Targeting phosphorylated p53 to elicit tumor-reactive T helper responses against head and neck squamous cell carcinoma

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ABSTRACT

The human T cell receptor is capable of distinguishing between normal and post-translationally modified peptides. Because aberrant phosphorylation of cellular proteins is a hallmark of malignant transformation, the expression of the phosphorylated epitope could be an ideal antigen to combat cancer without damaging normal tissues. p53 activates transcription factors to suppress tumors by upregulating growth arrest and apoptosis-related genes. In response to DNA damage, p53 is phosphorylated at multiple sites including Ser33 and Ser37. Here, we identified phosphorylated peptide epitopes from p53 that could elicit effective T helper responses. These epitope peptides, p53\textsubscript{22-41/Phospho-S33} and p53\textsubscript{22-41/Phospho-S37}, induced T helper responses against tumor cells expressing the phosphorylated p53 protein. Moreover, chemotherapeutic agents augmented the responses of such CD4 T cells via upregulation of phosphorylated p53. The upregulation of phosphorylated p53 expression by chemotherapy was confirmed in \textit{in vitro} and xenograft models. We evaluated phosphorylated p53 expression in the clinical samples of oropharyngeal squamous cell carcinoma and revealed that 13/24 cases (54\%) were positive for phosphorylated p53. Importantly, the lymphocytes specific for the phosphorylated p53 peptide epitopes were observed in the head and neck squamous cell cancer (HNSCC) patients. These results reveal that a combination of phosphorylated p53 peptides and chemotherapy could be a novel immunologic approach to treat HNSCC patients.

Introduction

Despite recent advances in treatment, the survival rate of head and neck squamous cell carcinoma (HNSCC) patients has not improved for decades. Because advanced stages are initially detected in most patients, their prognosis is poor. The clinical use of nivolumab, an immune checkpoint inhibitor, revealed that the immunotherapy could be a novel treatment for HNSCC patients. However, the biomarker for select responders has not yet been found, and the expensive cost of the reagents hinders the broad application of immune checkpoint inhibitors.\textsuperscript{1,3}

While some of the researchers have considered peptide vaccines as an old concept, optimal peptide conformation, immune adjuvants, and route of administration eliminated cancer in preclinical studies.\textsuperscript{4} Moreover, peptide vaccines have several advantages over other therapies such as cost-effectiveness, ease of synthesis, and a broad acceptance in clinical practice.\textsuperscript{5} Because CD4 helper T lymphocytes (HTLs) play a key role in direct killing of tumor cells in addition to their helper function for CD8 cytotoxic T lymphocytes (CTLs),\textsuperscript{6} the identification of a helper epitope from a tumor-associated antigen (TAA) is necessary to develop an effective peptide vaccine. Post-translational modifications, such as phosphorylation and acetylation, are unique targets in selecting a TAA.\textsuperscript{7} Andersen et al has described that CTLs can recognize the phosphorylated peptide as a completely different antigen compared to the non-phosphorylated peptide.\textsuperscript{8} In addition to CTLs, it has been reported that the T cell receptor (TCR) of HTLs has an ability to recognize a phosphorylated peptide derived from melanoma antigen (MART-1) recognized by T cells.\textsuperscript{9} Because phosphorylation is a hallmark of malignant transformation,\textsuperscript{10} phosphorylated peptide-targeted immunotherapy can be a novel approach to attack cancers without damaging normal tissues that weakly express TAA.\textsuperscript{7} The normal tissues that only express non-phosphorylated TAA might be spared in the phosphorylated antigen-targeted immunotherapy. Because MART-1 is only expressed in melanoma, the design of a phosphorylated peptide using a ubiquitous TAA would be valuable for the treatment of many types of cancer. p53 is an activator of proapoptotic signaling and widely expressed in tumors. More than 50\% of human malignancies have a p53 mutation,
and most of them are phosphorylated and acetylated. Accordingly, p53 is a candidate TAA to identify phosphorylated helper epitope peptides. Recently, we hypothesized that malignancy associated post-translated modifications could have a high immunogenicity and showed that acetylated p53 proteins served as the target of anti-tumor immunity. Not only hyperacetylation but also hyperphosphorylation have been observed in malignancies, and they occur at various stages of oncogenesis.

In the present study, we show that peptides p53$^{22-41}$/Phospho-S33 and p53$^{22-41}$/Phospho-S37 (referred as p-p53$^{S33}$ and p-p53$^{S37}$) were able to elicit antigen-specific, tumor-reactive HTL responses. The treatment of tumor cells with chemotherapeutic reagents enhanced these responses. These results suggest that the tumor-associated phosphorylated peptide vaccines with chemotherapy could be a promising approach in the HNSCC immunotherapy.

Results

Expression of phosphorylated p53 in oropharyngeal squamous cell carcinoma specimens

We first analyzed the expression of p53 and phosphorylated p53 in surgical specimens of oropharyngeal squamous cell carcinoma (OPSCC) by immunohistochemistry. The clinical parameters of 24 OPSCC patients are described in Supplemental Table 1. As shown in Fig. 1A, p53 was expressed in 18/24 cases (75%) of OPSCC. Phospho-p53$^{S33}$ and phospho-p53$^{S37}$ were expressed in 13/24 cases (54%) and 10/24 cases (42%), respectively (Fig. 1B and C). Ten cases (42%) of OPSCC were double positive for phospho-p53$^{S33}$ and phospho-p53$^{S37}$, indicating that the multiple serine residues of p53 are simultaneously modified in OPSCC. The expressions of HLA-DR and p16 (a surrogate marker of human papilloma virus infection) were found in 7/24 cases (29%) and 14/24 cases (58%), respectively (Supplemental Table 1). Phosphorylated p53 expression was not related to other parameters such as tumor stage, p16 expression, or HLA-DR expression (Supplemental Table 2). These results provide a rational basis for the development of immunotherapy to treat HNSCC by targeting phosphorylated p53 proteins.

In vitro induction of p53$^{22-41}$/Phospho-S33 and S37-specific CD4 T cell responses

The wild-type (wt) sequence peptide p53$^{25-35}$ was previously reported as a naturally processed epitope recognized by human HTLs. To create peptides containing the phosphorylated serine residues at position 33 or 37 in the p53 transactivation domain, the elongated peptide (p53$^{22-41}$) was assessed for its ability to elicit phosphorylated peptide-specific HTL responses. As shown in Fig. 2, the dual-phosphorylated peptide p53$^{22-41}$/Phospho-S33 and S37 (referred to as p-p53$^{S33}$ and S37) could induce phosphorylated peptide-specific HTL responses from two individuals. These HTLs only responded to p-p53$^{S33}$ and S37 and p-p53$^{S37}$ in a concentration-dependent manner but not to p-p53$^{S37}$ and non-phosphorylated wt p53$^{22-41}$, suggesting that the TCR of these HTLs is specific to phosphorylated Ser33. These responses were restricted to HLA-DR molecules (Fig. 2B), indicating that the phosphorylated peptide was sufficient to allow binding to MHC class II molecules. To determine which HLA class II molecules presented the phosphorylated p53 peptides to the HTLs, a panel of mouse fibroblasts expressing single HLA-DR molecules was used as antigen presenting cells (APCs). As shown in Fig. 2C, HTLs J5 and J16 recognized the
phosphorylated p53 peptide in the context of HLA-DR1, whereas the response of T20 was restricted by HLA-DR9. These findings demonstrate that the phosphorylated p53 peptides can bind to multiple HLA-DR molecules that cover a broad population of cancer patients. Taken together, these results revealed that HTL precursors that react to p-p53\textsubscript{S33} and S37 exist in human PBMCs, and phosphorylated Ser33 might be more immunogenic than phosphorylated Ser37.

\textbf{p53 Ser33 and Ser37 are immunogenic phosphorylation sites that induce CD4 T cell responses}

To confirm the immunogenicity of phosphorylated p53 peptides, we used the mono-phosphorylated peptides (p-p53\textsubscript{S33}, or p-p53\textsubscript{S37}) to elicit peptide-specific HTLs. As shown in Fig. 3A, we successfully isolated HTLs specific for phosphorylated peptide p53 Ser33 (K2 and J44 from donor 3 and donor1, respectively) and p53 Ser37 (H2 from donor 4) but not the non-phosphorylated wt p53\textsubscript{S22-41} peptide. HLA-DR molecules restricted the reaction of these HTLs (Fig. 3B). To define the restricting HLA class II molecules, L-cells transfected with individual HLA-DR genes as APCs to determine the restricting HLA class II molecules.

An increase in phosphorylated p53 was induced by treatment with chemotherapeutic reagents in vitro and in murine xenograft models

To test the ability of phosphorylated p53 peptide-reactive HTLs to directly recognize tumors, we first examined the expression of phosphorylated p53 in tumor cells. Western blot analysis showed that phosphorylated p53 was expressed in several tumor cell lines, including HNSCC (Fig. 4A-C). EBC1 (lung SCC) and SAS (HNSCC) cells expressed neither wt nor phosphorylated p53. Because DNA damage induces the phosphorylation of p53, tumor cells were treated with chemotherapeutic reagents. As previously reported, phosphorylated p53 expression was augmented by the addition of doxorubicin and cisplatin, a key drug for HNSCC treatment (Fig. 4C).
Figure 3. Induction of CD4 T cell responses using the mono-phosphorylated p53 peptide. (A) The phosphorylated peptide p-pS333-reactive CD4 T cells (K2 and J44) and p-pS337-reactive CD4 T cells (H2) were assessed for their ability to recognize various concentrations of phosphorylated peptides. Autologous PBMCs were used as APCs. (B) MHC restriction analysis of phosphorylated p53-reactive CD4 T cells. Peptide-induced T cell responses of p-pS333-reactive (K2 and J44) and p-pS337-reactive cells (H2) in the presence of anti-HLA DR mAb L243 or anti-HLA class I mAb 6/32 (negative control) were evaluated. Autologous PBMCs were used as APCs. (C) CD4 T cell responses to p-pS333-reactive (K2 and J44) and p-pS337-reactive cells (H2) were assessed using L-cells transfected with individual HLA-DR genes as APCs to determine the restricting HLA class II molecules.

Figure 4. Expression of total p53 protein and p53 carrying phosphorylated Ser33 or Ser37 in tumor cells. (A-C) The expressions of total p53 protein and phosphorylated p53 (phosphorylated Ser33 or Ser37) were examined by Western blotting. The tumor cell lines were treated with or without doxorubicin (1 μM) or cisplatin (5 or 10 μM) for 24 hours before assessment. HNSCC: HSC4, SAS, and Sa-3. Lung adenocarcinoma: LC-2 ad. Lung squamous cell carcinoma: EBC1. Bladder cancer: 5637.
In clinical settings, it is important to have an increase in phosphorylated p53 in tumors in vivo. Consistent with western blot findings, we observed that the positive staining of tumor cells for phosphorylated p53 (Fig. 5A-D), and total p53 levels (Supplemental Fig. 1A-D) were increased in murine xenograft tumors from cisplatin or doxorubicin treated mice. It is noteworthy that the chemotherapy induced a diffuse expression of phosphorylated p53 in the tumor. These findings suggest that such anti-cancer drugs enable not only an induction of immunogenic cell death (ICD) \cite{19,20}, but also enhancement of tumor antigens.

**Direct recognition of phospho-p53 positive tumor cells by phosphorylated peptide-reactive CD4 T cells**

Next, we evaluated whether phosphorylated p53-reactive HTLs could directly recognize phosphorylated p53 expressing tumor cells. The peptide-reactive HTLs and HLA-DR-matched phosphorylated p53-positive tumor cells were cocultured, and IFN-γ production in the culture supernatant was measured. As shown in Fig. 6A, all phosphorylated p53-specific HTLs (K2, J44, and H2) could directly recognize the HLA-DR-matched phosphorylated p53-positive tumor cells but not HLA-DR-matched phosphorylated p53-negative tumor cells in an HLA-DR dependent manner. These results indicate that the phosphorylation of p53 is maintained through the antigen processing machinery and the phosphorylated peptides in tumor MHC class II molecules are naturally presented to HTLs.

Because the Western blot analysis presented in Fig. 4 showed that p53 phosphorylated at Ser33 or Ser37 and total p53 protein expression levels in tumor cells were enhanced by the treatment with doxorubicin or cisplatin, tumor cells were pretreated with these reagents before coculturing with HTLs. As shown in Fig. 6B, the responses of p-p53\textsubscript{S33} or p-p53\textsubscript{S37}-reactive HTLs against tumor cells were augmented by treatment of the tumor cells with doxorubicin or cisplatin. Most importantly, granzyme B production from HTL clone J44 against tumor cells was observed with cisplatin treatment, indicating that the HTL had a cytotoxic effect on tumor cells (Fig. 6C). These results suggest that the accumulation of DNA damage by chemotherapy can act as an adjuvant to amplify expression of phosphorylated p53 and augment the efficacy of phosphorylated p53-targeted peptide vaccines.

**Recognition of phosphorylated p53 peptides by PBMCs from cancer patients**

The existence of precursor T cells specific for the phosphorylated peptides in the PBMCs of HNSCC patients is a
prerequisite for the application of a phosphorylated p53 peptide vaccine in the clinic. To evaluate the responses of T cells from HNSCC patients to p-p53\textsubscript{S33} and p-p53\textsubscript{S37}, we carried out a short-term culture using peptide-stimulated PBMC from five HNSCC patients. Since the volumes of blood obtained from these patients were small, we could not establish T cell lines to perform more detailed studies or HLA restriction analyses. A tetanus toxoid peptide (TT\textsubscript{830-843}) was used as a positive control because this peptide generates robust HTL responses regardless of the HLA-DR alleles.

As shown in Table 1, substantial T cell responses to phosphorylated p53 peptides were observed in HNSCC patients but not in healthy donors, suggesting that the precursor of phosphorylated p53-reactive HTLs exists in HNSCC patients. Moreover, HTL responses to p-p53\textsubscript{S33} was

![Figure 6. Direct tumor recognition by phosphorylated peptide-specific CD4 T-cells. Direct tumor recognition of naturally processed p53 antigen expressed in tumor cells by p-p53\textsubscript{S33}-reactive clones (K2 and J44) and the p-p53\textsubscript{S37}-reactive clone (H2). (A) p-p53\textsubscript{S33}-reactive T cells (K2 and J44, DR-1-restricted) were tested for their capacity to recognize HLA-DR1\textsuperscript{+}/p53 positive (HSC4, LC-2/ad and 5637) or DR1\textsuperscript{+}/p53 negative (EBC1) tumor cells, which was used as a negative control. The p-p53\textsubscript{S37}-reactive clone (H2) was tested for its capacity to recognize antigens directly on HLA-DR9\textsuperscript{+}/p53 positive tumor cells (Sa3, 5637) or DR9\textsuperscript{+}/p53 negative (SAS) tumor cells. (B) Tumor cells were treated with doxorubicin or cisplatin 24 hours before coculture with T cells (K2, J44 and H2). (C) Granzyme B production by the p-p53\textsubscript{S33}-reactive clone (J44) during coculture with tumor cells.](image)

Table 1. Assessment of T-cell responses to the phosphorylated p53 peptides in HNSCC patients and healthy donors

| No. | Sex | Age (y) | Pathological stage | Primary site | No antigen | p-p53\textsubscript{S33} | anti-HLA-DR mAb | p-p53\textsubscript{S37} | anti-HLA-DR mAb | TT\textsubscript{830-843} |
|-----|-----|---------|-------------------|-------------|------------|------------------|----------------|------------------|----------------|----------------|
| 1   | M   | 69      | T2N0M0            | Larynx      | <           | 124 ± 73         | <               | 67 ± 22          | <               | 195 ± 40       |
| 2   | M   | 71      | T2N0M0            | Larynx      | <           | 216 ± 34         | <               | 41 ± 20          | <               | 305 ± 157      |
| 3   | M   | 75      | T3NcM0            | Oropharynx  | <           | 155 ± 90         | <               | 57 ± 20          | <               | 167 ± 101      |
| 4   | M   | 47      | T2N0M0            | Oropharynx  | <           | 198 ± 89         | <               | 92 ± 39          | <               | 266 ± 163      |
| 5   | F   | 79      | T2N0M0            | Hypopharynx | <           | 236 ± 95         | <               | 97 ± 32          | <               | 388 ± 86       |
| 6   | M   | 37      | Normal Volunteer  |             | <           | 47 ± 5           | <               | 45 ± 7           | <               | 414 ± 296      |
| 7   | M   | 34      | Normal Volunteer  |             | <           | 56 ± 7           | <               | 43 ± 3           | <               | 721 ± 401      |
| 8   | F   | 52      | Normal Volunteer  |             | <           | 43 ± 5           | <               | 41 ± 11          | <               | 325 ± 133      |

<: less than the lower limit of detection (mean <30 pg/ml). No. 1–5 are HNSCC patients and No. 6–8 are healthy donors.
higher than that to p-pS33. This result indicates that phosphorylated Ser33 might be more immunogenic than phosphorylated Ser37, which is consistent with the results in Fig. 2A.

Discussion

In this study, we demonstrated that the phosphorylated peptides from p53 tumor suppressor proteins are candidates for a helper peptide vaccine to treat HNSCC. Since MDM2 tightly regulates the amount of p53 under normal conditions, the amount of p53 is maintained at a very low level. On the other hand, tumors frequently express an aberrant amount of p53. Consequently, it appears that self p53-reactive T cells, which have a low affinity for TCR, might only react to tumor (high burden of p53) but not to normal tissues (low burden of p53). Because only lymphocytes from HNSCC patients but not from healthy donors reacted to phosphorylated p53 peptides in a short-term culture (Table 1), the increased amount of p53 in tumors may spontaneously increase the number of p53-reactive HTLs. In response to DNA damaging reagents or ultraviolet irradiation, the phosphorylation of p53 at multiple sites including transactivation domain (Ser33 and Ser37) increases the stability and activity of p53 by decreasing its ability to bind MDM2 in tumors. Because T cells have been reported to precisely recognize phosphorylated epitopes and post-translationally modified epitopes on MHC molecules, the phosphorylated epitope peptides from p53 would be an ideal antitumor vaccine to combine with conventional therapies. Phosphorylated p53 peptides bound to multiple HLA-DR alleles (Fig. 2C), indicating that these peptides can be applied to a broad population.

There are multiple phosphorylated serine residues in p53, including Ser6, Ser9, Ser15, Ser18, Ser20, Ser33, Ser37, Ser46, Ser52, and Ser57. We targeted Ser33 and Ser37 because they are located close to the sequence previously reported for eliciting HTL responses against wt p53 peptides. There is an overlap between the peptide sequence that we selected and the p53S25-35 peptide previously defined as a CTL epitope, indicating that this peptide has a capacity to activate p53-reactive CTLs in addition to p53-reactive HTLs. Although the phosphorylation of p53 might induce phosphorylated peptide-specific CTL responses, it is also possible that the phosphorylation of the p53S25-35 peptide might attenuate the stability of the MHC-non-phosphorylated peptide-TCR complex to stimulate CTLs. Further research is required to examine whether our peptide has an ability to elicit phosphorylated p53-reactive CTLs. The stability of phosphorylated peptides in vivo also remains to be elucidated.

While T-cell based immunotherapy mainly focuses on CTLs, HTLs have cytotoxic activity against tumor cells in addition to their helper function. In addition to the pMART-1-specific HTL response, this report opens the avenue to utilize phosphorylated serine bearing peptides as helper epitope peptides. To induce robust responses to peptide vaccines in vivo, the appropriate selection of adjuvants is indispensable. Toll-like receptor (TLR) 3 or 7 ligand is one of the desirable adjuvants for an antitumor peptide vaccine. TLR3 and TLR7 ligands induce the expression of MHC Class II on DCs, which plays an indispensable role in activating HTLs through the presentation of peptide. Interestingly, imiquimod, a TLR7 ligand, induced apoptosis through p53 activation. However, p53 signaling increases the expression of TLR3, indicating that the TLR3 or 7 ligand elicits p53 expression in a positive feedback loop. Thus, these adjuvants may work in two different ways: 1) activation of APCs or 2) the upregulation of antigens (p53). Because we previously reported that the EGFR inhibition augments the expression of MHC Class II on HNSCC cells, an EGFR inhibitor that has been currently available as a treatment option in the patients with HNSCC could be an interesting adjuvant to combine with the phosphorylated p53 peptide vaccine.

During the malignant transformation, post-translational modifications play a pivotal role. A number of tumor cell types tend to accumulate the p53 protein, which mainly exhibits phosphorylation and acetylation. The DNA-damaging reagents such as cisplatin and doxorubicin augmented the expression of phosphorylated p53, the recognition of the tumor was augmented by phosphorylated p53-specific HTLs (Fig. 4, 6B, and 6C). Having been first-line treatments for HNSCC patients, platinum-based drugs are acceptable to use as an adjuvant. Cisplatin has been reported to upregulate the expression of antigen processing machinery components. Since the induction of ICD has been appreciated in doxorubicin, the effect of cisplatin in inducing ICD might be inferior to its derivative oxaliplatin. However, it is still possible that cisplatin stimulates APCs by inducing HMGB1 from dead tumor cells. Interestingly, p53 activation might also play a role in stimulating innate immunity via the induction of HMGB1 and ATP from tumor cells. Thus, chemotherapeutic reagents activate APCs and increase the amount as well as the presentation of antigens in tumors. Moreover, the production of IFN-γ from T cells can counteract platinum-resistance by altering the metabolism in cancer-associated fibroblasts, indicating that the cisplatin-induced infiltration of T cells renders tumors sensitive to chemotherapy. Collectively, non-lymphoablative chemotherapy and T-cell-targeted immunotherapy could attack tumors by complementing each other.

In conclusion, we have identified novel helper epitopes from phosphorylated tumor protein p53 (p-pS33 and p-pS37) that can induce antigen-specific HTL responses. The variant of amino acid sequences of these peptides has not been reported, suggesting that these peptides are well-conserved. Furthermore, cisplatin and doxorubicin may work as adjuvants for immunotherapy by upregulating phosphorylated p53. This approach could be a desirable therapy to treat HNSCC with chemotherapy, which expresses a high level of phosphorylated p53.

Materials and Methods

Cell lines

Mouse fibroblast cell lines (L-cells) expressing individual human HLA-DR molecules (DR1, DR4, DR9, DR15, and DR53) were kindly provided by Dr. Robert W. Karr (Karr Pharma, St. Louis, MO) and Dr. T. Sasazuki (International Medical Center of Japan, Tokyo, Japan). The HNSCC cell
lines HSC4 (tongue SCC), Sa-3 (gingival SCC), and the lung cancer cell lines LC-2/ad (adenocarcinoma) and EBC1 (squamous cell carcinoma) were supplied by the RIKEN Bio-Resource Center (Tsukuba, Japan). The HNSCC cell line SAS (tongue SCC) was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The bladder cancer cell line 5637 was obtained from Cell Resource Center for Biomedical Research Institute of Development (Aging and Cancer, Tohoku University, Sendai, Japan). HSC4, LC-2/a/d and 5637 cells possess mutant p53R248Q, p53S241 C and p53R280 T protein, respectively. EBC1 and SAS cells lack any detectable p53 protein. All cell lines were maintained in a tissue culture medium as recommended by the supplier and incubated at 37°C in a humidified atmosphere with 5% CO₂.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded (FFPE) samples were prepared from pretreatment biopsy tissues of oropharyngeal squamous cell carcinoma (OPSCC) patients or the subcutaneous tumors of BALB/c-nu/nu mice inoculated with HSC4 cells. An indirect immunoperoxidase technique (the streptavidin-biotin method) was performed. Polyclonal rabbit anti-human phospho-p53S33, phospho-p53S37 (1:100, CST, #2526 and #9289, Denver, CO), mouse anti-human p53 mAb (1:50, Santa Cruz, sc-126, DO-1, CA), monoclonal mouse anti-human HLA-DR monoclonal antibodies.

The p53 staining patterns were characterized into three categories: (a) ++: overexpression (at least 50% of the tumor cells express nuclear staining); (b): +: normal expression (no more than 49% of the tumor cells express nuclear staining); and (c): -: negative when there was a complete absence of staining in the tumor. The p53 expression in neighboring normal tissues served as an internal positive control. In order to quantify the number of DAB staining cells, we used a BZ-X710 microscope (Keyence, Tokyo, Japan) with the BZ-H3 hybrid cell count software (Keyence).

**Synthetic peptides**

We selected peptide epitopes from the previously described sequence. Synthetic peptides were purchased from GenScript (Tokyo, Japan). The wild-type (wt) p53S22-41 (LWKLLFLPENVLSPSLSAMQR), p53S22-41/Phospho-S33 and S37, p53S22-41/Phospho-S33 and S37, and p53S22-41/Phospho-S33 (referred as p-p53S33 and p-p53S37, p-p53S33, p-p53S37) peptides were used throughout this work. The tetanus toxoid (TT830-843; QYIKANSEKIGITE) peptide was used as a positive control that could bind to multiple HLA class II molecules.

**In vitro induction of peptide-reactive CD4 helper T cells**

The procedure utilized for the generation of peptide-reactive HTL lines from peripheral blood mononuclear cells (PBMCs) of healthy donors (donor 1: HLA-DR1/9, DR53, donor 2: HLA-DR9/14, 53, donor 3: HLA-DR1/15, 51, and donor 4: HLA-DR4/9, 53) was described in detail. In short, HTLs purified by CD4 MACS microbeads (Miltenyi Biotech, Auburn, CA) were stimulated with peptide-pulsed autologous dendritic cells (DCs) and followed by 2–3 cycles of stimulations with γ-irradiated autologous PBMCs and peptides (3 µg/mL). Peptide-reactive HTLs that showed at least a three-fold increase in cytokine production in response to peptide stimulation compared to the non-peptide control were expanded. HTL clones were isolated by limiting dilution. All blood samples from human healthy donors were collected after oral and signed informed consent (the institutional ethics committee gave approval number #16040).

**Measurement of antigen-specific responses with CD4 T cells**

An evaluation of HTL responses to diverse antigens was performed as previously described. The tumor cells were treated with IFN-γ (500 IU/ml, PeproTech, Rocky Hill, NJ) for 48 hours to induce the expression of HLA-DR before culturing with HTLs. To enhance phosphorylation of p53 in tumor cells, the tumor cells were treated with or without 1 µM doxorubicin (CST, Denver, CO), or cisplatin (5 or 10 µM; R&D Systems, Minneapolis, MN) during the last 24 hours of IFN-γ treatment.

**Granzyme B ELISA**

To evaluate the cytotoxic activity of HTL clones, the supernatant from antigen-specific responses by CD4 T cells were collected and assessed for Granzyme B by ELISA (MABTECH, Stockholm, Sweden) according to manufacturer’s instructions.

**Western blot analyses**

Expression of p53 was assessed by Western blotting after the treatment of tumor cell lines with or without 1 µM doxorubicin or cisplatin (5 or 10 µM) for 24 hours. The tumor cell lines were lysed in LDS sample buffer (Invitrogen, #NP0008, Carlsbad, CA), and the lysates were submitted to electrophoresis in NuPAGE bi-Tris SDS-PAGE gels (Invitrogen, #NP0322PK2, Carlsbad, CA). Antibodies used to detect specific protein expression were as follows: mouse anti-human p53 mAb (1: 1,000, Santa Cruz, sc-126, DO-1, CA), rabbit anti-human phospho-p53S33 mAb (1: 1,000, CST, #2526, Denver, CO), rabbit anti-human phospho-p53S37 mAb (1: 1,000, CST, #9289, Denver, CO), and mouse anti-β-actin (1:1,000, Santa Cruz, sc-47778, C4, Santa Cruz, CA).

**Analysis of wild-type p53 and phosphorylated p53 expression in a xenograft model**

Six-week-old female BALB/c-nu/nu mice were obtained from Charles River Japan (Yokohama, Japan) and were maintained in accordance with the animal facility at the Asahikawa Medical University. Five mice were injected subcutaneously with 2 × 10⁶ HSC4 cells in a flank. When the tumor reached
5 mm, PBS (control), cisplatin (7.5 mg/kg), or doxorubicin (5 mg/kg) was injected into the peritoneal cavity. Twenty-four hours later, mice were sacrificed, and tumor samples were collected for evaluating the p53 expression by immunohistochemistry. All animal experiments were approved by the Institutional Animal Care and Use Committee of Asahikawa Medical University.

Measurement of peptide-specific responses in cancer patients

Peripheral blood lymphocytes were isolated from fresh heparinized whole blood of HNSCC patients by Lymphoprep™ (Axis-Shield PoC AS, Oslo, Norway)-centrifugation, and cultured with peptides (10 μg/mL) in 96-well plates as described previously. 74 The lymphocytes were stimulated for 2 cycles (7 days/cycle). Seven days after the second peptide stimulation, production of IFN-γ or GM-CSF was assessed by ELISA (BD PharMingen, #555142). The institutional ethics committee approved this study protocol (approval number #16040).

Statistical analysis

The data were analyzed using Student’s t-test and Chi-squared test. p < 0.05 indicates statistical significance. Error bars in the data indicate the standard error of the mean (SEM). GraphPad Prism 6 was used for the analyses.

Disclosure of Potential Conflicts of Interest

Esteban Celis has filed patent applications based on the use of synthetic peptides and poly-IC combinatorial vaccines. The rights of the patent applications have been transferred to the Moffitt Cancer Center (Tampa, FL). The other authors declare no conflict of interest.

References

1. Vermorken JB, Remenar E, van Herpen C, Gorlia T, Mesia R, et al. Pembrolizumab in combination with gemcitabine and cisplatin compared to gemcitabine and cisplatin as first-line treatment for locally advanced or metastatic bladder cancer: results of the randomized phase III KEYNOTE-040 study. J Clin Oncol. 2018;36:11–9. doi:10.1200/JCO.2017.76.7591
2. DeLeo AB. The wild-type sequence (wt) p53(25-35) peptide stimulates anti-tumor CD4 T cells. J Immunol. 2006;176:6017–21. doi:10.4049/jimmunol.177.10.6795
3. Kumai T, Ishihashi K, Iikawa K, Matsuura Y, Aoki N, Kimura S, Hayashi S, Kitada J, Harabuchi Y, Celis E, et al. Induction of tumor-reactive T helper responses by a posttranslational modified epitope from tumor protein p53. Cancer Immunol Immunother. 2014;63:469–78. doi:10.1007/s00262-014-1533-z
4. Andersen MH, Bonfill JE, Neisig A, Arsequell G, Sondergaard I, Valencia G, Neefjes J, Zeuthen J, Ehmke T, Haane CSJ, et al. Phosphorylated peptides can be transported by TAP molecules, presented by class I MHC molecules, and recognized by phosphopeptide-specific CTL. J Immunol. 1999;163:3812–8. doi:10.1074/jimmunol.1998051206. PMID:95981576
5. Kumai T, Lee S, Cho HI, Sultan H, Kobayashi H, Harabuchi Y, Lewis ST, Engelhard VH, Hunt DF. Identification of class I MHC-bound phosphorylated peptides as targets for cancer immunotherapy. PNAS. 2009;106:12073–8. doi:10.1073/pnas.0903852106. PMID:19581576
6. Poeta ML, Manola J, Goldwasser MA, Benoit N, Califano JA, Ridge JA, Goodwin J, Kenady D, Saunders J, et al. TP53 mutations and survival in squamous-cell carcinoma of the head and neck. N Engl J Med. 2007;357:2552–61. doi:10.1056/NEJMoa073770. PMID:18094376
7. Levine AJ, p53, the cellular gatekeeper for growth and division. Cell. 1997;88:323–31. doi:10.1016/S0092-8674(00)81871-1
8. Andersen MH, Bonfill JE, Neisig A, Arsequell G, Sondergaard I, Valencia G, Neefjes J, Zeuthen J, Ehmke T, Haane CSJ, et al. Phosphorylated peptides can be transported by TAP molecules, presented by class I MHC molecules, and recognized by phosphopeptide-specific CTL. J Immunol. 1999;163:3812–8. doi:10.1074/jimmunol.1998051206. PMID:95981576
9. Poeta ML, Manola J, Goldwasser MA, Forastiere A, Benoit N, Califano JA, Ridge JA, Goodwin J, Kenady D, Saunders J, et al. TP53 mutations and survival in squamous-cell carcinoma of the head and neck. N Engl J Med. 2007;357:2552–61. doi:10.1056/NEJMoa073770. PMID:18094376
10. Levine AJ, p53, the cellular gatekeeper for growth and division. Cell. 1997;88:323–31. doi:10.1016/S0092-8674(00)81871-1
11. Minamoto T, Buschmann T, Habelhah H, Matuschek E, Tahara H, Boersensen-Dale AL, Harris C, Sidransky D, Ronai Z. Distinct pattern of p53 phosphorylation in human tumors. Oncogene. 2001;20:3341–7. doi:10.1038/sj.onc.1204458. PMID:11429384
12. Poeta ML, Manola J, Goldwasser MA, Forastiere A, Benoit N, Califano JA, Ridge JA, Goodwin J, Kenady D, Saunders J, et al. TP53 mutations and survival in squamous-cell carcinoma of the head and neck. N Engl J Med. 2007;357:2552–61. doi:10.1056/NEJMoa073770. PMID:18094376
13. Levine AJ, p53, the cellular gatekeeper for growth and division. Cell. 1997;88:323–31. doi:10.1016/S0092-8674(00)81871-1
14. Poeta ML, Manola J, Goldwasser MA, Forastiere A, Benoit N, Califano JA, Ridge JA, Goodwin J, Kenady D, Saunders J, et al. TP53 mutations and survival in squamous-cell carcinoma of the head and neck. N Engl J Med. 2007;357:2552–61. doi:10.1056/NEJMoa073770. PMID:18094376
15. Levine AJ, p53, the cellular gatekeeper for growth and division. Cell. 1997;88:323–31. doi:10.1016/S0092-8674(00)81871-1
16. Blagosklonny MV, An WG, Romanova LY, Trepel J, Fojo T, Slamon DJ. Cisplatin, doxorubicin, and paclitaxel induce hypoxia-inducible factor-stimulated transcription. Cancer Res. 1998;58:1327–34. doi:10.1158/0008-5472.CAN-98-0243. PMID:9517555
17. Appella E, Anderson CW. Post-translational modifications and cancer-old concept revisited. Curr Opin Immunol. 2001;13:159–65. doi:10.1016/S0952-7915(01)00022-X. PMID:11358490
18. Fraser M, Bai T, Tsang BK. Akt promotes cisplatin resistance in cancer. Clin Cancer Res. 2017;23:4065–77. doi:10.1158/1078-0432.CCR-16-0881. PMID:28380935
19. Janse JM, van Veelen PA, Melief CJ, van der Burg SH, Arens R. Tumor Eradication by Cisplatin Is Sustained by CD80/86-Mediated Antitumor CD4 T Cells. Cancer Immunol Res. 2017;5:72–83. doi:10.1158/2326-6066.CIR.2017-0104.
20. Galluzzi L, Buque A, Kepp O, Zitvogel L, Kroemer G. Immunogenic cell death in cancer and infectious disease. Nat Rev Immunol. 2017;17:97–111. doi:10.1038/nri.2016.107. PMID:27748397
21. Panina-Bordignon P, Tan A, Termijtelen A, Demotz S, Corradin G, Lanzavecchia A. Universally immunogenic T cell epitopes:
promiscuous binding to human MHC class II and promiscuous recognition by T cells. Eur J Immunol. 1989;19:2327–42. doi:10.1002/eji.1830191209. PMID:2481588

22. Olivier M, Hollstein M, Hainaut P. TP53 mutations in human cancers: origins, consequences, and clinical use. Cold Spring Harb Perspect Biol. 2010;2:a001008. doi:10.1101/cshperspect.a001008. PMID:20182602

23. Ichwan SJ, Yamada S, Sumrejkanchanakij P, Ibrahim-Auerkari E, Eto K, Ikeda MA. Defect in serine 46 phosphorylation of p53 contributes to acquisition of p53 resistance in oral squamous cell carcinoma cells. Oncogene. 2006;25:1216–24. doi:10.1038/sj.onc.1209158. PMID:16247456

24. Sakaguchi K, Herrera JE, Saito S, Miki T, Bustin M, Vassilev A, Anderson CW, Appella E. DNA damage activates p53 through a phosphorylation-acetylation cascade. Genes Dev. 1998;12:2831–41. doi:10.1101/gad.18.2831. PMID:9748680

25. Petersen J, Purcell AW, Rossjohn J. Post-translationally modified T cell epitopes: immune recognition and immunotherapy. J Mol Med. 2009;87:1045–51. doi:10.1007/s00109-009-0526-4. PMID:19763524

26. Engelhard VH, Altrich-Vanlith M, Ostankovitch M, Zarling AL, Weck MM, Grunebach F, Werth D, Sinzger C, Bringmann A, Theobald M, Biggs J, Dittmer D, Levine AJ, Sherman LA. Phosphorylated peptides are naturally processed and presented by major histocompatibility complex class I molecules in vivo. J Exp Med. 2000;192:1755–62. doi:10.1084/jem.192.12.1755. PMID:11120772

27. Zarling AL, Ficarro SB, White FM, Shabanowitz J, Hunt DF, Engelhard VH. Phosphorylated peptides are naturally processed and presented by major histocompatibility complex class I molecules in vivo. J Exp Med. 2000;192:1755–62. doi:10.1084/jem.192.12.1755. PMID:11120772

28. Dai C, Gu W. p53 post-translational modification: deregulated in tumorigenesis. Trends Mol Med. 2010;16:528–36. doi:10.1016/j.molmed.2010.09.002. PMID:20932800

29. Theobald M, Biggs J, Dittmer D, Levine AJ, Sherman LA. Targeting p53 as a general tumor antigen. PNAS. 1995;92:11993–7. doi:10.1073/pnas.92.26.11993. PMID:8618830

30. Weck MM, Grunebach F, Werth D, Sinzger C, Bringmann A, Brossart P. TLR ligands differentially affect uptake and presentation of cellular antigens. Blood. 2007;109:3890–4. doi:10.1182/blood-2006-04-15719. PMID:17218388

31. Huang SW, Chang SH, Mu SW, Jiang HY, Wang ST, Kao JK, Huang JL, Wu CY, Chen YJ, Shieh JJ. Imiquimod activates p53-dependent apoptosis in a human basal cell carcinoma cell line. J Dermatol Sci. 2016;81:182–91. doi:10.1016/j.jdermsci.2015.12.011. PMID:26775629

32. Shatz M, Menendez D, Resnick MA. The human TLR innate immune gene family is differentially influenced by DNA stress and p53 status in cancer cells. Cancer Res. 2012;72:5948–57. doi:10.1158/0008-5472.CAN-11-4134. PMID:22673234

33. Taura M, Eguma A, Suico MA, Shuto T, Koga T, Komatsu K, Komune T, Sato T, Saya H, Li JD, et al. p53 regulates Toll-like receptor 3 expression and function in human epithelial cell lines. Mol Cell Biol. 2008;28:6557–67. doi:10.1128/MCB.01202-08. PMID:18779317

34. Kumai T, Matsuda Y, Oikawa K, Aoki N, Kimura S, Harabuchi Y, Celis E, Kobayashi H. EGFR inhibitors augment antitumor helper T-cell responses of HER family-specific immunotherapy. Br J Cancer. 2013;109:2155–66. doi:10.1038/bjc.2013.577. PMID:24045666

35. Krüger KE, Srivastava S. Posttranslational protein modifications: current implications for cancer detection, prevention, and therapeutics. Mol Cell Proteomics. 2006;5:1799–810. doi:10.1074/mcp.R600009-MCP200. PMID:16844681

36. Haddad R, Colevas AD, Tishler R, Busse P, Goguen L, Sullivan C, Norris CM, Lake-Willcutt B, Case MA, Costello R, et al. Docetaxel, cisplatin, and 5-fluorouracil-based induction chemotherapy in patients with locally advanced squamous cell carcinoma of the head and neck: the Dana Farber Cancer Institute experience. Cancer. 2003;97:412–8. doi:10.1007/cincr.11063. PMID:12518365

37. Tran L, Allen CT, Xiao R, Moore E, Davis R, Park SJ, Spielbauer K, Van Waes C, Schmitt NC. Cisplatin Alters Antitumor Immunity and Synergizes with PD-1/PD-L1 Inhibition in Head and Neck Squamous Cell Carcinoma. Cancer Immunol Res. 2017;5(12):1141–51. doi:10.1158/2326-6066.CIR-17-0235. PMID:29097421

38. Xue W, Zender L, Miething C, Dickens RA, Hernando E, Krizhanovsky V, Gordon-Cardo C, Lowe SW. Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. Nature. 2007;445:656–60. doi:10.1038/nature05529. PMID:17251933

39. Guo G, Yu M, Xiao W, Celis E, Cui Y. Local Activation of p53 in the Tumor Microenvironment Overcomes Immune Suppression and Enhances Antitumor Immunity. Cancer Res. 2017;77:2292–305. doi:10.1158/0008-5472.CAN-16-2832. PMID:28280037

40. UniProt: the universal protein knowledgebase. Nucleic Acids Res. 2017;45:D158–d69. doi:10.1093/nar/gkw1099. PMID:27899622

41. Bouaoun L, Sonkin D, Ardin M, Hollstein M, Byrnes G, Zavadil J, Olivier M. TP53 Variations in Human Cancers: New Lessons from the IARC TP53 Database and Genomics Data. Hum Mutat. 2016;37:865–76. doi:10.1002/humu.23035. PMID:27328919

42. Kobayashi H, Wood M, Song Y, Appella E, Celis E. Defining promiscuous MHC class II helper T-cell epitopes for the HER2/neu tumor antigen. Cancer Res. 2000;60:5228–36. PMID:11016652

43. Kumai T, Ohkuri T, Nagato T, Matsuda Y, Oikawa K, Aoki N, Kimura S, Harabuchi Y, Kobayashi H, et al. Targeting HER-3 to elicit antitumor helper T cells against head and neck squamous cell carcinoma. Sci Rep. 2015;5:16280. doi:10.1038/srep16280. PMID:26538233