Mutated p53 Gene Encodes a Nonmutated Epitope Recognized by HLA-B*4601-restricted and Tumor Cell-reactive CTLs at Tumor Site

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ABSTRACT

Mutations of p53 gene occur in approximately 50% of human cancers, and accumulated p53 protein may be an appropriate target molecule to use for cancer immunotherapy. Indeed, mutated or nonmutated p53-derived peptides can induce HLA class I-restricted and tumor cell-reactive CTLs in vitro. However, to our knowledge, evidence that p53-derived peptides are truly recognized by CTLs at tumor sites has not yet been obtained. This study revealed that a mutated p53 gene encoded a nonmutated nonapeptide recognized by a HLA-B46-restricted and tumor cell-reactive CTL line that was established from T cells infiltrating a colon cancer lesion with the p53 mutation. This p53 peptide, at amino acid positions 99–107, had the ability to induce HLA-B46-restricted and peptide-specific CTLs reactive to the peripheral blood mononuclear cells of cancer patients, but not from those of healthy donors. These peptide-induced CTLs did not react to either HLA-B46+ tumor cells without the p53 mutation or to HLA-B46+ phagocytosed tumor cells. These results provide a scientific basis for the development of p53-directed specific immunotherapy for HLA-B46+ cancer patients.

INTRODUCTION

Mutations of the p53 gene occur in approximately 50% of human cancers (1–3). As a consequence of this mutation, dysfunctional p53 protein accumulates in cancer cells (3). In contrast, the half-life of the functional p53 protein in normal cells is so brief that normal cells have undetectable levels of p53 (4–6). Such findings suggest that accumulated p53 protein provides an appropriate target for cancer immunotherapy. Several reports showed that mutated or nonmutated p53-derived peptides were capable of inducing HLA-A allele-restricted and tumor cell-reactive CTLs from human PBMCs3 in vitro (6, 7). However, it remains to be clarified whether or not p53-derived epitopes are indeed recognized by CTLs in vivo.

Five hundred antigenic peptides recognized by HLA-A (HLA-A1, -A2, -A3, and -A24) allele-restricted CTLs have been reported during the past decade (8–11). In contrast, only a few peptides on the HLA-B alleles (HLA-B35 and -B53) have been reported (12, 13). The frequency of HLA-A2 or -A24 allele is relatively high in many ethnic groups, allowing prompt study of CTL-directed peptides from the viewpoint of their clinical implications. In contrast, the frequency of HLA-B alleles is generally low due to their great diversity. One such allele is the HLA-B46 allele, which is expressed exclusively in Asians. This allele is expressed in 30% of Singapore Chinese, 28% of the Thai population, 9% of Japanese, and 8% of Koreans, whereas it is expressed in <1% of Caucasians, blacks, and Indians (14, 15). We have reported in this study that a mutated p53 gene encodes a nonmutated nonapeptide recognized by a HLA-B46-restricted and tumor cell-reactive CTL line.

MATERIALS AND METHODS

Generation of HLA-B46-restricted CTLs. The HLA-B46-restricted and cell-reactive CTL line OKB-CTL was established from TILs of a patient with colon cancer (HLA-A*0207/3101, -B46/51, and -Cw1) by incubation with IL-2 (100 units/ml) alone for more than 50 days by the methods reported previously (11). The surface phenotypes of the CTLs were investigated by immunofluorescence assay with anti-CD3, -CD4, and -CD8 mAbs (Nichirei, Tokyo, Japan). To inhibit CTL activity, anti-HLA class I (W6/32, IgG2a), anti-HLA-A2 (BB7.2, IgG2b), anti-HLA-B/C (B1-23, IgG2a; Ref. 16), anti-CD8 (Nu-Ts/c, IgG2a), anti-HLA class II (H-DR-1, IgG2a), and anti-CD4 (N-Tul, IgG1) mAbs (20 μg/ml) were used (11). Anti-CD14 (JML-H14, IgG2a) mAb served as a control. A two-tailed Student’s t test was used for the statistical analysis in this study.

Identification of the cDNA Clone. The expression gene cloning method was used to identify genes coding for tumor antigens recognized by the OKB-CTL line obtained by the methods reported previously (10–11). In brief, cDNA of SW620 was inserted into the expression vector pCMV-SPORT-2 (Invitrogen, San Diego, CA). Wild-type p53, HLA-B*4601, HLA-B*5201, or HLA-A*0207 was amplified by reverse transcription-PCR and cloned into pcDNA3 (Invitrogen). DNA sequencing was performed with a dideoxynucleotide sequencing method using a DNA Sequencing kit (Perkin-Elmer, Foster, CA) and analyzed by an ABI PRISM 377 DNA Sequencer (Perkin-Elmer).

Construction of Deletion Mutants. The p53/CMV-SPORT2 plasmid was digested with BamHI and SpIol for the preparation of deletion mutants. ExonI11107 was used as a control. The mutant p53 was transduced into the cells by infection with retrovirus, and the transformants were selected by G418. The transformants were used to identify genes coding for tumor antigens recognized by the OKB-CTL line.

Peptides and CTL Assay. Eleven kinds of wild-type peptides (>70% purity; p531–19, p531–60, p5399–107, p53118–126, p53204–212, p53262–270, p5319–327, p53320–330, p53330–338, p53333–341, and p53377–385) with HLA-B46 binding motif (15) were synthesized for screening. For additional studies, two peptides (p5399–107 and p5330–313) with >90% purity were obtained. For detection of antigenic peptides, HLA-B*4601 or HLA-B*5201 CDNA (as negative control)-transfected COS-7 (5 × 105) cells were pulsed with a peptide at a final concentration of 10 μM for 2 h, and the CTLs were then added and incubated for 18 h. One hundred μl of supernatant were collected to measure IFN-γ by ELISA (limit of sensitivity, 10 pg/ml) in a duplicate assay.

CTL Induction from PBMCs of Cancer Patients. PBMCs from six HLA-B46+ cancer patients and three HLA-B46+ healthy donors served as subjects for the CTL induction assay. Informed consent was obtained from all patients. HLA class I PBMCs were serotyped by conventional serological methods, as reported previously (11). Nine HLA-B46 molecules from three healthy donors and six cancer patients were genotyped, and all those tested were HLA-B*4601 (data not shown). HLA-B*4601 would be the expected serological identification because the genotype is predominantly (>95%) HLA-B*4601 in the case of B46 (18). For induction of peptide-specific CTLs, 1 × 106 cells/well were incubated with 10 μM of each peptide in the wells of a 96-well microculture plate (Nunc, Roskilde, Denmark) in 200 μl of culture medium containing IL-2, as reported previously (17). On the 10th day, the cells were harvested, washed, and tested for their ability to produce IFN-γ.

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3 The abbreviations used are: PBMC, peripheral blood mononuclear cell; aa, amino acid(s); IL, interleukin; PHA, phytohemagglutinin; TIL, tumor-infiltrating lymphocyte; mAb, monoclonal antibody; LSAB, labeled streptavidin-biotin.

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in response to HLA-B*4601-transfected COS-7 cells pulsed with a corresponding peptide or a control peptide (p53_204-212) in duplicate assays. The peptide-stimulated PBMCs were further incubated for more than 10 days, and their cytotoxicity was tested by a standard 6-h ³Cr release assay, as reported previously (10). The cancer cell lines used in this study were HLA-B46⁺ p53 mutation-positive OSC20 (oral cancer), QG56 (lung squamous cell carcinoma), Ca9-22 (oral cancer); HLA-B46⁺, p53 mutation-negative MKN45 (stomach cancer); HLA-B46⁺, p53 mutation-positive Kuma-1 (head and neck cancer), SW620 (colon cancer), COLO320 (colon cancer), KWS (stomach cancer); and COS-7 (HLA-B46⁺, SV40-transformed African Green monkey kidney cells). PHA-blastoid T cells from PBMCs were also used as target cells. For the cold target inhibition assay, unlabeled HLA-B46⁺ PHA-blastoid T cells were incubated with a corresponding or a control peptide for 2 h, washed, and added to the ³Cr-labeled targets at a cold:hot target ratio of 10:1.

**Immunohistochemistry.** Anti-p53 mAb (DO-7, Dako, Glostrup, Denmark) was used for immunostaining. A Dako LSAB Kit (Dako, Carpinteria, CA), which is based on the LSAB method, was used for the immunohistochemical analysis. In brief, both the endogenous peroxidase activity and nonspecific staining of the specimens were blocked, and then the specimens were incubated with DO-7 mAb overnight at 4°C, followed by sequential 10-min incubation with biotinylated antimonine IgG and peroxidase-labeled streptavidin. Staining was completed after a 10-min incubation with the substrate-chromogen solution. Tumors were defined as positive for p53 when the relative amount of positive tumor cells exceeded 20. Tumor cell line cells were spun down on a slide, air-dried, permeabilized, fixed with acetone, and washed in PBS. Immunostaining was performed by the LSAB method using a DO-7 mAb, as outlined above. Two observers who were unaware of the examined lesion types determined the positivity.

**RESULTS**

Characterization of HLA-B46-restricted and Tumor Cell-reactive CTLs. The OKB-CTL line produced significant levels of IFN-γ in response to HLA-B46⁺ cancer cells (QG56 and Ca9-22), but not in response to HLA-B46⁻ cells (KWS, COLO320, and COS-7; Fig. 1A). CTL activity was inhibited by anti-HLA class I, anti-HLA-B/C, or anti-CD8 mAb, but not by any other mAbs tested (Fig. 1B). Anti-HLA-A2 mAb also failed to inhibit CTL activity (data not shown). The OKB-CTL line showed significant levels of cytotoxicity against HLA-B46⁺ cancer cells (QG56, Ca9-22, and OSC20) but failed to lyse HLA-B46⁻ cancer cells (COLO320, Kuma-1, and SW620), COS-7 cells, and HLA-B46⁺ PHA-blastoid T cells (Fig. 1C). Surface markers of OKB-CTL were mostly (>80%) CD3⁺ CD4⁻ CD8⁺ (data not shown).

**Identification of the Immunogenic Gene.** A total of 1 × 10⁵ cDNA clones from the cDNA library of SW620 tumor cells and HLA-B*4601 cDNA were cotransfected to COS-7 cells, followed by a test of their ability to stimulate IFN-γ production by the OKB-CTL line. After repeated experiments, one cDNA clone 7F (GenBank accession number AB082923) was identified (Fig. 1D). The sequence of clone 7F was identical to that of the p53, except for a missense point mutation G to A change at nucleotide position 886. Consequently, an aa at position 273 was changed from R to H. OKB-CTL recognized COS-7 cells cotransfected with clone 7F gene and the HLA-B*4601 gene in a dose-dependent fashion. OKB-CTL recognized COS-7 cells cotransfected with wild-type p53 and HLA-B*4601 gene (Fig. 1D). In contrast, OKB-CTL failed to recognize COS-7 cells cotransfected with clone 7F and the HLA-B*5201 gene, those cotransfected with clone 7F and the HLA-A*0207 gene, and those cotransfected with wild-type p53 and the HLA-B*5201 gene (Fig. 1D). The CTLs also failed to recognize COS-7 cells cotransfected with either the HLA-B*4601 gene or clone 7F alone (data not shown).

Immunostaining of p53 protein was performed on the cancer tissue from which the OKB-CTL line was generated, and accumulation of p53 protein in the nucleus was observed in more than 40% of the colon cancer cells (Fig. 2A). The tumor cell lines used in this study as target cells were also subjected to immunohistochemical analysis. OSC20, QG56, Ca9-22, KWS, Kuma-1, SW620, and COLO320 tumor cells were positive for accumulated p53 protein, whereas MKN45 tumor cells were negative (data not shown).

**Identification of CTL-directed Epitopes.** To identify peptides that could be recognized by the CTLs as epitopes of p53, we investigated the capability of deletion mutants of clone 7F to stimulate IFN-γ production by the OKB-CTL cells. Significant levels of IFN-γ production by OKB-CTL cells were observed when both the HLA-
**Mutated p53 Recognized by HLA-B46-Restricted TILs**

**Fig. 2.** A, expression of the p53 protein in cancer tissue. Immunostaining of p53 protein was performed on colon cancer tissue from which the OKB-CTL line was generated, and p53 was subjected to immunostaining by anti-p53 mAb (1:50 dilution, DO-7). Photomicrograph of colon cancer displays immunohistochromical localization of p53 confined to the nuclei of the cancer cells (right side; original magnification, ×400). However, p53 accumulation was not seen in the normal epithelial cells (left side). B, scheme of deletion mutants of clone 7F. Clone 7F had a missense point mutation at position 273 (R to H). 7F. Clone 7F had a missense point mutation at position 273 (R to H). C, the capability of wild-type p53 and also the deletion mutants to stimulate IFN-γ production by the OKB-CTL cells. cDNA of wild-type p53, clone 7F, or one of the four deletion mutants corresponding to p53Δ1–161, p53Δ1–147, p53Δ1–60, or p53Δ1–60 was cotransfected with the HLA-B*4601 gene into COS-7 cells, and IFN-γ production by the OKB-CTL cells was assessed. Values represent the mean of the triplicate assays.

**Table 1.** Immunodominant Peptides of HLA-B46 and HLA-B5201 EPTs and Recognition of These Peptides by OKB-CTL Cells

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Immunodominant Peptide Binding motifs</th>
<th>IFN-γ production (pg/ml)</th>
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<tr>
<td>p53Δ1–161</td>
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</tr>
<tr>
<td>p53Δ1–147</td>
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<td>2451 (273aa)</td>
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<tr>
<td>p53Δ1–60</td>
<td>1147–1245</td>
<td>2451 (273aa)</td>
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**Fig. 3.** A, B, and C, protective activity of the p53Δ1–161 peptide in vivo. A, comparison of IFN-γ production by the OKB-CTL cells. The PBMCs preloaded with the corresponding peptide, but not by those preloaded with a control peptide (p53Δ99–107). Unstimulated but IL-2-activated PBMCs did not show such cytotoxicity (data not shown). This CTL activity was inhibited by anti-CD8 and anti-HLA-B/C mAbs, but not by any other mAbs tested (Fig. 3B). This CTL activity increased when the OSC20 cells were preincubated with a corresponding peptide (p53Δ99–107), but not with a control peptide (p53Δ201–212; Fig. 3C). Furthermore, this CTL activity against OSC20 cells was neutralized by the addition of unlabeled PHA-blastoid T cells preloaded with the corresponding peptide, but not by those preloaded with a control peptide (Fig. 3C). Taken together, these results support the hypothesis that the p53Δ99–107 peptide on the groove

B*4601 gene and wild-type p53, the HLA-B*4601 gene, and one of the deletion mutants corresponding to p53Δ1–161 (full length), p53Δ1–147, p53Δ1–60, or p53Δ1–60 was cotransfected with the HLA-B*4601 gene into COS-7 cells, and IFN-γ production by the OKB-CTL cells was assessed. Values represent the mean of the triplicate assays.

\*P < 0.05 by Student’s t test.

D, recognition of a p53-derived peptide by the OKB-CTL cells. Various doses of peptide were loaded onto HLA-B*4601-transfected COS-7 cells for 2 h, followed by the addition of OKB-CTL cells at an E/T ratio of 10:1. After an 18-h incubation, the culture supernatant was collected for measurement of IFN-γ. The values represent the means of duplicate assays.

Among them, p53Δ99–107-stimulated PBMCs from patients 1 and 2 produced significant levels of IFN-γ in response to OSC20 cells (HLA-B46+, p53 mutation positive) but not in response to Kuma-1 cells (HLA-B46−, p53 mutation positive; Table 1) or COLO320 cells (HLA-B46−, p53 mutation positive; data not shown).

The p53Δ99–107 peptide-induced CTL activity against tumor cells in the PBMCs from patients 1 and 2 was confirmed by a 6-h 51Cr release assay, and representative results are shown in Fig. 3A. The PBMCs stimulated by the p53Δ99–107 peptide showed significant levels of cytotoxicity against the OSC20 cells (HLA-B46−, p53 mutation positive) but failed to lyse Kuma-1 cells (HLA-B46−, p53 mutation positive), MKN45 cells (HLA-B46−, p53 mutation negative), and PHA-blastoid T cells (HLA-B46−; Fig. 3A). Unstimulated but IL-2-activated PBMCs did not show such cytotoxicity (data not shown). This CTL activity was inhibited by anti-CD8 and anti-HLA-B/C mAbs, but not by any other mAbs tested (Fig. 3B). This CTL activity increased when the OSC20 cells were preincubated with a corresponding peptide (p53Δ99–107), but not with a control peptide (p53Δ201–212; Fig. 3C). Furthermore, this CTL activity against OSC20 cells was neutralized by the addition of unlabeled PHA-blastoid T cells preloaded with the corresponding peptide, but not by those preloaded with a control peptide (Fig. 3C). Taken together, these results support the hypothesis that the p53Δ99–107 peptide on the groove
of HLA-B46 molecules of OSC20 tumor cells is recognized by the PBMCs stimulated with the corresponding peptide. In contrast to the p53<sub>99–107</sub> Peptide, the p53<sub>330–338</sub> Peptide failed to induce HLA-B46-restricted and tumor cell-reactive CTLs in PBMCs from any of the cancer patients tested (data not shown).

**DISCUSSION**

We have newly established a HLA-B<sup>*A4601</sup>-restricted and tumor cell-reactive CTL line (OKB-CTL) from the TILs of a colon cancer patient. The OKB-CTL cells produced significant levels of IFN-γ in response to HLA-B<sup>46</sup> cancer cells (QG56 and Ca9-22) but failed to produce IFN-γ in response to HLA-B<sup>46</sup> cancer cells (WKS and COLO320). The OKB-CTL cells also showed significant levels of cytotoxicity against HLA-B46<sup>+</sup> cancer cells (QG56, Ca9–22, and OSC20) with p53 mutation, but they failed to lyse either HLA-B46<sup>+</sup> SW620, Kuma-1, and COLO320 cells with a p53 mutation or HLA-B46<sup>+</sup> MKN45 cells without a p53 mutation. Furthermore, OKB-CTL activity was inhibited by anti-HLA-B/C mAb. All of these results, when taken together, indicate that the OKB-CTL cells recognized HLA-B46<sup>+</sup> tumor cells with a mutated p53 gene.

The p53 protein has been divided into five domains (19, 20), The immunogenic epitope identified in this study at aa positions 99–107 was located between a proline-rich domain and a sequence-specific DNA-binding domain, and its biological activity has not yet been reported. Although the sites of p53 mutations vary greatly, most of them are single-base mutations, and they are mostly restricted to the regions within the sequence-specific DNA-binding domain (1, 2). Subsequently, nonmutated CTL epitopes other than those with single-base mutations could be maintained in many types of tumor cells. If this is the case, nonmutated epitopes in conserved regions may become common antigens recognized by the host CTLs in many cancer patients whose tumors display the p53 mutation. Because the immunogenic epitope identified in this study, p53<sub>99–107</sub>, is encoded in a conserved region, this peptide could be an appropriate molecule for use as a part of a peptide-based cancer vaccine for HLA-B46<sup>+</sup> cancer patients with p53 mutation.

We constructed deletion mutants of the p53 gene to determine the antigenic peptide recognized by HLA-B46-restricted CTLs. The results of the experiments using these genes suggest that antigenic epitopes may be located within 60–630 bp (1–188 aa) of p53. The peptide p53<sub>99–107</sub> was identified as a CTL-directed epitope using synthesized peptides. These results from two separate experiments suggest that peptide p53<sub>99–107</sub> is an immunogenic peptide recognized by OKB-CTL cells. In addition, p53<sub>330–338</sub> was also recognized by the OKB-CTL cells when it was loaded on COS-7 cells transfected with HLA-B<sup>*A4601</sup>. Subsequently, the two peptides (p53<sub>99–107</sub> and p53<sub>330–338</sub>) were tested for their ability to induce HLA-B46-restricted CTL activity in the PBMCs of cancer patients and healthy donors. Both peptides stimulated IFN-γ production in the PBMCs of cancer patients, but not in those of healthy donors, in response to HLA-B46-transfected COS-7 cells. This discrepancy may be that higher or lower activity with regard to the binding to HLA-B46 molecules may be observed in the p53<sub>99–107</sub> and p53<sub>330–338</sub> peptides, respectively. This assumption is supported by the result that the highest IFN-γ production was observed when the HLA-B<sup>*A4601</sup>-transfected COS-7 cells were loaded with 0.1 or 10 μM p53<sub>99–107</sub> or p53<sub>330–338</sub> peptide, respectively. Additional studies are needed to clarify this issue.

Mutations of the p53 gene occur in approximately 50% of human cancers, and our study demonstrated that HLA-B46-restricted and p53-specific CTLs reactive to tumor cells with the p53 mutation were

<table>
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<th>Donors</th>
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<th>IFN-γ production (pg/ml) in response to&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>OSC20 HLA-B46 Kuma-1 HLA-B6244</td>
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<sup>a</sup> PBMCs from six HLA-B46<sup>+</sup> cancer patients and three HLA-B46<sup>+</sup> healthy donors were stimulated in vitro with 10 μM peptide and tested for their ability to produce IFN-γ by recognition of various target cells at an E:T ratio of 5.

<sup>b</sup> Values represent the means of duplicate determinations. The background IFN-γ production in response to HLA-B46-transfected COS-7 cells (50–100 pg/ml) has been subtracted from the experimental values shown in the table.
Fig. 3. Cytotoxicity of p53\textsubscript{99–107}-induced CTL cells. A. p53\textsubscript{99–107}-stimulated PBMCs from patients 1 and 2 were tested for their cytotoxicity against OSC20 (HLA-B46\textsuperscript{+}, p53 mutation positive), Kuma-1 and KWS (HLA-B46\textsuperscript{+}, p53 mutation positive), and MKN45 (HLA-B46\textsuperscript{+}, p53 mutation negative) tumor cells. PHA-blastoid T cells (HLA-B46\textsuperscript{+}, p53 mutation negative) were also used as a negative control. Six-h \textsuperscript{51}Cr release assay was performed at three E:T ratios. *, P < 0.05 by Student’s t test. B. IFN-γ production by the p53\textsubscript{99–107}-stimulated PBMCs in response to OSC20 tumor cells was tested in the presence of 20 μg/ml anti-HLA class II, anti-CD4, anti-CD8, anti-CD14, or anti-HLA-B/C mAb. Values represent the mean of the triplicate determinations. *, P < 0.05 by Student’s t test. C, an excess amount (20 μg/ml) of a corresponding peptide (p53\textsubscript{99–107}) or a control peptide (p53\textsubscript{304–312}) was preloaded onto \textsuperscript{51}Cr-labeled OSC20 tumor cells, which were used as target cells in a 6-h \textsuperscript{51}Cr release assay. Unlabeled PHA-blastoid T cells preloaded with either a corresponding peptide (p53\textsubscript{99–107}) or a control peptide (p53\textsubscript{304–312}) were added to wells containing the \textsuperscript{51}Cr-labeled OSC20 tumor cells at a cold:hot cell ratio of 10:1. Values represent the mean of the triplicate determinations. *, P < 0.05 by Student’s t test.

induced by \textit{in vitro} stimulation with the p53\textsubscript{99–107} peptide of PBMCs from cancer patients with various types of tumors. Because the mutation of p53 is generally associated with tumors cells resistant to chemotherapy and radiotherapy, specific immunotherapy using the p53 peptides may provide an attractive new strategy for the treatment of HLA-B46\textsuperscript{+} cancer patients with p53 mutation in various histological types of cancers.

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