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Cytotoxic T lymphocytes
Sniping cancer stem cells

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Abbreviations: CSC, cancer stem-like cell; CIC, cancer-initiating cell; CTL, cytotoxic T lymphocyte; TAA, tumor-associated antigen

Cancer stem cells (CSCs)/cancer-initiating cells (CICs) are characterized as a small population of cancer cells that have high tumor-initiating ability. CSCs/CICs are resistant to several cancer therapies, and eradication of CSCs/CICs is essential to cure cancer. How can we eradicate CSCs/CICs? Cytotoxic T lymphocytes (CTLs) might be a promising answer.

Cancer stem-like cells (CSCs)/cancer-initiating cells (CICs) are defined as a small population of cancer cells that have (1) high tumor-initiating ability, (2) self-renewal ability and (3) differentiation ability (**Fig. 1A**).[1] In recent studies, CSCs/CICs have shown to be resistant to cancer therapies by their senescence state, high expression of transporters to efflux anti-cancer drugs, high expression of apoptosis inhibitors, low expression of reactive oxygen species.[2] Thus, the action of CSCs/CICs are regarded as major mechanisms of cancer recurrence, distant metastasis and treatment resistance. However, effective cancer treatment targeting CSCs/CICs effectively have not been reported so far.

The prominent nature of the acquired immune system is its antigen specificity due to antigen-specific receptors including T cell receptors and B cell receptors, and isolation of human tumor-associated antigens (TAAs) has enabled us to target caner cells specifically in an antigen-specific manner.[3] Cancer immunotherapy trials using TAAs have recently been performed in several facilities and significant results have been obtained.[4] However, it is still not clear whether the immune system can recognize therapy-resistant CSCs/CICs or not. Some reports on immunity and CSCs/CICs have recently been published, and natural killer (NK) cells and γδT cells have been shown to recognize CSCs/CICs derived from human colon cancer and gliomas; however CTLs, which are a major component of the acquired immune system, have not been characterized yet.[5]

We analyzed the relation between CTLs and CSCs/CICs.[6] We isolated CSCs/CICs from human colon cancer cells using a side population (SP) technique. Since CTLs recognize antigenic peptides derived from TAAs, we evaluated the expression of TAAs in colon CSCs/CICs and non-CSCs/CICs. Colon CSCs/CICs expressed *CEP55*, one of the TAAs, at the same level as did non-CSCs/CICs. In a further study, we evaluated the expression of several TAAs in both CSCs/CICs and non-CSCs/CICs, and we found that the expression pattern can be classified into the following groups (**Fig. 1B**, unpublished data): (1) **CSC/CIC antigens**, which are expressed in CSCs/CICs but not in non-CSCs/CICs (e.g., MAGE-A3 and MAGE-A4); (2) **shared antigens**, which are expressed in both CSCs/CICs and non-CSCs/CICs (e.g., CEP55, SURVIVIN); and (3) **non-CSC/CIC antigens**, which are expressed in only non-CSCs/CICs but not in CSCs/CICs (e.g., AMACR, HIFPH3). Therefore, CEP55 is one of the (2) shared antigens.

Since we have established CTL clone #41 which is specific for CEP55-derived antigenic peptide,[7,8] we evaluated the reactivity of CTL clone #41 for colon CSCs/CICs and non-CSCs/CICs. Interestingly, CTL clone #41 recognized both colon CSCs/CICs and non-CSCs/CICs at the same level in vitro. Furthermore, CTL clone #41 inhibited the tumor-initiating ability of colon CSCs/CICs in vivo. These findings clearly indicate that treatment-resistant colon CSCs/CICs, as well as non-CSCs/CICs are sensitive to CTLs. Therefore, CTL-based immunotherapy is a promising approach to target CSCs/CICs.

In the next stage, another question has emerged. Which are the best TAAs for CSC/CIC-targeting cancer immunotherapy: (1) CSC/CIC antigens, (2) shared antigens or (3) non-CSC/CIC antigens? Non-CSC/CIC antigens do not seem to be suitable for targeting CSCs/CICs since they are not expressed in CSCs/CICs. Further analyses are under way to address thes questions, and we have found that targeting CSC/CIC antigens was more effective than targeting shared antigens in a CTL adoptive transfer model and a DNA vaccination model (unpublished data). Both CSC/CIC antigens and shared antigens are expressed in CSCs/CICs; however, the anti-tumor effects are

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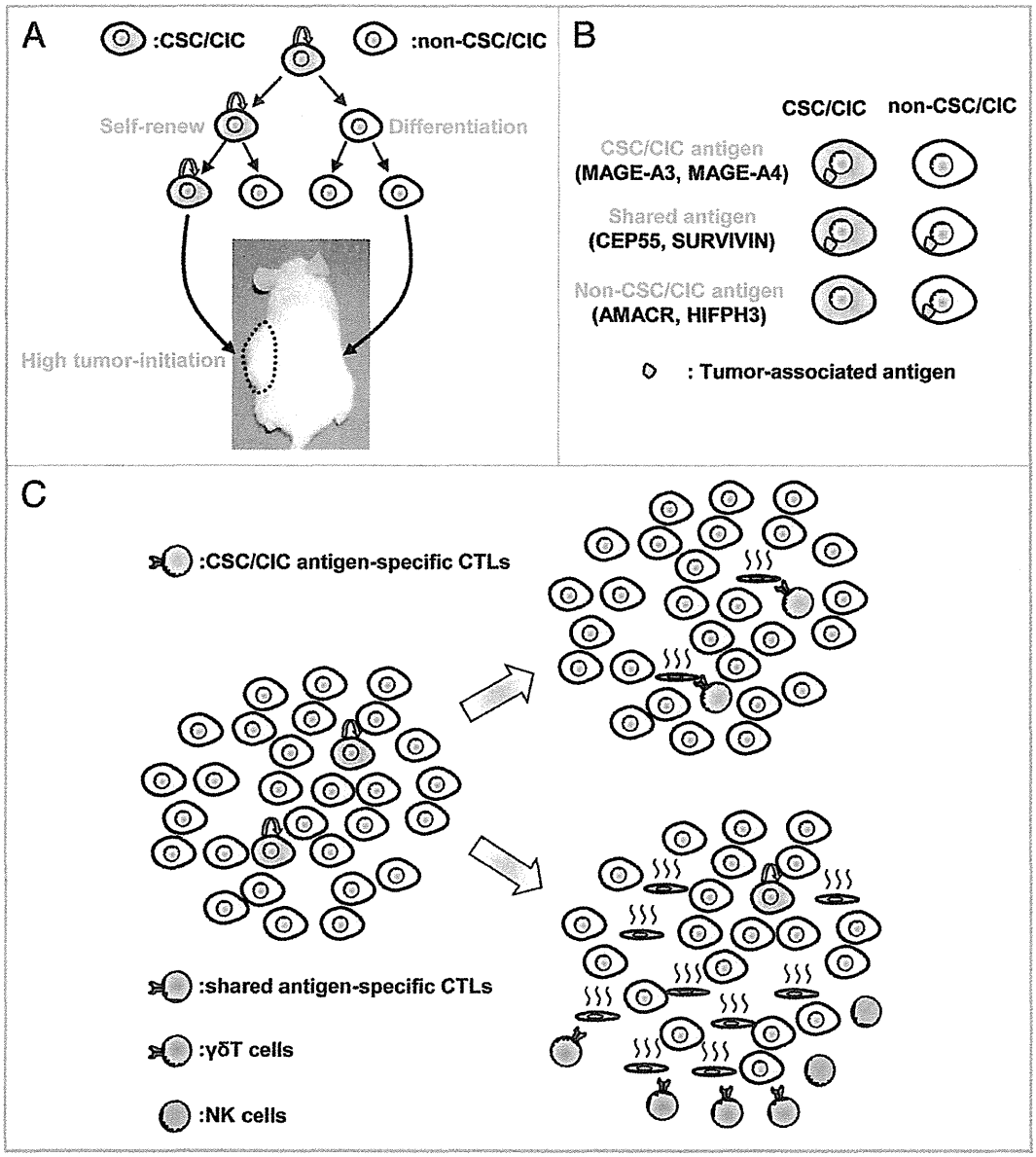

Figure 1. CSC/CIC targeting immunotherapy. (A) Characters of CSC/CIC. CSC/CIC has three distinct characteristics: (1) high tumor-initiating ability, (2) self-renewal ability and (3) differentiation ability. (B) Three groups of tumor-associated antigens. Tumor-associated antigens can be classified into 3 groups according to the expression in CSC/CIC and non-CSC/CIC: (1) CSC/CIC antigens, which are expressed in CSCs/CICs but not in non-CSCs/CICs (e.g., MAGE-A3 and MAGE-A4); (2) shared antigens, which are expressed in both CSCs/CICs and non-CSCs/CICs (e.g., CEP55, SURVIVIN); and (3) non-CSC/CIC antigens, which are expressed in only non-CSCs/CICs but not in CSCs/CICs (e.g., AMACR, HIFPH3). (C) CSC/CIC-targeting immunotherapy. CSC/CIC antigen specific CTLs recognize only higher tumorigenic CSCs/CICs, whereas shared antigen specific CTLs, NK cells and γδT cells recognize both CSCs/CICs and non-CSCs/CICs. CSCs/CICs might be eliminated most efficiently by CSC/CIC antigen-specific CTLs.

different. We are not sure about the exact mechanisms and we are now analyzing; however, these data indicate that targeting CSC/CIC specific antigens is more effective than targeting shared antigens.

The numbers of CTL clones are very restricted and limited in vivo, and the maximum numbers of one CTL clone might be about 10^7 to 10^8 cells in the whole body. On the other hand, cancer tissues contain 5×10^8 cancer cells per gram,[9] and advanced cancer tissues may therefore contain more than 10^{10} cancer cells. It is easy to imagine the difficulty in eliminating all cancer cells with such a limited number of CTLs (Estimated effector/target ratio is about 0.001 in the case of 10^7 CTL and 10^{10} cancer cells.). On the other hand, if we focus on just CSCs/CICs targeting CSC/CIC antigens, the situation will be improved (Estimated

effector/target ratio is about 0.1 in the case of 10^7 CTL, 10^{10} cancer cells and 1% frequency of CSCs/CICs.). Therefore, targeting CSC/CIC antigens might be a more effective approach to eradicate higher tumorigenic CSCs/CICs and may bring about greater anti-tumor effects (Fig. 1C).

As stated above, NK cells and γδT cells have been reported to recognize CSSs/CICs. However, these immune cells belong to the innate immune system and do not recognize target cells in an antigen-specific manner. Thus, activation of these cells in vivo may not be more effective than CSC/CIC antigen-specific CTLs (Fig. 1C). CTL adoptive transfer therapy has recently been described in detail,[10] and huge numbers of CTLs can be obtained by in vitro culture. Therefore, (2) shared antigens may also be suitable candidates for CTL adoptive transfer therapy using high numbers of CTLs.

In summary, CTLs can recognize CSCs/CICs as well as non-CSCs/CICs, and targeting CSC/CIC antigens with CTLs may be a reasonable approach for CSC/CIC targeting therapy.

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Cancer Research

HSP DNAJB8 Controls Tumor-Initiating Ability in Renal Cancer Stem–like Cells

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Abstract

Cancer stem–like cells (CSC) are a small population of cancer cells with superior tumor initiating, self-renewal, and differentiation properties. In this study, we show that the cancer-testis antigen and HSP40 family member DNAJB8 contributes to the CSC phenotype in renal cell carcinoma (RCC). DNAJB8 overexpression increased the percentage of side population (SP) cells representing CSCs in RCC cells, enhancing their tumor-initiating ability. Conversely, attenuation of DNAJB8 decreased SP cells and reduced tumor-initiating ability. The utility of DNAJB8 as an immunologic target was established in DNA vaccination experiments. Compared with immunization with the tumor-associated antigen survivin, which was expressed in both CSCs and non-CSCs in RCC, immunization with Dnajb8 expression plasmids yielded stronger antitumor effects. Together, our findings suggest that DNAJB8 plays a role in CSC maintenance and that it offers a candidate for CSC-targeting immunotherapy in RCC. *Cancer Res; 72(11); 2844–54. ©2012 AACR.*

Introduction

Renal cell carcinoma (RCC) is one of the most resistant forms of cancers to both radiotherapy and chemotherapy. In recent years, molecular targeted therapies have been developed and have shown significant objective responses (1–3), and they have been incorporated into current standard therapies of metastatic RCC; however, these molecular targeted therapies have not provided durable responses. RCC is regarded as an immunogenic malignancy and has well-documented responses to some cytokines such as interleukin-2 (IL-2) and IFN-α, and some patients have shown significant responses to treatments with these cytokines (4–6). However, the results have been limited by such nonspecific immunotherapy; therefore, cancer-specific immunotherapy may become a new modality for patients with metastatic RCC. Tumor-associated antigens (TAA) that can be recognized by CTLs have been investigated (7, 8), and some of these antigens induced objective tumor regression in patients with malignant melanoma (9, 10). In advanced cases, however, complete elimination of tumor cells by limited numbers of effector CTLs is difficult because a solid tumor contains an enormous number of tumor cells (about 5×10^8 cells per gram; ref. 11).

Cancer cells in solid carcinomas display heterogeneity in many aspects of their phenotypes, and only a small population of cells, called cancer stem–like cells/cancer-initiating cells (CSC/CIC), express stem cell phenotype and have high tumor-initiating ability (cancer stem cell hypothesis; refs. 12–14). CSCs/CICs are resistant to chemotherapy and radiotherapy by various mechanisms, and these characteristics of CSCs/CICs are thought to be related to posttherapeutic recurrence (15). Thus, therapy targeting the small population of CSCs/CICs might be a reasonable approach for treatment of resistant and advanced cancers. Some immunologic effector cells, including natural killer (NK) cells and γδT cells, have been reported to be able to efficiently recognize the CSC/CIC population (14). Furthermore, we have reported that CTLs can efficiently recognize human colon CSCs/CICs (16). We have categorized TAAs that can be recognized by CTLs into 3 groups: (i) CSC antigens, which are expressed in CSCs/CICs but not in non-CSCs/CICs (e.g., MAGE-A3 and MAGE-A4); (ii) shared antigens, which are expressed in both CSCs/CICs and non-CSCs/CICs (e.g., CEP55, SURVIVIN); and (iii) non-CSC antigens, which are expressed in non-CSCs/CICs but not in CSCs/CICs (e.g., AMACR, HIFPH3; ref. 17).

In this study, we found that a HSP40 family protein, DnaJ (Hsp40) homolog, subfamily B, member 8 (DNAJB8), is expressed preferentially in the CSC/CIC population cancer cells, including RCC and the testis among normal tissues, indicating

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that DNAJB8 is a novel cancer-testis (CT) antigen and also a novel CSC antigen. DNAJB8 has a role in the maintenance of RCC CSCs/CICs. We compared the potency of DNAJB8 as an immunologic target with that of Survivin, which is expressed in both CSC/CIC and non-CSC/CIC populations (shared antigen), and we found that DNAJB8 is more effective than Survivin. Our results suggest that targeting a CSC antigen is more effective than targeting a shared antigen and that DNAJB8 is a candidate for CSC/CIC-targeting immunotherapy.

Materials and Methods

Cell lines

RCC cell lines ACHN, Caki-1, SMKTR2, and SMKTR3 and the murine RCC cell line RenCa of BALB/c mouse origin were maintained in RPMI1640 (Sigma) supplemented with 10% FBS. HEK293T cells and the murine fibroblast cell line BALB/3T3 of BALB/c mouse origin were grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) supplemented with 10% FBS. PLAT-E and PLAT-A cells (kind gifts from Dr. T. Kitamura, Division of Stem Cell Signaling, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan) were grown in DMEM supplemented with 10% FBS, 10 μg/mL blasticidin, and 1 μg/mL puromycin.

Mice

All mouse procedures were carried out in accordance with institutional protocol guidelines at Sapporo Medical University School of Medicine. BALB/c female mice were purchased from Clea Japan and nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were purchased from Charles River Laboratory Japan at the age of 6 to 8 weeks.

Reverse transcriptase PCR analysis

Reverse transcriptase PCR (RT-PCR) analysis was carried out as described previously (18). Human Multiple Tissue cDNA Panels I and II (Clontech) were used as templates of normal adult tissue cDNAs. Except for *SOX2* and *Sox2*, PCR amplification was done in 20 μL of PCR mixture containing 0.25 μL of the cDNA mixture, 0.1 μL of Taq DNA polymerase (Qiagen), and 12 pmol of primers. The PCR mixture was initially incubated at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 15 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds. PCR amplification of *SOX2* and *Sox2* was carried out in 20 μL of PCR mixture containing 0.4 μL of the cDNA mixture, 0.2 μL of PrimeSTAR HS DNA polymerase (Takara), and 12 pmol of primers. The PCR mixture was initially incubated at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 15 seconds and annealing and extension at 68°C for 30 seconds. Primers used in experiments are summarized in Supplementary Table.

Development of anti-DNAJB8 monoclonal antibody and Western blot analysis

A monoclonal antibody (mAb) against DNAJB8 (clone #EMR-DNAJB8.214-8) was generated, as described previously (19), by immunizing mice 4 times weekly with recombinant His-tag DNAJB8 protein produced and purified by a Ni-NTA agarose column (Qiagen). Cell lysate with SDS sample buffer was separated by denaturing SDS-PAGE. Separated proteins were transferred onto nitrocellulose membranes and probed with mouse anti-FLAG M2 antibody (Sigma) or anti-DNAJB8 antibody. β-Actin was used as a loading control and was detected with a mouse mAb (Sigma). Anti-DNAJB8 antibody was used at 200 times dilution. Anti-FLAG antibody and anti-β-actin antibodies were used at 2,000 times dilution.

Side population analysis

Side population (SP) cells were isolated as described previously using Hoechst 33342 dye (Lonza) with some modifications (20). Briefly, cells were resuspended at 1×10^6/mL in prewarmed DMEM supplemented with 5% FBS. Hoechst 33342 dye was added at a final concentration of 2.5 μg/mL in the presence or absence of verapamil (50 μmol/L; Sigma-Aldrich), and the cells were incubated at 37°C for 90 minutes with intermittent shaking. Analyses and sorting were carried out with an FACSAria II cell sorter (Becton Dickinson).

Retroviral gene transduction and generation of stable transformants

Transduction of genes into cells was carried out by a retrovirus-mediated method as described previously (21). PLAT-A and PLAT-E cells, which are amphotropic and ecotropic packaging cells, respectively, were transiently transduced with a pMXs-puro (kind gift from Dr. T. Kitamura, Tokyo, Japan) retroviral vector expressing FLAG-tagged DNAJB8, Dnajb8, and Survivin, V5-tagged DNAJB8 ΔSSF-SST mutant, and a control plasmid using FuGENE HD transfection reagent (Roche) following the manufacturer's protocol. Retroviral supernatants were harvested 48 hours after transfection. The supernatant was used for infection of BALB/3T3 cells, ACHN cells, or RenCa cells in the presence of 8 μg/mL of polybrene (Sigma) overnight. For the generation of stable transformants, the infected cells were selected with 1 μg/mL puromycin. DNAJB8, Dnajb8, or Survivin expression was confirmed by Western blot analysis.

siRNA-mediated knockdown

DNAJB8 siRNAs (HSS136480, HSS136482, and HSS176060) were purchased from Invitrogen and transfected using Lipofectamine RNAi MAX reagent (Invitrogen) according to the protocol of the manufacturer. Cells were transfected with siRNA 72 hours before analysis. Nontargeting siRNA (Stealth RNAi Negative Control; Invitrogen) was used as a negative control. DNAJB8 knockdown was confirmed by Western blot analysis.

Site-directed mutagenesis

Plasmids including the target site for mutation as a template and primers that contain the desired mutation encoding the same amino acids as those of the wild-type sequence were used for site-directed mutagenesis. The sequences of primers are listed in Supplementary Table. Mutated plasmids were generated by 20 cycles of PCR amplification with KOD plus DNA polymerase (TOYOBO) and then digested by restriction enzyme *Dpn*I for 2 hours. The generated nicked dsDNA was transformed into BL21

strain of *Escherichia coli* and amplified. The mutations were verified by DNA sequencing.

Xenograft transplantation

Sorted SP and non-SP cells, and gene-transfected ACHN cells were injected into the subcutaneous space of the back of syngeneic BALB/c mice (for RenCa cells) or NOD/SCID mice (for ACHN cells). Tumor growth was monitored weekly, and tumor volume was calculated by $XY^2/2$ (X = long axis, Y = short axis).

DNA vaccine constructs

Murine *Dnajb8* and *Survivin* cDNAs were amplified by PCR from RenCa cells and subcloned into *Bam*HI and *Xho*I cloning sites of the modified pcDNA3.1(+) vector (Invitrogen), which contains the murine immunoglobulin kappa chain signal sequence at the N terminus [inserted into *Nhe*I and *Hind*III cloning sites of pcDNA3.1(+)] and a FLAG epitope at the C terminus [inserted into *Xba*I and *Pme*I cloning sites of pcDNA3.1(+)] of the expression antigen. A blank vector, modified pcDNA3.1(+), was used as control. The sense sequences and antisense sequences of the inserted oligo DNA are listed in Supplementary Table.

***In vitro* stimulation of $CD8^+$ T cells**

One week after the DNA vaccination, mice were sacrificed and a single-cell suspension was prepared from the spleen. Erythrocytes were lysed by osmotic shock (0.1 mol/L NH_4Cl), and $CD8^+$ T cells were isolated from splenocytes by using a MACS separation system (Miltenyi Biotech). A total of 5×10^6 $CD8^+$ or $CD8^-$ cells per well were, respectively, seeded in a 24-well plate with RPMI-1640 medium (Sigma) supplemented with 10% FBS. Phytohemagglutinin (PHA) blasts were generated from $CD8^-$ cells by using 50 IU/mL IL-2 and 2 μg/mL PHA for 3 days, and then *Dnajb8* or *Survivin* genes were transduced with a retrovirus vector. On day 7, 5×10^5 *Dnajb8*- or *Survivin*–expressing PHA blasts were irradiated (100 Gy), washed once, and added onto $CD8^+$ cells. On day 8, IL-2 was added to each well at a concentration of 50 IU/mL. On day 14, the antigen-specific IFN-γ release of $CD8^+$ T cells was assessed by an ELISPOT assay as described previously (19).

Tumor growth inhibition assay and rechallenge study

To measure protective immunity, BALB/c female mice were weekly immunized with DNA plasmid 4 times by injection into their footpad followed by injection of 1×10^5 RenCa tumor cells. Tumor growth was monitored weekly. RenCa/Dnajb8 cells (1×10^6) were injected subcutaneously into the back of BALB/c female mice that had rejected a previous injection of 1×10^5 RenCa cells.

***In vivo* depletion of T-cell subsets**

Mice were injected intraperitoneally with 500 μg of either anti-CD4 (clone: GK 1.5, rat IgG) or anti-CD8 (clone: 2.43, rat IgG) mAb 2 days before immunization and then immunized with cDNA plasmid vaccines once weekly for 4 weeks. Tumor cells were challenged 1 week after the fourth immunization. Depletion of $CD4^+$ and $CD8^+$ T cells was verified by flow cytometry.

Statistical analysis

Data are presented as means ± SD. Differences in variables were assessed using Student *t* test. Survival curves were constructed according to the Kaplan–Meier method. Statistical significance was determined by the log-rank test. $P < 0.05$ was considered significant. Statistical analysis was done with Stat Mate III (ATMS Co., Ltd.).

Results

DNAJB8 is a potential cancer-testis antigen

DNAJB8 has been reported to inhibit the aggregation of missfolding proteins and to protect cells from cell death (22); however, its role in cancer cells is not clear. We first investigated *DNAJB8* mRNA distributions in human normal adult tissues and cancer cells by RT-PCR. *DNAJB8* mRNA is barely expressed in normal mature tissues except for the testis (Fig. 1A), and it is expressed in all cells of human cancer lines (Fig. 1B).

We generated a DNAJB8-specific mAb (clone: #EMR-DNAJB8.214-8) to investigate DNAJB8 protein expression. The mAb #EMR-DNAJB8.214-8 showed DNAJB8-specific reaction using FLAG-tagged DNAJB8-overexpressed ACHN cells by Western blotting (Fig. 1C). Interestingly, the mAb #EMR-DNAJB8.214-8 was also reactive for FALG-tagged mouse Dnajb8-transduced RenCa cells (Fig. 1C). Therefore, the mAb #EMR-DNAJB8.214-8 is reactive for both human DNAJB8 and mice DNAJB8 proteins. DNAJB8 proteins were detectable in human sperm and mice testis tissues (Fig. 1D). Immunohistochemical staining also confirmed protein expression in human testis and mouse testis. Matured sperm and spermatid were positive for #EMR-DNAJB8.214-8, whereas spermatogonia and spermatocytes were negative for #EMR-DNAJB8.214-8 (Fig. 1E). DNAJB8 protein was also detectable in human RCC (clear cell carcinoma) tissues, whereas it was undetectable in counterpart human renal tubules (Fig. 1F). These expression profiles strongly indicated that DNAJB8 is a novel cancer-testis antigen (23).

Isolation and analysis of RCC stem-like population from human and mouse RCC cells

To address DNAJB8 functions in RCC CSCs/CICs, we carried out SP analysis using the human RCC cell lines ACHN, Caki1, SMKTR2, and SMKTR3 and the mouse RCC cell line RenCa. SP cells were detectable in ACHN, Caki1, and RenCa cells, and those SP cells were completely inhibited by verapamil (Fig. 2A and Supplementary Fig. S1), indicating that SP cells were specific for ABC transporter activity. The ratios of SP cells in ACHN, Caki1, and RenCa cells were 2.6%, 0.2%, and 18%, respectively. The ratios of SP cells in Caki1, SMKTR2, and SMKTR3 cells were too low for further analysis, and we therefore used ACHN and RenCa in the following experiments. It has been shown that SP cells were not enriched with CSCs/CICs in some types of cancer cells (24), and thus it is essential to validate that SP cells are enriched with CSCs/CICs. ACHN SP cells initiated tumor formation with 10^3 cells, whereas ACHN

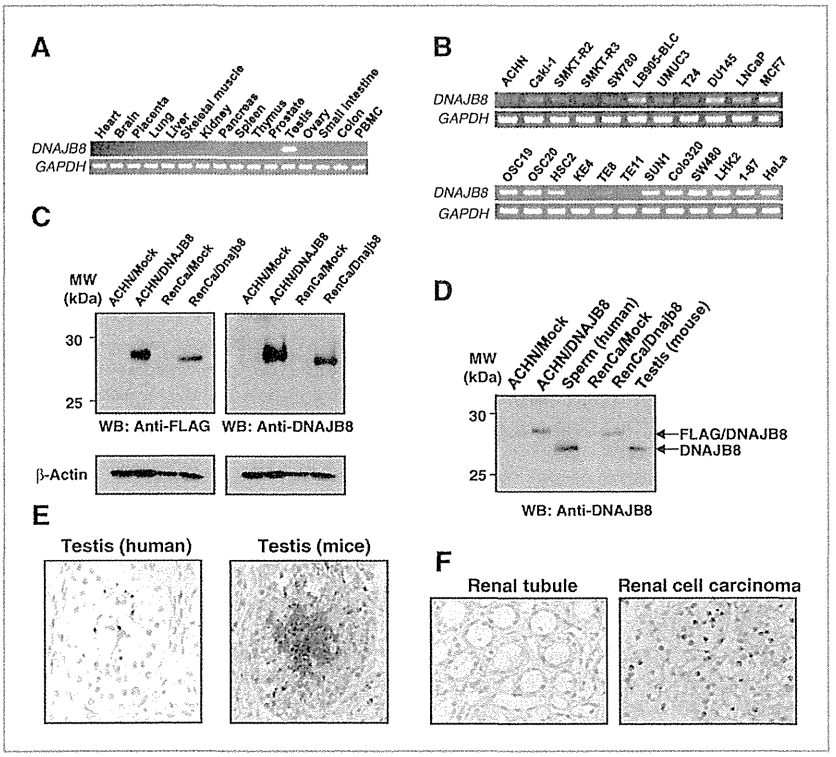

Figure 1. Expression of DNAJB8. A, RT-PCR analysis with normal organs. Expression of *DNAJB8* mRNA was examined by RT-PCR. *GAPDH* was used as a positive control. B, RT-PCR analysis with cancer cells. Expression of *DNAJB8* mRNA was examined by RT-PCR. *GAPDH* was used as a positive control. C, Western blot analysis. DNAJB8-transduced ACHN cells and Dnajb8-transduced RenCa cells were analyzed with anti-FLAG antibody and anti-DNAJB8 mAb (clone: #EMR-DNAJB8.214-8). ACHN/DNAJB8 and RenCa/Dnajb8 represent ACHN and RenCa cells transduced with FLAG-tagged DNAJB8 and Dnajb8, respectively. D, Western blot analysis. Endogenous DNAJB8 protein expression was addressed in human sperm and mouse testis using anti-DNAJB8 mAb (#EMR-DNAJB8.214-8). ACHN/DNAJB8 and RenCa/Dnajb8 were used as positive controls. E, immunohistochemical staining of human testis and mouse testis. Human testis and mouse testis tissues were stained with mAb #EMR-DNAJB8.214-8. Magnification, ×200. F, immunohistochemical staining of human normal kidney and RCC. Human normal kidney and RCC (clear cell carcinoma) tissues were stained with mAb #EMR-DNAJB8.214-8. Magnification, ×200. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; WB, Western blot.

main population (MP) cells needed 10^4 cells to initiate tumor formation (Fig. 2B and Table 1). RenCa SP cells initiated tumor formation with 10^2 cells, whereas RenCa MP cells needed 10^3 cells to initiate tumor formation (Fig. 2B and Table 1). These data indicated that SP cells derived from ACHN and RenCa cells were enriched with CSCs/CICs.

We carried out RT-PCR analysis to evaluate genetic characteristics of SP and MP cells derived from ACHN and RenCa cells (Fig. 2C). SP cells derived from ACHN and RenCa cells showed higher expression levels of *SOX2/Sox2* and *POU5F1/Pou5f1* than did MP cells, indicating that they present stem cell–like phenotypes (Fig. 2C). Recently, induction of epithelial to mesenchymal transition (EMT) in transformed mammary epithelial cells has been shown to result in the creation of populations of cells that are highly enriched in CSCs/CICs (25); therefore, we analyzed EMT-related gene expression in SP and MP cells. Both ACHN and RenCa SP cells showed repressed *CDH1/Cdh1* expression. ACHN SP cells preferentially expressed *SNAI2* and RenCa SP cells preferentially expressed *Snai1* and *Twist1*, representative EMT-inducing transcription factors. These findings suggested that SP cells of RCC are enriched with CSCs/CICs and have a partial EMT feature.

DNAJB8/Dnajb8 mRNA was predominantly expressed in SP cells derived from both ACHN and RenCa cells (Fig. 2D). DNAJB8 mRNA was also predominantly expressed in SP cells derived from colon carcinoma cells (KM12LM and SW480 cells), lung carcinoma cells (LHK2 cells), and breast carcinoma cells (MCF7 cells; Supplementary Fig. S2). Western blotting and immunostaining using SP cells and MP cells derived from ACHN and RenCa cells also revealed preferential expression of DNAJB8 protein in SP cells (Fig. 2E and F and Supplementary Fig. S3A and B). DNAJB8 protein was also preferentially expressed in SP cells derived from LHK2 and SW480 cells (Supplementary Fig. S4). These findings indicated that DNAJB8/Dnajb8 is preferentially expressed in the CSC/CIC population and that DNAJB8 is therefore a novel CSC antigen.

DNAJB8 function in maintenance of CSCs/CICs

To address the functions of DNAJB8 in RCC CSCs/CICs, we generated stable transformants expressing DNAJB8 (Fig. 1C).

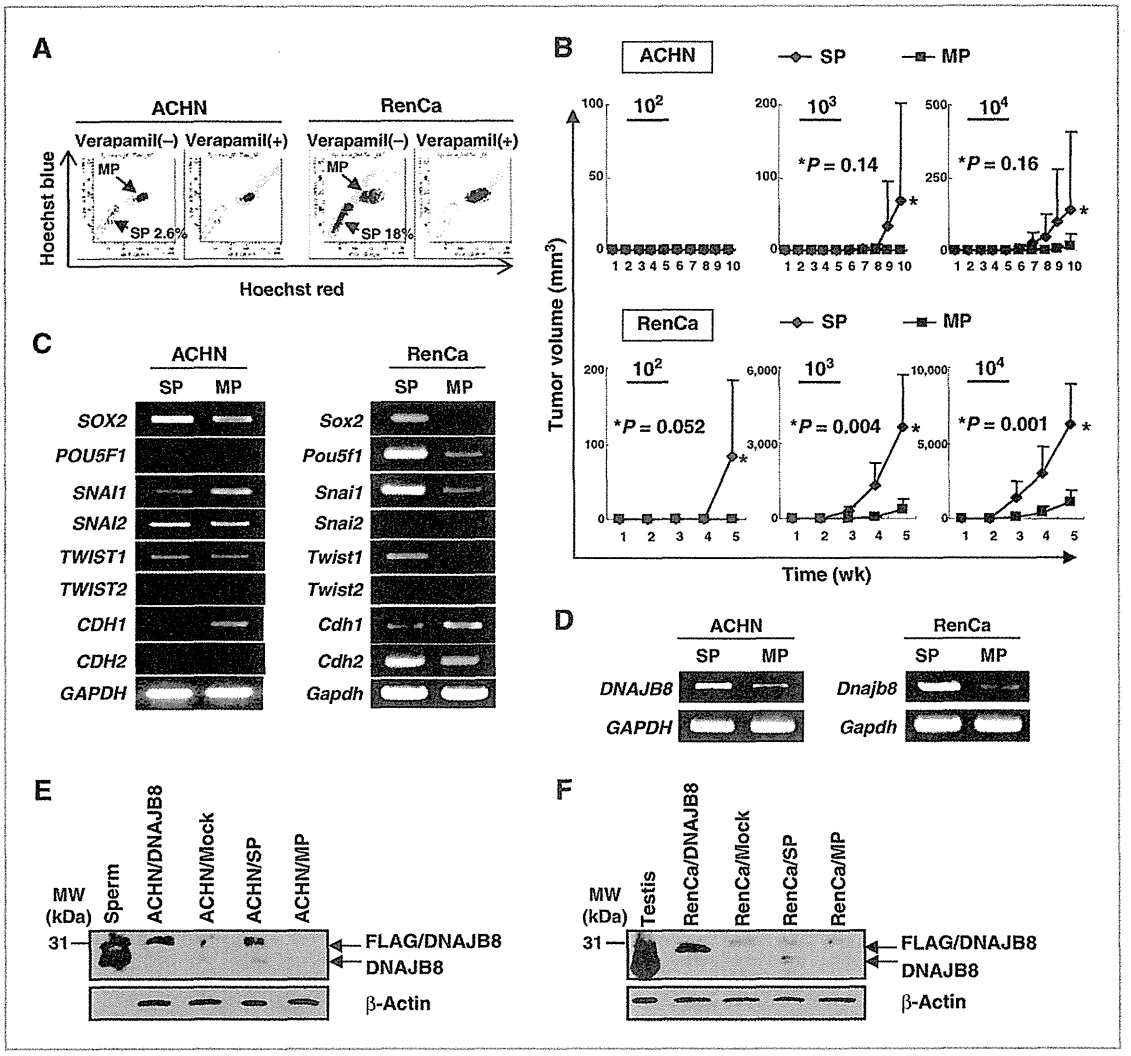

Figure 2. Isolation of CSCs/CICs from human and mouse RCC cells. A, SP analysis. ACHN human RCC cells and RenCa mouse RCC cells were stained with Hoechst 33342 dye with or without verapamil and analyzed with an FACSAria II cells sorter. The percentages represent the ratios of SP cells. B, tumor-initiating ability. SP cells and MP cells (10^2–10^4 cells) derived from ACHN cells were injected into NOD/SCID mice and tumor growth was observed. Data are means + SD. C, RT-PCR analysis. SP and MP cells derived from ACHN and RenCa cells were analyzed by RT-PCR. *GAPDH* was used as a positive control. D, RT-PCR analysis. SP and MP cells were derived from ACHN, and RenCa cells were examined for expression of *DNAJB8* and *Dnajb8*, respectively. *GAPDH* was used as a positive control. E, Western blot analysis. Expression of DNAJB8 proteins in human sperm, in ACHN/DNAJB8 and ACHN/Mock cells, and in SP and MP cells derived from ACHN cells was detected by Western blotting using anti-DNAJB8 mAb #EMR-DNAJB8.214-8. F, Western blot analysis. Expression of DNAJB8 proteins in the mouse testis, in RenCa/Dnajb8 and RenCa/Mock cells, and in SP and MP cells derived from RenCa cells was detected by Western blotting using anti-DNAJB8 mAb #EMR-DNAJB8.214-8.

The percentages of SP cells in DNAJB8-transduced ACHN and RenCa cells (ACHN/DNAJB8 and RenCa/DNAJB8) were increased compared with those in control cells (ACHN/Mock and RenCa/Mock cells; Fig. 3A). Similar phenomena were observed in several other cell lines including RCC (Caki1), prostate carcinomas (LNCaP and DU145), and bladder carcinoma cells (T-24; Supplementary Fig. S5). Furthermore, DNAJB8-transduced ACHN cells showed higher tumor-initiating ability than that of Mock-transduced ACHN cells (Fig. 3B and Table 1). DNAJB8 contains an N-terminal J-domain, which is responsible for association with HSP70 families, and a C-terminal serine-rich region, which is capable of interacting with histone deacetylases (HDAC; HDAC4, HDAC6, and SIRT2) and has a role in suppression of protein aggregation. A DNAJB8 mutant that lacks the C-terminal serine-rich region (ΔSSF-SST) showed a smaller SP augmentation effect (Supplementary Fig.

Table 1. Tumor initiating ability of RCCs

Cells		Tumor initiation (injected cell number)[a]			
		10	10^2	10^3	10^4
ACHN	SP cells	n.d.	0/5	3/6	4/6
	MP cells	n.d.	0/5	0/6	1/6
RenCa	SP cells	0/5	3/5	5/5	5/5
	MP cells	0/5	0/5	5/5	5/5
ACHN	DNAJB8 transduced	n.d.	0/5	3/5	9/9
	Mock transduced	n.d.	0/5	0/5	7/9
ACHN/DNAJB8	SP cells	n.d.	0/4	2/2	n.d.
	MP cells	n.d.	0/4	0/2	n.d.

Abbreviation: n.d., not determined.
[a]The tumor-initiating abilities were evaluated at day 70 postcell injection for ACHN cells and at day 35 for RenCa cells.

S6), suggesting that the C-terminal serine-rich region has a role induction of CSCs/CICs.

DNAJB8 gene knockdown experiments were carried out using gene-specific siRNAs. Three different *DNAJB8*-specific siRNAs (siRNA A, B, and C) could suppress the expression of DNAJB8 (Fig. 3C); therefore, we used siRNA A in the following experiments. Transfection of siRNA A almost completely eliminated the SP cell population in both wild-type ACHN and ACHN/DNAJB8 cells (Fig. 3D). Furthermore, siRNA A transfection significantly inhibited the tumorigenicity of ACHN cells in NOD/SCID mice (Fig. 3E). Gene knockdown of DNAJB8 by siRNA transfection almost completely decreased the numbers of SP cells. Thus, we generated a DNAJB8 mutant (DNAJB8 mt) by site-directed mutagenesis to confirm the siRNA results (Fig. 3F). DNAJB8 mt contains 6 DNA point mutations within the iRNA target sequence and codes the same amino acid sequence as that of the wild type. DNAJB8 mt was insensitive to siRNA A transfection, whereas DNAJB8 wt was inhibited by siRNA transfection (Fig. 3G). The siRNA transfection into ACHN/DNAJB8 cells decreased the numbers of SP cells (Fig. 4D); however, the DNAJB8 mt stable transformant showed no reduction of SP cells by siRNA transfection (Fig. 3H). These results indicated that the effects of siRNA transfection were specific for targeting *DNAJB8* mRNA, and DNAJB8 therefore has a role in the maintenance of RCC CSCs/CICs.

DNAJB8-targeting immunotherapy

Because DNAJB8 is one of the cancer-testis antigens and one of the CSC antigens and has a role in the maintenance of RCC CSCs/CICs, we hypothesized that DNAJB8 is a suitable immunologic target of RCC CSCs/CICs-targeting immunotherapy. However, it is not clear which will bring about a better antitumor effect: targeting CSC antigens or targeting shared antigens. On the basis of this point of view, we compared the immunogenicity of DNAJB8 with that of the well-characterized TAA Survivin (26–29), as Survivin is expressed in both CSCs/CICs and non-CSCs/CICs at the same levels (Fig. 5A) and it is a shared antigen.

We subcloned FLAG-tagged Dnajb8 and Survivin into the pcDNA3.1 expression vector and confirmed the expression by Western blot analysis (Fig. 4B). We carried out an ELISPOT assay to verify the immunization of DNA vaccine. Anti-Dnajb8- or anti-Survivin–specific IFN-γ spots could be observed with $CD8^+$ T cells derived from Dnajb8- or Survivin-immunized mice, respectively (Fig. 4C). To address the antitumor effects, we injected RenCa cells into Dnajb8- and Survivin-immunized mice. Antitumor effects were observed in both Dnajb8- and Survivin-immunized mouse compared with control vector- or PBS-immunized mouse (Fig. 4D; P values of 0.002 and 0.026, respectively). Dnajb8-immunized mouse showed significantly greater antitumor effect than that in Survivin-immunized mouse ($P = 0.03$; Fig. 4D).

To evaluate the subtype of T cells for antitumor activities, we depleted murine $CD4^+$ or $CD8^+$ T cells in immunized mice by 4 intraperitoneal injections of anti-CD4 or CD8 antibody (Fig. 5A). $CD4^+$ T-cell depletion significantly inhibited the antitumor effect of Dnajb8 immunization ($P = 0.046$), whereas $CD8^+$ T-cell depletion did not (Fig. 5B). However, the survival period of RenCa cell-injected mice was significantly shorter for both $CD4^+$ T-cell–depleted mice and $CD8^+$ T-cell–depleted mice than the survival period of Dnajb8-immunized mice (Fig. 5C). These observations indicated that both $CD8^+$ T cells and $CD4^+$ T cells might have a role in the antitumor effect. Three of 5 Dnajb8-immunized mice showed complete inhibition of tumor formation initiation, and the mice were therefore rechallenged with 10 times larger numbers of higher tumorigenic RenCa/DNAJB8 cells. Initiation of tumor formation was completely inhibited in those mice, suggesting a strong tumor-inhibitory effect of Dnajb8 immunization (Fig. 5D).

Discussion

In this study, we investigated the distributions of DNAJB8 mRNA and protein and found that DNAJB8 is expressed only in the testis among normal tissues. DNAJB8 protein expression was detected in postmeiotic sperm and spermatid. Previous studies have shown that DNAJB6, DNAJB1, and DNAJB13 are also expressed in the testis (30–32), and Dnaja1 was reported to

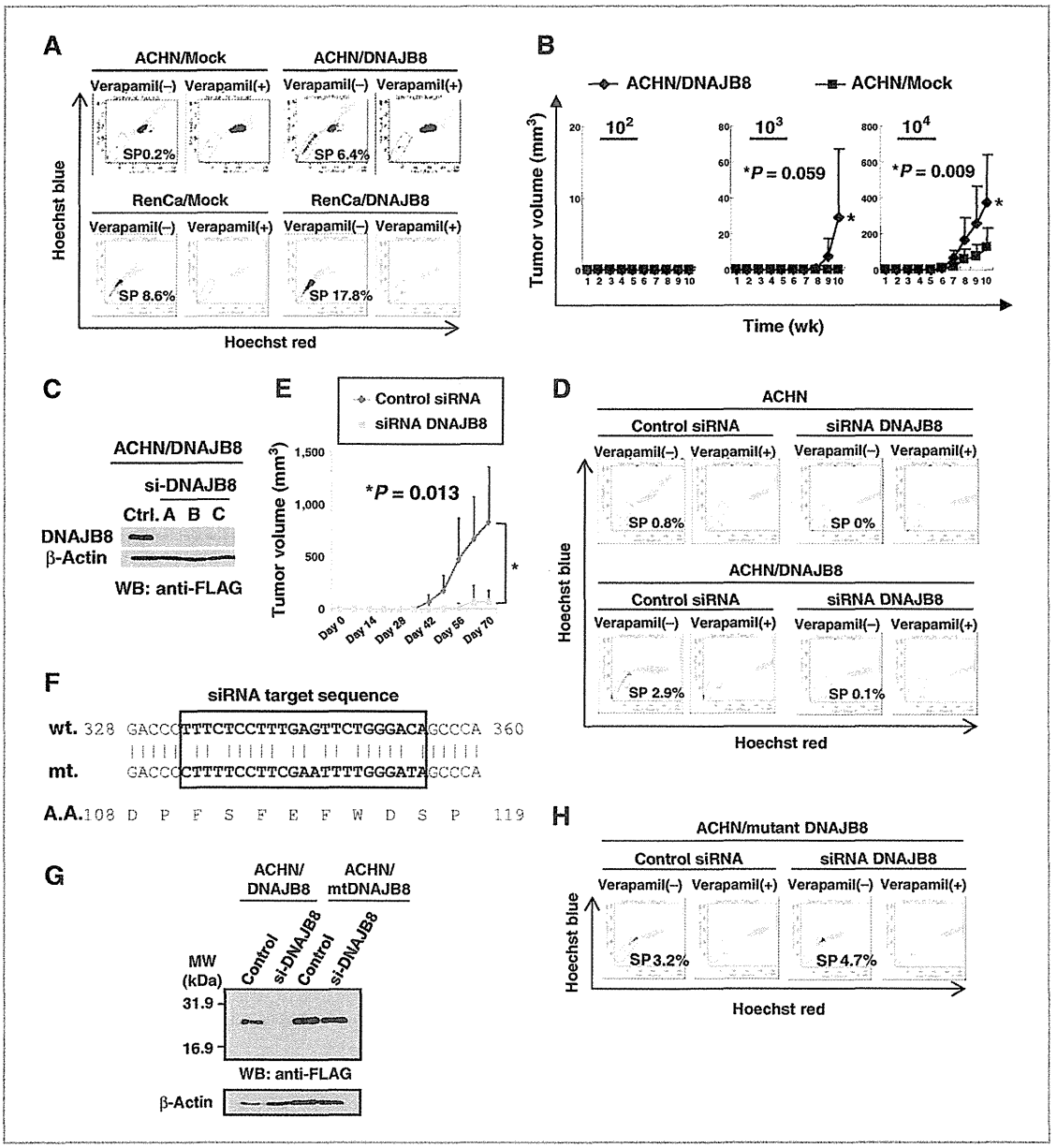

Figure 3. DNAJB8 function in maintenance of RCC CSCs/CICs. A, SP analysis of DNAJB8-transduced RCC cells. DNAJB8-transduced ACHN cells (ACHN/DNAJB8) and Dnajb8-transduced RenCa cells (RenCa/DNAJB8) were analyzed for SP cells. Mock-transduced ACHN and RenCa cells were used as controls (ACHN/Mock and RenCa Mock). The percentages represent the ratios of SP cells. B, tumor-initiating ability of ACHN/Mock cells and ACHN/DNAJB8 cells. ACHN/Mock cells and ACHN/DNAJB8 cells (10^2–10^4 cells) were injected into NOD/SCID mice. Data are means + SD. C, Western blot analysis of DNAJB8-specific siRNA-transduced ACHN/DNAJB8 cells. ANCH/DNAJB8 cells were transduced with 3 different siRNAs specific for DNAJB8 (siRNA A, B, and C). Two days after transduction, ACHN/DNAJB8 cells were analyzed by Western blotting using anti-FLAG mAb. D, SP analysis of DNAJB8-knockdown RCC cells. ACHN and ACHN/DNAJB8 cells were transduced with DNAJB8-specific siRNA A. Two days after siRNA transduction, cells were analyzed for SP cells. The percentages represent the ratios of SP cells. E, tumor-initiating ability of DNAJB8-knockdown RCC cells. DNAJB8-specific siRNA or control siRNA was transduced into ACHN cells. Two days after transduction, cells were injected into NOD/SCID mice. Data are means + SD. F, DNAJB8-mutant sequence. DNAJB8-mutant (mt) gene was constructed by site-directed PCR. The square indicates siRNA target sequence. Numbers indicate DNA sequence and amino acid sequence, respectively. G, Western blotting of mt DNAJB8. DANJB8 siRNA was transduced into ACHN/DNAJB8 and ACHN/DNAJB8 mt cells. Expression of DNAJB8 was evaluated by Western blotting using anti-FLAG mAb. H, canceling test. DNAJB8-siRNA–transduced ACHN/DNAJB8 mt cells were analyzed for SP cells. The percentages represent the ratios of SP cell.

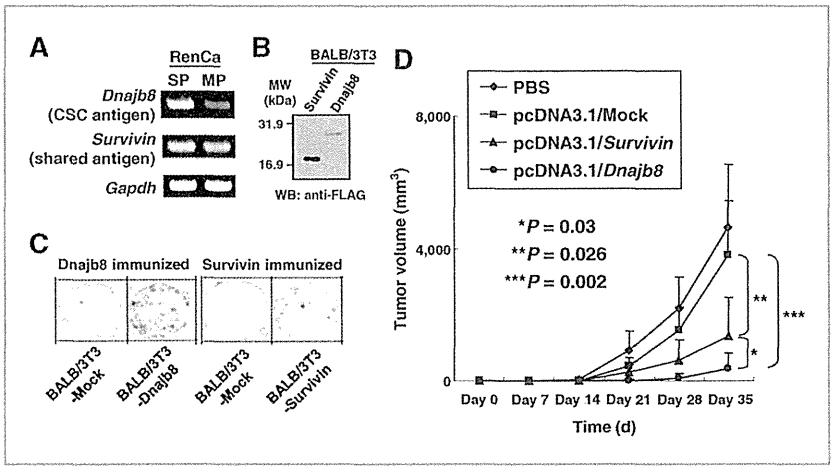

Figure 5. Immunogenicity of DNAJB8. A, RT-PCR analysis. SP and MP cells derived from RenCa cells were analyzed for expression of *Dnajb8* (CSC antigen) and *Survivin* (shared antigen) by RT-PCR. *Gapdh* was used as a positive control. B, Western blot analysis using Dnajb8- and Survivin-transduced BALB/3T3 cells. Dnajb8- or Survivin-transduced BALB/3T3 cells were analyzed by Western blotting using anti-FLAG mAb. C, immunoreactions to Dnajb8 and Survivin. Spleen cells were isolated from mice immunized with Dnajb8 plasmid or Survivin plasmid. Spleen cells were stimulated *in vitro* and then immuoreactivity to Dnajb8 or Survivin was evaluated by an ELISPOT assay using Dnajb8-transduced BALB/3T3 (BALB/3T3/Dnajb8) cells and Survivin-transduced BALB/3T3 (BALB/3T3/Survivin) cells. D, antitumor effect of DNA vaccine. Dnajb8, Survivin, Mock plasmid, and PBS-immunized mice were challenged with RenCa cells by injecting 1×10^5 RenCa cells subcutaneously. Data are means + SD.

have a role in spermatogenesis by regulating androgen receptor signals (33). Therefore, DNAJB8 is another testis-expressing HSP40 family protein and may have a role in spermatogenesis, especially in the postmeiotic stage.

We isolated RCC CSCs/CICs as SP cells. SP cells have been reported to be enriched with CSCs/CICs in several kinds of malignancies (34–36). SP cells derived from RCC and normal renal tubule epithelia have also been reported (37, 38); however, the tumor-initiating ability of those RCC SP cells has not been characterized yet. Tumor-initiating ability is one of major characteristics of CSCs/CICs, and this characteristic makes CSCs/CICs a reasonable target of cancer therapy. In this study, SP cells derived from ACHN human RCC cells and RenCa mouse RCC cells showed higher tumor-initiating ability than that of MP cells, and these SP cells are suitable for analysis of RCC CSCs/CICs.

HSPs, especially HSP90, are overexpressed in a wide range of human cancers, allowing mutant proteins to be retained in cancer cells and to confer resistance to cytotoxic therapies (39). On the other hand, most of the HSP40 family proteins have been reported to be inversely correlated with high-grade malignancy, and HSP40 family proteins have been reported to function as tumor suppressors (40). In this study, we found that DNAJB8 was expressed in several kinds of cancer cells, including RCCs, and that it was preferentially expressed in the CSC/CIC population. DNAJB8 has a role in maintenance of RCC CSCs/CICs as shown by siRNA and mutant DNAJB8 canceling experiments, and DNAJB8 is thus an HSP40 family member that has oncogenic potential. CSCs/CICs are known to be resistant to several stresses, including chemotherapy and radiotherapy (15), and overexpression of DNAJB8 might be related to the antistress ability and resistance to treatments. We observed that overexpression of DNAJB8 increased the percentage of SP cells; however, we still detected MP cells in ACHN/DNAJB8 cells. With regard to tumor-initiating ability, ACHN/DNAJB8 SP cells showed higher tumor-initiating ability than that of ACHN/DNAJB8 MP cells (Table 1). Because both ACHN/DNAJB8 SP cells and ACHN/DNAJB8 MP cells expressed DNAJB8 at almost same levels (data not shown), these observations suggest the existence of other cofactor for induction of CSCs/CICs. In a previous study, DNAJB8 was shown to have role in inhibition of cytotoxic protein aggregation (22), and it is important for association with HDACs through the C-terminal serine-rich region. We also found that the serine-rich region of DNAJB8 has a role in maintenance of CSCs/CICs. These observations suggest that association with HDACs (HDAC4, HDAC6, and SIRT2) might be important for DNAJB8 functions in CSCs/CICs, and that HDACs are possible candidates of cofactors of DNAJB8.

In this study, we showed for the first time that DNAJB8 can be a target of immunotherapy. Mutated and wild-type HSP70 proteins have been reported to be targets of CTLs (41–43), whereas other HSP family proteins have never been reported to be targets of immunocytes. HSP family proteins are often expressed in several kinds of normal organs, and immunocytes might be immunologically tolerated to those proteins. On the other hand, DNAJB8 is expressed only in the testis, and it is thus a novel CT antigen that is regarded as an ideal immunologic target.

HSP family proteins work as molecular chaperones and bind to their client proteins. Antigenic peptide-bound HSP 70 and 90 family proteins are known to work as immune modulators that enhance the cross-priming pathway and enhance antitumor immunity (44–47). In this study, we confirmed anti-DNAJB8

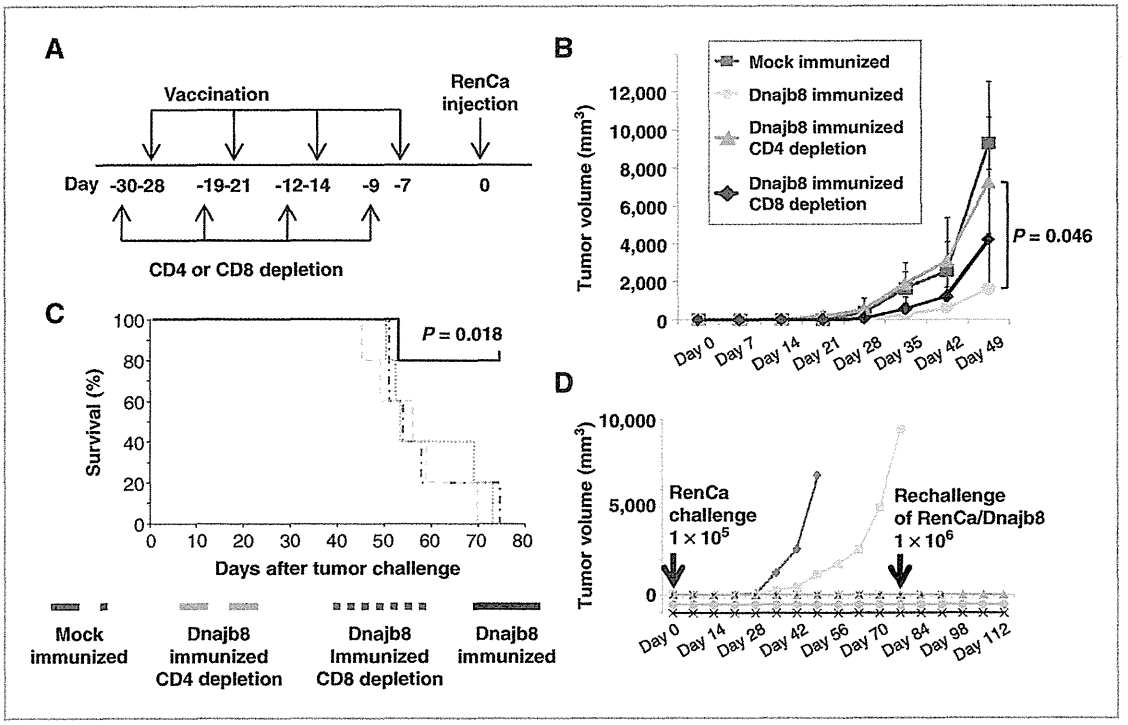

Figure 5. Immunogenicity of DNAJB8. A, time course of $CD4^+$ or $CD8^+$ T cell depletion. $CD4^+$ or $CD8^+$ cells were depleted 2 days before DNA vaccination by injecting anti-CD4 or anti-CD8 mAb into the peritoneal cavity. B, antitumor effects with CD4 or CD8 T-cell depletion. Tumor growth curves of the Mock-immunized group, Dnajb8-immunized group, Dnajb8-immunized CD4-depleted group, and Dnajb8-immunized CD8-depleted group are shown. Data are means + SD. C, survival curve with CD4 or CD8 T-cell depletion. Mouse survival curves of Mock-immunized group, Dnajb8-immunized group, Dnajb8-immunized CD4-depleted group, and Dnajb8-immunized CD8-depleted group are shown. D, rechallenge test with RenCa/DNAJB8 cells. Tumor growth curves of individual mice in the Dnajb8-immunized group are shown. Each curve represents tumor growth in each mouse. Three of 5 mice immunized with Dnajb8 did not show initiation of tumor formation with injection of 1×10^5 RenCa cells at day 70. Thereafter, the 3 mice were rechallenged with 10 times larger numbers of RenCa/DNAJB8 cells (1×10^6) at day 77 and were observed.

immunity with DNAJB8-immunized mouse spleen cells. However, there remains the possibility that DNAJB8 also binds to another antigen protein/peptide and induces antigen-specific immunity, and further investigation should be carried out.

A DNA vaccination experiment revealed that targeting CSC antigen (Dnajb8) was more effective than targeting a shared antigen (Survivin). In a previous study, we found that colon CSCs/CICs can be recognized by an established CTL clone at the same level as non-CSCs/CICs both *in vitro* and *in vivo* (16). We therefore suggest that CTLs are promising tools to target CSCs/CICs. Expression of TAAs is essential for CTL recognition, and we categorized TAAs into 3 groups (CSC antigens, shared antigens, and non-CSC antigens; ref. 17). In a recent study, we found that TAA expression in the CSC/CIC population is necessary to achieve an antitumor effect and that the antitumor effect of a shared antigen is greater than that of non-CSC antigens (48). Then, which is the best target, CSC antigens or shared antigens? In this study, we showed for the first time that a CSC antigen is more effective than a shared antigen. Our results indicate that a CSC antigen has the greatest antitumor effect and a non-CSC antigen has the smallest antitumor effect and that evaluation of the distribution in CSCs/CICs and non-CSCs/CICs is important to predict the efficiency of antitumor effects of novel TAAs.

We showed that targeting CSCs/CICs is an effective approach for cancer immunotherapy. On the other hand, glioma-associated CSCs/CICs have been reported to be related to immunosuppression through B7-H1 and soluble Galectin-3 and STAT3 signaling (49, 50). These observations suggest that CSCs/CICs might suppress the CTL induction phase but might not affect the CTL effector phase. In this study, we observed a significant antitumor effect with immunization using *Dnajb8*-coding plasmid. This is a prophylactic model and CTL induction might not be affected by CSCs/CICs included in RenCa cells and thus bring about preferable results. Therefore, immunotherapy targeting CSCs/CICs might be useful for suppression of posttreatment tumor recurrence and/or adoptive transfer of established CTLs.

In summary, we identified an HSP40 family protein, DNAJB8, as one of the CT antigens and also as a CSC antigen. DNAJB8 has a role in maintenance of RCC CSCs/CICs. Targeting a CSC antigen is more effective than targeting a shared antigen, and DNAJB8 is a possible candidate for CSC/CIC-targeting immunotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Development of methodology: Y. Hirohashi, T. Torigoe, A. Takahashi, K. Kamiguchi, A. Sokolovskaya, R. Fujii

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Nishizawa, Y. Hirohashi, T. Torigoe, A. Takahashi, Y. Tamura, H. Asanuma

Writing, review, and/or revision of the manuscript: S. Nishizawa, Y. Hirohashi, T. Torigoe, T. Kanaseki, R. Fujii

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Hirohashi, T. Torigoe, T. Mori, H.H. Kampinga, T. Kondo, I. Hara

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Rapid Communication

Establishment of a monoclonal anti-pan HLA class I antibody suitable for immunostaining of formalin-fixed tissue: Unusually high frequency of down-regulation in breast cancer tissues

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A novel monoclonal anti-pan human leukocyte antigen (HLA) class I heavy chain antibody, EMR8-5, was established. It could detect HLA-A, -B, and -C antigens in formalin-fixed paraffin embedded tissues. By immunohistochemical staining using the EMR8-5 antibody, various cancer tissues from 246 cases were examined for HLA class I expression. It was found that HLA class I expression was decreased in 20% to 42% of the cases of lung cancer, hepatocellular carcinoma, colon cancer, renal cell carcinoma, and urothelial carcinoma. In contrast, 85% of breast cancer cases had loss of or decreased HLA class I expression. Of the 35 breast cancer cases that had decreased HLA class I heavy chain expression, 33 (94%) also had decreased beta2-microglobulin expression detected by immunohistochemical staining. It was suggested that HLA class I down-regulation might be a common characteristic of breast cancer mostly caused by the down-regulation of beta2-microglobulin expression.

Key words: antigen presentation, beta2-microglobulin, breast cancer, Immune escape, MHC class I

Human leukocyte antigen (HLA) class I molecules have a central role in the cell-mediated immune system, especially as antigen-presenting molecules for cytotoxic T lymphocytes (CTL). The CTL can recognize antigenic peptides presented on the cell surface by HLA class I molecules, and kill the target cells. Recently, a large number of CTL epitope peptides were identified in various tumor antigens, and CTL-based immunotherapy has been widely tested for various cancer patients.[1–4] Favorable outcomes have been obtained in some clinical trials for melanoma patients, however, most of the CTL-based immunotherapies for non-melanoma cancer patients have fallen short of expectations.[4] Precise examination for the insufficiency revealed immune escape of cancer cells, including down-regulation of HLA class I molecules, secretion of immunosuppressive cytokines and infiltration of immunosuppresive cells.[5–8] Though immune escape phenotypes such as tumor HLA class I down-regulation have been demonstrated mostly in primary cultured tumor cells or frozen tumor tissues, they are rarely found in formalin-fixed paraffin-embedded tissues because of the limited availability of anti-HLA class I antibodies that can react to the denatured molecules in the fixed tissues. Since most surgical tumor specimens are examined and stored after formalin fixation, development of novel monoclonal antibodies that are capable of detecting specific denatured antigens in the fixed tissues should contribute to extensive histological examinations using large numbers of archival tumor specimens.

In the present study, we introduce a novel monoclonal anti-pan HLA class I antibody termed EMR8-5, which is suitable for immunohistochemical staining of formalin-fixed paraffin-embedded tissue sections. Immunostaining of various tumor tissues revealed an unusually high frequency of HLA class I down-regulation in breast cancer as compared with lung, colon, kidney, liver, and bladder cancers. Involvement of beta2 microglobulin down-regulation is also addressed as the major cause of the HLA down-regulation in breast cancer.

METHODS

Development of monoclonal anti-pan HLA class I heavy chain antibody

A recombinant His-tagged extracellular domain of HLA-A*2402 heavy chain protein was produced and purified by

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using Ni-NTA agarose (QIAGEN, Valencia, CA, USA) as described previously.[9] The purified protein was dialyzed in PBS, and approximately 0.2 mg of protein per 500 μl of PBS was emulsified with 500 μl of either complete Freund's adjuvant (for the first immunization) or incomplete Freund's adjuvant (after the second immunization). BALB/c mice were immunized subcutaneously with the emulsion eight times every week. Four days after the final immunization, spleen cells were fused with NS-1 myeloma cells by using polyethylene glycol #4000 (Kanto Kagaku, Tokyo, Japan) and plated into 96-well plates. Hybridoma supernatants were initially screened using ELISA with a recombinant extracellular domain of HLA-A*2402 heavy chain protein denatured in 8 M urea buffer (8 M urea, 20 mM HEPES [pH 8.0]) as the antigen. The positive supernatants were then screened by Western blotting with cell lysates from OSC20 cells (HLA-A*2402-negative oral cancer cell line),[10] OSC20 transfectants with HLA-A*2402 cDNA, K562 cells, and recombinant HLA-A*2402 protein. The third screening of the supernatants was performed by immunostaining of formalin-fixed paraffin-embedded human tissue sections. The resulting hybridoma EMR8 was cloned by limiting dilution, and finally its subclone EMR8-5, which produced a monoclonal anti-HLA class I heavy chain antibody with the IgG1 subclass and κ chain, was established.

Western blotting

The monoclonal anti-HLA class I antibody HC10 was kindly provided by Dr. S. Ferrone (Roswell Park Cancer Institute, Buffalo, NY, USA).[11,12] Recombinant extracellular domains of various HLA class I allele proteins were kindly provided by MBL Co. Ltd. (Nagoya, Japan). Cultured cells were washed in ice-cold PBS, homogenized in ice-cold RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris-HCL [pH 8.0], and protease inhibitor cocktail [Complete, Roche Diagnostics, Basel, Switzerland]) for 30 min and clarified by centrifugation at 12 000 g for 20 min at 4°C. The lysates and 0.5 μg of recombinant HLA heavy chain proteins were boiled for 5 min with SDS sample buffer and then separated by 7.5% SDS-polyacrylamide gel electrophoresis. The proteins were transferred electrophoretically to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore, Billerica, MA, USA). The membranes were incubated with blocking buffer (5% nonfat dry milk in PBS) at room temperature and then incubated for 60 min with EMR8-5 culture supernatant. After washing three times with PBS-T buffer (0.05% Tween-20 in PBS), the membrane was reacted with a peroxidase-labeled goat anti-mouse IgG antibody (KPL, Gaithersburg, MD, USA) for one hour. Finally, the signals were visualized using an enhanced chemiluminescence (ECL) detection system (Amersham Life Science, Arlington Heights, IL, USA) according to the manufacturer's protocol.

Tissue specimens

Formalin-fixed paraffin-embedded tissue specimens of breast cancer (41 cases), lung cancer (35 cases), hepatocellular carcinoma (57 cases), colon cancer (15 cases), renal cell carcinoma (45 cases), and bladder cancer (53 cases) that were resected from patients at the Sapporo Medical University Hospital between 1995 and 2005 were used in the immunohistochemical examination. The study was approved by the Institutional Review Board for Clinical Research of our university.

Immunohistochemistry

Tissue specimens were cut into 5–μm-thick sections and deparaffinized. Antigen retrieval was performed using TT-mega Milestone (ESBE Scientific, Markham, Ontario, Canada) in 10 mM citrate buffer (pH 6.0) at 120°C for 15 min. After incubation in 0.3% hydrogen peroxide for 30 min, tissue slides were reacted with 10 μg/mL purified EMR8-5 antibody or polyclonal anti-beta2-microglobulin antibody (DAKO) for 1 h, washed three times with PBS-T buffer, and then incubated with SimpleStainMax-PO (Nichirei, Tokyo, Japan), followed by reaction with 3,3'-diaminobenzidine tetrahydrochloride-hydrogen peroxide as a chromogen for 1 min. After washing with distilled water, the slides were stained with hematoxylin solution for 1 min, rinsed, dehydrated through graded alcohols into non-aqueous solution, and cover-slipped with mounting media.

Tumor HLA class I expression levels were classified according to the criteria established by the HLA and Cancer component of the 12th International Histocompatibility Workshop.[13] According to these criteria, expression levels were defined as strongly positive (2+) if the cell membrane was stained as strongly as stromal lymphocytes or endothelial cells in more than 75% of the tumor cells. If membrane staining was heterogenous in 25% or more of the constituent tumor cells, it was defined as weak positive (1+). If membrane staining was lost in more than 75% of the tumor cells, it was defined as negative (0). Cases with a level 1+ or less were judged as down-regulated.

RESULTS

Characterization of monoclonal antibody EMR8-5

OSC20 is an HLA-A*2402-negative cell line.[10] HLA-A*2402 cDNA was cloned into plasmid pcDNA3.1 and transfected

into OSC20 cells. The resulting stable transfectant OSC20-A*2402 cells expressed HLA-A24 antigen on the cell surface. K562 is an HLA class I-negative cell line. Western blotting with the EMR8-5 antibody and HC10 antibody indicated that the EMR8-5 antibody could react to both immunized recombinant HLA-A*2402 heavy chain protein and endogenous non-HLA-A*2402 heavy chains expressed in OSC20 cells, whereas the HC10 antibody could react to endogenous non-HLA-A*2402 heavy chains, but not to the HLA-A*2402 heavy chain (Fig. 1).

Western blotting analysis with deletion mutant HLA-A*2402 proteins revealed that the epitope of EMR8-5 was located within a conserved α3 domain (Fig. 2).

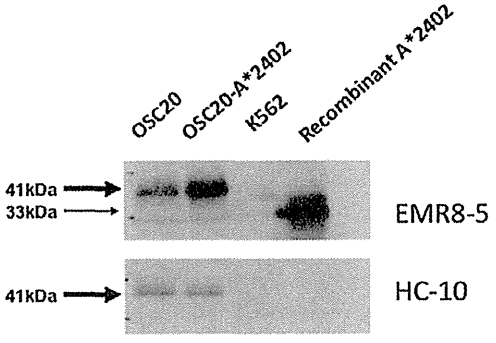

Figure 1 Western blot analysis with antibodies EMR8-5 (upper panel) and HC10 (lower panel). OSC20 cells are HLA-A*2402 negative. OSC20-HLA*2402 cells are OSC20 transfectant cells with pcDNA3.1-HLA-A*2402 cDNA. K562 cells are HLA class I-negative cells. Recombinant A*2402 indicates 0.5 μg of recombinant extracellular domain protein derived from HLA-A*2402 that was used as the immunogen.

To clarify the specificity of these antibodies, reactivity to recombinant extracellular domains of various HLA class I allele proteins was examined by Western blotting (Fig. 3). It was demonstrated that EMR8-5 could react to all the HLA-A, B, and C alleles examined, whereas HC10 reacted preferentially to HLA-B and C alleles as reported previously.[11,12] The data indicate that EMR8-5 was a monoclonal pan-HLA class I heavy chain antibody.

Immunohistochemical staining of formalin-fixed tumor tissues with EMR8-5 antibody

To determine the frequency of down-regulation of HLA class I expression in various tumors, surgically resected tissue specimens from 246 cases were immunostained with the EMR8-5 antibody. Representative immunostaining data of HLA class I-positive cases of colon cancer and breast cancer are shown in Figure 4a,d, respectively, and HLA class I down-regulated cases of colon cancer and breast cancer are shown in Figure 4b,c,e,f, in which stromal lymphocytes and endothelial cells are strongly stained by the antibody, serving as internal positive control. A summary of the frequency of HLA class I down-regulation in each tumor is presented in Table 1. A striking difference in the frequency of HLA class I expression was noted between breast cancer (85%) and other cancers (20% to 42%). HLA class I expression was down-regulated in a larger proportion of breast cancer cases than in other cancers such as lung cancer, colon cancer, hepatocellular carcinoma, renal cell cancer, and bladder cancer.

To determine the mechanism involved in the down-regulation of membrane expression of HLA class I in breast

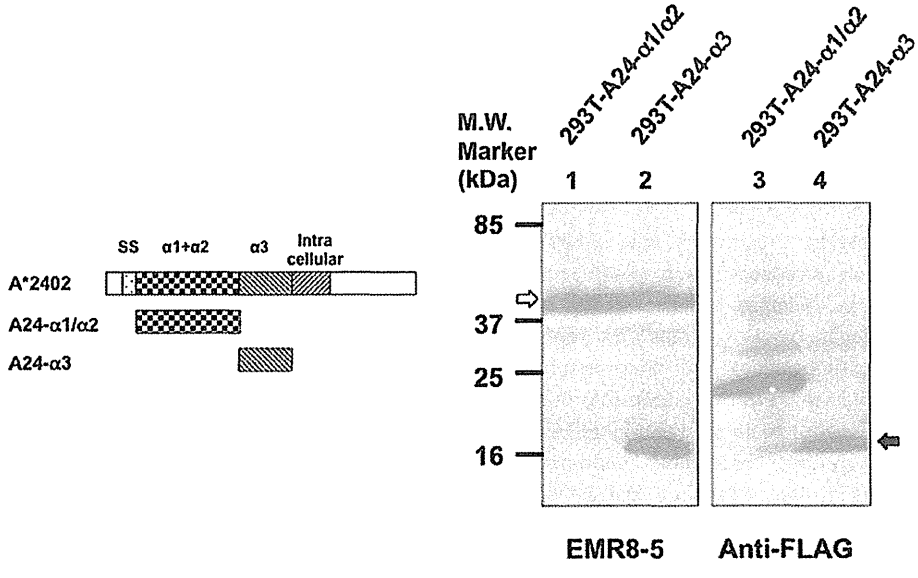

Figure 2 293T cells were transfected with cDNA encoding FLAG-tagged deletion mutant HLA-A*2402 proteins, A24-α1/α2 (lanes 1 and 3) and A24-α3 (lanes 2 and 4). Reactivity of EMR8-5 to the deletion mutant proteins was analyzed by Western blot analysis (lanes 1 and 2). Both of deletion mutant proteins were detected by anti-FLAG antibody (lenes 3 and 4). EMR8-5 reacted only to 16 kDa A24-α3 (lane 2, black arrow). White arrow indicates 41 kDa endogenous HLA class I protein in 293T cells.

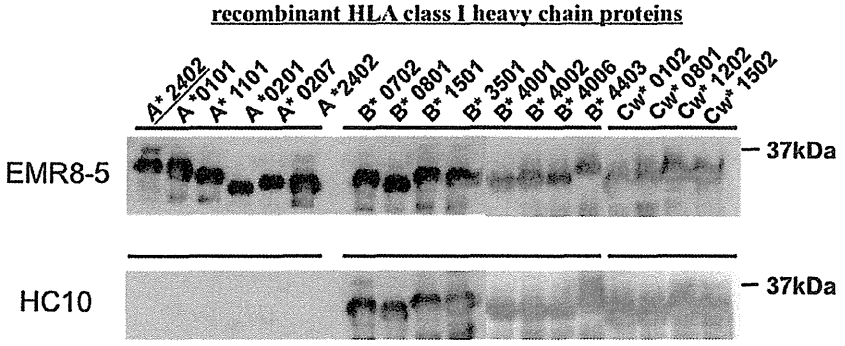

Figure 3 Western blot analysis with antibodies EMR8-5 (upper panel) and HC10 (lower panel). Recombinant extracellular domain proteins (0.5 μg per lane) derived from the indicated HLA class I alleles were run on the SDS-PAGE and immunoblotted with the indicated antibody. Underlined protein in italics indicates the immunogen of our preparation. Other proteins were gifts from Medical & Biological Lab. Co. Ltd. (Nagoya, Japan).

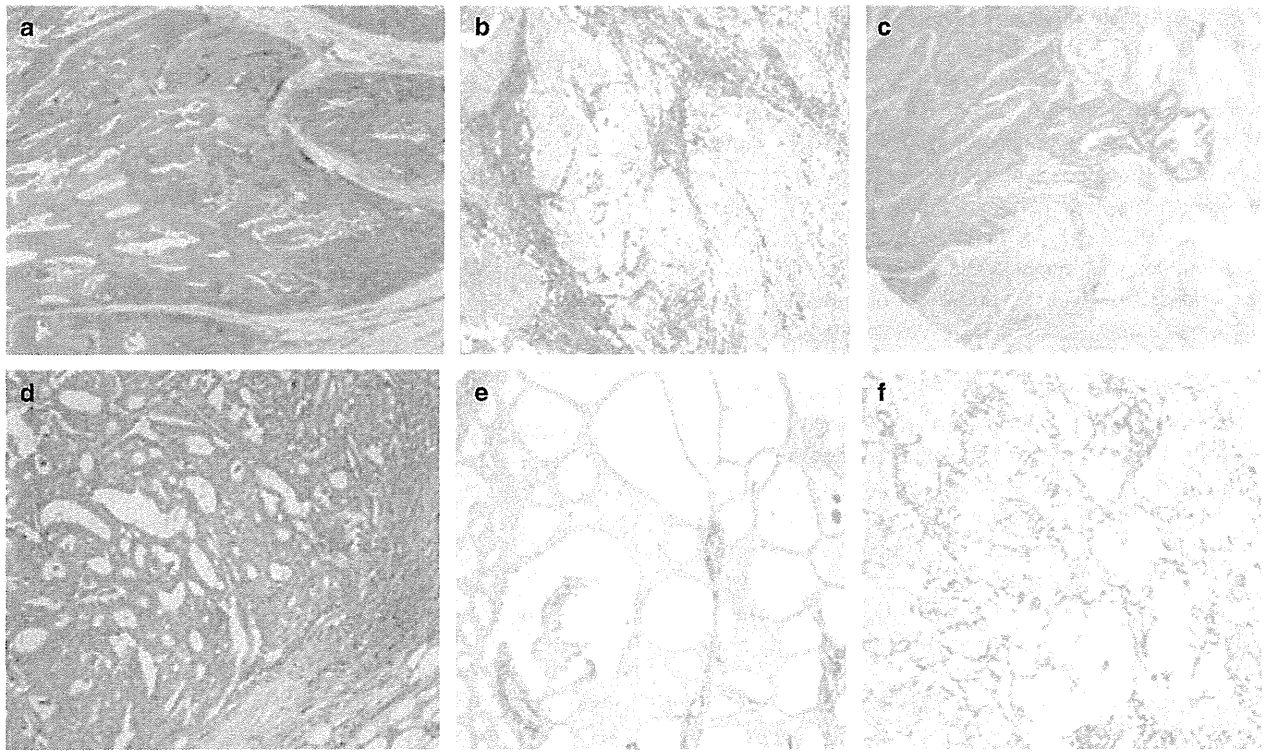

Figure 4 Representative immunostaining pictures with EMR8-5 antibody. Panels **a**, **b** and **c** are colon cancer cases that are HLA class I-positive (2+, Panel **a**), negative (0, Panel **b**) and heterogeneous staining (1+, Panel **c**). Panels **d**, **e** and **f** are breast cancer cases that are HLA class I-positive (2+, Panel **d**), negative (0, Panel **e**) and weak positive staining (1+, Panel **f**). Note that stromal cells such as lymphocytes, endothelial cells and fibroblasts are HLA class I-positive in Panel **b**, **c**, **e** and **f**, serving as internal positive control.

cancers, the same tissue sections were examined for the expression of beta2-microglobulin (HLA class I light chain) by immunostaining. As shown in Table 2, 33 of the 35 cases (94%) of HLA class I weak or negative breast cancer had loss of beta2-microglubulin expression demonstrated by immunostaining. These data indicated that HLA class I down-regulation in breast cancer cells was mostly accompanied by the down-regulation of beta2-microglobulin expression, which might be one of the major causes of the impairment of membrane expression of HLA class I.

Table 1 Human leukocyte antigen (HLA) class I expression levels detected by immunostaining with antibody EMR8-5

Cancer origin	Total cases	2+	1+/0 (%)
Breast cancer	41	6	35 (85%)
Lung cancer	35	28	7 (20%)
Hepatocellular carcinoma	57	33	24 (42%)
Colon cancer	15	11	4 (27%)
Renal cell carcinoma	45	29	16 (35%)
Bladder cancer	53	35	18 (34%)

Expression levels 2+, 1+, and 0 are strongly positive, weakly positive, and negative, respectively.

Table 2 Human leukocyte antigen (HLA) class I heavy chain expression and beta2-microglobulin expression in the breast cancer cases

	Total cases	β2-MG +	β2-MG −
HLA class I 0	15	0	15
HLA class I 1+	20	2	18
HLA class I 2+	6	6	0

Expression levels 2+, 1+, and 0 are strongly positive, weakly positive, and negative, respectively.

DISCUSSION

There are many reports showing the down-regulation of HLA class I and immune escape of cancer cells.[14–22] Since most anti-HLA class I antibodies recognize the allele-specific native structure of cell-surface HLA class I molecules or heterodimeric structure of a heavy chain and beta2-microglobulin, these antibodies cannot react with denatured HLA class I molecules in formalin-fixed paraffin-embedded tissue sections. Two monoclonal anti-HLA class I antibodies, HC10 and HCA2, have been well characterized and are available for the immunostaining of formalin-fixed tissue specimens. However, as shown in our results (Figs 1,3), HC10 can barely react with HLA-A allele proteins, and HCA2 reacts to some HLA-A allele proteins, but not to most HLA-B or C allele proteins.[11,12] In the present study, we demonstrated a novel monoclonal pan-HLA class I heavy chain antibody suitable for the immunostaining of formalin-fixed tissue specimens. The epitope was located within a conserved α3 domain, and the antibody reacted to 17 HLA class I allele proteins examined. Allele frequencies are shown in Table 3. The antibody enabled us to examine HLA class I expression at the tissue level in a large number of archival tissue specimens that were surgically resected and stored for a long time.

By immunostaining of various tumor specimens from 246 cases using EMB8-5, it was found that breast cancer might have a unique immune escape phenotype as compared with other tumors. Our findings are essentially compatible with studies of Ferrone *et al.*[6] and other groups,[16,20] although the frequency was higher in our study. Although we could not find a correlation between the prognoses of the cancer patients and HLA class I expression level, it was noted that all the cases of micropapillary type breast cancer (10 cases) were HLA class I negative, which subtype is known to be extremely metastatic and have a poor prognosis. Of the breast cancer cases with decreased HLA class I heavy chain expression, 94% were accompanied by loss of beta2-microglobulin expression. No beta2-microglobulin gene mutation was detected by sequencing analysis of three breast cancer cases with beta2-microglobulin negative phenotype. It is likely that beta2-microglobulin gene expression may be regulated by epigenetic mechanisms such as gene methylation and histone deacetylation. Actually, we have found that beta2-microglobulin expression of breast cancer cell lines was up-regulated in the presence of a histone deacetylase inhibitor, leading to the restoration of cell surface HLA class I expression (data not shown). Further analysis of the mechanism of HLA class I gene regulation should lead to a novel therapeutic strategy for breast cancer.

Table 3 Human leukocyte antigen (HLA) class I Allele Frequency (Phenotype frequency)

	Allele	Frequency % (Japanese)	Frequency % (Asian in USA)	Frequency % (Caucasoid in USA)
1	A 0101	4	3–10	22–29
2	A 1101	16–22	36–46	12–14
3	A 0201	21–23	18–19	40–48
4	A 0207	4–8	8–13	0
5	A 2402	65–76	36–38	13–26
6	B 0702	10–13	5	20–31
7	B 0801	0	3	13–23
8	B 1501	13–17	7–10	12–16
9	B 3501	4–18	8	9–14
10	B 4001	6–12	16–18	8–14
11	B 4002	6–17	6	2–3
12	B 4006	8–13	4–7	0
13	B 4403	12–24	8–9	6–16
14	Cw 0102	30–40	27	4–7
15	Cw 0801	15–22	22	0
16	Cw 1202	20–24	6	2
17	Cw 1502	3–5	5	3–5

Percentage of individuals who have the allele or gene.

Phenotype frequencies were calculated from the data of 'The Allele Frequency Net Database' (http://www.allelefrequencies.net/).[23]

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