New peptides of the polycomb group protein enhancer of zeste homolog 2 with the potential to induce cancer-reactive cytotoxic T lymphocytes in human leukocyte antigen-A2$^+$ prostate cancer patients

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Abstract. The polycomb group protein enhancer of zeste homolog 2 (EZH2) is linked to aggressive prostate cancer and could be an appropriate target in specific immunotherapy. In this study, we attempted to identify EZH2-derived peptides that have the potential to generate cancer-reactive cytotoxic T lymphocytes (CTLs) in human leukocyte antigen (HLA)-A2$^+$ prostate cancer patients. Twelve EZH2-derived peptides were prepared based on the HLA-A2 binding motif. These peptide candidates were screened first by their ability to be recognized by immunoglobulin G (IgG), and then by their ability to induce peptide-specific cytotoxic T lymphocytes (CTLs). As a result, five EZH2 peptides recognized by IgG (EZH2 120-128, EZH2 165-174, EZH2 569-577, EZH2 665-674, and EZH2 699-708) were frequently detected in the plasma of prostate cancer patients. Among them, the EZH2 120-128 and EZH2 165-174 peptides effectively induced HLA-A2-restricted and cancer-reactive CTLs from prostate cancer patients. The cytotoxicity was mainly dependent on EZH2 peptide-specific and HLA-A2-restricted CD8$^+$ T cells. These results indicate that these EZH2 120-128 and EZH2 165-174 peptides could be promising candidates in peptide-based immunotherapy for HLA-A2$^+$ prostate cancer patients.

Introduction

Prostate cancer is one of the most frequent cancers diagnosed in the elderly male population (1). Prostate cancer frequently metastasizes to bone, and androgen withdrawal therapy has been applied for such patients. However, there is no efficient therapy against hormone-refractory and metastatic prostate cancer. Therefore, novel therapeutic modalities for the treatment of hormone refractory prostate cancer are urgently needed, and immunotherapy appears to be one candidate. Identification of useful target molecules for specific immunotherapy against prostate cancer has been attempted (2,3).

Enhancer of zeste homolog 2 (EZH2) has been reported to be overexpressed in metastatic prostate cancer (4). EZH2 is a polycomb group protein homologue to the *Drosophila* enhancer of zeste, and is involved in gene silencing (5). Dysregulation of this gene-silencing machinery can lead to cancer (4,6-8). EZH2 participates in epigenetic gene silencing by directly controlling DNA methylation (9). In addition, EZH2 has been reported to be a tumor-associated antigen (10). These lines of evidence suggest that EZH2 could be a promising target molecule in specific immunotherapy for prostate cancer patients. We previously identified EZH2-derived peptide epitopes which can be useful for human leukocyte antigen (HLA)-A24$^+$ prostate cancer patients (11). In this study, we further attempted to identify EZH2-derived peptides that have the potential to generate cancer-reactive cytotoxic T lymphocytes (CTLs) in HLA-A2$^+$ prostate cancer patients because of the higher worldwide frequency of this allele (12).

Materials and methods

Patients. All prostate cancer patients in this study provided their informed consent before enrollment. None of these participants was infected with human immunodeficiency virus (HIV). Twenty milliliters of peripheral blood was obtained, and the peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll-Conray density gradient centrifugation. The expression of HLA-A2 molecules on the PBMCs of cancer patients was determined by flow cytometry. In some patients, the HLA-A2 genotypes were determined using sequence-specific oligonucleotide DNA typing after PCR.

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Cell lines. T2 is an HLA-A*0201-expressing cell line. PC93 is an HLA-A2-negative prostate cancer cell line which was established by Dr K. Ohiishi, Kyoto University. PC93-A2 is a subline which was stably transfected with the *HLA-A*0201* gene (13). All cell lines were maintained in RPMI-1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% FCS.

Peptides. Twelve EZH2-derived peptides (Table I) were prepared based on the HLA-A2 binding motif (14). All peptides were of >90% purity and were purchased from Biologica Co., Nagoya, Japan. Influenza (Flu) virus-derived (GILGFVFTL) and HIV-derived peptides (SLYNTVATL) with the HLA-A2 binding motif were used as controls. All peptides were dissolved with DMSO at a dose of 10 mg/ml.

Detection of peptide-specific IgG. Peptide-specific IgG levels in the plasma were measured by the Luminex™ system as previously reported (15). In brief, plasma was incubated with 25 μl of peptide-coupled color-coded beads for 2 h at room temperature on a plate shaker. After incubation, the mixture was washed with vacuum manifold apparatus and incubated with 100 μl of biotinylated goat anti-human IgG (γ chain-specific) for 1 h at room temperature. The plate was then washed, followed by the addition of 100 μl of streptavidin-PE into each well, and was incubated for 30 min at room temperature on a plate shaker. The bound beads were washed three times followed by the addition of 100 μl of Tween-PBS into each well. Of the sample, 50 μl was detected using the Luminex™ system.

Assay for peptide-specific CTLs in PBMCs. The assay for the detection of peptide-specific CTLs in PBMCs was performed according to a previously reported method (16). In brief, PBMCs ($1x10^5$ 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 culture medium. The culture medium consisted of 45% RPMI-1640, 45% AIM-V medium (Gibco BRL), 10% FCS, 100 U/ml of interleukin (IL)-2, and 0.1 mM MEM nonessential amino acid solution (Gibco, BRL). Half of the culture medium was removed and replaced with new medium containing a corresponding peptide (20 μg/ml) every 3 days. On the 15th day of culture, the cultured cells were separated into 4 wells, two of which were used for EZH2 peptide-pulsed T2 cells, and the other two of which were used for the HIV peptide-pulsed T2 cells. After an 18-h incubation period, the supernatants were collected and the level of IFN-γ was determined by ELISA.

Cytotoxicity assay. After *in vitro* stimulation with the EZH2 peptides, the peptide-stimulated PBMCs were additionally cultured with 100 U/ml IL-2 for approximately 10 days in 96-round-well plates in order to obtain a sufficient number of cells to carry out a cytotoxicity assay. For cytotoxicity assay, $CD8^+$ T cells were purified using a CD8 isolation kit (DYNAL, Oslo, Norway). These 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. In some experiments, either anti-HLA class I (W6/32: mouse IgG2a) or anti-HLA-DR (L243: mouse IgG2a) monoclonal antibody was added to the wells at a dose of 20 μg/ml at the start of the assay.

Table I. EZH2-derived peptide candidates binding to the HLA-A2 molecules.

Position	Amino acid sequence	Length	Binding score
48-56	KILERTEIL	9	31
95-104	VIPLKTLNAV	10	37
120-128	FMVEDETVL	9	119
165-174	FINDEIFVEL	10	105
222-230	KIFEAISSM	9	83
438-446	SMFRVLIGT	9	46
569-577	KQCPCYLAV	9	37
661-669	YMCSFLFNL	9	3598
665-674	FLFNLNNDFV	10	3190
668-677	NLNNDFVVDA	10	43
699-708	VMMVNGDHRI	10	47
729-737	SQADALKYV	9	366

The peptide binding score was calculated based on the predicted half-time of the HLA-A2 molecules as obtained from the website (Bioinformatics and Molecular Analysis Section, Computed Bioscience and Engineering Laboratory, Division of Computer Research and technology, NIH).

Cold inhibition assay. The specificity of EZH2 peptide-stimulated CTLs was confirmed by a cold inhibition assay. In brief, ^{51}Cr-labeled target cells ($2x10^3$ cells/well) were cultured with the CTLs ($4x10^4$ cells/well) in 96-round-well plates with $2x10^4$ cold target cells. T2 cells that were pre-pulsed with either the HIV peptide or a corresponding EZH2 peptide were used as cold targets.

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

Results

IgGs reactive to EZH2-derived peptides. We prepared 12 kinds of EZH2-derived peptides based on their binding affinity to the HLA-A*0201 molecules (Table I). We first screened EZH2-derived peptide candidates that can be recognized by the humoral immune system in cancer patients for the following reasons. First, we previously reported that IgGs reactive to peptides derived from cancer-associated antigens were frequently detectable in prostate cancer patients (13,17,18). Second, the assay for peptide-specific IgG is much simpler and easier than the *in vitro* sensitization experiment to induce peptide-specific CTLs in cases in which only a limited number of PBMCs are available. Therefore, we determined whether IgGs reactive to each of the 12 EZH2-derived peptides could be detected in the plasma of 20 prostate cancer patients

Table II. EZH2 peptide-specific IgG in the plasma of prostate cancer patients.

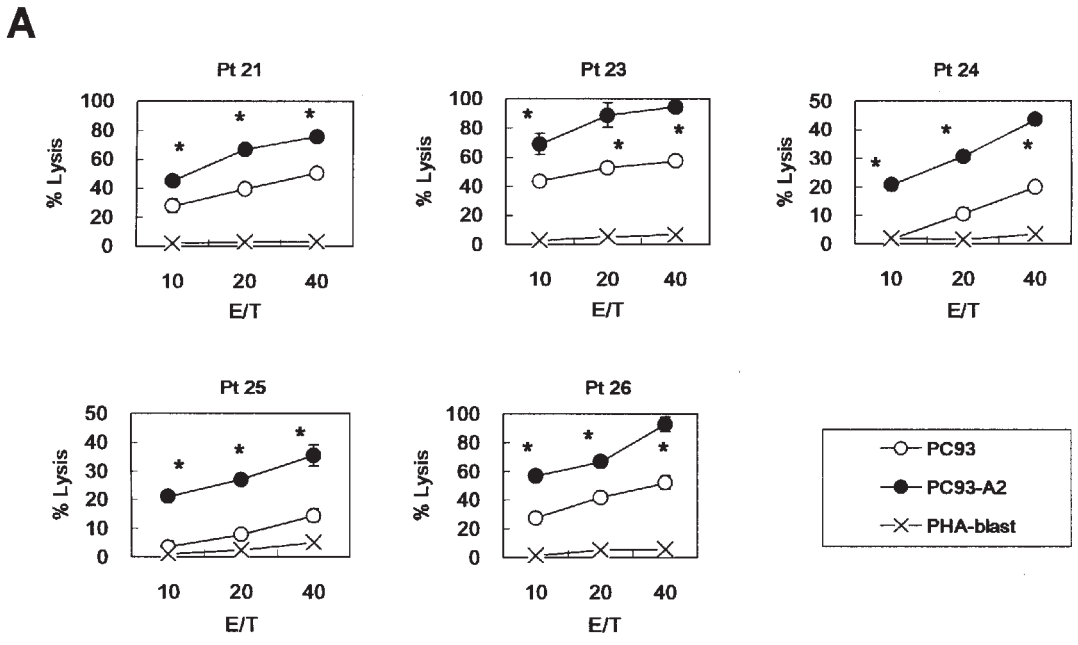
Patient	EZH2 peptide											
	48-56	95-104	120-128	165-174	222-230	438-446	569-577	661-669	665-674	668-677	699-708	729-737
	Immunofluorescence intensity											
1	-	-	-	-	-	-	-	129	184	-	-	-
2	-	-	-	-	-	-	-	-	127	-	-	-
3	-	-	-	-	-	-	-	-	186	-	-	-
4	-	-	-	-	-	-	394	-	74	-	-	-
5	-	-	-	-	-	-	464	-	89	-	-	-
6	-	-	-	-	-	-	-	-	95	-	-	-
7	-	-	-	-	-	-	-	-	114	-	56	-
8	-	-	-	55	-	-	-	-	118	-	-	-
9	-	-	-	343	-	-	-	-	76	63	-	-
10	-	163	53	-	-	-	-	-	131	-	-	-
11	204	-	395	214	100	-	320	-	1482	282	233	-
12	-	-	209	-	-	-		-	123	-	-	-
13	65	-	169	243	-	-	95	-	214	-	239	-
14	-	-	-	-	-	-	-	-	152	-	-	-
15	-	-	-	-	-	-	-	-	229	-	-	-
16	-	-	-	-	-	-	-	-	168	-	-	-
17	-	-	-	67	-	-	-	-	240	-	-	-
18	222	-	118	187	146	-	-	-	1675	195	204	124
19	217	-	329	544	56	102	236	-	657	113	280	-
20	-	-	-	56	-	-	-	-	126	-	-	-
Total	4/20	1/20	6/20	8/20	3/20	1/20	5/20	1/20	20/20	4/20	5/20	1/20

An IgG reactive to a corresponding peptide was judged to be positive when the immunofluorescence intensity in l:100-diluted plasma was >50 times higher than that of a peptide-unloaded negative control. Only the positive results are shown.

Table III. Induction of EZH2 peptide-specific CTLs from the PBMCs of of HLA-A2$^+$ prostate cancer patients.

Patient	HLA-A2 genotype	EZH2 peptide						Flu
		120-128	165-174	569-577	665-674	699-708	95-104	
		IFN-γ (pg/ml)						
21	0201	272	241	0	0	0	0	0
22	0207	236	0	0	0	127	0	236
23	0201	168	270	0	0	0	0	325
24	0207	3367	90	0	0	0	0	438
25	0201	2339	179	137	0	0	133	574
26	0207	2055	60	0	0	0	0	1073
27	0201	0	181	0	0	0	0	734
28	0207	0	0	0	0	0	0	53
29	0206	0	0	0	0	0	0	0
30	0206	0	81	53	0	0	0	0
Total		6/10	7/10	2/10	0/10	1/10	1/10	7/10

The PBMCs from HLA-A2$^+$ prostate cancer patients were stimulated *in vitro* with the indicated EZH2 peptide as described in the Materials and methods. On day 15, the cultured PBMCs were tested for their reactivity to T2 cells, which were pre-pulsed with the corresponding EZH2 peptide or the HIV peptide. The values represent the mean of 4 wells, and the background IFN-γ production in response to the HIV peptide was subtracted. The values which showed >50 pg/ml and $P<0.05$ by Student's t-test were judged to be positive.



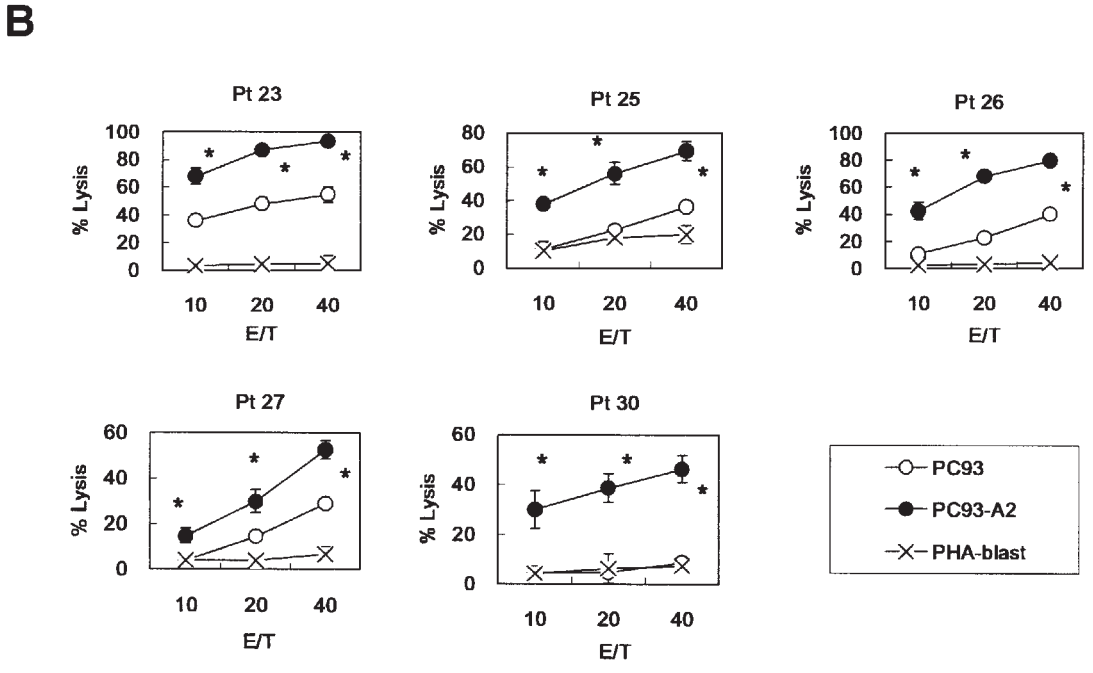


Figure 1. Cytotoxicity of EZH2 peptide-stimulated PBMCs from prostate cancer patients. $CD8^+$ T cells from the PBMCs of 5 $HLA\text{-}A2^+$ prostate cancer patients which were stimulated *in vitro* with each of the EZH2 120-128 (A) and EZH2 165-174 (B) peptides were tested for their cytotoxicity toward 3 different targets by a 6-h ^{51}Cr-release assay. *Statistically significant at $P<0.05$.

(Table II). The levels of peptide-specific IgG were determined by using the Luminex method (15). These patients were not limited to the $HLA\text{-}A2^+$ subjects, because the peptide-specific IgGs were not restricted to MHC class I molecules, as reported previously (19). An IgG reactive to a corresponding EZH2 peptide was judged to be positive when the immunofluorescence intensity in a 1:100-diluted plasma was more than 50 times higher than that of a peptide-unloaded negative control. As a result, IgGs reactive to the EZH2 120-128, 165-174, 569-577, 665-674, and 699-708 peptides were detected in the plasma of 6, 8, 5, and 5 of 20 patients, respectively. These 5 EZH2 peptides were recognized by IgGs more efficiently than the other 7 EZH2 peptides, and were employed in the following assays of CTLs.

Induction of EZH2 peptide-specific CTLs from cancer patients. We next determined whether or not these 5 EZH2 peptides had the potential to generate peptide-specific CTLs from the PBMCs of 10 $HLA\text{-}A2^+$ cancer patients (Table III). Their HLA-A2 genotypes were determined, and are shown in

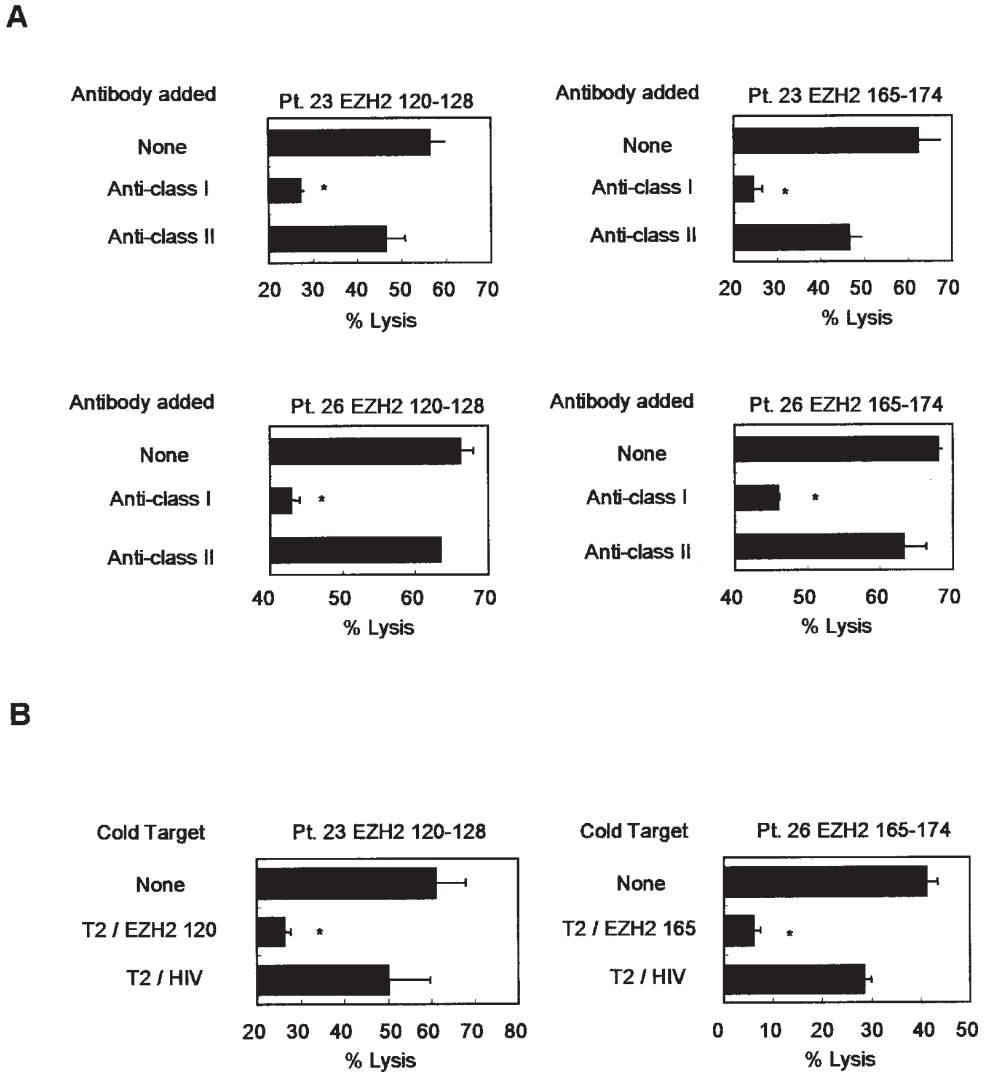


Figure 2. Peptide-specific and CD8+ T cell-dependent cytotoxicity of EZH2 peptide-stimulated PBMCs from prostate cancer patients. (A) CD8+ T cells from EZH2 peptide-stimulated PBMCs of 2 patients were tested for their cytotoxicity against the PC93-A2 cells. A 6-h cytotoxicity assay was performed in the presence of the indicated monoclonal antibodies. The assay was performed at an effector/target ratio of 10/1. *Statistically significant at P<0.05. (B) CD8+ T cells from EZH2 peptide-stimulated PBMCs of 2 patients were tested for their cytotoxicity against the PC93-A2 cells in the presence of unlabeled T2 cells, which were pre-loaded with either the corresponding EZH2 peptide or the HIV peptide. The assay was performed at an effector/target ratio of 10/1. *Statistically significant at P<0.05.

the table. The EZH2 95-104 peptide was employed as a control that was recognized by IgG less frequently. The PBMCs were stimulated *in vitro* with each of the EZH2 peptides and examined for their IFN-γ production in response to the corresponding peptide-pulsed T2 cells. The assay was carried out in 4 wells, and the values that showed >50 pg/ml IFN-γ production, compared to the control, and P<0.05 by the Student's t-test were considered to be positive. The result was that the EZH2 120-128 and EZH2 165-174 peptides induced peptide-specific CTLs in 6 and 7 of 10 cancer patients, respectively, and the induction efficiency was at the same level as that of the control Flu peptide. The EZH2 120-128 peptide induced peptide-specific CTLs from HLA-A*0201 and HLA-A*0207 patients, and the EZH2 165-174 peptide induced peptide-specific CTLs from HLA-A*0201, HLA-A*0207, and HLA-A*0206 patients. The other EZH2 peptides induced peptide-specific CTLs less efficiently. The EZH2 95-104 peptide induced peptide-specific CTLs in 1 of 10 cancer patients. These results indicate that both the EZH2 120-128 and EZH2 165-174 peptide have the potential to effectively induce peptide-specific CTLs from HLA-A2+ prostate cancer patients.

Peptide-specific and CD8+ T cell-dependent cytotoxicity of EZH2 peptide-stimulated PBMCs. We next determined whether CTLs which were induced by the EZH2 120-128 and EZH2 165-174 peptides could show cytotoxicity against prostate cancer cells. To confirm their HLA-A2-restricted cytotoxicity, we utilized a PC93 prostate cancer cell line and its HLA-A2-transfectant, PC93-A2 (13). In addition, purified CD8+ T cells from peptide-stimulated PBMCs were used for the cytotoxicity assay. As a result, the EZH2 120-128 peptide-stimulated PBMCs which were induced from 5 patients (no. 21, 23, 24, 25, and 26) showed a higher level of cytotoxicity against PC93-A2 cells than against PC93 cells (Fig. 1A). The EZH2 165-174 peptide-stimulated PBMCs which were induced from 5 patients (no. 23, 25, 26, 27, and 30) showed a higher level

of cytotoxicity against PC93-A2 cells than against PC93 cells (Fig. 1B). In both cases, the levels of cytotoxicity against HLA-A2 positive PHA-stimulated T cell blasts were low or negligible.

We further tried to confirm which effector cells were responsible for the cytotoxicity. The cytotoxicity of $CD8^+$ T cells from PBMCs of 2 prostate cancer patients (no. 23 and 26), which were stimulated *in vitro* with each of the EZH2 120-128 and EZH2 165-174 peptides, against the PC93-A2 cells was significantly inhibited by the addition of anti-class I mAb, but not by the addition of anti-class II (anti-HLA-DR) (Fig. 2A). In addition, the cytotoxicity of these EZH2 peptide-stimulated PBMCs against the PC93-A2 cells was significantly suppressed by the addition of the relevant EZH2 peptide-pulsed unlabeled T2 cells, but not by the addition of the HIV peptide-pulsed unlabeled T2 cells. Collectively, the results of these antibody blocking and cold inhibition assays indicated that the cytotoxicity of these EZH2 peptide-stimulated PBMCs was mainly ascribable to peptide-specific and HLA-A2-restricted $CD8^+$ T cells.

Discussion

Immunotherapy could be a novel therapeutic modality for the treatment of prostate cancer (2,3). In recent years, our group has identified a panel of epitope peptides that are able to generate prostate cancer-reactive CTLs from prostate cancer patients (13,17,18). Nevertheless, we have been searching for more useful peptide candidates for peptide-based immunotherapy against prostate cancer. In this study, we focused on the recently identified EZH2 protein, because this protein is overexpressed in metastatic prostate cancer (4) and is suggested to be a tumor-associated antigen (10). These lines of evidence indicate that EZH2 could be a good target for the development of a specific immunotherapy against prostate cancer. We previously identified EZH2-derived peptide epitopes which can be useful for the treatment of $HLA\text{-}A24^+$ prostate cancer patients (11). In this study, we further attempted to identify EZH2-derived peptides that have the potential to generate cancer-reactive CTLs in $HLA\text{-}A2^+$ prostate cancer patients because of the higher worldwide frequency of this allele (12). As a result, we identified two new EZH2-derived peptides, the EZH2 120-128 and EZH2 165-174. Although a different EZH2-derived peptide having the potential to induce HLA-A2-restricted CTLs has been identified recently (10), these two new EZH2 peptides are potentially useful in specific immunotherapy for $HLA\text{-}A2^+$ prostate cancer patients.

We first investigated whether or not IgGs against 12 EZH2 peptide candidates would be detectable in the plasma of prostate cancer patients for the following reasons. First, we previously reported that IgGs reactive to peptides derived from cancer-associated antigens were frequently detectable in prostate cancer patients (13,17,18). Second, the assay for peptide-specific IgG is much simpler and easier than the *in vitro* sensitization experiment to induce peptide-specific CTLs when the availability of a patient's PBMCs is limited. In this study, after screening CTL-directed peptide candidates by their ability to be recognized by IgGs, the 5 selected EZH2-derived peptides were further examined for their ability to induce peptide-specific CTLs from the PBMCs of prostate cancer patients. As a consequence, the EZH2 120-127 and EZH2 165-174 peptides were found to be good candidates for peptide-based vaccine for $HLA\text{-}A2^+$ prostate cancer patients. This result means that these two peptides were recognized efficiently by both the cellular and the humoral immune system. Interestingly, the induction of IgGs reactive to vaccinated peptides was positively correlated with longer survival of patients with advanced lung or gastric cancer (20,21). In addition, the induction of IgGs reactive to the administered peptides was also correlated with a clinical response among patients with recurrent gynecologic cancer (22). Therefore, vaccination with the EZH2 120-128 and EZH2 165-174 peptides into $HLA\text{-}A2^+$ prostate cancer patients could efficiently elicit the induction of both prostate cancer-reactive CTLs and peptide-specific IgG, and subsequently lead to clinical responses. We recently observed that peptide vaccination with a 9-mer peptide could induce peptide-specific and HLA-DR-restricted $CD4^+$ T cells *in vivo* (23). Such $CD4^+$ T cells may participate in the augmentation of peptide-specific humoral responses in vaccinated patients. However, we do not yet have a clear understanding of the roles played by peptide-specific IgGs in anti-tumor immune responses. Further study could shed light on this matter.

We identified two EZH2-derived peptides that have the potential to generate cancer-reactive CTLs in $HLA\text{-}A2^+$ prostate cancer patients. Most Caucasians are HLA-A*0201-positive, but HLA-A2 subtypes vary considerably in Japanese (12). EZH2-derived peptides were prepared based on the binding motif to HLA-A*0201 molecules (14), and HLA-A*0201 molecule-expressing T2 cells were used for assays in this study. As a consequence, two EZH2 peptide candidates were found to effectively induce HLA-A2-restricted and tumor-reactive CTLs from patients with several HLA-A2 subtypes, including HLA-A*0201 and HLA-A*0207. The EZH2 165-174 peptide induced peptide-specific CTLs from an $HLA\text{-}A*0206^+$ prostate cancer patient. Therefore, two newly identified EZH2 peptides could be applicable for the immunotherapy of a vast majority of prostate cancer patients with different HLA-A2 subtypes. This information might increase the possibility of treating $HLA\text{-}A2^+$ prostate cancer patients using peptide-based immunotherapy.

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