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

Koichi Azuma, Shigeki Shichijo, Yoshiki Maeda, Tetsuya Nakatsuma, Yoichi Nonaka, Teruhiko Fujii, Kenta Koike, and Kyoko Itoh

INTRODUCTION

Mutations of 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.

A large number of 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 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 HLA-B46, 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 peptide 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 tumor 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 (B-1–23, IgG2a; Ref. 16), anti-CD8 (Nu-Tsc, IgG2a), anti-HLA class II (H-DR-1, IgG2a), and anti-CD4 (Nu-Th1, IgG1) mAbs (20 μg/ml) were used (11). Anti-CD14 (JHL-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 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-Sport2 (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/pCMV-Sport2 plasmid was digested with BamHI and SpeI for the preparation of deletion mutants. ExoIII nuclease/Mung bean nuclease was used for the digestion according to the manufacturer’s instructions (TaKaRa, Otsu, Japan) to obtain four deletion mutants of p53 (p531–60 corresponding to nucleotide positions 1–60, p5361–80, p5381–113, and p53114–1122).

Peptides and CTL Assay. Eleven kinds of wild-type peptides (>70% purity; p531–19, p5320–49, p5350–99, p53100–157, p53158–215, p53216–270, p53271–327, p53328–328, p53329–388, p53339–341, and p53373–385) and HLA-B46-binding motif (15) were synthesized for screening. For additional studies, two peptides (p5399–107 and p53108–117) with >90% purity were obtained. For detection of antigenic peptides, HLA-B*4601 or HLA-B*5201 cDNA (as negative control) transfect 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 (17). For induction of peptide-specific CTLs, PBMCs (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.
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 3 days, and their cytotoxicity was tested by a standard 6-h ³¹Cr 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). 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 ³¹Cr-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 antimouse 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-
and also the deletion mutants to stimulate p53 position 273 (R to H).

C, 7F. Clone 7F had a missense point mutation at C and left side (mutation was not seen in the normal epithelial cells).

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 immunohistochemical 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 C and left side (mutation was not seen in the normal epithelial cells). The highest IFN-γ production by the OKB-CTL cells was 2451 bp (full length), 22451/bp (full length), 2451 bp (full length), and 22451/bp (full length), respectively (Table 1). AMONG them, p53204–212 peptide showed significant levels of IFN-γ production by the OKB-CTL cells transfected with the HLA-B*4601 gene, respectively (Table 1). Within them, p53204–212-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).

This CTL activity against OSC20 cells was confirmed by a 6-h 51Cr release assay. The PBMCs stimulated by the p53204–212 peptide on the groove of HLA-B46 restricted and tumor cell-reactive CTLs from the PBMCs of six HLA-B46+ cancer patients and three healthy donors. p53204–212 and p53330–338 peptide-stimulated PBMCs from three and four of six cancer patients produced significant levels of IFN-γ by recognition of the corresponding peptide-loaded COS-7 cells transfected with the HLA-B*4601 gene, respectively (Table 1). Among them, p53204–212-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).

By comparison, the production of IFN-γ by the OKB-CTL cells failed to recognize COS-7 cells cotransfected with the HLA-B*4601 gene. This CTL activity was inhibited by anti-CD8 and anti-HLA-B/C mAbs, but not by any other mAbs tested (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 (p53204–212), but not with a control peptide (p53204–212; 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 p53204–212 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*4601-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-B46<sup>+</sup> cancer cells (QG56 and Ca9-22) but failed to produce IFN-γ in response to HLA-B46<sup>+</sup> cancer cells (KWS 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 recognize 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*B4601. 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 transfected with HLA-B*B4601. We, therefore, tested only the p53<sub>99–107</sub> peptide, but not the p53<sub>330–338</sub> peptide, induced HLA-B46-restricted and tumor cell-reactive CTLs in the PBMCs of cancer patients. One explanation for this discrepancy may be that higher or lower activity with regard to the binding to HLA-B46<sup>+</sup> macrophages occurs 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
induced by in vitro stimulation with the p53 <sub>99-107</sub> 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<sup>+</sup> cancer patients with p53 mutation in various histological types of cancers.

ACKNOWLEDGMENTS

We thank Dr. Pierre G. Coulie (Catholiche de Louvain University, Brussels, Belgium) and Dr. H. Ikeda (Sapporo Medical School, Sapporo, Japan) for providing the anti-HLA-B/C (B1–23, IgG2a) mAb.

REFERENCES

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

Koichi Azuma, Shigeki Shichijo, Yoshiaki Maeda, et al.


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/63/4/854

Cited articles  This article cites 18 articles, 13 of which you can access for free at: http://cancerres.aacrjournals.org/content/63/4/854.full.html#ref-list-1

Citing articles  This article has been cited by 4 HighWire-hosted articles. Access the articles at: /content/63/4/854.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.