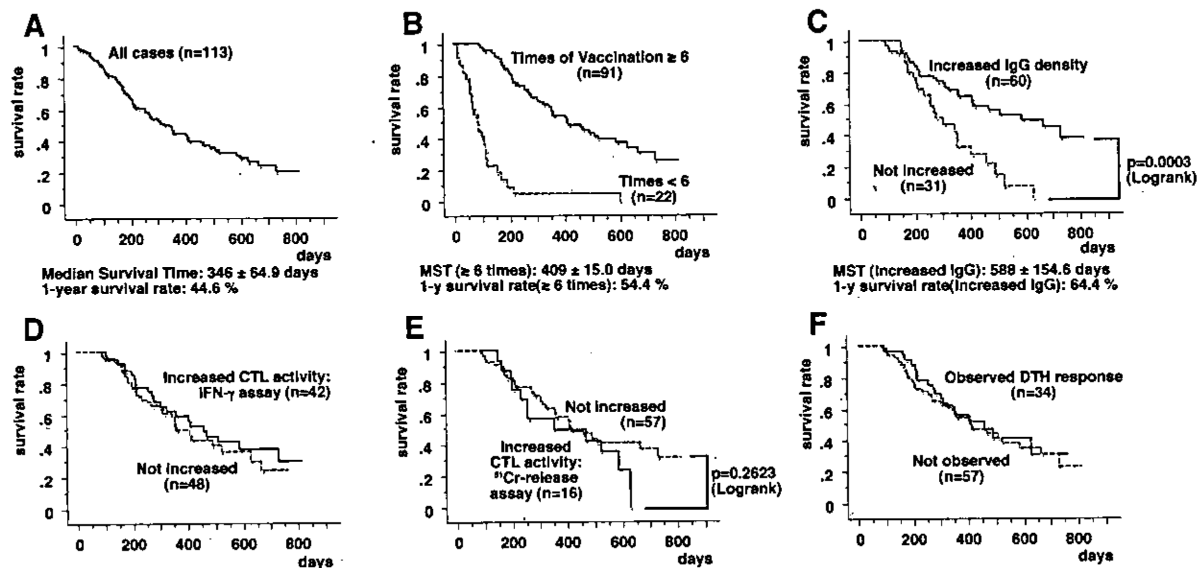

Fig. 1 Overall survival and laboratory markers. In *A*, overall survival of all of the enrolled cases (n = 113) is shown. In *B*, 91 cases received six more vaccinations (one cycle), and 22 cases could not achieve one cycle because of disease progression. In *C*, 60 of the 91 cases, whose postvaccination (third and sixth) sera showed increased levels of peptide-specific IgG, had longer survival (P = 0.0003) than 31 cases, whose sera did not show such increment. *D* and *E*, overall survival curves of cases who had increased CTL activity to the vaccinated peptides (*D*) and cancer cells (*E*). *F*, overall survival curve of cases with observation of delayed type hypersensitivity (*DTH*) response until the sixth vaccination is shown.

this study, a substantial number of cases have been receiving the peptide vaccination for >18 months with stable disease conditions in most cases, and the consistently higher levels are observed in sera of these cases (data not shown).

In regard to numbers of positive peptides, positive antibody responses to only one peptide and at least two peptides were seen in sera of the 35 and 25 cases, respectively. There was also no apparent difference of the survival time between the two groups (Fig. 3*C*). In detail, 35, 18, 6, and 1 showed increased humoral responses to one, two, three, and four peptides among all of the vaccinated peptides (at maximum four), respectively. However, because of too few cases for the analysis, the positive response to more than three peptides was not seen to contribute to overall survival.

In regard to the magnitude of humoral responses, the strong antibody responses by means of increase of IgG levels from the baseline to >0.5 OD values at a serum dilution of 1:100 were seen in of sera 2, 15, 6, and 1 cases after the 3rd, 6th, 9th, and 12th vaccinations, respectively. A part of the results are shown in Fig. 2. The peptides involved in the stronger responses were mainly UBE_{43}, SART3 $_{109}$, and lck_{486}.

The 91 cases who received the vaccination for more than six times were divided into the four groups based on their CTL and antibody responses. There was no apparent difference in the overall survival between the cases showing both CTL and antibody responses (n = 30) and those showing only antibody response (n = 30). Similarly, there was no apparent difference in the overall survival between the cases showing only CTL response (n = 12) and those showing neither CTL nor antibody response (n = 19; Fig. 3*D*).

We lastly studied the correlation between antibody response and overall survival in each of the four diseases. Antibody response did not significantly correlate with overall survival in colorectal cancer patients (Fig. 3*E*) or gastric cancer patients (P = 0.6059; data not shown), whereas it well correlated in hormone refractory prostate cancer patients (P = 0.0374; Fig. 3*F*) and lung cancer patients (P = 0.0486; Fig. 3*G*).

DISCUSSION

The detailed results of a Phase I study of CTL precursor-oriented peptide vaccines to each type of cancer have been reported elsewhere (21–25) or are currently under submission for colorectal cancer.[9] In this study, immune responses and clinical benefits were mainly analyzed in all of the patients with various types of cancers under the same regimen to discover a laboratory marker for prediction of prognosis. The results of 91 patients who received more than six vaccinations (one cycle) were used for statistical analysis. Patients (n = 60) whose sera showed increased levels of humoral responses to at least one vaccinated peptide had significantly better prognosis than those whose sera did not show such increased levels. We reported previously that some CTL-directed peptides have the ability to elicit both cellular and humoral immune responses in Phase I clinical studies (20–24). It is well known that humoral response is important for tumor regression. Indeed, we have reported that levels of antipeptide antibodies in postvaccination sera seemed

[9] Y. Sato *et al.*, submitted for publication.

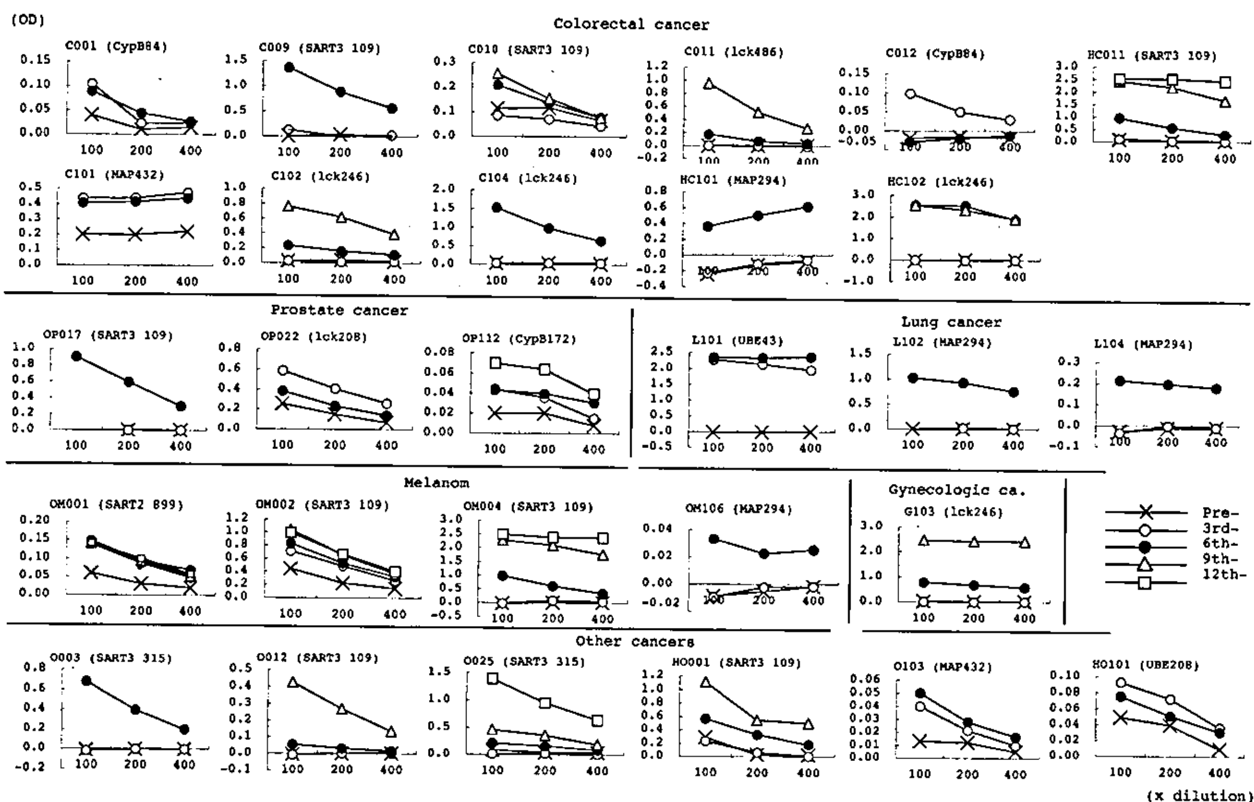

Fig. 2 Measurements of IgG reactive to the vaccinated peptides. Representative results of measurements of IgG reactive to the peptides for ≤12 vaccinations in the patients whose sera showed the positive responses to the vaccinated peptides are shown. It was judged as positive when postvaccination serum showed at least significant levels of IgG (>0.02 of net optimal density (*OD*) value at a serum dilution of 1:100) specific to the vaccinated peptides from a negative level in prevaccination serum. It was also judged as positive when postvaccination serum showed a ≥2-fold increase of the IgG level at a serum dilution of 1:100 than that of the prevaccination serum.

to correlate with the overall survival of advanced lung cancer patients who received peptide vaccination (21). The results shown in this study along with those from the initial results regarding lung cancer suggest that the elevation of humoral response to vaccinated peptides is a favorite factor for patients who received peptide vaccination beyond their different tumor origins. The correlation of humoral responses with better prognosis has also been reported in the other vaccination regimens in which whole tumor cells or epidermal growth factor is used for vaccination in melanoma patients or non-small cell lung cancer patients, respectively (30, 31).

We reported previously that IgG reactive against CTL epitope peptides is often detected in prevaccination sera of cancer patients and also in sera of healthy donors (21–25, 31). We also reported that IgG reactive to these CTL epitope peptides is either lacking or unbalanced in the sera of patients with atopic disease (32, 33). The results shown in this study along with those from noncancerous subjects suggest that these peptide-reactive IgGs play a role in host defense against various diseases, although the underlying mechanism in antitumor immune responses in cancer patients is unclear. These antipeptide IgGs did not react to the mother proteins and also failed to show either the direct inhibition of tumor cell growth *in vitro* or elicit antibody-dependent, cell-mediated cytotoxicity to tumor cells as far as tested (data not shown). Additional studies are needed to clarify their biological role, as well as their mechanism of action.

DTH response is a simple method with high reproducibility and has often been used as a laboratory marker to monitor immune responses *in vivo* for vaccination against infectious diseases and also malignant diseases. However, controversial results have been obtained regarding DTH response as a laboratory marker for either measuring immune responses to antigens or in the prediction of clinical benefits for vaccinated patients (15–28, 30, 31). In this study, DTH response did not correlate with either clinical course or overall survival. In addition, measurements of increased cellular immunity to either peptide or tumor cells did not correlate with overall survival. Collectively, none of the three assays for cellular immunity correlated closely with overall survival, regardless of the fact that the vaccinated peptides were screened by CTL precursor assay in prevaccination PBMCs. There may be several reasons for this unexpected result. One of them could have to do with reproducibility. CTL precursor frequency analysis, enzyme-linked immunospot assay, and cytotoxicity assays are generally used as

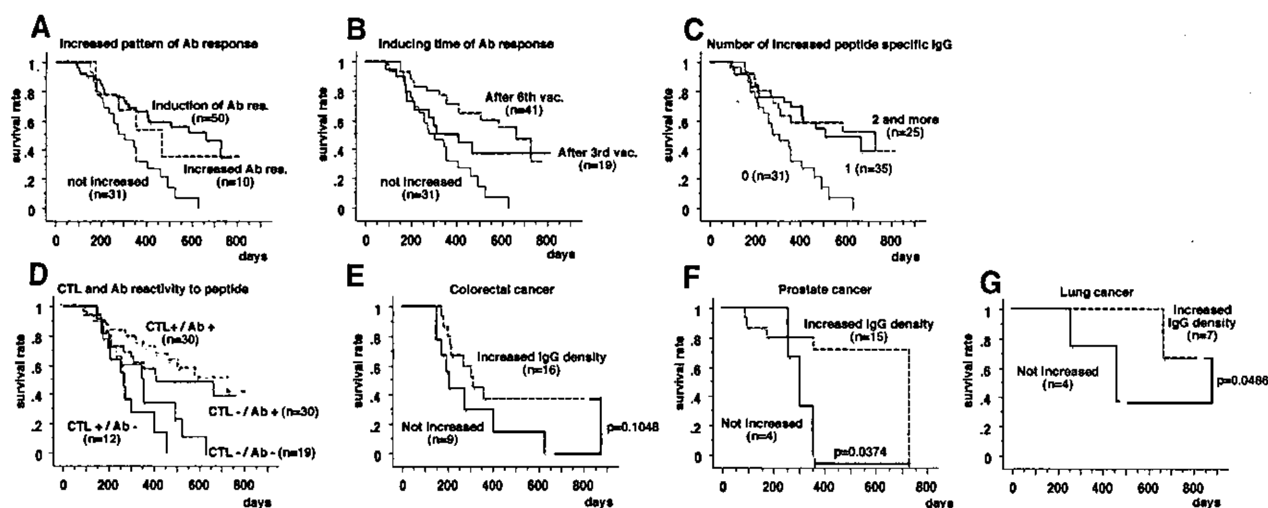

Fig. 3 Analysis of antibody responses and survival time. In *A*, 50 of 60 cases showed induction of peptide-specific IgG antibody response from negative to positive (>0.02) optimal density level, and the remaining 10 cases showed increased peptide-specific IgG antibody. In *B*, positive antibody responses were induced in sera after the third vaccinations in 19 of 60 cases and after the sixth vaccinations in the remaining 41 cases, respectively. In *C*, positive antibody responses to only one peptide and at least two peptides were seen in sera of the 35 and 25 cases, respectively. In *D*, 91 cases, who received the vaccination for more than six times, were divided into four groups based on their CTL and antibody responses. There was no apparent difference in the overall survival between the cases showing both CTL and antibody responses (n = 30) and those showing only antibody response (n = 30). *E*, antibody response and overall survival in colorectal cancer patients. *F*, antibody response and overall survival in prostate cancer patients (P = 0.0374). *F*, antibody response and overall survival in lung cancer patients (P = 0.0486).

laboratory markers to measure cellular immune responses to vaccinated peptides (15–31, 34). Although these assays are well-established monitoring systems, none of them is highly reproducible, mainly because the CTL precursors in PBMCs are usually very low, and the precursor frequency is between 1/1000 and 1/100,000, whereas the limit of sensitivity of these assays for detection is ~1/3000 to 1/10,000 cells (15–31, 34, 35). The lower reproducibility of these cellular assays might also be attributable to *in vitro* biases, including the cells' condition of cryopreservation, the culture medium, culture conditions, and the numbers and viability of cells at the time of harvesting. In contrast, the measurement of humoral immune responses can be relatively reproducible, because antibody molecules reactive to peptides are generally stable and abundant in serum samples (21–25, 32, 33). One of the other reasons is that these CTL assays use PBMCs, not tumor-infiltrating lymphocytes, and thus do not necessarily reflect the CTL activity at tumor sites. It is well known that T cells in the circulation rarely infiltrate into tumor sites. In contrast, IgG molecules might easily reach either peri-tumor or intratumor sites. This assumption is in part supported by the recent observation that inflammatory responses were observed around prostate cancers at the time of surgery in patients who received peptide vaccinations based on information regarding antibodies reactive to peptides before radical prostatectomy.[10]

The feasibility of ELISA as a laboratory marker for monitoring immune responses to vaccinated peptides could be superior to any of the CTL assays from several different points of view. Serum samples are much easier to preserve than PBMCs. A small amount of sera (10 μl/peptide) is needed for the assay, whereas relatively large numbers of PBMCs ($\sim\geq 10^6$ cells/peptide) are needed for CTL assays. The occupation time for ELISA is only 1 day, whereas CTL assays need 14–30 days. Running costs are another advantage of ELISA.

We have shown in this study new evidence that measurement of peptide-specific IgG in postvaccination sera is a suitable laboratory marker to predict prolonged survival for advanced cancer patients who received peptide vaccination based on pre-existing CTL precursors in the circulation. This evidence should be confirmed by other clinical trials regarding peptide-based immunotherapy for cancer patients.

REFERENCES

1. van der Bruggen, P., Traversari, C., Chomez, P., Lurquin, C., De Plaen, E., van den Eynde, B., Knuth, A., and Boon, T. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science (Wash. DC), *254:* 1643–1647, 1991.

2. Kawakami, Y., Eliyahu, S., Sakaguchi, K., Robbins, P. F., Rivoltini, L., Yannelli, J. R., Appella, E., and Rosenberg, S. A. Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. J. Exp. Med., *180:* 347–352, 1994.

3. Brichard, V., Van Pel, A., Wolfel, T., Wolfel, C., De Plaen, E., Lethe, B., Coulie, P., and Boon, T. The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. J. Exp. Med., *178:* 489–495, 1993.

[10] M. Noguchi *et al.*, unpublished data.

4. Peoples, G. E., Goedegebuure, P. S., Smith, R., Linehan, D. C., Yoshino, I., and Eberlein, T. J. Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. Proc. Natl. Acad. Sci. USA, *92:* 432–436, 1995.

5. Kikuchi, M., Nakao, M., Inoue, Y., Matsunaga, K., Shichijo, S., Yamana, H., and Itoh, K. Identification of a SART-1-derived peptide capable of inducing HLA-A24-restricted and tumor-specific cytotoxic T lymphocytes. Int. J. Cancer, *81:* 459–466, 1999.

6. Nakao, M., Shichijo, S., Imaizumi, T., Inoue, Y., Matsunaga, K., Yamada, A., Kikuchi, M., Tsuda, N., Ohta, K., Takamori, S., Yamana, H., Fujita, H., and Itoh, K. Identification of a gene coding for a new squamous cell carcinoma antigen recognized by the CTL. J. Immunol., *164:* 2565–2574, 2000.

7. Yang, D., Nakao, M., Shichijo, S., Sasatomi, T., Takasu, H., Matsumoto, H., Mori, K., Hayashi, A., Yamana, H., Shirouzu, K., and Itoh, K. Identification of a gene coding for a protein possessing shared tumor epitopes capable of inducing HLA-A24-restructed CTLs in cancer patients. Cancer Res., *59:* 4056–4063, 1999.

8. Gomi, S., Nakao, M., Niiya, F., Imamura, Y., Kawano, K., Nishizaka, S., Hayashi, A., Sobao, Y., Oizumi, K., and Itoh, K. A cyclophilin B gene encodes antigenic epitopes recognized by HLA-A24-restricted and tumor-specific cytotoxic T lymphocytes. J. Immunol., *163:* 4994–5004, 1999.

9. Harashima, N., Tanaka, K., Sasatomi, T., Shimizu, K., Miyagi, Y., Yamada, A., Tamura, M., Yamana, H., Itoh, K., and Shichijio, S. Recognition of the Lck tyrosine kinase as a tumor antigen by cytotoxic T lymphocytes of cancer patients with distant metastases. Eur. J. Immunol., *31:* 323–332, 2000.

10. Nishizaka, S., Gomi, S., Harada, K., Oizumi, K., Itoh, K., and Shichijo, S. A new tumor-rejection antigen recognized by CTLs infiltrating into a lung adenocarcinoma. Cancer Res., *60:* 4830–4837, 2000.

11. Kawano, K., Gomi, S., Tanaka, K., Tsuda, N., Kamura, T., Itoh, K., and Yamada, A. Identification of a new endoplasmic reticulum-resident protein recognized by HLA-A24-restricted tumor-infiltrating lymphocytes of lung cancer. Cancer Res., *60:* 3550–3558, 2000.

12. Imai, N., Harashima, N., Ito, M., Miyagi, Y., Harada, M., Yamada, A., and Itoh, K. Identification of Lck-derived peptides capable of inducing HLA-A2-restricted and tumor-specific CTLs in cancer patients with distant metastases. Int. J. Cancer, *94:* 237–242, 2001.

13. Tamura, M., Nishizaka, S., Maeda, Y., Ito, M., Harashima, N., Harada, M., Shichijio, S., and Itoh, K. Identification of cyclophilin B-derived peptides capable of inducing histocompatibility leukocyte antigen-A2-restricted and tumor-specific cytotoxic T lymphocytes. Jpn. J. Cancer Res., *92:* 762–767, 2001.

14. Ito, M., Shichijo, S., Tsuda, N., Ochi, M., Harashima, N., Saito, N., and Itoh, K. Molecular basis of T cell-mediated recognition of pancreatic cancer cells. Cancer Res., *61:* 2038–2046, 2001.

15. Jager, E., Gnjatic, S., Nagata, Y., Stockert, E., Jager, D., Karbach, J., Neumann, A., Rieckenberg, J., Chen, Y. T., Ritter, G., Hoffman, E., Arand, M., Old, L. J., and Knuth, A. Induction of primary NY-ESO-1 immunity: CD8+ T lymphocyte and antibody responses in peptide-vaccinated patients with NY-ESO-1+ cancers. Proc. Natl. Acad. Sci. USA, *97:* 12198–12203, 2000.

16. Marchand, M., van Baren, N., Weynants, P., Brichard, V., Dreno, B., Tessier, M. H., Rankin, E., Parmiani, G., Arienti, F., Humblet, Y., Bourlond, A., Vanwijck, R., Lienard, D., Beauduin, M., Dietrich, P. Y., Russo, V., Kerger, J., Masucci, G., Jager, E., De Greve, J., Atzpodien, J., Brasseur, F., Coulie, P. G., van der Bruggen, P., and Boon, T. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. Int. J. Cancer, *80:* 219–230, 1999.

17. Rosenberg, S. A., Yang, J. C., Schwartzentruber, D. J., Hwu, P., Marincola, F. M., Topalian, S. L., Restifo, N. P., Dudley, M. E. Schwarz, S. L., Spiess, P. J., Wunderlich, J. R., Parkhurst, M. R., Kawakami, Y., Seipp, C. A., Einhorn, J. H., and White, D. E. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat. Med., *4:* 321–327, 1998.

18. Valmori, D., Dutoit, V., Rubio-Godoy, V., Chambaz, C., Lienard, D., Guillaume, P., Romero, P., Cerottini, J. C., and Rimoldi, D. Frequent cytolytic T-cell responses to peptide MAGE-A10(254–262) in melanoma. Cancer Res., *61:* 509–512, 2001.

19. Gohara, R., Imai, N., Rikimaru, T., Yamada, A., Hida, N., Ichiki, M., Kawamoto, M., Matsunaga, K., Ashihara, J., Yano, S., Tamura, M., Ohkouchi, S., Yamana, H., Oizumi, K., and Itoh, K. Phase I clinical study of cyclophilin B peptide vaccine for patients with lung cancer. J. Immunother., *25:* 439–444, 2002.

20. Miyagi, Y., Imai, N., Sasatomi, T., Yamada, A., Mine, T., Katagiri, K., Nakagawa, M., Muto, A., Okouchi, S., Isomoto, H., Shirouzu, K., Yamana, H., and Itoh, K. Induction of cellular immune responses to tumor cells and peptides in colorectal cancer patients by vaccination with SART3 peptides. Clin. Cancer Res., *7:* 3950–3962, 2001.

21. Mine, T., Gouhara, R., Hida, N., Imai, N., Azuma, K., Rikimaru, T., Katagiri, K., Nishikori, M., Sukehiro, A., Nakagawa, M., Yamada, A., Aizawa, H., Shirouzu, K., Itoh, K., and Yamana, H. Immunological evaluation of CTL precursor-oriented vaccines for advanced lung cancer patients. Cancer Sci., *94:* 548–556, 2003.

22. Tanaka, S., Harada, M., Mine, T., Noguchi, M., Gohara, R., Azuma, K., Tamura, M., Yamada, A., Morinaga, A., Nishikori, M., Katagiri, K., Itoh, K., Yamana, H., and Hashimoto, T. Peptide vaccination for patients with melanoma and other types of cancer based on pre-existing peptide-specific ctotoxic T-lymphocyte precursors in the periphery. J. Immunother., *26:* 357–366, 2003.

23. Noguchi, M., Kobayashi, K., Suetsugu, N., Tomiyasu, K., Suekane, S., Yamada, A., Itoh, K., and Noda, S. Induction of cellular and humoral immune responses to tumor cells and peptides in HLA-A24 positive hormone-refractory prostate cancer patients by peptide vaccination. Prostate, *57:* 80–92, 2003.

24. Sato, Y., Shomura, H., Maeda, Y., Mine, T., Une, Y., Akasaka, Y., Kondo, M., Takahashi, S., Shinohara, T., Katagiri, K., Sato, M., Okada, S., Matsui, K., Yamada, A., Yamana, H., Itoh, K., and Toda, S. Immunological evaluation of peptide vaccination for patients with gastric cancer based on pre-existing cellular response to peptide. Cancer Sci., *94:* 808–902, 2003.

25. Noguchi, M., Itoh, K., Suekane, S., Yao, A., Suetsugu, N., Katagiri, K., Yamada, A., Yamana, H., and Noda, S. Phase I trial of patient-oriented vaccination in HLA-A2 positive patients with metastatic hormone refractory prostate cancer. Cancer Sci., 2003, in press.

26. Berd, D., Maguire, H. C., Jr., and Mastrangelo, M. J. Induction of cell-mediated immunity to autologous melanoma cells and regression of metastases after treatment with a melanoma cell vaccine preceded by cyclophosphamide. Cancer Res., *46:* 2572–2577, 1986.

27. Kirkwood, J. M., Ibrahim, J. G., Sosman, J. A., Sondak, V. K., Agarwala, S. S., Ernstoff, M. S., and Rao, U. High-dose interferon alfa-2b significantly prolong relapse-free and overall survival compared with the GM2-KLH/QS-21 vaccine in patients with resected stage IIb-III melanoma: results of intergroup trial E1694/S9512/C509801. J. Clin. Oncol., *19:* 2370–2380, 2001.

28. Salgia, R., Lynch, T., Skarin, A., Lucca, J., Lynch, C., Jung, K., Hodi, F. S., Jaklitsch, M., Mentzer, S., Swanson, S., Lukanich, J., Bueno, R., Wain, J., Mathisen, D., Wright, C., Fidias, P., Donahue, D., Clift, S., Hardy, S., Neuberg, D., Mulligan, R., Webb, I., Sugarbaker, D., Mihm, M., and Dranoff, G. Vaccination with irradiated autologous tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor augments antitumor immunity in some patients with metastatic non-small-cell lung carcinoma. J. Clin. Oncol., *21:* 624–630, 2003.

29. Maeda, Y., Hida, N., Niiya, F., Katagiri, K., Harada, M., Yamana, H., Kamura, T., Takahashi, M., Sato, Y., Todo, S., and Itoh, K. Detection of peptide-specific CTL-precursors in peripheral blood lymphocytes of cancer patients. Br. J. Cancer, *87:* 796–804, 2002.

30. Gonzalez, G., Crombet, T., Torres, F., Catala, M., Alfonso, L., Osorio, M., Neninger, E., Garcia, B., Mulet, A., Perez, R., and Lage, R. Epidermal growth factor-based cancer vaccine for non-small-cell lung cancer therapy. Ann. Oncol., *14:* 461–466, 2003.

31. DiFronzo, L. A., Gupta, R. K., Essner, R., Foshag, L. J., O'Day, S. J., Wanek, L. A., Stern, S. L., and Morton, D. L. Enhanced humoral immune response correlates with improved disease-free and overall survival in American Joint Committee on Cancer stage II melanoma patients receiving adjuvant polyvalent vaccine. J. Clin. Oncol., *20:* 3242–3248, 2002.

32. Ohkouchi, S., Yamada, A., Imai, N., Mine, T., Harada, K., Shichijo, S., Maeda, Y., Saijo, Y., Nukiwa, T., and Itoh, K. Non-mutated tumor-rejection antigen peptides elicit type-I allergy in the majority of healthy individuals. Tissue Antigens, *59:* 259–272, 2002.

33. Kawamoto, N., Yamada, A., Ohkouchi, S., Maeda, T., Tanaka, S., Hashimoto, T., Saijo, Y., Saijo, S., Nukiwa, T., Shichijo, S., Aizawa, H., and Itoh, K. IgG reactive to CTL-directed epitopes of self-antigens is either lacking or unbalanced in atopic dermatitis patients. Tissue Antigens, *61:* 352–361, 2003.

34. Scheibenbogen, C., Lee, K. H., Stevanovic, S., Witzens, M., Willhauck, M., Waldmann, V., Naeher, H., Rammensee, H. G., and Keilholz, U. Analysis of the T cell response to tumor and viral peptide antigens by an IFN gamma-ELISPOT assay. Int. J. Cancer, *71:* 932–936, 1997.

35. Hida, N., Maeda, Y., Katagiri, K., Takasu, H., Harada, M., and Itoh, K. A simple culture protocol to detect peptide-specific cytotoxic T lymphocyte precursors in the circulation. Cancer Immunol. Immunother., *51:* 219–228, 2002.

Identification of Peptide Vaccine Candidates Sharing among HLA-A3$^+$, -A11$^+$, -A31$^+$, and -A33$^+$ Cancer Patients

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ABSTRACT

Purpose: **Only a few studies have been reported on CTL epitope peptides restricted with alleles other than *HLA-A2* and *-A24*. The *HLA-A11*, *-A31*, and *-A33* alleles share similar binding motifs with *HLA-A3* and *-A68* alleles, and, thus, are classified as an *HLA-A3* supertype. This study tried to identify CTL epitope peptides as vaccine candidates sharing by HLA-A3$^+$, -A11$^+$, -A31$^+$, and -A33$^+$ cancer patients.**

Experimental Design: **Seven peptides possessing the ability to induce HLA-A31-restricted and tumor-reactive CTLs were examined for their ability to induce HLA-A3-, -A11-, and -A33-restricted and tumor-reactive CTLs from peripheral blood mononuclear cells (PBMCs) of 18 epithelial cancer patients. The five reference peptides all have the ability to induce CTL activity restricted with one of the HLA-A3 supertypes, and, thus, were also examined as positive controls.**

Results: **Three peptides (2 from β-tublin5- and 1 from CGI37-derived peptides) induced tumor-reactive CTLs in PBMCs of HLA-A3$^+$, -A11$^+$, and -A33$^+$ cancer patients with various frequencies (17–50%). One RLI- or KIAA0036-derived peptide induced tumor-reactive CTLs in PBMCs of HLA-A3$^+$ and -A11$^+$ or HLA-A11$^+$ and -A33$^+$ cancer patients also with various frequencies (22–67%), respectively, whereas the other peptide induced CTL activity in only HLA-A33$^+$ patients. Among the five reference peptides tested, one peptide, TRP2–197, induced CTL activity in both HLA-A11$^+$- and -A33$^+$-restricted manners.**

Conclusions: **We identified new peptide vaccine candidates for HLA-A3, -A11, -A31, and -A33 positive cancer patients. This study may facilitate the development of both basic and clinical studies of peptide-based immunotherapy for cancer patients with other alleles of *HLA-A2* and *-A24*.**

INTRODUCTION

CTLs play an important role in elimination of cancer cells (1). Many tumor antigens that are recognized by CTLs have been identified from various malignant cells such as melanocyte-related antigens (2, 3), cancer-testis-antigens (4), prostate-specific antigens (5), lymphoma/leukemia specific antigens (6), and self-antigens involved in cellular proliferation (7, 8). Subsequent clinical applications of these peptides as a form of peptide-based immunotherapy are now in progress; however, most of these peptides are limited to *HLA-A2* or *-A24* allele$^+$ cancer patients (2, 9), primarily because of the higher worldwide frequency of these alleles. We identified previously seven peptides capable of inducing HLA-A31-restricted and tumor-reactive CTLs (10). The relatively low frequency of the *HLA-A31* allele in major ethnic groups (5–12.5%) may hamper the clinical application of these peptides in cancer patients with this allele. On the other hand, based on the structural similarities of the group of HLA alleles and the peptide binding motif analysis, the following supertypes have been proposed, *HLA-A2*, *-A3*, *-B7*, and *-B44* supertype alleles (11). Among them, the A3 supertype includes the allelic products of at least five common HLA-A alleles, *A3*, *A11*, *A31*, *A33*, and *A68*. The *HLA-A3* supertype allele is found in 38% of Caucasians, 53% of Chinese, 46% of Japanese, and 43% of North American African-Americans and Hispanics (11). It has been shown that the same epitope peptides, derived from viral protein and melanoma antigen, were recognized by different *HLA-A3* supertype alleles (3, 12). Subsequently, we extended our previous study, and investigated here whether or not the seven CTL-epitope peptides with an HLA-A31-restriction would be able to induce tumor-reactive CTLs from peripheral blood mononuclear cells (PBMCs) of HLA-A3$^+$, -A11$^+$, and -A33$^+$ cancer patients.

MATERIALS AND METHODS

Patients and Cell Lines. PBMCs from HLA-A3 supertype$^+$ cancer patients were obtained after the written informed consent was obtained. The patient supertype alleles included HLA-A3 (n = 3), -A11 (n = 9), and -A33 (n = 6), but HLA-A68$^+$ patients were not available because of their extremely low frequency (0.5%) in the Japanese population (13). Only 3 HLA-A3$^+$ patients were provided for the study because of its low frequency (1.6%) in the Japanese population. None of the participants was infected with HIV. Twenty ml of peripheral blood were obtained, and PBMCs were prepared by Ficoll-Conray density gradient centrifugation. All of the samples were cryopreserved until they were used for the experiments. HLA class I genotyping was examined by PCR-sequenced-based typing (SRL, Tokyo, Japan).

The following tumor cell lines were used in this study,

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Table 1 Summary of the binding affinity of antigenic peptides to the HLA-A3 supertype alleles

Peptides	Sequence	Reference	Bind to[b]	Binding Score[a]				
				A3	A11	A31	A33	A68.1
RLI-522	FIMATYLADR	10	A31	2.7	0.16	12	15	10
β-tublin5-154	KIREEYPDR	10	A31	1.8	0.24	6	4.5	5
β-tublin5-232	TMSGVTTCLR	10	A31	6	0.08	2	15	5
β-tublin5-309	RYLTVAAVFR	10	A31	0.01	0.36	3.6	4.5	1
CGI37-72	KFTKTHKFR	10	A31	0.01	0.06	0.9	0.9	0.5
KIAA0036-241	HQEILILLR	10	A31	0.36	0.24	8	3	5
KIAA0036-356	RQRAMRLSR	10	A31	0.24	0.72	12	0.9	5
MAGE1-95	ESLFRAVITK	12	A11	0.27	0.02	0.01	1.5	54
MAGE2-73	TTINYTLWR	12	A11	1.8	0.6	4	3	100
PAP-274	ATQIPSYKK	12	A11	4.5	2	0.5	0.1	30
gp100-87	ALNFPGSQK	13	A3/A11	30	0.4	0.2	0.3	3
TRP2-197	LLGPGRPYR	3	A31/A33	6	0.08	2	9	15
EBV	IVTDFSVIK	12	A11	10	4	0.6	0.5	240
Flu	NVKNLYEKVK	12	A11	3	1	0.1	0.5	180
HIV	RLRDLLLIVTR	14	A31	-	-	-	-	-

[a] The peptide binding score was calculated based on the predicted half-time of dissociation from HLA class I molecules, as obtained from a Website (Bioinformatics and Molecular Analysis Section, Computational Bioscience and Engineering Laboratory, Division of Computer Research & Technology, NIH).[3]

[b] Previously reported HLA class I alleles in which the peptide have immunogenicity are shown.

esophageal cancer cell line KE5 (A1101, B1501/5504, Cw0303/0401); lung adenocarcinoma cell lines 1-87 (A0207/1101, B4601/5401, Cw0102), LC1 (A3101/3303, B1511/44031, Cw0303/1403), 11-18 (A0201/2402, B5201/5401, Cw0102/1202), and QG56 (A2601, B4601, Cw0102); gastric adenocarcinoma cell line MKN28 (A3101, B5101, Cw0304); colon adenocarcinoma cell line SW620 (A0201/2402, B0702/1518, Cw0702/0704); cervical cancer cell line RKN (A3303, B44/51, Cw1403); and astrocytoma cell line Becher (A0301/6802). All of the cell lines were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% FCS. Phytohemagglutinin-activated normal T cells were also used as a negative control. To generate HLA-A11- and -A33-transfected C1R cell lines, an *HLA-A1101* or *-A3303* cDNA clone was inserted into the eukaryotic expression vector pCR3.1 (Invitrogen) by a method reported previously (8). Electroporation was performed using a Gene Pulser (Bio RAD, Richmond, CA). G418-resistant single clones were selected by limiting dilution. To generate HLA-A3 transfected COS-7 cells, 100 ng of *HLA-A0301* cDNA, which was inserted into pCR3.1, were mixed in 100 μl of Opti-MEM (Invitrogen) with 0.3 μl of Fugene 6 (Invitrogen) and incubated for 30 min. The mixture was then added to the COS-7 cells (1×10^3 cells), which were then incubated for 3 h. One hundred μl of RPMI 1640 containing 20% FCS was added, and COS-7 cells were cultured for 2 days. The surface expression of HLA-A3, -A11, or -A33 molecules was examined by anti-HLA-A3 (IgM; One Lambda, Canoga Park, CA), anti-HLA-A11 (A11.1M, IgG3), and anti-HLA-A33 mAb (IgM; One Lambda), respectively. Surface phenotypes of the CTLs were analyzed by fluorescence-activated cell sorter with FITC-conjugated anti-CD3, anti-CD4, anti-CD8, and anti-CD14 monoclonal antibody (mAb; Nichirei, Tokyo, Japan).

Peptides. Eleven different peptides, each with the ability to induce peptide-specific and tumor-reactive CTL activity with an HLA-A3-, -A11-, -A31-, or -A33-restricted manner, all belonging to the HLA-A3 supertype, were used in this study (3, 12, 13). We recently reported 7 of these peptides as being capable of inducing HLA-A31-restricted and tumor-reactive CTLs (10). The remaining 5 peptides that were used as reference peptides in this study were reported from the other laboratories as CTL epitope peptides with the ability to induce CTLs restricted to one of the HLA-A3 supertypes (3, 12, 13). EBV- and Influenza virus-derived peptides with an HLA-A11-binding motif were used as positive controls, whereas an HIV-derived peptide with an HLA-A31-binding motif was used as a negative control (14, 15). The peptide binding affinities for these alleles of the HLA-A3 supertype were calculated based on a predicted half-time of dissociation to HLA class I molecules, as reported previously (16); the binding scores obtained from the bioinformatics and molecular analysis section website[3] are given in Table 1. All of the peptides (>90% purity) used in this study were purchased from Biosynthesis (Lewisville, TX) and were dissolved with DMSO at a dose of 10 mg/ml.

Assay for Peptide-Specific CTLs. The method used for the detection of peptide-specific CTLs has been reported elsewhere (17). In brief, PBMCs (1×10^5 cells/well) were incubated with 10 μM of each peptide in the wells of a U-bottomed-type 96-well microculture plate (Nunc, Roskilde, Denmark) in 200 μl of culture medium. The medium consisted of 45% RPMI 1640, 45% AIM-V (Invitrogen), 10% FCS, 100 units/ml of interleukin 2, and 0.1 mM MEM nonessential amino acid solution (Invitrogen). On the third, sixth, and ninth days, half of the medium was removed and replaced with new medium containing the corresponding peptide (20 μg/ml). On day 12 of culture, the harvested cells were tested for their ability to produce IFN-γ in response to HLA-A3, -A11, or -A33 expressing COS-7 cells or C1R cells, designated as COS-7-A3, C1R-A11, and -A33 respectively; the cells were preloaded with either a correspond-

[3] Internet address: http://bimas.dcrt.nih.gov/.

Table 2 Induction of peptide-reactive CTLs from peripheral blood mononuclear cells (PBMCs) of HLA-A11$^+$ cancer patients

The PBMCs from patients were tested for their reactivity to a corresponding peptide after *in vitro* stimulation with each peptide for 12 days. Values represent the IFN-γ production by the effector PBMCs in response to C1R-A11 cells prepulsed with the corresponding peptide. Background IFN-γ response to C1R-A11 cells prepulsed with the HIV peptide was subtracted (<50 pg/ml). Significant values ($P < 0.05$ by two-tailed Student's *t* test) are underlined.

	Patient										
Peptide	#1 Lung	#2 Lung	#3 Prostate	#4 Prostate	#5 Prostate	#6 Prostate	#7 Prostate	#8 Bladder	#9 Melanoma	Total	
RLI-522	11	0	0	102	0	0	267	0	31	2/9	22%
β-tublin5-154	226	44	156	107	54	1		149	0	4/8	50%
β-tublin5-232	0	60	10	0	0	141	0	0	0	1/9	11%
β-tublin5-309	152	0	0	0	289	39	0	0	0	2/9	22%
CGI37-72	0	65	106	0	34	11	0	307	0	2/9	22%
KIAA0036-241	18	0	0	0	0	7	0	52	0	0/9	0%
KIAA0036-356	10	0	114	9	0	0	590	371	155	4/9	44%
MAGE1-95	395	27	0	0	63	0	0	848	0	2/9	22%
MAGE2-73	1717	0	137	0	25	44	243	1835	196	5/9	56%
PAP-274	541	47	0	16	366	300	0	85	0	3/9	30%
gp100-87	2358	3	0	0	1479	2	0	310	0	3/9	30%
TRP2-197	0	210	4	44	35	50	0	443	0	2/9	22%
EBV	1297	34		7	487	32	228	1436	531	5/8	63%
Flu	1363	244		7	533	269	0	950	0	5/8	63%
HIV	62	0	0	5	0	1	10	0	29	0/9	0%

ing peptide or an HIV peptide, used as a negative control. Four wells were prepared for each peptide, and the assays were performed in duplicate. The background IFN-γ production (<50 pg/ml) in response to the HIV peptide was subtracted from the values given in the data. The concentration of IFN-γ was assessed by ELISA as reported previously (16). For the inhibition assay, the peptide-reactive CTLs were positively purified using a CD8 Isolation kit (DYNAL, Oslo, Norway), and their peptide-specific IFN-γ production was measured in the presence of 20 μg/ml of anti-HLA class I (W6/32, IgG2a), anti-CD8 (Nu-Ts/c, IgG2a), or anti-HLA class II (H-DR-1, IgG2a) mAbs, as reported previously (17). The background IFN-γ production (<100 pg/ml) was subtracted from the data. A two-tailed Student's *t* test was used for the statistical analysis throughout the study.

Cytotoxicity Assay. The peptide-stimulated PBMCs were incubated for >21 days, and cytotoxicity was tested by a standard 6-h ^{51}Cr release assay (17). A two-tailed Student's *t* test was used for the statistical analysis. For the cold target inhibition assays, unlabeled C1R-A11 or C1R-A33 cells were incubated with the corresponding peptide or with an HIV-peptide for 2 h, and then the cells were added to the ^{51}Cr-labeled targets at a cold:hot target cell ratio of 10:1.

Recognition of MHC/Peptide Complexes. One hundred ng each of *HLA-A* cDNA, which were inserted into pCR3.1, or 100 ng of *HLA-A* cDNA with 100 ng of each clones (RLI, β-tublin5, CGI37, and KIAA0036) were mixed in 100 μl of Opti-MEM (Invitrogen) with 0.3 or 0.6 μl of Fugene 6 (Invitrogen), respectively, and incubated for 30 min. The mixture was then added to the COS-7 cells (1×10^3 cells), which were then incubated for 3 h. One hundred μl of RPMI 1640 containing 20% FCS was added and COS-7 cells were cultured for 2 days. The peptide-stimulated PBMCs were then added and incubated for 12 h. Then, their ability to produce IFN-γ was measured by ELISA.

RESULTS

CTL Induction by Peptides. Seven different peptides capable of inducing HLA-A31-restricted and tumor-reactive CTLs were tested for their ability to induce HLA-A11 or -A33-restricted and tumor-reactive CTLs from the PBMCs of 9 HLA-A11 cancer patients (2 lung, 5 prostate, 1 bladder cancer, and 1 melanoma), 6 HLA-A33 cancer patients (2 lung, 2 prostate, 1 pancreas, and 1 cervical cancer), and 3 HLA-A3 cancer patients (1 lung, 1 cervical, and 1 prostate cancer), respectively. Tables 2, 3, and 4 show the representative results of CTL induction by IFN-γ production in response to peptide-loaded C1R-A11, C1R-A33 cells, and HLA-A3-transfected COS-7 cells in HLA-A11, -A33, and -A3 cancer patients, respectively. RLI-522, β-tublin5–154, β-tublin5–232, β-tublin5–309, CGI37–72, KIAA0036–241, and KIAA0036–356 peptides were shown to have the ability to induce peptide-reactive CTL activity in PBMCs from 2, 4, 1, 2, 2, 0, and 4 of 9 HLA-A11$^+$ cancer patients tested, respectively (Table 2). Similarly, these peptides possessed the ability to induce peptide-reactive CTL activity in PBMCs from 0, 2, 1, 1, 1, 4, and 4 of 6 HLA-A33$^+$ cancer patients tested, respectively (Table 3). RLI-522, β-tublin5–154, β-tublin5–232, β-tublin5–309, and CGI37–72, but not KIAA0036–241 and KIAA0036–356 peptides, were shown to have the ability to induce peptide-reactive CTL activity in a PBMCs from at least 1 of the 3 HLA-A3$^+$ cancer patients tested (Table 4).

We demonstrated previously the ability of these seven peptides to induce peptide-reactive CTL activity in PBMCs from 5, 3, 4, 4, 5, 7, and 5 of 10 HLA-A31$^+$ cancer patients tested, respectively (10). Collectively, four peptides (three from β-tublin5-derived peptides and CGI37–72) induced tumor-reactive CTLs in PBMCs of HLA-A3$^+$, -A11$^+$, and -A33$^+$ cancer patients with various frequencies (11–50%). RLI-522 or KIAA0036–356 peptide induced tumor-reactive CTLs in

Table 3 Induction of peptide-reactive CTLs from the peripheral blood mononuclear cells (PBMCs) of HLA-A33$^+$ cancer patients

The PBMCs from patients were tested for their reactivity to a corresponding peptide after *in vitro* stimulation with each peptide for 12 days. Values represent the IFN-γ production by the effector PBMCs in response to C1R-A33 cells prepulsed with the corresponding peptide. The background IFN-γ response to C1R-A33 cells prepulsed with the HIV peptide was subtracted from the value (<50 pg/ml). Significant values ($P < 0.05$ by two-tailed Student's *t* test) are underlined.

	Patient							
Peptide	#10 Lung	#11 Lung	#12 Prostate	#13 Prostate	#14 Pancreas	#15 Cervical	Total	
RLI-522	0	0	0	0	0	0	0/6	0%
β-tublin5-154	0	0	141	0	0	104	2/6	33%
β-tublin5-232	4	0	0	0	0	500	1/6	17%
β-tublin5-309	0	20	0	306	0	0	1/6	17%
CGI37-72	0	0	228	0	2	0	1/6	17%
KIAA0036-241	0	381	142	130	0	122	4/6	67%
KIAA0036-356	432	239	5	113	0	124	4/6	67%
MAGE1-95	325	39		27	0	0	1/5	20%
MAGE2-73	0	0		134	213	0	2/5	40%
PAP-274	582	16		14	0	2726	2/5	40%
gp100-87	0	0		12	219	0	1/5	20%
TRP2-197	124	590		27	135	0	3/5	60%
EBV	305	0		0	116	105	3/5	60%
Flu	234	4		0	0	803	2/5	40%
HIV	0	0		0	0	0	0/5	0%

PBMCs of HLA-A3$^+$ and -A11$^+$ or HLA-A11$^+$ and -A33$^+$ cancer patients also with various frequencies (22–67%), respectively. The remaining KIAA0036–241 peptide induced CTL activity in only HLA-A33$^+$ patients (4 of 6 patients). In regard to each peptide, the β-tublin5–154 is the most widely shared peptide possessing the ability to induce CTLs restricted to HLA-A3 supertypes under the present conditions, because it induced HLA-A3-, -A11-, -A33-, and -A31-restricted CTL activity in 33%, 50%, 33%, and 30% of HLA-A3$^+$, -A11$^+$, -A33$^+$, and -A31$^+$ cancer patients, respectively. Although β-tublin5–232, β-tublin5–309, and CGI37–72 also induced HLA-A3-, -A11-, -A31- and -A33-restricted CTL activity, these frequencies were relatively low. Although KIAA0036–356, failed to induce HLA-A3-restricted CTL activity in any of the 3 cases tested, it is also a widely shared peptide possessing the ability to induce CTL activity in 44%, 67%, and 50% of HLA-A11$^+$, -A33$^+$, and -A31$^+$ cancer patients, respectively. The RLI-522 peptide failed to induce HLA-A33-restrictred CTL activity, whereas the KIAA0036–241 peptide failed to induce HLA-A3 and -A11-restricted CTL activity, respectively.

Table 4 Induction of peptide-reactive CTLs from the peripheral blood mononuclear cells (PBMCs) of HLA-A3$^+$ cancer patients

The PBMCs from patients were tested for their reactivity to a corresponding peptide after *in vitro* stimulation with each peptide for 12 days. Values represent the IFN-γ production by the effector PBMCs in response to HLA-A3 transfected COS-7 cells prepulsed with the corresponding peptide. The background IFN-γ response to target cells prepulsed with the HIV peptide (<50 pg/ml) was subtracted from the value. Significant values ($P < 0.05$ by two-tailed Student's *t* test) are underlined.

	Patient				
Peptide	#16 Lung	#17 Cervical	#18 Prostate	Total	
RLI-522	2988	43	107	2/3	66%
β-tublin5-154	1421	35	31	1/3	33%
β-tublin5-232	645	7	18	1/3	33%
β-tublin5-309	726	31	0	1/3	33%
CGI37-72	11	11	126	1/3	33%
KIAA0036-241	0	18	3	0/3	0%
KIAA0036-356	15	2	45	0/3	0%
TRP2-197	112	12	0	1/3	33%
HIV	0	0	0	0/3	0%

IFN-γ production by these peptide-stimulated PBMCs in response to C1R-A11 or -A33 cells pulsed with a corresponding peptide was inhibited by both the anti-HLA class I mAb and the anti-CD8 mAb, but not by the anti-HLA class II mAb in all of the tested cases. Representative data for each of HLA-A11 and -A33 from patients 8 and 11, respectively, are shown in Fig. 1.

These peptide-reactive PBMCs were further cultured for >2 weeks *in vitro* with interleukin 2 alone, and their cytotoxicity against various tumor cells was determined by a standard 6-h ^{51}Cr release assay at three E:T ratios. These peptide-reactive PBMCs, except for those stimulated with β-tublin5–232, exhibited significant levels of cytotoxicity against HLA-A allele-matched cancer cells, but not against HLA-A allele-mismatched cancer cells (Fig. 2*A* for HLA-A11 patients, Fig. 2*B* for HLA-A33 patients, and Fig. 2*C* for HLA-A3 patients). None of them were cytotoxic against HLA-A allele-matched phytohemagglutinin blastoid T cells (Fig. 2*A* for HLA-A11 patients and Fig. 2*B* for HLA-A33 patients). In contrast, PBMCs cultured with HIV peptide (Fig. 2, *A* and *B*) or flu peptide (data not shown), both of which were used as negative controls for an antitumor response, failed to display HLA-A11- or -A33-restricted cytotoxicity against cancer cells, respectively.

A cold-target inhibition assay was then performed to determine the specificity of the peptide-induced CTL responses. The cytotoxicity of β-tublin5–309 or KIAA0036–356-reactive CTLs against the HLA-A11$^+$ KE5 tumor cell line was blocked by unlabeled C1R-A11 cells loaded with the corresponding peptide, but not by unlabeled C1R-A11 cells loaded with the HIV peptide (Fig. 3, *A-1*). The similar results were obtained

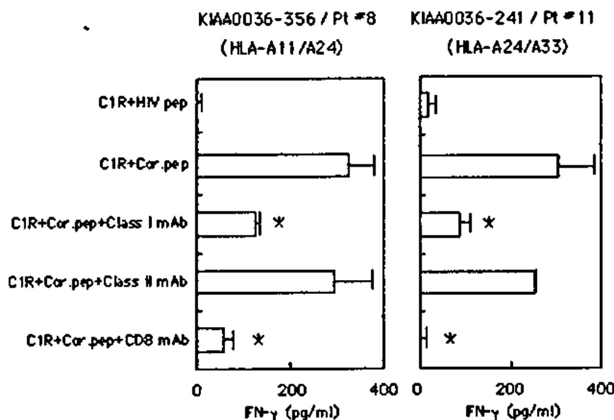

Fig. 1 Induction of peptide-reactive CTLs. CD8$^+$ T cells were purified from peripheral blood mononuclear cells after stimulation *in vitro* with the indicated peptides, followed by determination of their IFN-γ production by ELISA in response to C1R-A11 or -A33 cells that had been preloaded with a corresponding peptide. The assay was carried out at an E:T ratio of 10:1, and the indicated monoclonal antibody (*mAb*) was added at a dose of 20 μg/ml. The representative results of an HLA-A11$^+$ bladder cancer patient (*#8*) and an HLA-A33$^+$ lung cancer patient (*#11*) are shown. These values represent the means of triplicate assays. The background IFN-γ production by the effector cells alone (<50 pg/ml) was subtracted from the values. A two-tailed Student's *t* test was used for the statistical analysis. *, $P < 0.05$; *bars*, ± SD.

with RLI-522, β-tublin5–154, and CGI37–72 peptides (Fig. 3, *A-2*). In the inhibition test, the cytotoxicity against the HLA-A33$^+$ LC1 tumor cell line mediated by each of the 5 peptides (2 from β-tublin5, CGI37–72, and 2 from KIAA0036) was blocked by unlabeled C1R-A33 cells loaded with the corresponding peptide, but not by unlabeled C1R-A33 cells loaded with the HIV peptide (Fig. 3*B*). The cytotoxicity against the HLA-A11$^+$ lung cancer 1-87, HLA-A33$^+$ lung cancer LC1, or HLA-A3$^+$ astrocytoma cell lines mediated by each of the peptide reactive-PBMCs was blocked by anti-CD8 and anti-HLA class I but not by the other mAbs (Fig. 2*C* and Fig. 3*C*). All of these results indicate that CD8$^+$ T cells from the peptide-stimulated PBMCs, except for those with β-tublin5–232, exhibited peptide-specific CTL activity reactive to tumor cells with an HLA class I-restricted manner.

We then used transfection experiments to confirm the recognition of MHC/peptide complexes by each of the six peptide-stimulated PBMCs. In regard to HLA-A11 molecules, the four peptides were tested. Namely, the HLA-A11$^+$ PBMCs stimulated with each of RLI-522, β-tublin5–154, CGI37–72, or KIAA0036–356 produced significant levels of IFN-γ by recognition of COS-7 cells that were cotransfected with both *HLA-A11* and the corresponding cDNA clone (Fig. 4*A*). In contrast, these PBMCs failed to produce IFN-γ either by recognition of COS-7 cells that were cotransfected with any of the irrelevant *HLA class I* (*A33, A31,* or *A2*) and corresponding clones (β-*tublin5, CGI37, RLI,* or *KIAA0036*), or by recognition of COS-7 cells that were transfected with any of the *HLA class I* (*A33, A31, A11,* or *A2*) clones with an irrelevant (*ppMAPkkk*) cDNA clone (Fig. 4*A*). β-tublin5–309 peptide was not examined by transfection study because of the sample limitation.

In regard to HLA-A33 molecules, the three peptides were initially tested (Fig. 4, *B-1*). Namely, the HLA-A33$^+$ PBMCs stimulated with each of β-tublin5–154, β-tublin5–309, or CGI37–72 produced significant levels of IFN-γ by recognition of COS-7 cells that were cotransfected with both *HLA-A33* and the corresponding cDNA clone. In contrast, these PBMCs failed to produce IFN-γ either by recognition of COS-7 cells that were cotransfected with any of the irrelevant *HLA class I* (*A31, A11,* or *A2*) and β-*tublin5* or CGI37 cDNA clones or by recognition of COS-7 cells that were transfected with any of the *HLA class I* (*A33, A31, A11,* or *A2*) clones and with an irrelevant (*ppMAPkkk*) cDNA clone (Fig. 4, *B-1*).

Secondly, we tested the six peptides. Namely, HLA-A33$^+$ PBMCs stimulated with each of the six peptides tested (β-tublin5–154 or CGI37–72, 2 from KIAA0036, and MAGE1–95 and MAGE1–73 as reference peptides) produced significant levels of IFN-γ by recognition of COS-7 cells that were transfected with the *HLA-A33* cDNA clone, followed by loading of the corresponding peptide (Fig. 4, *B-2*). In contrast, these PBMCs failed to produce IFN-γ by either of the two following experiments, by recognition of COS-7 cells that were transfected with any of the irrelevant *HLA class I* cDNA clones, followed by loading of the corresponding peptide or by recognition of COS-7 cells that were transfected with any of the *HLA class I* (*A33, A31, A11,* or *A2*) clones, followed by loading of an irrelevant peptide (HIV).

CTL Induction by the Reference Peptides. The following five reference peptides of the HLA-A3 supertype were also tested in this study, MAGE1-, MAGE2-, and PAP-derived peptides capable of inducing HLA-A11-restricted CTLs (14); a gp100-derived peptide capable of inducing HLA-A3 and -A11-restricted CTLs (18); and a TRP2-derived peptide capable of inducing HLA-A31 and -A33-restricted CTLs (3). In addition, EBV- and flu-derived peptides with an HLA-A11-restricted type of immunogenicity were used as the other reference peptides in this study (14). HIV-derived peptide was used as a negative control (15). The representative results regarding CTL induction in PBMCs from HLA-A11 and -A33 cancer patients are shown in Tables 2 and 3, respectively. The MAGE1–95, MAGE2–73, PAP-274, gp100–87, TRP2–197, EBV, flu, and HIV-derived peptides had the ability to induce peptide-reactive CTL activity in PBMCs from 2, 5, 3, 3, 2, 5, 5, and 0 of 9 HLA-A11$^+$ cancer patients tested, respectively. Similarly, these peptides had the ability to induce peptide-reactive CTL activity from 1, 2, 2, 1, 3, 3, 2, and 0 of 5 HLA-A33$^+$ cancer patients tested, respectively. Although most of these results are consistent with those of previous studies (3, 14, 18), it is of note that the TRP2–197 peptide, which can induce HLA-A31- and-A33-restricted CTLs, also induced HLA-A11-restricted CTL activity in 2 of 9 HLA-A11$^+$ and 1 of 3 cancer patients tested.

DISCUSSION

We demonstrated that each of the three (β-tublin5–154, β-tublin5–309, and CGI37–72) HLA-A31-restricted CTL-epitope peptides had the ability to induce HLA-A3-, -A11-, and -A33-restricted and tumor-reactive CTLs, from the PBMCs of epithelial cancer patients. These four alleles (*HLA-A0301, -A1101, -A3101,* and *-A3301*) along with *HLA-A6801* allele share the similar binding motifs, and, thus, are classified under

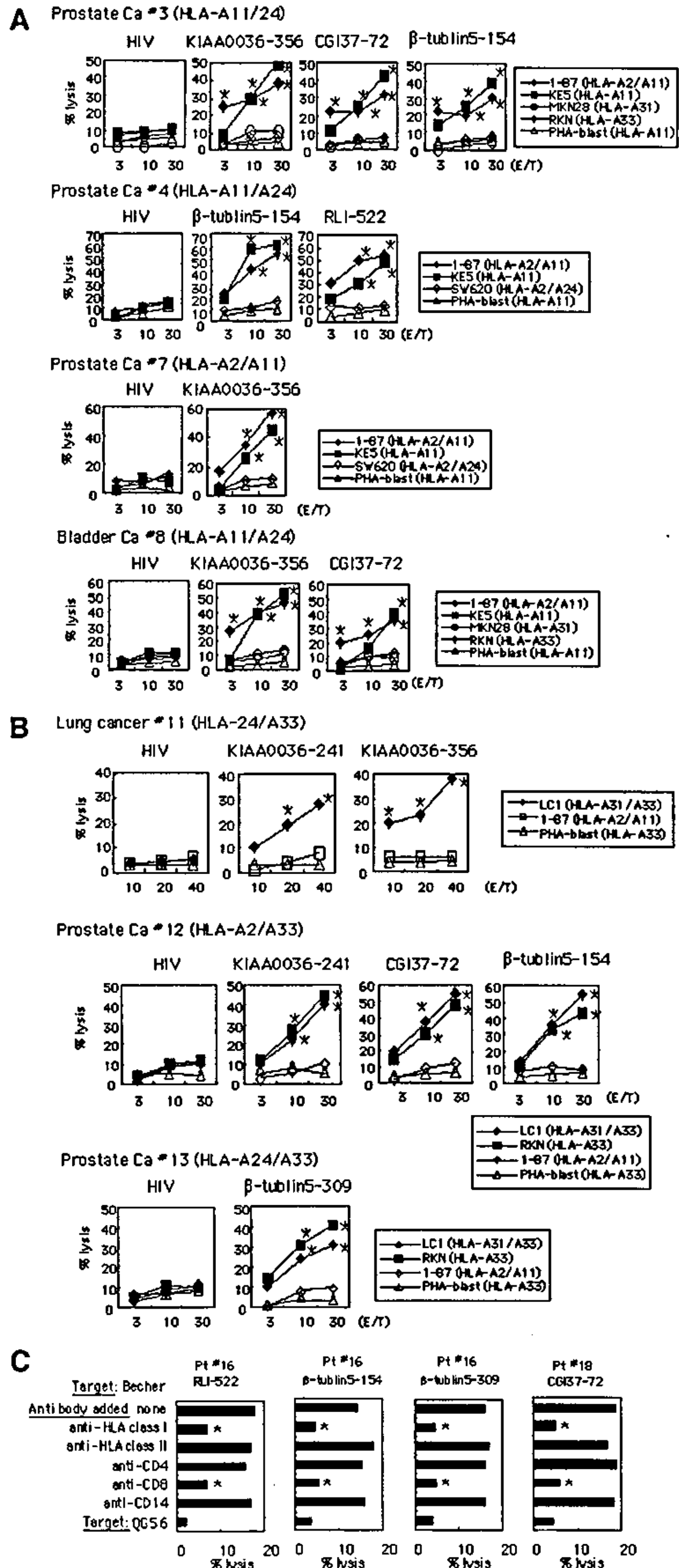

Fig. 2 Cytotoxicity of peptide-induced CTLs. *A*, cytotoxicity of peptide-specific CTLs was determined by 6-h ^{51}Cr release assay at three different E:T ratios. Values represent the means of triplicate assays. The target cells were HLA-A11$^+$ lung cancer cells 1-87, HLA-A11$^+$ esophagus cancer cells KE5, HLA-A11$^-$ gastric cancer cells MKN28, HLA-A11$^-$ cervical cancer cells RKN, HLA-A11$^-$ colon cancer cells SW620, and HLA-A11$^+$ PHA-blastoid T cells. A two-tailed Student's *t* test was used for the statistical analysis of the percentage of lysis of 1-87 or KE5 cells *versus* that of RKN or SW620 cells. *, $P < 0.05$. *B*, targets cells were HLA-A33$^+$ LC1, HLA-A33$^+$ cervical cancer cells RKN, HLA-A33$^-$ lung cancer cells 1-87, and HLA-A33$^+$ PHA-blastoid T cells. A two-tailed Student's *t* test was used for the statistical analysis of the percentage of lysis of LC1 or RKN cells *versus* that of 1-87 cells. *, $P < 0.05$. *C*, target cells were HLA-A3$^+$ astrocytoma Becher cells and HLA-A3$^-$ lung cancer QG56 cells. Indicated monoclonal antibody (mAb) was added at a dose of 20 μg/ml. These values represent the means of triplicate assays. A two-tailed Student's *t* test was used for the statistical analysis of the inhibition of lysis by mAbs in response to Becher cells. *, $P < 0.05$.

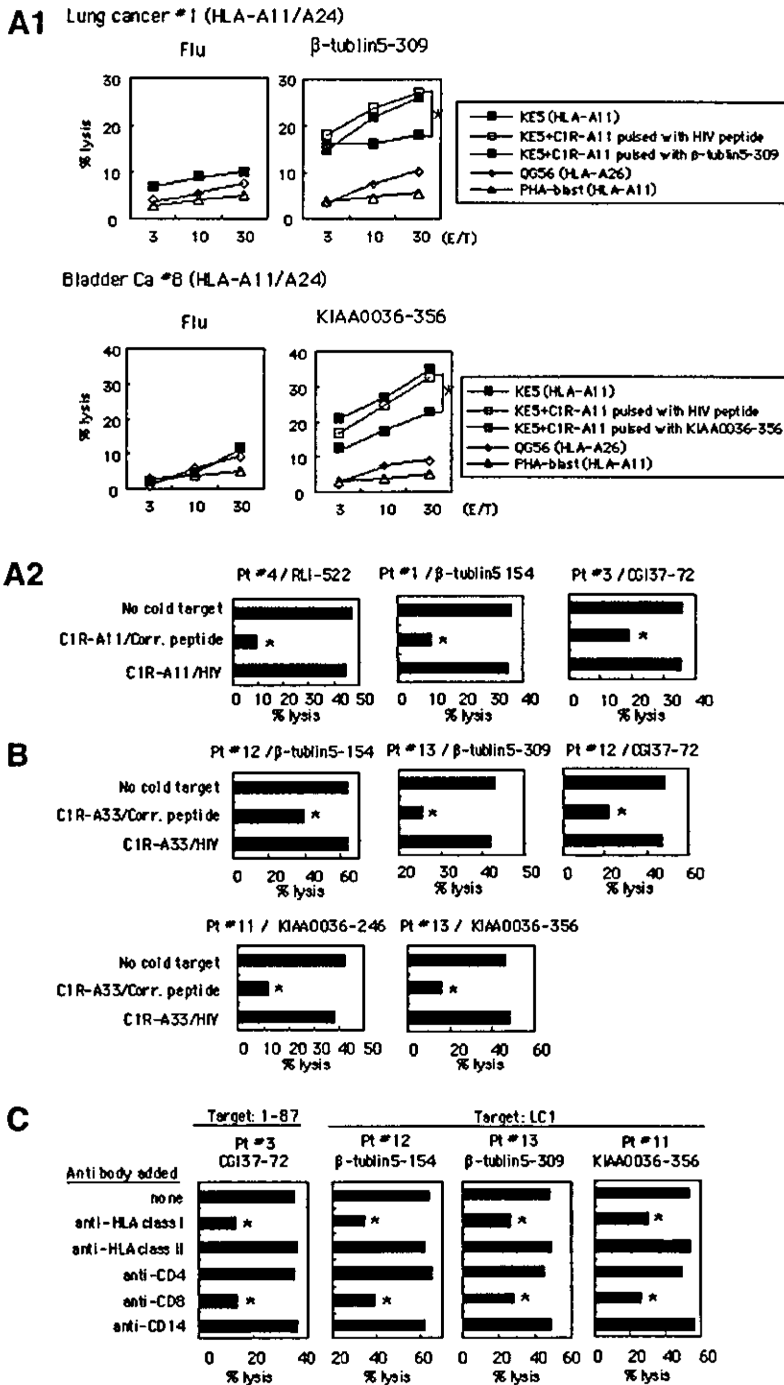

Fig. 3 Peptide specificity. Cold inhibition assay was carried out to confirm the peptide specificity of the CTLs. Unlabeled C1R-A11 or C1R-A33 cells that were preloaded with a corresponding peptide or a control HIV-peptide were added to the culture and tested to confirm the peptide specificity of the CTL activity against HLA-A11$^+$ KE5 tumor cells or HLA-A33$^+$ LC1 tumor cells. *A-1*, the effector cells were β-tublin5–309, KIAA0036–356, or Flu-stimulated peripheral blood mononuclear cells (PBMCs) from HLA-A11$^+$ cancer patients. The ratio of cold cells to hot cells was 10:1. *A-2*, the effector cells were RLI-522, β-tublin5–154, or CGI37–72-stimulated PBMCs from HLA-A11$^+$ cancer patients. The ratio of cold cells to hot cells was 20:1. *B*, the effector cells were PBMCs from HLA-A33$^+$ cancer patients, which were stimulated with each of the five peptides shown in the figure. The ratio of cold cells to hot cells was 20:1. A two-tailed Student's *t* test was used for the statistical analysis of these cold inhibition assays. *, $P < 0.05$. *C*, the indicated monoclonal antibody (mAb) was added at a dose of 20 μg/ml. The representative results are shown. These values represent the means of triplicate assays. A two-tailed Student's *t* test was used for the statistical analysis of the inhibition of lysis by mAbs in response to the target cells. *, $P < 0.05$.

the *HLA-A3* supertype, which is dominant in the human population (11). Namely, the phenotypic frequency of the *HLA-A3* supertype except for *HLA-A6801* allele is ~30%, 35%, 44%, 52%, and 35% among Caucasians, North American African-Americans, Japanese, Chinese, and Hispanics, respectively (11). Although *HLA-A3303* is not included in the A3 supertype classically, it was reported that HLA-A3303 binding peptides shared the same anchor residues for HLA-A3101 binding peptides (19, 20). We used *HLA-A3303*$^+$ PBMCs in this study, instead of *HLA-A3301* for the experiments, because it is a predominant allele in Asian population. Therefore, β-tublin5–154, β-tublin5–309, and CGI37–72 peptides, along with the TRP2–197 reference peptide, might be appropriate for use in a cancer vaccine for HLA-A3 supertype$^+$ cancer patients except with HLA-A6801$^+$ patients. Among them, the β-tublin5–154 is the most widely shared peptide possessing the ability to induce CTLs among HLA-A3 supertype under the present conditions. On the other hand, RLI-522 or KIAA0036–356 peptide induced tumor-

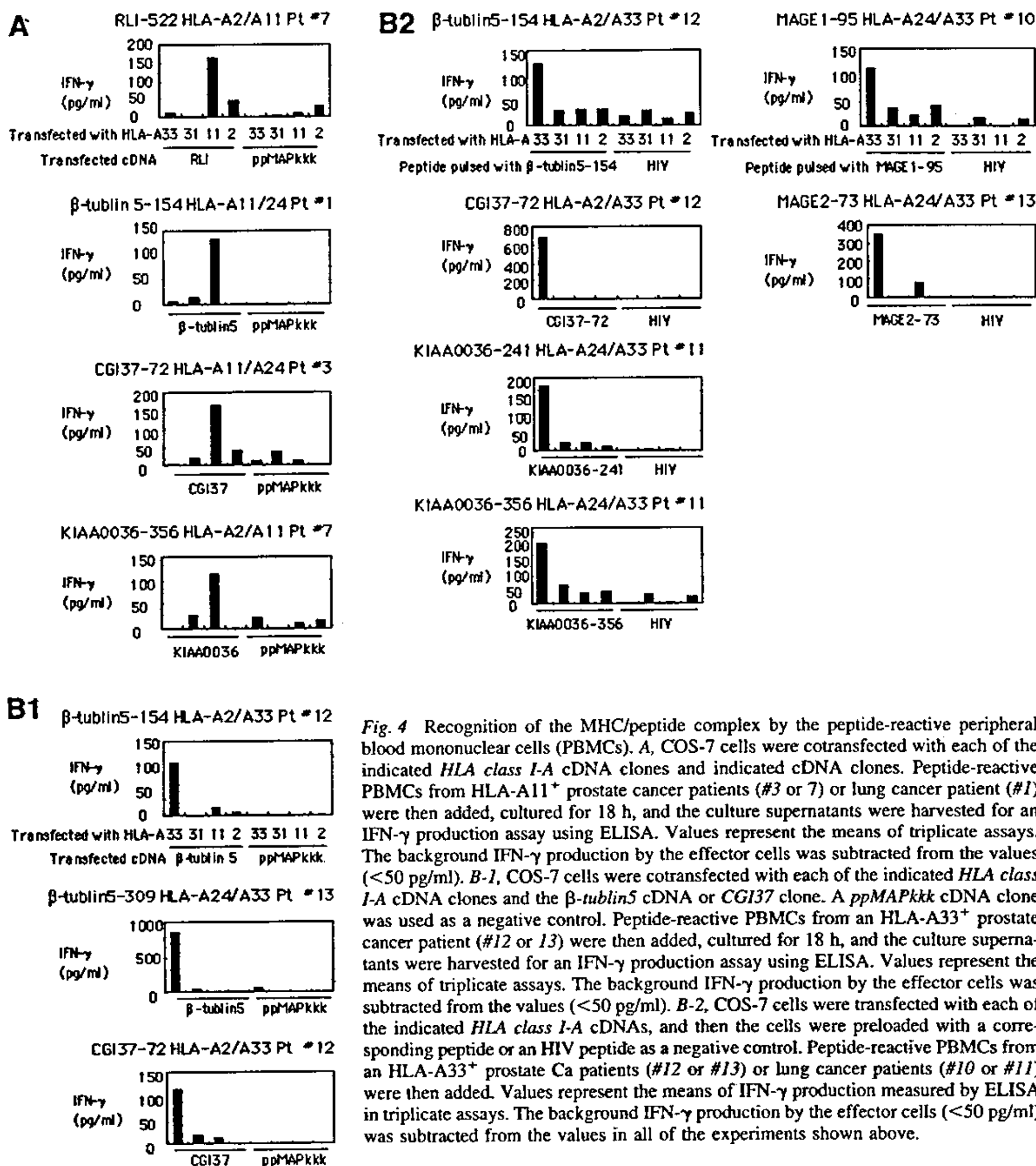

Fig. 4 Recognition of the MHC/peptide complex by the peptide-reactive peripheral blood mononuclear cells (PBMCs). *A*, COS-7 cells were cotransfected with each of the indicated *HLA class I-A* cDNA clones and indicated cDNA clones. Peptide-reactive PBMCs from HLA-A11$^+$ prostate cancer patients (*#3* or 7) or lung cancer patient (*#1*) were then added, cultured for 18 h, and the culture supernatants were harvested for an IFN-γ production assay using ELISA. Values represent the means of triplicate assays. The background IFN-γ production by the effector cells was subtracted from the values (<50 pg/ml). *B-1*, COS-7 cells were cotransfected with each of the indicated *HLA class I-A* cDNA clones and the β-*tublin5* cDNA or *CGI37* clone. A *ppMAPkkk* cDNA clone was used as a negative control. Peptide-reactive PBMCs from an HLA-A33$^+$ prostate cancer patient (*#12* or *13*) were then added, cultured for 18 h, and the culture supernatants were harvested for an IFN-γ production assay using ELISA. Values represent the means of triplicate assays. The background IFN-γ production by the effector cells was subtracted from the values (<50 pg/ml). *B-2*, COS-7 cells were transfected with each of the indicated *HLA class I-A* cDNAs, and then the cells were preloaded with a corresponding peptide or an HIV peptide as a negative control. Peptide-reactive PBMCs from an HLA-A33$^+$ prostate Ca patients (*#12* or *#13*) or lung cancer patients (*#10* or *#11*) were then added. Values represent the means of IFN-γ production measured by ELISA in triplicate assays. The background IFN-γ production by the effector cells (<50 pg/ml) was subtracted from the values in all of the experiments shown above.

reactive CTLs in HLA-A3$^+$ and -A11$^+$ or HLA-A11$^+$ and -A33$^+$ cancer patients with various frequencies (22–67%), respectively. Thus, these two peptides might also be useful for relatively large numbers of cancer patients. However, the PBMCs of only three HLA-A3$^+$ patients were tested, and -A68.1$^+$ patients were not tested, because these allele frequencies are extremely low in Japan (1.6 and 0.5%). Therefore, additional studies with more subjects with *HLA-A0301* and *-A6801* alleles are needed to confirm the results of this small-scale study.

Although the estimated binding affinities of the majority of peptides tested were relatively low in each of the HLA-A3 supertypes, these peptides, including KIAA0036–356 and β-tublin5–154, induced HLA-A3-supertype-restricted and tumor-reactive CTL activity in PBMCs from cancer patients tested with relatively high frequencies. No correlation between binding affinity and the ability to induce CTLs was obtained in this study; this result is consistent with those from previous studies (3, 21). These results suggest that the binding affinity for MHC class I molecules is not the only factor that is determinative of immunogenicity.

In regard to biological function of genes coding the peptides studied herein, RLI is a member of the superfamily of ATP-binding cassette transporters and blocks the activity of

protein synthesis in the 2–5A/RNase L system, the central pathway for viral IFN action (22, 23). RLI regulates mitochondrial mRNA stability by inhibiting antiproliferative action after IFN-α treatment (22). Thus, RLI appears to become activated under cytokine influence near tumor sites, along with enhancing intrinsic antitumor immune responses. It has been shown that β-tubulin is an integral component of microtubules, and that it plays important roles in mitosis, cell signaling, and motility (24). The elevated expression of β-tubulin is reported more often in cancer cells than in normal cells (25, 26). Thus, β-tubulin is one of the major targets of anticancer therapies (24). The function of CGI37 or KIAA0036 has not yet been determined. However, the mRNA expression levels of these genes are higher in cancer cells than in normal tissues except for testis (10).

As mentioned in the introduction, clinical trials of peptide vaccination are currently underway, although most are limited to use in either HLA-A2$^+$ or -A24$^+$ cancer patients (2, 9). This limitation is largely due to the relative infrequency of *HLA-A* alleles other than *HLA-A2* and *-A24* (<20%). The present results suggest potential vaccine candidates for HLA-A3 supertype$^+$ cancer patients, and, thus, may facilitate the development of both basic and clinical studies of peptide-based immunotherapy for cancer patients with alleles other than *HLA-A2* and *-A24*.

REFERENCES

1. Wang, R. F., and Rosenberg, S. A. Human tumor antigens recognized by T lymphocytes: implications for cancer therapy. J. Leukoc. Biol., *60:* 296–309, 1996.

2. Rosenberg, S. A., Yang, J. C., Schwartzentruber, D. J., Hwu, P., Marincola, F. M., Topalian, S. L., Restifo, N. P., Dudley, M. E., Schwarz, S. L., Spiess, P. J., Wunderlich, J. R., Parkhurst, M. R., Kawakami, Y., Seipp, C. A., Einhorn, J. H., and White, D. E. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat. Med., *4:* 321–327, 1998.

3. Wang, R. F., Johnston, S. L., Southwood, S., Sette, A., and Rosenberg, S. A. Recognition of an antigenic peptide derived from tyrosinase-related protein-2 by CTL in the context of HLA-A31 and -A33. J. Immunol., *160:* 890–897, 1998.

4. Martelange, V., De Smet, C., De Plaen, E., Lurquin, C., and Boon, T. Identification on a human sarcoma of two new genes with tumor-specific expression. Cancer Res., *60:* 3848–3855, 2000.

5. Correale, P., Walmsley, K., Zaremba, S., Zhu, M., Schlom, J., and Tsang, K. Y. Generation of human cytolytic T lymphocyte lines directed against prostate-specific antigen (PSA) employing a PSA oligoepitope peptide. J. Immunol., *161:* 3186–3194, 1998.

6. Molldrem, J., Dermime, S., Parker, K., Jiang, Y. Z., Mavroudis, D., Hensel, N., Fukushima, P., and Barrett, A. J. Targeted T-cell therapy for human leukemia: cytotoxic T lymphocytes specific for a peptide derived from proteinase 3 preferentially lyse human myeloid leukemia cells. Blood, *88:* 2450–2457, 1996.

7. Fisk, B., Blevins, T. L., Wharton, J. T., and Ioannides, C. G. Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. J. Exp. Med., *181:* 2109–2117, 1995.

8. Yang, D., Nakao, M., Shichijo, S., Sasatomi, T., Takasu, H., Matsumoto, H., Mori, K., Hayashi, A., Yamana, H., Shirouzu, K., and Itoh, K. Identification of a gene coding for a protein possessing shared tumor epitopes capable of inducing HLA-A24-restricted cytotoxic T lymphocytes in cancer patients. Cancer Res., *59:* 4056–4063, 1999.

9. Noguchi, M., Kobayashi, K., Suetsugu, N., Tomiyasu, K., Suekane, S., Yamada, A., Itoh, K., and Noda, S. Induction of cellular and humoral immune responses to tumor cells and peptides in HLA-A24 positive hormone-refractory prostate cancer patients by peptide vaccination. Prostate, *57:* 80–92, 2003.

10. Takedatsu, H., Shichijo, S., Azuma, K., Takedatsu, H., Sata, M., and Itoh, K. Detection of a set of peptide vaccine candidates for use in HLA-A31$^+$ epithelial cancer patietns. Int. J. Oncol., *24:* 337–347, 2004.

11. Sette, A., and Sidney, J. Nine major HLA class I supertypes account for the vast preponderance of HLA-A and -B polymorphism. Immunogenetics, *50:* 201–212, 1999.

12. Missale, G., Redeker, A., Person, J., Fowler, P., Guilhot, S., Schlicht, H. J., Ferrari, C., and Chisari, F. V. HLA-A31- and HLA-Aw68-restricted cytotoxic T cell responses to a single hepatitis B virus nucleocapsid epitope during acute viral hepatitis. J. Exp. Med., *177:* 751–762, 1993.

13. Aizawa, M. The Proceedings of the 3rd Asia-Oceania Histocompaatibility Workshop Conference, pp. 1080–1103. Oxford: Oxford University Press, 1986.

14. Alexander, J., Oseroff, C., Sidney, J., Wentworth, P., Keogh, E., Hermanson, G., Chisari, F. V., Kubo, R. T., Grey, H. M., and Sette, A. Derivation of HLA-A11/Kb transgenic mice: functional CTL repertoire and recognition of human A11-restricted CTL epitopes. J. Immunol., *159:* 4753–4761, 1997.

15. Safrit, J. T., Andrews, C. A., Zhu, T., Ho, D. D., and Koup, R. A. Characterization of human immunodeficiency virus type 1-specific cytotoxic T lymphocyte clones isolated during acute seroconversion: recognition of autologous virus sequences within a conserved immunodominant epitope. J. Exp. Med., *179:* 463–472, 1994.

16. Rammensee, H. G., Friede, T., and Stevanoviic, S. MHC ligands and peptide motifs: first listing. Immunogenetics, *41:* 178–228, 1995.

17. Maeda, Y., Hida, N., Niiya, F., Katagiri, K., Harada, M., Yamana, H., Kamura, T., Takahashi, M., Sato, Y., Todo, S., and Itoh, K. Detection of peptide-specific CTL-precursors in peripheral blood lymphocytes of cancer patients. Br. J. Cancer, *87:* 796–804, 2002.

18. Kawashima, I., Tsai, V., Southwood, S., Takesako, K., Celis, E., and Sette, A. Identification of gp100-derived, melanoma-specific cytotoxic T-lymphocyte epitopes restricted by HLA-A3 supertype molecules by primary in vitro immunization with peptide-pulsed dendritic cells. Int. J. Cancer, *78:* 518–524, 1998.

19. Takiguchi, M., Matsuda, T., and Tomiyama, H. Polarity of the P1 anchor residue determines peptide binding specificity between HLA-A*3101 and HLA-A*3303. Tissue Antigens, *56:* 501–506, 2000.

20. Takiguchi, M., Matsuda, T., Tomiyama, H., and Miwa, K. Analysis of three HLA-A*3303 binding peptide anchors using an HLA-A*3303 stabilization assay. Tissue Antigens, *55:* 296–302, 2000.

21. Deng, Y., Yewdell, J. W., Eisenlohr, L. C., and Bennink, J. R. MHC affinity, peptide liberation, T cell repertoire, and immunodominance all contribute to the paucity of MHC class I-restricted peptides recognized by antiviral CTL. J. Immunol., *158:* 1507–1515, 1997.

22. Martinand, C., Montavon, C., Salehzada, T., Silhol, M., Lebleu, B., and Bisbal, C. RNase L inhibitor is induced during human immunodeficiency virus type 1 infection and down regulates the 2–5A/RNase L pathway in human T cells. J. Virol., *73:* 290–296, 1999.

23. Bisbal, C., Martinand, C., Silhol, M., Lebleu, B., and Salehzada, T. Cloning and characterization of a RNAse L inhibitor. A new component of the interferon-regulated 2–5A pathway. J. Biol. Chem., *270:* 13308–13317, 1995.

24. Jordan, M. A., and Wilson, L. Microtubules and actin filaments: dynamic targets for cancer chemotherapy. Curr. Opin. Cell Biol., *10:* 123–130, 1998.

25. Hashimoto, Y., Tajima, O., Hashiba, H., Nose, K., and Kuroki, T. Elevated expression of secondary, but not early, responding genes to phorbol ester tumor promoters in papillomas and carcinomas of mouse skin. Mol. Carcinog., *3:* 302–308, 1990.

26. Kato, K., Ito, H., Inaguma, Y., Okamoto, K., and Saga, S. Synthesis and accumulation of αB crystallin in C6 glioma cells is induced by agents that promote the disassembly of microtubules. J. Biol. Chem., *271:* 26989–26994, 1996.

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ORIGINAL ARTICLE

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Identification of new prostate stem cell antigen-derived peptides immunogenic in HLA-A2$^+$ patients with hormone-refractory prostate cancer

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Abstract *Purpose* Prostate cancer refractory to hormonal manipulation requires new treatment modalities. In the present study we attempted to identify prostate stem cell antigen (PSCA)-derived peptides immunogenic in HLA-A2$^+$ prostate cancer patients in order to develop peptide-based immunotherapy against hormone-refractory prostate cancer (HRPC).
Methods Eleven different PSCA-derived peptides, which were prepared based on the HLA-A2 binding motif, were examined to determine whether they would be recognized by cellular and humoral immune responses in 12 HLA-A2$^+$ patients (11 with HRPC and 1 with non-HRPC).
Results Among the PSCA-derived peptides, PSCA 7–15 and PSCA 21–30 peptides effectively induced HLA-A2-restricted peptide-specific and tumor-reactive cytotoxic T lymphocytes (CTLs) from peripheral blood mononuclear cells (PBMCs) of HLA-A2$^+$ patients. The PSCA 21–30 peptide was capable of inducing peptide-specific CTLs in both cancer patients and healthy donors, whereas the PSCA 7–15 peptide was immunogenic in only cancer patients. Immunoglobulin G (IgG) reactive to the PSCA 21–30 peptide was detected in plasma of most patients and healthy donors, whereas IgG reactive to PSCA 7–15 was undetectable in all cases. These results indicate that the former peptide elicits both cellular and humoral immune responses in both patients and healthy donors, whereas the latter elicits only cellular responses in patients.
Conclusion These two PSCA peptides should be considered for use in clinical trials of immunotherapy for HLA-A2$^+$ HRPC patients.

Keywords Prostate cancer · PSCA · CTLs · Peptide · Antibody

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Introduction

Prostate cancer is one of the most common cancers in older men [5]. Although hormone ablation therapy can temporarily palliate patients with advanced disease, the progression to hormone-refractory prostate cancer (HRPC) is inevitable in most cases [17]; therefore, the development of novel therapeutic modalities for the treatment of HRPC is necessary. One such therapy could be peptide-based immunotherapy, as recent advances in tumor immunology have enabled us to identify many genes encoding tumor antigens and their peptides that are recognized by cytotoxic T lymphocytes (CTLs) [1, 28]. Several prostate-specific antigens and antigen-derived peptides have also been reported, including prostate-specific antigen (PSA) [2, 3, 31] prostate-specific membrane antigen (PSMA) [10, 30], and prostatic acid phosphatase (PAP) [12, 25]. Some of these antigen-derived peptides have been used in the treatment of prostate cancer patients, but the clinical responses observed thus far have been unsatisfactory [19, 20, 21]; therefore, new antigens and peptides suitable for use in specific immunotherapy for patients with HRPC are needed.

The prostate stem cell antigen (PSCA) is a recently identified antigen expressed on the cell surface of prostate cancer cells [27]. This antigen is a glycosylphosphatidylinositol-anchored protein, and is overexpressed by both androgen-dependent and androgen-independent prostate cancers [27], suggesting that this antigen could be a good candidate for specific immunotherapy for patients with HRPC. In addition, immunotherapy targeting on PSCA could be promising for the treatment of patients with bone metastases, as the expression of PSCA in prostate cancer is higher in metastases than in primary tumors [6].

We report in this study new PSCA-derived peptides that can be recognized by both cellular and humoral immune responses in HLA-A2$^+$ prostate cancer patients.

Materials and methods

Patients

Twelve HLA-A2$^+$ prostate cancer patients (11 with HRPC and 1 with non-HRPC) and 5 HLA-A2$^+$ healthy volunteers were enrolled in this study after informed consent was obtained. None of these participants were infected with human immunodeficiency virus (HIV). Twenty milliliters of peripheral blood was obtained, and peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll-Conray density-gradient centrifugation. The expression of HLA-A2 molecules on PBMCs of cancer patients and healthy donors was first determined by flow cytometry, and HLA-A2 subtypes were determined by the sequence-specific oligonucleotide probe method.

Cell lines

T2 is an HLA-A*0201-expressing line. PC93 is an HLA-A2-negative prostate cancer cell line (HLA-A*6802$^+$) that was established by K. Ohishi (Department of Urology, Kyoto University, Japan). To establish PC93 cells stably expressing HLA-A2 molecules, pCR3.1 vector (Invitrogen, Calif.), which was inserted with the *HLA-A*0201* gene, was electroporated into PC93 cells and selected with neomycin (Gibco BRL, Grand Island, N.Y.) at a dose of 0.75 mg/ml. An established cell line was designated as PC93-A2. LNCap is a prostate carcinoma cell line. Both colo201 and colo302 are HLA-A2$^+$ and HLA-A2$^-$ colon carcinoma cell lines, respectively. All cell lines were maintained in RPMI-1640 medium (Gibco BRL) supplemented with 10% FCS.

RT-PCR

Total RNA was isolated from cancer cell lines using RNAzol B (Tel-Test, Friendswood, Texas). The cDNA was prepared using the SuperScript Preamplification System for First Strand cDNA Synthesis (Invitrogen, Calif.), and it was amplified using the following primers. The primer pair used for PSCA was as follows:

- Sense primer, 5'-GCAAGAAGAACATCACGTGC-3'
- Antisense primer, 5'-TAGGATGTGCCTCAGGAACC-3'.

The primer pair used for glyceraldehydes 3-phosphate dehydrogenase (GAPDH) was as follows:

- Sense primer, 5'-ACAACAGCCTCAAGATCATCAG-3',
- Antisense primer, 5'-GGTCCACCACTGACACGTTG-3'.

The PCR was performed using Taq DNA polymerase in a DNA thermal cycler (iCycler, Bio-Rad Laboratories, Calif.) for 30 cycles (at 94°C for 1 min, 60°C for 2 min, and 72°C for 1 min).

Flow cytometric analysis

The expression of PSCA on tumor cell lines were examined using anti-PSCA mAb (1G8: mouse IgG1) [6], which was kindly provided by R.E. Reiter (Department of Urology, University of California). To examine the expression of HLA-A2 molecules on tumor cell lines, cells were stained by anti-HLA-A2 mAb, followed by FITC-conjugated goat anti-mouse IgG. The results were analyzed by the CELLQuest program (Becton Dickinson, Calif.).

Peptides

Eleven PSCA-derived peptides, which are listed in Table 1, were prepared based on the HLA-A*0201 binding motif. All peptides were of >90% purity and were purchased from Biologica (Nagoya, Japan). Influenza virus-derived (GILGFVFTL), Epstein-Barr virus (EBV)-derived (GLCTLVAML), and HIV-derived (SLYNTYATL)

Table 1 Reactivity of prostate stem cell antigen (PSCA) peptide-stimulated peripheral blood mononuclear cells (PBMCs) from HLA-A2$^+$ patients with prostate cancer. *EBV* Epstein-Bar virus. *ND* not determined

Peptides	Amino acid sequence	Score[a]	Prostate cancer patients												Total
			1 A0201	2 A0201	3 A0206	4 A0206	5 A0206	6 A0206	7 A0206	8 A0206	9 A0206	10 A0207	11 A0207	12 A0210	
			IFN-γ production (pg/ml)[b]												
PSCA4–13	VLLALLMAGL	309	0	*415*	4	5	19	*629*	20	86	0	25	26	19	2 of 12
PSCA5–13	LLALLMAGL	84	0	7	52	15	39	*136*	66	63	0	*116*	60	14	2 of 12
PSCA7–15	ALLMAGLAL	79	5	0	*101*	50	10	*104*	*121*	*133*	0	*198*	56	19	5 of 12
PSCA21–30	LLCYSCKAQV	118	4	*124*	0	*302*	89	*1342*	0	60	0	*291*	86	2	4 of 12
PSCA43–51	OLGEQCWTA	153	20	0	32	27	10	*274*	43	2	21	24	*131*	22	2 of 12
PCA70–79	CVDDSQDYYV	55	0	54	40	50	16	*253*	25	0	64	74	47	*121*	2 of 12
PSCA106–115	LLALLPALGL	36	0	5	35	34	34	*143*	*116*	27	61	34	*147*	55	3 of 12
PSCA108–117	ALLPALGLLL	79	7	20	46	40	39	28	15	0	50	11	59	46	0 of 12
PWA109–117	LLPALGLLL	36	10	8	63	40	1	58	14	23	36	57	92	93	0 of 12
PSCA14–22	ALQPGTALL	21	0	4	0	5	6	ND	ND	0	0	20	67	0	0 of 10
PSCA105–113	ALLALLPAL	25	0	0	24	29	5	ND	ND	0	17	50	79	17	0 of 10
EBV	GLCTLVAML	–	2	*312*	21	21	17	*142*	44	*847*	0	0	53	*173*	4 of 12
Flu	GILGFVFTL	–	0	0	*554*	22	*292*	*236*	*395*	34	26	*105*	57	*134*	5 of 12

[a]Scores represent the estimated half-time of dissociation of the PSCA peptides binding HLA-A2 molecules
[b]The PBMCs of HLA-A2$^+$ prostate cancer patients were in vitro stimulated with the indicated PSCA peptides, as described in "Materials and methods"
On day 15, the cultured PBMCs were tested for their reactivity to T2 cells, which were pre-pulsed with a corresponding peptide in quadruplicate
Values represent the results of the best of the four wells, and background IFN-γ production in response to the HIV peptide was subtracted
Successful induction of peptide-specific CTLs was judged to be positive when more than 100 pg/ml IFN-γ was produced in response to corresponding peptide-pulsed T2 cells compared with HIV peptide-pulsed T2 cells. Positive responses are italicized

peptides with the HLA-A2 binding motif were used as a control. All peptides were dissolved with dimethyl-sulfoxide at a dose of 10 mg/ml.

Assay for peptide-specific CTLs in PBMCs

The assay for the detection of peptide-specific CTLs was performed according to a previously reported method with several modifications [9]. In brief, PBMCs ($1{\times}10^5$ cells/well) were incubated with 10 μg/ml of each peptide in a U-bottom-type 96-well microculture plate (Nunc, Roskilde, Denmark) in 200 μl of culture medium. The culture medium consisted of 45% RPMI 1640, 45% AIM-V medium (Gibco BRL), 10% FCS, 50 U/ml of interleukin-2 (IL-2), and 0.1 mM MEM nonessential amino acid solution (Gibco BRL). Half of the culture medium was removed and replaced with the new medium containing a corresponding peptide (20 μg/ml) every 5 days. On the fifteenth day of culture, the harvested cells were tested for their ability to produce interferon (IFN)-γ in response to T2 cells ($1{\times}10^4$ cells/well), which were pre-loaded with either a corresponding peptide or the HIV peptide as a negative control. After an 18-h incubation, the supernatant was collected, and the level of IFN-γ was determined by enzyme-linked immunosorbent assay (ELISA; limit of sensitivity: 10 pg/ml).

A small number of PBMCs ($1{\times}10^5$/well) were applied in microwell plates, and were separately stimulated with corresponding peptides in an assay for peptide-specific CTLs. Under these conditions, the reactivity varied considerably among individual wells, as reported previously [9]; therefore, each well was separately estimated to screen for the presence of peptide-specific CTL precursors.

In vitro culture for CTL assay

The PBMCs from patients ($1{\times}10^5$ cells/well) were incubated with 10 μg/ml of each peptide in a U-bottom-type 96-well microculture plate (Nunc, Roskilde, Denmark) at a volume of 200 μl of the culture medium containing 50 U/ml of IL-2. Half of the culture medium was removed and replaced with the new medium containing a corresponding peptide (20 μg/ml) and IL-2 (50 U/ml) every 5 days. On the fifteenth day of culture, half of the cultured cells were harvested, washed, and divided into three wells. The cultured cells in each well were cultured with or without PC93 or PC93-A2 cells ($1{\times}10^4$/well). After an 18-h culture, the supernatants were collected, and the levels of IFN-γ were determined by ELISA. Then, the cells in the wells producing IFN-γ in an HLA-A2-restricted manner were collected and further cultured in order to obtain a sufficiently large number of cells to carry out a CTL assay.

Cytotoxicity assay

Cultured cells were tested for cytotoxicity against both PC93 and PC93-A2 by a 6-h ^{51}Cr-release assay.Two thousand ^{51}Cr-labeled cells per well were cultured with effector cells in 96 round-well plates at the indicated effector/target ratios. The specific ^{51}Cr-release was calculated according to the following formula:

$$\frac{\text{test c.p.m.} - \text{spontaneous c.p.m.}}{\text{test c.p.m.} - \text{spontaneous c.p.m.}} \times 100$$

Spontaneous release was determined by the supernatant of the sample incubated with no effector cells, and the total release was then determined by the supernatant of the sample incubated with 1% Triton X (Wako Pure Chemical Industries, Osaka, Japan). In some experiments, $CD8^+$ T cells were positively isolated using CD8 Positive Isolation Kit (Dynal, Oslo, Norway) from the PSCA peptide-stimulated PBMCs. The positive percentage of $CD8^+$ T cells was >97%. In other experiments of cytotoxicity assay, 10 μg/ml of either anti-HLA class I (W6/32: mouse IgG2a) or anti-HLA-DR (L243: mouse IgG2a) mAb was added into wells at the initiation of the culture.

Cold inhibition assay

The specificity of PSCA peptide-stimulated CTLs was confirmed by a cold inhibition assay. In brief, ^{51}Cr-labelled target cells ($2{\times}10^3$ cells/well) were cultured with the CTLs ($2{\times}10^4$ cells/well) in 96 round-well plates with $4{\times}10^4$ cold target cells. T2 cells, which were pre-pulsed with either the HIV peptide or the corresponding PSCA peptide, were used as cold target cells.

Detection of peptide-specific immunoglobulin G

Peptide-specific immunoglobulin G (IgG) levels in plasma were measured by ELISA, as previously reported [22, 23]. Briefly, a peptide (20 μg/well)-immobilized plate was blocked with Block Ace

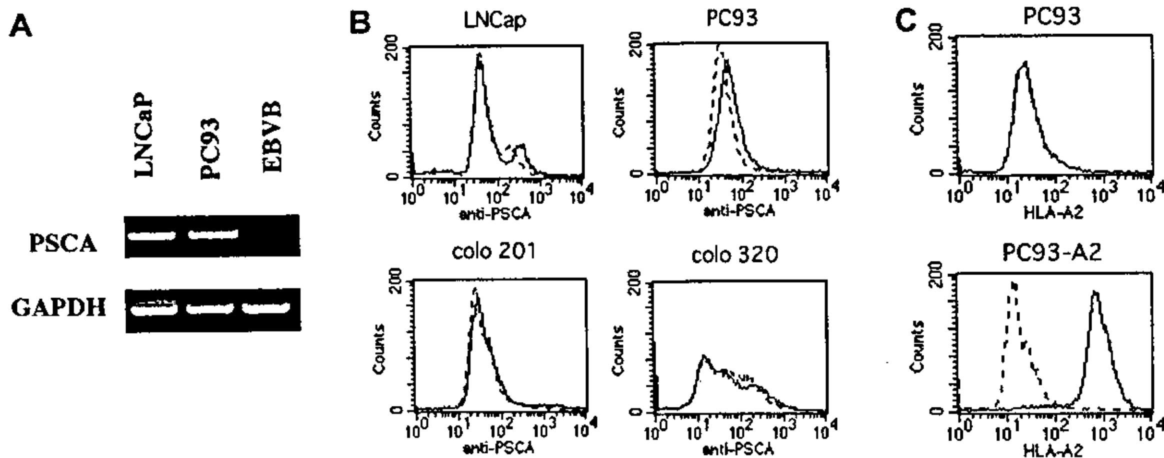


Fig. 1A–C Expression of prostate stem cell antigen (*PSCA*) on PC93 cells and an HLA-A2-expressing transfectant cell line. **A** RT-PCR was carried out using cDNA from either LNCaP, PC93, or Epstein-Barr virus (EBV)-transformed B cells. Thirty PCR cycles were carried out. *GAPDH* glyceraldehyde 3-phosphate dehydrogenase. **B** Flow cytometric analysis was performed on LNCap, PC93, colo201, and colo320 cells. These cells were stained with anti-PSCA mAb, followed by FITC-conjugated anti-mouse IgG mAb. The *dashed lines* represent the staining without the first mAb. **C** Flow cytometric analysis was performed on PC93 and PC93-A2 cells. These cells were stained with anti-HLA-A2 mAb, followed by FITC-conjugated anti-mouse IgG mAb. The *dashed lines* represent the staining without the first mAb

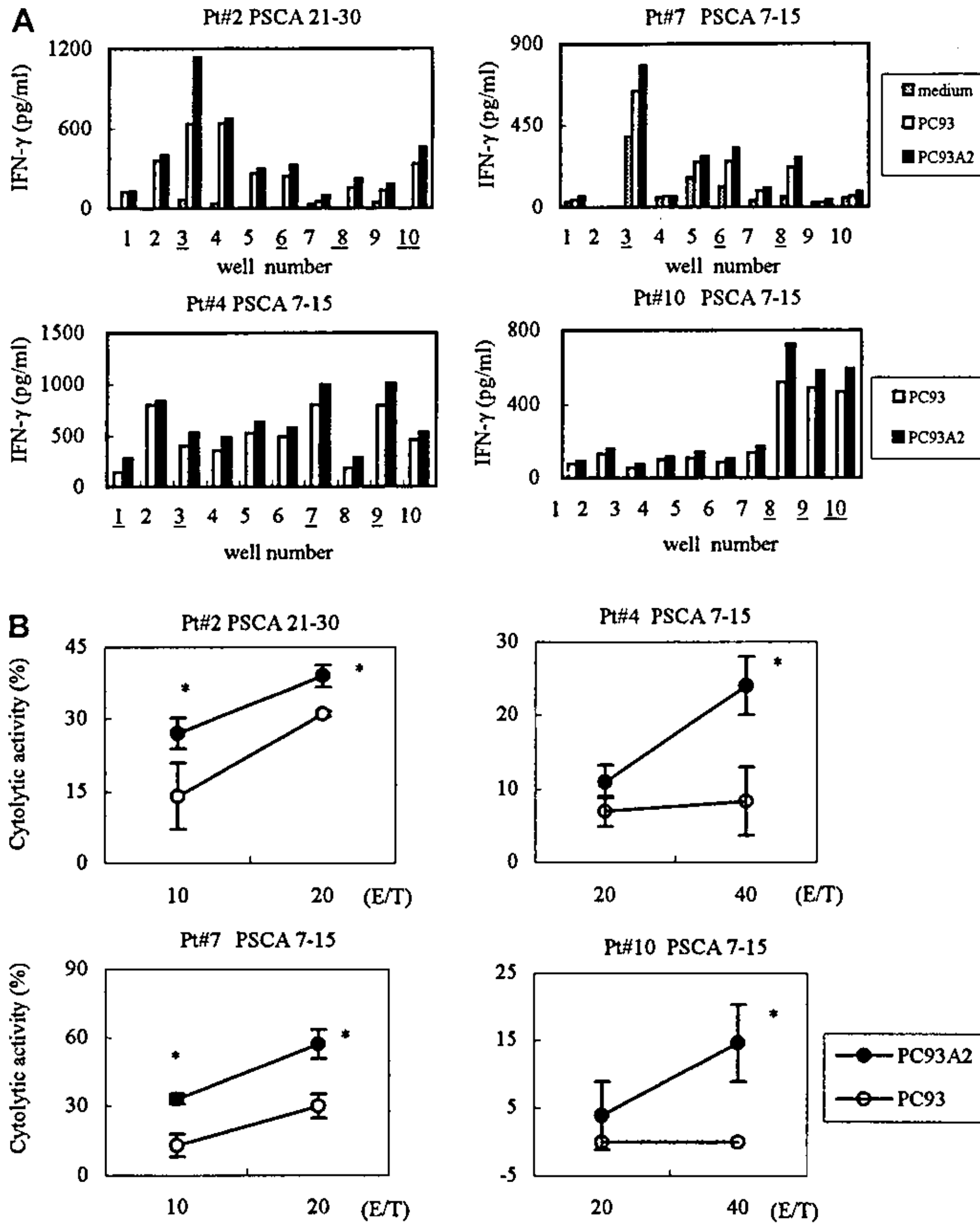


Fig. 2A, B Induction of HLA-A2-restricted and prostate cancer-reactive cytotoxic T lymphocytes (CTLs) from peripheral blood mononuclear cells (PBMCs) of cancer patients. **A** The PBMCs from 4 HLA-A2$^+$ prostate cancer patients were in vitro stimulated with the indicated PSCA peptides in 10 wells, as described in "Materials and methods." On day 15, half of the cultured cells were separately harvested and cultured with or without PC93 or PC93-A2 cells for 18 h. The levels of IFN-γ in supernatants were then determined by ELISA. The cultured cells in wells, which are underlined at the well number, were further cultured with IL-2 alone for approximately 10 days for the CTL assay. **B** An assay of cytotoxicity carried out for 6-h was performed against PC93 and PC93-A2. Values represent the mean of triplicate assays. * $p < 0.05$ was considered statistically significant

(Yukijirushi, Tokyo, Japan) and washed with 0.05% Tween 20-PBS, after which 100 μl /well of plasma sample diluted with 0.05% Tween20-Block Ace was added to the plate. After a 2-h incubation at 3°C, the plates were washed and further incubated for 2 h with a 1:1000-diluted rabbit anti-human IgG (γ-chain specific; Dako, Glostrup, Denmark). The plate was washed, and then 100 μl of 1:100-diluted goat anti-rabbit IgG-conjugated horseradish peroxidase (En Vision, Dako) was added to each well, and the plate was incubated at room temperature for 40 min. After the plate was washed again, 100 μl/well of tetramethyl benzidine substrate solution (KPL, Guildford, UK) was added, and the reaction was stopped by the addition of 1 M phosphoric acid. To estimate peptide-specific IgG levels, we compared the optical density (OD) values of each sample with those of serially diluted standard samples, and the values are shown as OD units/ml. To confirm the specificity of IgG to the PSCA 21–30 peptide, sample plasma was cultured with plates coated with either the PSCA 21–30 peptide or the control HIV peptide; thereafter, the levels of PSCA 21–30 peptide-specific IgG in the resultant supernatant were determined by ELISA.

Statistics

The statistical significance of the data was determined using a two-tailed Student's *t* test. A *p* value of less than 0.05 was considered to be statistically significant.

Results

Induction of PSCA peptide-specific CTLs from prostate cancer patients

The PBMCs from 12 patients (11 with HRPC and 1, patient 2, with non-HRPC) were incubated with each of 11 kinds of PSCA-derived peptides or control peptides, and were examined for their IFN-γ production in response to corresponding peptide-pulsed T2 cells (Table 1). The assay was carried out in quadruplicate, and the results of the best well are shown. Successful induction of peptide-specific CTLs was judged to be positive when more than 100 pg/ml of IFN-γ was produced in response to the corresponding peptide-pulsed T2 cells compared with HIV peptide-pulsed T2 cells. The PSCA 14–22 and PSCA 105–113 peptides, which have been reported to be capable of inducing peptide-specific CTLs from HLA-A2$^+$ cancer patients [4, 14], failed to induce peptide-specific CTLs with the employed culture system. The PSCA 108–117 and PSCA 109–117 peptides failed to induce peptide-specific CTL from any patients. Each of five PSCA peptides, including PSCA 4–13, PSAC 5–13, PSCA 43–51, PSCA 70–79, and PSCA 106–115 peptide, induced peptide-specific CTLs in 2 or 3 of 12 patients. The PSCA 7–15 and PSCA 21–30 peptides induced peptide-specific CTLs in 5 and 4 of 12 patients, respectively. Based on these findings, the PSCA 7–15 and PSCA 21–30 peptides were the focus in the subsequent experiments.

Induction of prostate cancer-reactive CTLs by the PSCA 7–15 and PSCA 21–30 peptides

It is important to determine whether or not peptide-induced CTLs show any reactivity against prostate cancer cells. The reactivity to either a parental PC93 cell line or its HLA-A2-transfectant, PC93-A2, was compared in order to test HLA-A2-restricted CTL responses to prostate cancer cells. RT-PCR revealed that PC93 was positive for the mRNA expression of PSCA, similar to LNCaP, which is widely used as a prostate cancer cell line (Fig. 1A). The expression of PSCA on tumor cell lines was examined using anti-PSCA mAb (Fig. 1B). In accordance with a previous report [6], LNCap cells were negative, although a part of them was non-specifically stained. PC93 was lowly but positively stained with anti-PSCA mAb. In contrast, both colo201 and colo320 tumor cell lines were negative. In addition, as shown in Fig. 1C, PC93 was negative for the expression of HLA-A2 molecules, whereas PC93-A2 was positive for HLA-A2 molecules on their cell surface.

The PBMCs from 4 HLA-A2$^+$ patients were cultured in 10 wells in the presence of the PSCA 7–15 or PSCA 21–30 peptide to determine whether PSCA peptide-reactive CTLs from HLA-A2$^+$ patients show reactivity against prostate cancer. After 15 days of culture, half of the cultured cells were used to test the reactivity against prostate cancer cells, and representative results are shown in Fig. 2A. Among 10 wells, the cultured cells in 3 or 4 wells produced a higher level of IFN-γ in response to PC93-A2 than to PC93. The cells in these positive wells, which are underlined in Fig. 2A, were further cultured with IL-2 for a cytotoxicity assay. The peptide-stimulated CTLs exhibited a higher level of cytotoxicity against PC93-A2 than against PC93 (Fig. 2B). These results indicate that PBMCs, which were in vitro stimulated with either the PSCA 7–15 or PSCA 21–30 peptide, can show HLA-A2-restricted cytotoxicity against prostate cancer cells.

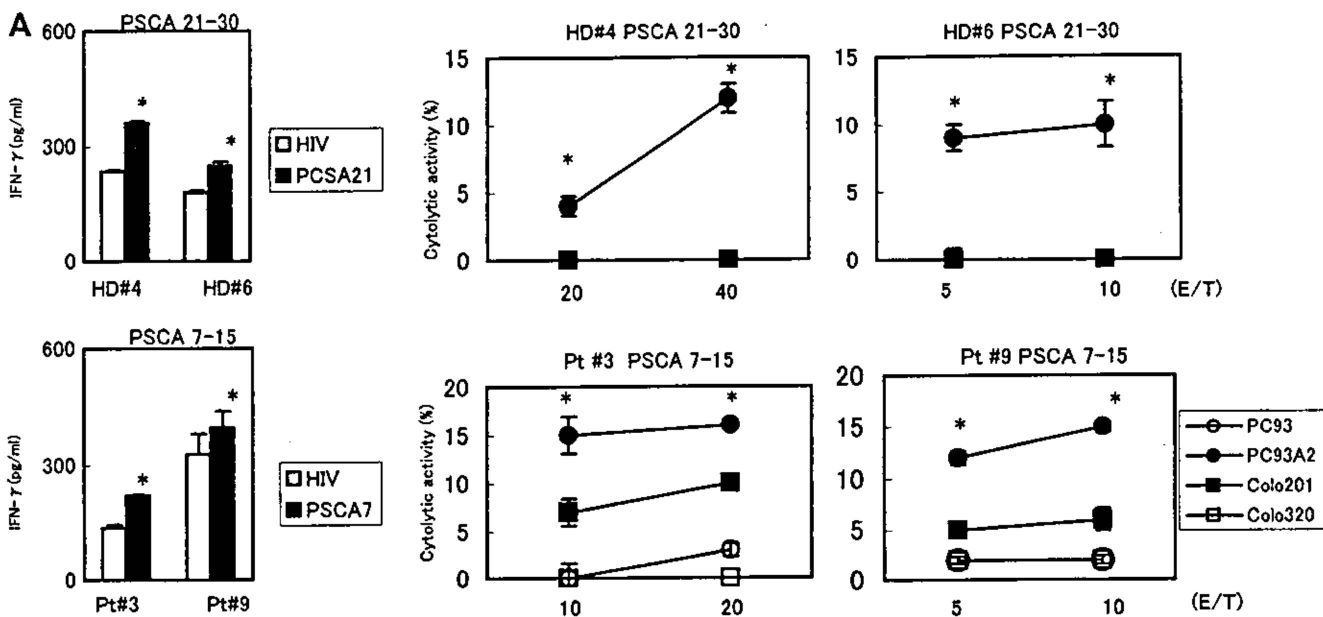
Table 2 Reactivity of PSCA peptide-stimulated PBMCs from HLA-A2$^+$ healthy donors

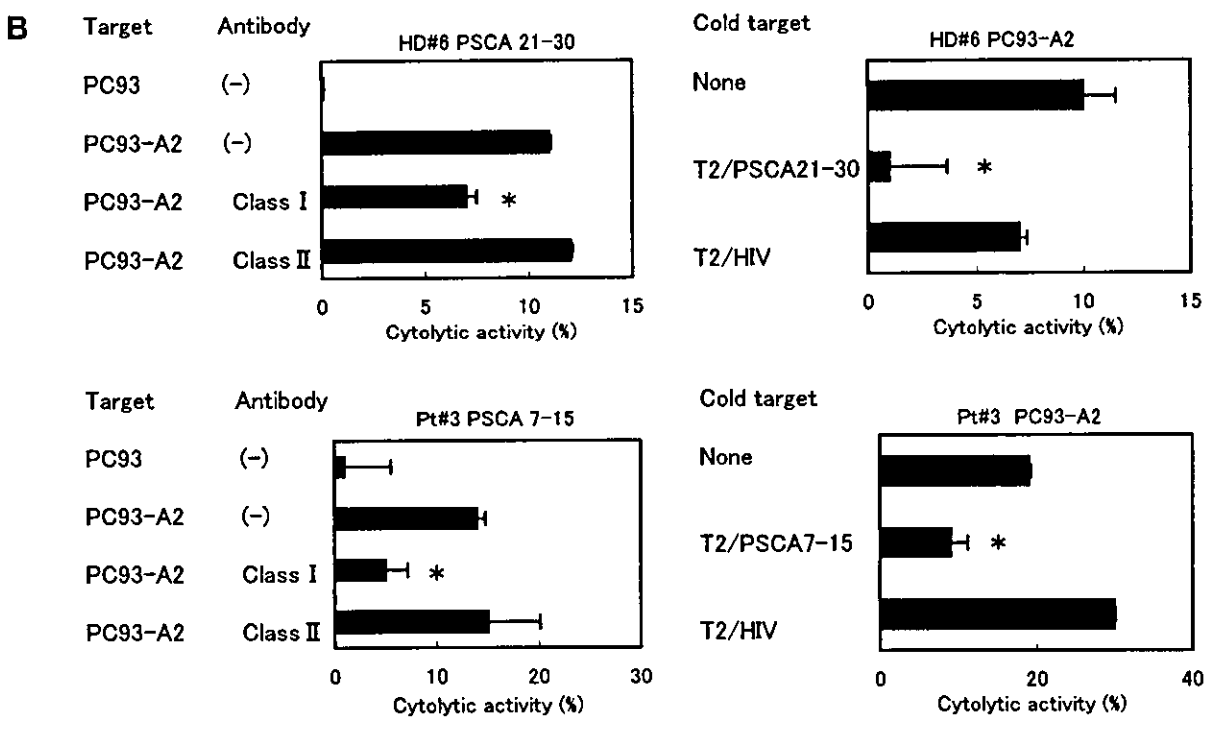
Peptides	Healthy donors					
	1 A0201	2 A0202	3 A0203	4 A0206	5 A0207	Total
	IFN-γ production (pg/ml)[a]					
PSCA4–13	*255*	10	12	0	*263*	2 of 5
PSCA5–13	14	24	70	0	5	0 of 5
PSCA7–15	5	45	3	0	0	0 of 5
PSCA21–30	*1016*	*106*	*250*	0	0	3 of 5
PSCA43–51	*129*	11	5	9	*568*	2 of 5
PSCA76–79	*143*	20	5	16	*530*	2 of 5
PSCA106–115	50	20	43	2	*455*	1 of 5
PSCA108–117	5	33	*106*	*121*	0	2 of 5
PSCA109–117	24	14	*2179*	4	*2006*	2 of 5
Flu	*272*	62	33	*179*	*741*	3 of 5

[a] The PBMCs of HLA-A2$^+$ healthy donors were in vitro stimulated with the indicated PSCA peptides, as described in "Materials and methods." On day 15, the cultured PBMCs were tested for their reactivity to T2 cells, which were pre-pulsed with a corresponding peptide in quadruplicate

Values represent the results of the best of the four wells, and background IFN-γ production in response to the HIV peptide was subtracted

Successful induction of peptide-specific CTLs was judged to be positive when more than 100 pg/ml IFN-γ was produced in response to corresponding peptide-pulsed T2 cells compared with HIV peptide-pulsed T2 cells. Positive responses are italicized





Induction of PSCA peptide-reactive CTLs from healthy donors

Some prostate-related antigens have the potential to induce peptide-specific CTLs even from healthy donors [7, 8]; therefore, we determined whether PSCA-derived peptides could induce peptide-specific CTLs from PBMCs of HLA-A2$^+$ healthy donors (Table 2). The PSCA 21–30 peptide induced peptide-specific CTLs in PBMCs of 3 of 5 healthy donors. Each of the PSCA 4–13, PSCA 43–51, PSCA 70–79, PSCA 108–117, and PSCA 109–117 peptides induced peptide-specific CTLs in PBMCs of two healthy donors. The PSCA 106–115 peptide induced peptide-specific CTLs in 1 patient. The PSCA 5–13 and PSCA 7–15 peptides failed to induce peptide-specific CTLs in any patients. The inability of the PSCA 7–15 peptide of inducing peptide-specific CTLs from PBMCs of HLA-A2$^+$ healthy donors was in sharp contrast to the results of peptide-specific CTL induction from PBMCs of cancer patients.