A Wild-type Sequence p53 Peptide Presented by HLA-A24 Induces Cytotoxic T Lymphocytes that Recognize Squamous Cell Carcinomas of the Head and Neck1

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ABSTRACT

Evidence has accumulated indicating that HLA-A2-restricted CTLs specific for human wild-type sequence p53 epitopes lyse tumor cells expressing mutant p53. To explore the possibility that wild-type sequence p53 peptides could also be used in vaccines for patients expressing HLA-A24 antigen, another frequent HLA class I allele, we investigated the induction of HLA-A24-restricted p53-specific CTLs from the peripheral blood lymphocytes of normal donors. Of six p53-derived peptides possessing an HLA-A24 binding motif, the p53 peptide 125–134 (p53125–134)1 was found to have a high binding capacity and induced peptide-specific CTLs from peripheral blood mononuclear cells, using peptide-pulsed autologous dendritic cells and subsequent cultivation with cytokines interleukin 2 and interleukin 7. Bulk CTL populations lysed peptide-pulsed HLA-A24+ targets as well as HLA-A24+ squamous cell carcinoma of the head and neck (SCCHN) cell lines. However, IFN-γ pretreatment of HLA-A24+ SCCHN cell lines was necessary for lysis, suggesting that a ligand density higher than that normally expressed by tumor cells is required for these CTLs to mediate lysis. Moreover, a cloned CTL, designated TH899, isolated from the bulk population by limiting dilution, lysed HLA-A24+ SCCHN targets more efficiently than the bulk CTL population. Lysis was inhibited by anti-HLA class I monoclonal antibody but not by anti-HLA-DR monoclonal antibody. These results indicate that HLA-A24-restricted CTLs recognizing the wild-type sequence p53125–134 can be generated using autologous dendritic cells from precursors present in peripheral blood lymphocytes obtained from normal HLA-A24+ donors. This finding suggests that vaccine strategies targeting wild-type sequence p53 epitopes can be extended to a wider range of cancer patients.

INTRODUCTION

The identification of TAAs recognized by CTLs has progressed rapidly in the last few years (1–7). The p53 gene has been shown to be frequently mutated in a wide range of human cancers, including SCCs (8–10). In most cases, mutations in the p53 gene are associated with p53 accumulation in tumors and enhanced potential of MHC class I presentation of nonmutated (wild-type sequence) p53 epitopes derived from mutant p53 molecules. Therefore, the p53 protein is an attractive candidate for the development of broadly applicable vaccine therapies. Several studies relating identification of TAAs have demonstrated that peptides derived from p53 molecules can act as TAAs. In murine models, immunization of mice with mutant- or wild-type p53 peptide-pulsed DC-based vaccines induced tumor p53-specific CTLs (11–13). Furthermore, CTLs recognizing a murine wild-type p53 peptide were generated by immunizing p53-deficient C57BL/6 mice with a syngeneic tumor expressing mutant p53, and adoptive transfer of these CTLs into tumor-bearing p53+/− nude mice caused tumor eradication (14). With respect to human p53, some reports have shown that CTLs specific for the human wild-type sequence p53 epitope p53264–272 could be established from either HLA-A2 transgenic mice or healthy HLA-A2 donors (15–23). Moreover, these CTLs lysed certain p53-mutated tumor cell lines expressing mutant p53.

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3 The abbreviations used are: TAA, tumor-associated antigen; PBMC, peripheral blood mononuclear cell; DC, dendritic cell; SCCHN, squamous cell carcinoma of the head and neck; SCC, squamous cell carcinoma; IL, interleukin; mAb, monoclonal antibody; FBS, fetal bovine serum; MFI, mean fluorescence intensity; BL50, half-maximal binding level; GM-CSF, granulocyte macrophage colony-stimulating factor; EBV-B, EBV transformed B.
HLA-A molecules. HLA-A24 antigen is one of the most frequently expressed HLA-A common alleles worldwide (24). Therefore, identification of HLA-A24-restricted wild-type sequence p53 CTL epitopes would extend the potential of p53-based vaccine therapies. To identify such epitopes, the p53 amino acid sequence was screened for sequences expressing the known HLA-A24 binding motifs, and potential peptides were synthesized and tested for their binding affinity to HLA-A24 antigen. We then investigated which HLA-A24-binding p53 peptides could induce CTLs from the PBMCs of HLA-A24

Table 1  HLA-A24 binding affinity of peptides derived from wild-type p53 peptides

<table>
<thead>
<tr>
<th>Position</th>
<th>Sequence</th>
<th>BL_{50} (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18–26</td>
<td>TFSDLWKLL</td>
<td>7.4 × 10^{-5}</td>
</tr>
<tr>
<td>106–114</td>
<td>SYGFRGLFL</td>
<td>1.7 × 10^{-3}</td>
</tr>
<tr>
<td>204–212</td>
<td>EYLDDRNTF</td>
<td>2.4 × 10^{-7}</td>
</tr>
<tr>
<td>340–348</td>
<td>MFRELNEAL</td>
<td>2.8 × 10^{-4}</td>
</tr>
<tr>
<td>102–111</td>
<td>TGYQGSGFRFL</td>
<td>6.2 × 10^{-5}</td>
</tr>
<tr>
<td>125–134</td>
<td>TYSPLNKMFM</td>
<td>1.5 × 10^{-9}</td>
</tr>
</tbody>
</table>

MATERIALS AND METHODS
Cell Lines. SCC cell lines used in this study were HSC-3, HSC-4, HSQ-89, Ca9-22, FS-1, Kuma-3 (head and neck), and TE-11 (esophagus). FS-1 and Kuma-3 were established in our laboratory (25), and the other four cell lines were obtained from the Japanese Cancer Research Bank (Tokyo, Japan). The HLA-A24 EBV-B cell line MO24 was also established, and it was used in cytotoxicity assays and for stimulation of effector cells. These cell lines were cultured in RPMI 1640 (Iwaki, Chiba, Japan) supplemented with 10% FBS (Life Technologies, Inc.), L-glutamine, and antibiotics, as was the K562 erythroleukemic cell line used in this study. HLA-A typing was done using the molecular PCR sequence-specific oligonucleotide probe method described by Date et al. (26).

p53 Mutation Analysis. p53 mutation analysis of SCC cell lines used in this study was performed using the yeast functional assay, as described previously (27). Briefly, first-strand cDNA was synthesized from mRNA with Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) and random primers (Life Technologies, Inc.). cDNA was amplified by PCR with recombinant Pfu polymerase (Stratagene, La Jolla, CA) and the P3 and P4 primers (P3, 5’-ATTGATGCTGTCCTCCCGAGATATTGAA-3’; P4, 5’-ACCCTTTGACATCCAGTGCGCTGAGTG-3’). The PCR product was confirmed by a 1% agarose gel electrophoresis.
Crude PCR product and linearized p53 expression vector were cotransfected into the yeast reporter strain yIG397, which was blocked with 10% normal goat serum. p53 immunohistochemistry was performed to detect p53 protein with the streptavidin-biotin peroxidase for 30 min. Nonspecific conjugation was performed from PBMCs using a modification of the method described previously (28, 29). Briefly, RMA-S-A*2402 cells were incubated at 26°C for 18–24 h. Cells (2 × 10^5) in 50 μl of PBS supplemented with 20% FBS (PBS-FBS) were incubated at 26°C for 3 h with a 50-μl solution of peptides at 10^{-7} to 10^{-8} μM and then at 37°C for 3 h. After washing with PBS-FBS, cells (2 × 10^6) were incubated for 30 min on ice with an appropriate dilution of mAb TP 25.99 (HLA-class I-specific mAb; a generous gift from Dr. S. Ferrone, New York Medical College, Valhalla, NY). After two washes with PBS-FBS, the cells were incubated for 30 min on ice with an appropriate dilution of FITC-conjugated IgG of sheep antibody specific to mouse immunoglobulin (Silenus Laboratories, Howthorn, Australia). Cells were then washed three times with PBS-FBS, and the fluorescence intensity of the cells was measured using a FACScan.

**Peptide Binding Assay by Flow Cytometry.** The binding of peptides to HLA-A*2402 molecules was tested as described previously (28, 29). Briefly, RMA-S-A*2402 cells were incubated at 26°C for 18–24 h. Cells (2 × 10^5) in 50 μl of PBS supplemented with 20% FBS (PBS-FBS) were incubated at 26°C for 3 h with a 50-μl solution of peptides at 10^{-7} to 10^{-8} μM and then at 37°C for 3 h. After washing with PBS-FBS, cells (2 × 10^6) were incubated for 30 min on ice with an appropriate dilution of mAb TP 25.99 (HLA-class I-specific mAb; a generous gift from Dr. S. Ferrone, New York Medical College, Valhalla, NY). After two washes with PBS-FBS, the cells were incubated for 30 min on ice with an appropriate dilution of FITC-conjugated IgG of sheep antibody specific to mouse immunoglobulin (Silenus Laboratories, Howthorn, Australia). Cells were then washed three times with PBS-FBS, and the fluorescence intensity of the cells was measured using a FACScan. RMA-S-A*2402 cells cultured at 26°C or at 37°C and stained with mAb TP25.99 under the same experimental conditions served as controls.

**Peptides.** Peptides were synthesized using standard N-(9-fluorenyl)methoxycarbonyl methodology and purified, and their sequences were confirmed by mass spectral analysis. Peptides were dissolved in DMSO at a concentration of 2 mg/ml and stored at −80°C.

**Cytokines.** Cytokines used in this study were obtained from the following sources: (a) GM-CSF, R&D Systems (Minneapolis, MN); (b) IL-1α, Genzyme (Cambridge, MA); (c) IL-7, Sigma (St. Louis, MO); and (d) IFN-γ, Endogen (Woburn, MA).

**Immunohistochemistry.** Immunohistochemical staining was performed to detect p53 protein with the streptavidin-biotin method using the Histofine SAB-PO kit (Nichirei, Tokyo, Japan). Tumor cell suspensions were placed on silane-coated slides, and then the slides were fixed with 95% ethanol. Endogenous peroxidase activity was blocked in methanol with 3% hydrogen peroxidase for 30 min. Nonspecific conjugation was blocked with 10% normal goat serum. p53 immunohistochemical staining was performed with murine mAb DO-7 (Novocastra) and rabbit antibody RSP53 (Nichirei), which binds to both wild-type and mutant p53 protein.

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Technologies, Inc.) and distributed in T-75 flasks (Falcon, Becton Dickinson, NJ). After 2 h of incubation, nonadherent cells were removed by gentle washing, and AIM-V medium containing GM-CSF (1000 units/ml) was added. After 6 days of incubation, IL-1α (50 units/ml) was added to the medium. One day later, nonadherent DCs were harvested and used as antigen-presenting cells. The DCs were resuspended in AIM-V medium containing 40 μg/ml peptide and incubated at 37°C for 4 h. Subsequently, the peptide-pulsed DCs were irradiated (3000 rad) and washed. At day 0, 3 × 10^6 PBMCs and 3 × 10^5 peptide-pulsed DCs were cocultured in 24-well tissue culture plates in a final volume of 2 ml of AIM-V medium supplemented with 5% human AB serum and IL-7 (5 ng/ml). On day 7, and weekly thereafter, responder cells were restimulated with peptide-pulsed autologous adherent PBMCs. These cells were prepared as follows: irradiated PBMCs (4 × 10^6) were incubated for 2 h in a final volume of 0.5 ml well AIM-V medium containing 10 μg/ml peptide in a 24-well plate; and then nonadherent cells and excess peptide-containing medium were removed. The responder lymphocytes in culture medium

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**Fig. 4** A. Cytolytic activity of bulk populations of anti-p53_{125-134} peptide-specific CTLs against HLA-A24^+ tumor cell lines. Target cell lines were incubated for 48 h with IFN-γ (1000 units/ml) before the lysis assay. B, cytolytic activity of three cell lines (HSC-4, Kuma-3, and Ca9-22) is inhibited by anti-HLA class I mAbs, but not by anti-HLA-DR mAbs. CTLs were added at an E/T ratio of 80:1.

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**Fig. 5** Lysis of MO24 cells pulsed with p53_{125-134} peptide (TYSPAL-NKMF) by cloned anti-p53_{125-134} CTL TH#99.

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**Fig. 6** Comparative analysis of the reactivity of the bulk population and cloned anti-p53_{125-134} peptide-specific CTLs against MO24 target cells pulsed with various concentrations of the p53_{125-134} peptide. CTLs were added at an E/T ratio of 20:1.
supplemented with IL-2 (10 IU/ml) and IL-7 (5 ng/ml) were added to the 24-well plate containing peptide-pulsed autologous adherent PBMCs. After five rounds of stimulation, responder cells were analyzed for their phenotype and tested for their specificity.

The CTLs were cloned from the bulk cell line at a density of 1 cell/well in 96-well plates. Each well contained 5 × 10^4 irradiated PBMCs from two different healthy donors in 0.2 ml/well AIM-V medium containing 5% AB serum, IL-2 (100 IU/ml), IL-4 (1 ng/ml), and 1 × 10^4 peptide-pulsed allogeneic HLA-A24^+ EBV-B cells. The cloned CTLs were stimulated with irradiated allogeneic HLA-A24^+ EBV-B cells that had been incubated with peptide (10 μg/ml) for 2 h and washed. Peptide-specific CTL clones were then restimulated every week and expanded using irradiated allogeneic PBMC mixtures and A24^+ EBV-B cells pulsed with peptide as feeder and stimulator cells, respectively. After transferring to 24-well plates, irradiated allogeneic EBV-B cells were added as feeder cells to the clones instead of PBMC mixtures.

**Flow Cytometric Analysis.** The effector cells were incubated with FITC-conjugated mAbs to CD3, CD4, CD8, or CD16 (all purchased from Becton Dickinson, Mountain View, CA) for 30 min on ice and washed twice. All incubations and washes were performed in PBS containing 0.1% NaN_3 and 2% FBS. Cells were analyzed on a FACScan (Becton Dickinson).

**Chromium Release Assay.** Chromium release assays were performed as described previously (31). Briefly, 1 × 10^6 target cells were incubated with 51Cr for 1 h. Effector cells were added to 2 × 10^3 target cells in triplicate wells of V-bottomed microtiter plates (Limbro; Flow Laboratories, McLean, VA) in a final volume of 0.2 ml. After a 4-h incubation, the supernatant was harvested, and chromium release was measured. Tumor cell lines were pretreated for 48 h with IFN-γ (1000 units/ml) before the lysis assay.

Antigen specificity of tumor cell lysis was further determined in a cold target inhibition assay by analysis of the ability of the peptide-pulsed unlabeled (cold) HLA-A24^+ EBV-B cell line (MO24) to inhibit lysis of 51Cr-labeled tumor cell lines.

**RESULTS**

**Binding Capacity of Wild-type Sequence p53 Peptides to HLA-A24 Molecules.** Kubo et al. (32) reported the HLA-A24 allele-specific motif Y, F, M, or W at position 2 and F, L, I, W, or M at position 9 or 10. On the basis of the predicted HLA-A24 allele-specific motifs, nonamer or 10-mer peptides were searched for in the wild-type p53 protein. Six peptides were identified that possessed an HLA-A24 binding motif. These peptides were synthesized, and their binding affinities were analyzed in an HLA-A24 stabilization assay. HLA-A24-restricted HIV-1 CTL epitope RYLRDQQLGI (amino acid position in HIV envelope, 584–594) and HLA-B35 binding peptide VPVKLKPGM derived from HIV polymerase (amino acid position in HIV polymerase, 163–171) served as positive and negative controls, respectively. As shown in Fig. 1, all of the peptides synthesized bound to HLA-A24 molecules, but with different affinities. Two peptides, p53125–134 and p53204–212, bound to HLA-A24 with high affinity, and four peptides were intermediate to low binders (Table 1).

![Figure 7](https://clincancerres.aacrjournals.org)
**Induction of CTL Cell Lines Specific for p53 Peptide.**

To achieve optimal induction of CTLs specific for the p53 peptides, we used DCs generated from PBMCs obtained from a healthy donor. The DCs were generated in the presence of GM-CSF and IL-4, pulsed with p53 peptide, irradiated, and used as stimulator cells. The CTL cultures were initiated in the presence of IL-7, and the responding cells were restimulated on day 7 with peptide-pulsed adherent PBMCs in medium supplemented with IL-2 and IL-7. Thereafter, the responding cells were continually restimulated with peptide-pulsed adherent PBMCs in the cytokine-supplemented medium. Of the six peptides studied, one high binder peptide (p53<sub>125–134</sub>) was able to elicit CTLs. After five rounds of stimulation, the bulk effector effectively lysed HLA-A24<sup>+</sup> EBV-B cell line (MO24) target cells pulsed with relevant peptide. Irrelevant peptide (p53<sub>204–212</sub>)-pulsed and untreated target cells, as well as K562 cells, were not lysed by these effector cells (Fig. 2). A flow cytometric analysis of the responding cells was performed. On day 40, most responding cells were CD3<sup>+</sup> and CD8<sup>+</sup> (CD3<sup>+</sup>, 99.7%; CD4<sup>+</sup>, 3.7%; CD8<sup>+</sup>, 96.4%; CD16<sup>+</sup>, 0.2%). The other peptide with high affinity to HLA-A24 molecules, p53<sub>204–212</sub>, did not elicit peptide-specific CTLs from these donors using this culture system.

**Recognition of HLA-A24<sup>+</sup> SCCHN by Anti-p53<sub>125–134</sub> CTLs.**

After five rounds of stimulation, the reactivity of anti-p53<sub>125–134</sub> CTLs against three HLA-A24<sup>+</sup> tumor cell lines was evaluated. These tumor cell lines have mutations in the p53 gene and accumulate p53 protein (Table 2). As shown in Fig. 3, none of these HLA-A24<sup>+</sup> tumor target cells were lysed by the bulk anti-p53<sub>125–134</sub> CTL cell line. In parallel to these assays, the tumor cells were pulsed with relevant peptide and shown to be effectively lysed, indicating that the anti-p53<sub>125–134</sub> CTLs can mediate cytolysis of targets presenting artificially high levels of the epitope (Fig. 3). These results suggested that the expression of epitope on tumor cells was too low to allow efficient recognition of tumor cell lines by CTLs. Therefore, to enhance HLA-class I and antigen presentation, target cells were pretreated with IFN-γ as described by Theobald et al. (18). Of five A24<sup>+</sup> SCCHN cell lines tested, three (HSC-4, Kuma-3, and Ca9-22) were lysed, and two (HSQ-89 and FS-1) were not (Fig. 4A). The lysis of the three SCCHN cell lines was blocked by anti-HLA-class I mAb, but not by anti-HLA-DR mAb (Fig. 4B).

To facilitate the further analysis of anti-p53 CTLs and possibly isolate a potent CTL, cloned CTLs were isolated from the bulk population of effectors by limiting dilution. After analysis of the reactivity of numerous CTL clones against peptide-pulsed MO24 cells, one of the more potent clones, designated TH#99, was selected for additional experiments. As shown in Fig. 5, this CTL showed strong reactivity against peptide-pulsed target cells at E:T cell ratios similar to the bulk population. Moreover, a comparative analysis of the CTL clone and the bulk population showed that the affinities of both effector cells are in the intermediate range and are comparable. They mediated half-maximal lysis of peptide-pulsed target cells at approximate concentrations of 10<sup>−7</sup> to 10<sup>−8</sup> M, respectively (Fig. 6). Three HLA-A24<sup>+</sup> SCCHNs that were sensitive to the bulk anti-p53 CTL cell line were pretreated with IFN-γ and tested as targets. As expected, the cloned CTL TH#99 efficiently lysed these SCCHN targets (Fig. 7A), and the lysis was blocked by anti-HLA-class I mAb, but not by HLA-DR mAb (Fig. 7B). The antigen specificity of the killing of HSC-4 tumor cells by this CTL clone was confirmed by showing that cold (unlabeled) targets that were pulsed with peptide p53<sub>125–134</sub> but not targets pulsed with an irrelevant peptide p53<sub>204–212</sub>, specifically inhibited the lysis of HSC-4 cells (Fig. 7C). These results confirm that the cloned CTLs can recognize the naturally occurring epitope but that a ligand density higher than that normally expressed by tumor cells is required for these CTLs to mediate cytolysis.

**DISCUSSION**

Naturally occurring p53-derived CTL epitopes presented by HLA-A2.1 molecules on the cell surface of tumors have been confirmed and p53 has become an attractive target for immunotherapy (16, 18, 21, 23). The HLA-A2.1 allele is the most frequently expressed class I allele among the different ethnic populations, but over 50% of the populations do not express the HLA-A2.1 allele. Therefore, identifying MHC class I-restricted epitopes derived from p53 other than those presented by HLA-A2.1 would allow coverage of many ethnic populations and broaden the applicability of p53-based immunotherapy. Besides the HLA-A2.1 allele, HLA-A24 is one of the most frequently expressed HLA-A alleles (24).

In the present study, we used peptide-pulsed autologous DCs as antigen-presenting cells and succeeded in generating an HLA-A24-restricted CTL cell line from PBMCs of a healthy donor in vitro that recognizes the wild-type sequence p53<sub>125–134</sub> epitope. The peptide-specific CTL cell line could lyse some HLA-A24<sup>+</sup> SCCHN cell lines. Moreover, a cloned CTL, TH#99, isolated from the bulk population of effectors by limiting dilution, lysed HSC-4, Kuma-3, and Ca9-22 cells more efficiently than the bulk CTL cell line. The killing activity was blocked by anti-HLA class I mAb. These results indicate that the p53<sub>125–134</sub> peptide is endogenously processed and presented on the surface of those tumor cells in association with HLA-A24 molecules. Interestingly, HSC-4 and Ca9-22 have point mutations at the same p53 codon (Table 2). However, HSC-4 was more sensitive to the CTLs than was Ca9-22. Treatment of tumor target cells with IFN-γ has been reported to enhance the expression of MHC class I antigens and proteasomes (33–36). In this study, three tumor cell lines that were sensitive to the CTLs expressed the HLA-A24 molecule, and their expression was enhanced by treatment with IFN-γ (data not shown). The HLA-A24 antigen expression of HSC-4 was much higher level than that of Ca9-22 (data not shown). These results may explain the difference in sensitivity to the CTLs. It is worthwhile to note that HSC-4, Kuma-3, and Ca9-22 were sensitive to the CTLs, whereas FS-1 and HSQ-89, which also express and accumulate mutant p53 molecules, were not sensitive to the CTLs. The relation between p53 mutations and presentation of CTL-defined p53 epitopes still remains to be elucidated. Ropke et al. (19) reported that accumulation of p53 protein was not an absolute requirement for killing because SCC-9 carcinoma cells, in which p53 protein was not detectable, could induce both tumor necrosis factor secretion and killing by p53-specific CTLs. The proteasome system represents a major source for the generation of MHC class I ligands (33, 34), and degradation of
p53 protein is dependent on proteasomal processing (35). CTL epitopes are produced via proteasomal processing and can be affected by mutations outside of epitope in addition to mutational alterations within epitopes. For example, the p53 mutation at residue 273 affected p53 264–272-specific CTL recognition of tumor cells carrying this mutation (36). Namely, because the R to H mutation at residue 273 inhibits proteolytic cleavage between residues 272 and 273, the flanking peptide epitope p53 264–273 is not produced sufficiently in tumor cells with this type of p53 mutation to be recognized by the epitope-specific and HLA-A*0201-restricted CTLs. In contrast, the same cells transfected with p53 genes harboring mutations at residues 143 or 175 were sensitive to lysis by the same effector cells. In our studies, two SCCIN cell lines (FS-1 and HSQ-89), which accumulate mutant p53 expressing missense mutation outside of the codons encoding the epitope, were not recognized by anti-p53 125–134 CTLs. The alterations in these tumor cells that might interfere with the processing and presentation of this epitope are presently unknown. As additional MHC class I and II-restricted p53 T-cell-defined epitopes are identified (37), and as tumor cell lines are characterized for their antigen processing and presentation, a more definitive pattern concerning p53 alterations/mutations that influence the presentation of CTL-defined epitopes will become evident. This relatively novel aspect of p53 alterations will become evident. This relatively novel aspect of p53-based immunotherapy. The election of p53 antigen-loss variants and the applicability of epitopes will become evident. This relatively novel aspect of p53 mutations in squamous cell carcinoma of the head and neck predominate in a subgroup of former and present smokers with a low frequency of genetic instability. Cancer Res., 57: 4070–4074, 1997.


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