**Identification of the HLA-Cw*0702-Restricted Tumor-Associated Antigen Recognized by a CTL Clone from a Lung Cancer Patient**

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**Abstract**

**Purpose:** A large number of tumor-associated antigens have been used in vaccination trials for mainly melanomas. Our purpose of this study is to identify a novel tumor antigen useful for immunotherapy of lung cancer patients.

**Experimental Design:** Analysis of an autologous tumor-specific CTL clone F2a that was established from regional lymph node lymphocytes of a patient with lung cancer (A904) by a mixed lymphocyte-tumor cell culture.

**Results:** F2a recognized and killed autologous tumor cells (A904L), whereas it did not respond to autologous EBV-transformed B cells, phytohemagglutinin-blastoid T cells, and K562 cells. cDNA clone 31.2 was isolated by using cDNA expression cloning method as a gene encoding antigen. This gene was identical to the reported gene whose function was unknown. The antigen encoded by the cDNA was recognized by the CTL in a HLA-Cw*0702-restricted manner. Furthermore, a 9-mer peptide at positions 659 to 685 in cDNA clone 31.2 was identified as a novel epitope peptide. The CTL recognized some allogeneic cancer cell lines with HLA-Cw*0702 as well as some HLA-Cw*0702-negative cell lines when transfected with HLA-Cw*0702, thus indicating that the identified antigen was a cross-reactive antigen.

**Conclusions:** Although exact mechanism to process the encoded protein and present the antigen in the context of HLA class I remains to be elucidated, the CTL recognized some of tumor cells in the context of HLA-Cw*0702 but did not recognize a variety of normal cells and also autologous EBV-transformed B cells. These results indicated that the antigen identified in this study may therefore be a possible target of tumor-specific immunotherapy for lung cancer patients.

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**Lung cancer** is one of the most common cancers, and its incidence and mortality continue to increase worldwide. Although the optimal management of lung cancer is a surgical resection in the early stages, the overall 5-year survival is ~50% after a surgical resection (1). Many clinical trials with chemotherapy and radiotherapy have been applied to establish standard treatments for patients with advanced non–small cell lung cancer. Nonetheless, these treatments are still not satisfactory due to the high rates of both distant and local recurrences (2). Therefore, the prognosis of advanced lung cancer is extremely poor.

Tumor-specific CTLs against tumor-associated antigens (TAA) have been generated from patients with such solid tumors as malignant melanoma (3–7). Some TAAs encoded by melanoma-associated antigen genes (MAGE) have been shown to be expressed in not only melanoma but also in various tumors but not in normal tissues, except for testis. As a result, MAGE peptides have been applied for clinical trials as cancer vaccines in patients with malignant melanoma, and such clinical responses as tumor shrinkage and regression have been observed in a significant proportion of patients (8–10). The immunologic monitoring of patients revealed that CTL precursor frequency increased after vaccinations in patients receiving such treatment (11). However, an elevated CTL frequency did not necessarily correlate with the clinical response (12).

Although identification of new tumor antigens may provide new insight into tumor-specific immunotherapy, there is still little information regarding tumor antigens of lung cancers. This may be ascribed to difficulties in both establishing lung cancer cell lines and inducing CTLs against human lung cancer cells, as we have reported the establishment of only 15 lung cancer cell lines and inducing CTLs against human lung cancer cell lines from 570 lung cancer specimens (13). To analyze the specific immune responses against autologous tumor cells, we reported previously the induction of tumor-specific CTL from patients with adenocarcinoma (14) and adenosquamous cell carcinoma of the lung (15) using CD80-transfected tumor cells as stimulators (16).

In the present study, we identified an autologous TAA recognized by a CTL clone from a patient with large cell carcinoma of the lung in the context of HLA-Cw*0702.
Patients and Methods

Culture medium. Culture medium consisted of RPMI 1640 (Life Technologies, Grand Island, NY) containing 5% or 10% heat-inactivated FCS (EQUITech-Bio, Inc. Ingram, TX) with 10 mmol/L HEPES, 100 units/mL penicillin G, and 100 mg/mL streptomycin sulfate.

Patient A904. Patient A904 was a 51-year-old male with primary lung cancer. The histologic type was large cell carcinoma. The first operation for primary lung cancer was done in September 1996. A metastatic tumor in the right adrenal gland was detected and a right adrenalectomy was done in October 1997 (13 months after the first operation). Two courses of antitumor systemic chemotherapy consisted of cisplatin, mitomycin C, and vindesine sulfate.

Culture of CTL clone. RLNLs (107 cells) were rapidly thawed and 1.0 × 106 cells/well of a 24-well plate (Iwaki Glass, Tokyo, Japan) were cultured with CD80 (B7-1) cDNA using LipofectAMINE reagent (Life Technologies, Gaithersburg, MD) and maintained in culture medium with 5% FCS. COS7 cells grew well in DMEM (Life Technologies, Inc., Gaithersburg, MD) with 5% FCS.

HLA typing of A904L and cloning of the HLA DNAs. HLA genotypes of the tumor cell lines were determined by PCR (Shionogi Biomedical Laboratories, Osaka, Japan). The genotypes of A904 EBV-B were HLA-A*2402, 2603, HLA-B*0702, 3901, and HLA-Cw*0702, 0702. However, A904L lost a haplotype of the HLA alleles. Total RNA extracted from A904L using RNeasy Mini kit (Qiagen, Hilden, Germany) was converted to cDNA using a computer analysis (20, 21). The synthesized peptide 1 and peptide 2 from protein encoded by the HLA class I–specific forward and reverse primers and was cloned into pcDNA3 (Invitrogen) were transformed by electroporation with the recombinant plasmids and selected with ampicillin (50 μg/mL). The library was divided into 734 pools each containing 100 cDNA clones. Each pool was amplified for 4 hours and plasmid DNA was extracted using QiAprep 8 Plasmid kit (Qiagen).

Cytokine production of CTL clone. A total of 3 × 104 CTLs were added to microwells each containing 1 × 106 stimulator cells or 3 × 104 recipient cells transfected with cDNA of A904L and HLA-B*0702/Cw*0702 cDNA mixture in 100 μL RPMI 1640 supplemented with 10% FCS and 25 units/mL interleukin-2. After 24-hour incubation, the TNF content was determined using an IFN-γ assay (19). At the same time, supernatants (50 μL) were also collected to measure IFN-γ production using an IFN-γ ELISA test kit (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer’s instruction.
peptide. CTLs were then added at a 10:1 effector:tumor ratio. The cytotoxic activity was calculated based on the above-described formula.

**Real-time reverse transcription-PCR of antigen-coding gene.** Each RNA sample extracted from cell line A904L, A904 phytohemagglutinin (PHA)-blast, and A904 EBV-B was analyzed by real-time reverse transcription-PCR. PCR amplification was done using TaqMan probe (Applied Biosystems, Foster City, CA). This probe was labeled with a reporter dye 6-carboxyfluoroscein at the 5’ end and with the quencher dye 6-carboxytetramethyl rhodamine at the 3’ end. The primers and probes were designed based on the sequences obtained from Genbank. Reverse transcription-PCR was carried out in the ABI PRISM 7000 Sequence Detector (Applied Biosystems). After measuring the threshold cycle (Ct; the number of cycles at which PCR products reach a certain amount) of each RNA concentration, transcriptional levels of each gene were quantified using the standard curve. The transcriptional level of β-actin was used as an internal control to determine the relative transcriptional levels of the target genes in each sample.

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**Results**

**Induction of CTL clone and cytotoxic activity of the CTL clone F2a.** A CTL line was established from RLNLs of a patient with large cell carcinoma after stimulation with the autologous tumor cell line (A904L) as described in Patients and Methods. A limiting dilution of the bulk CTL line was done to obtain CTL clones. Among 12 CTL clones obtained, the CTL clone F2a lysed A904L in a dose-dependent manner but not autologous EBV-B, PHA-blastoid T cells, pokeweed mitogen–stimulated B lymphoblasts, immature and mature dendritic cells, or K562 cells (Fig. 1A and B). Moreover, F2a recognized A904L both with and without IFN-γ treatment at almost the same level (Fig. 1B). The phenotype of F2a was positive for CD3 and CD8. CTL activity of F2a measured by TNF production against A904L was inhibited by the addition of anti-HLA class I mAb or anti-HLA-B/C mAb but not by anti-HLA class II mAb or

![Fig. 1. Cytotoxicity and HLA restriction of CTL clone F2a.](image-url)
anti-HLA-A24 mAb (Fig. 1C). A904L expressed HLA-A*2402, HLA-B*0702, and HLA-Cw*0702 with a HLA haplotype loss. These results suggested that F2a recognized A904L in the context of HLA-B*0702 or HLA-Cw*0702.

Identification of cDNA clone 31.2 coding for the antigen recognized by F2a. The cDNA library of A904L was divided into 734 pools each containing 100 cDNA clones as described in Patients and Methods. Plasmid DNA was extracted from each pool and then cotransfected with HLA-B*0702/Cw*0702 plasmid into duplicate microplates of COS7 cells. The screening of cDNA library was done using TNF production of F2a in response to the cotransfected COS7 cells. One positive pool of cDNA reacted with F2a was obtained. The positive pool was subcloned from 100 to 12 cDNA clones. Finally, a single positive clone (cDNA clone 31.2) was isolated and proven to be 1,593 bp, including poly(A) tail by a direct sequencing. As shown in Fig. 2, F2a produced TNF in response to COS7 cells only when cotransfected with HLA-Cw*0702 and cDNA clone 31.2. This result suggested that CTL clone F2a recognized cDNA clone 31.2 coding for antigenic peptide in HLA-Cw*0702-restricted manner. cDNA clone 31.2 with a length of 1,593 bp showed a 100% homology with a gene already registered in Genbank (accession no. AL137255) with a length of 2,645 bp. The functions of this gene, however, are still unknown. The 2,645-bp mRNA was isolated from a human cDNA clone DKFZp434B1813 in a German Genome Project. The sequence of cDNA clone 31.2 has an open reading frame encoding 231 amino acids.

Identification of the antigenic peptide recognized by F2a. Several PCR fragments of different lengths of the cDNA clone 31.2 (minigenes) were amplified to identify an epitope peptide.
These constructs were cotransfected into COS7 cells with HLA-Cw*0702 plasmid. The TNF production by F2a was assessed against COS7 cells cotransfected with each minigene, and the location of the epitope was presumed between minigene A3 and minigene A4 (positions 676-685). Based on a HLA-Cw*0702-binding motif search using a computer analysis [BioInformatics and Molecular Analysis Section (20, 21)], two different peptides [peptide 1, VYPEYVIQY (positions 659-685) and peptide 2, EKHQVYPEY (positions 647-673)] with high HLA-Cw*0702-binding score were selected as candidates and synthesized (Fig. 3). F2a was incubated with A904 EBV-B cells pulsed with each peptide at various concentrations (1 fg/mL-100 ng/mL) to detect IFN-γ production. The mutated p53 peptide was an irrelevant control peptide that has been known to bind to HLA-Cw*0702 (22). Peptide 1 induced a significant level of IFN-γ production of F2a in a dose-dependent manner, and half of the maximal response was obtained at 30 pg/mL (Fig. 4).

**Expression of the cDNA clone 31.2 among cancer cell lines and normal tissues.** The expression of the gene at the mRNA level was investigated by reverse transcription-PCR method in the panels of cDNAs of normal tissues (Clontech, Palo Alto, CA). The expression of cDNA clone 31.2 was observed in various tissues (fetal liver, trachea, lung, spleen, placenta, heart, brain, testis, kidney, and whole brain) among 20 normal tissues tested. This gene was also expressed in 15 tumor cell lines (79%) composed of lung adenocarcinoma (10 of 11), lung squamous cell carcinoma (2 of 2), lung large cell carcinoma (0 of 3), lung adenosquamous cell carcinoma (1 of 1), breast cancer (1 of 1), and esophageal cancer (1 of 1) among 19 tumor cell lines tested. There was no significant difference in the transcriptional levels of A904L, A904 EBV-B, and A904 PHA-blast for the identified gene as shown in Fig. 5.

**Cross-reaction of F2a against allogeneic tumor cell lines.** TNF production of F2a against HLA-Cw*0702-positive allogeneic tumor cell lines were assessed as shown in Fig. 6A. A high level of cytokine production was observed against two of three cell lines with expression of the cDNA clone 31.2. However, it was not observed against B901L lung cancer cells in spite of the expression of this gene. The PC13 cell line was used as a negative control. When HLA-Cw*0702 was transfected into seven cancer cell lines with the expression of the cDNA clone 31.2, F2a responded to four of them, except for B1203L.
F1121L, and H122Eso cell lines, as shown in Fig. 6B. When the antigen peptide was loaded into these three cell lines, F2a could respond to these cell lines. It was suggested that the HLA-Cw*0702 transfection was successful.

**Discussion**

Because MAGE was identified as an antigen-coding gene recognized by CTLs (3–5), numerous TAAs recognized by CTLs have been identified (23–28). These antigens are classified into four categories as follows: (a) cancer-testis antigens; proteins expressed in testis, placenta, and various types of cancers (MAGE-1, MAGE-3, BAGE, GAGE, and NY-ESO-1); (b) melanocyte differentiation antigens; (MART-1, gp100, tyrosinase, and TRP-1); (c) amplification or overexpression antigens (carcinoembryonic antigen, α-fetoprotein, and HER-2/neu); and (d) tumor-specific mutated antigens (β-catenin and MUM-1). Because cancer-testis antigens are expressed in various cancer tissues (29) but not in normal tissue, except for the testis and placenta, they might thus seem to be suitable targets for immunotherapy (30).

The establishment of permanent lung cancer cell lines is quite difficult; however, we have reported previously the establishment of only 15 lung cancer cell lines from 570 lung cancer specimens (2.6%; ref. 13). In addition, it is also difficult to induce and maintain CTL clones that recognize autologous cancer cells. Consequently, there are a few reports about the tumor antigens identified in autologous lung cancers and CTL systems. For example, α-actinin-4 gene (31) and mutated elongation factor-2 gene (32) are both tumor antigens that have been identified from such systems. Both α-actinin-4 gene and mutated elongation factor-2 gene were categorized as tumor-specific mutated antigens, and such a mutation was unique to individual patient.

In this study, we defined a new gene encoding TAA recognized by CTL derived from a patient with large cell...
protein through post-translational modifications (37). For reviewed about the unusual peptides derived from ubiquitous at sufficient levels to be recognized by CTL (36). Engelhard system might result in presentation of peptide on the cell surface b epitope peptide and (38). Processing of the protein caused higher expression levels of immunoproteasome for antigen processing. The CTL F2a recognized A904L and two of three tumor cell lines possessing HLA-Cw*0702 and cDNA clone 31.2 but not A904 EBV-B and 901T cells as shown in Fig. 6A. Therefore, we suppose the existence and involvement of different antigen processing machinery from immunoproteasome that works in some tumor cells but not in normal cells, EBV-B cells, and some of tumor cell lines (35). Robbins et al. also reported TAA, which was expressed in a variety of normal tissues and autologous EBV-B cells. The CTL that recognized the antigen, however, lysed only tumor cells but not EBV-B cells. As a cause of this phenomenon, they speculated the possibilities of (a) the post-transcriptional regulation of the gene expression as well as alterations in the processing of the protein caused higher expression levels of epitope peptide and (b) overexpression in transient expression system might result in presentation of peptide on the cell surface at sufficient levels to be recognized by CTL (36). Engelhard reviewed about the unusual peptides derived from ubiquitous protein through post-translational modifications (37). For more details, Hanada et al. described a peptide antigen derived from the post-translational splicing of two discontinuous segments of the fibroblast growth factor-5 protein (38). The occurrence of protein splicing has important implications for the mechanism of the proteasome and for the immune recognition of self and foreign peptides.

We have isolated several CTL clones against autologous lung cancer cells from the RLNLs of patient A904. One of them (CTL clone F2b) recognized a cross-reactive antigen in the context of HLA-A*2402.1. The tumor-specific CTLs may control progression of the tumor; consequently, the patient was free from recurrence for 7 years after the second operation for adenal metastasis. These results indicate that it is possible to induce several CTL clones against different TAAs of one tumor from RLNLs in the same patient and that these CTL clones may play an important role in the rigorous surveillance for metastasis. In the same patient, we reported previously the identification of the 38 antigens recognized by antibody derived from tumor-infiltrating B lymphocyte.2 Furthermore, anti-p53 antibodies were detected in the patient’s sera 2 years after surgery (39). Moreover, the CTL clone against mutated p53 155-161 peptide could be induced from the RLNLs of the patient (22). The CTL clone recognized the mutated p53 peptide in the context of HLA-Cw*0702, thus indicating the presence of two different CTL clones in RLNLs that recognize different tumor antigens, which are independently expressed on the same HLA allele. To our knowledge, there has only been one report regarding HLA-Cw*0702-restricted tumor antigen, MAGE-A12, induced from human bladder carcinoma line LB831-BLC (40). HLA-Cw7 is common allele expressed in 41% of Caucasians, 40% of Blacks, and 28% of Orientals (41) and 20% of Japanese (42). These results suggest that cDNA clone 31.2 may be a possible target for CTL-based immunotherapy in lung cancer patients. It remains to be elucidated, however, whether F2a may play a crucial role in the clinical evolution of patient A904.

References

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