Identification of a Novel Cancer-Testis Antigen CRT2 Frequently Expressed in Various Cancers Using Representational Differential Analysis

Emiko Hayashi,1 Yuriko Matsuzaki,1 Go Hasegawa,1 Tomonori Yaguchi,1 Sachiko Kurihara,1 Tomonobu Fujita,1 Toshiro Kageshita,3 Makoto Sano,2 and Yutaka Kawakami1

Abstract

Purpose: Cancer-testis antigens are promising targets for cancer immunotherapy. Identification of additional cancer-testis antigens with frequent expression in various cancers was attempted using representational differential analysis (RDA) and immunogenicity evaluation.

Experimental Design: cDNAs preferentially expressed in testis were enriched using RDA by subtraction between testis and normal tissues. Thirty clones showing cancer-testis-like expression based on EST database analysis were evaluated by reverse transcription-PCR. A potential antigen, CRT2, was identified and its expression was analyzed with a newly generated anti-CRT2 antibody. The immunogenicity of CRT2 was examined based on reactivity with serum immunoglobulin G (IgG) from cancer patients, using Western blot and ELISA analysis, and on in vitro induction of tumor-reactive CTLs from HLA-A24 transgenic mice and human peripheral blood lymphocytes.

Results: CRT2 was expressed in elongated spermatids of testis among normal tissues and in various cancer cell lines and tissues. The recombinant CRT2 protein was recognized by serum IgG from patients with various cancers in Western blot and ELISA analyses. A CRT2-derived peptide was identified as an HLA-A24-restricted T-cell epitope that induced tumor-reactive CTLs.

Conclusion: CRT2 was identified as a new cancer-testis antigen expressed in elongated spermatids of testis and in cancer tissues (particularly melanoma) that is recognized by serum IgG from cancer patients. An HLA-A24-restricted T-cell epitope capable of inducing tumor-reactive CTLs was identified, suggesting that CRT2 may be useful for cancer diagnosis and immunotherapy.

Identification of human tumor antigens recognized by T cells is important in development of cancer immunotherapy (1–4). A variety of tumor antigens have been identified, and among these, cancer-testis antigens are promising targets (5–7). Cancer-testis antigens are preferentially expressed in various cancers and in some normal tissues including testis, ovary, and placenta. Such antigens have previously been isolated using cDNA expression cloning with tumor-reactive T cells or with serum immunoglobulin G (IgG) antibodies from cancer patients (SEREX; refs. 8–10); DNA homology searches using public gene databases; and cDNA subtraction between cDNA libraries from testis and other normal tissues. Representational differential analysis (RDA) is a cDNA subtraction method that uses suppression PCR to identify preferentially expressed genes, even if these genes have low expression (11, 12).

In this study, we attempted to identify additional cancer-testis antigens that are frequently expressed in various cancers by evaluating the immunogenicity of proteins identified by RDA to be expressed in cancers and normal testis. A candidate antigen, CRT2, was identified based on immunoreactivity with serum IgG of cancer patients and induction of tumor-reactive CTLs by HLA-binding antigen-derived peptides. CRT2, which is homologous to calreticulin (CRT; ref. 13), may be useful for cancer diagnosis and immunotherapy.

Materials and Methods

Cell lines, tissue samples, and total RNA. The cell lines used in the study were melanoma, SKmel23, SKmel28, 888mel, A375mel, 1363mel, 928mel, 864mel, 526mel, 501mel, 397mel, 1362mel, 115mel, 938mel, 1102mel, C32mel, MMG1, MMG3, G361, HT144, RPMI7951, Malme3M, HS294T, WM266mel, and DC201 MEL [Surgery Branch, National Cancer Institute, NIH and American Type Culture Collection (ATCC)]; colon cancer COLO205 (ICRB); breast cancer HS578 (ATCC); endometrial cancer SNGII (Keio
University); renal cell cancer Caki-1 (ATCC) and RCC7 (Surgery Branch, National Cancer Institute); prostate cancer LNCaP (ATCC); bladder cancer KU7 (Keio University); and brain tumor U87MG (ATCC). These cell lines were maintained in 10% fetal bovine serum RPMI 1640. NIH3T3, K4B, 293, and COS7 cells were purchased from ATCC, 888EBV-B, 1088EBV-B, and LG2 EBV-B (EBV-transformed B cell lines) have previously been described (14). Melanocytes and keratinocytes were purchased from Kurabo. Primary cultured fibroblasts and activated T cells were generated in our laboratory.

Total RNA from normal tissues including brain, heart, kidney, spleen, liver, small intestine, muscle, lung, testis, uterus, placenta, ovary, bladder, prostate, pancreas, esophagus, stomach, colon, adipose, bone marrow, retina, fetal brain, fetal thymus, thymus, and fetal liver were purchased from Clontech. Total RNA from esophagus was purchased from Biochain Institute, Inc. The malignant melanoma, esophageal cancer, gastric cancer, pancreatic ductal adenocarcinoma, colon cancer, endometrial cancer, cervical cancer, renal cancer, and brain tumor samples used in the study were surgically resected at Kumamoto University, Tohoku University, Shinshu University, Keio University Hospital, or the National Cancer Center Hospital, after informed consent was obtained according to the institution guidelines. These specimens were stored at -80°C until use.

Subcloning of cDNAs preferentially expressed in testis. cDNAs identified by RDA to have preferential expression in testis compared with other normal tissues (Human Testis-Specific PCR-Selected cDNA, Clontech) were used for subcloning. For amplification and enrichment, an aliquot of the reaction mixture was PCR amplified with nested primer 1 (5′-TGACGCCTGAGCGGCGGCTCT-3′) and primer 2 (5′-AGGCCGAGCGGCTCTGCTTG-3′). The resulting cDNAs were cloned into the pCR II vector (Invitrogen). Plasmid DNA was sequenced on an ABI Prism 3100 sequencer (Applied Biosystems).

Reverse transcription-PCR. Reverse transcription-PCR (RT-PCR) of CRT2 was done using the following primers: forward 1 (5′-CTAGACGAGACGACATGAGAAG-3′), forward 2 (5′-GGAATTCAATAAGGCAGCAATCCTCTGAG-3′), forward 3 (5′-AAGAATGAAAGGCAGCCTCTG-3′), and reverse 1 (5′-TAGACGAGACGACATGAGAAG-3′). cDNA was synthesized with an oligo-dT primer using total RNA from various normal tissues, normal cell lines, tumor tissues, and tumor cell lines. RT-PCR was done for 35 cycles, each consisting of 30 s of denaturation at 94°C, 30 s of annealing at 57°C, and 30 s of extension at 72°C. CRT2-RT-PCR was also done after treatment with 5 μmol/L 5-aza-2′-deoxycytidine (Sigma-Aldrich) for 2 and 4 days.

Preparation of CRT2 bacterial recombinant proteins, production of a polyclonal antibody, and generation of CRT2-transfected cells. PCR products containing the full-length human CRT2 and the splicing variant (CRT2Δ3) resulting from skipping of exon 3 were used to generate bacterial and mammalian recombinant CRT2. The PCR primers were 5′-tagggacctcgacctggcggcgccctttgtgcc-3′ (forward) and 5′-atggcggcctcaatagaaatcttcctcctgc-3′ (reverse). The PCR products were digested with EcoRI and NotI. To generate bacterial recombinant CRT2 proteins, the digested product was subcloned into the pET16b plasmid (Novagen), which was modified to contain multiple cloning sites, and the recombinant plasmids were introduced into the E. coli strain DH5α (Novagen). The recombinant CRT2 and CRT2Δ3 proteins were purified using the affinity resin HitTrap Chelating (Amersham Biosciences). Rabbit polyclonal antibody to the recombinant CRT2Δ3 protein was generated by the Protein Purification Company. To generate CRT2-transfected cell lines, the digested product was subcloned into the pcDNA3.1 vector (Invitrogen). The pcDNA3.1-CRT2Δ3 vector was transfected into NIH3T3 cells using Effectene transfection reagent (Qiagen). After incubation for 48 h at 37°C, the transfected cells were cultivated for immunostaining.

Immunocytochemistry and immunohistochemistry. For immunocytochemistry, cultured cells were fixed with acetone for 15 min and with 4% paraformaldehyde for 1 min. After reaction with 1% H2O2 in PBS for 30 min, cells were blocked for 15 min with 5% normal swine serum and then incubated for 1 h with rabbit anti-CRT2 antibody against CRT2 (diluted 1:250). After incubation for 30 min with horseradish peroxidase-conjugated swine anti-rabbit immunoglobulin antibody (diluted 1:100; DakoCytomation), staining was developed with a 3,3′-diaminobenzidine or TrueBlue peroxidase substrate (KPL). Slides were counterstained with hematoxylin or nuclear fast red.

For immunohistochemistry, formalin-fixed and paraffin-embedded tissue sections were deparaffinized using xylene and dehydrated with ethanol. Tissues were pretreated with 0.05% protease (Sigma-Aldrich) for 15 min and incubated in 1% H2O2 in PBS for 30 min. After blocking with 5% normal goat serum for 30 min, the slides were incubated overnight at 4°C with anti-CRT2 antibody (diluted 1:500). After washing with PBS containing 0.1% Tween 20 (PBST), the slides were incubated for 1 h with horseradish peroxidase-labeled goat anti-rabbit immunoglobulin antibody (Envision Plus, DakoCytomation).

Western blot screening for serum IgG antibodies specific for CRT2. The bacterial recombinant CRT2 and CRT2Δ3 proteins (1 μg/well) were loaded on 10% SDS-PAGE gels. After transfer onto a nitrocellulose membrane (Hybond Extra C, Amersham Biosciences), the membrane was cut into 15 strips and each strip was incubated overnight at 4°C with a patient serum sample (diluted 1:100) or mouse monoclonal anti-histidine antibody (diluted 1:4,000) as a positive control (Amersham Biosciences). The strips were washed in TBS containing 0.1% Tween 20 and incubated for 1 h with goat anti-human IgG Fc antibody conjugated with alkaline phosphatase or goat anti-mouse IgG Fc antibody conjugated with alkaline phosphatase (each diluted 1:4,000; Cappel).

ELISA for detection of anti-CRT2 IgG antibodies. The bacterial recombinant CRT2Δ3 protein and junction peptide (Invitrogen) were diluted in 10 mmol/L CAPS buffer or PBS containing 1% DMSO to a final concentration of 2 μg/mL, dispersed into 96-well plates (100 μL/well), and incubated overnight at 4°C. Sera from cancer patients and healthy controls (100 μL; 1:10 dilution) were added to the junction peptide solution or a negative peptide solution (10 μg/mL) and incubated overnight at 4°C. Serum samples were added to each well and incubated for 2 h at room temperature. After incubation with 100 μL of goat anti-human IgG Fc labeled with horseradish peroxidase (1:5,000 dilution; Cappel), the plates were washed with PBST and developed with tetramethylbenzidine solution for 20 min. After washing the plates with 1 mol/L H2SO4, the absorbance was measured at 450 nm. All serum samples were measured in duplicate and were randomly dispensed on the plates.

Induction of CRT2-specific T cells using HLA-A*2402 transgenic mice. Four potential T-cell epitopes of CRT2 (p48: corresponding to amino acids 48-56, sequence HFRLSSGRF; p128: amino acids 128-136, YYIMGF; p240: amino acids 240-248, RSGTIFDNE; and p299: amino acids 299-307, KNRHELHV) were predicted by three programs [Qbag (15), BIMAS, and SYFPEITHI] based on HLA-A*2402 binding affinities. Each candidate peptide (100 μg) and a Λ5-restricted helper peptide (100 μg) from tetanus toxoid (amino acids 947-967, FNFTISVSWFVLRVPVSASHL) were mixed and emulsified in incomplete Freund's adjuvant. Six- to eight-week-old HLA-A*2402 transgenic mice ( kindly provided by Dr. H. Takezaki, Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan; ref. 16) were immunized twice in the hind footpad with a 14-day interval. Seven days after final boosting, CD8+ cells were separated from splenocytes using mouse CD8a (Ly-2) Microbeads (Miltenyi Biotec GmbH), and the peptide-specific response was estimated by a murine IFN-γ ELISPOT assay (17). CD8+ cells (2 × 106) were cultured with HLA-A*2402/K0-transfected Jurkat cells (1 × 106; ref. 16) along with peptide (1 μg/mL) and interleukin-2 (10 units/mL) for 40 h, and spots were counted. MYEOV p252 peptide (amino acids 252-260, LPLRVAGSW) was used as a negative control.

In vitro induction of CTLs from human peripheral blood mononuclear cells by stimulation with synthetic peptides. In vitro CTL induction using peripheral blood mononuclear cells (PBMC) from healthy volunteers was done as previously described (18). PBMC were divided into 10 to 12 wells (3 × 104/well) and cultured. The peptide-specific T cells were evaluated using an IFN-γ-ELISA on day 21 against a

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peptide-pulsed T2-A24 (refs. 19, 20; provided by Dr. Tsunoda, Tokyo University, Tokyo, Japan) after the third round of peptide stimulation. The HLA-A24 binding PRAME p301 peptide (amino acids 301-309, LYVDSLFFL) was used as a negative control.

Specific lysis of tumor cells by T cells was evaluated using a standard 4-h 51Cr release assay with some modifications (21). Briefly, target cells were labeled with Na 51CrO4 for 2 h, washed thrice, and mixed with effector T cells at various E/T ratios. After 4-h incubation, the supernatant radioactivity was measured using a Top Counter, and percent specific lysis was calculated. Melanoma and colon cell lines expressing HLA-A24/02 (501mel and DC201 MEL), HLA-A01/10 (397mel), and HLA-A01/02 (Colo205) were used as target cells. To block the HLA class I/CD8 interaction, target cells were preincubated with antihuman HLA class I antibody (W6/32) for 1 h at 4°C before mixing with T cells.

Statistical analysis. Unpaired Mann-Whitney U tests were used for evaluation of the significance of differences.

Results

Identification of CRT2 expressed in normal testis and cancer cells. Cancer-testis antigens expressed in cancer cells and germ line–related normal tissues, including testis, ovary, and placenta, are attractive candidates as targets for immunotherapy. In this study, cDNAs preferentially expressed in testis were first isolated using representative differentiation analysis (RDA) to identify new cancer-testis antigens. cDNAs from Human Testis-Specific PCR-Selected cDNA (Clontech), a cDNA library enriched in cDNAs preferentially expressed in testis by differential hybridization between testis and other normal tissues, were subcloned into pCRII plasmids. The DNA sequences of 850 clones obtained from the subtracted testis library were compared with the EST database. Thirty clones were identified to have either testis-specific or cancer-testis antigen–like expression, including the known cancer-testis antigens SCP1 (22) and OY-TES-1 (23) and sperm proteins such as NYD-sp10 (24). Actual expression of these 30 genes was evaluated by RT-PCR analysis using cDNA samples from cancer cell lines and normal tissues. One of the clones coded for a 426-bp cDNA, CRT2, which was expressed in testis and in many cancer cell lines. The properties of CRT2 were further examined in the study.

CRT2 was previously isolated as an isoform of CRT expressed in normal testis (13), as also shown in Fig. 1A. RT-PCR analysis with forward 1 and reverse 1 primers showed two bands corresponding to a full-length CRT2 and a smaller cDNA with

Fig. 1. Cancer-testis antigen—like expression of CRT2. A, structures of mRNAs for CRT, CRT2, and a short splice variant CRT2Δ3, in which exon 3 is deleted, showing the following characteristic regions: N-domain, a globular structure in the NH2-terminal; P-domain, a proline-rich region; C-domain, a Ca2+ storage domain in the COOH-terminal; ss, signal sequence for localization to the endoplasmic reticulum; ss, signal sequence for localization to the endoplasmic reticulum; KDEL and RNEL, signals for retention in the endoplasmic reticulum. Three sets of primers were designed for detection of CRT2 (forward 1 and reverse 1, forward 2 and reverse 1, and forward 3 and reverse 1). B, RT-PCR analysis (35 cycles) for CRT2 using specific primers (forward 2 and reverse 1). CRT2 was strongly expressed in normal testis and melanoma cell lines and tissues. Glyceroldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. C, induction of CRT2 by treatment with demethylating reagent 5-aza-2'-deoxycytidine. CRT2 mRNA was induced in renal cancer cell lines Caki-1 and RCC7 by treatment with 5 µmol/L 5-aza-2'-deoxycytidine for 2 and 4 d.
The CRT2 expression was induced by treatment of renal cell cancer cell lines with no or a faint CRT2 expression with DNA demethylation. Among them, 5 of 6 metastasis samples were CRT2 positive. 0 of 2 stage I, 1 of 5 stage II, 5 of 7 stage III, and 2 of 4 stage IV expressed in advanced melanomas, similar to the other cancer tissues detected in many cancer cell lines, including 23 of 25 melanoma, 14 of 15 esophageal cancer, and 11 of 14 melanoma tissues, including the primary and metastatic melanomas shown in Fig. 2C (e, h, and k), but was not detected in melanocytes. CRT2 seemed to locate in both the cytoplasm and plasma membrane of melanoma cells.

**Immunogenicity of CRT2 in patients with various cancers.** To elucidate the immunogenicity of CRT2, the presence of serum IgGs specific for CRT2 was evaluated in patients with cancer. In Western blot analysis, specific bands for both CRT2 and CRT2Δ3 recombinant proteins were detected using sera from melanoma patients, indicating the presence of IgGs specific for both CRT2 and CRT2Δ3. Thus, CRT2 is an immunogenic tumor antigen in cancer patients (Fig. 3A). Because the CRT2Δ3 band had a higher density than the CRT2 band, the presence of an IgG specific for CRT2Δ3 was investigated. The effect of absorption of sera with the 20-amino-acid CRT2Δ3-specific exon 2 to 4 junction peptide was evaluated, but the properties of the treated and untreated sera did not differ for the reactivity to the CRT2Δ3 protein (data not shown). In addition, specific binding of sera to the junction peptide was not found in an ELISA using peptide-coated plates (data not shown). These results indicate that an IgG specific for the CRT2Δ3-specific exon 2 to 4 junction peptide was not present in the serum samples.

The CRT2Δ3 recombinant protein was used for evaluation of serum IgG from cancer patients. The expected CRT2 band was detected frequently by serum IgG from patients with various cancers, including 30 of 55 melanoma, 27 of 33 esophageal cancers, including 23 of 25 melanoma, 14 of 15 esophageal cancer, and 11 of 14 melanoma tissues, including the primary and metastatic melanomas shown in Fig. 2C (e, h, and k), but was not detected in melanocytes. CRT2 seemed to locate in both the cytoplasm and plasma membrane of melanoma cells.

**Table 1. Frequency of CRT2 expression in cancer cell lines and tissues.**

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Cell lines positive/total (%)</th>
<th>Tissues positive/total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanoma</td>
<td>23/25 (92)</td>
<td>8/18 (44)</td>
</tr>
<tr>
<td>Esophageal cancer</td>
<td>14/15 (93)</td>
<td>NT*</td>
</tr>
<tr>
<td>Gastric cancer</td>
<td>5/6 (83)</td>
<td>2/5 (40)</td>
</tr>
<tr>
<td>Pancreas cancer</td>
<td>4/8 (50)</td>
<td>NT</td>
</tr>
<tr>
<td>Colon cancer</td>
<td>11/16 (69)</td>
<td>10/62 (16)</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>13/13 (100)</td>
<td>8/24 (33)</td>
</tr>
<tr>
<td>Endometrial cancer</td>
<td>7/10 (70)</td>
<td>6/21 (32)</td>
</tr>
<tr>
<td>Cervical cancer</td>
<td>2/2 (100)</td>
<td>1/2 (50)</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>3/4 (75)</td>
<td>NT</td>
</tr>
<tr>
<td>Renal cell cancer</td>
<td>4/10 (40)</td>
<td>4/5 (80)</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>3/4 (75)</td>
<td>NT</td>
</tr>
<tr>
<td>Bladder cancer</td>
<td>5/8 (63)</td>
<td>NT</td>
</tr>
<tr>
<td>Brain tumor</td>
<td>2/5 (40)</td>
<td>NT</td>
</tr>
<tr>
<td>Lymphoma and leukemia</td>
<td>7/8 (88)</td>
<td>NT</td>
</tr>
</tbody>
</table>

Abbreviation: NT, not tested.
cancer, 19 of 30 pancreatic cancer, 21 of 43 colon cancer, 11 of 25 lung cancer, 28 of 45 endometrial cancer, 13 of 33 cervical cancer, 8 of 10 ovarian cancer, 13 of 29 renal cell cancer, 4 of 12 prostate cancer, 10 of 12 bladder cancer, 5 of 7 brain tumor, and 20 of 30 lymphoma/leukemia patients; faint bands were also detected in serum IgG from 6 of 44 (14%) healthy individuals (Table 2). To examine the quantitative difference in the specific IgG titer, an ELISA was developed using the recombinant CRT2 protein. As shown in Fig. 3B, the titer of IgG reacting with CRT2 was significantly higher ($P < 0.01$) in many cancer patients, including those with melanoma, colon cancer, lung cancer, endometrial cancer, cervical cancer, renal cell cancer, bladder cancer, and brain tumor, compared with healthy controls (Fig. 3B). These results indicate that CRT2 is an immunogenic tumor antigen that is present in many patients with various cancers.

**Induction of HLA-A$^*2402$–restricted tumor-reactive CTLs with a CRT2 peptide.** To evaluate whether CRT2 can function as a tumor antigen recognized by T cells, we attempted to identify T-cell epitopes restricted to HLA-A24, which is frequently expressed in the Japanese (a rate of $\sim 60\%$) and Caucasian (a rate of $\sim 20\%$) populations. HLA-A24–binding peptides of CRT2 were predicted using the computer programs Qbag (15), BIMAS, and SYFPEITHI. Four possible HLA-A24–binding peptides, CRT2-p48, CRT2-p128, CRT2-p240, and CRT2-p299, were selected. HLA-A$^*2402$ transgenic mice were immunized with each of these peptides and the I-Ab–restricted tetanus toxoid helper peptide. HLA-A24–restricted T-cell responses were evaluated by a murine IFN-$\gamma$ ELISPOT assay using CD8$^+$ T-cell responder cells isolated from splenocytes of the immunized mice and HLA-A$^*2402$/K$^b$–transfected Jurkat antigen-presenting cells. The response to p128 was highest among the four peptides (Fig. 4A). The p128 peptide is located at the junction of exons 3 and 4 in CRT2. To evaluate whether CRT2-p128 can induce tumor-reactive CTLs, we attempted to induce CTLs in vitro from PBMCs of five healthy donors. p128 peptide–specific IFN-$\gamma$ secretion was observed in all five healthy donors. The representative experiment in Fig. 4B shows

### Table 2. Frequency of anti-CRT2 IgG antibody in sera from cancer patients

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Positive/total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanoma</td>
<td>30/55 (55)</td>
</tr>
<tr>
<td>Esophageal cancer</td>
<td>27/33 (82)</td>
</tr>
<tr>
<td>Pancreas cancer</td>
<td>19/30 (63)</td>
</tr>
<tr>
<td>Colon cancer</td>
<td>21/43 (49)</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>22/51 (40)</td>
</tr>
<tr>
<td>Endometrial cancer</td>
<td>28/45 (62)</td>
</tr>
<tr>
<td>Cervical cancer</td>
<td>19/33 (58)</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>8/10 (80)</td>
</tr>
<tr>
<td>Renal cell cancer</td>
<td>13/29 (45)</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>4/12 (33)</td>
</tr>
<tr>
<td>Bladder cancer</td>
<td>10/12 (83)</td>
</tr>
<tr>
<td>Brain tumor</td>
<td>5/7 (71)</td>
</tr>
<tr>
<td>Lymphoma and leukemia</td>
<td>20/30 (67)</td>
</tr>
<tr>
<td>Healthy individuals</td>
<td>6/44 (14)</td>
</tr>
</tbody>
</table>

![Fig. 3. Presence of an IgG antibody specific for CRT2 in sera from patients with various cancers.](https://clincancerres.aacrjournals.org/content/13/21/6272/F3.large.jpg)
CRT2 p128–specific IFN-γ secretion in 2 of 12 independent culture wells (Fig. 4B). The p128-reactive T cells lysed melanoma cell lines (501mel and DC201MEL) expressing both HLA-A24 and CRT2, but did not lyse cell lines (397mel or Colo205) with no expression of HLA-A24 or CRT2 (Fig. 4C). Lysis was inhibited by the anti–HLA class I antibody W6/32 (Fig. 4D). These results indicate that CRT2-p128 is an immunogenic peptide that is naturally processed and expressed with HLA-A24 on tumor cells, indicating that it is a potential target for T-cell–based immunotherapy.

**Discussion**

Cancer-testis antigens are promising targets for immunotherapy. In this study, we identified CRT2 as a new cancer-testis antigen that is frequently expressed in various cancers, particularly in melanoma. An immunohistochemical study using a newly generated polyclonal anti-CRT2 antibody indicated the presence of CRT2 protein in the cytoplasm and plasma membrane of melanoma cells in tumor tissues but not in melanocytes. CRT2 has an endoplasmic reticulum signal sequence in the NH2 terminus for endoplasmic reticulum localization and a "RNEL" sequence similar to the endoplasmic reticulum retention sequence "KDEL" in the COOH terminus of CRT (25).

Among normal tissues, CRT2 expression was detected in testis, and immunohistochemistry showed that CRT2 is expressed in elongate spermatids. This restricted expression indicates that CRT2 may be useful for cancer diagnosis through detection in tumor tissues and migrated tumor cells in lymph nodes and peripheral blood.

CRT2 was previously identified as a gene encoding a novel 384-amino-acid human CRT isoform with 53% amino acid sequence similarity to CRT (13). CRT2 has a similar structure to CRT, which has a signal sequence for endoplasmic reticulum localization at the NH2 terminus, a globular structure in the NH2-terminal region, a proline-rich region, a COOH-terminal Ca2+ storage domain, and an endoplasmic reticulum retention signal KDEL, which is changed to RNEL in CRT2. In this study, a novel 316-amino-acid CRT2 variant with deletion of exon 3 (CRT2D3) was also identified. This variant is a minor transcript in testis, but is expressed in some cancer cell lines.

CRT is a multifunctional calcium-binding protein that mainly functions as a chaperone in the endoplasmic reticulum and also participates in Ca2+ signaling, cell adhesion, and gene transcription in the cytoplasm, at the cell surface, and in the extracellular space (30). The function of CRT2 in normal testis and cancer cells remains unclear. Translocation of CRT from the endoplasmic reticulum to the plasma membrane of cancer cells induced by anthracycline drugs has recently been reported to be essential for uptake by dendritic cells and subsequent induction of antitumor T cells (31). CRT on dendritic cells and macrophages has been reported to be involved in efficient cross presentation of the cancer-testis antigen NY-ESO-I by its direct binding to NY-ESO-I (32). These intriguing findings indicate the possible use of CRT in immunotherapy. CRT2 seems to be expressed in the cytoplasm and plasma membrane in some
melanoma tissues based on our immunohistochemical study. The role of CRT2 in the plasma membrane is currently under investigation in our laboratory, and the general roles of CRT2 and CRT2Δ3 in cancer cell development, testicular germ cell differentiation, and interactions with immune cells require further studies.

CRT2 was identified as an antigen recognized by IgGs and CD8+ CTLs. Western blot analysis showed that IgGs against the bacterial recombinant CRT2 are present in sera from patients with various cancers, including melanoma, brain tumor, endometrial cancer, cervical cancer, ovarian cancer, lymphoma, pancreatic cancer, esophageal cancer, and bladder cancer. Furthermore, an ELISA showed that the IgG titer in sera from cancer patients was significantly higher than that of healthy individuals. These results indicate that CRT2 is an immunogenic antigen in various cancers. An autoantibody to CRT has been reported in patients with systemic lupus erythematosus, rheumatoid arthritis, and Sjögren syndrome (33), but the antibody against CRT2 was not associated with these collagen diseases in this study.

In Western blot analysis with sera from patients, the bacterial recombinant CRT2Δ3 protein showed more intense bands than recombinant CRT2, and in ELISA the titer of serum IgG against CRT2 was not associated with these collagen diseases. Thus, CRT2 may be useful in cancer diagnosis and immunotherapy.

However, we found no evidence for the presence of an IgG specific for a CRT2Δ3-specific peptide located at the junction of exons 2 and 4. However, a disulfide bridge between cysteines in exons 3 and 4, as found in CRT2, may lead to different conformations for CRT2 and CRT2Δ3, leading to differential IgG recognition. In addition to the IgG responses, we identified a novel HLA-A24-binding T-cell epitope peptide of CRT2 by computational prediction of peptide binding to HLA-A24, with testing in HLA-A24 transgenic mice and by in vitro CTL induction from human PBMCs. The identified peptide CRT2Δ3-p128 is located at the junction of exons 3 and 4 and is able to induce HLA-A24-restricted tumor-reactive CD8+ CTLs from human PBMCs. These results indicate that CRT2 may be an attractive antigen for induction of both helper CD4+ T cells and cytotoxic CD8+ T cells. In summary, CRT2 is a novel cancer-testis antigen that is expressed in elongate spermatids in normal testis and in various cancer cells and is recognized by IgG and HLA-A24-restricted CD8+ CTLs. Therefore, we conclude that CRT2 may be useful in cancer diagnosis and immunotherapy.

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References

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