Identification of a New Cancer/Germline Gene, KK-LC-1, Encoding an Antigen Recognized by Autologous CTL Induced on Human Lung Adenocarcinoma

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Abstract

The purpose of our present study is to identify a tumor-specific antigen capable of inducing a specific cellular immune response in lung cancer patients. We established a lung adenocarcinoma cell line, designated as F1121L, and induced tumor-specific CTL clone H1 from regional lymph node lymphocytes of patient F1121. CTL clone H1 lysed autologous tumor cells in an HLA-B*1507-restricted manner, but not autologous EBV-B, phytohemagglutinin-blast cells, and K562. The CTL clone also recognized allogeneic lung cancer cell lines in the HLA-restricted manner. Using the CTL clone, we identified an antigen-coding gene by cDNA expression cloning technique. The gene consisted of 556 bp, including an open reading frame consisted of 113 amino acids, designated as Kita-kyushu lung cancer antigen 1 (KK-LC-1). A 9-mer peptide (KK-LC-176-84; RQKRILVNL) was identified as an epitope peptide. The genomic DNA of this antigen was located in chromosome Xq22. A reverse transcription-PCR analysis revealed that the mRNA of this gene was only expressed in the testis among normal tissues. It was expressed in 9 of 18 (50%) allogeneic lung cancer tissues, but not in normal tissues except for the testis.

Introduction

Lung cancer is the leading cause of cancer deaths in the developed countries, accounting for ~30% of all cancer deaths. The long-term prognosis of patients with lung cancer is poor and the overall survival rate has been reported to be as low as 11% to 14% (1). Although surgical treatment can sometimes be a curative option for lung cancer, the poor prognosis of lung cancer patients depends, in part, on the relative low sensitivity of lung cancers to both radiotherapy and chemotherapy (2). Moreover, two thirds of lung cancer patients tend to present at an advanced stage at the time of diagnosis. The results of combined modality therapy, such as a surgical resection, chemotherapy, and radiotherapy, are still not satisfactory for patients in advanced stage. As a result, the development and application of new therapeutic strategies are essential for improving the prognosis of this disease.

Since the discovery of the first human tumor-specific antigen in 1991 (3), a large number of targets for tumor-specific CTLs have been identified (4, 5). Some of these antigens have been applied for cancer vaccination, and clinical effectiveness was induced in a certain population of melanoma patients (6, 7). However, the candidates for vaccine trials are limited because they must express a particular HLA molecule and their tumors must also express a corresponding tumor-associated antigen. Therefore, it is very important to identify a number of antigens capable of binding to a variety of HLA molecules. The development of effective vaccine-based immunotherapy for lung cancer patients depends on (a) the identification of appropriate antigens expressed selectively in lung cancers; (b) the presence of T-cell precursors that recognize tumor cells expressing relevant antigens; (c) the ability to deliver activated effector cells to tumor site; and (d) the overcoming of immunosuppressive circumstance around the tumor site.

We previously established 15 lung cancer cell lines from surgical specimens (8). We reported that tumor-specific CTL clones could be induced from regional lymph node lymphocytes in lung cancer patients (9–13). In this study, we identified a new cancer/germline antigen recognized by CTL using cDNA expression cloning method. The new antigen, designated as Kita-kyushu lung cancer antigen 1 (KK-LC-1), should be a promising target for a specific immunotherapy because they are expressed in a substantial proportion of allogeneic lung cancer tissues, but not in normal tissues except for the testis.

Materials and Methods

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

Culture medium. Culture medium consisted of RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated FCS (Equitech-bio, Ingram, TX), 10 mmol/L HEPES, 100 units/ml of penicillin G, and 100 mg/mL of streptomycin sulfate (11).

Establishment of the tumor cell line. F1121L (HLA-A*0201/2402, B*1507/4006, Cw*0303/0801), a lung adenocarcinoma cell line, was established from the surgical specimen obtained from a 69-year-old patient (F1121). The method used to establish the lung cancer cell line has previously been described (13). Briefly, fresh tumor tissue was excised from surgical specimen and then was minced into small pieces with scissors. The minced tissue specimens were shaken with a mixture of 0.1 mg/mL of...
DNase type I, 1 mg/mL of collagenase type IV, and 0.5 mg/mL of hyaluronidase type V (Sigma, St. Louis, MO) in culture medium at 150 rpm at 37 °C for 1 hour. Thereafter, the cells were placed in a flask and have been maintained as a monolayer culture by serial passages in RPMI 1640 with 10% FCS.

The histologic types of other lung cancer cell lines used are adenocarcinoma (A110L, A129L, A925L, B203L, B901L, C422L, D611L, E522L, G82L, and H124L), squamous cell carcinoma (B1203L and H1215L), large cell carcinoma (A904L and C831L), adenosquamous carcinoma (A529L), large cell neuroendocrine carcinoma (J206L), and pleomorphic carcinoma (G603L). F1121 EBV transformed B cells (EBV-B; HLA-A*0201/2402, B*1507/4006, Cw*0303/0801) were produced from this patient’s (F1121) peripheral blood mononuclear cells by infection with supernatant from EBV producer line Bo9.58, F1121 phytomemagglutinin-blast cells were induced by stimulating the peripheral blood mononuclear cells with 1 μg/mL of phytomemagglutinin-P (Difco Laboratories, Detroit, MI) and 200 units/mL of recombinant interleukin (IL)-2 (Takeda Chemical Industries, Osaka, Japan) for 48 hours. Rosi EBV-B (HLA-A*2402/3201, B*3503/4403, Cw*0401/0401), tumor necrosis factor (TNF)-sensitive WEHI-164L cells, and 293-EBNA cells were kindly donated by Dr. P.G. Coule (Cellular Genetics Unit, Université Catholique de Louvain, Brussels, Belgium). WEHI-164L cells were maintained in culture medium with 5% FCS; 293-EBNA cells were maintained in DMEM (Life Technologies, Gaithersburg, MD) with 5% FCS; and the other cell lines were maintained in culture medium with 10% FCS.

Preparation of regional lymph node lymphocytes and induction of CTL. The regional lymph node lymphocytes from F1121 patient were obtained at the time of surgery. Each lymph node was divided into two parts for the histologic diagnosis and for this study. The latter part of each lymph node was mixed and prepared for regional lymph node lymphocytes as previously described (11). Regional lymph node lymphocytes were frozen in a deep freezer at -130 °C until use.

Regional lymph node lymphocyte obtained as described above were rapidly thawed, washed twice, and stimulated weekly with irradiated (100 Gy) F1121L at tumor cell-to-lymphocyte ratio of 1:10 in culture medium with 20 units/mL of IL-2 (kindly donated by Takeda Chemical) for 4 weeks in 24-well plates (Iwaki Glass, Tokyo, Japan) at 37 °C in a 5% CO2 atmosphere as previously reported (11). The CTL activity was assessed at 7 days after the fourth stimulation.

To generate T-cell clones, limiting dilution was done from the bulk CTL line 7 days after the last stimulation as previously described (14). The cells were seeded at 0.1, 0.3, 1, or 3 per well in 96-well round-bottomed plates and stimulated under the following culture condition: culture medium with irradiated F1121L cells and Rosi EBV-B cells as a feeder in the presence of IL-2 (100 units/mL), IL-2 (5 ng/mL), and IL-2 (5 ng/mL). Thereafter, the irradiated stimulator cells and feeder cells were added to each well for restimulation of the lymphocytes once a week with culture medium containing IL-2, IL-4, and IL-7.

Cytotoxicity activity of the CTL clone. The cytotoxic activity of CTL was assessed by a standard 51Cr release assay. Briefly, the target cells were labeled for 90 minutes with 100 μCi of 51Cr at 37 °C. The labeled target cells were washed twice and seeded at 1,000 per well in 96-well round-bottomed plates. The CTLs were cocultured at an indicated effector/target ratio (ratio, 1:1, 3:1, 10:1, and 30:1) for 4 hours at 37 °C. The supernatant (100 μL) was collected and then counted for 1 minute in a gamma counter. The percent specific lysis was calculated with the following formula: % lysis = (observed cpm of test sample – spontaneous cpm of target cells with medium) / (maximum cpm of target cells with 0.5% Triton X-100 – spontaneous cpm of target cells with medium) × 100.

Measurement of cytokine production by the CTL clone. To assess the activity of CTL clone in response to target tumor cells, both TNF and IFNγ were measured. The TNF production of the CTL clone was measured by the WEHI assay using TNF-sensitive WEHI cells (15). Briefly, CTLs (6 × 104/mL) were added to 96-well flat-bottomed plates and incubated with tumor cell lines (6 × 103/mL). After 6 hours, the supernatant was collected and WEHI cells (6 × 105/mL) were added for evaluation of the cytotoxic effect (16). IFNγ production in the supernatant was measured using a Human IFNγ ELISA Test Kit (BioSource, Camarillo, CA) according to the instruction manual.

Monoclonal antibody used for blocking assay. Hybridomas (HB-145, HB-95, and HB-82) were purchased from American Type Culture Collection (Rockville, MD). C7709.A26 (anti-HLA-A2) and B1.232 (anti-HLA-B, C) were kindly donated by Dr. P.G. Coule. The culture supernatants of ATCC HB-145 (I4212; anti-HLA-DR, DP, DQ), HB-95 (W6/32; anti-HLA-A, B, C), HB-82 (BB7.2; anti-HLA-A2), C7709.A26, and B1.232 were used for the analysis of the HLA restriction of T-cell clones.

Flow cytometry for expression of HLA class I antigens. For evaluation of expression of HLA class I on the cell surface, the tumor cells (2 × 106) were incubated with either fluorescein-conjugated HLA-A, B, and C, and monoclonal antibody (mAb) or control immunoglobulin G for 30 minutes at 4 °C. Thereafter, they were washed and assessed using a flow cytometer (EPICS XL, Coulter International, Fullerton, CA) as previously described (17).

Assessment of HLA class I restriction of the CTL. The cDNA derived from F1121-EBV-B cell was served as a template for PCR amplification with Pfu DNA polymerase (Stratagene, Alameda, CA) using each HLA-B, C specific primers (HLA-B6, CCGGATCCGCCGAGATGCGGGTCA and ACTG-CGGCCATTTCTCTAGTCCTCAACAAAGGCAGCTGTC; HLA-Cw, CGGGATCCGCCGAGATGCGGGTCA and CGGAAGTCTCAGGGTCACTGAAGGGTGA). The PCR products were cloned and ligated into the pCDS3A by a TA Expression Kit (Invitrogen, Carlsbad, CA).

To determine the HLA restriction of the CTL clone, 100 ng of each HLA molecule possessed by F1121L were separately transfected into B1203L using a Lipofectamine reagent (Invitrogen, Carlsbad, CA). According to the instruction manual, CTL and these HLA transfectants were cocultured overnight at 37 °C in 5% CO2 atmosphere. Supernatant was applied for measurement of TNF as described above.

Construction and screening of the cDNA library. The CTL clone also recognized B203L; therefore, cDNA library was constituted from mRNA of B203L, an allogeneic lung cancer cell line. Approximately 5 μg of polyadenylated RNA extracted from B203L with the FastTrack kit (Invitrogen, Carlsbad, CA) were converted to cDNA with Superscript Choice System (Life Technologies, Grand Island, NY) using an oligo(dt) primer [5′-ATAAGAATGCGGCCGCTAAACTA(T)18VZ-3′; V = G, A, or C; Z = G, A, T or C] containing a NotI site at its 5′ end. The cDNA was ligated to HindIII-EcoRI adaptors (Stratagene, Heidelberg, Germany), phosphorylated, digested with NotI, and inserted at the HindIII and NotI sites of expression vector pCEP4 (Invitrogen, Carlsbad, CA). This plasmid contains the EBV origin of replication, resulting in episomal multiplication of transfected with EBV EBNA-1 gene. Escherichia coli TOP10 (Invitrogen, Carlsbad, CA) was transformed by electroporation with the recombinant plasmids and selected with ampicillin (50 μg/mL). The library was divided into 1,544 bacteria pools, each containing 100 clones. Each pool was amplified for 4 hours and plasmid DNA was extracted using QIA prep 8 plasmid Kit (Qiagen, Hilden, Germany). 293 EBNA cells, plated in flat-bottomed 96-well plates (3.0 × 104 per well) 24 hours before transfection, were duplicate microcultures cotransfected with 1.1 μL of Lipofectamine reagent (Invitrogen, San Diego, CA), plasmid DNA (100 ng) of each pool in CDNA library, and 50 ng of HLA cDNA. After 24 hours, CTLs (3.0 × 105 per well) were cocultured with these transfectants for 24 hours at 37 °C in 5% CO2 atmosphere. The supernatant was collected to measure the TNF as described above.

Identification of antigenic peptide. To determine the antigenic peptide, the affinity of the peptide to the HLA was predicted by using peptide anchor motif for HLA-B*4101 and a computer algorithm from the Bioinformatics and Molecular Analysis Section of NIH (BIMAS; ref. 18), which ranks the potential MHC-binding peptides according to the competitive one-half-time dissociation of peptide/MHC complexes. The critical motifs for binding with HLA-B15 are glutamine at position 2, isoleucine (hydrophobic residue) at position 5, and phenylalanine or tyrosine at position 9. We selected two nonapeptides (L6RLDLNLF; KK-LC-1G-16, and RKQRKLVLN; KK-LC-1G-16) for candidate on the basis of anchor motif for HLA-B15. According to the prediction score by BIMAS, binding prediction scores for HLA-B15 of KK-LC-1G-16, and KK-LC-1G-16 were 6.6
and 28,8, respectively. On the basis of these data, one nonapeptide (IKQRILVLN: KK-LC-176-84) was synthesized. The autologous EBV-B was loaded with the nonapeptide and cytolytic activity of CTL clone against the peptide loaded EBV-B was determined by H-1 release assay.

RNA isolation and reverse transcription-PCR analysis of antigen-coding gene. The total RNA from the cell lines, specimens of lung cancer, and normal tissues was isolated by using RNeasy Mini Kit (Qiagen), and it was converted to cDNA using an oligo(dT) primer. Screening of the identified antigen and β-actin was done by PCR. PCR amplification was done in 20 µL of PCR mixture containing 1 µL cDNA template, deoxynucleotide triphosphate (Takara, Shiga, Japan), and 500 nmol/L of primers described below. The PCR mixture was initially incubated at 94°C for 5 minutes, followed by a cycle of denaturation at 94°C for 30 seconds, annealing for 30 seconds, and extension at 72°C for 1 minute. The cycles of PCR, annealing temperature, and size of PCR product are described below. Reverse transcription-PCR (RT-PCR) for β-actin was done with GGCACTGTGATGGACTCCG and GCTGGAAGGTGGACAGCGA as primers at an annealing temperature of 68°C and 24 cycles, yielding a 615-bp product. For RT-PCR of KK-LC-1, ATGAACTTCTATTTACTCCCG and TTAGGTTGATTTCCGGTGAGG were used as specific primers at an annealing temperature of 67°C and 35 cycles.

Quantitative RT-PCR was carried out in ABI PRISM 7000 (Applied Biosystems, Foster, CA). The relative amount of KK-LC-1 mRNA was measured by means of detection of intercalated SYBR green. PCR was done with 25 µL of SYBR Green PCR Master Mix (Applied Biosystems), either 1 µL of cDNA or 1 µL of water and each primer set described below in a total volume of 50 µL. The PCR cycles were 95°C for 10 seconds, followed by 45 cycles of amplification of 15 seconds and 67°C for 1 minute. The primer sequences of KK-LC-1 for quantitative RT-PCR were ATGAACTTCTATTTACTCCCG and CTACAAATATGAGTGTGGGAAATTATTTAA. The quantitative PCR primer sequences of β-actin were the same as those used in the screening of β-actin. The concentration of each primer set was 200 and 100 nmol/L for the identified gene and β-actin. The threshold cycle number (C_T) was defined as a fractional cycle number at which the amount of amplified target product reaches a fixed threshold. ΔC_T was obtained by comparing C_T of KK-LC-1 with C_T of β-actin in same amount of templates. Relative quantitation was achieved by comparisons with ΔC_T of F1121L. The relative expression was calculated by the following formula: relative expression = 2^(-ΔΔC_T), where ΔΔC_T = (ΔC_T sample) - (ΔC_T calibrator).

Results

Generation of the CTL clone recognizing the autologous adenocarcinoma cell line. Regional lymph node lymphocytes (4 × 10^7) were stimulated with irradiated F1121L (tumor-to-lymphocyte ratio, 1:10) in the presence of IL-2 (20 units/mL). After four weekly tumor stimulations, the bulk CTL line was induced from the regional lymph node lymphocyte. The bulk CTL was cloned by a limiting dilution, and then CTL clone H1 was finally established. CTL clone H1 responded to the autologous tumor cell line F1121L, but not to F1121 EBV-B, F1121 phytohemagglutinin-blast, or K562 (Fig. 1A).

Determination of HLA class I restriction. IFN-γ production of CTL clone H1 in response to F1121L was inhibited by the addition of anti-MHC class I mAb or anti HLA-B/C mAb (Fig. 1B). To determine HLA class I restriction of CTL clone H1, HLA-B*1507, B*4006, Cw*0303, and Cw*0801 were cloned from F1121 L, and then each of them was transfected into the allogeneic tumor cell line B1203L. CTL clone H1 produced TNF in response to the allogeneic B1203L after HLA-B*1507 transfection (Fig. 1C). These results suggested that the target antigen was shared by the allogeneic tumor (B1203L), except for the autologous one, and that CTL clone H1 was restricted by HLA-B*1507.

Identification of the cDNA clone encoding specific antigen. A cDNA library prepared from mRNA of B203L, to which CTL clone H1 could respond as shown in Table 1, was cloned into expression vector pCEP4. The screening of the cDNA library was done by measuring the TNF production of CTL clone H1 in response to 293 EBNA cells cotransfected with HLA-B*1507 and each cDNA fraction. One positive pool of cDNA was obtained. The cDNA pool
was subcloned from 100 to 12 clones, and finally a single cDNA clone (cDNA clone 2B35) was isolated. The isolated cDNA clone was proved to be 556bp with polyadenylate tail by direct sequence. CTL clone H1 showed TNF production in response to 293 EBNA cells cotransfected with cDNA clone 2B35 and HLA-B*1507, but not with any of other cDNA (Fig. 2 A). The identified cDNA has been reported to be a function of an unknown gene expressed in testis according to databank (BLAST accession no. BC062223; ref. 19). This gene was designated as **KK-LC-1**.

**Identification of the antigenic peptide recognized by the CTL clone.** To identify epitope peptide, two minigenes of different lengths (**KK-LC-1**/252 and **KK-LC-1**/81) were amplified by PCR. The TNF production of CTL clone H1 was assessed against 293 EBNA cells cotransfected with each minigene and HLA-B*1507. CTL H1 responded to **KK-LC-1**/252 but not to **KK-LC-1**/81. Therefore, the portion of cDNA encoding the antigen epitope was narrowed down to a 171-bp sequence (**KK-LC-1**/76-84). Based on an HLA-B*1507 binding motif search by a computer analysis (BIMAS, NIH) and peptide anchor motif of HLA-B15, the peptide (RQKRILVNL: **KK-LC-1**/76-84) was synthesized as mentioned in Materials and Methods. CTL clone H1 recognized the EBV-B pulsed with **KK-LC-1**/76-84 in a dose-dependent manner. One half of maximal lysis was obtained at 10 ng/mL (Fig. 2B). The mutated p53 peptide (TRVLMAIY), which has been identified as a tumor antigen restricted by HLA-Cw*0702 (14), was used as a negative control.

**Expression of KK-LC-1 in non–small-cell lung cancer and recognition of the CTL against allogeneic lung cancer cell lines.** We evaluated the mRNA expression of **KK-LC-1** coding gene **Table 1.**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>HLA alleles</th>
<th>HLA-B*1507 transfection</th>
<th>IFN-γ production of the CTL in response to target tumor cells pretreated without or with IFN-γ</th>
<th>Relative expression level of KK-LC-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>A locus B locus Cw locus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B203L †</td>
<td>2402/31012 1501/5401 0102/0304</td>
<td>–</td>
<td>456 ‡</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>791</td>
<td>9,784</td>
<td></td>
</tr>
<tr>
<td>G603L †</td>
<td>2602/31012 1501/4002 0303/0304</td>
<td>–</td>
<td>0</td>
<td>18.86</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>118</td>
<td>1,653</td>
<td></td>
</tr>
<tr>
<td>B1203L</td>
<td>2402/2402 5201/5401 0102/1202</td>
<td>–</td>
<td>0</td>
<td>2.44</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>108</td>
<td>155</td>
<td></td>
</tr>
<tr>
<td>A110L</td>
<td>2402/— 5201/— 1202/—</td>
<td>–</td>
<td>0</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>G821L</td>
<td>2602/— 5101/— 1402/—</td>
<td>–</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>CTL alone</td>
<td></td>
<td></td>
<td>0</td>
<td>—</td>
</tr>
</tbody>
</table>

*Relative expression of KK-LC-1 was calculated on the basis of KK-LC-1 expression in F1121L as 1.0.
†HLA-B*1501-positive cell lines.
‡IFN-γ production was assessed as CTL response against each cell line.

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**Figure 2.** Identification of the antigen-coding gene and antigenic peptide recognized by the CTL clone H1. **A**, 293 cells were transfected with cDNA plasmid of HLA-B*1507 and/or cDNA clone 2B35. The cDNA clone 1 as irrelevant cDNA (one of the B203L cDNA clones used in cDNA expression cloning method) was also transfected with HLA-B*1507. A high level of TNF production of CTL clone H1 was observed against 293 cells cotransfected with cDNA clone 2B35 and HLA-B*1507. B, 51Cr-labeled F1121 EBV-B cells were incubated for 1 hour at room temperature with indicated concentrations of the peptide. CTL clone H1 recognized the EBV-B pulsed with the peptide **KK-LC-1**/76-84 in a dose-dependent manner. Mutated p53 peptide (TRVLMAIY) was used as an irrelevant peptide.
in allogeneic non–small-cell lung cancer cell lines and tumor tissues by RT-PCR method. KK-LC-1 was expressed in autologous F1121L but not in F1121 EBV-B (Fig. 3A). The expression of the mRNA was detected in 9 of 18 allogeneic non–small-cell lung cancer cell lines (Fig. 3B). Among KK-LC-1-positive lung cancer cell lines, relative expression levels were determined by quantitative RT-PCR. G821L was used for negative control. The expression levels were evaluated by calculating relative ratio on the basis of the antigen expression in the F1121L. The relative expression levels of KK-LC-1 in two cell lines (B1203L and G603L) were higher than in autologous KK-LC-1 in two cell lines (B1203L and G603L) were higher than in allogeneic non–small-cell lung cancer tissues. In the tumor tissue specimens, 40 of 100 (40%) were positive for F1121L gene expression. According to histologic types, 23 of 60 (38%) adenocarcinomas and 14 of 31 (45%) squamous cell carcinomas were positive for KK-LC-1 expression. However, it could not be detected in all normal lung tissue specimens. In a panel of normal tissues, the gene was not expressed in any normal organs except for the testis (Fig. 3C).

The CTL clone H1 also recognized allogeneic tumor cell lines expressing HLA-B*1501 or 1507. The CTL clone H1 produced IFN-γ in response to B203L, which was HLA-B*1501 positive (Table 1). This result means that the antigenic peptide recognized by CTL clone H1 could also be presented on HLA-B*1501. This may be due to the difference of only one amino acid between HLA-B*1501 and 1507 (20). On the other hand, the CTL clone H1 did not produce IFN-γ at all in response to another HLA-B*1501-positive cell line, G603L, without IFN-γ treatment, although it expressed the highest level of KK-LC-1. However, the transfection of HLA-B*1507 rendered G603L to be sensitive to CTL clone H1. The relative expression levels of KK-LC-1 among lung cancer cell lines were not likely to affect magnitude of response of the CTL (Table 1).

These findings suggested that KK-LC-1-76-84 is not sufficiently expressed on the cell surface of G603L in the context of HLA-B*1501. IFN-γ treatment (100 units/mL for 48 hours) was done in four cell lines that express the KK-LC-1 gene and in G821L as a negative control. After IFN-γ treatment, the HLA class I expression on the cell surface was enhanced in all lung cancer cell lines tested. The magnitude of augmentation of HLA expression was different among lung cancer cell lines as shown in Table 2. The CTL response was also augmented by IFN-γ treatment of target cells. The augmentation of HLA expression and IFN-γ production by CTL clone H1 was approximately correlative against B203L (11.8- and 13.2-fold, respectively). However, in G603L, the augmentation rate of IFN-γ production (14.0-fold) was more than that of HLA expression (at most 2.1-fold for HLA-B*1501 but none for transfected HLA-B*1507; Table 2).

### Discussion

We previously reported that regional lymph node lymphocyte was a potential source of precursor CTL because regional lymph node lymphocyte of lung cancer was more activated than peripheral blood lymphocyte and it also included higher frequency of autologous tumor reactive lymphocytes (17). CTL clone H1 was induced by the weekly stimulation of autologous regional lymph node lymphocyte with autologous F1121L. The establishment of the CTL clone showed that precursor CTL resided in regional lymph node lymphocyte and it was activated by a sufficient quantity of peptide-HLA complex as previously reported (11).

### Table 2. Elevation of HLA expression of tumor cell lines by IFN-γ treatment

<table>
<thead>
<tr>
<th>Cell line</th>
<th>HLA intensity on the cell surface (MFI)</th>
<th>Fold of augmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFN-γ treatment</td>
<td>No treatment</td>
</tr>
<tr>
<td>B203L</td>
<td>197.7</td>
<td>16.7</td>
</tr>
<tr>
<td>G603L</td>
<td>14.2</td>
<td>6.8</td>
</tr>
<tr>
<td>B1203L</td>
<td>22.2</td>
<td>19.6</td>
</tr>
<tr>
<td>A110L</td>
<td>56.1</td>
<td>16.6</td>
</tr>
<tr>
<td>G821L</td>
<td>109.6</td>
<td>60.4</td>
</tr>
</tbody>
</table>

NOTE: These cell lines were treated with 100 units/mL IFN-γ for 48 hours. Abbreviation: MFI, mean fluorescence intensity.
An analysis of immune responses to autologous tumor cells in clinical cancer patients has allowed the identification of various tumor-associated antigens (3, 21–26). However, there have been several reports about the tumor antigens identified by using CTL from lung cancer patients (27–31). Three of such reports were about tumor-specific mutated antigens, which was unique to the individual patient. On the other hand, the shared tumor-associated antigens, as recognized by CTls, have also been mainly identified in melanoma. Among these shared tumor-associated antigens, cancer/germline antigens may be a suitable target for tumor immunotherapy. They are expressed in tumor cells but not in most somatic normal tissues, except for the testis which does not express MHC genes (32). The tumor-restricted expression of antigen thus seems to be important for the immunotherapy of cancer to prevent autoimmunodisease. During the last decade, 44 cancer/germline genes have been identified (33). According to the mRNA expression of each cancer/germline gene in a panel of normal tissues, Scanlan et al. categorized cancer/germline genes to four groups: (I) testis-restricted transcripts (BAGE, MAGE-B2, SSX-2, etc.); (II) tissue-restricted cancer/germline genes expressed in two or fewer of nongametogenic tissues (MAGE-A1, MAGE-C1, NY-ESO-1, etc.); (III) differentially expressed cancer/germline genes expressed in three to six of the nongametogenic tissues (XAGE-1, etc.); and (IV) ubiquitously expressed cancer/germline genes (OY-TES-1, etc.). The most favorable candidate for specific immunotherapy may be the antigen shared with various cancers and the expression of normal organs should be restricted in testis such as group I. An evaluation of mRNA expression showed that the antigen (KK-LC-1) coding gene was not expressed at all in normal lung tissues, but only selectively expressed in tumor cell lines, lung cancer tissues, and the testis. Most of cancer/germline genes such as MAGE gene family were located on the X chromosome (34). A cluster of 12 MAGE-A genes is located in the q28 region of the X chromosome. MAGE-B and MAGE-C genes were reported to be located in Xp21 and Xq26 region, respectively (34). NY-ESO-1 maps to chromosome Xq28 near MAGE-A subfamily (25). Five BAGE genes have been reported to map to the juxtacentromeric regions of human chromosomes 13 and 21; nine BAGE gene fragments map to the juxtacentromeric regions of chromosomes 9, 13, 18, and 21 (21); and OY-TES-1 maps to 12p12-13 (35). The KK-LC-1 coding gene is located on chromosome Xq22 and consists of 113 amino acids. However, the function of this gene has not yet been clarified.

Scanlan et al. (33) reported the expression of the cancer/germline genes in non-small-cell lung cancer tissues to be 49% for MAGE-A1, 47% for MAGE-A3, 4% for BAGE, 16% for SSX-2, and 17% for NY-ESO-1. These cancer/germline antigens may be able to elicit a cellular immune response against lung cancer cells (33). Regarding KK-LC-1 coding gene, the frequency of a positive expression in lung cancer tissues was 40% (adenocarcinoma, 38%; squamous cell carcinoma, 45%) and was 50% in our lung cancer cell lines. This high expression rate in lung cancer indicates that the gene coding KK-LC-1 might have the similar advantages in cellular immunotherapy as the other cancer/germline antigens in lung cancer patients.

B203L, which did not express HLA-B*1507 but did express HLA-B*1501, was recognized by CTL clone H1. The finding implied that CTL clone H1 recognized KK-LC-1. In the context of HLA-B*1507 and/or HLA-B*1501 because only one amino acid is different between HLA-B*1501 and HLA-B*1507. The 121st amino acid in HLA-B*1507 changes from arginine to serine as compared with HLA-B*1501 (20). Percentage of HLA-B*1501- or 1507-positive population is ~15% among Japanese (36) and 12% among Caucasians (37).

Some of these cancer/germline antigens have been applied for peptide-based immunotherapy (7, 38, 39). These studies showed not only the safety of peptide vaccination but also its clinical efficacy. Some investigators reported ~10% to 20% efficacy of vaccine therapy, which included complete, partial, and minor responses (40, 41). Several clinical trials have been also conducted using immature or mature dendritic cells pulsed with peptides derived from such cancer/germline antigens as MAGE (42). The CTL response by dendritic cell vaccination seemed to be better than those observed by peptide or ALVAC vaccination (43). As a significant feature, polyclonal CTL responses were often observed after dendritic cell vaccination (43).

CTL clone H1 responded to allogeneic lung cancer cell lines expressing the gene encoding KK-LC-1 after transfection of HLA-B*1507 and IFN-γ treatment, as shown in Table 1. Among HLA-B*1501-positive cancer cell lines, CTL clone H1 produced the highest amount of IFN-γ in response to B203L but not to G603L. The mRNA expression of KK-LC-1 coding gene in G603L exhibited the highest amount among all cell lines tested (Table 1). However, in terms of the HLAA expression on cell surface, G603L was the lowest among the lung cancer cell lines tested (Table 2). The down-regulation of MHC class I expression in G603L may be one of the immune escape mechanisms. The IFN-γ treatment of G603L augmented HLAA expression and rendered them minimally sensitive to the CTL (Table 1). After IFN-γ treatment of lung cancer cell lines, the HLAA expression increased 11.8-fold in B203L and 2.1-fold in G603L (Table 2). CTL clone H1 increased the IFN-γ production in proportion to the augmentation of the HLAA expression against B203L. Although HLAA expression increased 2.1-fold at most for HLA-B*1501 but none for transfected HLA-B*1507, CTL clone H1 showed a 14.0-fold augmentation of IFN-γ production to IFN-γ-treated-G603L (Table 1). This enhancement of CTL recognition might be mainly due to an increased antigen expression by means of the change of antigen processing machinery by IFN-γ treatment from standard proteasome to immunoproteasome (44) and also partially due to the up-regulation of HLAA expression. In terms of KK-LC-1 gene expression, G603L was 100-fold more than B203L. However, our results indicated that B203L was a far more suitable stimulator for CTL clone H1 as shown in Table 1. This may be ascribed to difference of transcriptional and posttranslational modification or alterations in the processing of the protein (45), in addition to difference of expression levels of HLAA.

We identified a new antigen recognized by the tumor-specific CTL clone from a lung cancer patient. The CTL clone recognized several tumor cell lines and the antigen was expressed in 40% of lung cancers but not in any normal tissues except for the testis. The new tumor antigen encoded by cancer/germline gene KK-LC-1 may thus be a promising target for tumor-specific immunotherapy.

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References

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