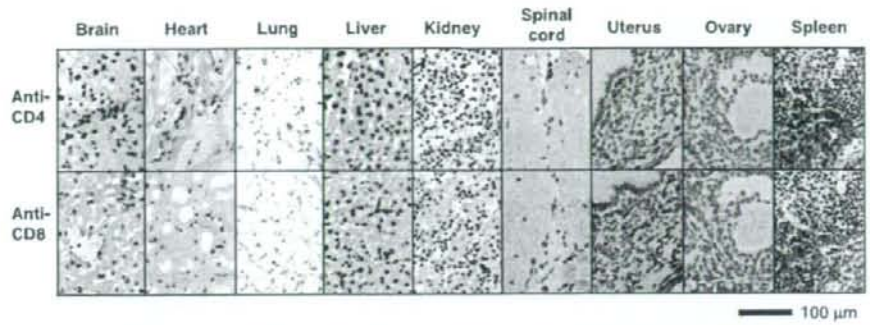


**Fig. 3.** Immunohistochemical staining with anti-CD4 or anti-CD8 monoclonal antibody in tissue specimens of BALB/c mice twice immunized with BMDC pulsed with the mixture of H2-K$^d$-restricted SPARC-derived peptides twice. These tissue specimens were removed and analyzed 7 days after the second dendritic cells (DC) vaccination (original magnification, ×200).

such as weight loss, diarrhea and skin abnormalities was observed (data not shown). These results indicate that the immunization of mice with SPARC-4$_{225-234}$ peptide does not induce autoimmune diseases.

**Inhibition of tumor growth in BALB/c mice by vaccination of SPARC-4$_{225-234}$ peptide-pulsed BMDC.** We investigated whether the immunization of mice with the SPARC epitope peptide inhibit the growth of tumor expressing SPARC *in vivo* or not. The BALB/c mice were injected i.p. twice at 7-day intervals with SPARC-4$_{225-234}$ peptide-pulsed BMDC, peptide-unpulsed BMDC or PBS ($n = 8$, each group). During the vaccination period, none of the treated mice showed any abnormalities. Subcutaneous inoculation of N2C cells ($3 \times 10^4$) into the right flank was given 7 days after the last vaccination. Growth curves of N2C tumor mass are shown in Fig. 4(A,B). The N2C tumor appeared 25 days after the inoculation in the PBS-injected group. Measurement of tumor size was continued until 62 days after inoculation of the tumor cells when one mouse in the PBS-injected group died. Mean tumor size ($1024.8 \pm 1820.7$ mm$^3$) on day 62 observed in the mouse group inoculated with SPARC-4$_{225-234}$ peptide-pulsed BMDC was significantly smaller ($5343.6 \pm 3117.2$ mm$^3$, $P < 0.01$) than that observed in the mice inoculated with peptide-unpulsed BMDC and in those injected with PBS ($6623.1 \pm 3883.9$ mm$^3$, $P < 0.01$). Complete tumor rejection was observed in four out of the eight mice in the SPARC-4$_{225-234}$ peptide-pulsed BMDC group. Although one out of the eight mice in the peptide-unpulsed BMDC group also completely rejected the tumor growth, there was no statistical significance in difference of tumor growth between the mice inoculated with peptide-unpulsed BMDC and those injected with PBS ($P = 0.48$). All mice injected with PBS died within 125 days after inoculation of the tumor cells. Mice inoculated with the SPARC-4$_{225-234}$ peptide-pulsed BMDC lived significantly longer than the mice of the other two groups (Fig. 4C). No significant abnormalities including neurological disorders were observed in four tumor-free mice in the SPARC-4$_{225-234}$ peptide-pulsed BMDC group for over 150 days after vaccination. This experiment was repeated twice with similar results. These findings indicate that vaccination of mice with BMDC pulsed with the SPARC epitopes resulted in significant inhibition of tumor growth and prolongation of overall survival *in vivo*.

## Discussion

In this study, we demonstrated that: (i) *SPARC* was overexpressed in mouse cancer cell lines; (ii) the CTL induced by the SPARC-derived peptides were reactive to tumor overexpressing SPARC; and (iii) the immunization of BALB/c mice with SPARC peptide-pulsed BMDC protected mice from tumor growth and induced prolonged survival without causing autoimmune diseases.

Although immunohistochemical staining of these tissues with anti-SPARC antibody was negative (Fig. 1C), *SPARC* mRNA was expressed in several important normal tissues including brain and spinal cord using RT-PCR. Therefore, we needed to investigate whether induction of SPARC-specific CTL could induce antitumor immunity without causing autoimmune diseases in the mouse model system. One of the reasons for lack of autoimmune diseases might be that the H2-K$^d$-positive SPARC epitope peptide complexes are more densely expressed on tumor cells in comparison to those expressed on normal tissues including brain and spinal cord, if any. These possibilities must be evaluated in a future study.

The HLA-A24 is positive in 60% of the Japanese population (95% of whom are genotypically *A*2402*), 20% of Caucasians and 12% of Africans.[29] It is important especially for the Japanese to identify HLA-A24-restricted CTL epitope peptides. Structural motifs of peptides bound to human HLA-A24 and BALB/c mouse H2-K$^d$ are similar,[30-32] and the amino acid sequences of human and mouse SPARC protein exhibit a 92% homology.[33] SPARC-derived and H2-K$^d$-restricted CTL epitopes identified in BALB/c mice may well be applicable to induce human HLA-A24-restricted CTL. Therefore, in this study, we used BALB/c mice, and searched for SPARC-derived peptides having amino acid sequences shared between mouse and human SPARC. We could identify the SPARC-derived and H2-K$^d$-restricted CTL epitopes. According to these findings, we tried to induce the HLA-A24-restricted human CTL reactive to these peptides by stimulating peripheral blood mononuclear cells (PBMC) of healthy donors or various cancer patients *in vitro* with the peptides. In a preliminary study, these SPARC peptide-specific and HLA-A24-restricted human CTL were also generated *in vitro* (unpublished observation, 2008).

We observed the inhibitory effect of immunization of mice with BMDC pulsed with SPARC-derived epitope peptide on growth of the inoculated N2C tumor cell line in BALB/c mice. N2C expressing SPARC is a mouse breast cancer cell line originating from Her-2/neu transgenic BALB/c mice.[34] It was reported that SPARC was overexpressed in human breast cancers, and associated with poor prognosis or invasive phenotype type.[35-38] N2C tumors grew as solid nests forming lobules embedded in dense, well-vascularized, connective tissue and surrounded by the stromal septa.[34] The stromal cells in N2C tumors also expressed SPARC. N2C tumor grown in SPARC

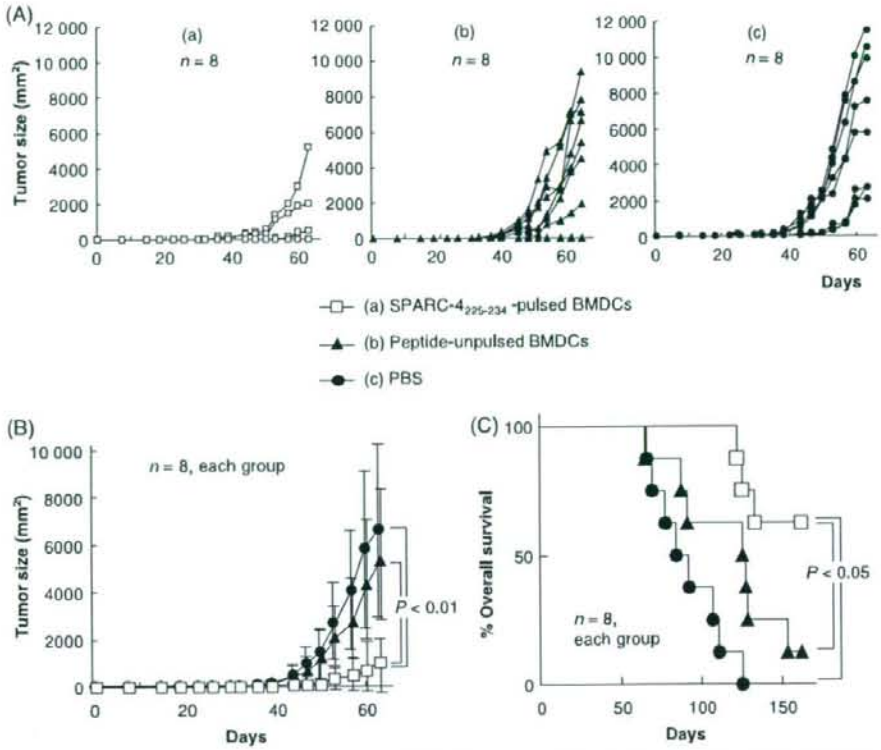


**Fig. 4.** Inhibition of tumor growth in BALB/c mice by vaccination of SPARC-$4_{225-234}$ peptide-pulsed bone marrow-derived dendritic cells (BMDC) *in vivo*. The BALB/c mice were injected i.p. twice at 7-day intervals with SPARC-$4_{225-234}$ peptide-pulsed BMDC, peptide-unpulsed BMDC or phosphate-buffered saline (PBS) only. Subcutaneous inoculation of N2C cells ($3 \times 10^4$/mouse) into the right flank was given 7 days after the last vaccination. (A) Growth curves of N2C tumor mass in individual mice in each group: (a) SPARC-$4_{225-234}$ peptide-pulsed BMDC inoculated group; (b) peptide-unpulsed BMDC inoculated group; (c) PBS injected group ($n = 8$, each group). (B) The mean tumor volumes ± standard deviation in three groups were compared in this panel. Statistical significance of the differences between each group were evaluated using the unpaired Student's *t*-test. (C) Survival rate of mice in each group. Mice in the SPARC-$4_{225-234}$ peptide-pulsed BMDC group lived significantly longer than the mice in the other two groups. Statistical significance of the differences between each group were evaluated using the Wilcoxon rank sum test.

knockout mice were smaller and histologically characterized by undefined lobules, frequently presenting necrotic central areas. The lobules were not completely delineated by the stromal septa, which appeared generally thin and sometimes heavily infiltrated by leukocytes. Therefore, the destruction of tumor stromal cells by immunity directed against SPARC may well be a possible mechanism for inhibition of N2C tumor cell proliferation observed in this study. This possibility awaits evaluation in a future study.

Taken together, these findings indicate that the antitumor immunity stimulated with SPARC-derived peptide is effective and safe at least in a preclinical study using a mouse cancer-prevention model system.

## Acknowledgments

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# HLA-A2 and -A24-restricted glypican-3-derived peptide vaccine induces specific CTLs: Preclinical study using mice

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**Abstract.** We previously reported that glypican-3 (GPC3) is uniquely overexpressed in human hepatocellular carcinoma and melanoma and that it is an ideal tumor antigen for immunotherapy in mouse models. We recently identified both HLA-A24 (*A*2402*) and H-2K$^d$-restricted $GPC3_{298-306}$ (EYILSLEEL) and HLA-A2 (*A*0201*)-restricted $GPC3_{144-152}$ (FVGEFFTDV), both of which can induce GPC3-reactive cytotoxic T cells (CTLs). The present study was a preclinical study in a mouse model that was conducted in order to design an optimal schedule for clinical trial of GPC3-derived peptide vaccine. When BALB/c mice were intradermally vaccinated at the base of the tail with K$^d$-restricted $GPC3_{298-306}$ peptide mixed with incomplete Freund's adjuvant (IFA), the peptide-specific CTLs were induced. But the peptide alone could not induce peptide-specific CD8$^+$ T cells. Furthermore, proteomic analyses showed that IFA protected the peptide against degradation in the human serum. Peptide-reactive CTLs were induced by peptide vaccine in a dose-dependent manner. In addition, at least two vaccinations with a single dose >10 $\mu$g were needed for the induction of $GPC3_{298-306}$-specific CTLs. But repeated vaccination with a lower dose of $GPC3_{298-306}$ did not induce peptide-specific CTLs. Similarly, induction of an Ag-specific immune response by HLA-A2 $GPC3_{144-152}$ depended on the dose administered. The results of this study suggested that IFA is one of the indispensable adjuvants for peptide-based immunotherapy, and that the immunological effect of peptide vaccines depends on the dose of peptide injected.

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*Abbreviations*: BM-DC, bone marrow-derived dendritic cell; mAb, monoclonal antibody; M/W, molecular/weight

*Key words*: cancer immunotherapy, GPC3, peptide vaccine, hepatocellular carcinoma

## Introduction

Hepatocellular carcinoma (HCC) is one of the most common tumors worldwide, especially in Asian and Western countries (1). Despite advances in diagnosis and treatment, the overall survival of patients with HCC has not significantly improved in the last two decades (2). The effective treatments currently available are only indicated in a relatively small proportion of early stage cases. When patients presents with clinical manifestations of HCC, the tumor is usually advanced, and there are few treatment options. Many HCC patients have type B or C hepatitis or cirrhosis, so patients treated surgically or by other therapies are also at high risk for recurrence. Furthermore, the liver function of such patients is often very poor, so treatment for recurrence is often restricted. As a result, the prognosis of HCC remains poor and new therapies for cancer development and recurrence, i.e., adjuvant therapy, are urgently needed.

We previously reported that glypican-3 (GPC3), glycosylphosphatidylinositol (GPI)-anchored membrane protein, is specifically overexpressed in human HCC and melanoma, and that among normal tissues it is slightly expressed in placenta and embryonic liver (3). We found that GPC3 is useful not only as a novel tumor marker, but also as a target antigen for immunotherapy in several studies with mice (4,5). In addition, we identified CTL epitope peptides: HLA-A24-restricted $GPC3_{298-306}$ (EYILSLEEL) and HLA-A2-restricted $GPC3_{144-152}$ (FVGEFFTDV) (6). To design the schedule for the phase I clinical study of these GPC3-derived peptide vaccines, many factors need to be taken into consideration: the adjuvant, dosage, number of doses, vaccination interval, etc. Many investigators have reported various vaccination schedules (7,8). There is no world-wide consensus concerning the schedule to use for administration of peptide

vaccines. In this study, we attempted to identify a more effective vaccine regimen that would induce a strong cell-mediated immune response. Ten years ago Salgaller *et al* reported that they did not observe any dose dependency between 1 and 10 mg in the capacity of gp100 peptide to enhance immunogenicity in humans (9). The results of our present study, however, showed dose-dependency in the immunizing effect of a peptide vaccine.

## Materials and methods

*Mice.* Female BALB/c mice at 6-8 weeks of age were obtained from Japan SLC (Hamamatsu, Japan) or Charles River Laboratories. *HLA-A2.1* (HHD) Tgm; *H-2D*$^{b-/-}$*h2m*$^{-/-}$ double knockout mice transfected with a human h2m-*HLA-A2.1* (a1 a2)-*H-2D*$^{b}$ (a3 transmembrane cytoplasmic) (HHD) monochain construct gene were prepared in the Department SIDA-Retrovirus, Unité d'Immunité Cellulaire Antivirale, Institut Pasteur, France (10), and kindly provided by Dr F.A. Lemonnier. The mice were maintained under specific-pathogen-free conditions. The mouse experiments were approved by the Animal Research Committee of the National Cancer Center Hospital East.

*Cells lines.* A subline of BALB/c-derived colorectal adenocarcinoma cell line Colon 26, C26 (C20) (11) was provided by Dr Kyoichi Shimomura (Astellas Pharmaceutical Co., Tokyo, Japan). Colon 26/GPC3 (C26/GPC3) is an established stable GPC3-expressing cell line (4). RMA-HHD cells were kindly provided by Dr Masanori Matsui of Saitama Medical School, Saitama, Japan. A human ß2m-HLA-A2.1 (α1α2)-H-2D$^{b}$ (α3 transmembrane cytoplasmic) (HHD) monochain construct was transfected into RMA lymphoma cells [transporter associated with antigen presentation (TAP) positive] to establish RMA-HHD cells (10). The cells were cultured in RPMI-1640 medium supplemented with 10% FCS. To obtain GPC3-expressing RMA-HHD (RMA-HHD-GPC3) cells, RMA-HHD cells were transfected with pCAGGS-GPC3-internal ribosomal entry site (IRES)-puromycin-resistant gene with Lipofectamine 2000 reagent (Invitrogen Corp., Carlsbad, CA), selected with puromycin, and then subjected to cloning by limiting dilution in drug-free medium in 96-well culture plates (12,13). Dendritic cells were obtained from bone marrow cells (BM-DCs) as described previously (4). Irradiated BM-DCs pulsed with peptide were used for *in vitro* CTL culture or as target cells for Elispot assays.

*Vaccination.* HLA-A24- and K$^{d}$-restricted GPC3$_{298-306}$ (EYILSLEEL) and HLA-A2-restricted GPC3$_{144-152}$ (FVGEFFTDV) were dissolved in 7% $NaHCO_3$ and the solution was diluted with saline. For peptide vaccination, mice were intradermally injected at the base of the tail with peptide solution emulsified in incomplete Freund's adjuvant (IFA). Different doses of peptide were administered at 7-day intervals, and mice were sacrificed to obtain inguinal lymphocytes and spleen cells seven days after the final vaccination.

*IFN-γ Elispot assays.* Female BALB/c mice were intradermally vaccinated with GPC3$_{298-306}$ or GPC3$_{144-152}$/IFA. Their inguinal lymphocytes were stimulated with peptide-pulsed BM-DCs *in vitro* for five days. The proportion of cells producing IFN-γ against target cells (BM-DCs pulsed with or without GPC3 peptide) was assessed by an Elispot assay as described previously (14). The spots were automatically counted and subsequently analyzed with the Eliphoto system (Minerva Tech, Tokyo, Japan).

*Analysis of peptide degradation.* GPC3$_{298-306}$ was mixed with human serum, and the solution was applied to the surfaces of a Q10 (strong anion exchange) ProteinChip (Bio-Rad Laboratories, CA). We sequentially examined the solution with a SELDI-TOF mass spectrometer (Bio-Rad). Female BALB/c mice were intradermally vaccinated with GPC3$_{298-306}$/IFA at the base of the tail. A week later, we collected the residual peptide vaccine at the base of the tail into buffer in a tube, and the tube was centrifuged at 4°C at 10,000 rpm for 15 min. The supernatant was applied to a ProteinChip and the surface of the chip was examined with the spectrometer.

*Induction of GPC3-specific CTLs and cytotoxicity assay.* Mice were intradermally vaccinated twice with 50 μg GPC3 peptide/IFA 7 days apart. Seven days after the second vaccination, inguinal lymph nodes were excised and the lymphocytes were cultured in 24-well culture plates ($5x10^6$ per well) with GPC3 peptide-pulsed BM-DCs ($1x10^5$ per well) in RPMI medium supplemented with 10% horse serum, recombinant human interleukin (IL)-2 (100 units/ml), and 2-mercaptoethanol (50 μmol/l). After culture for 5 days, the cells were recovered and analyzed for their cytotoxic activity against target cells with the TERASCAN VPC system (Minerva Tech) as previously described (15). Briefly, C26, C26/GPC3, RMA-HHD and RMA-HHD-GPC3 cells were used as target cells and labeled with calcein-AM solution for 30 min at 37°C. The labeled cells were washed three times and distributed to the 96-well culture plate ($1x10^4$ per well), and they were then incubated with effector cells for 5-6 h. Fluorescent intensity was measured before and after the 5-6-h culture, and Ag-specific cytotoxic activity was calculated by using this formula: cytotoxicity (%) = [(sample release) - (spontaneous release)]/[(maximum release) - (spontaneous release)] x 100. In some experiments, CD8$^{+}$ T cells were isolated from effector cell preparations with a magnetic cell sorting system (Miltenyi, Bergisch Gladbach, Germany). Positively selected CD8$^{+}$ T cells were 95% pure as determined by flow cytometry.

*Histologic and immunohistochemical analysis.* Mice were injected twice with GPC3 peptide vaccine, and seven days later tumor cells were subcutaneously implanted in their shaved backs. Seven days after the tumor challenge, frozen sections of tumor tissue were prepared. The frozen tissue sections were immunohistochemically analyzed using monoclonal antibody (mAb) specific for CD4 (L3T4; BD PharMingen, San Diego, CA) or CD8 (Ly-2; BD PharMingen) as described previously (4).

*Statistical analysis.* The 2-tailed Student's t-test was used to determine the statistical significance of differences in the

data obtained by ELISPOT assay. P<0.05 was considered to be significant. Statistical analyses were made using the StatView 5.0 software package (Abacus Concepts, Calabasas, CA).

## Results

*IFA is an effective adjuvant for the peptide vaccine that induced strong immune responses and maintained the stability of the peptide.* We attempted to verify whether emulsions of GPC3 peptide in IFA could induce a peptide-specific immune response in mouse models. The results showed that only GPC3 peptide emulsified in IFA elicited a T-cell-mediated immune response, whereas vaccination with peptide alone failed to induce any detectable immune response (Fig. 1A). In addition, we investigated the stability of GPC3 peptide alone in human serum, with the Surface Enhanced Laser Desorption/Ionization SELDI system (Bio-Rad). We applied the sample of GPC3 peptide mixed with human serum to a ProteinChip and detected the peak of GPC3 peptide. The peak value of $GPC3_{298-306}$ in buffer was about 1,000, whereas the peak of $GPC3_{298-306}$ in serum had decreased to 200, 3 min after mixing it with the serum (Fig. 1B). This finding indicated that GPC3 peptide was immediately degraded in serum. Moreover, we collected the white residue of peptide/IFA emulsions that were still present at the base of the tail of the vaccinated mice, and after applying the peptide/IFA emulsions to a ProteinChip, quantified the peak of $GPC3_{298-306}$. Mass spectrometric analysis demonstrated that the peptide was still present in a stable form in the peptide/IFA emulsions (Fig. 1C). IFA not only induced a potent immune response, but protected the peptide from various enzymes in the serum.

*Dose-dependent effects of GPC3-derived peptide vaccine emulsified in IFA.* Next we examined whether a more peptide-specific response was induced, when a higher dose of peptide was used. The proportion of peptide-specific CTLs among $5x10^4$ $CD8^+$ T cells was evaluated by IFN-γ Elispot assays, when mice were vaccinated with $GPC3_{298-306}$ $K^d$-restricted peptide doses of 5, 10, 20, or 50 μg. Peptide-specific $CD8^+$ T cell responses were observed when vaccinated with $GPC3_{298-306}$ $K^d$-restricted peptide doses above 10 μg (Fig. 2A). Additionally, as the peptide dose increased, peptide reactive CTLs were detected more frequently (Fig. 2A). We vaccinated A2 Tg mice with HLA-A2-restricted $GPC3_{144-152}$ peptide in the same manner. The results indicated that the higher doses of the peptide induced a greater peptide-specific immune response (Fig. 2B). We therefore, concluded that the higher the dose of peptide injected, the more peptide-specific $CD8^+$ T cells were induced. But Elispots by vaccinations of >5 μg $GPC3_{144-152}$ been seen to reach a plateau level.

*Marked infiltration of subcutaneous tumor tissue by $CD8^+$ T cells in mice vaccinated with the 50 μg dose of GPC3 peptide.* Immunohistochemical analysis of the tumor tissue specimens showed more intense infiltration by $CD8^+$ T cells, but not by $CD4^+$ T cells, in and/or around C26/GPC3 (Fig. 3D) or RMA-

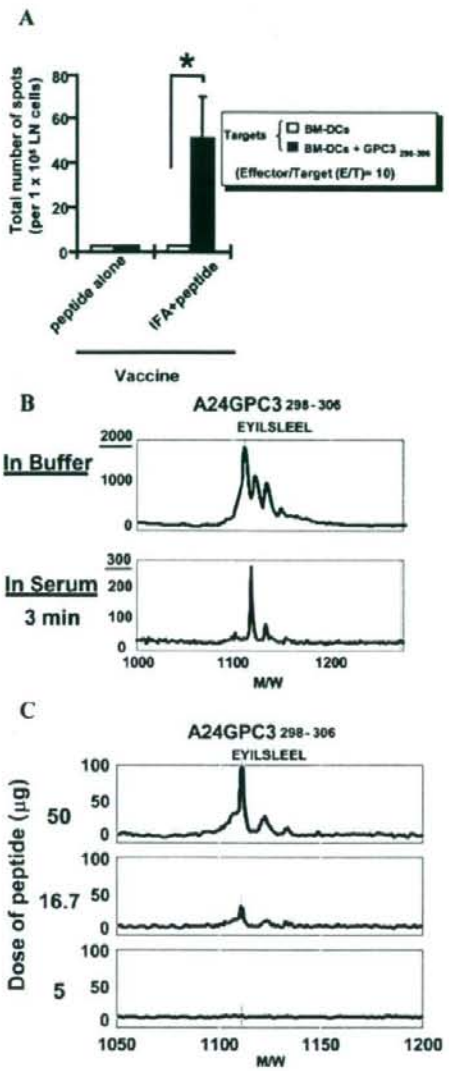


Figure 1. IFA is an appropriate adjuvant for the peptide vaccine. (A) The $GPC3_{298-306}$-specific immune response was induced by peptide emulsified in IFA. Female BALB/c mice were vaccinated twice by intradermal injection of 50 μg of $GPC3_{298-306}$ (EYILSLEEL) with IFA. Seven days after the second injection, inguinal lymph node cells were isolated and cultured with $GPC3_{298-306}$-pulsed BM-DCs for 5 days. The cells were recovered, and their Ag-specific activity was analyzed by IFN-γ Elispot assays against BM-DCs pulsed or not pulsed with $GPC3_{298-306}$. *P<0.05, statistically significant difference in response. (B) Stability of $GPC3_{298-306}$ (EYILSLEEL) in human serum. We measured $GPC3_{298-306}$ in the human serum 3 min after the mixture of $GPC3_{298-306}$ and human serum by SELDI-TOF mass spectrometry. The discriminating peaks of M/W 1111 represent $GPC3_{298-306}$. When GPC3 peptide was mixed with human serum, the peptide was immediately degraded by various proteases. (C) Detection of GPC3 peptide in emulsions collected from vaccinated mice. BALB/c mice were intradermally injected at the base of the tail with $GPC3_{298-306}$ emulsified in IFA. A week later we collected peptide/IFA emulsions at the base of the tail and measured GPC3 peptide contained in the emulsions. When injected with 50 μg of peptide, GPC3 peptide was detected clearly. Data are representative of 3 independent experiments with similar results in (A-C).

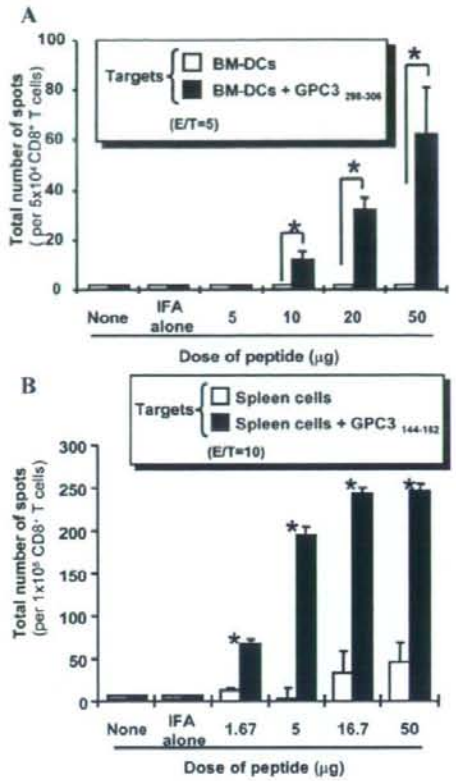


Figure 2. The higher the dose of peptide injected, the more peptide-specific CD8⁺ T cells were induced. Groups of mice were vaccinated twice at 7-day intervals with one of the dose levels of H-2K$^d$-restricted GPC3$_{298-306}$ peptide (A) or HLA-A2-restricted GPC3$_{144-152}$ peptide (B) emulsified with IFA (each group; n=3). Inguinal lymphocytes were restimulated *in vitro* with each GPC3 peptide-pulsed BM-DCs for 5 days. The recovered cells were sorted to the CD8⁺ T cells fraction by MACS and IFN-γ Elispot assays were performed. Peptide-specific CD8⁺ T cells were induced dose-dependently. Data are representative of 3 independent experiments with similar results. *P<0.05, difference in response was statistically significant.

HHD-GPC3 (Fig. 3H) tumor tissue of mice vaccinated with the 50 μg dose of GPC3 peptide than with IFA alone, and the 1.67 μg dose of GPC3 peptide (Fig. 3). This phenomenon was also observed in metastatic inguinal lymph nodes tissues (data not shown). These results also suggest that the higher the dose of peptide, the more peptide-specific CD8⁺ T cells were induced and infiltrated the GPC3-expressing tumor. However, 1.67 μg dose of GPC3$_{144-152}$ had seemed to induce a few peptide-specific CD8⁺ T cells, which corresponded to the result shown in Fig. 2B.

*A second vaccination is needed to induce a peptide-specific response.* Next we attempted to determine how many vaccinations were required to induce a peptide-specific immunological response. BALB/c mice were vaccinated with a 1.67, 5, 16.7, 50 μg dose of peptide once a week for 1-4 weeks, respectively. A single vaccination did not elicit a peptide-specific immune response at any of the

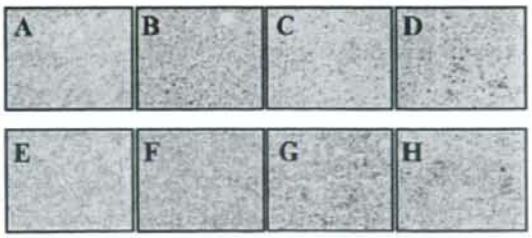


Figure 3. Infiltration by CD8⁺ T cells around and into subcutaneous C26/GPC3 (A-D) or RMA-HHD-GPC3 (E-H) tumor tissue is shown. We estimated infiltration by CD8⁺ T cells immunohistochemically: no treatment (A and E), IFA (B and F), 1.67 μg (C), 50 μg (D) of GPC3$_{298-306}$, 1.67 μg (G), 50 μg (H) of GPC3$_{144-152}$. In mice treated with 50 μg GPC3 peptide, a larger number of CD8⁺ T cells had clearly infiltrated into and/or around the tumor (D and H). But even in 1.67 μg dose of GPC3$_{144-152}$, a few peptide-specific CD8⁺ T cells were induced (original magnification, x200).

GPC3$_{298-306}$ dose levels (Fig. 4A). Induction of a peptide-specific T-cell response required at least two vaccinations and >16.7 μg dose of peptide, and no expansion of peptide-specific T cells occurred after repeated vaccinations with lower doses of peptide (Fig. 4B-D). We also compared immunological responses induced by two and five vaccinations, with 1.67 and 50 μg doses of HLA-A2 GPC3$_{144-152}$, but the same as with HLA-A24 GPC3$_{298-306}$, five vaccinations did not increase a peptide-specific response (Fig. 5).

*Cytotoxity of CD8⁺ T cells primed with GPC3 peptide vaccines.* We analyzed the cytotoxity of CD8⁺ T cells primed with GPC3 peptide vaccines. Their killing activity against target cells that expressed or did not express GPC3 was analyzed. The effector cells primed with the GPC3 vaccines showed a significantly higher killing activity against C26/GPC3 cells than against C26 cells, and significantly higher killing activity against RMA-HHD-GPC3 cells than against untransfected RMA-HHD cells (Fig. 6). These results suggest that the CD8⁺ T cells induced by GPC3 peptide vaccinations have cytotoxic activity against tumor cells that express GPC3 naturally.

## Discussion

The stability of antigens and the immunogenicity of ISA 720 based on Western blot experiments (16) have been verified, and in the present study we showed that IFA is one of the indispensable adjuvants for peptide vaccines.

We previously reported that vaccination with GPC3$_{298-306}$ peptide-pulsed BM-DCs induced complete rejection of a C26/GPC3 tumor challenge in a mouse model (4), but in the present study, C26/GPC3 tumors in a prophylactic model were not rejected after two intradermal vaccinations with GPC3 peptide/IFA at the base of the tail even though CD8⁺ T cells by GPC3 peptide vaccine was demonstrated by immunological and immunohistological analysis (data not shown). Comparison of the capacity of peptide-pulsed BM-DCs vaccine to induce peptide-specific CTLs with the

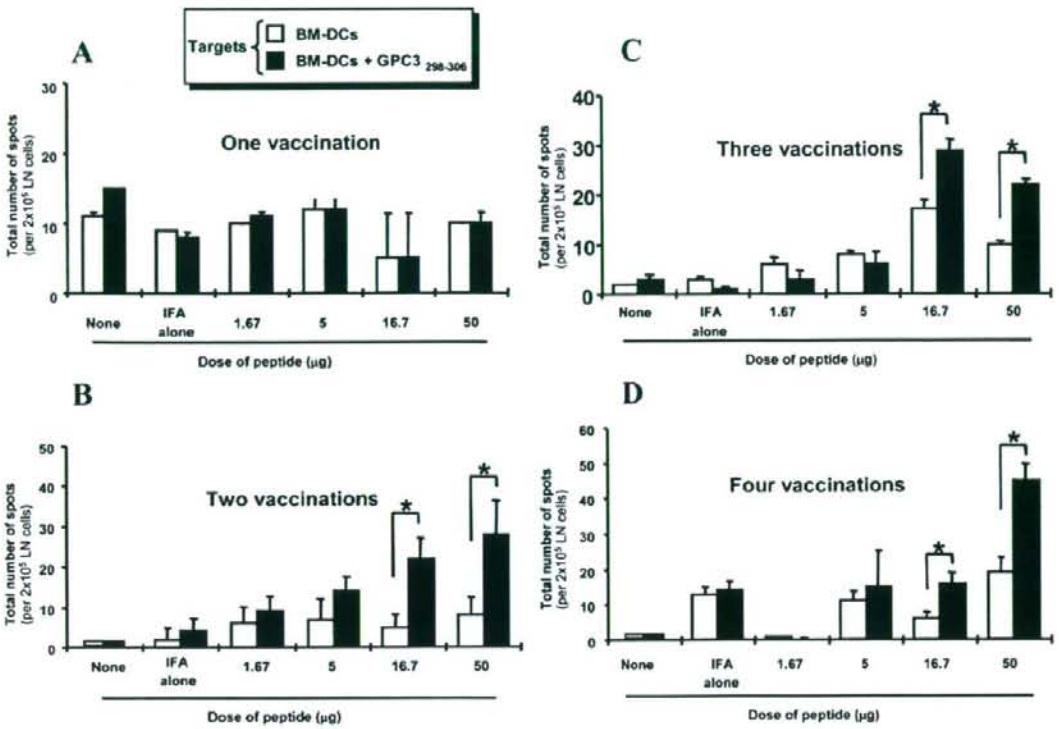


Figure 4. A second vaccination is needed to induce peptide-specific T cells. The immune responses to one (A), two (B), three (C) and four (D) vaccinations with each dose of peptide are shown. BALB/c mice (each group: n=3) were vaccinated with 1.67, 5, 16.7, or 50 $\mu$g $GPC3_{298-306}$. Seven days after the final vaccination, bilateral inguinal LNs were excised. Each lymphocyte was restimulated *in vitro* with $GPC3_{298-306}$ peptide-pulsed BM-DCs for 5 days, and IFN-$\gamma$ Elispot assays were then performed against BM-DCs pulsed or not pulsed with $GPC3_{298-306}$ to count $GPC3_{298-306}$ peptide-specific CTLs. Data are representative of 3 independent experiments with similar results (A-D).

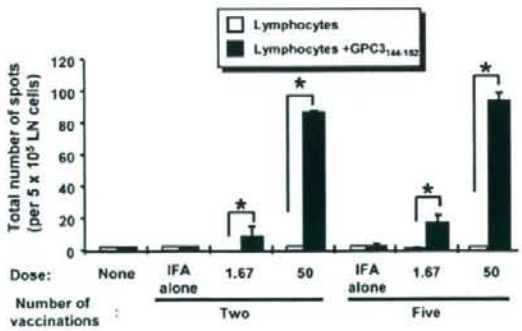


Figure 5. Comparison of immune responses after two or five vaccinations with a 1.67 $\mu$g or 50 $\mu$g dose of $GPC3_{144-152}$. A2 Tg mice (n=3) were vaccinated with 1.67 $\mu$g or 50 $\mu$g $GPC3_{144-152}$ in the same manner in Fig. 3, but the IFN-$\gamma$ Elispot assay was performed using whole lymph node cells without *in vitro* culture. Data are representative of 3 independent experiments with similar results. *P<0.05, difference in response was statistically significant.

capacity of peptide/IFA vaccine by *in vitro* IFN-$\gamma$ Elispot assays demonstrated that peptide-pulsed BM-DCs vaccine induced more peptide-specific CTLs (data not shown). There have been few reports of induction of tumor regression *in vivo* by peptide vaccine. Pilar *et al* recently reported finding that a combination of peptide vaccine and CpG induced stronger anticancer responses not only in a prophylactic model, but also in a therapeutic model. They reported in the same study that vaccination with peptide p66 in IFA in the absence of CpG resulted in insignificant CTL responses (17). Although other adjuvants, including CpG, were not effective in the present study (data not shown), peptide/IFA with CpG may be effective. Further study is needed.

The results of the present study showed that at least two vaccinations were necessary to elicit immunological effects. A comparison between HLA-A2-restricted $GPC3_{144-152}$/IFA and $K^d$-restricted $GPC3_{298-306}$ showed that $GPC3_{144-152}$ induced more peptide-specific CTLs at a lower dose. Moreover, HLA-A2-restricted $GPC3_{144-152}$ specific CTLs were induced without *in vitro* stimulation with $GPC3_{144-152}$ peptide. That may have been attributable to the difference in mouse strain. It is usually said that C57BL/6 and BALB/c mice are a prototypical Th1-type strain and a prototypical Th2-type mouse strain, respectively (18,19) and the difference in genetic background seemed to affect their susceptibility to each of the peptide vaccines.

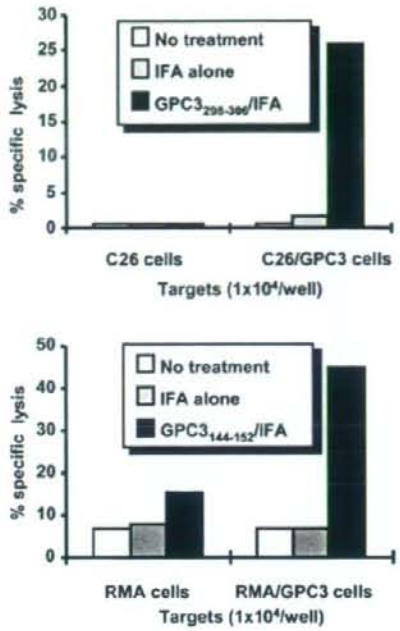


Figure 6. Cytotoxity of $CD8^+$ T cells primed with GPC3 peptide vaccine. BALB/c or C57BL/6 mice (n=2-3) were vaccinated with IFA alone or 50 $\mu$g peptide/IFA in the same manner as described above. The inguinal LN cells were cultured with $1x10^5$ peptide-pulsed BM-DCs for 5 days. The cells obtained were sorted to the $CD8^+$ T cell fraction with microbeads. Cytotoxic assays were performed with the cells to evaluate their capacity to kill $1x10^4$ C26, C26/GPC3, RMA-HHD, or RMA-HHD-GPC3 cells. Data are representative of 3 independent experiments with similar results.

In 1996, Salgaller *et al* reported that they did not detect dose dependency between 1 and 10 mg in the capacity of gp100 peptide to enhance immunogenicity in humans (9). A dose-dependent effect of peptide vaccine was shown in the present study. We are conducting a phase I clinical trail of GPC3-derived peptide vaccine in patients with advanced HCC at National Cancer Center Hospital East, and we are waiting for the results to determine whether a dose-dependent effect of peptide/IFA was shown in humans, the same as in the mouse model.

## Acknowledgments

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# Differential expression of heat shock protein 105 in melanoma and melanocytic naevi

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**The objective of this study is to assess the expression of heat shock protein 105 (HSP105) in melanoma and benign melanocytic lesions. The expression of HSP105 in 62 human melanoma samples – 46 primary and 16 metastatic lesions – and 42 melanocytic naevi samples, was assessed by immunohistochemistry. Western blotting was performed on melanoma cell lines, melanoma tissues with matched normal skin and melanocytic naevi. The Mann–Whitney test was used for statistical analysis and significance was considered to be *P* less than 0.05. Seventy-four per cent of the primary melanoma lesions and 88% of the metastatic lesions overexpressed HSP105 by immunohistochemistry. The majority of melanocytic lesions (95%) were negative ($P<0.05$). Western blotting detected high expression of HSP105 in melanoma cell lines and tissues. The expression of HSP105 was related to the invasiveness of the lesions. Melanocytic naevi expressed HSP105 at a level that was similar to that of normal skin. Our results show that high expression of HSP105 is associated with malignant melanoma especially advanced and metastatic lesions. The results suggest that HSP105 analysis may be a helpful tool as a poor prognostic indicator and as a diagnostic aid in problematic lesions; in addition, melanoma can be included in the growing list of tumours overexpressing HSP105 to be targeted for potential HSP105-based therapeutic strategies. *Melanoma Res* 18:166–171 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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**Keywords: heat shock protein105, melanoma, melanocytic naevus**

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## Introduction

Malignant melanoma results in the highest number of skin cancer-related deaths. Several strategies have been used in the treatment of melanoma, with no significant improvement in prognosis. It is essential to identify molecules that are highly expressed in melanoma and to target them in future treatment options. It is also important to find molecules that can aid in the differential diagnosis of malignant melanoma and benign lesions that might mimic it clinically and histologically. We report here differential expression of heat shock protein 105 (HSP105) in malignant and benign melanocytic lesions and the analysis of the relationship of this expression to the clinical characteristics of the patients.

HSP105 is a mammalian stress protein in the HSP105/HSP110 family. It was discovered from murine FM3A cells [1] and was shown by immunofluorescence to be localized in the cytoplasm and nuclei of cells under both stressed and nonstressed conditions [2]. It has been shown to be involved in malignant cell transformation by protecting tumour cells from apoptosis [3]. Mechanisms by which HSP105 suppresses apoptosis have been elucidated in HeLa cells [4].



HSP105 is expressed constitutively at very low levels in normal tissues and overexpressed in several cancers [5,6]; however, there is no report on the expression of HSP105 in melanocytic lesions. In this study, we investigated the expression of HSP105 in malignant melanoma and in melanocytic naevi by immunohistochemistry and western blot analyses.

## Materials and methods

### Tissue samples

Tissue specimens were obtained from patients who underwent surgery in the Department of Dermatology and Plastic and Reconstructive Surgery at Kumamoto University Hospital. Written informed consent was obtained from all participants and the study was approved by the institutional review board.

Sixty-two archival paraffin-embedded specimens of melanoma were obtained from 50 patients ranging in age from 22 to 95 years (median 71.5 years). Forty-eight per cent of the patients were male and 52% were female. The lesions consisted of 46 primary [acral lentiginous melanoma (ALM), $n=25$; lentigo maligna melanoma, $n=9$; superficial spreading melanoma (SSM), $n=7$;

mucosal melanoma, $n = 3$; and nodular melanoma, $n = 2$] and 16 metastatic tumours (obtained from 13 patients). Fifty-two per cent of the primary lesions consisted of ALM, which is the commonest in the Japanese population. Staging was carried out according to the International Union against Cancer/American Joint Committee on Cancer Tumour Node Metastasis Classification [7]. Primary tumours varied in thickness from *in situ* to 7.20 mm. Analysis of the clinical data was carried out based on the following classification, T1 ($\leq 1.00$), T2 (1.01–2.00 mm), T3 (2.01–4.00 mm) and T4 ($\geq 4.00$ mm). Melanocytic naevi ($n = 42$) consisted of five junctional, eight intradermal, 22 compound and seven Spitz naevi, obtained from 16 male and 26 female patients ranging in age from 2 to 66 years. Frozen samples consisted of SSM ($n = 2$), ALM ($n = 2$), metastatic melanoma ($n = 1$), melanoma *in situ* ($n = 1$) and melanocytic naevi ($n = 4$).

### Cell lines

Eleven melanoma cell lines: CRL1579, G361, HMV-I, SK-MEL-28, 888mel, 526mel, COLO 38, SK-MEL-19, MEWO, HM3 KO and 164 mel were kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan) and Dr Y Kawakami, Keio University (Japan).

### Immunohistochemistry

Four-micrometer sections were cut from paraffin-embedded archival blocks, dried at 37°C, deparaffinized in xylene and rehydrated in graded alcohols. Antigen retrieval was carried out by heating the sections in citric acid (0.01 mmol/l, pH 6) for 10 min and cooling for 60 min at room temperature. Immunohistochemical staining was performed as described previously [5]. The primary antibody, HSP105 (Santa Cruz Biotechnology, Santa Cruz, California, USA) was used at a dilution of 1 : 100 and normal rabbit immunoglobulin-G (IgG; Upstate Cell Signaling Solutions, Temecula, California, USA) was used as a nonspecific IgG control at a dilution of 1 : 100. Colour was achieved using the diamino benzidine system and counter-staining was performed using 0.5% Giemsa's solution in phosphate buffered saline. HSP105 expression was graded into –, + and + + when an average of < 25, 26–50 and > 50% of cells per high-power field were positive, respectively. The results were viewed by two authors (F.C.M. and T.K).

**Table 1 Expression of HSP105 in melanoma and melanocytic naevi**

| Lesion | Positive cases | (%) | (%) |
|---|---|---|---|
| Primary melanoma | 34/46 | | 76 |
| T1 | 7/15 | 47 | |
| T2 | 7/7 | 100 | |
| T3 | 10/12 | 83 | |
| T4 | 10/12 | 83 | |
| Metastatic melanoma | 14/16 | | 87.5 |
| Melanocytic naevus | 2/42 | | 5 |

HSP105, heat shock protein 105; T, tumour thickness; T1, ≤ 1.00 mm; T2, 1.01–2.00 mm; T3, 2.01–4.00 mm; T4, ≥ 4.00 mm.

**Table 2 Clinical data and HSP105 expression of melanoma patients**

| ID | Age/sex | Melanoma type | Breslow (mm) | HSP105 expression |
|---|---|---|---|---|
| 20 | 65/M | Melanoma IS | 0 | – |
| 16 | 82/F | Melanoma IS | 0 | + |
| 50 | 50/M | Melanoma IS | 0 | – |
| 48 | 62/F | Melanoma IS | 0 | – |
| 46 | 78/F | Melanoma IS | 0 | – |
| 15 | 85/F | Melanoma IS | 0 | + + |
| 40 | 70/M | LMM | 0.2 | – |
| 22 | 76/F | ALM | 0.4 | + |
| 17 | 63/F | ALM | 0.4 | + |
| 8 | 70/F | Mucous | 0.5 | – |
| 44 | 72/M | LMM | 0.5 | – |
| 36 | 56/F | ALM | 0.6 | – |
| 2 | 74/F | ALM | 0.6 | + + |
| 31 | 88/M | ALM | 0.9 | + |
| 3 | 61/M | ALM | 0.9 | + + |
| 25 | 87/F | ALM | 1 | + |
| 12 | 83/E | ALM | 1.3 | + |
| 34 | 49/F | SSM | 1.6 | + |
| 35 | 60/F | ALM | 1.8 | + + |
| 10 | 69/M | SSM | 2 | + |
| 14 | 75/M | ALM | 2 | + + |
| 4 | 65/M | ALM | 2 | + + |
| 27 | 69/M | ALM | 2.1 | + + |
| 49 | 75/M | ALM | 2.2 | – |
| 24 | 88/F | ALM | 2.3 | + |
| 1 | 53/M | ALM | 2.3 | + + |
| 9 | 88/M | ALM | 2.6 | + |
| 11 | 79/F | ALM | 2.6 | + + |
| 41 | 76/M | LMM | 2.8 | + + |
| 19 | 70/M | ALM | 2.9 | + |
| 18 | 74/F | SSM | 3.2 | + |
| 30 | 56/M | LMM | 3.4 | + + |
| 45 | 91/F | ALM | 3.6 | – |
| 32 | 81/M | SSM | 3.8 | + |
| 26 | 72/F | ALM | 4.1 | – |
| 23 | 86/F | ALM | 4.1 | + |
| 39 | 54/M | Mucous | 4.3 | – |
| 38 | 22/M | ALM | 4.4 | + |
| 37 | 47/F | NM | 4.4 | + + |
| 6 | 75/F | Mucous | 4.4 | + + |
| 13 | 50/F | SSM | 4.6 | + + |
| 33 | 71/M | SSM | 4.7 | + + |
| 7 | 57/M | ALM | 5.4 | + + |
| 42 | 86/M | LMM | 6.7 | + |
| 28 | 91/M | ALM | 7.2 | + + |
| 47 | 49/F | NM | | + + |
| 5 | 95/F | LN metastasis | | + + |
| 21 | 62/F | LN metastasis | | + + |
| 29 | 44/M | LN metastasis | | + + |
| 29 | 44/M | Skin metastasis | | + + |
| 43 | 86/F | LN metastasis | | + + |
| 10 | 69/M | LN metastasis | | – |
| 11 | 79/F | LN metastasis | | + + |
| 41 | 76/M | Skin metastasis | | + + |
| 30 | 56/M | LN metastasis | | + + |
| 32 | 81/M | Skin metastasis | | + + |
| 37 | 47/F | LN metastasis | | + |
| 33 | 71/M | LN metastasis | | + + |
| 33 | 71/M | Skin metastasis | | – |
| 7 | 57/M | LN metastasis | | + |
| 7 | 57/M | Skin metastasis | | + + |
| 42 | 86/M | Skin metastasis | | + + |

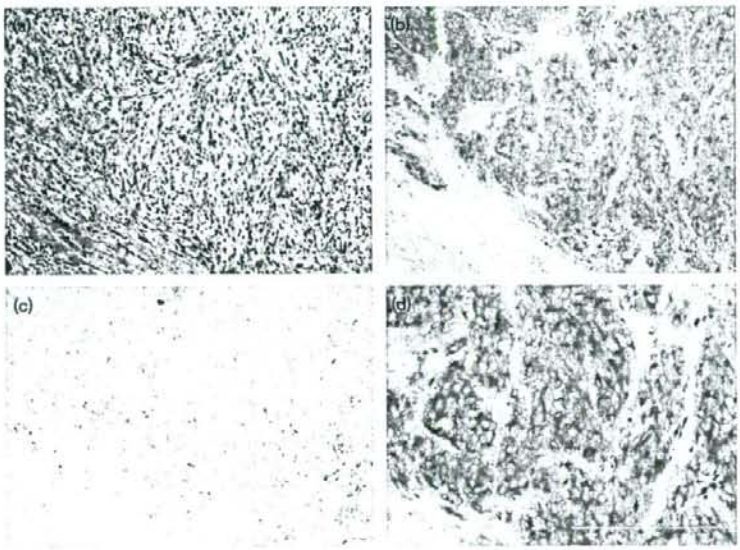
ALM, acral lentiginous melanoma; HSP105, heat shock protein 105; IS, in-situ; LMM, lentigo maligna melanoma; LN, lymph node; NM, nodular melanoma; SSM, superficial spreading melanoma.
–, negative; +, positive; ++, strongly positive.

### Western blotting

Tissue samples were obtained immediately after excision, snap frozen in liquid nitrogen and stored at −80°C. Primary SSM and ALM samples were classified according to the growth phase. After careful examination of the histological sections; the microinvasive, nontumorigenic parts of the tumour (radial growth phase) and the invasive tumorigenic parts (vertical growth phase) were then treated as separate sections of the same sample. Uninvolved skin from the surgical margins was also included for comparison by western blot analysis.

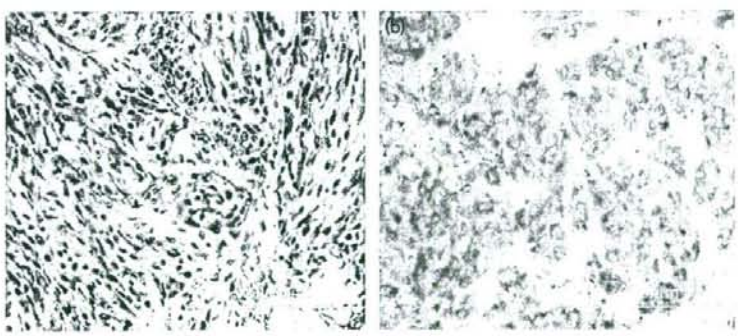
Cell lines were cultured in Dulbecco's modified Eagle's medium or Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal calf serum. The samples were homogenized and the lysate exposed to 10% SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Blocking was

**Fig. 1**



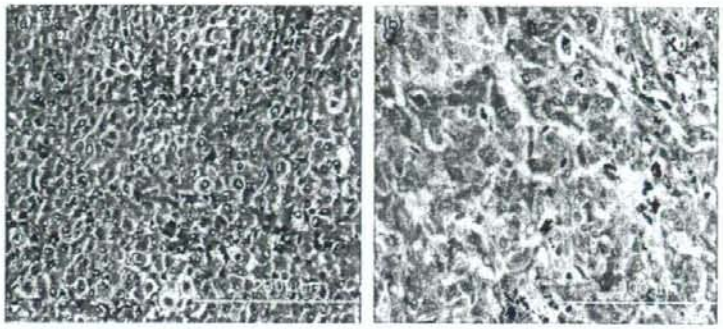

Representative immunohistochemical staining for heat shock protein 105 (HSP105) in malignant melanoma (b, d) nodular melanoma in a 47-year-old female patient, Breslow thickness of 4.4 mm (case ID 37 in Table 2). Haematoxylin and eosin stain shows the atypical, mitotically active malignant melanocytes (a). HSP105 immunohistochemical staining (b; × 100, d; × 200 original magnification, respectively) shows a nuclear and cytoplasmic staining. Normal rabbit IgG control antibody was used to eliminate nonspecific staining (c).

**Fig. 2**




Heat shock protein 105 immunohistochemical staining (b) of advanced acral lentiginous melanoma, thickness, 7.2 mm (case 28 in Table 2). The corresponding histological section is also shown (a). Bar 200 μm.

Fig. 3



High expression of heat shock protein 105 in metastatic melanoma lesion (b), case 29 in Table 2. The histological section is shown (a). Bar 200 μm.

achieved by incubating the membrane in 5% skimmed milk/Tris buffered saline 0.2% Twin-20 (TBST) overnight. HSP105 anti-rabbit polyclonal IgG was applied at a dilution of 1:500 and incubated for 60 min at room temperature, after which the membrane was incubated with goat anti-rabbit IgG-HRP (Biorad, Hercules, California, USA) for 30 min. Membranes were washed thoroughly with TBST and signals detected using the Enhanced Chemiluminescence system (Amersham Biosciences, Piscataway, New Jersey, USA). ß-actin was used as a loading control.

### Statistical analysis

To compare the HSP105 expression between malignant melanoma and melanocytic naevus, the nonparametric Mann–Whitney test was performed. Samples graded –, + and + + were assigned a number (1, 2 and 3, respectively), and the two groups, melanoma and melanocytic naevus, were assigned into groups A and B, respectively. The raw data were assessed by computational analysis. The significance level was considered as $P$ less than 0.05.

## Results

### Immunohistochemistry

HSP105 was highly expressed in melanoma but it showed minimal or no expression in melanocytic naevi by imunohistochemical analysis. Staining was observed in both the nuclei and cytoplasm. A total of 48 of 62 melanomas (77%) highly expressed (+ and + +) HSP105 as outlined in Table 1. Of the primary lesions, 34 of 46 (74%) overexpressed HSP105, whereas 14 of the 16 (88%) metastatic lesions were strongly positive. Increased staining was noted to be associated with an advanced stage of melanoma (Table 2); in addition, primary and metastatic lesions from the same patient maintained the same high expression of HSP105. Representative immunohistochemical staining for HSP105 in a case of nodular melanoma with a Breslow thickness of 4.4 mm, in a 47-year-old female patient, is shown in Fig. 1. Intensity of immunohistochemical staining was very high in invasive primary and metastatic lesions as shown in Figs 2 and 3. No association was,

Table 3 Clinical data and HSP105 expression of melanocytic naevi patients

| ID | Age/Sex | Type | HSP105 expression |
|---|---|---|---|
| 1 | 66/F | Junctional | – |
| 2 | 10/M | Junctional | – |
| 9 | 45/F | Junctional | – |
| 17 | 10/M | Junctional | – |
| 26 | 30/F | Junctional | – |
| 29 | 33/F | Intradermal | – |
| 31 | 14/M | Intradermal | – |
| 32 | 35/F | Intradermal | – |
| 13 | 34/F | Intradermal | – |
| 3 | 39/M | Compound | – |
| 4 | 5/F | Compound | – |
| 6 | 5/F | Compound | – |
| 7 | 28/F | Compound | – |
| 8 | 33/M | Compound | – |
| 10 | 15/F | Compound | – |
| 11 | 9/M | Compound | – |
| 12 | 32/F | Compound | – |
| 14 | 54/F | Compound | – |
| 15 | 44/F | Compound | – |
| 18 | 9/F | Compound | – |
| 19 | 10/M | Compound | – |
| 20 | 2/F | Compound | – |
| 21 | 11/M | Compound | – |
| 22 | 4/M | Compound | – |
| 23 | 4/F | Compound | + |
| 24 | 20/F | Compound | – |
| 25 | 25/F | Compound | – |
| 30 | 20/F | Compound | – |
| 33 | 9/M | Compound | – |
| 34 | 29/M | Compound | – |
| 35 | 2/M | Compound | – |
| 36 | 20/M | Spitz | – |
| 37 | 13/F | Spitz | – |
| 38 | 35/M | Spitz | – |
| 39 | 24/M | Spitz | – |
| 40 | 3/F | Spitz | – |
| 41 | 24/M | Spitz | – |
| 42 | 14/F | Spitz | + + |

HSP105, heat shock protein 105; F, female; M, male.

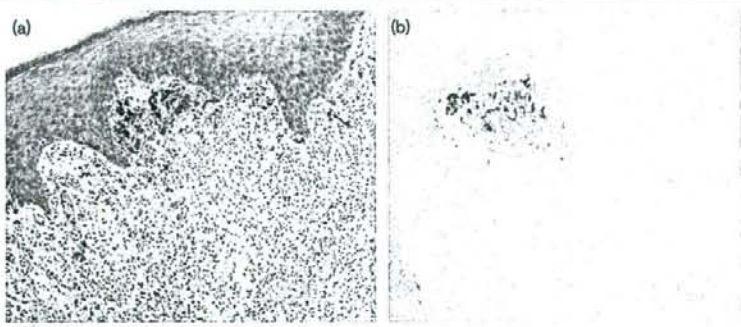
however, observed between the immunohistochemistry results and the sex or age of the patients. Only 5% (two of 42) of benign melanocytic naevi showed increased staining of HSP105 (Table 3). These consisted of a compound naevus from a 4-year-old girl and a Spitz naevus from a 14-year-old girl. No abnormality or recurrence was noted on follow-up of these cases. A representative staining of melanocytic naevus is shown in Fig. 4. A significant difference in the expression of HSP105 was observed between melanocytic naevi and malignant melanoma ($P < 0.05$).

### Western blotting

Western blot analysis of four primary and one metastatic melanomas detected high expression of HSP105 in the vertical growth phase of the tumour, and much less expression in the radial growth phase of the same tumour in comparison (Fig. 5a); in addition, skin (epidermis) obtained from the surgical margins of the same patient showed a minimal expression of the protein. Melanoma *in situ* expressed HSP105 to a low level. These results are in concordance with the immunohistochemical observations, which show higher expression of HSP105 with increasing tumour thickness.

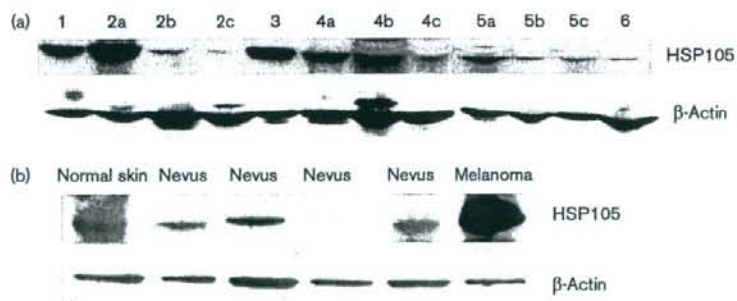
Western blotting was also performed to compare the expression of HSP105 in melanoma and melanocytic naevi. Results showed high HSP105 expression in melanoma and reduced expression in melanocytic naevi (Fig. 5b).

**Fig. 4**




Representative staining for melanocytic naevus, case 15 (Table 3). Haematoxylin and eosin stain shows a compound naevus with multiple naevus nests (a). Immunohistochemical staining (b) shows a negative expression of heat shock protein 105. Bar 200 μm.

**Fig. 5**




(a) Western blot analysis of melanoma tumours and skin from the surgical margins of the same tumour; metastatic lesions and the vertical growth phase of primary lesions show an increased expression of heat shock protein 105 (HSP105) compared with the radial growth phase and uninvolved skin from the excision margins obtained from the same patient. 1, skin metastasis; 2a, superficial spreading melanoma (SSM) vertical growth phase; 2b, SSM radial growth phase; 2c, SSM NS. 3, SSM; 4a, acral lentiginous melanoma (ALM) vertical growth phase; 4b, ALM radial growth phase; 4c, ALM NS; 5a, ALM vertical growth phase; 5b, ALM radial growth phase; 5c, ALM NS; 6, melanoma *in situ*. NS, uninvolved skin from the surgical margins. (b) Western blotting demonstrating reduced expression of HSP105 in melanocytic naevus compared with malignant melanoma. Equal loading was verified by β-Actin immunoblotting.

Eleven human melanoma cell lines, CRL1579, G361, HMV-I, SK-MEL-28, 888mel, 26mel, COLO 38, SK-MEL-19, MEWO, HM3 KO and 164mel, showed a uniformly high expression of HSP105 by western blot analysis (results not shown).

## Discussion

In this study, we have, for the first time, characterized the expression of HSP105 in melanocytic lesions. We have shown an increased expression correlated with aggressive primary tumours and metastatic lesions, and markedly reduced expression in benign melanocytic naevi. Therefore, HSP105 is a useful marker and may be an unfavourable prognostic indicator in malignant melanoma. In an earlier study, we showed that HSP105 was overexpressed in squamous cell carcinoma of the skin and extramammary Paget's disease. We demonstrated a higher expression in metastatic squamous cell carcinoma compared with primary lesions. In contrast, the more indolent tumour, basal cell carcinoma, did not overexpress HSP105 [5]. A variety of other tumours have also been shown to overexpress HSP105, including colon and pancreatic adenocaricnomas, thyroid, oesophageal, breast, bladder carcinoma and others; however, the testis is the only normal tissue that overexpresses HSP105 [6].

HSP105 is a member of the heat shock family of proteins, whose functions include: acting as in-vivo chaperons of tumour-associated antigen epitopes [8], inhibition of aggregation of denatured proteins [9], assisting in protein folding and refolding of misfolded proteins, protecting cells from the cytotoxic effects of stressors that induce their transcription, and suppression of stress-induced apoptosis [4]. In the absence of overexpression of other stress proteins, HSP105 is still effective in protecting cells against potentially lethal heat exposures by inhibiting heat-induced aggregation [10]. HSP105 overexpression in cancer cells is therefore essential for their survival. Some studies have demonstrated the ability of HSP105 to prevent apoptosis by suppressing its expression using HSP105 short interfering RNA; the cancer cells underwent apoptosis [3]. Therefore, HSP105-mediated inhibition of apoptosis promotes tumorigenesis of cancer cells.

Metastatic melanoma remains resistant to therapy; therefore, it is essential to identify other molecules present in melanoma cells that can be targeted for treatment. We observed that high HSP105 expression is related to aggressive primary tumours and metastatic lesions; showing increased expression with increasing stage and thickness of melanoma. Several studies have investigated the potential use of HSP105 as an antitumor agent. In-vivo studies on HSP105/DNA vaccination resulted in a reduction of melanoma and colorectal cancer tumour bulk using BALB/c and C57Bl/6 mice models [11]. Furthermore, in a later study, HSP105-pulsed dendritic cell immunization induced an even stronger tumour rejection response in similar mice models [12,13]. Therefore, identification of tumours that overexpress HSP105 may be important for the future treatment of these tumours.

We have shown here that HSP105 is overexpressed in melanoma and is associated with advanced clinical stage and therefore may be a poor prognostic indicator. Our findings also suggest that HSP105 may have a role in the diagnosis of challenging or problematic melanocytic lesions. In addition, advanced and metastatic melanoma, which we have shown to overexpress HSP105, may be a potential target for HSP105-based treatment options.

## Acknowledgements

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# HLA-A2-restricted CTL epitopes of a novel lung cancer-associated cancer testis antigen, cell division cycle associated 1, can induce tumor-reactive CTL

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**Toward the development of a novel cancer immunotherapy, we have previously identified several tumor-associated antigens (TAAs) and the epitopes recognized by human histocompatibility leukocyte (HLA)-A2/A24-restricted cytotoxic T lymphocyte (CTL). In this study, we tried to identify a TAA of lung cancer (LC) and its HLA-A2 restricted CTL epitopes to provide a target antigen useful for cancer immunotherapy of LC. We identified a novel cancer testis antigen, cell division cycle associated gene 1 (CDCA1), overexpressed in nonsmall cell LC using a cDNA microarray analysis. The expression levels of CDCA1 were also increased in the majority of small cell LC, cholangiocellular cancer, urinary bladder cancer and renal cell cancers. We used HLA-A2.1 transgenic mice to identify the HLA-A2 (*A*0201*)-restricted CDCA1 epitopes recognized by mouse CTL, and we investigated whether these peptides could induce CDCA1-reactive CTLs from the peripheral blood mononuclear cells (PBMCs) of HLA-A2-positive donors and a NSCLC patient. Consequently, we found that the $CDCA1_{65-73}$ (YMMPVNSEV) peptide and $CDCA1_{351-359}$ (KLATAQFKI) peptide could induce peptide-reactive CTLs in HLA-A2.1 transgenic mice. In HLA-A2$^+$ donors, *in vitro* stimulation of PBMC with these peptides could induce peptide-reactive CTLs which killed tumor cell lines endogenously expressing both HLA-A2 and CDCA1. As a result, CDCA1 is a novel cancer-testis antigen overexpressed in LC, cholangiocellular cancer, urinary bladder cancer and renal cell cancers, and CDCA1 may therefore be an ideal TAA useful for the diagnosis and immunotherapy of these cancers.**


**Key words:** tumor immunology; cancer testis antigen; CDCA1; CTL; HLA-A2

Lung cancer (LC), especially nonsmall cell lung cancer (NSCLC), is one of the most common cancers in the world, and more than 1 million people are killed by LC annually. The most common cause of cancer death is LC among men and women aged 60 years and older.[1]

Currently, new combination therapies are prescribed for patients with advanced NSCLC, but LC is relatively resistant to the currently available chemotherapy and radiotherapy regimens.[2] Most current regimens for NSCLC provide a limited survival benefit and are often considered to be ineffective or excessively toxic.[3]

Recently, the presence of lymphocytic infiltrates in murine and human tumors or generation of cytotoxic T lymphocytes (CTLs) recognizing lung tumor antigens suggest that an immune reaction could potentially help to eliminate tumor cells.[2] Adoptive immunotherapy using *in vitro* expanded tumor antigen-specific $CD8^+$ CTLs has been considered as a feasible therapy for *in vivo* eradication of tumors.[4] There have been reports on the clinical efficacy of immunotherapy for advanced cancer, but little clinical data have been reported in cases of advanced NSCLC.[5–7]

Tumor-associated antigens (TAAs) are proteins known to be overexpressed in and broadly distributed among malignant cells of various origins.[8,9] The molecular identification and characterization of expressed TAAs has rapidly evolved because of the availability of new technologies. Many TAAs in certain human malignancies were identified using methods of cDNA expression cloning.[10–12]

Recently, cDNA microarray technologies have been developed and the systematic analysis of the expression levels of thousands of genes is an effective method for the identification of TAAs overexpressed in cancer tissues.[13] We used a genome-wide expression profile analysis of LCs with the cDNA microarray containing 27,648 genes and investigated the biological and clinicopathological significance of the respective gene products.[13] This systematic approach revealed that cell division associated 1 (CDCA1) was frequently overexpressed in various histologic types of LC but not in normal adult tissues including lung tissues, except normal testis, and that CDCA1 was essential for growth or survival of LC cells.[13]

The protein CDCA1 has a specific function at the kinetochores to stabilize microtubule attachment in Hela cells. CDCA1 is a part of a molecular linker between the kinetochore attachment site and the tubulin subunits within the lattice of the attached plus ends. Therefore, the depletion of CDCA1 results in a strong prometaphase blockade with an active spindle checkpoint and it causes aberrant chromosome segregation. These CDCA1-suppressed cells displayed reduced tension at kinetochores of chromosomes.[14,15] CDCA1 was one of cell cycle-associated genes that was coexpressed with known cell cycle genes, including cell division cycle 2 (CDC2), cyclin, topoisomerase II and others.[16] On the other hand, CDCA1 is involved in the process of pulmonary carcinogenesis. The NSCLC patients with highly CDCA1 expressing cancerous tissue showed poorer prognosis in comparison to patients with low CDCA1 expression.[13] Therefore, we have considered that CDCA1 may prove to be applicable as a candidate target for cancer immunotherapy.

The gene frequency of human histocompatibility leukocyte (HLA)-A2 (A*0201) is high among various ethic groups, includ-

*Abbreviations:* ADC, adenocarcinoma; BM-DCs, bone marrow-derived dendritic cell; CDCA1, cell division cycle associated 1; CTL, cytotoxic T lymphocyte; ELISPOT, enzyme-linked immunospot; GM-CSF, granulocyte-macrophage colony-stimulating factor; HLA, human histocompatibility leukocyte antigen; IL, interleukin; LC, lung cancer; mAb, monoclonal antibody; NSCLC, nonsmall cell lung cancer; PBMCs, peripheral blood mononuclear cells; RT-PCR, reverse transcription-PCR; SqCC, squamous cell carcinoma; TAAs, tumor-associated antigens; Tgm, transgenic mice.

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ing Asians, Africans, Afro-Americans and Caucasians.[17] It is suggested that the HLA-A2-restricted and CDCA1-derived CTL epitopes might be very useful for the immunotherapy of many patients with NSCLC all over the world. In this study, we identified human CDCA1-derived CTL epitopes restricted by HLA-A2 using HLA-A2.1 (HHD) transgenic mice (Tgm) and examined whether these HLA-A2 restricted epitope peptides could induce CDCA1-reactive CTLs from the peripheral blood mononuclear cells (PBMCs) of healthy donors.

## Material and methods

### *Mouse*

HLA-A2.1 (HHD) Tgm; H-2D$^{-/-}$ β2m$^{-/-}$ double knockout mice introduced with human β2m-HLA-A2.1 (α1, α2)-H-2D$^{b}$ (α3 transmembrane cytoplasmic) (HHD) monochain construct gene were a generous gift from Dr. F.A. Lemonnier of the Department SIDA-Retrovirus, Unite d' Immunite Cellulaire Antivirale, Institut Pasteur, France.[18,19]

### *cDNA microarray and acquisition of data*

A genome-wide cDNA microarray was fabricated by the Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, containing 27,648 cDNAs selected from the UniGene Database (build No. 131) of the National Center for Biotechnology Information. Briefly, the microarray system containing 27,468 cDNAs was established previously.[20] Cancer cells were selectively collected from the preserved samples using laser microbeam microdissection (LMM) method.[21] Extraction of total RNA and T7-based amplification were performed as described previously. As a control probe, normal human lung poly(A) RNA (BD Biosciences Clontech, Palo Alto, CA) was amplified using the same amplification condition; 2.5 μg aliquots of amplified RNAs (aRNAs) from each cancerous tissue and from the control were reverse transcribed in the presence of Cy5-dCTP and Cy3-dCTP, respectively. The relative expression ratio was derived from the value of the expression of CDCA1 mRNA in cancer cells divided by that in normal counterpart (Fig. 1*a*). In Figure 1*b*, the relative expression ratio of normal tissues was derived from the value of the expression of CDCA1 mRNA in each normal tissue divided by the mean value of the expression of CDCA1 mRNA in the mixture of an equal amount of RNA derived from all normal tissues tested.

### *Northern blot analysis and reverse transcription-PCR*

Northern blot analysis was done as described previously.[22] The integrity of RNA in the formalin-Mops gels was checked using electrophoresis. Gels with 20 μg of total RNA per lane were blotted onto a nylon membrane (Hybond N; Amersham, Piscataway, NJ). Poly(A) RNA blots of human tissues were also used. The membranes were hybridized with a CDCA1-specific cDNA probe (1113–1924 bp) labeled with [32P]dCTP. RT-PCR analysis of cancer cell lines and NSCLC tissues was done as described previously.[23] CDCA1 gene-specific PCR primer sequences were as follows: sense, 5′-CCCAGATATAATGTAGCTGAGATT-3′; antisense, 5′-CTCCTGGTGTGCGGCGTTTA-3′, and used RT-PCR reactions consisting of initial denaturation at 94°C for 5 min and 32 amplification cycles at an annealing temperature of 55°C.

### *Histologic and immunohistochemical analysis*

Immunohistochemical examinations of the CDCA1 protein in patients' cancer tissues were also done as described previously.[24,25] The primary antibody used in this study, mouse monoclonal antibody specific to Nuf2 (synonymous to CDCA1), was purchased from Abcam (Cambridge, UK).

### *Patients, blood samples and cell lines*

Blood samples from patients with NSCLC were obtained during routine diagnostic procedures after obtaining informed consent from the patients in Kumamoto University Hospital from September 2006 to December 2007. A TAP-deficient and HLA-A*0201-positive cell line (T2) and the CDCA1$^{+}$ human pancreas cancer cell line (PANC1) were purchased from Riken Cell Bank in Tsukuba, Japan. CDCA1-negative human colon cancer cell line COLO201, CDCA1-positive LC cell lines EBC-1 and LC-1sq were kindly provided by Health Science Research Resources Bank in Tsukuba, Japan. The expression of HLA-A2 on the cell surface was examined using flow cytometry with an anti-HLA-A2 monoclonal antibody (mAb), BB7.2 (One Lambda, Canoga Park, CA), to select HLA-A2-positive blood donors and target cell lines for CTL assay.

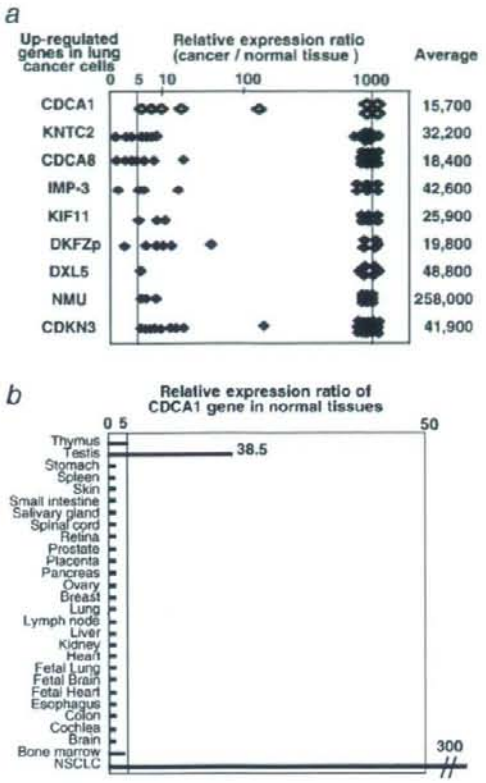


FIGURE 1 – Markedly and frequently enhanced expression of a novel cancer testis antigen, CDCA1, in NSCLC tissues as based on cDNA microarray analysis. (*a*) A list of upregulated genes in NSCLC tissues. These genes were overexpressed in cancer cells when compared with normal counterpart. All 9 patients, who could be investigated by microarray analysis, showed high expression of *CDCA1* in cancer cells. The relative expression ratio was derived from the value of the expression of *CDCA1* mRNA in cancer cells divided by that in normal counterpart. (*b*) The relative ratio (RR) of expression of human *CDCA1* mRNA in 9 NSCLC tissues and disease-free tissues examined by cDNA microarray analysis. *CDCA1* gene was highly expressed only in testis among normal tissues, as based on cDNA microarray analysis. The relative expression ratio of normal tissues was derived from the value of the expression of *CDCA1* mRNA in each normal tissue divided by the mean value of the expression of *CDCA1* mRNA in the mixture of equal amount of RNA isolated from all normal tissues tested.

TABLE I – EXPRESSION OF CDCA1 IN NSCLC AND OTHER CANCEROUS TISSUES[1]

| Cancerous tissue | Frequency of tumor overexpressing CDCA1 | | Average of relative expression ratio |
|---|---|---|---|
| | *N* | % | |
| Small cell lung cancer | 15/15 | 100 | 10,299 |
| Nonsmall cell lung cancer | 9/9 | 100 | 15,694 |
| Cholangiocellular cancer | 12/12 | 100 | 6,885 |
| Bladder cancer | 28/28 | 100 | 3,880 |
| Renal cell cancer | 7/7 | 100 | 15 |
| Prostate cancer | 17/20 | 85 | 24,653 |
| CML | 14/17 | 82 | 27,898 |
| Malignant lymphoma | 7/9 | 78 | 26,159 |
| Cervical cancer | 12/16 | 75 | 10,068 |
| Osteosarcoma | 14/19 | 74 | 88,855 |
| Breast cancer | 25/39 | 64 | 3,070 |
| Sarcoma | 18/36 | 50 | 6,134 |
| Colon cancer | 4/10 | 40 | 2,996 |
| Esophageal cancer | 4/18 | 22 | 3.4 |
| AML | 0/16 | 0 | 1.6 |
| Gastric cancer | 0/5 | 0 | 0 |

[1]The expression level of CDCA1 also increased in all cancerous tissues of small cell lung cancer, cholangiocellular cancer, bladder cancer and renal cell cancer based on cDNA microarray analysis.[21,28–30]

*Lentiviral gene transfer*

A lentiviral vector-mediated gene transfer was performed as described.[26] Briefly, 17 μg of CSII-CMV-RfA and CSIIEF-RfA self-inactivating vectors[27] carrying CDCA1 cDNAs and 10 μg of pCMV-VSV-G-RSV-Rev and pCAG-HIVgp were transfected into the 293T cells grown in the 10-cm culture dish using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). After 60 hr of transfection, the medium was recovered and the viral particles were pelleted by ultracentrifugation (50,000*g*, 2 hr). The pellet was suspended in 50 μl of RPMI 1640 medium and then 10 μl of viral suspension was added to $5 \times 10^4$ COLO201 cells per well in a U-bottom 96-well plate. The expression of the transfected *CDCA1* gene was confirmed by Western blot analysis.

*Induction of CDCA1-reactive mouse CTLs and IFN-γ enzyme-linked immunospot assay*

Human CDCA1-derived peptides carrying binding motifs for HLA*0201-encoded molecules were identified using the BIMAS software program (BioInformatics and Molecular Analysis Section, Center for Information Technology, NIH, Bethesda, MD) and 40 peptides (purity > 90%) carrying HLA-A2 (A*0201) binding motifs were synthesized (Table I).[21,28–30] The immunizations of mice with peptides were done as previously described.[22] Then, 6 days after the start of *in vitro* culture, an enzyme-linked immunospot (ELISPOT) assay was done, according to the manufacturer's recommendations. Mouse IFN-γ ELISPOT kit (BD Biosciences, San Jose, CA) was used for the detection of CDCA1 epitope reactive mouse CTLs secreting IFN-γ in response to the syngenic BM-DC pulsed or unpulsed with each peptides.[31,32]

*Induction of CDCA1-reactive human CTLs*

Monocyte-derived DCs were used as antigen-presenting cells to induce CTL responses against peptides presented in the context of HLA. The DC were generated by *in vitro* culture as previously described.[33,34] Briefly, PBMC isolated from a healthy volunteers or a NSCLC patient positive for HLA-A*0201 using Ficoll-Plaque (GE Healthcare UK, Buckinghamshire, UK) solution were sorted to the $CD8^-$ population and the $CD14^+$ population with microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). To generate DCs, the $CD14^+$ population was cultured in the presence of 100 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF; provided by PeproTec, NJ) and 10 ng/ml interleukin (IL)-4 (PeproTec) in AIM-V (Invitrogen) containing 2% heat-inactivated autologous plasma. After 4 days of culture, OK-432 was added into the dish to make the DCs mature. After 5 days, we started to culture the cytokine-generated DCs, and they were pulsed with 20 μg/ml HLA-A2-binding peptides in the presence of 4 μg/ml β2-microglobulin (Sigma-Aldrich, St. Louis, MO) for 2 hr at 37°C in AIM-V. These peptide-pulsed DCs were then irradiated (3,500 cGy) and mixed at a 1:50 ratio with autologous $CD8^+$ T cells, obtained by positive selection of PBMCs with anti-CD8 microbeads (Miltenyi Biotec). These cultures were set up in 48-well plates; each well contained $1 \times 10^4$ peptide-pulsed DCs, $5 \times 10^5$ $CD8^+$ T cells and 10 ng/ml human IL-7 (Wako, Osaka, Japan) in 0.5 ml AIM-V with 2% autologous plasma. After 3 days, these cultures were supplemented with human IL-2 (PeproTec) to a final concentration of 20 IU/ml. On days 12 and 19, the T cells were further restimulated with the peptide-pulsed autologous DCs. We prepared the DCs each time in the same way as described earlier. The antigen-specific responses of the CTLs were investigated using chromium release assay and IFN-γ ELISPOT assay on 6 days after the third round of peptide stimulation was performed on day 25.

HLA-A*0201 tetramers labeled with PE and bound by the $CDCA1_{351-359}$ peptide was obtained from Medical and Biological Laboratories, Nagoya, Japan.

*CTL responses against target cells*

The CTLs were cocultured with each cancer cell line or peptide-pulsed/unpulsed T2 cells as a target cell at the indicated effector/target ratio, and a $^{51}Cr$ release assay and IFN-γ ELISPOT assay were done as described.[32,35,36] Briefly, the target cells were labeled with 3.7 KBq $Na_2{}^{51}Cr_4$ (Perkin Elmer Life Sciences, Waltham, MA) for 1 hr at 37°C in a $CO_2$ incubator. The labeled target cells were rinsed 3 times, and the peptide-pulsed target cells were prepared by incubating the cells with 20 μg/ml peptide for 3 hr at 37°C. The target cells were mixed with the effector cells in a final volume of 200 μl in flat-bottomed microtiter plates and incubated. After 6 hr incubation, 50 μl of the supernatant was collected from each well and the radioactivity was quantified using a gamma counter. The specific cytotoxicity was evaluated by calculating the percentage of specific $^{51}Cr$ release as described.[37] An ELISPOT assay was carried out as described previously.[32]

## Results

*Markedly enhanced expression of CDCA1 mRNA in NSCLC and other cancerous tissues and cell lines*

Using a cDNA microarray representing 27,648 genes, we chose 9 genes which were overexpressed in the great majority of NSCLC tissues among 37 cases of NSCLC in comparison to their adjacent normal counterpart. The relative expression ratios (cancer/normal counterpart) of all 9 genes were more than 15,000 (Fig. 1*a*). Thereafter, we analyzed the expression of these genes using a cDNA microarray analysis in 28 kinds of normal tissues (including 4 embryonic tissues) (Fig. 1*b*). The relative expression ratio of CDCA1 was more than 15,000 (mean: 15,694; range: 6.5–54,778) in cancerous tissues isolated from 9 patients with NSCLC available from the cDNA microarray data (Fig. 1*a*). In addition, the *CDCA1* gene was strongly expressed only in the testis among 24 adult normal tissues, as based on cDNA microarray analysis (Fig. 1*b*). Consequently, we identified CDCA1 to be an ideal target for immunotherapy of NSCLC patients.

Moreover, to confirm the results obtained from the cDNA microarray analyses, we examined the expression of the *CDCA1* gene in NSCLC cell lines and normal tissues at the mRNA level by using RT-PCR and Northern blot analysis. The *CDCA1* gene was significantly expressed only in the testis among adult normal tissues in both the RT-PCR and Northern blot analyses (Figs. 2*a* and 2*b*), in accordance with the results of the cDNA microarray analysis. The LC cell lines including 1–89, A549, PC14, RERF-LC-KO (adenocarcinoma: ADC), EBC-1, LC-1sq (squamous cell

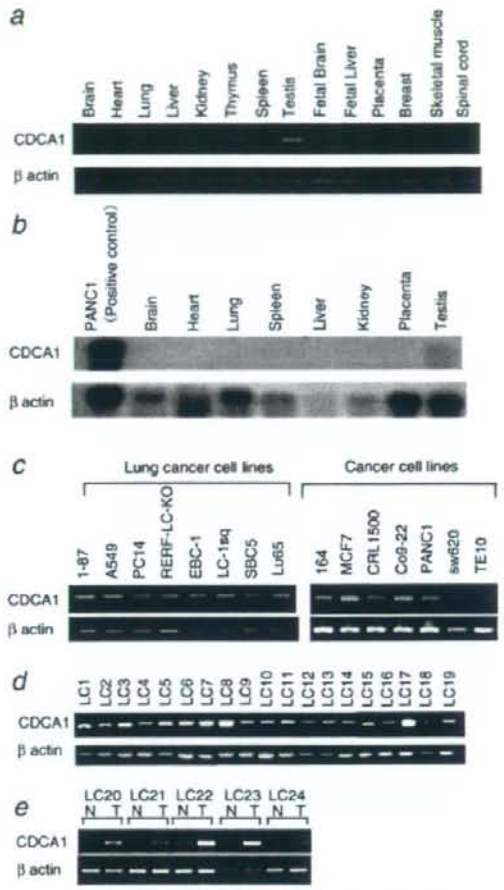


FIGURE 2 – Expression of *CDCA1* mRNA in human normal tissues detected by RT-PCR and Northern blot analysis. Expression of the *CDCA1* mRNA in normal tissues was also analyzed using RT-PCR (*a*) and Northern blot analysis (*b*). *CDCA1* mRNA was strongly expressed in the normal testis and very weakly expressed in thymus, but not in brain, heart, lung, spleen, liver, kidney and placenta. *CDCA1* gene expression detected by RT-PCR in cancer cell lines (*c*) and cancer tissues of NSCLC patients (*d*). (*c*) *CDCA1* mRNA was expressed in all 8 lung cancer cell lines tested and other cancer cell lines, including 1-87, A549, PC14, RERF-LC-KO (ADC), EBC-1, LC-1sq (SqCC), SBC5, LU65 (SCLC), 164 (melanoma), MCF7 (breast cancer), CRL1500 (breast cancer), Co9-22 (oral cancer), PANC1 (pancreas cancer), sw620 (colon cancer) and TE10 (esophageal cancer). (*d*) In 18 cases out of 19 NSCLC tissues, *CDCA1* gene was expressed in cancer tissue. (e) *CDCA1* mRNA was strongly expressed in lung cancer tissues compared with noncancerous lung tissues of NSCLC patients.

carcinoma: SqCC), SBC5 and Lu65 (small cell LC: SCLC) strongly expressed *CDCA1* mRNA (Fig. 2*c*). The other cancer cell lines including 164 (melanoma), MCF7 (breast cancer), CRL1500 (breast cancer), Co9-22 (oral cancer), PANC1 (pancreas cancer), sw620 (colon cancer) and TE10 (esophageal cancer) revealed high CDCA1 expression at mRNA level (Fig. 2*c*). In 18 of 19 NSCLC patients, *CDCA1* mRNA was highly expressed in cancer tissues (Fig. 2*d*). We also performed RT-PCR analysis of CDCA1 mRNA expression in both cancerous and normal lung tissues isolated from 5 LC patients in Figure 2*e*. These results indicated the expression of CDCA1 to be higher in LC tissues in comparison to that of normal lung tissues in all the 5 patients investigated.

Furthermore, we analyzed the expression level of *CDCA1* mRNA in other kind of cancer tissues by using microarray analysis[21,28–30] (Table I). *CDCA1* mRNA was overexpressed in all cases of SCLC ($n = 15$), cholangiocellular cancer ($n = 12$), bladder cancer ($n = 28$) and renal cell cancer ($n = 7$) in comparison to their adjacent normal counterparts.

### *Expression of CDCA1 protein in testis and cancer cell lines*

To investigate the expression of CDCA1 protein, we then examined many paraffin-embedded normal tissues and 12 cases of paraffin-embedded NSCLC tissues. CDCA1 did not stained in the normal brain, liver, lung, kidney and spleen (Figs. 3*a*–3*e*), except for normal testis (Fig. 3*f*). In this study, we investigated 8 samples of lung ADC and 4 samples of lung SqCC, and strong CDCA1 staining was observed in 9 cases, and no staining was observed in any of their adjacent normal lung tissues (Figs. 3*g*–3*i*).

### *Identification of HLA-A2-restricted mouse CTL epitopes of human CDCA1 by using HLA-A2.1 (HHD) Tgm*

As the candidates of HLA-A2-restricted and human CDCA1-derived CTL epitopes, we selected 40 kinds of peptides having high predicted binding scores to HLA-A2 (A*0201) calculated by using the BIMAS software program (Table II). We tried to identify HLA-A2-restricted CTL epitopes by using HLA-A2.1 HHD Tgm. CD4 negative spleen cells isolated from HLA-A2.1 (HHD) Tgm immunized i.p. twice with BM-DCs pulsed with the mixture of 4 peptides selected from these 40 CDCA1 peptides were again stimulated *in vitro* with BM-DCs pulsed with each peptide (Fig. 4*a*). As a result, we found that CD4-negative spleen cells stimulated *in vitro* with the $CDCA1_{65-73}$ and $CDCA1_{351-359}$ peptides produced a significant amount of IFN-γ in a peptide-specific manner in ELISPOT assays (Figs. 4*b* and 4*c*). These CD4 negative spleen cells ($2 \times 10^4$/well) showed $106 \pm 13.1$ spot counts/well in response to the BM-DCs pulsed with the $CDCA1_{65-73}$ peptide, whereas they showed $42.0 \pm 9.64$ spot counts/well in the presence of the BM-DCs without peptide loading ($p < 0.05$) (Fig. 4*b*). These assays were done 3 times with similar results. Secondly, the CD4-negative spleen cells stimulated with BM-DC pulsed with $CDCA1_{351-359}$ peptides showed $42.3 \pm 4.02$ spot counts/well, whereas they showed $24.6 \pm 7.19$ spot counts/well in the presence of BM-DCs without peptide loading ($p < 0.05$) (Fig. 4*c*).

As for other peptides, no significant peptide-specific response was observed (data not shown). These results suggest that the $CDCA1_{65-73}$ and $CDCA1_{351-359}$ peptides could be HLA-A2-restricted CTL epitope peptides in HLA-A2.1 (HHD) Tgm, and we also expected these peptides to be epitopes for human CTLs.

### *Induction of CDCA1-reactive CTLs from PBMCs of HLA-A2-positive healthy donors and a NSCLC patient*

We evaluated the CDCA1-specific immune responses of CTLs that were generated by the stimulation with the $CDCA1_{65-73}$ or $CDCA1_{351-359}$ peptide of PBMCs isolated from healthy donors and NSCLC patient. The PBMCs were isolated from these donors positive for HLA-A2 (A*0201), and the $CD8^+$ T cells sorted from the PBMCs were cocultured with autologous monocyte-derived DCs pulsed with each peptide. This stimulation of $CD8^+$ T cells was repeated 3 times in every week (Fig. 5*a*).

CTLs induced from donors were cocultured with target cells, and ELISPOT assay and $^{51}Cr$ release assay were done by using $CDCA1_{65-73}$ or $CDCA1_{351-359}$ peptide-pulsed T2 cell as a target cell. In the cancer patient 1, the IFNγ production of CTLs stimulated with $CDCA1_{65-73}$ peptide-pulsed T2 (HLA-A2+) was significantly greater than that of stimulated with T2 cells pulsed with the HLA-A2-binding irrelevant HIV-derived peptide (Fig. 5*b*).

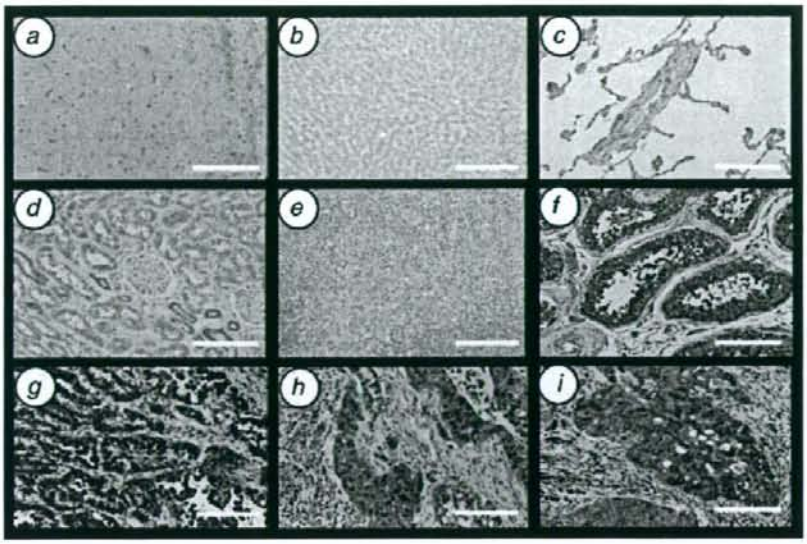


FIGURE 3 – Immunohistochemical staining of CDCA1 protein. Normal brain (*a*), normal liver (*b*), normal lung (*c*), normal kidney (*d*), normal spleen (*e*), normal testis (*f*) and NSCLC (*g*, *h*: ADC; *i*: SqCC) tissues were analyzed. The scale bars represent 100 μm.

The CTLs induced from the healthy donor 1 produced a considerable amount of IFNγ in response to the T2 cells pulsed with the $CDCA1_{351-359}$ peptide (more than 300 spot counts/well), but not to T2 cells without peptide loading (64.3 ± 12.9 spot count/well) by ELISPOT assay (Fig. 5*c*). The CTLs induced from PBMCs of the cancer patient 1 also showed cytotoxic activity to T2 cells pulsed with the $CDCA1_{65-73}$ peptide, but not to T2 cells pulsed with the HLA-A2-binding irrelevant HIV-derived peptide or peptide unpulsed T2 cells in $^{51}Cr$ release assay, and similar responses were observed in the healthy donor 1 (Fig. 5*d*). These results indicated that these CTLs had peptide-specific cytotoxic activity. In Figure 5*e*, CDCA1-peptide-induced CTLs were stimulated with T2 cells pulsed with various concentrations of CDCA1 peptide. We found the CTLs respond to CDCA1 peptide-pulsed T2 cells in a peptide dose-dependent manner, and that CTLs produced a significant amount of IFN-γ in response to the T2 cells pulsed with more than 0.2 μg/ml of the peptide in comparison to the responses observed in the presence of T2 cells unpulsed with the peptide or T2 cells pulsed with the HLA-A2-binding irrelevant HIV-derived peptide.

Furthermore, we used CDCA1-transfected COLO201 (COLO201/CDCA1, CDCA1+, HLA-A2+; Fig. 6*a*) as target cells and examined whether we could find a CDCA1-specific immune response of CTLs. As shown in Figure 6*b*, the IFNγ production of CTLs generated from the healthy donor 1 by using the $CDCA1_{65-73}$ peptide in response to COLO201/CDCA1 was significantly larger than that stimulated with mock transfected tumor cell line negative for CDCA1 gene expression, COLO201/Mock. The CTLs induced from PBMCs of healthy donor 2 by stimulation with the $CDCA1_{351-359}$ peptide also showed specific immune response against COLO201/CDCA1, but not against COLO201/Mock, using ELISPOT assay (Fig. 6*c*). In addition, the CTLs generated from the healthy donor 1 showed immune response to PANC1 (CDCA1+, HLA-A2+) but not to A549 (CDCA1+, HLA-2−) (Fig. 6*d*).

When we think about the application of CDCA1-derived peptides to cancer immunotherapy, the most important point is that these CDCA1 peptide-reactive CTLs can exhibit specific cytotoxicity to the tumors endogenously expressing CDCA1. We thus investigated whether these CTLs could kill human cancer cell lines expressing endogenous CDCA1. As shown in Figure 6*e*, we could generate CDCA1-reactive CTLs by the stimulation of PBMC with the $CDCA1_{63-75}$ peptide and these CTLs exhibited cytotoxic activity to PANC1 (CDCA1+, HLA-A2+), but not to A549 (CDCA1+, HLA-2-) or COLO201 (CDCA1-, HLA-A2+) in the cancer patient 1 (Fig. 6*e*). Similarly, we could generate CDCA1-reactive CTLs by the stimulation of PBMCs of healthy donor 1 with the $CDCA1_{351-359}$ peptide and these CTLs exhibited cytotoxic activity to PANC1, but not to A549 (Fig. 6*f*). These findings indicate that these peptides can be processed naturally in cancer cells, and the peptides in the context of HLA-A2 can be expressed on the cell surface of cancer cells to be recognized by the CTLs. We have produced a HLA-A2-CDCA1 351–359 peptide tetramer to detect HLA-A2-restricted and CDCA1-specific CTLs. By using this tetramer, we observed a strong correlation between the frequency of the CDCA1 351–359 peptide-reactive CTLs and the frequency of the tetramer-positive CTLs observed in a given $CD8^+$ T cell population (Fig. 6*g*). These observations strongly proved the presence of HLA-A2-restricted and CDCA1 peptide-specific CTLs in $CD8^+$ T cells used in this study.

## Discussion

The identification of TAA-derived peptides naturally processed in and presented on tumor cells is important for the establishment of peptide-based cancer immunotherapy. We identified a novel cancer-testis antigen, CDCA1, using a cDNA microarray analysis of NSCLC and normal tissues. CDCA1 was strongly expressed in NSCLC and in normal testis, but not in other normal tissues tested at both the mRNA and protein levels. Because the testis is an immune-privileged site, we think that CTLs reactive to CDCA1 peptides can attack only NSCLC cells without injuring normal tissues in the case of immunotherapy targeted on CDCA1. Thus, we chose CDCA1 as a candidate of TAA for the immunotherapy of patients with NSCLC.

We wanted to identify a TAA, which is indispensable for proliferation and survival of NSCLC, as a target for immunotherapy, because the use of such TAAs may minimize the well-described