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Heat Shock Protein 105 as an Immunotherapeutic Target for Patients With Cervical Cancer

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Abstract. *Background/Aim: Heat shock protein 105 (HSP105) is overexpressed in various cancers, but not in normal tissues. We investigated the expression levels of HSP105 in cervical cancer and the efficacy of immunotherapy targeting HSP105. Materials and Methods: Previously, we established human leukocyte antigen-A*02:01 (HLA-A2) restricted HSP105 peptide-specific cytotoxic T lymphocyte (CTL) clones from a colorectal cancer patient vaccinated with an HSP105 peptide. Herein, we evaluated the expression of HSP105 in cervical cancer and cervical intraepithelial neoplasia. Moreover, we tested the effectiveness of an HLA-A2-restricted HSP105 peptide-specific CTL clone against cervical cancer cell lines. Results: HSP105 was expressed in 95% (19/20) of examined cervical cancer tissues. Moreover, the HSP105 peptide-specific CTL clone recognized HSP105- and HLA-A*02:01-positive cervical cancer cell lines and also showed that cytotoxicity against the cervical cancer cell lines depends on HSP105 peptide and HLA class I restricted manners. Conclusion: HSP105 could be an effective target for immunotherapy in patients with cervical cancer.*

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Key Words: Heat shock protein 105, cervical cancer, cytotoxic T lymphocyte clone, cancer immunotherapy, cancer germline antigen.

Cervical cancer is the fourth most common malignancy among women worldwide in terms of incidence and mortality, and is the most common gynecologic cancer (1). The standard treatments for patients with early-stage disease are radical hysterectomy and radiation therapy, which are highly effective, while those with advanced and metastatic cervical cancer have traditionally been treated with chemoradiation, which is not adequate (2). Furthermore, radical trachelectomy is a viable option for selected patients with early-stage cancers, although its impairment of fertility is a serious consequence (3). Thus, the development of new cervical cancer treatments that lack severe adverse effects is an urgent need.

Cancer immunotherapy represents a remarkable development over the past 2 decades. Tumor-specific antigens that are highly expressed in cancer tissues have been discovered (4, 5), as have T cells that recognize them (6). Using serological analysis of recombinant cDNA expression libraries (SEREX), we found that heat shock protein 105 (HSP105) was overexpressed in various cancers but not in normal tissues other than the testis (7, 8). HSP105 is a stress protein that belongs to the HSP105/110 family (9) and functions to suppress apoptosis in various cancer cells but not in normal tissues (10). This renders the tumor germline antigen HSP105 a potential cancer-specific target for immunotherapy.

We previously identified HLA-A*02:01 restricted peptides derived from HSP105, and cytotoxic lymphocytes (CTLs) that recognized these peptides were effective against colorectal cancer and melanoma cells *in vivo* (11). Furthermore, we conducted a phase I trial of an HSP105-derived peptide vaccine for patients with advanced esophageal and colorectal cancer (12), where we found that HSP105-derived peptide-specific CTLs expanded in the peripheral blood mononuclear cells of some patients received the peptide vaccine. Human leukocyte antigen-A*02:01 (HLA-A2) restricted HSP105 peptide-specific cytotoxic T lymphocyte (CTL) clones were successfully

established from patients with colorectal cancer who were vaccinated with HSP105 peptide. Recently, we found that HSP105 was overexpressed in cervical cancer; hence, we performed the current study to investigate the suitability of HSP105 as a tumor-specific antigen and determine whether the HSP105-specific CTL clone could target cervical cancer cells.

Materials and Methods

HSP105 peptide-specific CTL clone. The HLA-A*02:01 restricted CTL clone that recognizes the peptide derived from HSP105 (RLMNDMTAV) was established from tumor-infiltrating lymphocytes derived from patients with colorectal cancer who were vaccinated with a HSP105 peptide in a phase I trial (12).

Cell lines. The human cervical cancer cell lines Caski ($HSP105^{high}$, HLA-A*02:01/A03:01), HeLa ($HSP105^{high}$, HLA-A*68:02), and SiHa ($HSP105^{high}$, HLA-A*24:02); the human liver cancer cell line HepG2 ($HSP105^{low}$, HLA-A*02:01/A*24:02); and the human lymphoblastoid cell line T2 (which is 'transporter associated with antigen processing'-deficient and HLA-A*02:01-positive) were used as target cells. Human fibroblasts ($HSP105^{low}$, HLA-A*02:01/A*24:02) established from normal lung tissues of patients with lung cancer were used as a control target. This study was approved by the Institutional Review Board of the National Cancer Center and conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from each patient. The cells were cultured in our laboratory and maintained in RPMI-1640 or DMEM media (Sigma, St Louis, MO, USA) supplemented with 10% fetal calf serum at 37˚C in a humidified atmosphere containing 5% CO_2.

Tissue samples and arrays. Twenty cervical cancer tissues and 7 normal cervical epithelial tissues from patients with primary gynecologic cancer who underwent surgery at Nagoya University Hospital between January 2004 and September 2013 were collected and evaluated. The tissue microarray cervical intraepithelial neoplasia (CIN), BB10011, purchased from US Biomax, Inc. were used. This study was approved by the Institutional Review Board at the Nagoya University School of Medicine (approval number 2017-0053) and written informed consent was obtained from all patients in this study.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue sections were prepared from surgical resection specimens. The tissue sections were sliced at 4-μm thickness using a microtome. After standard deparaffinized and rehydrated, the sections were performed to antigen retrieval in 10 mM sodium citrate (pH6.0) for 15 min at 95˚C in a microwave, and treated with 0.3% H_2O_2 solution for 10 min to inactivate endogenous peroxidase. Then, the sections were blocked with 10% goat serum include in Histofine SAB-PO(MULTI) kit according to the manufacture's protocol (Nichirei, Tokyo, Japan), and incubated overnight with a first antibody at 4˚C. The following monoclonal antibodies: rabbit anti-human HSP105 (dilution at 1:50; clone EPR4576, Abcam), mouse anti-human CD8 (dilution at 1:100; clone 1A5, Novocastra), and mouse anti-human HLA class I (dilution at 1:1000; clone EMR8/5, Hokudo) were used as first antibodies, as previously described (13). After washing with PBS, the sections were incubated with an appropriate secondary antibody, and then peroxidase labeled streptavidin including the kit. Then, the sections were rinsed with PBS and stained with the 3, 3'-deaminobenzidine (DAB) substrate-chromogen. After washing with water, the sections were incubated with hematoxylin, dehydrated, and mounted. The extent of staining was scored according to the percentage of tumor cells positively stained for HSP105 and HLA class I as follows: 0, negative; 1+, weak; 2+, moderate; and 3+, strong.

Quantitative real-time PCR. RNA was extracted from cells using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. Gene validation was performed *via* relative quantification using real-time PCR. 5 μg of total RNA was synthesized complementary DNA (cDNA) with a PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa, Kyoto, Japan) according to the manufacturer's instructions. The synthesized cDNA was then used as the template for subsequent real-time PCR with the following primers: *HSP105* forward 5-ATGAAGTGATGGAATGGATG-3 and reverse 5-TTTGGTTTCGGTTGTGTTAC-3; *GAPDH* forward 5-GAAGG TGAAGGTCGGAGTC-3 and reverse 5-GAAGATGGTGATGGG ATTTC-3. Gene expression levels under all conditions were normalized to those of *GAPDH*. All *HSP105* gene expression levels were compared to those of Caski cells at baseline. PCR was performed using the 96-well Gene Amp PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA). The amplification condition was for 20 cycles of denaturation at 98˚C for 10sec, annealing at 64˚C for 30 sec and extension at 72˚C for 30 sec, followed by a step of 72˚C for 10 sec. All samples were run in duplicate (14).

Flow cytometry. Cells were stained in fluorescence-activated cell sorting buffer for 20 min at 4˚C using FITC mouse anti-human HLA-A2 (clone BB7.2; MBL), FITC mouse IgG2b (clone 3D12; MBL), FITC mouse anti-human HLA-A, B, C (clone W6/32; BioLegend), and FITC mouse IgG2a (clone MOPC-173; BioLegend) monoclonal antibody. For intracellular staining, cells were fixed and permeabilized according to the manufacturer's protocol of the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) and stained with primary mouse anti-human HSP105 monoclonal antibody (clone J1G12; MyBioSource) and Alexa Fluor 488-conjugated secondary Ab (Invitrogen). Cytoplasmic staining was performed to quantify the expression of HSP105. For the CD107a degranulation assay, HSP105 specific CTL clones and target cells at a 2:1 ratio were co-cultured with APC mouse anti-human CD107a (clone H4A3; BioLegend) for 3.5 h at 37˚C. After co-culturing, HSP105-specific CTL clones were stained with FITC mouse anti-human CD8 (clone LT8; BIORAD) and degranulation was analyzed using flow cytometry. Stained cells were acquired on a Becton Dickinson FACSCanto™ II and analyzed using the FlowJo software (Tree Star).

Interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) analysis. Antigen-specific IFN-γ production of T cells were detected using the ELISPOT kit (BD Biosciences) according to the manufacturer's protocols. Briefly, HSP105-specific CTL clones were co-cultured with target cells at a 2:1 ratio for 20 h at 37˚C and 5% CO_2. For HLA class I block, the CTL clone was co-cultured with each cancer cell line as a target cell at the indicated ratio of effector/target, and the IFN-γ ELISPOT assay was then performed. HLA-class I blocking was performed with the mouse anti-human HLA-A, B, C monoclonal antibodiy (clone W6/32; BioLegend) and mouse IgG2a (clone MOPC-173; BioLegend) as previously described (15). The resulting spots were automatically counted using the ELIPHOTO system (Minerva Tech, Tokyo, Japan).

Cytokine measurement. HSP105-specific CTL clones and target cells were cultured at a 2:1 ratio for 48 h at 37˚C. Interleukin-2(IL-2), tumor necrosis factor (TNF)-α, and granzyme B levels in the supernatants after co-culture were detected with Cytometric Bead Array Flex Sets (BD Bioscience) according to the manufacturer's protocol. The detection results were analyzed with the FCAP Array Software 3.0.

Cytotoxicity assays. The cytotoxicity of HSP105-specific CTL clones was analyzed using the Terascan VPC system (Minerva Tech), as previously described (15). Briefly, for labeling, target cells were incubated with calcein AM (Dojindo, Kumamoto, Japan) for 30 min at 37˚C. Labeled target cells were incubated with effector cells for 5 h at the indicated dffector/target ratio. Fluorescence intensity before and after incubate was measured and compared, and specific cytotoxic activity was calculated using the following formula: % cytotoxicity=(1- [{average fluorescence of the sample wells}{average fluorescence of the maximal release control wells}/{average fluorescence of the minimal release control wells}{average fluorescence of the maximal release control wells}]) · 100%.

Mouse model. CB17.Cg-Prkdc^{scid}Lyst^{bg-J}/CrlCrlj mice (SCID Beige mice) were purchased from Charles River Laboratories. Experiments using 6–8-week-old female mice were conducted according to the institutional guidelines of the Animal Research Committee of the National Cancer Center Hospital East, Japan. Caski cells (10^6) were introduced subcutaneously into the flank. HSP105 peptide-specific CTLs, cytomegalovirus peptide-specific CTLs, or phosphate-buffered saline alone were injected intravenously on the 12th day post-transplantation 3 times (5×10^5, 1×10^6, and 2×10^6 cells, respectively) with 2 weeks between each injection. Tumor volumes were assessed using the formula (length×[width]^2)/2.

Cold inhibition assay. As previously described (16), HSP105-specific CTL clones and target cells labeled with calcein AM were cultured with or without cold target cells for 5 h, and the cytotoxic activity against the hot target was then determined. T2 cells, prepared to pulse with either $HIV_{19\text{-}27}$ or $HSP105_{169\text{-}177}$ peptide, were used as cold target cells.

A0201 transfection. HeLa and SiHa cells were transfected with an HLA-A2 expression plasmid using Lipofectamine 3000 Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer's protocol.

Statistical analysis. Statistical significance between 2 groups was determined using the Student's *t*-test. The Kruskal-Wallis test with post hoc analysis was performed to compare the results of immunohistochemistry in different cervical lesion tissues. One-way analysis of variance and Tukey's test were used to compare tumor volumes *in vivo*. Two-tailed statistical significance was defined as $p<0.05$.

Ethics approval and consent to participate. This study was approved by the Institutional Review Board at the Nagoya University School of Medicine (approval number 2017-0053) and written informed consent was obtained from all patients in this study. The *in vivo* experiments were conducted with the ethical approval of the Animal Experimentation Committee of the National Cancer Center East Hospital and in accordance with institutional guidelines.

Patient consent for publication. Written informed consent for publication was obtained from all patients, although Identifying information was removed.

Results

HSP105 was overexpressed in cervical cancer. To investigate the expression of HSP105 in cervical cancer, cervical intraepithelial neoplasia (CIN), and normal cervical epithelial tissue, we performed immunohistochemistry on formalin-fixed paraffin-embedded tissue sections representing 20 cervical cancer samples (7 stage I and 13 stage II) and 6 normal cervical epithelial tissues that were surgically resected at Nagoya University Hospital. CIN tissues stained for HSP105 were evaluated using a tissue array. HSP105 was expressed in 19 tumor samples but in only 2 of the normal myometrial tissue in the same cervical cancer patient samples (the expression levels and proportions in these samples were lower than those in cervical cancer tissues) (Table I and Figure 1A).

Additionally, we observed HLA class I expression in all samples, as well as ≥10 counts of tumor-infiltrating $CD8^+$ T cells per high-power field in 14 (70%) (Table I, Figure 1B and C). HSP105 expression data in normal cervical epithelial and cancer tissues are summarized in Table II.

Persistent human papillomavirus infection causes progression from CIN1 to CIN3 and ultimately to cervical cancer. Tissues representing different CIN stages have varying proportions of atypical cells, and it is difficult to evaluate the proportion of HSP105 expression in epithelial cells. Therefore, we scored immunostaining intensity as 0, 1+, 2+, and 3+ as described above. HSP105 expression levels were significantly different in normal cervical epithelial tissue versus high-grade CIN and cervical cancer tissues; representative data are shown in Figure 1A.

We next evaluated the expression of *HSP105* in fibroblasts and cancer cell lines using quantitative reverse transcription PCR and flow cytometry. *HSP105* RNA levels were higher in the SiHa and Caski cervical cancer cell lines than in other cells on quantitative reverse transcription-PCR; the cervical cancer HeLa cells showed a low level of gene expression (Figure 2A). In contrast, endogenous HSP105 protein expression was detected at high levels in all 3 cervical cancer cell lines on flow cytometry (Figure 2B). Hence, we used the 3 cervical cancer cell lines as target cells; fibroblasts established from normal lung tissues and HepG2 liver cancer cells both expressed HSP105 at low levels and were used as negative controls (Figure 2B). All tested cells expressed HLA-class I (Figure 2C); however, while fibroblasts, HepG2, and Caski cells expressed HLA-A0201, HeLa and SiHa did not (Figure 2D). Our finding that HSP105 and HLA-class I are expressed in almost all cervical cancer tissues indicated that HSP105 could be a tumor-specific antigen for cervical cancer.

Table I. *Characteristics and immunohistochemistry of cervical cancer patients.*

No	Age	TMN classification (UICC)	FIGO staging	HSP105*		HLA class I*	$CD8^+$ T cells#
				Tumor	Normal tissue		
1	51	pT2bN1M0	2B	1+	-	3+	-
2	20	pT2bN1M0	2B	2+	-	3+	-
3	47	pT2bN1M0	2B	3+	-	1+	+
4	34	pT2bN1M0	2B	2+	-	1+	+
5	60	pT2bN0M0	2B	3+	NA	2+	+
6	65	pT2bN0M0	2B	3+	-	2+	+
7	63	pT2bN1M0	2B	2+	-	2+	+
8	39	pT2bN1M0	2B	-	-	3+	+
9	38	pT2bN1M0	2B	3+	-	2+	-
10	34	pT2bN1M0	2B	2+	-	3+	+
11	62	pT1b12N0M0	1B2	3+	-	1+	+
12	41	pT2bN1M0	2B	1+	-	1+	-
13	68	pT1b1N0M0	1B1	2+	-	3+	+
14	62	pT1b1N0M0	1B1	3+	-	1+	-
15	39	pT1b1N0M0	1B1	3+	-	1+	+
16	26	pT1b1N0M0	1B1	2+	-	2+	+
17	73	cT2bN0M0	2B	3+	2+	-	-
18	39	pT1b1N0M0	1B1	1+	-	2+	+
19	34	pT1b1N0M0	1B1	2+	-	2+	+
20	41	pT2aN0M0	2A	2+	1+	3+	+

UICC, Union for International Cancer Control; FIGO, International Federation of Gynecology and Obstetrics; HSP105, Heat shock protein 105; HLA class I, Human Leukocyte Antigen class I. *The extent of staining of tumor cells for HSP105 and HLA class I: -, no reactivity; 1+, <10%; 2+, 10-49%; 3+, ≥50%; NA, not analyzed. #Quantification of TIL: +, ≥10 counts of CD8+ T cells/high power fields (HPF); -, <10 counts/HPF.

HSP105 peptide-specific CTL clone recognizes Caski cells. To assess whether HSP105 can be a target for immunotherapy in patients with cervical cancer, we investigated the ability of the CTL clone to recognize the HSP105 peptide. The HLA-A*0201-restricted CTL clone that recognizes the HSP105 (RLMNDMTAV) peptide was established from a colorectal cancer patient vaccinated with this peptide in a phase I trial (12). Caski ($HSP105^{high}$, HLA-A02:01), HepG2 ($HSP105^{low}$, HLA-A02:01), and fibroblasts ($HSP105^{low}$, HLA-A02:01) were used as target cells. HSP105-specific CTL clone response against Caski cells was investigated using the IFN-γ ELISPOT assay and by measuring cytokine levels in the supernatants of co-cultured effector and target cells. IFN-γ was detected in the supernatants of HSP105-specific CTL-exposed Caski cells, but not in the supernatants of fibroblasts and HepG2 cell counterparts (Figure 3A). The production of TNF and granzyme B following exposure to Caski cells was significantly higher than that after exposure to fibroblasts and HepG2. Interleukin-2 production following exposure to Caski cells was also markedly higher than that following fibroblast exposure, but was not significantly different from levels produced following HepG2 cell exposure (Figure 3B). Furthermore, the increases in CD107a expression levels of the CTL clone when exposed to Caski, HepG2, and fibroblasts were 43.9%, 0.29%, and 0.52%, respectively (Figure 3C). With respect to toxicity, the HSP105-specific cytotoxicity of the CTL clone toward Caski cells was markedly higher than that towards HepG2 cells and fibroblasts (Figure 3D). These results indicated that HSP105-specific CTL clones can recognize and attack cancer cells expressing high levels of HSP105.

HSP105 peptide-specific CTL clone recognizes cervical cancer cell lines. Next, we analyzed the responses of other cervical cell lines targeted by the HSP105-specific CTL clone. We transfected an HLA-A0201 expression plasmid into HeLa ($HSP105^{high}$, HLA-A*68:02) and SiHa ($HSP105^{high}$) for use as target cells; HLA-A02 expression on the surfaces of these cells was confirmed using flow cytometry (Figure 4A). The CTL clone responses against HeLa/A*02:01 and SiHa/A*02:01 were detected as described above. IFN-γ production was detected after CTL clone exposure to HeLa/A*02:01 and SiHa/A*02:01 cells, but not to HeLa/mock and SiHa/mock cells (Figure 4B). CD107a expression following CTL clone exposure to HeLa/A*02:01 and SiHa/A*02:01 was higher than that following exposure to HeLa/mock and SiHa/mock (Figure 4C). Cytotoxicity against SiHa/A*02:01 was markedly high, while cytotoxicity against HeLa/A*02:01 was weak (Figure 4D). These results indicated that HSP105-specific CTL responses were restricted to cervical cancer cell lines expressing HSP105 and HLA-*A02:01.

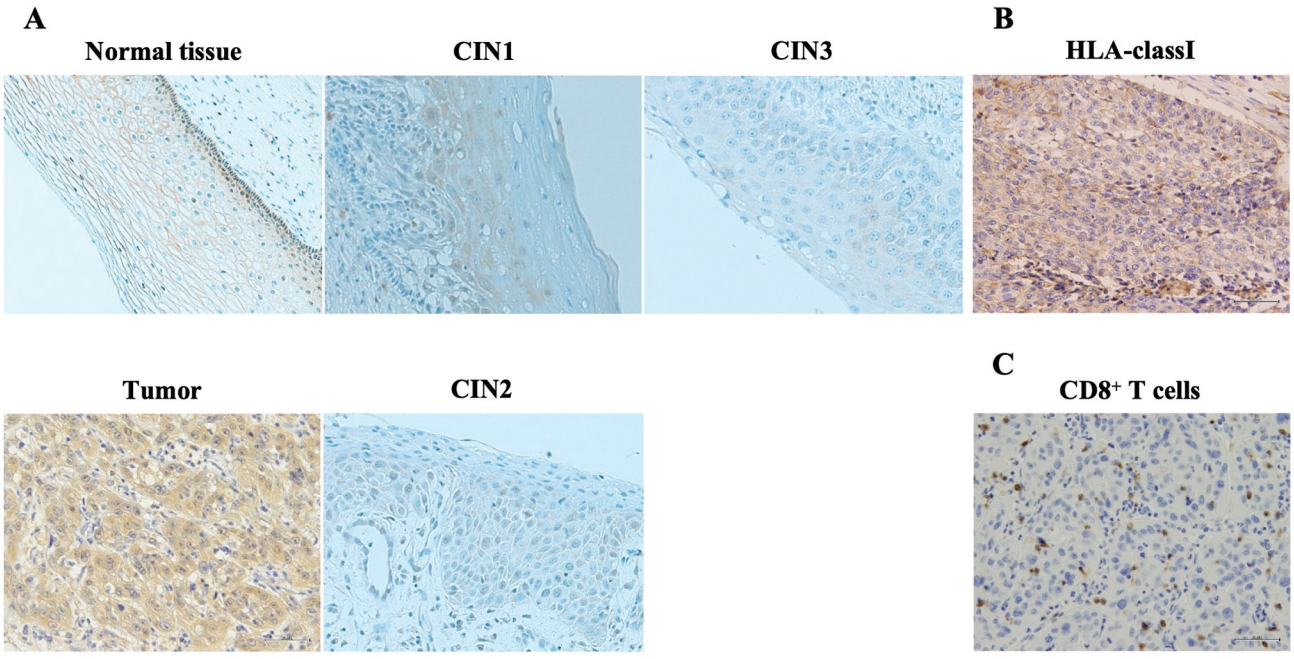

Figure 1. *HSP105 expression is high in cervical cancer, but not in normal tissues. (A) Representative images showing high expression of HSP105 in cervical cancer and cervical intraepithelial neoplasia (CIN), while this the protein is absent in normal cervical tissues [the panels represent high-power fields (×400)]. HLA class I expression (B) and tumor infiltrating CD8 T cells (C) are shown. The tissues shown in all panels are from the same patient sample or a tissue microarray.*

Analysis of HLA-A2 and HSP105 restriction. The aforementioned results suggested that HSP105-specific CTL clones attack cervical cancer cell lines, but whether these interactions were restricted by HLA type and tumor antigen was not ascertained. Therefore, we examined IFN-γ production after exposure of HSP105 peptide-specific CTL clones to Caski cells carrying either an anti-HLA-class I monoclonal antibody or IgG2a isotype control. IFN-γ production was suppressed by the anti-HLA-class I monoclonal antibody in a concentration-dependent manner, but was not suppressed by the isotype control (Figure 5A). To verify the HSP105 antigen-specific response, we performed a cold target inhibition assay to investigate the cytotoxicity of Caski cells exposed to the HSP105 peptide-specific CTL clone by pulsing T2 cells with either the HSP105 or HIV peptide. The CTL clone-induced cytotoxicity was inhibited by the addition of HSP105 peptide-pulsed T2 cells, but not by the addition of HIV peptide-pulsed counterparts (Figure 5B). These results indicated that the effect of the HSP105 peptide-specific CTLs on Caski cells was specifically caused by the HSP105 peptide and HLA-A2.

HSP105 peptide-specific CTL clone elicits Caski xenograft tumor regression. Transduced HSP105 peptide-specific CTL clones were tested for their ability to control Caski tumors in SCID Beige mice. Twelve days after tumor cell introduction, mice were treated intravenously 3 times with HSP105 peptide-specific CTLs or cytomegalovirus peptide-specific CTLs over 2-week intervals, or left untreated. Mice treated with HSP105 peptide-specific CTLs experienced delayed tumor growth compared to the other groups; however, the differences were not significant (Figure 6). As we were unable to obtain a sufficient number of CTL clones,

Table II. *Percentage of HSP105 positivity in immunohistochemistry.*

	Case	Positive N			%pos cases
		1+	2+	3+	
Normal tissue	6	1	0	0	17
CIN1	16	7	4	0	69
CIN2	5	1	4	0	100
CIN3	12	9	2	0	92
Cervical cancer	20	3	8	8	95

HSP105, Heat shock protein 105; CIN, cervical intraepithelial neoplasia. *The extent of staining of tumor cells for HSP105: -, no reactivity; 1+, weak; 2+, moderate; 3+, strong.

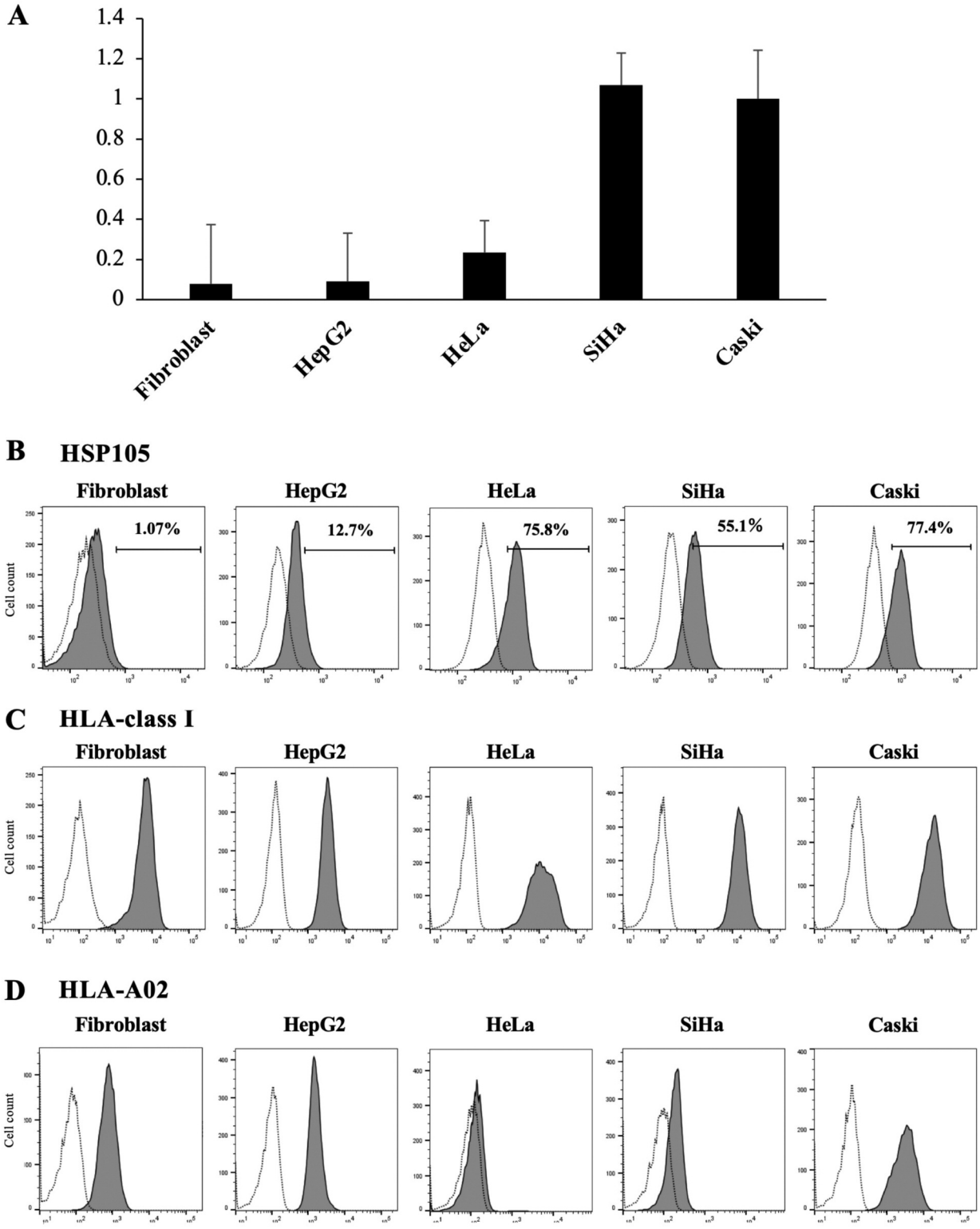

Figure 2. *HSP105 and HLA class I expression in human cell lines. (A) Gene expression of HSP105 in various cells was compared to that of the cervical cancer cell line Caski (baseline) using quantitative reverse transcription-PCR. (B) Endogenous HSP105 expression was investigated by flow cytometry. The gray-shaded areas represent HSP105 expression while the non-shaded areas represent isotype controls. (C, D) HLA class I and HLA-A02 expression was assessed using flow cytometry; the gray-shaded areas represent HLA-class I and HLA-A02 expression while the non-shaded areas represent isotype controls.*

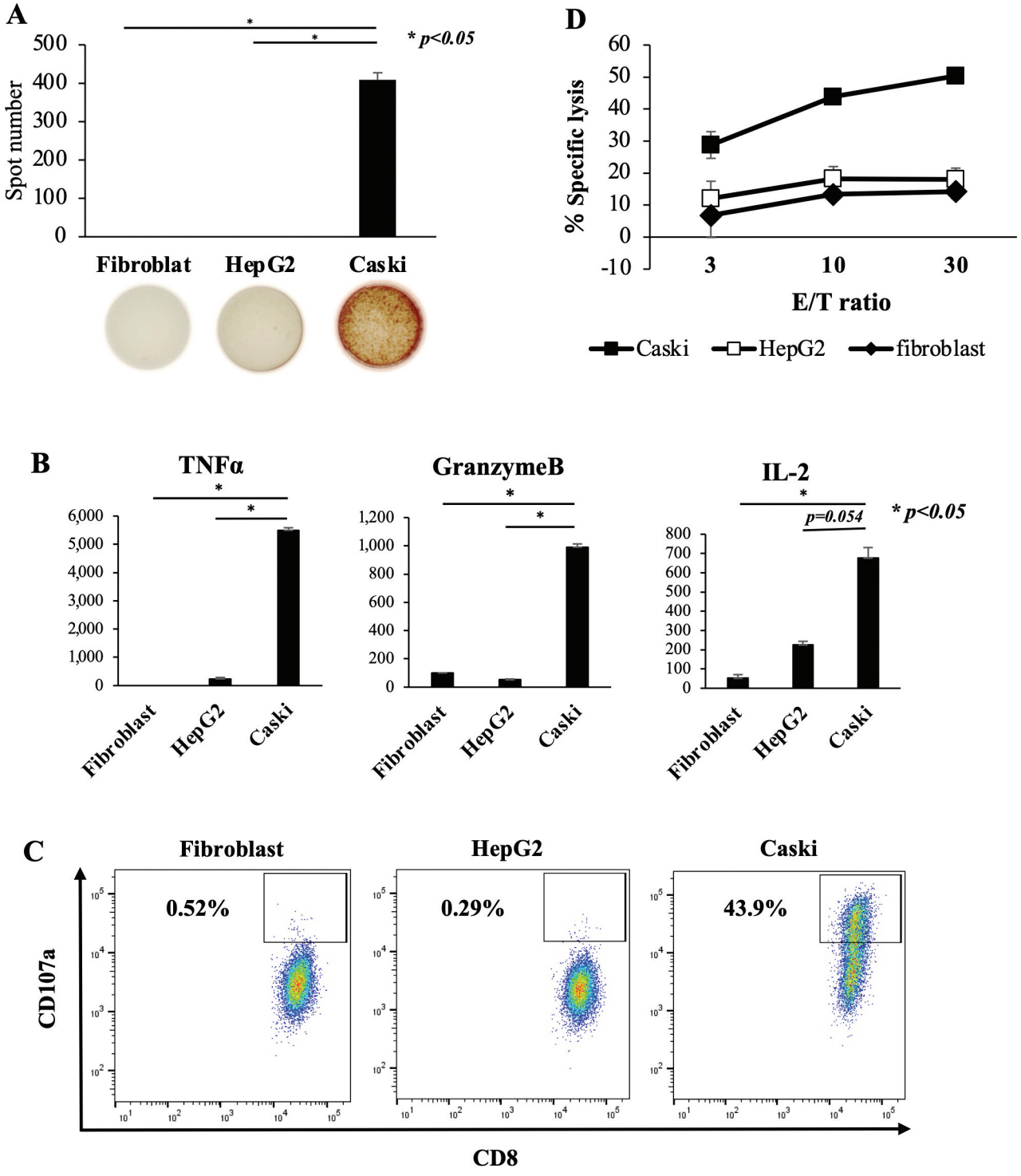

Figure 3. *HLA-A2-restricted HSP105 peptide-specific cytotoxic T lymphocyte (CTL) clone recognizes the cervical cancer cell line Caski. (A) Interferon-γ ELISPOT assay data of cells exposed to the HSP105-specific CTL are plotted, and representative experimental images are shown below the graph. (B) Tumor necrosis factor-alpha (TNFα), granzyme B, and interleukin-2 (IL-2) following CTL exposure as evaluated using Cytometric Bead Array Flex Sets. Each sample in (A) and (B) was examined in duplicate. (C) Externalized CD107a analysis of HSP105-specific CTLs exposed to fibroblasts, HepG2, and Caski cells. (D) Cytotoxicity of HSP105-specific CTL against Caski cells; fibroblasts and HepG2 cells were used as negative controls, and Caski pulsed with the HSP105 peptide was used as a positive control (p<0.05). Technical triplicates were performed for each sample. The effectors/targets ratio=2 in all panels.*

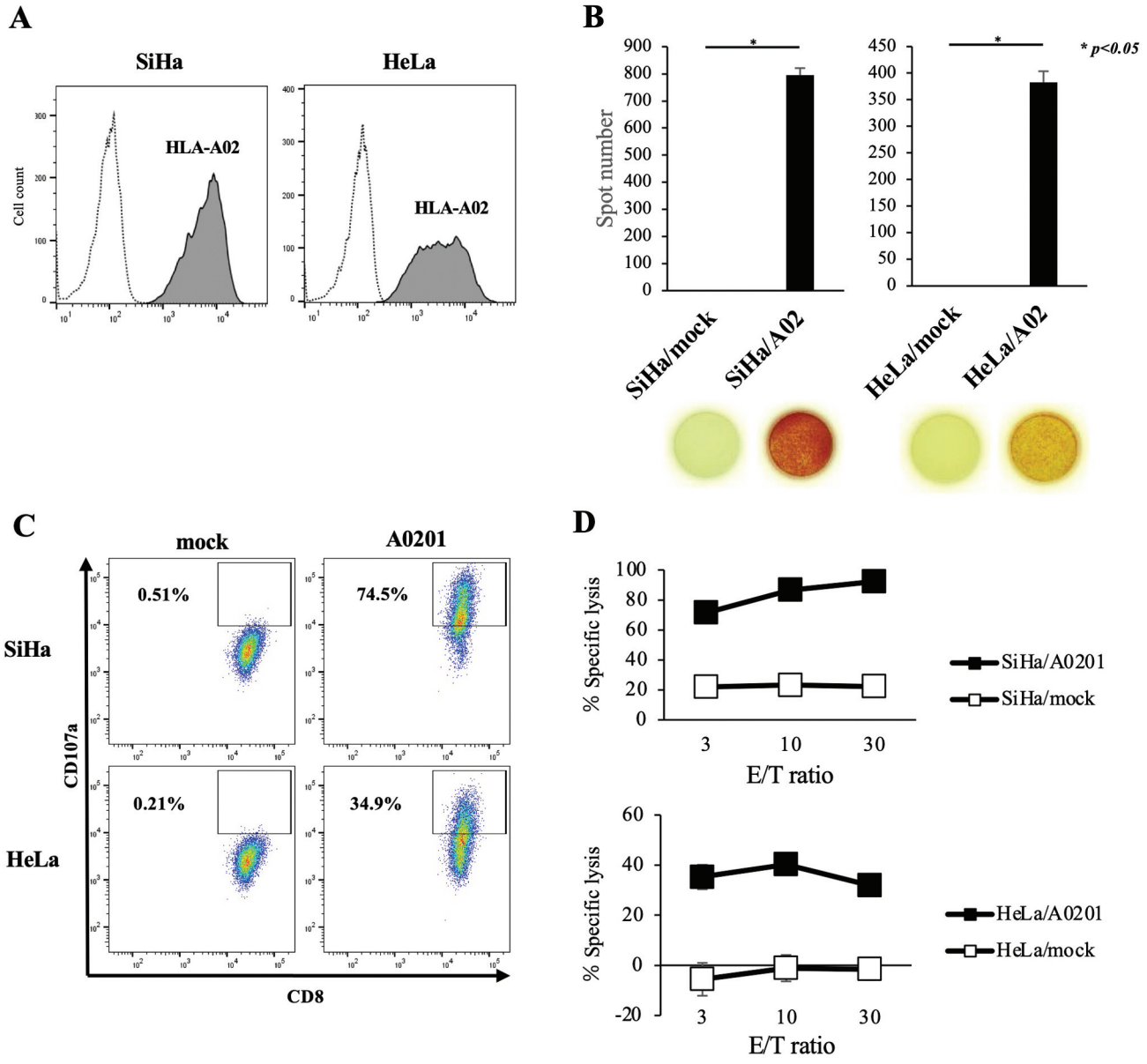

Figure 4. *HLA-A2-restricted HSP105 peptide-specific cytotoxic T lymphocyte (CTL) clone recognizes cervical cancer cell lines. (A) HLA-A02 expression of HeLa/A0201 and SiHa/A0201 after the transfection of HLA-A2-expressing plasmids was assessed using flow cytometry. The gray-shaded areas represent HLA-A2 expression while the non-shaded area represents the isotype control. (B) Interferon-γ ELISPOT assay analysis of HSP105-specific CTLs are shown, with representative experiments shown below the graph. Externalized CD107a analysis (C) and the cytotoxicity (D) of HSP105-specific CTLs against HeLa/A0201 and SiHa/A0201 are shown. The effector/target ratio=2 in (B-D). HeLa/mock and SiHa/mock were used as negative controls (p<0.05).*

tumor regression may have been more pronounced had a greater number of CTLs been administered.

Discussion

We previously identified HSP105 and glypican-3 as cancer-specific antigens, and conducted clinical trials involving peptide vaccination in patients with several cancer types (7, 8, 12, 13, 15, 16). To date, we identified HSP105 as a cancer-specific antigen and performed peptide vaccination clinical trials of patients with colorectal cancer (12, 13). We also successfully induced HSP105-specific CTLs (as well as CTLs for other antigens) from the peripheral blood mononuclear cells (PBMCs) of patients with colorectal cancer vaccinated

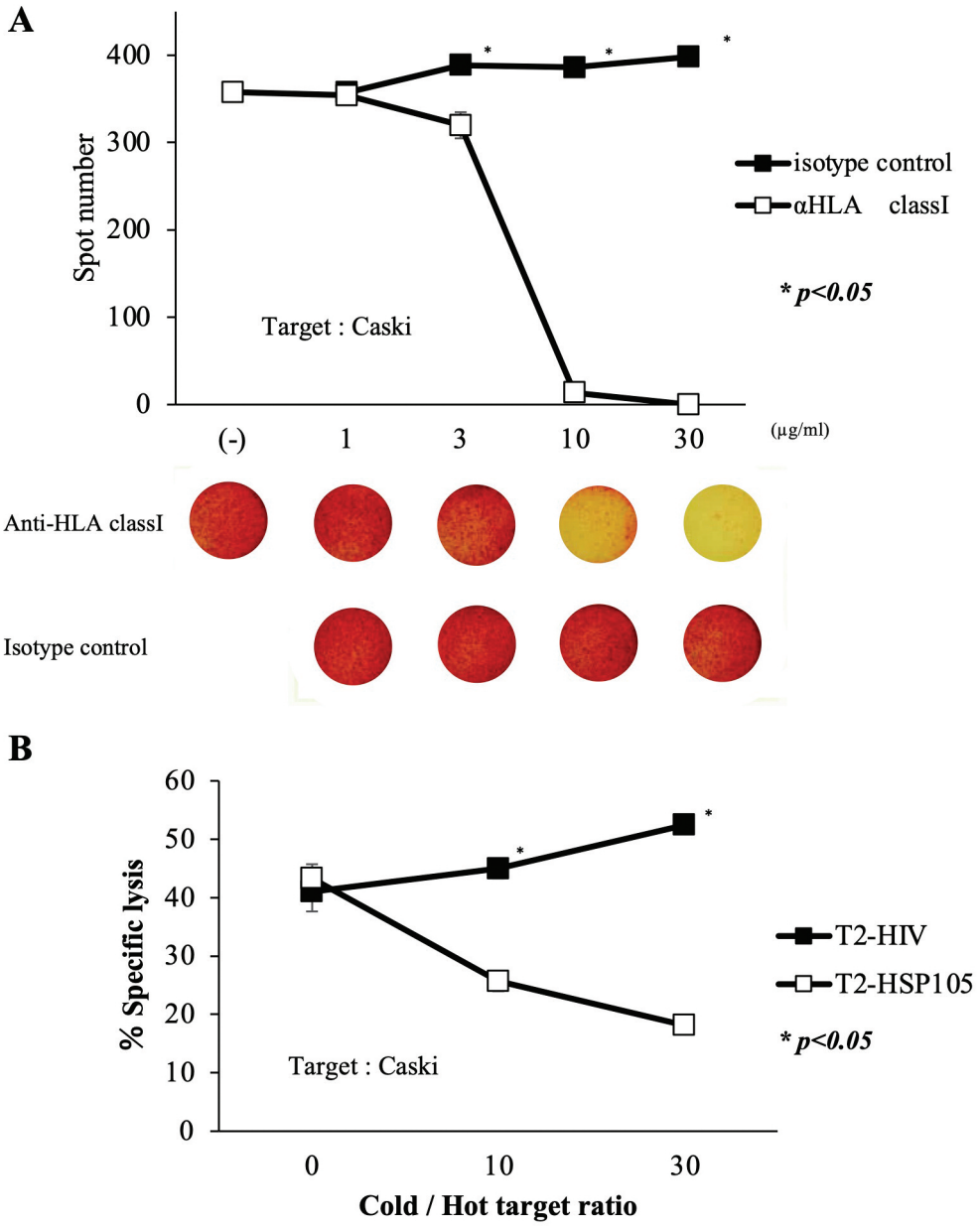

Figure 5. *Analysis of HLA-A2 and HSP105 restriction. (A) Inhibition of interferon-γ production owing to cytotoxic T lymphocyte (CTL) clone exposure to Caski cells via anti-HLA class I monoclonal antibodies (effector/target ratio=2). Representative experiments are shown below the graph. Interferon-γ production was inhibited by an anti-HLA class I monoclonal antibody but not by an isotype control (p<0.05). (B) Cold target inhibition assay of HSP105-peptide-specific CTL clones exposed to Caski cells. T2 cells pulsed with HIV19–27 peptide or with HSP105 peptide were used as cold target cells. The cytotoxic effect of HSP105-specific CTLs against Caski cells was inhibited by HSP105 peptide-pulsed T2 cells but not by HIV19–27 peptide-pulsed counterparts. Data represent the mean±standard deviation from the 5h cytotoxicity assay (p<0.05).*

with HSP105 peptide. In the current study, we confirmed HSP105 expression in almost all cervical cancer tissues and found that an HSP105 peptide-specific CTL clone actively attacks cervical cancer cell lines but not normal cells *in vitro*. Notably, we had already demonstrated the safety of HSP105 peptide vaccine therapy in clinical trials for patients with colorectal cancer (12), wherein cytotoxicity caused to normal tissues was negligibly low. These results suggest that germ cell antigens are promising targets for immunotherapy to treat patients with cervical cancer stemming from viral infections.

Cancer immunotherapy, which particularly depends on immune checkpoint inhibition, has improved markedly; such

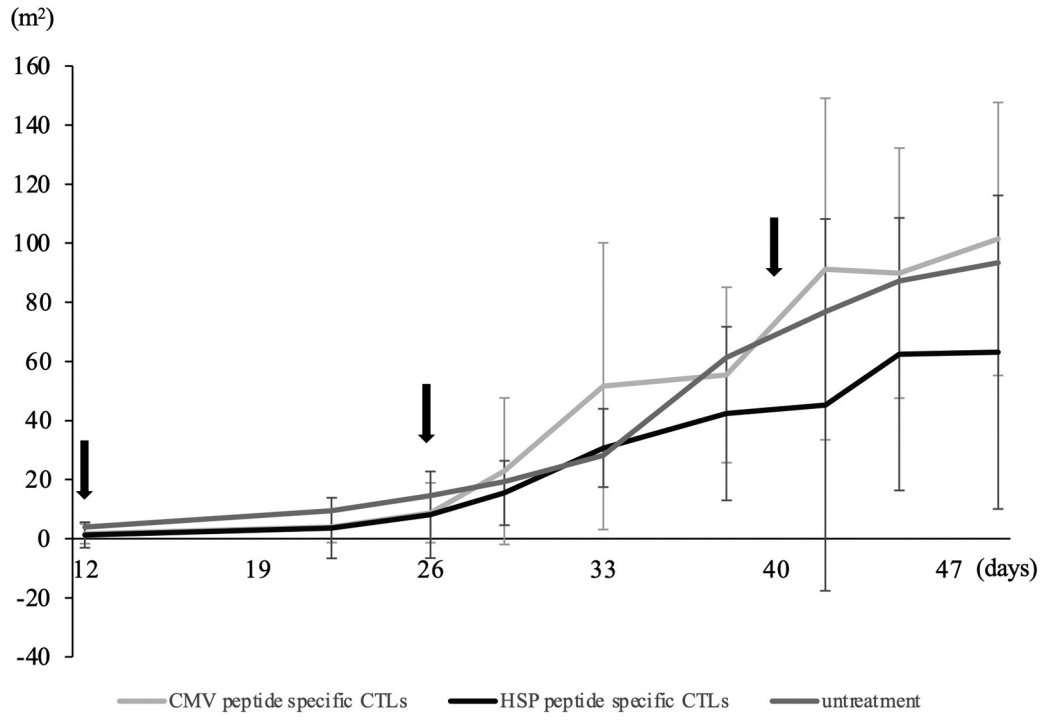

Figure 6. *HSP105 peptide-specific cytotoxic T lymphocyte (CTL) clone mediates Caski xenograft tumor regression. Caski cells were implanted subcutaneously into SCID Beige mice. Twelve days post-transplantation, mice were treated intravenously 3 times with HSP105 peptide-specific CTLs or cytomegalovirus peptide-specific CTLs in 2-week intervals, or were left untreated. HSP105 peptide-specific CTLs caused a greater delay in tumor growth compared to the other treatments, but with no significant difference.*

immune checkpoint inhibitors have shown dramatic benefits in patients with various cancers. It appears that this contributes to the activation of CTLs, which then recognize various neoantigens. Importantly, conventional cancer immunotherapy is considered to have a limited benefit given that it targets a single antigen. However, recent advances in analytical techniques have made it possible to analyze the properties of a single tumor cell; the diversity of cancer cells in a tumorous tissue is known as "intra-tumor heterogeneity". Treatments that target a single molecule can be devised for patients with cancers that exhibit less intra-tumoral heterogeneity, but may be inadequate otherwise.

In this study, we found that the HSP105 peptide-specific CTLs were effective against cervical cancer. Furthermore, HSP105 peptide-specific CTLs (and the associated vaccine therapy) may also be effective against CIN given that HSP105 expression was also found to be significantly increased in high-grade CIN, which carries fewer gene mutations and exhibits less heterogeneity than cervical cancer tissue (17). To date, the only effective treatment for CIN lesions is surgery, which increases the risk of perinatal complications in women who become pregnant. Immunotherapy targeting HSP105 as an antigen is an ideal non-invasive treatment that may provide new therapeutic options for young women.

In summary, this is the first study that evaluated the expression of HSP 105 and HLA class I in the same case in not only cervical cancer, but also CIN. The frequency and intensity of the HSP 105 expression was higher in cervical cancer cases than low-grade CIN and normal tissues. Furthermore, we demonstrated that HSP105 peptide-specific CTLs show marked cytotoxicity in cervical cancer cell lines; hence, HSP105 may be a target for immunotherapy in patients with cervical cancer. However, this study had several limitations. First, since there is no preclinical model, the effect of HSP105 peptide-specific CTLs on CIN lesions has not been evaluated. Second, it is unclear whether functional HSP105 peptide-specific CTLs are also induced in cervical cancer patients. We next planned to conduct a clinical trial of the HSP105 peptide vaccine in patients with high-grade CIN and cervical cancer. We would like to verify the efficacy of HSP105-targeted immunotherapy for CIN lesions and cervical cancer, and to confirm the induction of HSP105-specific CTL in the PBMCs of these patients. Additionally, as we established an HLA-A24-restricted HSP105-specific CTL clone, we will strive to develop HSP105 peptide-specific T cell receptor-transfer T cell therapy against cervical cancer.

Conflicts of Interest

The co-author, TN is the inventor of the peptide vaccine patents. TN received fundamental research funding from Takeda Pharmaceutical Co., Ltd. and MEDINET Co., Ltd. The funders had no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. The remaining Authors report no conflicts of interest.

Authors' Contributions

KN, SS, TY, MS and TN contributed significantly to the design of the experiments and the immunological analysis of HSP105. KN, KK, KY, MY, FK and HK analyzed the histological examination of the cervix and collected the experimental data. SS, TY, TN and HK confirm the authenticity of all the raw data and contributed by critical revision of the manuscript for intellectual content. All Authors read and approved the final manuscripts.

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