

A β -tubulin 5-derived peptide induces cytotoxic T lymphocytes restricted to the HLA-A24 allele in prostate cancer patients

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Abstract. To facilitate the development of a peptide-based cancer vaccine for prostate cancer patients, we examined whether any of the 13 peptides previously reported to induce HLA-class I-restricted cytotoxic T lymphocyte (CTL) activity in HLA-A3 supertype (-A3, -A11, -A31 and -A33)-positive prostate cancer patients are also capable of inducing CTLs restricted to HLA-A2, HLA-A24 or HLA-A26 alleles. Among the 13 peptides tested, a peptide at positions 309 to 318 of β -tubulin 5 exhibited binding activity to the HLA-A*2402 molecule and induced HLA-A24-restricted CTL activity against prostate cancer cells derived from peripheral blood mononuclear cells of prostate cancer patients. The CTL activity was determined to be specific to this peptide and was mediated by CD8⁺ T cells in an HLA-class I-restricted manner. These results suggest that this peptide could be applicable as a peptide vaccine, not only for HLA-A3 supertype-positive, but also for HLA-A24-positive prostate cancer patients.

Introduction

A peptide-based cancer vaccine is one of the new treatment modalities for cancer. We recently reported that it has a clinical benefit for advanced prostate cancer patients in a randomized

clinical trial (1). However, peptide-based immunotherapy for cancer patients is highly restricted by HLA-A alleles, which in turn is hampering the development of peptide-based cancer vaccines at the commercial level. Therefore, the identification of candidate peptides widely applicable for patients with different HLA-A alleles is required. We previously found and reported (2-5) such epitope peptides, which bind to more than one HLA-class IA allele. Therefore, in the present study we examined whether or not the 13 different peptides that have been reported to induce HLA-A3 supertype-restricted cytotoxic T lymphocyte (CTL) activity (2,3,6-10) also induce CTL activity restricted to the HLA-A2, HLA-A24 and HLA-A26 alleles in the peripheral blood mononuclear cells (PBMCs) of prostate cancer patients.

Materials and methods

Peptide-HLA stabilization assay. To assess the binding and stabilizing activity of peptides to HLA-A*0201, -A*0206, -A*0207, -A*2402 and -A*2601 molecules, a previously reported method was employed, with several modifications (2,3,6-10). Briefly, RMA-S-A*0201, -A*0206, -A*0207, -A*2402 and -A*2601 (5x10⁵ cells/well in a 24-well plate) were incubated in 500 μ l RPMI-1640 (Invitrogen) supplemented with 20% fetal bovine serum (FBS) (MP Biomedicals Inc., Eschwege, Germany) for 20 h at 26°C in 5% CO₂. Then, the cells were incubated in 500 μ l Opti-MEM (Invitrogen, Carlsbad, CA, USA) containing 0.1-100 μ M peptides and human β 2 microglobulin (2 μ g/ml) at 26°C for 2 h, and then for 3 h at 37°C in 5% CO₂. Cells were washed and incubated for 30 min on ice with an appropriate dilution of anti-HLA-A24 or BB7.2 supernatant (anti-HLA-A2). After being washed with phosphate-buffered saline (PBS), the cells were stained by Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) for 30 min on ice. The mean fluorescence intensity (MFI) was measured by flow cytometry, and peptides which exhibited a >25% increase in the MFI were defined as positive binding peptides.

Cell lines. The cell lines used as target cells for cytotoxicity were the PC3 (HLA-A*2402) and LNCaP (HLA-A*0201) prostate cancer cell lines, and LNCaP transfected with the HLA-A*2402 gene (LNCaP-A24) as previously reported (11). PC3 and LNCaP-A24 tumor cells were used as relevant

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Abbreviations: CTL, cytotoxic T lymphocyte; DMSO, dimethyl sulfoxide; EBV, Epstein-Barr virus; FBS, fetal bovine serum; HDs, healthy donors; HIV, immunodeficient virus; IFN- γ , interferon- γ ; PAP, prostate acid phosphatase; PBMCs, peripheral blood mononuclear cells; PHA, phytohemagglutinin

Key words: β -tubulin 5, peptide, cytotoxic T lymphocyte, HLA-A24, cancer vaccine, peripheral blood mononuclear cell, prostate cancer

Table I. Characteristics of the prostate cancer patients.

Patient no.	Age	Gender	TMN category	GS	PSA (before surgery)	PSA (after surgery)
1	74	Male	cT2aN0M0	3+4=7	9.99	0.348
2	65	Male	T2bN0M0	3+4=7	4.11	0.529
3	76	Male				
4	58	Male	cT2aN0M0	4+4=8	5.05	0.028
5	65	Male	cT1cN2M0	4+3=7	9.09	<0.005
6	66	Male	cT2aN0M0	3+4=7	14.70	0.006
7	75	Male	cT3bN0M0	4+5=9	46.92	<0.005
8	81	Male	cT1cN0M0	3+3=6	4.70	0.144
9	70	Male	cT1cN0M0	3+4=7	7.45	0.049

GS, Gleason score; PSA, prostate-specific antigen.

tumor cells for the measurement of HLA-A24-restricted CTL activity, whereas LNCaP cells were used as irrelevant target cells. The cell cultures were maintained in RPMI-1640 medium supplemented with 10% FBS. For the pulsing of peptides to the assessed induction of peptide-specific CTLs from PBMCs as reported previously (1,8,11), we used C1R-A*2402 cells (an HLA-A*2402 gene-stable transfectant of the B lymphoblastoid cell line, kindly provided by Dr M. Takiguchi, Kumamoto University, Japan), as reported previously (11,12). RMA-S cells were derived from a mouse mutant cell line deficient in antigen processing, which showed decreased cell surface expression of MHC class I molecules. The HLA-A*0201, -A*0206, -A*0207, -A*2402 and -A*2601 genes were also individually transfected into RMA-S cells using the FuGENE transfection reagent (Roche, Mannheim, Germany). Clones of stably HLA gene-transfected cells were established from a separate well in the presence of geneticin (0.5 mg/ml). The detailed methods for establishing these transfectants have been reported previously (7).

Peptides. Peptides with >90% purity were purchased from Hokkaido System Science (Sapporo, Japan) or Genenet (Fukuoka, Japan) and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 μ g/ml. Fourteen peptides that were previously shown to be capable of inducing HLA-A3 supertype (-A3, -A11, -A31 and -A33)-restricted CTLs (2,3,6-10) were used in this study. In addition, Epstein-Barr virus (EBV)-derived and human immunodeficient virus (HIV)-derived peptides were used as controls binding to the HLA-A2 and -A24 alleles, as reported previously (13-16). A peptide derived from positions 155 to 163 of prostate acid phosphatase (PAP) was used as a positive control for binding to the HLA-A*0201, -A*0206 and -A*2402 alleles, but not the other alleles, as reported previously (2). NS3₁₅₈₂₋₁₅₉₀ was also used as a positive-control peptide for HLA-A26 (2).

Patients. The Institutional Ethical Review Board of Kurume University approved the study protocol, which conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Informed written consent was obtained from all participants

who donated PBMCs for this study. PBMCs were obtained from 9 prostate cancer patients and from 3 healthy donors (HDs) who were homozygous for the HLA-A24 allele. None of the participants were infected with HIV. The patient characteristics are presented in brief in Table I. PBMCs were isolated from blood samples by density centrifugation using Ficoll-Conray (density 1.077), and were cryopreserved until use. The expression of HLA-A24 molecules on PBMCs was discriminated by staining with anti-HLA-A24 mAb and analyzed by flow cytometry (2).

Induction of peptide-specific CTLs from PBMCs. The induction of peptide-specific CTLs and the detection of interferon (INF)- γ produced by CTLs were carried out according to a previously reported method with several modifications (6). Briefly, PBMCs (1×10^5 cells per well in a 96-well U-bottom-type plate) were incubated with 10 μ g/ml of each peptide in culture medium. The culture medium consisted of 45% RPMI-1640, 45% AIM-V medium (Invitrogen, Gaithersburg, MD, USA), 10% FBS, 100 U/ml interleukin-2 and 0.1 mM MEM Non-Essential Amino Acids Solution (Life Technologies) at 37°C in 5% CO₂. On Day 15 of culture, the cells were divided into four wells. Two of these wells were mixed with the corresponding peptide-pulsed C1R-A*2402 cells, while the other two were mixed with the irrelevant (HIV) peptide and incubated for 18 h at 37°C in 5% CO₂. The IFN- γ production of CTLs was determined by an enzyme-linked immunosorbent assay. Discrimination of the induction of peptide-specific CTLs was considered to be successful when the P-value was <0.05 and when the difference in IFN- γ production compared to the control HIV peptide exceeded 50 pg/ml.

Cytotoxicity assay. Peptide-stimulated PBMCs were tested for their cytotoxicity against PC3, LNCaP and LNCaP-A24 prostate cancer cells by a standard 6-h ⁵¹Cr-release assay (2). Phytohemagglutinin (PHA)-activated T cells from HLA-A24-positive patients were used as a negative control. The PBMCs were also tested for their cytotoxicity against C1R-A*2401 cells that were pre-pulsed with either a corresponding peptide or the HIV peptide.

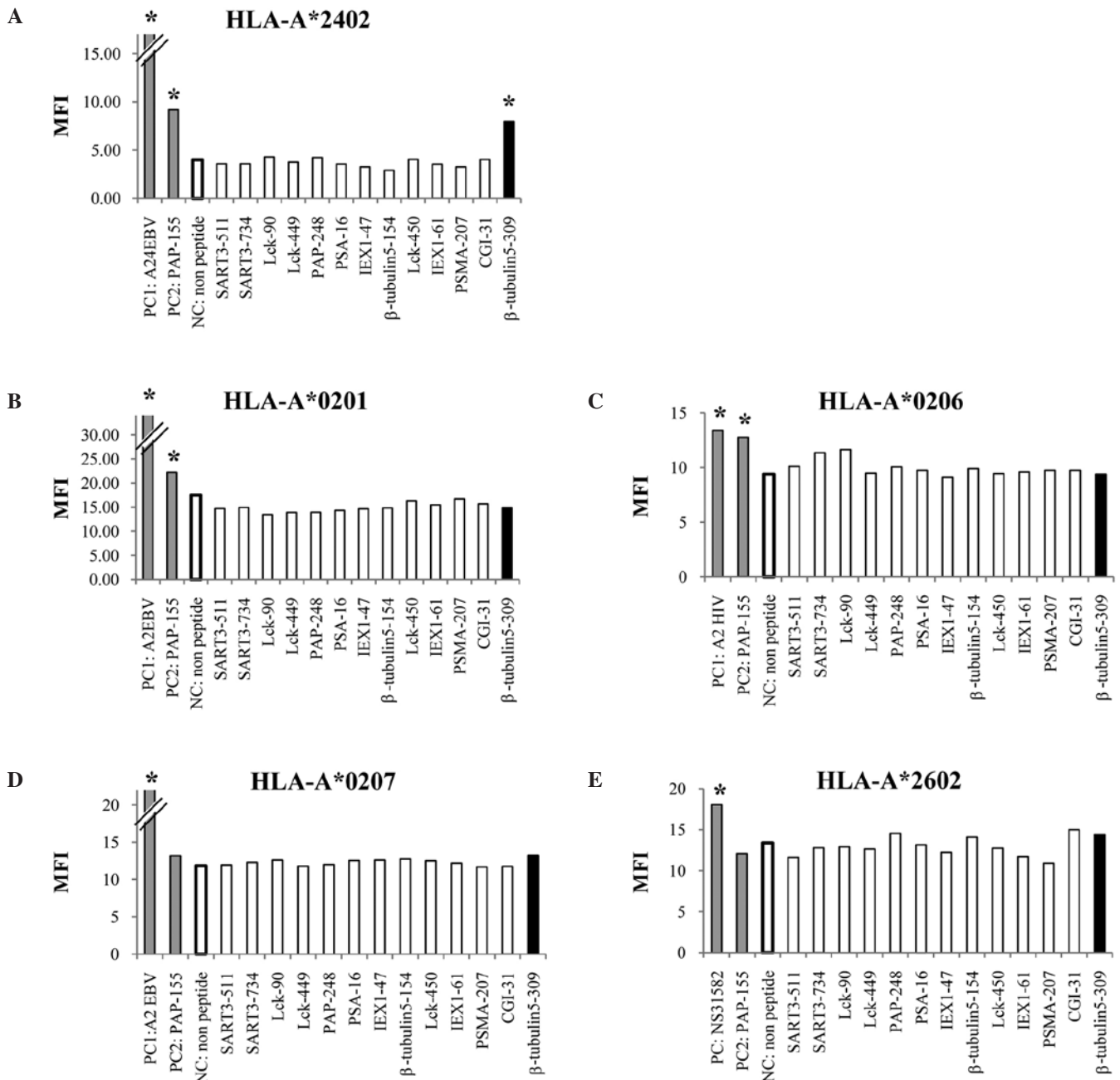


Figure 1. Stabilization assay of the β -tubulin 5₃₀₉₋₃₁₈ peptide for various HLA alleles. The binding activities of the β -tubulin 5₃₀₉₋₃₁₈ peptide to various HLA-A alleles were examined using the stable transfectant cell lines RMA-S-A*0201 (A), -A*0206 (B), -A*2402 (C), -A*0207 (D) and -A*2601 (E) with a positive-control peptide and negative control (DMSO). The positive-control peptides used for each HLA were HIV-A2 (A and B), HIV-A24 (C), EBV-A2 (D) and NS3₁₅₈₂₋₁₅₉₀ (E). A peptide derived from positions 155 to 163 of prostate acid phosphatase (PAP) was used as a positive control for binding to the HLA-A*0201, -A*0206 and -A*2402 alleles, but not the other alleles, as reported previously (4). The mean fluorescence intensity (MFI) was indicated at 100 μ M of the peptide against HLA-A*2402 (A), HLA-A*0201 (B), HLA-A*0206 (C), HLA-A*0207 (D) and HLA-A*2602 (E). Representative results from at least three separate experiments are shown. *Statistically significant at $P < 0.05$.

⁵¹Cr-labeled target cells (2,000 cells/well) were mixed with effector cells at the indicated effector-to-target (E/T) ratios in 96 round-well plates. Immediately before the cytotoxicity assay, CD8⁺ T cells were positively isolated using a CD8 Positive Isolation kit (Dyna, Oslo, Norway) according to the manufacturer's manual. After incubation for 20 h, the plates were centrifuged and the supernatant was collected to measure radioactive quantitation by a gamma counter. The specific ⁵¹Cr release was according to the formula (test cpm - spontaneous cpm). Spontaneous ⁵¹Cr release was calculated

by measuring the radioactive quantitation of the ⁵¹Cr-labeled target cell supernatant alone, and the total ⁵¹Cr release was then calculated by measuring the radioactive quantitation of ⁵¹Cr-labeled target cell lysis by 1% Triton X-100 (Wako Pure Chemical Industries, Osaka, Japan). For the blocking assay, 10 μ g/ml of either anti-HLA class I (W6/32: mouse IgG2a), anti-HLA-DR (L243: mouse IgG2a) or anti-HLA-B,C (B1-23, IgG2a; kindly donated by Dr Pierre G. Coulie, Catholique de Louvain University, Brussels, Belgium) was added to the medium at the initiation of the mixed culture.

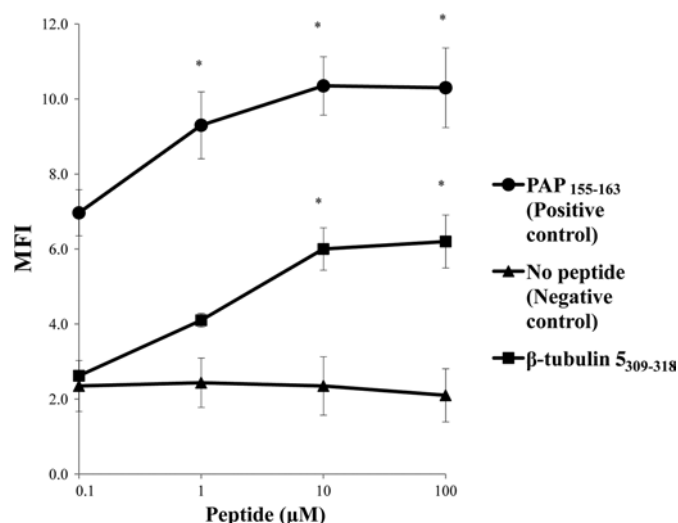


Figure 2. Dose-dependence in the stabilization assay of the β -tubulin 5₃₀₉₋₃₁₈ peptide for the HLA-A*2402 allele. The mean fluorescence intensity (MFI) was recorded at 0.1, 1, 10 and 100 μ M of the peptide or DMSO. The MFI increase induced by the β -tubulin 5₃₀₉₋₃₁₈ peptide compared to DMSO was calculated. Representative results from at least three separate experiments are shown. A PAP-derived peptide at positions 155 to 163 was used as a positive control for binding to the HLA-A*0201, -A*0206 and -A*2402 alleles, but not the other alleles, as reported previously (2). *Statistically significant at $P < 0.05$.

The peptide-stimulated CTLs were confirmed by specific peptide recognition using a cold inhibition assay. In brief, ^{51}Cr -labeled target cells (2×10^4 cells per well) were mixed with the effector cells (2×10^4 cells per well) in 96 round-well plates with 2×10^4 cold target cells and peptide-pulsed C1R-A*2402 cells.

Statistical analysis. The Student's t-test was used to test statistical significance, and P-values of < 0.05 were considered significant.

Results

HLA stabilization assay. We first screened the binding activity of each of the 13 different HLA-A3 supertype peptides (100 μ M) to the HLA-A*0201, -A*0206, -A*0207, -A*2402 and -A*2601 alleles by means of an HLA stabilization assay using RMA-S cells expressing each HLA molecule. A PAP-derived peptide consisting of the amino acid sequence from positions 155 to 163 was used as a positive control for binding to the HLA-A*0201, -A*0206 and -A*2402 alleles, but not the other alleles, as reported previously (4). As a result, one peptide from positions 309 to 318 of β -tubulin 5 (β -tubulin 5₃₀₉₋₃₁₈) showed binding activity to HLA-A*2402 molecules, but not to any of the other molecules tested (Fig. 1). The surface expression of the HLA-A*2402 molecules on RMA-S-A*2402 cells was stabilized in a dose-dependent manner when cells were cultured with either a positive control or the β -tubulin 5₃₀₉₋₃₁₈ peptide (Fig. 2).

Induction of peptide-specific CTL activity. We attempted to determine by means of an IFN- γ production assay whether or not the β -tubulin 5₃₀₉₋₃₁₈ peptide has the potential

Table II. Interferon- γ production in peptide-stimulated prostate cancer patient peripheral blood mononuclear cells.

Patient no.	β -tubulin 5-309	Positive (EBV)	Negative (HIV)
1	86	51	NS
2	ns	242	NS
3	ns	539	NS
4	50	ns	NS
5	50	1,501	NS
6	272	ns	NS
7	178	ns	NS
8	ns	457	NS
9	ns	50	NS

EBV, Epstein-Barr virus; HIV, immunodeficient virus; NS, not significant.

to generate peptide-specific CTLs from prostate cancer patients and HDs. PBMCs from HLA-A24/A24 homozygotes were stimulated *in vitro* with the β -tubulin 5₃₀₉₋₃₁₈ peptide, a positive (EBV) control peptide or a negative (HIV) control peptide, followed by measurement of IFN- γ production in response to the appropriate peptide-pulsed cells. The results showed that this peptide induced peptide-specific CTL activity in the PBMCs from 5 of the 9 patients tested (Table II), but not in any of the 3 HDs tested (data not shown). Of the 9 patients, 6 showed CTL activity reactive to the EBV-derived peptide (a positive control), while none showed CTL activity reactive the HIV-derived peptide (a negative control) (Table II).

Cytotoxicity assay. We then determined whether or not the CTLs induced by *in vitro* stimulation with the β -tubulin 5₃₀₉₋₃₁₈ peptide showed cytotoxicity against prostate cancer cells in PBMCs from 4 of the 5 patients (pt. 1, 4, 6, 7) who exhibited a positive CTL response as indicated by the IFN-release assay (Table II). The peptide-stimulated PBMCs from all 4 of the patients exhibited significant levels of cytotoxicity against both PC3 and LNCaP-A24 cells, but not against LNCaP cells or HLA-A24⁺ PHA-stimulated T-cell blasts as indicated by the ^{51}Cr release assay (Fig. 3). By contrast, as shown in Table II, PBMCs from none of the 3 patients (pt. 3, 8 and 9) whose samples responded negatively in the IFN- γ assay showed detectable levels of CTL activity with this assay (data not shown). The PBMCs from the remaining 3 patients were not eligible for the assay.

We then attempted to identify the cells responsible for the cytotoxicity of β -tubulin 5₃₀₉₋₃₁₈ peptide-stimulated PBMCs. Purified CD8⁺ T cells were used in the following experiments. The levels of cytotoxicity by CD8⁺ T cells purified from the peptide-stimulated PBMCs against PC3, as well as LNCaP tumor cells, were significantly decreased by the addition of anti-HLA class I mAb (W6/32), but not by the addition of either anti-HLA class II (HLA-DR) or anti-

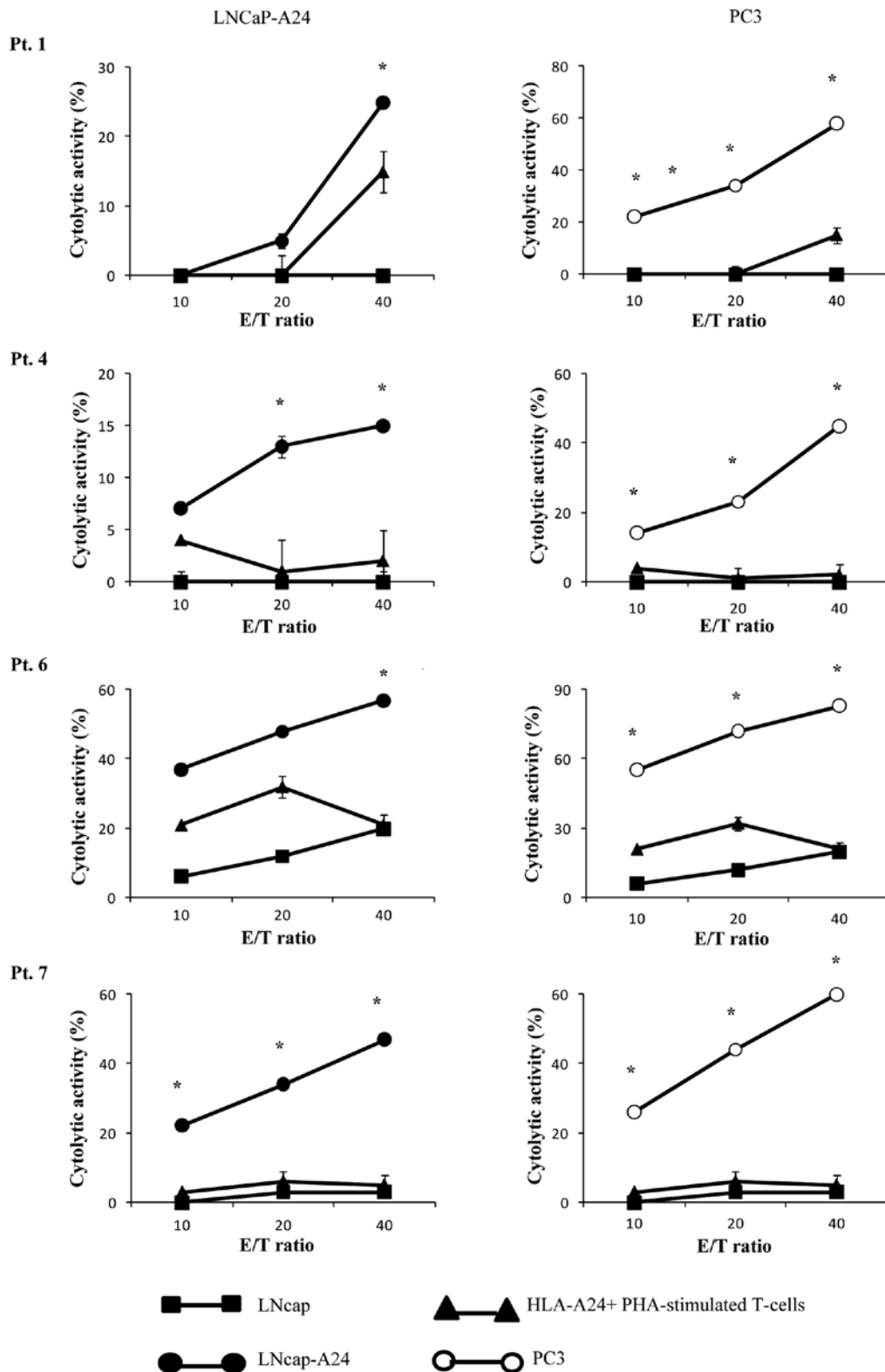


Figure 3. The peptide-stimulated PBMCs exhibited significant levels of cytotoxicity against both PC3 and LNCaP-A24 cells. Peptide-stimulated PBMCs from the 4 patients (pt. 1, 4, 6 and 7 of Table II) were tested for their cytotoxicity towards three different targets by a 6-h ^{51}Cr -release assay. Phytohemagglutinin (PHA)-stimulated T-cell blasts were derived from PBMCs of HLA-A24⁺ HDs. *Statistically significant at $P < 0.05$.

HLA-B,C (B1-23, IgG2a) mAbs. Representative cases are shown in Fig. 4A. In addition, cytotoxicity was significantly inhibited by the addition of a corresponding peptide-pulsed unlabeled C1R-A*2402, but not by the addition of an HIV

peptide-pulsed unlabeled C1R-A*2402. Representative cases are shown in Fig. 4B. These results indicate that CTL activity was determined to be specific to this peptide, and was mediated by CD8⁺ T cells in an HLA-class I-restricted manner.

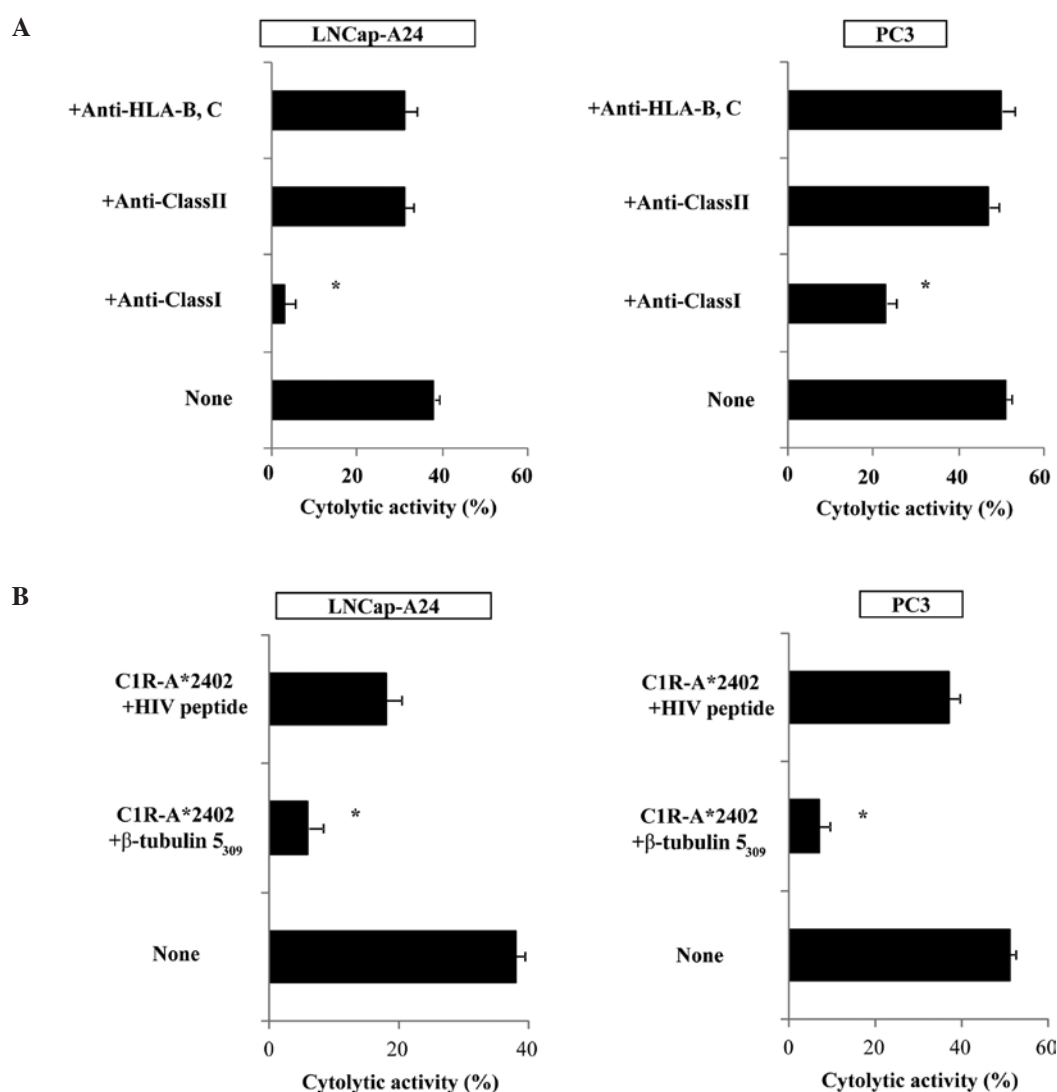


Figure 4. Inhibition assay of peptide-stimulated PBMCs with Abs. (A) Peptide-stimulated PBMCs from 4 patients (patient no. 1, 4, 6 and 7 of Table II) were tested for their cytotoxicity against LNCaP-A24 and PC3 cells in the presence of the indicated monoclonal antibodies. The results of patient no. 1 are presented, and similar results were obtained in the remaining 3 patients (data not shown). (B) Peptide-stimulated PBMCs from 4 patients (patient no. 1, 4, 6 and 7 of Table II) were tested for their cytotoxicity against LNCaP-A24 and PC3 cells in the presence of unlabeled C1R-A*2402 cells, which were pre-loaded with either the corresponding peptide or the HIV peptide. The results of patient no. 6 are presented, and similar results were obtained in the remaining 3 patients (data not shown). *Statistically significant at $P < 0.05$.

Discussion

The binding score of the β -tubulin 5₃₀₉₋₃₁₈ peptide (RYLTVAAVFR) to HLA-A*2402 was lower than it was to HLA-A*3101 and -A*3302, but higher than it was to HLA-A*3 or -A*1101, based on information from the Bioinformatics and Molecular Analysis Section (BIMAS) website (2). In this study, we showed that the β -tubulin 5₃₀₉₋₃₁₈ peptide, a previously reported peptide capable of inducing HLA-A*3 supertype-restricted CTLs (7), did bind to HLA-A*2402, one of the dominant HLA-A types in Asian populations, including the Japanese. HLA-A*24 binding peptides are characterized by the presence of Y or F residues at amino acid position 2; and of L, F, I or W residues at position 9 (12,17). These findings suggest that the peptide binds to HLA-A*2402 molecules. On the other hand, no binding of this peptide to HLA-A*0201-transfected cells was expected, since its binding score to HLA-A*0201 on BIMAS is zero. Indeed, β -tubulin 5₃₀₉₋₃₁₈

showed no binding activity to the HLA-A*0201, -A*0206 or -A*0207 molecules.

We previously reported that the use of the CTL assay with a 14-day incubation period and with stimulation administered five times did not detect CTL precursors by *de novo* sensitization to an epitope peptide (18). The sensitivity of this employed CTL assay was 1 out of 3,000 to 1 out of 5,000 CTL precursors. Thus, it is likely that the immune response against the β -tubulin 5₃₀₉₋₃₁₈ peptide is relatively restricted in cancer patients whose tumors overexpress β -tubulin 5 antigen (19-21). This is primarily because naïve T cells from HDs do not induce CTL activity as readily as those from prostate cancer patients. Indeed, PBMCs from any of the three HDs homozygous for the HLA-A*24 allele showed CTL activity (data not shown). By contrast, it was relatively easy to induce β -tubulin 5₃₀₉₋₃₁₈-specific CTLs in prostate cancer patients, and such CTLs were detectable in 5 of the 9 patients tested. CTL precursors were not detectable in the remaining 4 patients, which may have

been due, in part, to the immune suppression associated with prostate cancer. Alternatively, the employed CTL assays may not have been sufficiently sensitive, based on the finding that the CTL precursors to the EBV-derived peptide, which were used as a positive control, were also detectable in some of the patients tested.

Significantly higher fractions of β -tubulin class II and V mRNA were reported as compared to the other isotypes in lung tumor samples (22). In regard to biological function, β -tubulin 5, which is located in the cytoplasm and one of the structural subunit of microtubules, is important for cell proliferation (19). Tubulin is one of the major target molecules of anticancer drugs such as docetaxel, based on the fact that the expression of tubulin is reported more often in cancer cells than in normal cells (19-21).

Together with the results presented herein, these findings suggest that the β -tubulin 5 peptide has potential utility as a cancer vaccine, both in prostate and other types of cancer.

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