Heterogeneous Expression of the Tumor-associated Antigens RAGE-1, PRAME, and Glycoprotein 75 in Human Renal Cell Carcinoma: Candidates for T-Cell-based Immunotherapies1

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Abstract

It has recently been shown that tumor-associated antigens (TAAs) can evoke tumor-specific T-cell-defined immune responses in cancer patients, thereby offering the possibility of treating patients with such antigens. To develop T-cell-based immunotherapeutic approaches for renal cell carcinoma (RCC), we studied the mRNA expression profile of the TAAs RAGE-1, tyrosinase, MAGE-1, MAGE-2, NY-ESO-1, Melan-A/MART-1, glycoprotein (gp) 75, gp100, β-catenin, PRAME, and MUM-1 in 14 human RCC cell lines and in tissue specimens of 37 primary RCCs, 2 related metastases, and 33 specimens of normal renal epithelium. Reverse transcription-PCR was performed with TAA-reactive primers, and the specificity of the PCR products was confirmed by Southern blot and/or direct sequencing. PRAME (10 of 14 cell lines), RAGE-1 (7 of 14 cell lines), and gp75 (4 of 14 cell lines) antigens were expressed in a high percentage of RCC cell lines, although the level of TAA expression varied among the different RCC cell lines. However, low levels of TAA expression in RCC cells are sufficient for recognition by TAA-specific CTLs. Transcription of tyrosinase, Melan-A/MART-1, MAGE-1, MAGE-2, NY-ESO-1, gp100, β-catenin, and MUM-1 was not detected in any RCC cell line. Approximately 50% of surgically removed neoplasias expressed at least one TAA. RAGE-1 mRNA expression was found in 8 of 39 (21%) RCC samples, PRAME mRNA expression was found in 15 of 39 (40%) RCC samples, and gp75 mRNA expression was found in 4 of 39 (11%) RCC samples, but the expression levels of these TAAs were heterogeneous in the different RCC lesions. One RCC specimen expressed MAGE-2, whereas transcription was not detected in any RCC specimen for MAGE-1, NY-ESO-1, tyrosinase, Melan-A/MART-1, gp100, β-catenin, and MUM-1. The normal kidney epithelium specimens were negative for any TAA tested. Thus, RAGE-1, PRAME, and gp75 expression is found with a different frequency in surgically removed lesions and in RCC cell lines, suggesting that a subgroup of RCC patients could be selected for immunotherapeutic strategies that may benefit from immunization against the RAGE-1, gp75, and/or PRAME antigens. However, additional targets for T-cell-based immunotherapy of RCC have yet to be identified.

Introduction

In recent years, a number of TAAs1 recognized by CTLs in the context of appropriate MHC class I molecules have been characterized, giving new impetus to the treatment of patients with immunotherapy (1, 2). Thus far, three main categories of CTL-defined determinants exist (1, 3): (a) differentiation-specific antigens that are expressed only in melanoma and in normal melanocytes, such as tyrosinase (4, 5), Melan-A/MART-1 (6), gp75 (7), and gp100 (8); (b) cancer/testis antigens, such as the MAGE gene family, BAGE, GAGE, and NY-ESO-1, which are expressed in a significant proportion of tumors of distinct histological origin but are silent in normal adult tissue, except in the testis (9-14); and (c) antigens caused by point mutations of ubiquitously expressed genes that are present in a variety of individual tumors (15-17). Due to their expression pattern in cancers and the existence of antigen-mediated autoimmunity, TAAs represent attractive targets for antigen-specific immune responses. Indeed, a number of melanoma-associated antigens are currently being tested in clinical trials for the treatment of metastatic melanoma (18-20). Immunization with peptides derived from gp100, Melan-A/ MART-1, and tyrosinase-induced delayed-type hypersensitivity reactions, peptide-specific CTL responses, and, in some cases, objective tumor regressions (19, 20).

RCC represents the most common malignancy of the kidney. The morphological classification according to Thoenes et al. (21), which was confirmed by cytogenetic analysis (22), distinguishes between clear cell RCC and chromophilic/papillary RCC, both of which originate from the proximal tubulus, and chromophobe RCC, which originates from the distal tubulus. In contrast to chromophobe tumors, clear cell RCC and chromophilic/papillary RCC possess a high metastatic potential, and the prognosis of RCC patients in these subgroups is very poor. Until now, the benefit of conventional therapies, such as surgical, radiological, or chemotherapeutic approaches, has been quite limited. Due to a remarkable spontaneous regression rate, the existence of lymphocytic infiltrates, and some objective remission in clinical trials with immune response modifiers, RCC seems to represent an attractive target for immunotherapeutic strategies using TAAs (2, 23, 24).

The main prerequisites for the successful implementation of TAAs are: (a) adequate expression of the target antigen(s) in tumor lesions; (b) a tumor antigen concentration adequate for efficient CTL recognition; and (c) conservation of the MHC class I restriction element(s) throughout tumor progression (25-27). The absence or low expression of these genes can abrogate or lead to poor immune responses (28). Therefore, it is of interest to evaluate the expression pattern of TAAs in human tumors (29, 30). To gain insight into the suitability of TAA-based immunotherapy for RCC patients, it is essential to evaluate the abundance of TAA expression in an appropriate number of RCC cell lines and tumor specimens of RCC. Here we determine the expression of MAGE-1, MAGE-2, NY-ESO-1, tyrosinase, Melan-A/MART-1, gp75, gp100, MUM-1, β-catenin, RAGE-1, and PRAME in samples of primary RCC, metastases, and normal kidney epithelium. Data from studies like this one will be mandatory to estimate the potential of specific RCC immunotherapy.
The chronic myelogenous leukemia cell line K562; and (?) the acute T-cell gastric carcinoma cell line MKN-45: (c) the RCC cell line LE921 IRC (33); (d) Germany; Table 2).

Unpublished observations.

The RCC cell lines designated with the prefix LE were a kind gift of could not be performed because the anti-PRAME CTLs were HLA-A24-restricted. Lysis of PRAME-positive RCC cell lines and allogeneic HLA-B7. RAGE mRNA-positive RCC cell lines was analyzed by 51Cr release assay as described previously (36, 37). Lysis of PRAME-positive RCC cell lines

Table 2. TAA mRNA expression in various RCC cell lines

Total cellular RNA was subjected to RT-PCR analyses using the respective TAA-specific primers as described in “Materials and Methods.” K562 cells served as a positive control for FRAME expression, and LE921 IRC served as a positive control for RAGE-1

MAGE-1 f CGAGGAGTCCTGCACTGCA 54°C One-step RT-PCR 10 TCCCACTACCATCAACTCACACTCTGACAGAG
MAGE-2 f AGCGAGGATCTCATAGGAGAG 64°C One-step RT-PCR 34 AGAGGACTCATCCTGGACTGAGAGGAG
RAGE-1 f GTGATATCTGCACGGATGAC 57°C Classical RT-PCR 35 GTGGAAGAAGGAGGCTCAGAG
PRAME f GTCCCTCGG CCTCTCCTCTTACTTA 54°C One-step RT-PCR 36 GAGAACTCTTATACCTGGACCTGAT
NY-ESO-1 f GCATGATGTTGTGTTGTTT 59°C One-step RT-PCR 4 GCTCTTGATTTCTCTGGCATTGCT
Tyrosinase f GCCGCCCTGGTCATCAGTTATT 65°C One-step RT-PCR 37 GCACCCGCTCTCCTCAAGATAGATTCAC
Melan-A/MART-1 f ATCTAGTGGAGAAGTGGAGAAG 60°C One-step RT-PCR 8 TCACTGGCTTGTGATTTGGAAGAG
gp100 f GCCATGCTGCCGTGGACTGCTG 52°C One-step RT-PCR 38 ATTACTACCATGTAAGAGAAGATAC
gp75 f CTTGAAGGGATCTGACTACAT 52°C One-step RT-PCR 39 CTGAAACAGGATGCTCAGCTGCT
β-catenin f AGCGCTGGAGAGAG 53°C RT-PCR followed by digestion with XmnI 14 GTGAGCAAGTATAAGCTGAGATCGCTC
MUM-1 wt f GCCCTTCTTCAATAAGGGATTT 52°C RT-PCR using mutation-specific primers 15 GTGAGAACCCAAACAGGACTATTCTG
mut f GCCCTTCTTCAATAAGGGATTT

Table 1 Primer sequences used for PCR analyses

RANGE-1

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<th>Tumor antigen</th>
<th>Primers from 5' to 3'</th>
<th>Annealing temperature</th>
<th>Method</th>
<th>Ref. no.</th>
<th>Oligonucleotide used for hybridization</th>
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<td>MAGE-2</td>
<td>f AGCGAGGATCTCATAGGAGAG 64°C One-step RT-PCR 34 AGAGGACTCATCCTGGACTGAGAGGAG</td>
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<td>RAGE-1</td>
<td>f GTGATATCTGCACGGATGAC 57°C Classical RT-PCR 35 GTGGAAGAAGGAGGCTCAGAG</td>
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<td>PRAME</td>
<td>f GTCCCTCGG CCTCTCCTCTTACTTA 54°C One-step RT-PCR 36 GAGAACTCTTATACCTGGACCTGAT</td>
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<td>NY-ESO-1</td>
<td>f GCATGATGTTGTGTTGTTT 59°C One-step RT-PCR 4 GCTCTTGATTTCTCTGGCATTGCT</td>
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<td>Melan-A/MART-1</td>
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<td>f GCCATGCTGCCGTGGACTGCTG 52°C One-step RT-PCR 38 ATTACTACCATGTAAGAGAAGATAC</td>
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<td>β-catenin</td>
<td>f AGCGCTGGAGAGAG 53°C RT-PCR followed by digestion with XmnI 14 GTGAGCAAGTATAAGCTGAGATCGCTC</td>
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<tr>
<td>MUM-1 wt</td>
<td>f GCCCTTCTTCAATAAGGGATTT 52°C RT-PCR using mutation-specific primers 15 GTGAGAACCCAAACAGGACTATTCTG</td>
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<tr>
<td>MUM-1 mut</td>
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Materials and Methods

Cell Lines and Tissue Culture. Fourteen cell lines derived from primary RCCs and designated with the prefix MZ or NW were established by Knuth et al. (31). The RCC cell lines designated with the prefix LE were a kind gift of Prof. P. Schrier (University Hospital of Leiden, Leiden, the Netherlands). The HLA phenotype of the different RCC cell lines, as determined by standard PCR analyses, was kindly provided by E. Hilmes (University Hospital. Mainz, Germany). All cell lines were maintained in RPMI 1640 (Biochrom KG, Berlin, Germany) supplemented with 10% (v/v) FCS (Greiner GmbH Frickenhausen, Germany), 2 mM L-glutamine, 20 units/ml penicillin, and 200 units/ml streptomycin. The cells were harvested by trypsinization and washed with PBS before RNA extraction.

Patients and Tissue Samples. Representative tissue samples from RCC tumors and normal epithelial kidney tissue at a site distant from the tumor were obtained from patients who had undergone radical nephrectomy. In total, 37 primary RCCs, 2 metastases, and 33 normal kidney specimens were collected before RNA extraction.

Table 3. HLA phenotype of the different RCC cell lines

According to the criteria proposed by Thoenes et al. (21). These data, including gender, stage of disease, tumor invasion, and lymph node involvement according to the TNM classification system, are listed in Table 3. All tissue samples

leukemia cell line Jurkat. MKN-45, Jurkat, and K562 cells were obtained from the American Type Culture Collection (Rockville, MD).

All cell lines were maintained in RPMI 1640 (Biochrom KG, Berlin, Germany) supplemented with 10% (v/v) FCS (Greiner GmbH Frickenhausen, Germany), 2 mM L-glutamine, 20 units/ml penicillin, and 200 units/ml streptomycin. The cells were harvested by trypsinization and washed with PBS before RNA extraction.

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The following cell lines were used as positive controls in PCR analyses: (a) the melanoma cell lines Mel-624, MZZ-Mel (10), and SK29Mel-1 (32); (b) the gastric carcinoma cell line MKN-45; (c) the RCC cell line LE921 IRC (33); (d) the chronic myelogenous leukemia cell line K562; and (e) the acute T-cell leukemia cell line Jurkat. MKN-45, Jurkat, and K562 cells were obtained from the American Type Culture Collection (Rockville, MD).

Unpublished observations.

Fig. 1. Representative RT-PCR analyses of RAGE-1 from RCC cell lines. RT-PCR analyses were performed as described in “Materials and Methods.” For detailed information on the primers, see Table 1. A representative RT-PCR analysis using RAGE-1- and β-actin-specific primers is shown. A. PCR products visualized on ethidium bromide-stained agarose gels. Top panel, the β-actin control; bottom panel, the corresponding RAGE-1-specific RT-PCR products. B. RAGE-1-specific PCR products visualized after Southern blotting.
were immediately frozen in liquid nitrogen after surgical resection and stored at −70°C until RNA extraction.

RT-PCR Analysis and Sequencing. Total cellular RNA was extracted by the guanidinium isothiocyanate/cesium chloride procedure, as described previously (31). All oligonucleotides used for PCR amplification and sequence analysis were generated using the Primer Designer program (Version 2.01; Scientific & Educational Software), synthesized by the triester method, and purchased from MWG Biotech GmbH (Ebersberg, Germany). The TAA-specific nucleotide primers and the PCR conditions used are listed in Table 1.

For the classical RT-PCR analysis, 1 µg of RNA was reverse-transcribed to cDNA with 10 units of avian myeloblastosis virus reverse transcriptase (United States Biochemicals, Bad Homburg, Germany) in the presence of 40 units of reverse-transcribed cDNA was used as a template in 50 µl of 1x PCR buffer [20 mM Tris-HCl (pH 8.3), 40 mM MgCl2, 250 mM NaCl, and 5 mM DTT (USB)], as described previously (34). For PCR amplifications, 100 ng of each deoxynucleotide triphosphate (Boehringer Mannheim), 50 pmol of each specific oligonucleotide primer, 250 µM of each deoxynucleotide triphosphate, and 1.5 units of Taq DNA polymerase (Boehringer Mannheim). Amplifications were performed with a hot start of 5 min at 94°C, and the PCR was terminated with an end extension for 10 min at 72°C. Primers for the housekeeping gene β-actin were used as a control for the amplifiability of the RNA used.

One-step PCR was performed using the Titan kit (One Tube RT-PCR System; Boehringer Mannheim) according to the manufacturer’s instructions. β-actin- and TAA-specific PCR was performed in the same reaction using the following conditions: 10 cycles of 20 s at 94°C, 30 s at the annealing temperature, and 1 min at 68°C followed by 25 cycles of 1 min at 94°C, 1 min at the annealing temperature, and 1 min at 68°C with a cycle elongation of 5 s for each cycle. The PCR products were fractionated by size and visualized on a 1% agarose gel containing ethidium bromide. To confirm specificity, the gels were blotted onto Hybond-N membranes (Amersham, Braunschweig, Germany), and filters were exposed to Reflection autoradiography film (New England Nuclear Life Science Products, Cologne, Germany). Samples were tested at least twice. In addition, the PCR products of tumor samples expressing gp75 and/or MAGE-2 were directly sequenced as described previously (35).

Cytotoxicity Assays. The sensitivity of target cells to lysis by the HLA-B7-restricted anti-RAGE-1 CTLs was evaluated by a standard 4-h 51Cr release assay (36, 37).

Results

Expression of RAGE-1, PRAME, and gp75 in RCC Cell Lines. Fourteen cell lines derived from primary RCCs were analyzed for the mRNA expression of tyrosinase, MAGE-1, MAGE-2, Melan-A/ MART-1, gp75, gp100, NY-ESO-1, β-catenin, PRAME, RAGE-1, and MUM-1. As shown in Table 2, a high percentage of RCC cell lines express RAGE-1 (7 of 14 cell lines) and PRAME (10 of 14 cell lines).
Expression of TAAs in Surgically Removed RCC Lesions. The results and the clinical and pathological features of the cases studied are summarized in Table 3. Based on the immunohistogenetic classification according to Thoenes et al. (21) and Störkel et al. (22), 39 surgically removed RCCs have been characterized as 25 primary clear cell RCCs, 2 metastases, 2 chromophobe RCCs, 6 chromophilic/papillary RCCs, 2 mixed RCCs, and 2 nonclassified RCCs. Samples of corresponding (23 samples) and unrelated (10 samples) normal kidney epithelium served as controls. These tissue samples were analyzed for mRNA expression of the different T-cell-defined TAAs. As shown in Table 3, more than 50% of primary RCCs expressed at least 1 TAA, 5 of 39 RCC lesions simultaneously expressed 2 TAs, and 2 of 39 RCCs expressed 3 TAs. RAGE-1 mRNA was expressed in 8 of 39 (21%) RCC specimens, and PRAME mRNA was expressed in 15 of 39 (40%) RCC specimens. MAGE-2 expression was only found in one RCC lesion (MZ2572T), which also expressed the antigens RAGE-1 and PRAME. Expression of the gp75 antigen was detected in 4 of 39 (11%) RCCs. False positive RT-PCR results could be excluded by direct sequencing of the gp75 PCR amplification products. PRAME, RAGE-1, MAGE-2, and gp75 were not expressed in any of the matched and nonmatched control samples of normal renal tissue. Furthermore, none of the tumors or normal kidney epithelium expressed MAGE-1, tyrosinase, Melan-A/MART-1, gp100, β-catenin, and MUM-1 (Table 3). A distinct TAA expression profile as well as heterogeneous levels of TAA mRNA expression are found in RCC lesions. This is shown for PRAME and gp75 in selected tumor specimens (Fig. 2). In all cases, the level of PRAME and gp75 expression was significantly higher than that of RAGE-1.

A comparison of TAA expression from primary clear cell RCC, chromophobe RCC, and chromophilic/papillary RCC demonstrated that both RAGE-1 and PRAME were often expressed in kidney tumors of distinct histology. In particular, chromophilic/papillary RCC and chromophobe RCC predominantly expressed RAGE-1, whereas PRAME expression seemed to be more pronounced in clear cell carcinoma. The data suggest a correlation between TAA expression and morphological subtypes. No significant association was found between TAA expression and TNM staging or tumor grade (Table 3).

Discussion

With the exception of RAGE-1 and NY-ESO-1, the T-cell-defined TAAs analyzed in this study have been isolated from human melanoma cell lines (1, 2). In contrast to the differentiation-specific antigens tyrosinase, Melan-A/MART-1, gp100, and gp75 (40), the cancer/testis antigens PRAME, RAGE-1, NY-ESO-1, and MAGE as well as antigens caused by point mutations (MUM-1, β-catenin) are expressed in tumors of distinct histology including various carcinomas and sarcomas as well as lymphocytic leukemias (14, 41–44). Little information was available on the expression of these TAAs in RCC. The present study demonstrates that individual TAAs were expressed both in surgically removed primary lesions and in RCC cell lines. A heterogeneous expression pattern of the PRAME, gp75, and RAGE-1 antigens was detected in a subset of RCCs, but in all cases, the expression of PRAME and gp75 was more pronounced compared with that of RAGE-1. In addition, the level and incidence of PRAME, gp75, and RAGE-1 expression were higher in RCC cell lines than they were in fresh tumors. For example, RAGE-1 transcription is present in >50% of RCC cell lines and in approximately 20% of the RCC tumors analyzed, and RAGE-1 mRNA expression is more pronounced in cell lines than it is in tumor lesions. The discrepancy in TAA expression observed between RCC cell lines and freshly isolated tumor specimens has not yet been classified. It could be due to clonal selection or a rapid turn-over or short half-life of TAA-specific mRNA in surgical specimens. Preliminary data using a RAGE-1-specific polyclonal antibody revealed RAGE-1 protein expression in RT-PCR-positive tumors, thereby arguing for clonal selection during the tissue culture of RCC. In addition, these first results of RAGE-1

3 B. Seliger, personal communication.
protein expression in RCC lesions demonstrate that the immunohistoch
tochemical data perfectly matched the RT-PCR results for RAGE-1 expression. A comparison of mRNA and protein expression of the antigens gp100 and Melan-A/MART-1 in melanoma revealed similar results (45).

The frequency of RAGE-1-expressing RCCs described here is in con
trast to the findings of Gaugler et al. (36), who reported only 1
RAGE-1-positive RCC specimen of 57 tumor samples. This discrep
ancy could be explained by differences in sensitivity of the RT-PCR
conditions, e.g., a different primer set and Southern blotting, which
was able to detect very low levels of RAGE-1 transcription in RCC
tumors.

Expression of the melanoma-associated antigens PRAME and gp75
was detected in a relatively high percentage of RCCs. The latter
antigen is of particular interest, because gp75 expression has mainly
been found in melanoma lesions or melanocytes and was found very
recently in normal brain tissue and glioblastoma multiforme (46).
However, the role and function of gp75 expression in RCC have yet
to be defined. It is noteworthy that MAGE-2 expression has been
found in 1 of 39 RCC tumor specimens, but not in any RCC cell line
analyzed. These overall results indicate that RCCs may exhibit a more
frequent expression of PRAME and RAGE-1 than do other tumors,
whereas MAGE is rarely expressed in RCC but is detected in a high
percentage of other carcinomas and sarcomas (41, 43, 44). However,
RT-PCR analysis does not provide information about the distributions
of the analyzed antigens in the lesions. Because no appropriate anti
bodies are available, it will be useful to develop the respective
TAA-specific antibodies, which could then be used for immunohis
tochemical staining of lesions.

The relationship of clinicopathological parameters and the expres
sion of these TAAas have been analyzed in melanomas and some
sarcomas (43, 44). The frequency of MAGE expression was shown
to be higher in metastatic lesions than it was in primary tumors. For
RAGE-1 and gp75, no such analysis has been documented (36),
whereas PRAME seemed to be associated with partial HLA loss (38),
which often correlates with the expression of a metastatic phenotype
(26). Our data demonstrate no correlation between the expression of
PRAME, gp75, and RAGE-1 and clinicopathological factors, includ
ing tumor invasion, lymph node metastasis, or disease stage (Table 3).
However, the expression of these TAAas is shared by different subsets
of RCC. The PRAME gene was found to be more frequently expressed
in clear cell RCC and chromophobipapillary RCC than it was in
chromophobe tumors. Because clear cell RCC and chromophbic/pap
illary RCC exhibit a significant tendency to metastasize (21, 22),
the expression of PRAME in both subtypes of RCC may be related to
the type of biological behavior observed in these kidney tumors. This
hypothesis was further strengthened by the analysis of nine oncocy
tomomas that were also negative for PRAME mRNA.3

An important question for T-cell-based immunotherapies is wheth
er low levels of TAA expression are sufficient to induce T-cell
responses. Autologous and allogeneic HLA-B7+ RCC cell lines expres
sing minimal levels of RAGE-1 were susceptible to lysis by anti-RAGE-1 CTLs (Table 2). This is in accordance with a recent report by Labarriere et al. (47) demonstrating that minimal expression of the Melan-A/MART-1 antigen in melanoma is sufficient to induce significant lysis by different Melan-A/MART-1-specific CTL clones. However, a higher threshold of antigen expression is required for
efficient cytokine release by CTLs. Although antigen density seemed
to constitute the most critical factor for optimal T-cell activation, the
expression of adhesion molecules and costimulatory factors is also
required for an efficient antigen response (48).

To date, no effective standard treatment for RCC is available, and the survival times of the patients are very short. It is therefore

Importance to develop new treatment strategies for these patients,
such as immunizations with tumor antigens. Pretreatment monitoring
tumor lesions for TAA expression may help in selecting patients for
an individually tailored antigen-specific vaccine therapy. Due to the
relatively high frequency of RAGE-1, gp75, and PRAME expression in
RCC lesions, these antigens alone or in combination may represent
possible candidates for the specific immunotherapy of patients with
kidney tumors.

Acknowledgments

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