Identification of a Novel Tumor-Associated Antigen, Cadherin 3/P-Cadherin, as a Possible Target for Immunotherapy of Pancreatic, Gastric, and Colorectal Cancers

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Abstract

Purpose: To establish cancer immunotherapy, it is important to identify the tumor-associated antigens (TAA) that are strongly expressed in the tumor cells but not in the normal cells. In this study, to establish an effective anticancer immunotherapy, we tried to identify the useful TAA of pancreatic cancer.

Experimental Design: Based on a previous genome-wide cDNA microarray analysis of pancreatic cancer, we focused on cadherin 3 (CDH3)/P-cadherin as a novel candidate TAA for anticancer immunotherapy. To identify the HLA-A2*0201-restricted CTL epitopes of CDH3, we used HLA-A2.1 (HHD) transgenic mice (Tgm). Furthermore, we examined the cytotoxicity against the tumor cells in vitro and in vivo of CTLs specific to CDH3 induced from HLA-A2-positive healthy donors and cancer patients.

Results: CDH3 was overexpressed in the majority of pancreatic cancer and various other malignancies, including gastric and colorectal cancers, but not in their noncancerous counterparts or in many normal adult tissues. In the experiment using HLA-A2.1 Tgm, we found that the CDH3-4655-663 (FILPVLGAV) and CDH3-7757-765 (FIENLKKAA) peptides could induce HLA-A2-restricted CTLs in Tgm. In addition, peptides-reactive CTLs were successfully induced from peripheral blood mononuclear cells by in vitro stimulation with these two peptides in HLA-A2-positive healthy donors and cancer patients, and these CTLs exhibited cytotoxicity specific to cancer cells expressing both CDH3 and HLA-A2. Furthermore, the adoptive transfer of the CDH3-specific CTLs could inhibit the tumor growth of human cancer cells engrafted into nonobese diabetic/severe combined immunodeficiency mice.

Conclusions: These results suggest that CDH3 is a novel TAA useful for immunotherapy against a broad spectrum of cancers, including pancreatic cancer.

Pancreatic cancer has a poor prognosis, with an overall 5-year survival rate of ~5% (1). A surgical resection remains the only option for a long-term survival, but patients with resectable pancreatic cancer are in the minority (9–22%; refs. 2–4). Even in these patients, however, the 5-year survival rate remains ~20% in spite of surgery with a curative intent (5, 6). Up to 80% of patients present with locally advanced or metastatic disease, and their median survival ranges from 6 to 9 months (7). It is generally thought that the presence of few signs or symptoms in the early stage, lack of an effective screening test, high rate of relapse, and poor response to current therapies contribute to the poor prognosis of this malignancy. Hence, the development of novel therapeutic modalities is an issue of great importance, and immunotherapy may be a potential treatment for pancreatic cancer.

To establish an effective antitumor immunotherapy, the identification of tumor-associated antigens (TAA) plays a key role. In the past, many TAAs in various malignancies have been identified using the method of cDNA expression cloning (8–10) and a serologic analysis of the recombinant cDNA expression library (11–15). Recently, cDNA microarray technologies have been developed, and the analyses of the gene expression profiles of cancer and normal cells have provided us an effective approach for the identification of the TAAs (16–21). As a result, we have identified the proliferation potential-related protein (16) and glypican-3 (20, 21) as ideal...
Translational Relevance

Efforts to find novel therapeutic modalities for pancreatic cancer patients have to be increased to improve poor patient prognosis, and immunotherapy could be a potentially effective treatment modality. In the current study, we identified a novel tumor-associated antigen, cadherin 3 (CDH3)/P-cadherin, which was overexpressed in various malignancies, including pancreatic cancer, whereas it was not expressed in most normal organs based on genomewide cDNA microarray analyses. We identified two HLA-A2–restricted epitopes that could induce CDH3-reactive CTLs in HLA-A2.1 (HHD) transgenic mice. In addition, CDH3-reactive CTLs were successfully induced from the peripheral blood mononuclear cells of healthy donors and cancer patients, and we showed the efficacy of the anticancer effect of CDH3-reactive CTLs in vitro and in vivo. These results suggested that CDH3 is a good candidate of immunotherapeutic target for not only pancreatic cancer but also various malignancies overexpressing CDH3. Based on the findings of the current study, we are now planning to take this study forward to the next stage, a phase I clinical trial of CDH3 peptide-based immunotherapy of broad-spectrum malignancies including pancreatic cancer. The final goal of this immunotherapy is established on an adjuvant immunotherapeutic regimen for various malignancies in combination with other therapies.

Materials and Methods

cDNA microarray analysis. A profiling of the gene expression by a cDNA microarray analysis was done, as described previously (30). The tissue samples from pancreatic cancers and adjacent noncancerous normal pancreatic tissues were obtained from surgical specimens, and all patients provided their written informed consent to participate in this study.

Mice. HLA-A2.1 (HHD) Tg; H-2Db(b–)/β2m−/− double knockout mice introduced with a human β2m-HLA-A2.1 (a1, a2)–H-2Db(b) (α3 transmembrane cytoplasmic; HHD) monochain construct gene were generated in the Département SIDA-Rétrovirus, Unite d’Immunologie Cellulaire Antivirale, Institut Pasteur (31, 32) and kindly provided by Dr. F.A. Lemonnier. Nonobese diabetic (NOD)/severe combined immunodeficiency (SCID) female mice at 6 wk of age were purchased from Charles River Japan. The mice were maintained at the Center for Animal Resources and Development of Kumamoto University, and they were handled in accordance with the animal care guidelines of Kumamoto University.

Cell lines and HLA expression. The human pancreatic cancer cell line PANC1, oral squamous cancer cell line HSC3, and a TAP-deficient and HLA-A2 (A∗0201)–positive cell line T2 were purchased from Riken Cell Bank. The human pancreatic cancer cell line PK8 was kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer, Tohoku University. The human colon cancer cell line HCT116 was kindly provided by Dr. B. Vogelstein, Johns Hopkins University. The human liver cancer cell line SK-Hep1 was kindly provided by Dr. Kyogo Ito, Kurume University. The expression of HLA-A2 was examined using flow cytometry with an anti–HLA-A2 monoclonal antibody (mAb), B72.2 (One Lambda, Inc.), to select the HLA-A2–positive blood donors and target cell lines for the cytotoxicity assays. These cells were maintained in vitro in RPMI 1640 or DMEM supplemented with 10% FCS in a 5% CO₂ atmosphere at 37°C.

Patients, blood samples, and tumor tissues. The clinical research using PBMCs from the donors was approved by the Institutional Review Board of Kumamoto University. The blood samples and the cancer and adjacent noncancerous tissues were obtained during routine diagnostic procedures after obtaining formal written informed consents by the patients in Kumamoto University Hospital. We also obtained blood samples from healthy donors after receiving their written informed consent. All samples were anonymized, numbered at random, and stored at -80°C until use. All patients and healthy donors were of Japanese nationality.

Reverse transcription–PCR and Northern blot analysis. The reverse transcription–PCR (RT-PCR) analysis of the normal and cancer tissues and cell lines was done as described previously (33). The primer sequences were as follows: CDH3, sense 5′-GTTCCCCTTCCCCAGA-GACTGA-3′ and antisense 5′-CCTCAATGATCCCTTGC-3′ and β-actin, sense 5′-CATCCAGCAAACTCCATCACT-3′ and antisense 5′-TCTCCCTGAAAGTGCCG-3′. After normalization by β-actin mRNA as a control, we compared the expression of CDH3 mRNA in the tissues and cell lines. A Northern blot analysis was done as described previously by using a CDH3 gene-specific cDNA probe (365–1198 bp; ref. 34).

Immunohistochemical staining. Immunohistochemical examinations of CDH3 protein were done as described previously (15, 16). The primary antibody used in this study, anti-CDH3 mAb, was purchased from BD Transduction Laboratories.

Lentiviral gene transfer. A lentiviral vector-mediated gene transfer was done as described (35). Briefly, 17 μg of CSII-CMV-RNA and CSIIEF-RNA self-inactivating vectors (36) carrying CDH3 cDNAs and 10 μg of pCMV-VSV-G-RSV-Rev and pCAG-HIVgp were transinfected into the 293T cells grown in the 10-cm culture dish using Lipofectamine 2000 reagent (Invitrogen Corporation). After 60 h of transfection, the medium was recovered and the viral particles were pelleted by ultracentrifugation (50,000 × g, 2 h). The pellet was suspended in 50 μL of RPMI 1640, and 10 μL of viral suspension were added to 5 × 10⁶ PANC1 cells or
CDH3 as a Novel Target for Anticancer Immunotherapy

Results

Identification of CDH3 gene up-regulated in pancreatic cancer and various malignancies based on a cDNA microarray. Using a genome-wide cDNA microarray containing 27,648 genes, we had previously examined the gene expression profiles of 18 pancreatic cancer tissues and their adjacent normal counterparts. After the analysis, we chose six genes of which the relative expression ratio was more than five times higher in pancreatic cancer tissues compared with their normal counterparts (CDH3, KIF20A, MICAl2, TRIM29, ABHD6, and EphA4). Among them, CDH3 was selected using the BIMAS software program (BioInformatics and Molecular Analysis Section, Center for Information Technology), and 18 peptides were synthesized (American Peptide Company; Supplementary Table S1). The immunizations of the mice with peptides were done as previously described (34). The frequency of cells producing IFN-γ/mL CD4⁺ spleen cells upon stimulation with syngeneic bone marrow–derived dendritic cells (BM-DC; 1 × 10⁶ per well) pulsed with or without each peptide was analyzed by an enzyme-linked immunospot (ELISPOT) assay as previously described (21).

Induction of CDH3-reactive human CTLs. We isolated the PBMCs from the hepatodermal blood of HLA-A2–positive Japanese patients with pancreatic, gastric, and colorectal cancers or healthy donors, by means of Ficoll-Conray density gradient centrifugation, and the peripheral monocyte-derived dendritic cells (DC) were generated as described previously (16, 21). The DCs were pulsed with 20 μg/mL of the candidate peptides in the presence of 4 μg/mL β2-microglobulin (Sigma-Aldrich) for 2 h at 37°C in AIM-V (Invitrogen) containing 2% heat-inactivated autologous plasma. The cells were then irradiated (40 Gy) and incubated with the CD8⁺ T cells. These cultures were set up in 24-well plates; each well contained 1 × 10⁵ peptide-pulsed DCs, 2 × 10⁶ CD8⁺ T cells, and 5 ng/mL human recombinant interleukin-7 (Wako, Osaka, Japan), in 2 mL AIM-V with 2% autologous plasma. After 2 d, these cultures were supplemented with human recombinant interleukin-2 (PeproTec, Inc.) to a final concentration of 20 IU/mL. Two additional weekly stimulations with peptide-loaded autologous DCs, using the same procedure, were carried out on days 7 and 14. Six days after the last stimulation, the antigen-specific responses of the induced CTLs were investigated by a ¹¹¹Indium release assay and an IFN-γ ELISPOT assay.

CTL responses against cancer cell lines. The CTLs were cocultured with each of the cancer cells or the peptide-pulsed T2 cells as a target cell (5 × 10⁵ per well) at the indicated effector/target ratio, and a standard ¹¹¹Indium release assay was done as described previously (21). The blocking of HLA class I or HLA-DR was done as described previously (21). In brief, before the coculture of the CTLs with a cancer cell line in the ¹¹¹Indium release assay or ELISPOT assay, the cancer target cells were incubated for 1 h with 10 μg/mL anti-class I mAb, W6/32, or 10 μg/mL anti–HLA-DR mAb, H-D1-1, or the effects of the mAbs on either the cytotoxic activity or the production of IFN-γ by the CTLs were examined as reported previously (37).

An adoptive immunotherapy model. An experimental adoptive immunotherapy was done as described previously (21). Briefly, we s.c. inoculated HCT116 cells (4 × 10⁶) positive for both HLA-A2 and endogenous CDH3 at the right flank of the NOD/SCID mice. When the tumor size became 25 mm² on day 7 after the tumor inoculation into the mice, the CDH3 peptide 4655-663 or 8757-763-specific CTL lines and/or without each peptide was analyzed by an enzyme-linked immunospot (ELISPOT) assay as previously described (21).

Statistical analysis. The two-tailed Student’s t test was used to evaluate the statistical significance of differences in the data obtained by the ELISPOT assay and in the tumor size between the treatment groups. A value of P < 0.05 was considered to be significant. The statistical analysis was done using a commercial statistical software package (SPSS for Windows, version 11.0).

Results

Identification of CDH3 gene up-regulated in pancreatic cancer and various malignancies based on a cDNA microarray. Using a genome-wide cDNA microarray containing 27,648 genes, we had previously examined the gene expression profiles of 18 pancreatic cancer tissues and their adjacent normal counterparts. After the analysis, we chose six genes of which the relative expression ratio was more than five times higher in pancreatic cancer tissues compared with their normal counterparts (CDH3, KIF20A, MICAl2, TRIM29, ABHD6, and EphA4). Among them, CDH3 was selected using the BIMAS software program (BioInformatics and Molecular Analysis Section, Center for Information Technology), and 18 peptides were synthesized (American Peptide Company; Supplementary Table S1). The immunizations of the mice with peptides were done as previously described (34). The frequency of cells producing IFN-γ/mL CD4⁺ spleen cells upon stimulation with syngeneic bone marrow–derived dendritic cells (BM-DC; 1 × 10⁶ per well) pulsed with or without each peptide was analyzed by an enzyme-linked immunospot (ELISPOT) assay as previously described (21).
including four embryonic tissues (Fig. 1B; refs. 16, 21). Consequently, we focused on CDH3 as a novel TAA of pancreatic cancer. The expression of the CDH3 gene in pancreatic cancer tissues was markedly enhanced in all of the 16 patients tested (average of the relative expression ratio, 1,900,000; range, 94,900–4,890,000). In addition, the CDH3 gene was faintly expressed only in ovary and mammary gland (Fig. 1B). The expression level of the CDH3 gene was also enhanced in the majority of various malignancies, including gastric and colorectal cancers, based on the previous cDNA microarray analyses (Table 1; refs. 30, 38–41).

Expression of CDH3 mRNA and protein in normal organs, cancer cell lines, and pancreatic, gastric, and colorectal cancer tissues. The expression of the CDH3 gene in normal tissues at the mRNA level was analyzed using RT-PCR and Northern blot analysis. A semiquantitative RT-PCR analysis of CDH3 in the normal tissues revealed that it was faintly expressed only in thymus and fetal brain (Fig. 2A, left). A Northern blot analysis in normal organs using CDH3 cDNA as a probe revealed that it was not expressed in all nine vital organs (Fig. 2A, right). The expression of the CDH3 gene was detected in the various cancer cell lines using an RT-PCR analysis (Fig. 2B).

Subsequently, we analyzed the expression of the CDH3 gene using an RT-PCR analysis in the cancer tissues and their adjacent normal counterparts, which were surgically resected. The expression of the CDH3 gene was detected in six of eight pancreatic cancer tissues, but little expression was detected in their normal counterparts (Fig. 2C). In addition, its expression was detected in the metastatic foci of the skin and peritoneum (Fig. 2D, a–d). Furthermore, strong staining was also observed in gastric and colorectal cancer tissues (Fig. 2D, g and h). No staining was detected in the tissue specimens of all three independent tumor-forming pancreatitis tested (Fig. 2D, i).

Identification of CDH3-derived and HLA-A2–restricted mouse CTL epitopes using HLA-A2.1 (HHD) Tgm. To identify the CDH3-derived and HLA-A2–restricted CTL epitopes, we selected a total of 18 different candidate 9 or 10 amino acid peptides that were expected to have a higher binding affinity to HLA-A2 (A*0201), the most common HLA allelic product worldwide, by the HLA Peptide Binding Predictions in the NIH BIMAS (Supplementary Table S1). To test which could induce peptide-reactive CTLs, the CD4+ spleen cells from HLA-A2.1 (HHD) Tgm, immunized i.p. twice with BM-DCs pulsed with the mixture of these 18 peptides, were again stimulated in vitro with BM-DCs pulsed with each peptide. We found that the CD4+ spleen cells, stimulated with CDH3-4655-663 (FIIENLKAA) and CDH3-7757-765 (FIENLKAA) peptide, produced a large amount of IFN-γ in a peptide-specific manner in an ELISPOT assay (Fig. 3A and B). These CD4+ spleen cells (2 × 10^4) showed 283.7 ± 40.0 spot counts per well in response to the BM-DCs pulsed with the CDH3-4655-663 peptide, whereas they showed 48.7 ± 11.9 spot counts per well in the presence of the BM-DCs without peptide loading (P < 0.05). Likewise, the CD4+ spleen cells stimulated with BM-DCs pulsed with CDH3-7757-765 peptide showed 79.3 ± 3.2 spot counts per well, whereas they showed 42.7 ± 2.5 spot counts per well in the presence of BM-DCs without peptide loading (P < 0.05). These results suggest that the CDH3-4655-663 and CDH3-7757-765 peptides could be the HLA-A2–restricted CTL epitope peptides in the HLA-A2.1 (HHD) Tgm, and we also expected these peptides to be epitopes for human CTLs.

### Table 1. Expression of CDH3 gene in pancreatic cancer and various malignancies investigated by cDNA microarray analyses

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Positive rate (%)</th>
<th>Average of relative expression ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic cancer</td>
<td>100</td>
<td>1,900,000</td>
</tr>
<tr>
<td>Testicular cancer</td>
<td>100</td>
<td>396,000</td>
</tr>
<tr>
<td>Soft tissue tumor</td>
<td>100</td>
<td>248,000</td>
</tr>
<tr>
<td>Cholangiocellular carcinoma</td>
<td>100</td>
<td>3,600</td>
</tr>
<tr>
<td>Non-small cell lung cancer</td>
<td>95</td>
<td>73,000</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>91</td>
<td>84,000</td>
</tr>
<tr>
<td>Cervical cancer</td>
<td>74</td>
<td>1,500</td>
</tr>
<tr>
<td>Gastric cancer</td>
<td>71</td>
<td>35,000</td>
</tr>
<tr>
<td>Urinary bladder cancer</td>
<td>71</td>
<td>30</td>
</tr>
<tr>
<td>Small cell lung cancer</td>
<td>21</td>
<td>7</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>4</td>
<td>1,500</td>
</tr>
<tr>
<td>Renal cell carcinoma</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Esophageal cancer</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

NOTE: Data are obtained from our previous studies (30, 38–41).

*The relative expression ratio (cancer/normal tissue) of >5 was considered to be positive.
Induction of CDH3-reactive CTLs from PBMCs of HLA-A2-positive healthy donors and cancer patients. We attempted to generate CDH3-specific CTLs from the PBMCs of healthy donors and various cancer patients positive for HLA-A2 by the stimulation with the CDH3-4655-663 and CDH3-7757-765 peptides. The CD8+ T cells sorted from the PBMCs were incubated with the autologous monocyte-derived DCs pulsed with each peptide. After three stimulations, the cytotoxic activity against the peptide-pulsed T2 cells was examined by a 51Cr release assay (Fig. 4A) and an IFN-γ ELISPOT assay (data not shown). The CTLs induced from the PBMCs of a healthy donor exhibited cytotoxic activity to the T2 cells pulsed with CDH3-4655-663 or CDH3-7757-765 peptide, but not to the T2 cells without peptide loading. Similar responses were observed in other donors (data not shown). These results indicate that these CTLs had a peptide-specific cytotoxicity.

Fig. 2. The analyses of CDH3 mRNA expressed in human normal tissues, cancer cell lines, and cancer tissues. A, expression of CDH3 mRNA was investigated in various normal tissues by using RT-PCR (left) and Northern blot analysis (right). CDH3 mRNA was faintly expressed only in thymus and fetal brain. B, RT-PCR analysis of the CDH3 expression in various cancer cell lines. The expression of the CDH3 gene was detected in six of eight pancreatic cancer tissues, two of four gastric cancer tissues, and six of seven colorectal cancer tissues. In contrast, little expression was detected in their normal counterparts. C, immunochemistry analysis of CDH3 protein in pancreatic, gastric, and colorectal cancer tissues. Positive staining signals are seen as brown. Scale bars, 100 μm.
and SKHep1/CDH3 (CDH3⁺, HLA-A2⁺), SKHep1 cells transfected with the CDH3 gene, as target cells to confirm that these peptides were processed naturally from the CDH3 protein in the cancer cells. As shown in Fig. 4C, the CTLs induced by stimulation with CDH3-4,655-663 or with CDH3-7,757-765 peptide revealed cytotoxicity against HCT116, PANC1/CDH3, and SKHep1/CDH3, but not to PANC1, SKHep1, or PK8. These results suggest that these peptides could be naturally processed and expressed on the surface of cancer cells in the context of HLA-A2 molecules. As a result, the CDH3-reactive CTLs had specific cytotoxicity to the cancer cells expressing both endogenous CDH3 and HLA-A2 molecules.

To confirm that the induced CTLs recognized the target cells in an HLA class I–restricted manner, we used the mAb against HLA class I (W6/32) to block the recognition by the CTLs. In this experiment, the anti–HLA class I antibody could markedly inhibit the IFN-γ production stimulated with SKHeP1/CDH3 cells in an ELISPOT assay of the CTLs generated by stimulation with CDH3-4,655-663 peptide, with statistical significance (Fig. 4D, left; P < 0.01), and could inhibit the cytotoxicity of the CTLs against the HTCT116 cells in a ⁵¹Cr release assay (Fig. 4D, middle). Similarly, the anti–class I antibody could markedly inhibit the IFN-γ production stimulated with HSCh3 cells in an ELISPOT assay of the CTLs generated by stimulation with CDH3-7,757-765 peptide (Fig. 4D, right; P < 0.05). These results clearly indicate that these induced CTLs recognized the target cells expressing CDH3 in an HLA class I–restricted manner.

In vivo antitumor activity of adoptively transferred CDH3-induced human CTLs in NOD/SCID mice. To assess the therapeutic efficacy of the CDH3-reactive CTL inoculation into the mice implanted with the CDH3⁺ human cancer cell, we inoculated s.c. HTCT116 positive for both CDH3 and HLA-A2 into NOD/SCID mice and injected i.v. human CTLs generated by stimulating the peripheral blood CD8⁺ T cells with CDH3-4,655-663 and CDH3-7,757-765 peptides or control-irrelevant HIV peptide into mice when the diameter of these tumors reached 5 × 5 mm in size, as described in Materials and Methods. The control HIV peptide–stimulated CD8⁺ T cells did not exhibit cytotoxicity against the HCT116 cells in vitro (data not shown). The tumor size of seven individual mice in each group (Fig. 5A) and the mean ± SD of the tumor sizes in each group (Fig. 5B) were evaluated. The control T-cell lines did not exhibit an inhibitory effect on the tumor growth, and PBS alone did not do so. The tumor size in the mice inoculated with the CDH3-stimulated CTLs was significantly smaller than those inoculated with the control HIV peptide–induced CD8⁺ T cells or with PBS alone (P < 0.001). These results clearly indicate the efficacy of adoptive transfer therapy of CDH3-reactive human CTLs against CDH3⁺ human tumor in NOD/SCID mice.

Discussion

In the current study, we identified a novel TAA, CDH3/P-cadherin, using a cDNA microarray analysis of pancreatic cancer. CDH3 was strongly expressed in pancreatic cancer cells and faintly expressed in ovary and mammary gland based on the cDNA microarray analysis. The CDH3 expression was barely detectable in other vital organs. Furthermore, our microarray and RT-PCR data showed that CDH3 was expressed in gastric and colorectal cancers, as well as in pancreatic cancer, and not in their

**Fig. 3.** Identification of HLA-A2–restricted mouse CTL epitopes of human CDH3 by using HLA-A2.1 (HHD) Tgm. A, we immunized the HLA-A2.1 (HHD) Tgm with 5 × 10⁵ syngeneic BM-DCs pulsed with 18 candidate peptides in vivo at days 7 and 14. On day 21, CD4⁺ spleen cells isolated from immunized mice were stimulated by BM-DCs pulsed with each peptide for 6 d. The CTL-producing IFN-γ was detected by an ELISPOT assay. We found that CDH3-4,655-663 (FILPVLGAV) and CDH3-7,757-765 (FIIENLKAA) were shown to induce peptide-reactive CTL. B, CD4⁺ spleen cells showed 283.7 ± 40.0 spot counts per well, in response to the BM-DC pulsed with the CDH3-4,655-663 peptide (top left), whereas they showed 48.7 ± 11.9 spot counts per well in the presence of BM-DC without peptide loading (bottom left; P < 0.05). Similarly, CD4⁺ spleen cells showed 79.3 ± 3.2 spot counts per well, in response to the BM-DC pulsed with the CDH3-7,757-765 peptide (top right), whereas they showed 42.3 ± 2.5 spot counts per well in the presence of BM-DC without peptide loading (bottom right; P < 0.05). These assays were done twice with similar results.

Subsequently, we asked whether these CTLs were able to kill human cancer cell lines expressing CDH3 and HLA-A2. As shown in Fig. 4B, the CDH3-reactive CTLs stimulated with CDH3-4,655-663 peptide exhibited cytotoxicity to HCT116 (CDH3⁺, HLA-A2⁺), HSCh3 (CDH3⁺, HLA-A2⁺), and PANC1/CDH3 (CDH3⁺, HLA-A2⁺), PANC1 cells transfected with CDH3 gene, but not to PANC1 (CDH3⁺, HLA-A2⁺), SKHep1 (CDH3⁺, HLA-A2⁺), and PK8 (CDH3⁺, HLA-A2⁻), in healthy donors. Similarly, the CTLs stimulated with CDH3-7,757-765 peptide exhibited cytotoxicity to HSCh3, but not to PANC1, PK8, or SKHep1.

These cytotoxic activities were observed in the CTLs derived from various cancer patients (Fig. 4C). We used PANC1/CDH3 and SKHep1/CDH3 (CDH3⁺, HLA-A2⁺), SKHep1 cells transfected with the CDH3 gene, as target cells to confirm that these peptides were processed naturally from the CDH3 protein in the cancer cells. As shown in Fig. 4C, the CTLs induced by stimulation with CDH3-4,655-663 or with CDH3-7,757-765 peptide revealed cytotoxicity against HCT116, PANC1/CDH3, and SKHep1/CDH3, but not to PANC1, SKHep1, or PK8. These results suggest that these peptides could be naturally processed and expressed on the surface of cancer cells in the context of HLA-A2 molecules. As a result, the CDH3-reactive CTLs had specific cytotoxicity to the cancer cells expressing both endogenous CDH3 and HLA-A2 molecules.

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normal counterparts. According to the findings of immunohis-tochemical analyses, CDH3 was overexpressed in the majority of pancreatic cancer cells, whereas the normal duct and the acinar cells in the pancreas showed a very weak expression at the protein level. These results suggest that targeting CDH3 could be a novel immunotherapeutic approach for these cancers, without the development of autoimmune diseases.

The cadherin family is divided into several subfamilies, including CDH1/E-cadherin, CDH2/N-cadherin, and CDH3/P-cadherin, designated by their tissue distribution. CDH1 is the predominant cadherin family member expressed in all epithelial tissues. It is well known that CDH1 is assumed to act as a tumor suppressor, negatively regulating the invasion and metastasis of tumor cells, in several malignancies (42–44). CDH2 is up-regulated in several invasive cancers and contributes to an invasive phenotype by interacting with fibroblast growth factor receptor and by downstream signaling (45). The expression and role of CDH3 in cancer are still poorly understood. In a previous study, Taniuchi et al. showed the up-regulation of CDH3 to be likely related to the biological aggressiveness of pancreatic cancer by interacting with p120ctn and activating Rho family GTPase, Rac1, and Cdc42 (46). Additional previous studies suggested the up-regulation of CDH3 to be a factor in the aggressive biological behavior and poor prognosis of both breast (26–28) and endometrial cancers (29). A recent report summarized that the objective response rate of cancer vaccine in clinical trials was low (2.6%; ref. 47). One possible reason is that the immune escape of cancer cells attributed to deletion, mutation, or down-regulation of the TAAs occurs as a consequence of therapeutically driven immune selection. Based from the standpoint that tumor cells cannot lose antigens which are required for tumorigenesis, we considered CDH3 as a candidate TAA useful for anticancer immunotherapy.

In this study, we identified two HLA-A2–restricted CDH3 epitope peptides, which could stimulate the generation of

**Fig. 4.** Induction of CDH3-specific human CTL from the PBMCs of HLA-A2+ positive healthy donors and cancer patients. A, the CDH3 peptide-reactive CTLs were generated from the PBMCs of HLA-A2+ positive healthy donors. After three stimulations with autologous monocyte-derived DCs pulsed with the CDH3-4655-663 (top) or CDH3-7757-765 (bottom) peptide, the cytotoxicity of the CTLs against T2 cells (HLA-A2, TAP deficient), pulsed with each peptide or peptide-unpulsed T2 cells, was detected by standard a ^51Cr^ release assay. These CTLs exhibited cytotoxicity to CDH3-4655-663 (top) or CDH3-7757-765 (bottom) peptide pulsed T2 cells, but not to peptide-unpulsed T2 cells. B, these CTLs exhibited cytotoxicity to the CDH3+ HLA-A2+ human colon cancer cell line HCT116 and oral squamous cancer cell line HSC5 and PANCl/CDH3, a CDH3+ HLA-A2+ human pancreatic cancer cell line transfected with the human CDH3 gene, but not to CDH3- HLA-A2+ human liver cancer cell line SKHep1, PANCl, nor CDH3+ HLA-A2+ human pancreatic cancer cell line PK8. C, the CDH3-reactive CTLs generated from the PBMCs of HLA-A2+ positive pancreatic and gastric cancer patients exhibited cytotoxicity to HCT116, PANCl/CDH3, and SKHep1/CDH3, a CDH3- HLA-A2+ human liver cancer cell line transfected with the human CDH3 gene, but not to PANCl, SKHep1, nor PK8. D, inhibition of cytotoxicity by anti-HLA class I mAb. After the target cells, SKHep1/CDH3 and HSC5 were incubated with anti-HLA class I mAb (W6/32, IgG2a) or anti-HLA-DR mAb (H-DR-1, IgG2a), respectively, for 1 h, the CTLs generated from the PBMCs of healthy donors by stimulation with CDH3-4655-663 (middle left) or CDH3-7757-765 (right) peptide were added. IFN-γ production (left and right; IFN-γ ELISPOT assay) and cytotoxicity (middle; ^51Cr^ release assay) were markedly inhibited by W6/32, but not by H-DR-1.
HLA-A2–restricted mouse CTLs by the vaccination of HLA-A2.1 (HHD) Tgm with the 18 candidate peptides predicted by the BIMAS algorithm. Furthermore, we found that the CDH3-reactive CTLs could be generated from PBMCs stimulated with these peptides in healthy donors and cancer patients (Fig. 4). These CTLs could kill not only the T2 cells pulsed with its corresponding peptide but also the cancer cell lines expressing CDH3 in an HLA-A2–restricted manner. These data suggest that these CDH3 peptides (CDH3-4655-663 and CDH3-7757-765) are naturally processed from CDH3 protein in cancer cells and presented onto the cell surface together with HLA-A2 molecules to be recognized by the CTLs.

HLA-A2 (A*0201) is one of the most common HLA alleles in various ethnic groups, including Asians, Africans, Afro-Americans, and Caucasians (48). The identification of the HLA-A2–restricted and CDH3-derived CTL epitopes has also been suggested to be useful for the immunotherapy of many patients with pancreatic cancer all over the world.

We confirmed the cytotoxicity of the CDH3-reactive CTLs not only in vitro by a 51Cr release assay but also in vivo by a CTL adoptive transfer model. As shown in Fig. 5, we observed that i.v. transferred CTLs inhibited the growth of tumor cells engrafted into NOD/SCID mice compared with the mice injected with control CD8+ T cells or PBS alone. Although the inhibition of tumor growth was observed for a while after the i.v. transfer of the CTLs, thereafter, the tumor gradually enlarged. We thought that it was important to continue the transfer of the CTLs again and again to obtain the continuous regression of the tumor. These data suggest that the adoptive transfer of CDH3-reactive human CTLs into mice bearing human tumors expressing CDH3 could effectively inhibit tumor growth, at least in the NOD/SCID mouse tumor model.

It is considered to be very important to investigate whether the targeting CDH3 could induce autoimmune diseases, either during or after anticancer immunotherapy. After performing vaccinations twice with the epitope peptides identified in this study, we checked the autoimmune phenomenon in HLA-A2.1 (HHD) Tgm. As a result, any clinical symptoms, such as weight loss, diarrhea, or skin abnormalities, or any pathologic changes, such as lymphocyte infiltration or tissue destruction, were not observed (data not shown). However, the amino acid sequences of these two epitope peptides are not conserved between human and mouse CDH3. There is one amino acid replacement between human and mouse CDH3-4655-663 peptide (human, FILPVLGAV; mouse, FILPILGAV) and CDH3-7757-765 peptide (human, FIIENLKAA; mouse, FIIENLKPA). Therefore, we could not accurately check the capacity of autoimmune disease-inducing activity of two CDH3-derived peptides in our mouse system.

In conclusion, our results suggest that CDH3 is a novel TAA of which epitope peptides could elicit CTLs to cancer cells expressing CDH3 in an HLA-A2–restricted manner. As CDH3 is highly expressed in a wide range of human malignancies, including pancreatic cancer, CDH3 is therefore suggested to be a promising target of peptide-based immunotherapy for a broad spectrum of malignancies, without causing any autoimmune phenomena.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Fig. 5. in vivo antitumor activity of the CDH3-induced human CTLs adoptively transferred into NOD/SCID mice transplanted with human cancer cells. A, marked inhibition of growth of a human colorectal cancer cell line, HCT116 (CDH3+, HLA-A2*), engrafted into NOD/SCID mice after the adoptive CTL transfer. When the tumor size reached 25 mm² on day 7 after s.c. tumor implantation, human CTLs (4 × 10⁶) reactive to CDH3-4655-663 (○) and to CDH3-7757-765 (■) peptide were inoculated i.v. On day 14, the same CTL inoculation was repeated. The control CD8+ T cells stimulated with irrelevant HLA-A2–restricted HIV peptide (△), which did not show cytotoxicity against HCT116 in vitro, were also inoculated into mice as a control. Tumor volumes in NOD/SCID mice given twice, on days 7 and 14, with CDH3-induced CTLs (n = 7), control CD8+ T cells (n = 7), or PBS alone (○, n = 7). Tumor size is expressed in square millimeters. B, comparison of mean tumor sizes in each group of mice; bars, SD (n = 7).
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