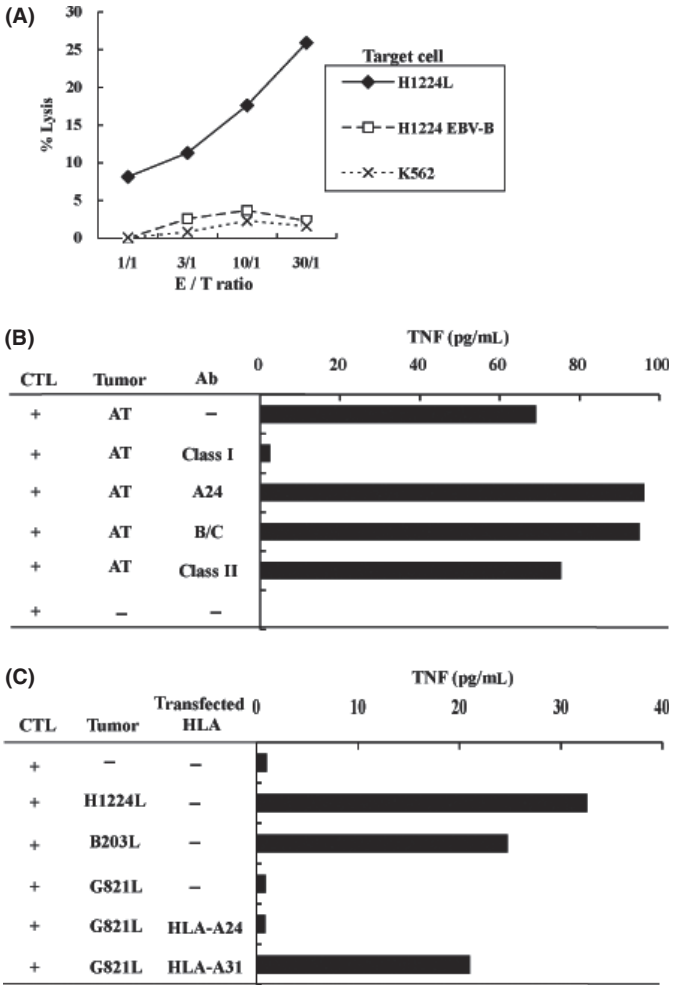

Fig. 1. Characteristics of the autologous tumor-specific cytotoxic T lymphocyte (CTL) clone L7/8. (A) Cytotoxic activities of CTL clone L7/8 were assessed by a standard 4-h ^{51}Cr release assay. CTL clone lysed autologous lung cancer cell line H1224L, but not H1224 Epstein–Barr virus (EBV)-B nor K562. (B) Tumor necrosis factor (TNF) production of the CTLs in response to H1224L was assessed in the presence of indicated blocking Abs in order to elucidate HLA class I restriction. The TNF production of CTL clone in response to H1224L was inhibited by anti-HLA class I mAb, but not anti-HLA-A24 mAb or anti-HLA-B/C mAb. (C) TNF production by the CTL in response to HLA-A31-positive lung cancer cell lines (H1224L, B203L) and HLA-A31-negative lung cancer cell line (G821L) transfected with HLA-A24 or HLA-A31 which is expressed in H1224L was assessed. The CTL clone produced TNF in response to HLA-A31-positive lung cancer cell lines (H1224L, B203L), but not the negative lung cancer cell line (G821L). Then, the CTL clones produced TNF in response to a negative lung cancer cell line (G821L) transfected with HLA-A31 but not with HLA-A24. The results shown are one representative of the findings from three experiments.

RPL19 mRNA expression of normal tissues and lung cancer. The expression level of the gene at the mRNA level was investigated by real-time PCR. The relative expression level of the *RPL19* gene in the lung cancer tissue from patient H1224 was 5.98 assuming that the RPL19 expression in the corresponding normal lung tissue is 1.0. The expression of RPL19 was analyzed in cDNA of normal tissues which were commercially available (Clontech, Palo Alto, CA, USA). The expression levels of RPL19 mRNA in each normal tissue were lower than that of H1224 lung cancer tissue. The expression levels of the *RPL19* gene in the heart and skeletal muscle were relatively high (1.72 and 2.84, respectively) among

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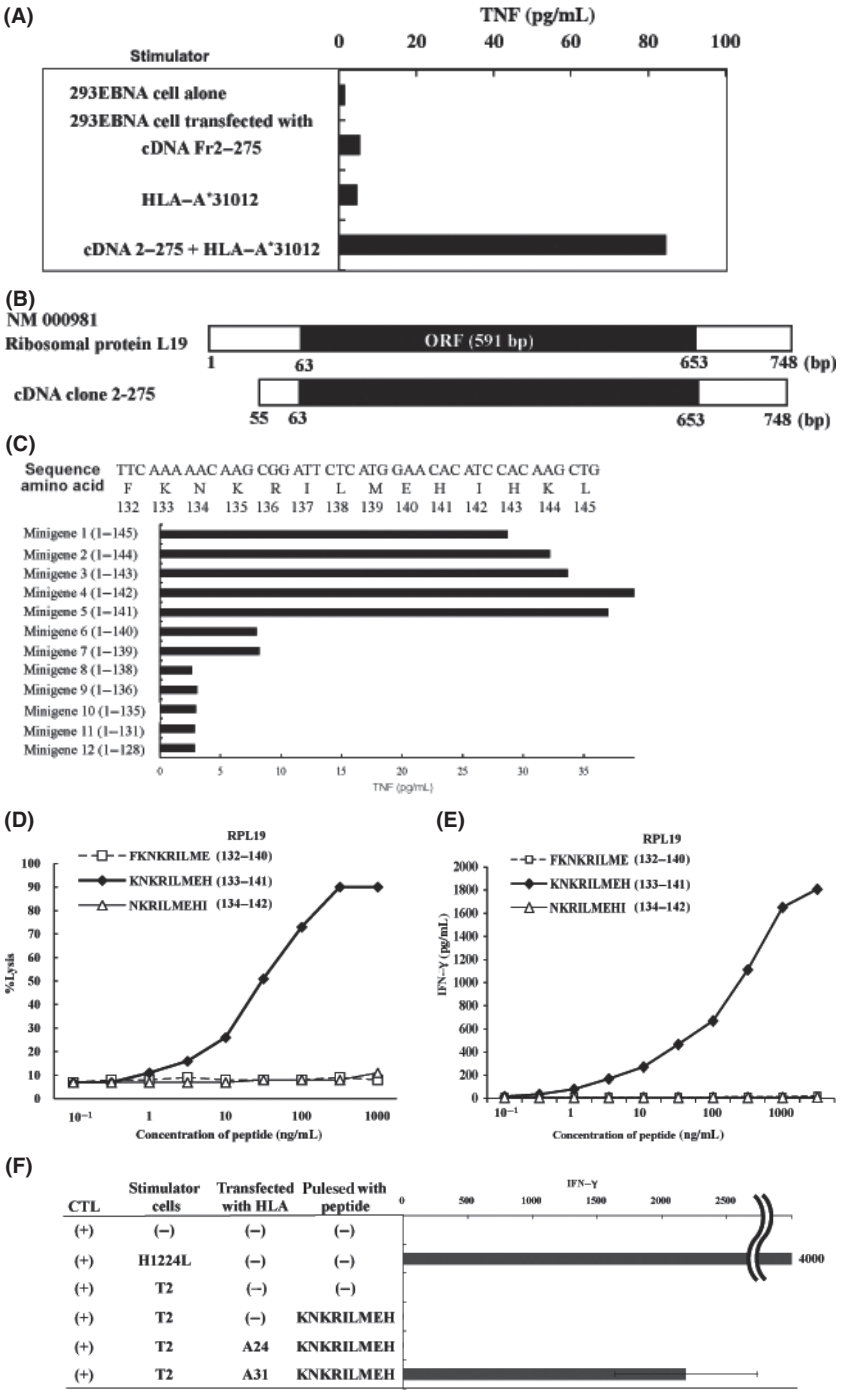

Fig. 2. Isolation of the antigen-coding gene by cDNA expression cloning and identification of an antigenic peptide recognized by the cytotoxic T lymphocyte (CTL) clone L7/8. (A) Tumor necrosis factor (TNF) production by the CTL clone L7/8 in response to 293 EBNA cells transfected with cDNA plasmid of HLA-A*31012 and/or cDNA clone 2-275. The CTL clone recognized cDNA clone 2-275 in conjunction with HLA- A*31012. (B) Isolated cDNA clone 2-275 recognized by the CTL clone L7/8 was a 694 bp length encoding 231 amino acid and included open reading frame of ribosomal protein L19 (RPL19) (BLAST accession no. NM 000981). (C) Several cDNA fragments (mini-genes) of cDNA clone 2-275 were amplified by PCR. TNF production by the CTL clone L7/8 in response to 293 EBNA cells transfected with the mini-genes and HLA-A*31012 was assessed and this data indicated that the antigenic peptide was located in the sequence coding for the 3′ end between 140 and 142 amino acid. (D) Three nonapeptides covering this region, FKNKRILME: $RPL19_{132\text{-}140}$, KNKRILMEH: $RPL19_{133\text{-}141}$, and NKRILMEHI: $RPL19_{134\text{-}142}$, were synthesized. ^{51}Cr-labeled T2 cells transfected with HLA- A*31012 were incubated for 1 h at room temperature with the indicated concentrations of the peptide. The CTL clone L7/8 recognized T2 cells transfected with HLA- A*31012 pulsed with the peptide $KNKRILMEH_{133\text{-}141}$ in a dose-dependent manner; (E) the peptide titration with interferon (IFN)-γ release assay. (F) T2 cells or T2 cells transfected with HLA-A*2401 or T2 cells transfected with HLA- A*31012 were incubated for 1 h at room temperature with 20 μg/mL of the nonapeptide KNKRILMEH: $RPL19_{133\text{-}141}$ and incubated with the CTL clone. T2 cells transfected with HLA- A*31012 and pulsed with the peptide KNKRILMEH: $RPL19_{133\text{-}141}$ were only recognized. The results shown are representative findings of three experiments.

normal tissues (Fig. 3A). The relative expression level of the *RPL19* gene in H1224L was 7.8 on the basis of that in the normal lung tissue of patient H1224.

Immunohistochemical analysis of RPL19 protein in cancer tissues and normal tissues. In patient H1224, the lung cancer tissue had a positive staining in comparison to the corresponding normal lung tissue in immunohistochemistry using anti-RPL19 mAb (Fig. 3B). The expression levels of RPL19 protein in 30 lung cancer tissues and normal lung tissues were further investigated. Overexpression of RPL19 was found in 12 of 30 (40%) NSCLC tissues, one of 10 (10%) breast cancer tissues, two of 10 (20%) esophageal cancer tissues, none of 10 (0%) gastric cancer tissues, and six of 10 (60%) colorectal cancer tissues in comparison to that of corresponding normal tissues. RPL19 protein was weakly expressed in the glandular epithelium of normal tissues and the basal layer of the esophageal epithelium. According to histological classification of NSCLC, RPL19 was found to be overexpressed in six of 18 adenocarcinomas, in five of 10 squamous cell carcinomas, in none of one adenosquamous cell carcinoma, and one of one pleomorphic carcinoma. Five of 12 lung cancer tissues showing positive expression of RPL19 were stained almost homogenously, while seven were stained heterogeneously.

Correlation between the RPL19 mRNA level of the cancer cell lines and the response of CTL against them. The correlation between production level of IFN-γ by CTL L7/8 in response to

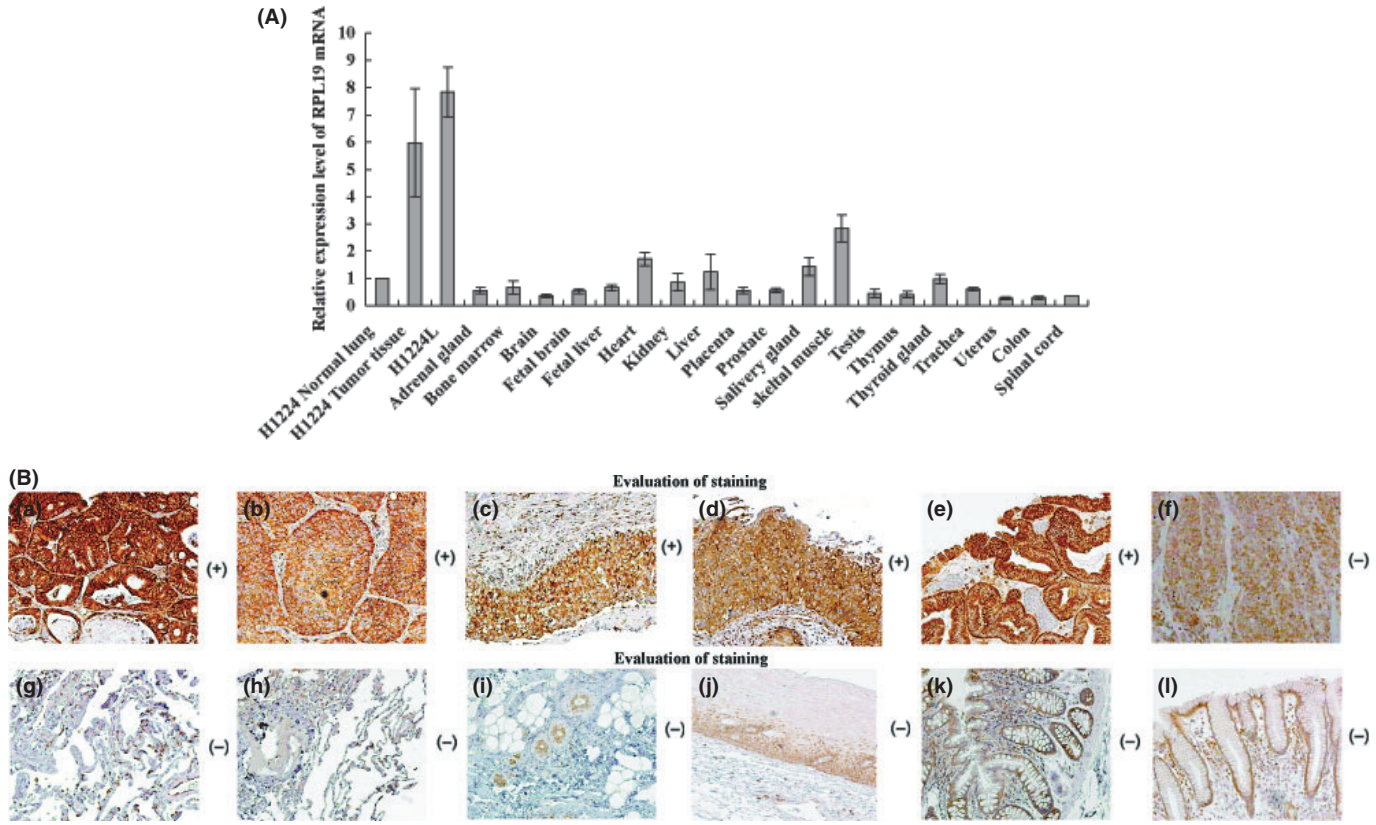

Fig. 3. Overexpression of ribosomal protein L19 (RPL19) in lung cancer in comparison to normal tissues. (A) The relative expression level of RPL19 mRNA determined by real-time PCR analysis. The expression level of RPL19 was determined by real-time RT-PCR (TaqMan probe method). The relative expression level of RPL19 was investigated among normal lung tissues and the lung cancer tissue in patient H1224 and H1224L on the basis of the RPL19 expression in the normal lung tissue from patient H1224 as 1.0. The results shown are representative findings of three experiments. (B) Immunohistochemical staining of RPL19 protein with anti-RPL19 mAb. RPL19 staining in lung cancer (a,b). (a) Adenocarcinoma H1224; (b) squamous cell carcinoma; (c) breast cancer; (d) esophageal cancer; (e) colon cancer; and (f) gastric cancer. The normal counterpart tissue from the same patient was stained and illustrated as (g), (h), (i), (j), (k), and (l), respectively. RPL19 protein was weakly expressed in the glandular epithelium of normal tissues (i,k,l) and in the basal layer of the esophageal epithelium (j).

10 lung cancer cell lines transfected with HLA-A*31012 and the expression level of RPL19 mRNA was investigated (Table 1). The relative expression levels of RPL19 mRNA in 10 tumor cell lines were positively correlated with the magnitude of IFN-γ production of the CTL clone L7/8 ($P = 0.0014$, $\gamma = 0.86$; Fig. 4).

Effect of siRNA against RPL19 on growth of lung cancer cell lines. To analyze the function of RPL19, lung cancer cell lines were transfected with control siRNA or three kinds of siRNA against RPL19. Transfection with either siRNA of RPL19 suppressed the synthesis of endogenous RPL19 protein in H1224L (Fig. 5A). Inhibition of RPL19 protein synthesis by transfection with these siRNA against RPL19 induced inhibition of proliferation of H1224L (Fig. 5B,C). To analyze the mechanism of the inhibition of proliferation, cell cycle– and apoptosis-related molecules were investigated in the tumor cells transfected with siRNA of RPL19. Transfection with siRNA of RPL19 suppressed the synthesis of cyclinD1 and cyclinD3, and at the

Table 1. Ribosomal protein L19 (RPL19) expression and response of CTL in lung cancer cell lines

Cell line	Lung cancer cell type	HLA-A*31012	Relative expression level of RPL19 mRNA†	IFN-γ production of CTL clone L7/8	
				Transfection (–)	Transfected with HLA-A*31012 (log10)
H1224L	Adenocarcinoma	Positive	7.84	3066	3972 (3.60)
B203L	Adenocarcinoma	Positive	2.48	170	454 (2.66)
A110L	Adenocarcinoma	Negative	2.62	4	25 (1.40)
G821L	Adenocarcinoma	Negative	1.86	5	23 (1.36)
A904L	Large cell carcinoma	Negative	0.73	2	16 (1.21)
K420L	Adenocarcinoma	Negative	2.42	7	8 (0.91)
QG56	Squamous cell carcinoma	Negative	1.98	6	6 (0.81)
PC9	Adenocarcinoma	Negative	0.62	8	5 (0.68)
F1121L	Adenocarcinoma	Negative	1.06	4	5 (0.68)
B1203L	Squamous cell carcinoma	Negative	1.00	2	4 (0.63)

†Relative expression level of RPL19 mRNA on the basis of RPL19 expression in normal lung tissue as 1.0. CTL, cytotoxic T lymphocyte; IFN, interferon.

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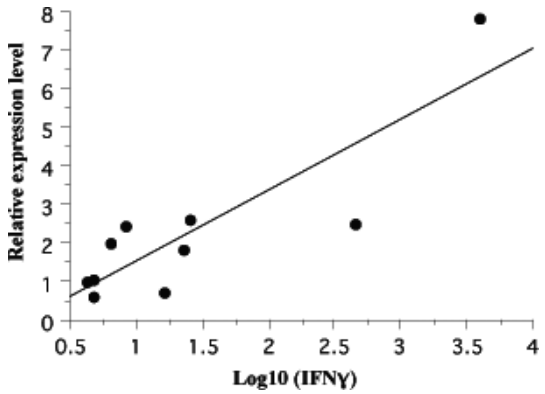

Fig. 4. Regression analysis of the relative expression level of ribosomal protein L19 (RPL19) mRNA in lung cancer cell lines and interferon (IFN)-γ secretion by the CTL clone L7/8 in response to lung cancer cell lines transfected with HLA-A*31012. Positive correlation between expression levels of RPL19 and IFN-γ production of the CTL in response to the tumor cells was observed ($P = 0.0014$, $\gamma = 0.86$).

same time augmented the synthesis of a negative cell cycle regulator (p27 kip1) and a CDK inhibitor (p16 INK4A) (Fig. 5D). Various apoptosis-related molecules such as cleaved caspase-3 and cleaved poly ADP-ribose polymerase (PARP) were analyzed. However, no significant change in the apoptosis-related molecules was observed. The H1224L cells transfected with siRNA were incubated, and the cell cycle phase distribution was determined by flow cytometry. When the H1224L cells were transfected with siRNA of RPL19, the fraction of cells showing the G1-phase increased from 36.6% to 48.4% and that of cells showing the S-phase decreased from 44.3% to 27.4% 24 h after the transfection.

Discussion

The long-term prognosis of patients with lung cancer is still poor. Therefore, new therapeutic strategies are expected to be developed. Our approach is to identify tumor-specific antigens recognized by autologous CTL, which may be useful for immunotherapy. For such purposes, it is necessary to establish lung cancer cell lines and autologous CTLs. However, the success rate for the establishment of permanent lung cancer cell lines was only 2.6% (29/1109) from 1994 to 2007 in our laboratory.[11,21] Until now, there have been limited numbers of reports on the identification of tumor-associated antigens in lung cancer. Mutated antigens (elongation factor 2, α-actinin-4, NF-YC, p53), overexpressed antigens (HER2/neu, Tara), cancer-testis antigens (KK-LC-1, MAGE family, NY-ESO-1), and antigens expressed during cancer-specific processing and presentation

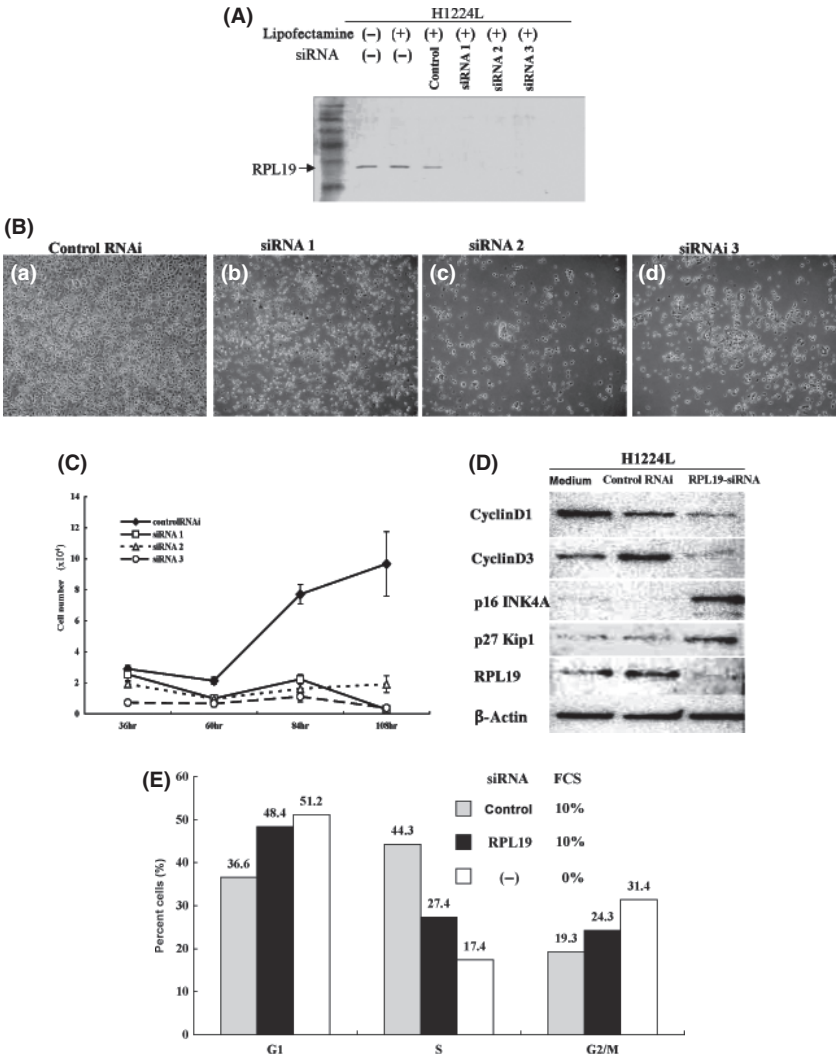

Fig. 5. Suppression of ribosomal protein L19 (RPL19) synthesis of H1224L by the siRNA. (A) RPL19 protein synthesis of H1224L cells transfected with the three siRNA was completely inhibited by a Western blot analysis. (B) The photos (×400) of H1224L cells in culture were taken 4 days after transfection with the indicated siRNA in a flat-bottomed plate. The proliferation of H1224L cells transfected with RPL19-siRNA was significantly suppressed in comparison to the control RNAi. (C) Proliferations of H1224L transfected with control RNAi or siRNA of RPL19 were compared at the indicated times. Marked growth suppression of H1224L cells transfected with RPL19-siRNA was observed. (D) The mechanism of growth suppression of H1224L by blocking of RPL19 synthesis. The levels of RPL19 and cyclin D1 and cyclin D3 and p16 INK4A and p27 Kip1 protein synthesis of H1224L cells transfected with siRNA were evaluated by Western blot analysis. Transfection with siRNA of RPL19 suppressed the synthesis of cyclin D1 and cyclin D3 and, in contrast, it augmented the synthesis of p27kip1, p16 INK4A. (E) A cell cycle analysis of the cells transfected with siRNA of RPL19. The change in the DNA pattern stained by propidium iodide was evaluated by flow cytometry. Transfection with siRNA of RPL19 led to an increase in the fraction of the G1-phase and a decrease in the fraction of the S-phase at 24 h after the transfection.

(SGT1, AL137255) were identified by CTL clones from lung cancer patients in our laboratory[15–20] and by others.[25,26] In this study, RPL19 was identified as a novel overexpressed tumor antigen in lung cancer by autologous CTL.

RPL19 is a member of family comprising over 70 different proteins that form the large and small ribosomal subunits.[27,28] RPL19 is located in chromosome 17q11.2-12.[29] The open reading frame of RPL19 encodes a protein consisting of 196 amino acids.[30] RPL19 is localized in the cytoplasm and is reported to function as an RNA chaperone during translation, coordinating interaction between the ribosome and RNA.[27,28] It has been reported that the augmentation of the RPL19 expression was observed in several cancers. Bee *et al.* reported that a significant augmentation of RPL19 expression was found in prostate cancer and that the augmentation was a powerful predictor of unfavorable prognosis of prostatic cancer.[31] The expression of RPL19 in the feces of colorectal cancer patients was associated with an advanced tumor stage and is a unfavorable prognostic factor.[32] Therefore, RPL19 may be associated with the poor prognosis of cancer patients. Henry *et al.* reported that human breast cancers with a high level of RPL19 expression also overexpressed erbB-2 (or HER-2/neu).[33] In the present study, transfection with siRNA of RPL19 suppressed the expression of RPL19 protein, but not erbB-2 in H1224L with immunohistochemical staining (data not shown). In addition, we analyzed the expression of both RPL19 and erbB-2 among 30 NSCLC tissues with immunohistochemical staining. The expression level of RPL19 protein did not correlate with the expression level of erbB-2 ($P = 0.23$, $\gamma = 0.22$), thus indicating that the RPL19 expression may have no direct association with erbB-2 expression in lung cancer.

HER2 and p53 are tumor antigens overexpressed in several tumors. HER2 was reported to be overexpressed in breast cancer, ovarian cancer, and NSCLC. A correlation between the expression of HER2/neu and sensitivity to HLA-A2-restricted CTL was reported in NSCLC and ovarian cancer.[34] The p53 mutation leads to change in p53 protein turnover and subsequent p53 protein accumulation in tumor cells.

Epitope peptides derived from wild-type p53 and mutated p53 were recognized by CTL in patients with head and neck carcinoma,[35–37] breast carcinoma, melanoma,[38] and lung cancer.[15,39] Melanocyte lineage-specific differentiation antigen glycoprotein (gp) 100 and MART-1 are expressed in melanocytes. However, these antigens are overexpressed only in melanoma cells.[40–42] Our study presents the first evidence that RPL19 was overexpressed in 40% of lung cancer tissues. Although the expression levels of RPL19 in the heart and skeletal muscle were relatively high, they were much lower than those observed in lung cancer tissue. Moreover, the expression level of RPL19 mRNA in lung cancer cell line was positively correlated with the response of the CTL clone. These results suggest that high expression of RPL19 may elicit tumor-specific immune responses in lung cancer patients. Spontaneous humoral immunity against RPL19 was evaluated in 39 lung cancer patients and 20 healthy individuals. The antigen-antibody reaction was evaluated by ELISA using commercially available RPL19 protein (Abnova). The optical density average of lung cancer patients and healthy individuals was 0.337 ± 0.123 and 0.398 ± 0.128 ($P = 0.66$). No positive lung cancer patient was found to show OD above the cut-off value (the average plus two times of the SD of the value in healthy individuals), indicating that Ab against RPL19 were not detected in lung cancer patients.

The sequential activation of cyclin-dependent kinases (CDK) regulates the eukaryotic cell cycle. Cyclin D/CDK4/6 activation occurs in the mid–late G1 phase of the cell cycle. This activation makes the hyperphosphorylation of the retinoblastoma gene product (pRb) and also allows for the release of S-phase-promoting transcription factors and it is also indicative of the ability of the cells to proliferate. This point in the cell cycle is known as the restriction point. CDK inhibitors (CKIs) negatively regulate CDKs, and CDK-bound G1 cyclins can activate CDKs. The CKIs can be subdivided into two families: the INK4 family, comprising p16INK4A, p15INK4B, p18INK4C, and p19INK4D; and the Cip/Kip family comprising p21Cip1, p27kip1, and p57Kip2.[43–45] In present study, transfection with siRNA of RPL19 suppressed the synthesis of cyclin D1 and cyclin D3, while at the same time it augmented a negative cell cycle regulator (p27 kip1) and a CDK inhibitor (p16 INK4A). In addition, transfection with siRNA of RPL19 led to an increase in the fraction of the G1-phase and a decrease in the fraction of the S-phase.

Since the identification of the *MAGE* gene from melanoma as a tumor antigen, immunotherapies using peptide vaccination have been applied mainly for patients with melanoma. Recently, adjuvant vaccination of MAGE-3 protein has been applied for completely resected NSCLC patients as a phase II randomized trial and resulted in reduction of the relative risk of cancer recurrence.[46] In the present study, the CTL clone L7/8 recognized an epitope peptide of RPL19 in an HLA-A*31012-restricted manner. The HLA-A31 allele is expressed in 5–10%[47] of the world population and in 19% of Japanese people.[48] Further investigation to identify a new epitope peptide of RPL19 presented in the context of HLA class I other than HLA-A*31012 should be undertaken for wide application of immunotherapy using RPL19.

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