Loss of tapasin in human lung and colon cancer cells and escape from tumor-associated antigen-specific CTL recognition

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ABSTRACT
Cytotoxic T-lymphocytes (CTLs) lyse target cells after recognizing the complexes of peptides and MHC class I molecules (pMHC I) on cell surfaces. Tapasin is an essential component of the peptide-loading complex (PLC) and its absence influences the surface repertoire of MHC class I peptides. In the present study, we assessed tapasin expression in 85 primary tumor lesions of non-small cell lung cancer (NSCLC) patients, demonstrating that tapasin expression positively correlated with patient survival. CD8\textsuperscript{+} T-cell infiltration of tumor lesions was synergistically observed with tapasin expression and correlated positively with survival. To establish a direct link between loss of tapasin and CTL recognition in human cancer models, we targeted the tapasin gene by CRISPR/Cas9 system and generated tapasin-deficient variants of human lung and colon cancer cells. We induced the CTLs recognizing endogenous tumor-associated antigens (TAA), survivin or cep55, and they responded to each tapasin-proficient wild type. In contrast, both CTL lines ignored the tapasin-deficient variants despite their antigen expression. Moreover, the adoptive transfer of the cep55-specific CTL line failed to prevent tumor growth in mice bearing the tapasin-deficient variant. Loss of tapasin most likely limited antigen processing of TAAs and led to escape from TAA-specific CTL recognition. Tapasin expression is thus a key for CTL surveillance against human cancers.

ARTICLE HISTORY
Received 22 July 2016
Revised 30 November 2016
Accepted 15 December 2016

KEYWORDS
CD8\textsuperscript{+} T cells; colon cancer; immune evasion; lung cancer; MHC class I; tapasin

Introduction
Cytotoxic T-lymphocytes (CTLs) recognize endogenously processed peptides presented by MHC class I molecules and lyse the target cells. MHC class I antigen processing consists of multiple steps across the cytosol and the endoplasmic reticulum (ER): The cytosolic proteasomes digest endogenous proteins, thereby generating short fragments of proteins, which are further transported into the ER through the transporter-associated with antigen processing (TAP).\textsuperscript{1,2} The ER aminopeptidase associated with antigen processing (ERAAP or ERAP1) trims the N-terminal extension of the short protein fragments, optimizing the length of the peptide for binding to MHC class I molecules.\textsuperscript{3,5} Meanwhile, the MHC I molecules associated with β2-microglobulin (β2m) form the peptide-loading complex (PLC) consisting of TAP, tapasin, ERP57, and calreticulin, which is necessary for stable pMHC I supply on the cell surface.\textsuperscript{6,7} Tapasin is an MHC I-specific chaperone tethering MHC I and TAP and plays an indispensable role in shaping the pMHC I repertoire. Many CD8\textsuperscript{+} T cell responses to tapasin-deficient cells are reduced or diminished in mouse models.\textsuperscript{8,9}

Loss of tapasin expression is a frequent event that has been reported in a wide variety of human cancers, including malignant melanoma, head and neck squamous cell carcinoma (HNSCC), renal cell carcinoma, colorectal carcinoma, glioblastoma, lung carcinoma, and neuroblastoma.\textsuperscript{10-18} Notably, tapasin expression associated with intratumoral T-cell infiltration has been reported as a prognostic marker of patient survival in ovarian carcinoma, HNSCC, glioblastoma, and colorectal carcinoma.\textsuperscript{12,13,19-21} It is also reported that loss of tapasin is more frequent among the other antigen-processing machinery (APM) components, strongly suggesting its central role in escape from CTL immune surveillance to tumors.\textsuperscript{15,22} Impaired CTL responses against tapasin-deficient cells have been well established in mouse models.\textsuperscript{8,9} In human cases, loss of tapasin alters the peptide repertoire presented by HLA-B*2705 and results in a decrease in alloreactive CTL responses.\textsuperscript{23} However, a direct evidence showing the processing defect of endogenous tumor-associated antigens (TAA) and following loss of cancer-specific CTL recognitions has been missing due to the lack of appropriate tapasin-deficient models. In the present study, we screened tapasin expression and CD8\textsuperscript{+} T-cell infiltration of tumor lesions in 85 primary tissue sections from non-small cell lung carcinoma (NSCLC), demonstrating that both tapasin expression and CD8\textsuperscript{+} T-cell infiltration positively correlate with patient survival. Moreover, we generated the genetically mutated tapasin-deficient human lung and colon cancer cells. Loss of tapasin influences the processing of endogenous TAAs and leads to escape from CTLs recognition.
Results

**Tapasin expression and CD8\(^+\) T-cell infiltration correlate with prognoses of NSCLC patients**

Tapasin expression levels and CD8\(^+\) T-cell infiltration in tumor lesions were assessed in 85 cases of human primary NSCLC patients (Table 1 and Table S1). A total of 48.2% of the patients had stage IA disease and stages of the remaining patients varied across IB to IIIB. Most patients were histologically diagnosed as adenocarcinoma (72.9%), followed by squamous cell carcinoma (SCC, 16.5%), and other histological types. Formalin-fixed and paraffin-embedded tumor sections obtained from the patients after surgery were stained with anti-tapasin or anti-CD8\(^+\) monoclonal antibodies (mAb) and then scored as indicated in Fig. 1. In summary, the tapasin expression was observed as follows: score 2 (>75% positive), 27.1%; score 1 (25–75% positive), 37.6%; score 0 (<25% positive), 35.3%. The results show that abundant tapasin expression is observed only in 27.1% of the tumor lesions and 72.9% cases heterogeneously lack tapasin expression. Meanwhile, CD8\(^+\) T-cell infiltration scores were as follows: score 2 (>75% positive), 63.5%; score 1 (25–75% positive), 18.8%; score 0 (<25% positive), 17.6%.

As expected, the overall survival rate (OS) of stage IA and IB patients was significantly higher than that of stage IIA to IIIB patients (Fig. 2A). We divided the patients into tapasin-positive (score 2) and tapasin-negative (score 1 and 0) groups and compared survival. Notably, the OS of the tapasin-positive group was significantly higher than that of the tapasin-negative group, suggesting that tapasin expression could be a good prognostic marker comparable to clinical stages in NSCLC patients (Fig. 2B). We also divided the patients into CD8\(^+\) T-cell infiltration-positive (score 2) and -negative (score 1 and 0) groups and the Kaplan–Meier survival analysis showed a statistically significant higher OS in the CD8\(^+\) T-cell infiltration-positive group (Fig. 2C). Tapasin expression and CD8\(^+\) T-cell infiltration were statistically correlated (Fig. 2D). Notably, combining tapasin expression and CD8\(^+\) T-cell infiltration scores provided further information: prognoses were comparably better among three groups positive to tapasin or CD8\(^+\), while only the tapasin and CD8\(^+\)-double negative group significantly impaired survival (Fig. 2E). These results suggest that losses of tapasin expression and CD8\(^+\) T-cell infiltration in tumor lesions synergistically correlate with and significantly influence NSCLC patient prognoses, consistent with the findings of former studies conducted in ovarian carcinoma, HNSCC, glioblastoma, and colorectal carcinoma patients.\(^{19-21,24}\)

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**Table 1. Summary of clinical annotations, tapasin scores and CD8\(^+\) T-cell infiltration scores of the NSCLC patients**

| Age (years) | n   | % total |
|------------|-----|---------|
| 66.6±9.42  | 85  | 100     |

**Gender**
- Female: 38 (44.7%)
- Male: 47 (55.3%)

**Stage**
- IA: 41 (48.2%)
- IB: 16 (18.8%)
- IIA: 6 (7.1%)
- IIB: 7 (8.2%)
- IIIA: 11 (12.9%)
- IIIB: 4 (4.7%)
- IV: 0 (0%)

**Histology**
- ADC: 62 (72.9%)
- SCC: 14 (16.5%)
- LCC: 4 (4.7%)
- PLE: 3 (3.5%)
- ADSQ: 2 (2.4%)

**Tapasin score**
- 2: 23 (27.1%)
- 1: 32 (37.6%)
- 0: 30 (35.3%)

**CD8 score**
- 2: 54 (63.5%)
- 1: 16 (18.8%)
- 0: 15 (17.6%)

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**Note:** ADC, adenocarcinoma; SCC, squamous cell carcinoma; LCC, large cell carcinoma; PLE, carcinoma with pleomorphic or sarcomatous elements; ADSQ, adeno-squamous cell carcinoma.

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Figure 1. Immunohistochemistry of human primary NSCLC. Representative staining patterns of formalin-fixed, paraffin-embedded tumor lesions with tapasin mAb (upper panels) and CD8\(^+\) mAb (lower panels). Tapasin scores and CD8\(^+\) scores are determined by percentages of positive tumor cells and by percentages of tumor-infiltrating positive cells in tumor lesions, respectively: 0, <25% positive; 1, 25% to 75% positive; 2, >75% positive. Arrows, tumor cells. Asterisks, stromal (non-tumor) cells. Magnification, x100 and x200.
Generation of a tapasin-deficient human lung cancer line

To establish a direct link between loss of tapasin and cancer-specific CTL evasion, we set about making a pair of human lung cancer lines that express a TAA with or without tapasin expression. Here we chose the LHK2 lung cancer line because it expresses the detectable tapasin protein as well as an endogenous TAA, survivin, which encodes a CTL epitope presented by HLA-A24. The CRISPR/Cas9-mediated genome engineering system allowed to introduce random heterozygous mutations at the gRNA targeting site in exon 2 of the tapasin gene, and we established the LHK2 variant, YS3 (Fig. 3A). In contrast to the wild type, which increased tapasin protein expression in an IFNγ dependent manner, the YS3 never expressed tapasin protein both in the absence and presence of IFNγ, most likely due to frameshift mutations taking place in both tapasin alleles (Fig. 3B). The LHK2 line was established from an HLA-A2402-positive patient and we confirmed the surface expression level, showing that both HLA-A/B/C and HLA-A24 levels significantly decreased on the YS3 variant (Fig. 3C and D). We thus concluded that the introduced tapasin-gene mutations diminished tapasin protein expression and resulted in loss of surface MHC class I expression as observed in tapasin-knockout mouse cases.

Loss of tapasin allows lung cancer cells to escape from TAA-specific CTL recognition

A TAA, survivin 2B, is a splicing variant derived from the survivin gene and its expression is widely observed among a variety of cancer lines but not in normal tissues. LHK2 and YS3 equally expressed the survivin 2B gene and survivin protein, demonstrating that introducing tapasin-gene mutation did not influence the endogenous expression of the survivin antigen
(Fig. 4A and B). We prepared the CTL clone H4 that recognized the survivin 2B epitope presented by HLA-A24 by stimulating cancer-patient PBMC with the synthetic peptide, followed by single-cell sorting as described in methods (Fig. 4C). The clone H4 produced IFN-γ in response to T2-A24 cells pulsed with survivin 2B peptides (Fig. 4D). The clone H4 recognized LHK2, suggesting that cancer-patient PBMCs contain survivin 2B-specific CTL precursors (Fig. 4E). By contrast, the CTLs failed to produce IFN-γ against YS3. These data indicate that loss of tapasin confers the capability to reduce TAA-specific CTL responses on cancer cells.

**Generation of a tapasin-deficient human colon cancer line**

To expand the finding over lung cancers and survivin 2B, we next used the human colon cancer line SW480 expressing both survivin 2B and CEPP55 gene, which is another TAA expressed in a wide variety of tumor cell lines including SW480 and encodes a CTL epitope presented by HLA-A24.26 CRISPR/Cas9 system again allowed to introduce random heterozygous mutations in exon 2 of the tapasin gene, and we established the SW480 variant, 3G12 (Fig. 5A). The tapasin-gene mutations resulted in loss of tapasin protein expression, demonstrating that the 3G12 clone was a tapasin-deficient variant (Fig. 5B). The SW480 was originally established from a patient with HLA-A’2402/A’0201 and expressed both HLA class I molecules on the surface (Fig. 5C). The survivin 2B-specific CTL clone H4 derived from cancer-patient PBMC in Fig. 4 was used for cytotoxic assay. The H4 lysed SW480 in an E/T ratio dependent manner consistent with clear survivin 2B-gene expression. In contrast, the H4 hardly lysed 3G12 as well as a MHC class I negative-K562 line despite survivin 2B expression equal to SW480 (Fig. 6D). We also used another CTL clone derived from cancer patient PBMC, the clone which specifically recognized the cep55...
epitope presented by HLA-A24. The CTL clone successfully lysed SW480 expressing the cep55 gene, while failed to recognize 3G12 despite cep55 gene expression equal to SW480 (Fig. 6E). The CTL failed to lyse another tapasin-deficient variants 1D4 and 3A9 as well (Fig. S2). Moreover, we found that loss of tapasin influenced the antitumor effect in vivo. SW480 and 3G12 were subcutaneously injected into NSG mice. The subsequent adoptive transfer of cep55-CTLs significantly prevented the growth of SW480 consistent with the CTL-mediated cell lysis observed in vitro (Fig. 6F). By contrast, the adoptive transfer did not reduce the sizes of tumors in mice bearing tapasin-deficient 3G12 (Fig. 6G).

**Loss of tapasin limits the antigen processing of endogenous TAAs**

We finally asked whether the impaired CTL responses against endogenous TAAs was caused by a decrease in peptide supply or by a decrease in HLA-A24 on tumor cell surfaces. Because direct CTL responses against SW480 and 3G12 can be influenced by both conditions, we first quantified the amount of CTL epitopes produced in the tumor cells using an exogenous presentation assay (Fig. 7A). Survivin 2B and cep55 CTL clones significantly prevented the growth of SW480 consistent with the CTL-mediated cell lysis observed in vitro (Fig. 6F). By contrast, the adoptive transfer did not reduce the sizes of tumors in mice bearing tapasin-deficient 3G12 (Fig. 6G).
difference may have contributed to the impaired TAA-specific CTL responses against 3G12 (Fig. 5C). However, the survivin 2B-CTL responses against $5 \times 10^4$ cells of SW480 and 3G12 were comparable, when the tumor cells were exogenously pulsed with synthetic survivin 2B peptides (Fig. 7D). Taken together, these results demonstrate that the impaired CTL responses caused by loss of tapasin was mainly due to a decrease in peptide supply but not a decrease in HLA-A24, suggesting that loss of tapasin limits the antigen processing of endogenous TAAs.

**Discussion**

This study aimed to investigate effect of loss of tapasin on TAA-specific CTL responses that plays a critical role in immune surveillance of human tumors *in vivo*. Loss of tapasin and its positive correlation to poorer patient prognoses have been reported among a variety of tumors. In a large-cohort study of human colorectal cancers, increased CD8$^+$ T-cell infiltration of tumors was acknowledged as a critical factor positively correlating with patient survival. In this study, significant correlation was observed only when cancer tissues lacked tapasin expression as well as CD8$^+$ T-cell infiltration, suggesting that CTL responses to cancer cells influence prognoses in a tapasin-dependent manner, consistent with previous studies in ovarian carcinoma, HNSCC, glioblastoma, and colorectal carcinoma. A former study has demonstrated that restoring tapasin gene expression in a mouse tumor model using viral vector transduction improved survival of model mice bearing lung carcinoma. In this study, we generated human lung and colon cancer variants that express TAAs, survivin, or cep55, genetically lacking tapasin protein expression. To our knowledge, this study is first to demonstrate that CTL responses to endogenous TAAs presented by HLA class I molecules are significantly reduced to undetectable levels solely by the absence of tapasin. Our results suggested the antigen processing of HLA-A24 endogenous TAAs is limited in cancer cells. Thus, tapasin expression is a key to regulate cancer-specific CTL surveillance, influencing patient survival.

It is well known that tapasin is the core component tethering MHC class I and TAP molecules and is indispensable to stabilize TAP heterodimers. Loss of tapasin would therefore result in disorganized PLC formation and influenced peptide transport into the ER. The molecular mechanisms underlying loss of tapasin in tumor cells have been elusive, but it most likely occurs due to several reasons such as deregulation of epigenetic control, transcriptional, and posttranscriptional modulation. Treatment of mouse melanoma and transformed lung cells with a DNA demethylating agent, 5-aza-2-deoxycytidine (5-AZA) or a histone deacetylase inhibitor, trichostatin A (TSA) restored tapasin expression. An alternative splicing form of tapasin, which failed to restore surface MHC class I levels in tapasin-deficient cells, has been reported in a human melanoma cell line. In addition, a recent report has shown a novel germ-line frameshift mutation in a single population of human melanomas, resulting in loss of tapasin, and expression could not be restored by IFN$\gamma$ treatment.

Our results raised another intriguing question concerning HLA types and tapasin dependency. It is known that loss of tapasin generally leads to a decrease in HLA surface expression, but the dependency on tapasin differs across HLA-class I
allotypes. Because loss of tapasin significantly decreases the quantity of naturally processed peptides that are stably bound to HLA-A2, the majority of peptide-HLA-A2 complexes on tapasin-deficient cell surfaces are potentially unstable.35 Despite that HLA-A*0201 has been reported as a tapasin-independent HLA type, 3G12 clearly showed a decrease in the HLA-A2 surface level in this study.36 The conflicting results between the widely used B-cell line (.220) and the colon cancer line (3G12) may imply an uncharacterized effects of targeting tapasin function and should be further investigated.

Recent clinical successes in immunotherapy using immune-checkpoint inhibitors that improved prognoses of a variety of cancer patients including melanoma, NSCLC, and renal cell carcinoma, strongly emphasize the potential of immune surveillance by endogenous T cells.37-39 NSCLC is indeed a good target for immune checkpoint blockade because of frequent gene mutations yielding neoantigens.40 In contrast, durable clinical effects are not always observed in every case and more than half are yet refractory. Frequent loss of tapasin in NSCLC implies that quite a few proportion of cancer cells are potentially immune-escape variants in vivo. Therefore, counteracting immune-escape variants that are resistant to immunotherapy would be an urgent issue of wide importance. A study with tapasin-deficient mice has showed...
that loss of tapasin gave rise to quantitative and qualitative change in the surface pMHC repertoire: The repertoire presented by tapasin-deficient cells consists of many lengthy/anchorless peptides, which are not presented by wild-type cells, thereby inducing wild-type CTL responses against tapasin-deficient cells although the antigens remain unknown.\(^4^1\),\(^4^2\) Therefore, endogenous CTL surveillance is potentially able to monitor tapasin deficiency and further investigation of such CTL responses may accelerate the drug development against tumor immune-escape variants.

**Materials and methods**

**Patients**

The study was performed with approval of the local ethics committee of Sapporo Medical University. Eighty-five NSCLC patients surgically treated at Sapporo Medical University Hospital, Sapporo, Japan, from 2008 and 2012 were retrospectively included in this study, with informed consent according to the guidelines of the Declaration of Helsinki. Histological diagnoses were determined in accordance with the World Health Organization criteria for histopathology classification. Clinical data were obtained from the patient records and OS was calculated from the day of the operation until October 7, 2015.

**Immunohistochemistry**

Formalin-fixed paraffin-embedded tumor sections from NSCLC patients or tumor-bearing mice were stained with anti-tapasin TO-3 mAb or anti-CD8\(^\text{+}\) mAb after epitope retrieval with Novocastra epitope retrieval solution (Leica). Endogenous peroxidase activity was blocked by immersion in 3% peroxidase. The
sections were then stained with a corresponding secondary Ab and visualized according to a standard protocol (Leica). All sections were counterstained with hematoxylin-eosin (HE) and cancer lesions were reviewed and verified by pathologists. For scoring CD8+ T-cell infiltration, we randomly assessed 10 high-power fields (HPF, x400) per section and counted the number of fields with >10 CD8+ cells migrating within cancer lesions.

**Cells and antibodies**

The human lung cancer LHK2, colon carcinoma SW480, HLA class I-null K562, and T2-A24 cells (T2 cells stably expressing HLA-A24) were cultured in RPMI-1640 or Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Biosera), 1% penicillin-streptomycin mixture (Gibco), 1% sodium pyruvate (Gibco), and 50 μM 2-mercaptoethanol (Gibco). The pan-HLA class I, HLA-A24, and HLA-A2 specific mAbs were prepared from W6/32 (ATCC), C7709A2 (a gift from Dr. P. G. Coulié), and BB7.2 (ATCC) hybridomas. The anti-tapasin TO-3 mAb (sc-80647; Santa Cruz), anti-human mAb were prepared from W6/32 (ATCC), C7709A2 (a gift from Dr. P. G. Coulié), and BB7.2 (ATCC) hybridomas. The anti-tapasin TO-3 mAb (sc-80647; Santa Cruz), anti-human mAb were prepared from W6/32 (ATCC), C7709A2 (a gift from Dr. P. G. Coulié), and BB7.2 (ATCC) hybridomas. The anti-tapasin TO-3 mAb (sc-80647; Santa Cruz), anti-human mAb were prepared from W6/32 (ATCC), C7709A2 (a gift from Dr. P. G. Coulié), and BB7.2 (ATCC) hybridomas. The anti-tapasin TO-3 mAb (sc-80647; Santa Cruz), anti-human mAb were prepared from W6/32 (ATCC), C7709A2 (a gift from Dr. P. G. Coulié), and BB7.2 (ATCC) hybridomas. The anti-tapasin TO-3 mAb (sc-80647; Santa Cruz), anti-human mAb were prepared from W6/32 (ATCC), C7709A2 (a gift from Dr. P. G. Coulié), and BB7.2 (ATCC) hybridomas. The anti-tapasin TO-3 mAb (sc-80647; Santa Cruz), anti-human mAb were prepared from W6/32 (ATCC), C7709A2 (a gift from Dr. P. G. Coulié), and BB7.2 (ATCC) hybridomas. The anti-tapasin TO-3 mAb (sc-80647; Santa Cruz), anti-human mAb were prepared from W6/32 (ATCC), C7709A2 (a gift from Dr. P. G. Coulié), and BB7.2 (ATCC) hybridomas. The anti-tapasin TO-3 mAb (sc-80647; Santa Cruz), anti-human mAb were prepared from W6/32 (ATCC), C7709A2 (a gift from Dr. P. G. Coulié), and BB7.2 (ATCC) hybridomas. The anti-tapasin TO-3 mAb (sc-80647; Santa Cruz), anti-human mAb were prepared from W6/32 (ATCC), C7709A2 (a gift from Dr. P. G. Coulié), and BB7.2 (ATCC) hybridomas. The anti-tapasin TO-3 mAb (sc-80647; Santa Cruz), anti-human mAb were prepared from W6/32 (ATCC), C7709A2 (a gift from Dr. P. G. Coulié), and BB7.2 (ATCC)

**RT-PCR and qPCR**

Total RNA was isolated from cancer cells using TRIzol reagent according to the manufacturer’s instructions (Thermo). Total RNA from human adult tissues was purchased (human total RNA master panel; Clontech). cDNA was synthesized from 2 μg of total RNA by reverse transcription with Superscript II and oligo(dT) primer (Life Technologies). PCR reactions were initially incubated at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 15 sec, annealing at 58°C for 30 sec, and extension at 68°C for 1 min. Primer pairs used for RT-PCR are as follows: G3PDH, 5'-ACTGTGGCTCACAATGCTTT-3' and 5'-GAGCAGCTTTCCGGTTC-3' (product size 450bp); cep55, 5'-ACTGTGGCTCCAAACTGCTT-3' and 5'-GAGCAGCTTTCCGGTTC-3' (product size 181bp); survivin 2B, 5'-TCAAGGACCACCGCATCTC-3' and 5'-GTGCTGGTATTACAGCGTAAG-3' (product size 221bp). Primer pairs used for quantitative PCR are as follows: cep55, 5'-TGAAGTGGCTCAAGAGG-3' and 5'-ATCTCCACGGA-CAAC-3'; survivin 2B, 5'-CTAAGGACCACCGCATCTC-3' and 5'-GTGCTGGTATTACAGCGTAAG-3'.

**Western blot**

5 × 106 cells were homogenized with buffer containing 20 mM Tris-HCl (pH 7.5), 1% NP40, 150 mM NaCl, and a complete EDTA-free protease inhibitor (Roche) for 30 min at 4°C. Cell lysates were mixed with an equal volume of 2x sample buffer (125 mM Tris-HCl (pH 6.8), 10% (v/v) 2-mercaptoethanol, 40 mg/mL SDS, 100 mg/mL sucrose, and 4 mg/mL bromophenol blue) and separated by SDS-PAGE. Proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes, which were blocked with 5% skim milk and 2% BSA in 0.1% PBS-Tween at 4°C overnight. Blots were incubated with anti-human tapasin TO-3 (1:1,000) mAb or anti-actin (1:5,000) mAb overnight at 4°C, followed by incubation with peroxidase-labeled goat anti-mouse immunoglobulin (1:10,000) for 30 min at room temperature. The membranes were treated with ECL™ Western Blotting Detection Reagents (GE Healthcare) and exposed to blue films (Fuji Photo Film). Odyssey FC Imaging System 2800 (LI-COR Biosciences) was used for the detection of survivin protein.

**Generation of tapasin-deficient human cancer cell lines**

Gene targeting with the CRISPR-Cas9 system has been described in detail elsewhere. Briefly, we designed guide RNAs (gRNAs; 5'-GGTGATCGAGTGGTTGTCG-3') with the online software CRISPRdirect, which predicted a unique site for targeting human tapasin, and introduced it into the GeneArt CRISPR Nuclease vector with orange-fluorescent protein reporter (OFP) (A21174; Thermo). We transfected SW480 cells according to the manufacturer’s instructions (Lipofectamine 2000; Thermo) and selected the OFP-positive clones via cell sorting (FACSArria II; BD). The tapasin-deficient clones 3G12, 1D4, and 3A9 were ultimately selected based on their tapasin-gene sequences.

**TAA-specific CTL induction and single-cell cloning**

PBMCs from an HLA-A24 positive cancer patient were cultured in AIM-V medium (Life Technologies) containing 10% human serum and 50 U/mL IL-2, and repeatedly stimulated with survivin 2B peptide (AYACN STL) (40 μg/mL) every 3–4 d. Two weeks later, the cells were stained with a PE-conjugated survivin 2B/HLA-A24 tetramers at 4°C for 20 min, followed by a PC5-conjugated anti-CD8+ mAb (Beckman Coulter) at 4°C for 30 min. A single-cell sorting using FACS (Aria II Special Order, BD, Houston, TX, USA) allowed to isolate the tetramer and CD8+ double-positive clones. The sorted clones were expanded in complete AIM-V medium containing 100 U/mL IL-2, 1 μg/mL PHA, and feeder cells (X-ray irradiated PBMCs) in a U-bottom 96-well plate (Corning). We used the cep55-specific CTL clone that was generated from PBMC of an HLA-A*2402-positive breast cancer patient with stimulation with synthetic cep55 peptide (VYVKGLLAKI) and described elsewhere.

**ELISPOT IFNγ assay**

About 1.0 × 105 survivin 2B- or cep55-CTL clones together with 5 × 104 cancer cells (LHK2 or YS3 cells) or 5 × 104 T2-A24 cells pulsed synthetic peptides were added to ELISPOT plates coated with an anti-human IFNγ antibody (BD Biosciences) and incubated in 5% CO2 at 37°C for 24 h. The wells were then incubated with a biotinylated anti-human IFNγ antibody for 2 h at room temperature followed by an ELISPOT streptavidin-HRP antibody for 1 h. IFNγ positive spots were visualized using the ELISPOT AEC Substrate Set according to the manufacturer’s instruction (BD Biosciences).
**LDH cytotoxicity assay**

Cytotoxicity against target cells was measured by release of cellular lactate dehydrogenase (LDH) an LDH cytotoxicity detection kit according to the manufacturer’s instruction (TaKaRa, Japan). The percentage of LDH release was calculated as follows: % release = 100 × (experimental LDH release – spontaneous LDH release)/(maximal LDH release – spontaneous LDH release). Cells treated with 1% Triton X-100 were used as positive controls for maximal LDH releases.

**Mice and in vivo tumor models**

NSG mice were purchased from the Jackson laboratory. 1 × 10^5 SW480 or 3G12 cells were subcutaneously injected into NSG mice. The major (x) and minor (y) axes of the tumors were routinely measured. Tumor volume was calculated as follows: volume = πxy^2/2. In tumor-rejection models, 2.0 × 10^6 cep55-specific CTLs or PBS was intravenously injected 10 and 17 d after tumor injection. The mice were maintained in the animal facility of Sapporo Medical University and all procedures were done in accordance with the institutional animal care guidelines.

**An exogenous presentation assay using cell extracts and APC**

Preparation of cell extracts is described in detail elsewhere. Briefly, peptide extracts of SW480 or 3G12 cells were prepared by acid extraction using 10% formic acid in the presence of D of an irrelevant martyr peptide followed by filtration using <10 kDa cut-off spin-column (amicon). The samples were then dried using a vacuum centrifugation for overnight, and assayed for antigenic activities by incubation with 5 × 10^4 cells of a survivin 2B-CTL clone along with 5 × 10^4 cells of T2-A24 cells. The produced IFNγ was detected using ELISPOT assay.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

**Funding**

This study is supported by Japan Society for the Promotion of Science (T.K.), Suhara Kinen Zaidan (T.K.), Grants-in-Aid for Regional R&D Proposal-Based Program from Northern Advancement Center for Science & Technology of Hokkaido Japan (T.K.), Japan Agency for Medical Research and development (T.T.), Grants-in-Aid of Ono Cancer Research Fund (T.T.), Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (N.S.), and Health and Labor Sciences Research Grants (N.S.).

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