The Renal Cell Carcinoma-associated Antigen G250 Encodes a Human Leukocyte Antigen (HLA)-A2.1-restricted Epitope Recognized by Cytotoxic T Lymphocytes


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ABSTRACT

Evidence has accumulated that the immune system can play a significant role in the defense against tumors in humans. Especially melanoma and renal cell carcinoma (RCC) are considered immunogenic tumors. In contrast to melanoma, hardly any RCC-associated antigens have been identified as targets for RCC-reactive T cells. Here, we report the identification of a human leukocyte antigen (HLA)-A2.1-restricted T-cell epitope within the G250 antigen. This antigen is expressed in 85% of RCCs but not by neighboring normal kidney tissue and has recently been molecularly defined and shown to be identical to MN/CA IX. Computer-aided motif prediction revealed the presence of 60 potential HLA-A2.1-binding peptides within the G250 antigen. Subsequent binding analysis showed that 13 of these peptides bound to HLA-A2.1 with high-to-intermediate affinity. Analysis of their immunogenicity in HLA-A2.1^Kb-transgenic mice indicated that 4 of the 13 peptides gave rise to cytotoxic T lymphocytes (CTLs) capable of lysing peptide-loaded target cells. However, only the G250 peptide 254–262 induced CTLs that recognized target cells that endogenously expressed the G250 antigen. Similarly, we were also able to raise human CTLs against the G250 peptide 254–262, which lysed target cells that endogenously expressed the G250 antigen. These findings and the high prevalence of this antigen in RCC patients makes G250 a potential target for anti-RCC immunotherapy.

INTRODUCTION

Numerous studies indicate that the immune system can react against tumor-associated antigens (1, 2). Various tumors of different origin are considered to be immunogenic, for example, melanoma, RCC, prostate cancer, head and neck cancer, and colon cancer (3). CTLs with antitumor reactivity have been isolated from both blood and TILs of cancer patients. Tumor antigens recognized by CTLs with antitumor reactivity can be divided into three groups (2, 4):

(a) the first group, the melanocyte differentiation antigens, are expressed in melanoma as well as in their normal counterpart, the melanocyte. Members of this group, including gp100, Melan A/MART-1, and tyrosinase, are expressed in most melanomas but not in tumors derived from other tissues;

(b) the second group comprises so-called shared tumor antigens expressed in a wide variety of tumors of different origins. Tumor antigens BAGE, GAGE, and MAGE are expressed in several distinct tumor types but not in normal tissue, except testis. Therefore, this particular group of antigens is often referred to as cancer-testis antigens;

(c) the third group consists of mutated antigens unique for each individual tumor such as the tumor-specific point mutation found in cyclin dependent kinase-4 (CDK-4) giving rise to a novel antigenic epitope.

Presently, several clinical trials aimed at the induction of anti-melanoma reactivity via vaccination against the tumor-associated antigens identified are ongoing (5–7).

Like melanoma, RCC is also relatively immunogenic. In a fair number of RCC patients, “spontaneous” partial or complete remissions have been observed and immunotherapies can increase the reactivity of the immune system against RCC (8–10). However, only a few specific CTL lines for autologous RCC have been identified so far (11–15). For two of these RCC-specific CTL lines, the target antigens have been identified. The first CTL-defined RCC-specific antigen (RAGE-1) was isolated by expression cloning (14). This gene is expressed in less than 2% of primary RCCs and is silent in normal tissue, except retina. The second CTL-defined antigen was a mutated HLA-A2 protein (15). In addition, CTLs induced against Her-2/neu-derived peptides in vitro were able to lyse RCC lines (16). Strikingly, the shared tumor antigens identified in melanoma that are also expressed in a variety of other tumors are not expressed in RCC.

For effective treatment of RCC patients with immunotherapy, high antigen expression and expression by all of the RCCs are first prerequisites. Previous studies have demonstrated that mAb G250 recognizes a RCC-associated antigen expressed on the cell surface of approximately 85% of RCCs but not on normal kidney tissue (17). In addition, G250 expression can be detected on the cell surface of colon, ovarian, and cervical carcinomas. Analysis of normal tissues indicated that the reactivity of the G250 mAb is limited to some gastric mucosal cells and to cells of the larger bile ducts. The staining observed in these normal tissues is relatively weak and cytoplasmic in nature (17–19). Clinical studies in RCC patients demonstrated exclusive targeting of radiolabeled mAb G250 to RCC (20). Recently, the cDNA encoding the RCC-associated antigen recognized by a mAb G250 was identified (17). The deduced amino acid sequence of the G250 antigen showed that the G250 protein is a transmembrane protein identical to the previously described tumor-associated antigen MN/CA IX that was identified in cervical carcinoma (18). Sequence comparison of the G250/MN/CA IX gene with RCC-derived cdNA of G250 demonstrated that the G250/MN/CA IX protein in RCC is nonmutated (21). The G250 antigen is the first widely expressed RCC-associated antigen identified and may constitute an interesting novel target for specific immunotherapy in RCC patients.

In this study, the immunogenicity of the RCC-associated antigen G250 was investigated using the reversed immunology approach (4). Applying this strategy, we now demonstrate that the G250 amino acid sequence from 254 to 262 is a HLA-A2.1-restricted CTL epitope that is both naturally processed and immunogenic.

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The abbreviations used are: RCC, renal cell carcinoma; mAb, monoclonal antibody; IL, interleukin; FL, fluorescence index; TAP, transporter associated with antigen processing; HLA, human leukocyte antigen; CTL, cytotoxic T lymphocyte; DC, dendritic cell; GM-CSF, granulocyte macrophage colony-stimulating factor; MCM, monocytodevated condition medium; PBMC, peripheral blood mononuclear cell; TIL, tumor-infiltrating lymphocyte.

MATERIALS AND METHODS

Peptides. Peptides were synthesized by Fmoc chemistry using a multiple peptide synthesizer. As determined by reversed-phase high-performance liquid chromatography the peptides were >90% pure. Peptides were dissolved in DMSO at a concentration of 10 μM, and stored at -20°C.

Unlabeled and fluorescein-labeled peptides were obtained from J. W. Drif- hout (Leiden University Medical Center, Leiden, the Netherlands).

Cell Culture. JY and T2 cells were cultured in Iscove’s medium enriched with 5% FCS and antibiotic-antimycotic (all of which were obtained from Life Technologies, Inc., Grand Island, NY). The melanoma cell line, BLM (expressing HLA-A2.1; Ref. 22) was transfected with the CDNA of either G250 or gp100 resulting in the cell lines BLM-G250 and BLM-gp100, respectively. The transfected cell lines BLM-G250 and BLM-gp100 were cultured in DMEM (Life Technologies, Inc.) enriched with 5% FCS and antibiotic-antimycotic in the presence of 1 mg/ml G418 (Life Technologies, Inc.). The mouse cell line EL-4 (American Type Culture Collection) was transfected with a DNA construct encoding for the HLA-A2.1κ chimeric molecule (23) with and without a DNA construct encoding G250 (EL-4/HLA-A2.1K/G250 and EL-4/HLA-A2.1K, respectively). These cell lines were maintained in Iscove’s medium with 5% FCS, G418 (1 mg/ml), and hygromycin (0.5 mg/ml).

HLA-A2.1 Up-Regulation on T2 Cells. To identify G250 HLA-A2.1-binding motifs in the G250 protein, a computer scoring program was used (24, 25). To determine whether the G250-derived peptides were able to bind to HLA-A2.1 molecules, peptide-induced HLA-A2.1 up-regulation on TAP-deficient T2B-cell hybrid T2 cells (26) was examined as described previously (27). Briefly, 10^6 T2 cells were incubated together with various concentrations of G250-derived peptides (final DMSO concentration 0.5%) for 14 h at 37°C. After extensive washing, chromium-labeled target cells labeled reference peptide (FLPSDC(27). Briefly, 10^5 T2 cells were incubated together with various concentrations of G250-derived peptides at similar concentrations.

Cytotoxicity Assay. CTL activity was measured using the chromium-release assay as described previously (22). Briefly, 10^6 target cells were incubated with 100 μCi of Na_2[^105]CrO_4 (Amersham, Bucks, United Kingdom) for 45 min at 37°C. After extensive washing, chromium-labeled target cells (2*10^6 cells/ml) were loaded with 10 μM peptide for 30 min at 37°C. Effector cells were added to 10^5 target cells in triplicate wells of a round bottom microtiter plate (total volume, 150 μl). After a 4-h incubation, 100 μl of the supernatant was harvested, and its radioactivity content was measured. The specific chromium release was defined as: [experimental release – spontaneous release (SR)] / maximum release – SR] × 100%.

Transgenic mice. HLA-A2.1K transgenic C57BL/6 mice were kindly provided by Dr. L. Sherman (Scripps Laboratories, San Diego, CA; Ref. 23). Mice were held under specified pathogen-free conditions at the Central Animal Laboratory, Nijmegen University (Nijmegen, the Netherlands). For experimental purposes, mice at the age of 8–12 weeks were used. HLA-A2.1κ expression (anti-HLA-A2 mAb BB7.2; Ref. 28) was analyzed by flow cytometry on PBMCs isolated from 4-week-old mice. Groups of three mice were vaccinated twice s.c. in the flank with 0.1 μmol of G250-derived peptide and hepatitis B virus core antigen-derived T-helper epitope (30) emulsified in incomplete Freund’s adjuvant (Difco Lab., Detroit, MI) with a 2-week interval. Mice were killed 14 days after the last immunization, and spleen cells (40*10^6 in 10 ml) were restimulated with peptide-loaded syngeneic-irradiated lipopolysaccharide (LPS)-stimulated B-cell lymphoblasts (ratio 4:1). Cells were cultured in Iscove’s medium supplemented with 5% FCS, antibiotic-antimycotic, and 50 μM β-mercaptoethanol. Six days after restimulation, responder T cells were harvested and tested for specific cytolytic activity.

In Vitro Induction of Human CTL Using Peptide-loaded DC. PBMCs of healthy individuals were separated using Percoll-density centrifugation and were allowed to adhere for 1 h at 37°C in RPMI 1640 (Life Technologies, Inc.) enriched with 2% human serum in 6-well tissue culture plates (Costar, Badhoevedorp, the Netherlands). Adherent monocytes were cultured in RPMI 1640 enriched with 10% FCS in the presence of IL-4 (500 units/ml, Schering-Plough, Amstelveen, the Netherlands) and GM-CSF (800 units/ml, Schering-Plough) for 6 days. Fresh cytokine-containing culture medium was added at day 3. At day 6, the immature DCs (MHC class I positive and low expression of MHC class II, B7.1 (CD80), B7.2 (CD86), and CD83) were cultured in the presence of 50% (v/v) MCM, obtained from IgG-activated monocytes (31). Mature monocyte-derived DCs, expressing MHC class I and II, B7.1 and B7.2, and CD83, were harvested at day 8. Mature DCs were pulsed with 50 μM peptide and 3 μg/ml β2-microglobulin (37°C, 2 h). Peptide-loaded and irradiated (2500 rad) DCs (2.5*10^6/well) were cocultured with enriched autologous CD8-positive T cells (5*10^5/well, depleted for CD4-positive and CD56-positive cells by magnetic sorting (Dynal, Oslo, Norway) in the presence of autologous irradiated peripheral blood lymphocytes (5*10^5/well) and IL-7 (10 ng/ml; Genzyme, Cambridge, MA) in a 24-well plate (Costar; Ref. 32). One day after stimulation, IL-10 (20 units/ml; Dynax, Palo Alto, CA) was added to the cell cultures. At day 7, responder T cells were restimulated with peptide-loaded autologous irradiated immature DCs (2.5*10^6/well). IL-10 was added at day 1, and IL-2 (15 units/ml; Cetus Corp., Emeryville, CA) was added at day 2 and 5 after restimulation. Restimulation of the responder T cells at day 14 was performed with immature peptide-loaded DCs, and IL-2 was added after 1 day. All of the wells containing responder T cells were kept separate. At day 21, half of the bulk CTLs of each well was used in a cytotoxicity assay with peptide-loaded T2 cells as target cells. Bulk CTLs that showed peptide-specific cytotoxic activity were stimulated weekly with 15 units/ml IL-2 and tumor cells endogenously expressing G250.

RESULTS

Identification of HLA-A2.1-binding Peptides Derived from G250. The G250 protein is highly expressed in primary and metastatic RCC. The ability to induce CTLs against G250 could, therefore, be of great importance for the treatment of RCC patients. Because HLA-A2.1 is a frequently expressed MHC molecule in Caucasians, the presence of HLA-A2.1-restricted CTL epitopes in the G250 antigen was investigated. Using a computer scoring program, we selected 60 potential HLA-A2.1 binding peptides (24, 25). These G250-derived peptides were first tested for their ability to bind to HLA-A2.1 molecules using the HLA-A2.1 up-regulation assay on TAP-deficient T2 cells (27). The T2 cells were incubated with 25 μM and 50 μM G250-derived peptide; 14 h later the number of HLA-A2.1 molecules on the surface of the cells was measured using anti-HLA-A2.1 mAbs. From the 60 predicted peptides, 18 G250-derived peptides were actually able to up-regulate HLA-A2.1 molecules (Table 1).

Binding Affinity of the G250-derived Peptides for HLA-A2.1 Molecules. Using the previously described competition-based HLA-A2.1 peptide-binding assay (29), we determined the binding affinity of the 18 selected peptides to HLA-A2.1 molecules. After mild acid elution, the CYJ cells with empty HLA-A2.1 molecules were incubated with titrated concentrations of G250-derived peptides and a standard concentration of a fluorescein-labeled reference peptide having a high binding affinity to HLA-A2.1 molecules. The concentration of the G250-derived peptides that inhibited the binding of the reference peptide by 50% (IC50) is shown in Fig. 1. From the 18 tested

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Table 1  

<table>
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<tr>
<th>Position</th>
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<th>HLA-A2.1 up-regulation*</th>
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<td>G250:205–213</td>
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<td>G250:254–262</td>
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<td>G250:271–279</td>
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<td>G250:415–423</td>
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<tr>
<td>G250:421–429</td>
<td>GLLFAYTVSV</td>
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G250-derived peptides, 4 peptides with a high (IC50 < 5 μM), 9 peptides with an intermediate (IC50 5-15 μM), and 5 peptides with a low binding affinity (IC50 > 15 μM) were found. The G250-derived peptides with high and intermediate affinity for HLA-A2.1 molecules were further studied to determine their immunogenicity in vivo and in vitro.

**Immunogenicity of G250-derived Peptides in HLA-A2.1Kb Transgenic Mice.** HLA-A2.1Kb transgenic mice were used to investigate whether the aforementioned G250-derived peptides were able to induce a CTL response in vivo. These transgenic mice express the chimeric HLA-A2.1Kb molecule, in which the α3 domain of HLA-A2.1 is replaced by the corresponding murine Kβ α3 domain. This results in a MHC molecule that can present peptides binding to the human HLA-A2.1 molecule and is still able to interact with the CD8 molecule present on murine T cells (23, 34, 35). Spleen cells of HLA-A2.1Kb mice vaccinated with G250-derived peptide were restimulated once in vitro and used in a cytotoxicity assay. In this assay, EL-4/HLA-A2.1Kb cells, loaded with relevant G250-derived peptide, were used to measure peptide-specific lysis of bulk CTLs and the same cells, loaded with irrelevant peptide, were used to measure nonspecific lysis. Of the 13 G250-derived peptides investigated, 4 were able to induce CTLs with peptide specificity in bulk cultures (Table 2). As shown in Fig. 2, the high-binding-affinity peptide G250:26–35 and the intermediate-binding-affinity peptides G250:254–262, G250:271–279, and G250:421–429 were able to induce a specific bulk CTL response.

To address whether these immunogenic epitopes are naturally processed and presented, EL-4 cells, stably transfected with cDNAs encoding HLA-A2.1Kb and G250, were used as target cells in a chromium-release assay using the four peptide-specific CTLs as effector cells. As shown in Fig. 3, only the CTLs directed against the peptide G250:254–262 were able to specifically lyse EL-4 cells expressing both HLA-A2.1Kb and G250. This indicates that the G250-derived peptide 254–262 is both an immunogenic and a naturally processed CTL epitope in HLA-A2.1Kb transgenic mice.

**Induction of Human CTL Responses against G250 In Vitro.** To investigate the immunogenicity of the aforementioned 13 G250-derived peptides in humans, they were analyzed for their ability to induce a CTL response in vitro. Each peptide was tested at least twice, using PBMCs from different donors. For this purpose, monocytes of healthy HLA-A2.1-positive donors were cultured in the presence of GM-CSF and IL-4 to generate immature DCs. After maturation with MCM, DCs were loaded with G250-derived peptides and cocultured with autologous CD8-positive T cells. After restimulating responder T cells twice with peptide-loaded immature DCs, the cytotoxicity of these bulk CTLs was tested against T2 cells loaded with either the relevant G250-derived peptide or an irrelevant peptide (Table 2). As

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One representative experiment is shown; upper left corner tested). In this study, we report the identification of a MHC class-I-restricted T-cell epitope within the RCC-associated antigen G250. The amino acid sequence from 254 to 262 (HLSTAFARV) is a CTL epitope that is both naturally processed and presented in the context of the HLA-A2.1 molecule. To characterize this epitope, we have selected, based on HLA-A2.1-binding motifs, G250-derived peptides using a computer scoring program, and subsequently these peptides were synthesized. The predicted G250-derived peptides were examined for their capacity to bind HLA-A2.1 molecules using a HLA-A2.1 up-regulation assay and a competition-based HLA-A2.1 peptide-binding assay. Of the 60 peptides studied, 4 G250-derived peptides with high binding affinity and 9 peptides with intermediate binding affinity for HLA-A2.1 molecules were defined. Using HLA-A2.1Kb transgenic mice and DCs of healthy HLA-A2.1-positive donors, we were able to demonstrate that CTLs induced against the G250-derived peptide G250:254–262 specifically lysed tumor cell lines positive for G250 and HLA-A2.1Kb or HLA-A2.1. This demonstrates that G250 contains an immunogenic epitope of amino acid 254–262 that is both endogenously processed and presented via HLA-A2.1.

In this study, we show that the reversed immunology approach is a potent method to identify CTL-restricted epitopes in tumor antigens. In addition to the naturally processed and presented G250:254–262 epitope, we found that two other G250-derived peptides, G250:26–35 and G250:421–429, gave rise to peptide-specific CTLs both in vivo and in vitro, whereas nine peptides did not yield any CTLs in either assay. The correlation between both approaches used to identify immunogenic peptides and naturally processed peptides confirms the value of these approaches (4, 34, 35). One immunogenic peptide (G250:271–279) gave rise to peptide-specific CTLs using HLA-A2.1Kb transgenic mice and DCs of healthy HLA-A2.1-positive donors. Possibly, this discrepancy can be explained by the fact that G250 is a self-antigen in humans. As a consequence, T cells recognizing this particular epitope may be deleted in the human T-cell repertoire.

Three G250-derived peptide-specific bulk CTLs were not able to lyse cells that endogenously expressed G250. Previously, this phenomenon has also been reported for in vitro induced CTLs recognizing estab-

shown in Fig. 4A, DCs loaded with peptides G250:26–35, G250:254–262, or G250:421–429 were all capable of inducing peptide-specific bulk CTLs.

To investigate whether any of these peptides are naturally processed and presented, peptide-specific CTLs were tested for their ability to lyse target cells endogenously expressing G250. As observed in HLA-A2.1Kb transgenic mice, two independent bulk CTL cultures against peptide G250:254–262 were able to specifically lyse HLA-A2.1-positive BLM cells transfected with the G250 cDNA but not BLM cells transfected with gp100 cDNA (Fig. 4B). In contrast, the CTLs induced against peptides G250:26–35 and G250:421–429 did not specifically lyse the BLM cells endogenously expressing the G250 antigen because the same background lysis was observed on BLM/gp100 transfectants (Fig. 4B).

Together, these data demonstrate that the G250-derived peptide 254–262 is immunogenic not only in HLA-A2.1Kb transgenic mice but also in humans. CTLs capable of specifically lysing cells that endogenously present this G250-derived T-cell epitope can be obtained in humans also.

DISCUSSION

In this study, we report the identification of a MHC class-I-restricted T-cell epitope within the RCC-associated antigen G250. The amino acid sequence from 254 to 262 (HLSTAFARV) is a CTL epitope that is both naturally processed and presented in the context of the HLA-A2.1 molecule. To characterize this epitope, we have selected, based on HLA-A2.1-binding motifs, G250-derived peptides using a computer scoring program, and subsequently these peptides were synthesized. The predicted G250-derived peptides were examined for their capacity to bind HLA-A2.1 molecules using a HLA-A2.1 up-regulation assay and a competition-based HLA-A2.1 peptide-binding assay. Of the 60 peptides studied, 4 G250-derived peptides with high binding affinity and 9 peptides with intermediate binding affinity for HLA-A2.1 molecules were defined. Using HLA-A2.1Kb transgenic mice and DCs of healthy HLA-A2.1-positive donors, we were able to demonstrate that CTLs induced against the G250-derived peptide G250:254–262 specifically lysed tumor cell lines positive for G250 and HLA-A2.1Kb or HLA-A2.1. This demonstrates that G250 contains an immunogenic epitope of amino acid 254–262 that is both endogenously processed and presented via HLA-A2.1.

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Together, these data demonstrate that the G250-derived peptide 254–262 is immunogenic not only in HLA-A2.1Kb transgenic mice but also in humans. CTLs capable of specifically lysing cells that endogenously present this G250-derived T-cell epitope can be obtained in humans also.
lished MHC class I epitopes (36, 37). Recent data indicated that exogenously added peptides may bind differently to HLA class I molecules as compared with endogenously generated peptides. However, in the case of the G250-derived peptides, it is possible that at least some of the peptides are either not properly processed by the proteasome or not properly transported by the TAP molecules (4, 38).

Previously, it has been demonstrated that peptides recognized by CTLs specific for tumor antigens are mostly intermediate HLA-A2.1 binders, whereas only a few examples of high- and low-binding peptides have been reported (2, 4). The finding that the naturally processed peptide G250:254–262 also binds with an intermediate affinity to HLA-A2.1 molecules is in line with the hypothesis that CTLs that recognize intermediate-binding peptides derived from self-antigens are still present in the T-cell repertoire.

Melanoma studies have revealed three classes of antigens: (a) melanocyte differentiation antigens; (b) shared tumor antigens; and (c) mutated antigens. The G250 antigen is expressed in a high percentage of RCCs and in some other carcinomas. G250 is expressed neither in normal kidney nor in testis (17). In addition, the G250 antigen is not mutated inasmuch as no sequence differences were found between an RCC-derived G250 cDNA and the G250/MN/CA IX gene characterized from a healthy individual (21). Weak cytoplasmic staining of some gastric mucosal cells and cells of the larger bile ducts by the G250 mAb has been noted (17, 19), which suggests that the G250 antigen may be an overexpressed differentiation antigen.

RCC belongs to the small group of immunogenic tumors. In a low percentage of RCC patients, a partial or complete remission has been observed, and additional immunotherapies can increase the immune reaction to the RCC (8–10). The presence of TILs in RCC supports observations that humoral responses against RCC do occur (39), which implies that CD4-positive T-helper cells are also involved. Moreover, CD4-positive TILs with antitumor reactivity have been isolated from RCCs (3). Thus far, only three antigens expressed in RCC that are recognized by human renal cell carcinoma T cells have been characterized (14–16). G250 is a novel target that is recognized by CTLs. Presently, we are screening RCC-derived TILs to investigate whether, in RCC patients, an immune response against G250 is mounted.

In conclusion, our results demonstrate that the RCC-associated antigen G250 encodes a HLA-A2.1-restricted CTL epitope (G250: 254–262) that is immunogenic and naturally processed. Together with the high prevalence of G250 in RCC patients, these results underline the potential usefulness of G250 in the development of immunotherapies for RCC and other G250-expressing tumors.

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The Renal Cell Carcinoma-associated Antigen G250 Encodes a Human Leukocyte Antigen (HLA)-A2.1-restricted Epitope Recognized by Cytotoxic T Lymphocytes


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