Gene and Peptide Analyses of Newly Defined Lung Cancer Antigens Recognized by HLA-A2402-restricted Tumor-specific Cytotoxic T Lymphocytes

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ABSTRACT

We investigated tumor antigens recognized by HLA-A2402-restricted CTLs established from T cells infiltrating into lung adenocarcinoma. We report here three newly identified tumor antigen genes, including one unreported gene, temporally referred to as clone 83, and two known genes, BTB domain containing 2 (BTBD2) and hairpin-binding protein. These genes were preferentially expressed in most of the cell lines of lung cancer and also of ovarian cancer and renal cell carcinoma at the mRNA level. The expression of these genes was confirmed in lung and other cancer tissue specimens. In normal tissues, clone 83 was expressed only in the colon, and hairpin-binding protein was not expressed at all, whereas BTBD2 was ubiquitously expressed. Clone 83, BTBD2, and hairpin-binding protein encoded two, one, and one epitope peptides that can be recognized by HLA-A2402-restricted tumor-specific CTLs after in vitro stimulation in a culture of peripheral blood mononuclear cells from patients with lung cancer. These results suggest that these genes and peptides are potential candidates for cancer vaccines in HLA-A241 patients with lung cancer.

INTRODUCTION

Lung cancer is among the most commonly occurring malignancies in the world and is one of the few that continues to show an increasing incidence (1). Prognosis of patients with unresectable progressive stages of non-small cell lung cancer is very poor because the response rate to anticancer drugs in these patients is low (1). Therefore, development of new therapeutic modalities for lung cancer is necessary. Specific immunotherapy is one of the most prominent modalities for treatment of these patients. We have previously investigated tumor antigens and their peptides recognized by HLA-A2402-restricted CTLs, respectively. These epitope peptides possessed the ability to induce HLA-A24-restricted tumor-specific CTLs after in vitro stimulation in a culture of peripheral blood mononuclear cells from patients with lung cancer. These results suggest that these genes and peptides are potential candidates for cancer vaccines in HLA-A241 patients with lung cancer.

MATERIALS AND METHODS

Cell Lines. A lung adenocarcinoma cell line, 11-18, was used for the preparation of a cDNA library. COS7, VA13 (fibroblast), and C1R-A2402 (an HLA-A2402 transfectant, generous gift of Dr. Takiguchi, Kumamoto University, Kumamoto, Japan) cells were used for the transfection and peptide-pulse experiments. The origins and HLA genotypes of cell lines used in this study have been described previously (4, 5).

Cloning and Identification of New Tumor Antigen Genes. The expression-gene cloning method was used to identify genes that encode tumor antigens recognized by the CTL line, GK-CTL, as reported elsewhere (4). The precise methods for preparation of a cDNA library of the 11-18 cells, screening of cDNA clones, and DNA sequencing have been described elsewhere (5). Briefly, mRNA of the 11-18 lung adenocarcinoma cells was converted to cDNA, ligated to a cDNA ligated to a random hexamer primer, and inserted into the expression vector pCMV-SPORT2.0 (Life Technologies, Inc., Gaithersburg, MD). A total of 10^3 cDNA clones from the cDNA library of the 11-18 cells were divided into 1000 wells (the expected number of clones/well was 100) and subjected to the first screening. Purified DNA from the divided pools and 100 ng of HLA-A2402 cDNA were cotransfected into VA13 cells (10^4 cells/well) and analyzed for their activity to stimulate IFN-γ production by the GK-CTLs. Assay protocol is described in the “Peptides and Assays” section. Full-length HBP1 cDNA was cloned into pCR3.1 Invitrogen, Carlsbad, CA) by TA-cloning method. A BTBD2 clone (KIAA1484) was purchased from Takara (Tokyo, Japan).

Northern Blot Analysis. Total RNA (5 μg/lane) extracted from various cells or tissue specimens (six lung cancers, two renal cell carcinomas, one colon cancer, four gastric cancers, two ovarian cancers, one esophageal cancer, and one oral cancer) using RNAzol B (TEL-TEST, Friendswood, TX) was separated on formaldehyde-agarose gel and transferred to nylon membranes (Hybond-N; Amersham, Buckinghamshire, United Kingdom). Total RNA preparations of normal tissues were purchased from Sawady Technology (Tokyo, Japan). The membranes were additionally hybridized with probes for mRNAs for p21, p53, and MUC1.

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3 The abbreviations used are: HBP, hairpin-binding protein; BTBD2, BTB domain containing 2; PBMC, peripheral blood mononuclear cell; nt, nucleotide; ORF, open-reading frame; mAb, monoclonal antibody.
or tryptophan at position 9, searched using BIMAS software (BioInformatics and Molecular Analysis Section, Center for Information Technology, NIH, Bethesda, MD; Ref. 21), were obtained from Sawady Technology. An HIV nef-derived peptide (RYPLTFGWCF) and an EBV membrane-protein-derived peptide (TYGPVFMCL) capable of binding to HLA-A2402 molecules were used as the source for a cDNA library. A total of 105 cDNA clones from the cDNA library of the 11-18 lung adenocarcinoma cells were used as the source for a cDNA library. A characteristics have been reported elsewhere (2, 4). The 11-18 lung adenocarcinoma cells were established from T cells infiltrating into a lung adenocarcinoma, and its expression was analyzed by northern blotting (Fig. 2B). The CTL line used in this study was the HLA-A24-restricted and tumor-specific CTL (GK-CTL) line with a CD3+CD4−CD8+ phenotype. This line was established from T cells infiltrating into a lung adenocarcinoma, and its characteristics have been reported elsewhere (2, 4). The 11-18 lung adenocarcinoma cells were used as the source for a cDNA library. A total of 105 cDNA clones from the cDNA library of the 11-18 cells were tested for their ability to stimulate IFN-γ production by GK-CTLs after cotransfection with HLA-A2402 cDNA into the VA13 cells. After repeated screening cycles, three clones, i.e., clones 50, 83, and 96, were confirmed to encode tumor antigens recognized by the GK-CTLs after cotransfection with HLA-A2402 but not with control HLA-A2601 (Fig. 1). Different reactivity of GK-CTL-derived sublines to each transfected antigen suggested that the presence of specific T cell clones to the above three gene products (data not shown).

Clone 50 contained a 750-bp-long cDNA insert in which the nt sequence was identical to that of the 3′ end of BTBD2/POZ (GenBank accession nos. AK000393 and AF355797), i.e., the nt sequence of clone 50 corresponds to positions 1708–2450 of the BTBD2 cDNA. The BTBD2 encodes a BTB/POZ-containing Kelch-like protein, BTBD2, which has been identified as a topoisomerase I-interacting zinc finger protein (23). However, clone 50 does not encode the BTBD2 protein because the ORF encoding BTBD2 is located at nt positions 758–1372 of the BTBD2 gene. Clone 83 (GenBank accession no. AF445025) contained a 4.2-kb-long cDNA insert, the nt sequence of which was not identical to those of any previously reported genes. The nt sequence of the 5′ end of clone 83 contained inverted repeats consisting of a 517-bp nt sequence that was partly identical to the 5′ end (positions 1–459) of Fe65L2 (GenBank accession no. AB024745); partial homology is 100%. Fe65L2 is a protein that interacts with Alzheimer’s beta-amyloid precursor protein (24). The nt sequence of the 3′ end of clone 83 (positions 1546–4180) was 86% identical to that of RIKEN cDNA 2610030J16 gene (GenBank accession no. NM08670.1), the function of which was unknown.

Clone 96 contained a 1.2-kb-long cDNA insert, the nt sequence of which was identical to that of histon HBP, also called stem-loop (histon) binding protein (GenBank accession no. Z71188; Ref. 25), but it lacked the 5′ end, i.e., the nt sequence of clone 96 corresponds to positions 658–1716 of the HBP cDNA.

Expression of the Genes at the mRNA Level. The expression of the three newly identified genes in lung and other cancer cell lines, cancer tissues, and normal tissues at the mRNA level was analyzed by northern blotting (Fig. 2). BTBD2/cDNA clone 50, and clone 83, and HPB clones 96 were expressed in almost all of the lung and other cancer cell lines tested to different degrees (Fig. 2A). It should be noted that the expression of these genes in VA13 cells was detectable, but expression levels were low.

The expression of each gene in lung and other cancer tissue specimens is shown in Fig. 2B. The expression of BTBD2/cDNA clone 50 was rarely observed in these cancer tissues, and only weak expression was detected in five lung cancer tissue specimens. In contrast, clone 83 and HBP/cDNA clone 96 were more frequently expressed in the cancer tissues. Expression of the three genes in normal tissues was also examined (Fig. 2C). BTBD2/cDNA clone 50 was ubiquitously expressed in the normal tissue specimens at different levels, i.e., relatively strong to intermediate expression was
detected in the brain, colon, kidney, and skeletal muscle, and weak expression was observed in the PBMC, heart, lung, stomach, small intestine, liver, spleen, placenta, and testis. In contrast to the ubiquitous expression of \textit{BTBD2/clone 50}, \textit{clone 83} was expressed only in the colon, and expression levels in the other normal tissues were almost same as the back ground level. Expression of \textit{HBP/clone 96} was not detected in the normal tissue specimens (data not shown).

**Identification of Antigenic Peptides Recognized by the CTL.**

Each of the 18 different synthetic peptides derived from \textit{clone 83}, \textit{clone 50}, and \textit{clone 96}, with binding motifs to HLA-A2402 molecules, was loaded onto an HLA-A2402 stable transformant, C1R-A2402 cells, and the ability of each peptide to induce IFN-\(\gamma\)/H9253 production by GK-CTL was tested. A total of 7 of 18 peptides derived from the genes induced significant levels of IFN-\(\gamma\)/H9253 production (Fig. 3A), and four (\textit{clone 50-1-767}, \textit{clone 83-3-297}, \textit{clone 83-3-301}, and \textit{clone 96-3-380}) of these peptides expressed the effect in a dose-dependent manner (Fig. 3B). However, remaining 3 of 7 peptides did not express such dose dependency. Therefore, we selected the former 4 peptides for additional analysis. The optimal concentration of the 4 peptides for loading C1R-A2402 cells varied, ranging from 0.1 to 10 \(\mu\)g/ml (compatible to 0.1–10 \(\mu\)M). The binding affinity of the 4 peptides to the HLA-A2402 molecules varied; the estimated half-time score of dissociation of each peptide ranged between 20 and 660 (Fig. 3A). Therefore, the binding affinity shows no relationship to the stimulatory property. This type of discordance has been observed in our previous study (2–8).

**Induction of CTLs by the Peptides.** The four peptides mentioned above were tested for their ability to induce CTLs in the PBMC cultures from eight HLA-A24\(^+\) patients with lung cancer (seven adenocarcinomas and one squamous cell carcinoma) and eight HLA-A24\(^+\) healthy donors. Representative results are shown in Fig. 4A. For example, \textit{clone 83-3-297} peptide induced significant levels of IFN-\(\gamma\) in PBMCs of patients 2 and 8 (left panel of patients). Control HIV peptide did not induce the specific T cells after being stimulated four times in any of the cases (data not shown). The induction rate of the peptides for peptide-specific T cells was as follows: 5 of 8 for \textit{clone 50-1-767}; 3 of 8 for \textit{clone 83-3-301} and 96-3-380; and 2 of 8 for \textit{clone 83-3-297}. Reactivity of the peptide-stimulated T cells to HLA-A24\(^+\) lung cancer cells (11–18) was also examined, and a similar tendency was observed, except for in the case of EBV (Fig. 4A, right panel of patients). It is noted that the magnitude of T-cell response to peptide-loaded cells and that to tumor cells cannot compare each other because the set of experiments were performed at different times and different culture conditions. The peptide-stimulated T cells also recognized HLA-A24\(^+\) other origin of cancer cells such as ovarian...
cancer (KOC-3S) and renal cell carcinoma (TUHR-1OTKB; Fig. 4B).

In contrast to the observed effects of peptides in the PBMCs of cancer patients, these peptides rarely induced the peptide-specific T cells in the PBMC cultures of healthy donors (Fig. 4A).

The cytotoxic activities of the peptide-induced T cells were additionally examined. All of the four peptide-induced T cells of patients lysed HLA-A24<sup>+</sup> lung cancer cells, 11-18 and Sq-1, whereas none of the T cells induced by the peptides killed the HLA-A24<sup>+</sup> QG56 lung cancer cells or the HLA-A24<sup>+</sup> EBV-transformed B cells (SS-EBB) and phytohemagglutinin blasts (Fig. 5A). Control EBV- or HIV-peptide-induced T cells did not show such cytolysis against either 11-18 or QG56 cells (data not shown). The cytotoxic activities of the CTLs against autologous lung cancer cells were also confirmed (Fig. 5B). Peptide specificity of the CTLs was additionally analyzed by a cold target inhibition assay (Fig. 5C). SS-EBB cells or peptide-loaded SS-EBB cells were used as cold targets, but not hot targets, because spontaneous release of 3<sup>11</sup>C from the cells was markedly increased after peptide loading. The cytotoxicity of each of the peptide-induced CTLs against 11-18 cells was not inhibited by addition of a 20-fold excess of cold SS-EBB cells or irrelevant control peptide-loaded SS-EBB cells. In contrast, corresponding peptide-loaded SS-EBB cells inhibited the cytotoxicity. Effects of mAbs on the IFN-γ production and cytotoxicity of the peptide-induced T cells were additionally examined. Representative results are shown in Fig. 5, D and E. IFN-γ production in response to the 11-18 cells (Fig. 5D), or the cytotoxicity against 11-18 cells (Fig. 5E) was inhibited by an addition of anti-CD8, anti-HLA-class I (W6/32), or anti-HLA-A24 mAb but not by that of anti-CD4 or anti-HLA-DR mAb. These results suggest that the four peptide-induced CD8<sup>+</sup> T cells specifically react to the HLA-A24<sup>+</sup> cancer cells, but not to nontumorous cells, through the recognition of the original peptide used for their induction; moreover, this reaction results in lysis of the tumor cells in a HLA-A24-restricted manner.


nt sequences of clone 50 and clone 96 are parts of BTBD2 and HBP, respectively. Therefore, we examined whether the transfectants of full-length BTR2 and HBP are similarly recognized by the CTLs. The cotransfectants of HLA-A2402 and full-length HBP were able to stimulate IFN-γ production of the GK-CTLs (Table 1). It makes sense because the CTL-epitope peptide encoded by clone 96 is included in the whole HBP protein. The cotransfectants of HLA-A2402 and full-length BTBD2 (KAIA4184) also stimulated IFN-γ production of clone 50-1-767-induced CTLs (Table 1). This result suggested that the first ORF-encoding clone 50-1-767 peptide was naturally translated from the full-length BTBD2 cDNA.

DISCUSSION

We identified in this study three new tumor antigen genes and four antigenic peptides derived from these genes that were recognized by the HLA-A2402-restricted tumor-specific CTLs of patients with lung cancer. An antigenic epitope-peptide coding region of clone 83 was located on the second unit of inverted repeat (nt position 617-1133), the nt sequence of which was similar to the 5'<sup>′</sup> end sequence of Fe65L2, although it was directionally opposite. An interacting site of Fe65L2 with Alzheimer’s β-amyloid precursor protein is located on the COOH-terminal of Fe65L2 (24) and is not encoded by the repeat units in either direction. A genomic sequence suggested that a chromosomal translocation did not occur between the second repeat unit (nt position 617-1133) and the down-stream sequence of the clone 83. However, the first unit (nt position 50–566) has not been identified on the genome sequence. Therefore, it is unclear whether the first inverted repeat unit is unique to the 11-18 lung cancer cells. The ORF (nt position 636-1166), including the CTL epitope peptides of clone 83, has the Kozak motif (26, 27) at the initiation codon, and Reinhardt’s method (28) for prediction of the subcellular location of proteins suggested that the putative protein of the ORF was a nuclear protein.

There are two putative ORFs in BTBD2/clone 50, and the first ORF (nt position 2038–2431) is present in both the BTBD2 and original clone 50. The first ORF possesses a Kozak motif at the initiation codon, and Reinhardt’s method suggested that the putative protein of the first ORF was a nuclear protein. The second ORF (nt position 758-1372) encodes BTBD2 protein, which has been identified as a topoisomerase I-interacting zinc finger protein (23). The second ORF
is only present in BTBD2 but not in the original clone 50, and it does not possess a Kozak motif at the initiation codon. The antigenic epitope peptide, clone 50-1-767, is encoded by the first ORF. Whether the first ORF was translated from the full-length BTBD2 cDNA, reactivity of clone 50-1-767-induced CTLs to a transfectant, which had been cotransfected with full-length BTBD2 and HLA-A2401 cDNA, was additionally examined, and the recognition of the transfectant by the CTLs was confirmed (Table 1). This result suggested that the first ORF-encoding clone 50-1-767 peptide was naturally translated from the full-length BTBD2 cDNA. Although the second ORF was not included in the original clone 50, we additionally attempted to find new epitope peptides encoded by the second ORF and identified another HLA-A2402-restricted epitope peptide (clone 50-3-380: MFKEPVEVL) in the BTBD2 protein (data not shown). These results suggest that both of the ORFs are translated to protein in these lung cancer cells.

HBP encodes HBP/stem-loop (histon) binding protein (25). The putative ORF of clone 96 encodes a partial HBP/stem-loop (histon) binding protein containing a COOH-terminal; thus, the epitope peptide, clone 96-3-380, is encoded by both the HBP and clone 96. Indeed, cells cotransfected with full-length HBP and HLA-A2402 cDNA were able to stimulate IFN-γ production of the GK-CTLs (Table 1). The HBP binds the stem-loop structure of replication-dependent histone pre-mRNAs and contributes to efficient 3' end processing by stabilizing the complex between pre-mRNA and U7 small nuclear ribonucleoprotein (25). Therefore, all of the putative proteins encoded by clone 83 and the first ORF of BTBD2/clone 50 and the HBP/stem-loop (histon) binding protein are localized in the nucleus. We previously reported that some of the transcription-related proteins located in the nucleus could be recognized by the CTLs as tumor antigens (6, 8).

Clone 83 and HBP/clone 96 were preferentially expressed in the cancer cells. In contrast, BTBD2/clone 50 was ubiquitously expressed in both normal tissues and cancer cells. These expressions in normal
Fig. 5. Cytotoxic activities of the peptide-induced CTLs. The peptide-induced CTLs shown in Fig. 4 were grown to obtain a sufficient number of cells for the assay. Representative results of PBMC culture of lung cancer patients are shown. A, cytotoxicity of the cells against HLA-A24+/ lung cancer (11-18, Sq-1), HLA-A24+/ lung cancer (QG56), HLA-A24+/ EBV-transformed B cells (SS-EBB), and HLA-A24+/ PHA-blast cells was measured by a 6-h 51Cr-release assay at different E:T ratios. Values represent the means of triplicate assays. Student’s t test was used for the statistical analysis between the percentage lysis of 11-18 cells and that of QG56 cells. *, P < 0.05. B, cytotoxicity of the cells against autologous lung cancer cells (11-18) and 11-18 cells at E:T ratio 20. Values represent the means of triplicate assays and background values against QG56 cells were subtracted. *, P < 0.05. C, cold target inhibition of the cytotoxicity. Indicated peptides (10 μg/ml) were loaded on SS-EBB cells and used as cold target cells for the cytotoxicity assay against 11-18 cells. Cold target cells/hot target (11-18) cells ratio was 20:1. Student’s t test was used for the statistical analysis between the percentage lysis of no cold target cells and that of peptide-loaded SS-EBB cells. *, P < 0.05. D and E, effects of mAbs on the IFN-γ production (D) and cytotoxicity (E) to the 11-18 cells. Indicated mAbs (final 1:100 dilution of ascites) were added to the assay culture. *, P < 0.05.
tissues could possibly induce adverse effects such as tissue distraction when the newly defined gene product-derived antigen peptides are used in treatment vaccines for patients with lung and other cancers. Therefore, clone83 and HBP/clone 96 are more suitable target molecules for cancer vaccines. However, it should be noted that no severe adverse effects in normal tissues or organs have been reported in the clinical trials of cancer vaccines specific to MAGE-1, MAGE-3, Melan-A, gp100, tyrosinase, and NY-ESO-1 in melanoma patients, although some of the target molecules are ubiquitously expressed in normal organs (11, 29). Subcellular traffic of antigenic molecules and/or subsequent processing of the antigenic peptides in the proteasomes of normal cells may differ from that of tumor cells in these cases. Alternatively, some molecules in normal cells, including a family of serpins (a group of serine-protease inhibitors), might be involved in normal cell resistance to CTL-mediated lysis (30). Therefore, BTBD2/clone 50 is also a possible candidate for the cancer vaccine. Study of CTL induction by the BTBD2/clone 50-derived peptide also suggests such a possibility, i.e., peptide clone 50-1-767-induced peptide-specific and tumor-specific CTLs in 5 of 8 PBMC cultures from lung cancer patients, whereas no such induction was observed in the PBMCs of healthy donors.

The HLA-A24 allele is found in 60% of Japanese (95% of these cases are genotypically A2402), in 30% of Chinese, and in 20% of Caucasians (31). The four peptides derived from the three genes were able to induce HLA-A24-restricted and tumor-specific CTLs in the PBMCs of lung cancer patients. The four peptides might therefore be appropriate vaccine candidates for use in specific immunotherapy for HLA-A24+ patients with lung cancer.

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