Characterization of Human Lymphocyte Antigen Class I Antigen-processing Machinery Defects in Renal Cell Carcinoma Lesions with Special Emphasis on Transporter-associated with Antigen-processing Down-Regulation

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ABSTRACT

The HLA class I antigen-processing machinery (APM) plays a crucial role in the generation of peptides from endogenously synthesized proteins and in their presentation to cytotoxic T lymphocytes. The potential role of defects of APM components in immune escape mechanisms used by malignant cells has prompted us to analyze their expression in renal cell carcinoma (RCC) lesions with special emphasis on TAP because of its critical role in the loading of HLA class I antigens with peptides. Immunohistochemical staining of 51 formalin-fixed RCC lesions and autologous normal renal epithelium detected transporter associated with antigen processing (TAP)1 and tapasin deficiencies in 63 and 80% of the tumor lesions. Impaired low molecular weight protein (LMP)2 and LMP7 expression was found in 73 and 80% of the tumor lesions. Impaired low molecular weight protein (LMP)2 and LMP7 expression was found in 73 and 80% of the tumor lesions. Impaired low molecular weight protein (LMP)2 and LMP7 expression was found in 73 and 80% of the tumor lesions. Impaired low molecular weight protein (LMP)2 and LMP7 expression was found in 73 and 80% of the tumor lesions.

In recent years, the MHC class I APM and antigen presentation machinery has been well characterized (1, 2). Several molecules participate in this process. These include (a) the subunits of the multicatalytic proteasome complex, generating antigenic peptides through the degradation of cytosolic and nuclear proteins (3), (b) the heterodimeric peptide transporter consisting of the subunits TAP1 and TAP2, translocating peptides from the cytosol into the lumen of the ER (4), and (c) several chaperones such as calnexin, calreticulin, ERP57, and tapn, which stabilize MHC class I molecules during their folding and/or assembly in the ER or facilitate their loading with peptides (5–8). Once formed, stable MHC class I/beta2m peptide complexes leave the ER and traffic via the Golgi to the cell surface where they display their peptides to CD8+ CTLs (9, 10).

Abnormalities in MHC class I antigen surface expression are frequently found in human malignancies of distinct histology (11, 12) and are often associated with reduced recognition by HLA class I antigen-restricted, tumor-associated antigen-specific CTL and disease progression. This altered HLA class I phenotype can be caused by either structural alterations or dysregulation of genes encoding the subunits of HLA class I antigens and/or components of the MHC class I APM (11, 13). In contrast to breast carcinoma, small cell lung carcinoma, cervical carcinoma, and melanoma, only limited information exists about the HLA class I antigen and APM component expression in surgically removed RCC lesions. Reduced HLA class I antigen expression has been reported in ~38% of the RCC lesions analyzed (14) with a selective allelic loss predominantly occurring in advanced RCC (15). In addition, analyses of RCC cell lines have demonstrated significant defects in the expression and/or function of HLA class I antigens, LMP and TAP subunits (16, 17). Overexpression of wild-type TAP1 in
RCC cell lines was able to partially reconstitute TAP function. HLA class I antigen surface expression and recognition of RCC cells by HLA class I antigen–restricted, tumor–associated antigen-specific CTL (18), suggesting that TAP plays a key role in controlling the immune surveillance of RCC cells.

Thus far, the significance and clinical relevance of TAP deficiencies as well as their underlying molecular mechanisms have not been analyzed in RCC lesions. In addition, tpn expression has not been investigated in tumors, including RCC. tpn augments peptide loading by bridging the HLA class I heavy chain complex to TAP. This association enhances TAP expression, increases peptide translocation, and stabilizes HLA class I complexes in a peptide-receptive conformation (7, 19). Thus, impaired tpn expression might also contribute to the TAP-deficient immune escape phenotype of tumors. In this study, the frequency of TAP, tpn, and other APM component deficiencies was studied. This information will contribute to the optimization of T-cell–based immunotherapeutic strategies for the treatment of RCC patients.

MATERIALS AND METHODS

Patients and Tissue Samples. Thirty clear cell carcinoma, 16 papillary (chromophilic) carcinoma lesions, and 5 chromophobic carcinoma lesions along with autologous normal kidney epithelium were obtained from patients who had undergone radical nephrectomy. None of the patients had received preoperative therapy (e.g., chemotherapy, immunotherapy). Histopathological classification of each tumor was performed according to the criteria proposed by Thoenes et al. (20, 21), which are in line with the recent classification of the WHO. The criteria include gender, tumor invasion, lymph node involvement, and tumor grade according to the TNM classification (22). Tissue samples were either formalin-fixed and paraffin-embedded or snap frozen.

mAbs and Polyclonal Antibodies. The mouse anti-TAP1 mAb 148.3 and the mouse mAb HC-10 recognizing a determinant expressed on β2-m-free HLA-A10, HLA-A28, HLA-A29, HLA-A30, HLA-A31, HLA-A32 and -HLA.A33 heavy chains as well as on all β2-m-free HLA-B heavy chains have been described elsewhere (23, 24). The mouse anti-tpn mAb TO-3 was generated from a BALB/c mouse immunized with a tpn-derived peptide and fusion protein. The specificity of the mAb was assessed by its reactivity with the immunizing peptide in ELISA and in Western blotting. The characteristics of the mAb will be described in detail elsewhere (Ferrone et al., personal communication). The rabbit anti-LMP2 and anti-LMP7 antibodies were purchased from Affinity (Manhead, United Kingdom), whereas the mouse anti-β2-m mAb and the biotinylated rabbit antimonute and goat antirabbit IgG antibodies were purchased from Dako (Hamburg, Germany). All antibodies were stored at 4°C or at −20°C and were diluted with sterile PBS (pH 7.2) before use.

Immunohistochemistry. Representative paraffin blocks of tumor and corresponding normal tissue were selected and serially cut into 5-μm sections using a cryostat. All sections were stained with H&E to identify tumor tissue. Antigens were retrieved by incubating consecutive tissue sections for 2–7 min in citrate buffer in a microwave oven at 750 W. After once washing with Tris-buffered saline (pH 7.4), tissue sections were incubated for 10 min at room temperature with 5-fold diluted normal swine serum. Immunohistochemical staining of paraffin-embedded tissue sections was performed by incubating them overnight at room temperature with the indicated antibodies. After rinsing twice with Tris-buffered saline, tissue sections were incubated for 30 min at room temperature with biotinylated rabbit antimonute IgG or goat antirabbit IgG antibodies. Tissue staining was detected using the commercially available LSAB-peroxidase kit and AEC (Dako).

The percentage of stained tumor cells was determined in each lesion. Results were classified according to the HLA workshop criteria as: (0, negative): <25% of stained tumor cells; (1, heterogeneous): >25–<75% of stained tumor cells; and (2, positive): >75% of stained tumor cells. Negative controls were performed by omitting primary antibodies.

Statistical analysis of LMP2, LMP7, TAP1, tpn, HLA class I heavy chain and β2-m expression in terms of positive, heterogeneous, and negative staining of primary RCC lesions was performed using χ2-test.

TAP1 Sequencing. Total cellular RNA from tumor and normal tissue was extracted using the Trizol method according to the manufacturer’s instructions (Life Technologies, Inc., Karlsruhe, Germany). Aliquots of 200–300 ng of RNA were used for one tube RT-PCR analysis (Titan kit; Roche Diagnostics, Mannheim, Germany) using overlapping TAP1-specific primer pairs as listed in Table 1. The amplification products were size fractionated by agarose gel electrophoresis, purified using the commercial PCR-purification kit (Qiagen, Hilden, Germany) employing an annealing temperature of 59°C followed by direct sequencing of the isolated amplification products as described in “Materials and Methods.”

<table>
<thead>
<tr>
<th>Primer and position</th>
<th>Sequence</th>
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<tr>
<td>TAP1A pos. 1</td>
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</tr>
<tr>
<td>TAP1A pos. 428</td>
<td>CTG ACA AGC AAG GCG GTA GG</td>
</tr>
<tr>
<td>TAP1B pos. 326</td>
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<tr>
<td>TAP1C pos. 862</td>
<td>GTA ACA GAG GAC ACG TCC AC</td>
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<td>TAP1D pos. 1397</td>
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<tr>
<td>TAP1E pos. 2246</td>
<td>CAT TCT GGA GCA TCT GCA GG</td>
</tr>
</tbody>
</table>

Table 1 TAP1 primers used for RT-PCR and sequencing reactions

The listed primers were used for one tube RT-PCR (Roche Diagnostics, Mannheim, Germany) employing an annealing temperature of 59°C followed by direct sequencing of the isolated amplification products as described in “Materials and Methods.”
the immunohistochemical reaction of the mAbs used in this study for staining of normal kidney cells. As shown in Fig. 1, the normal tissue sections demonstrated a moderate to strong staining intensity using the LMP2, LMP7, TAP1, HLA class I heavy chain, and β2-m-specific mAb. Staining of HLA class I heavy chains was localized on the cell membranes of the glomeruli and interstitial cells, whereas the β2-m staining was localized on the cell membrane of the proximal and distal tubule epithelium as well as in the glomeruli. LMP2, LMP7, and TAP1 staining was detected in the cytoplasm of the proximal and distal tubule epithelium as well as in the epithelium of the collecting duct system (Fig. 1). The anti-TAP1 mAb 148.3 showed a homogeneous cytoplasmic staining pattern with inconstant perinuclear foci of granular positivity of the proximal and distal tubule epithelium and of the epithelium of the collecting duct system. In contrast, anti-LMP2 and anti-LMP7 antibodies showed a uniform positive cytoplasmic staining pattern. No staining was detected in normal kidney tissue using the anti-tpn mAb TO-3. However, staining of the TILs, which served as control of the validity of the anti-tpn antibody, demonstrated a strong tpn reactivity. Regarding the tpn deficiency of normal kidney epithelium, it has to be considered that tpn is neither absolutely essential for MHC class I surface expression nor for recognition and stimulation of alloreactive CTL (25, 26). Concerning the heterogeneous APM component expression found in the different cell types of normal kidney, one could speculate that this might be attributable to or influenced by the tumor microenvironment.

**Frequency and Underlying Molecular Mechanisms of TAP Abnormalities in RCC Lesions.** To assess the frequency of TAP defects in RCC lesions, 51 formalin-fixed, paraffin-embedded primary RCC lesions, which included 30 clear cell carcinoma, 16 papillary, and 5 chromophobic RCC lesions as well as autologous normal kidney epithelium (e.g., distal tubule and collecting ducts), were immunohistochemically analyzed for TAP1 expression using the anti-TAP1 mAb 148.3. When compared with normal kidney epithelium, only 5.9% of the 51 RCC lesions analyzed showed a normal TAP1 expression profile. In contrast, TAP1 expression was down-regulated in 62.7% and heterogeneous in 31.4% of the RCC lesions tested (P < 0.05; Table 2). Representative examples of positive and negative TAP1 staining of RCC lesions in comparison to autologous normal kidney epithelium are shown in Fig. 2.

To investigate the molecular mechanism(s) underlying impaired TAP1 expression, TAP1-negative tumors were analyzed for structural changes within the TAP1 coding region using RT-PCR followed by direct sequencing. No mutations were found in the TAP1 mRNA isolated from RCC lesions with deficient TAP1 expression. Also, because no mutations in the dual TAP1/LMP2 promoter have been found in RCC lesions and RCC cell lines (27), TAP1 down-regulation in RCC lesions appears not to be caused by structural defects. It rather appears to be attributable to regulatory mechanisms, which could occur at the transcriptional or posttranscriptional level.

**Correlation of the Frequency of TAP Abnormalities with Down-Regulation of other APM Components.** Since APM components have been shown to be at least partially coordinately down-regulated in different tumor types, abnormalities of APM components in addition to TAP were determined by immunohistochemical staining with LMP2, LMP7, tpn, HLA class I heavy chains, and β2-m-specific mAb. A total of 72.5 and 74.5% (P < 0.05) of the RCC lesions tested displayed an immunoreactivity with the anti-HLA class I heavy chain and anti-β2-m mAb comparable with that of normal kidney epithelium, respectively (Table 2, Fig. 3). In contrast, HLA class I
heavy chain expression was markedly down-regulated or not detectable in 11.8% of tumor specimens. There exists a variable staining intensity of HLA class I antigens in the different RCC subtypes with a weak staining intensity in papillary and chromophobic RCC lesions but a moderate intensity in all RCC lesions of clear cell type.

Staining with anti-LMP2 and anti-LMP7 mAb was not detected in 72.6 and 33.4% of the RCC lesions, respectively (P \( < \) 0.05). Furthermore, the staining with the anti-tpn mAb TO-3 was negative or heterogeneous in 80.4% of the RCC lesions with a P \( < \) 0.05 (Fig. 3). In contrast, staining of tpn in TIL showed a strong reaction. Among the RCC specimens analyzed, 56.9% showed a coordinated loss or significant down-regulation of TAP1 and LMP2 expression, whereas 19.6% of the lesions were negative for LMPs, TAP1, and tpn (Table 2). A coordinated loss of LMP2, LMP7, TAP1, tpn, and HLA class I antigen was only found in 3 of 51 primary RCC lesions.

In terms of clinical significance of these results, the frequency of deficiencies varied among the RCC subtypes (Table 3). However, LMP subunit, TAP1, tpn, and HLA class I antigen down-regulation is not associated with tumor grading and TNM characteristics such as lesion dimension and lymph node metastasis (Table 2), suggesting that the APM component deficiencies are not relevant to disease progression.

**DISCUSSION**

Our results demonstrate that (a) the proteasomal subunits LMP2 and LMP7, TAP1, tpn, and HLA class I heavy chains are frequently down-regulated in primary RCC lesions, whereas \( \beta_2 \)-m expression in RCC lesions is comparable with that in normal kidney epithelium; (b) the frequency and the level of TAP1 and LMP2 down-regulation are more pronounced than those of LMP7 and HLA class I antigens and occurred in approximately 50% of RCC lesions; (c) tpn is not expressed in normal kidney epithelium and is strongly down-regulated in RCC lesions, whereas it is highly expressed in TIL; (d) the regulation of TAP gene expression, rather than mutations in TAP mRNA, is responsible for loss of TAP expression; and (e) TAP1, tpn, LMP2, LMP7, and HLA class I antigen down-

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**Table 2** APM component and HLA class I antigen subunit expression in RCC lesions

Tumor specimens and corresponding normal kidney tissue were obtained after radical nephrectomy from 51 patients with primary RCC. TNM staging and histopathological evaluation was performed according to the classification of Thoenes et al. (22). Informed consent was received from all patients. Paraffin-embedded tissue samples were subjected to immunohistochemical analysis using antibodies to APM components and to HLA class I antigen subunits. The results were summarized in terms of RCC subtype, tumor grading, and frequency of APM deficiencies in percentage. The APM deficiencies are scored as negative (−) and heterogeneous (+/−) as described in “Materials and Methods.”

<table>
<thead>
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<th>Grade</th>
<th>n</th>
<th>LMP2</th>
<th>LMP7</th>
<th>TAP1</th>
<th>tpn</th>
<th>( \beta_2 )-m</th>
<th>MHC class I</th>
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<td>60</td>
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<td>50</td>
<td>100</td>
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<td>33</td>
<td>66</td>
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<td></td>
<td>( \Sigma )</td>
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<td>37</td>
<td>9</td>
<td>17</td>
<td>20</td>
<td>32</td>
<td>41</td>
</tr>
</tbody>
</table>

\( n \), number of lesions analyzed; \( \Sigma \): total sum.

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Fig. 2 Staining of a RCC, clear cell type G2, for LMP2 \((a, \times 200)\) and TAP1 \((b, \times 200)\) as well as coordinated LMP2 \((c, \times 200)\) and TAP1 \((d, \times 200)\) loss in a RCC lesion of chromophobic type G2. Positive staining of TILs was found in \( c \) and \( d \).
regulation is not associated with tumor grading but significantly varied between the RCC subtypes.

TAP1 expression has been monitored in Hodgkin’s lymphoma as well as in various types of carcinoma and melanoma. TAP1 down-regulation or loss have been found in different tumor types with frequencies ranging from 10 to 84% (13, 28–31), but the molecular mechanisms underlying this defect have not been identified yet. We extended these data to surgically removed primary RCC lesions demonstrating TAP1 down-regulation or loss in 62.7% of the lesions analyzed (Table 2). Sequence analyses of TAP1 cDNAs revealed no mutations and deletions in any tumor specimen tested, suggesting that TAP1 abnormalities in RCC lesions are caused by defects in the regulatory mechanisms and not by structural alterations. This is certainly in line with the fact (a) that sequence abnormalities in the TAP1 coding region leading to loss of its expression and function have only been described in one lung cell carcinoma and one melanoma cell line (32, 33), (b) that no mutations were found in the TAP1/LMP2 promoter of a large series of RCC lesions and RCC cell lines analyzed (27), and (c) that cytokine treatment can often restore TAP1 expression in RCC and other tumor cell lines (28, 34).

Consistent with previous results in breast carcinoma and melanoma (30, 31), synchronous down-regulation or loss of TAP1 and LMP2, but not of LMP7, was found in a high percentage of RCC lesions (Table 2). The linkage between deficiencies of TAP1 and LMP2 expression could be explained by the common bidirectional promoter, which coordinately regulates the transcription of both genes (35).

It might be speculated that TAP abnormalities in tumors might be associated with impaired tpn levels. This hypothesis is based on data obtained from tpn knockout mice and tpn-negative cells, suggesting that tpn stabilizes the TAP heterodimer, which is associated with an increase in steady-state TAP levels and enhanced peptide translocation (19, 25, 26, 36, 37). This is attributable to an interaction between tpn and both TAP subunits and thereby enhancing the structural stability of TAP complexes (38). In addition, the diminished HLA class I surface expression could be restored in hepatoma cells by tpn gene transfer (39). As demonstrated in Table 2 and Fig. 3, loss of or heterogeneous tpn

![Image](https://example.com/image.png)

**Table 3** Summary of APM component and HLA class I antigen subunit deficiencies in terms of percentage of stained cells

<table>
<thead>
<tr>
<th>RCC subtype</th>
<th>Frequency of deficiencies (in %)</th>
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<tr>
<td></td>
<td>LMP2</td>
</tr>
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<td>Clear cell</td>
<td>76.6</td>
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<tr>
<td>Papillary</td>
<td>56.3</td>
</tr>
<tr>
<td>Chromophobic</td>
<td>100</td>
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</table>

Fig. 3  APM component expression in a RCC lesion of clear cell type, G2. Representative staining patterns for HLA class I heavy chain (a, ×200) and β2-m (b, ×200), heterogeneous (c, ×200) and negative (d, ×200) staining for tpn and strong staining for LMP7 in a RCC lesion of clear cell type G2 (e, ×200) are shown. LMP7 loss in another RCC lesion of clear cell type G2 while infiltrating lymphocytes show positive staining with the rabbit anti-LMP7 antibodies (f, ×200).
expression is found in RCC lesions, whereas it is highly expressed in TIL. This defect appears to be at least partially associated with TAP down-regulation. Thus, our results suggest a correlation between TAP and tpn deficiencies. On the other hand, it is noteworthy that the deficient tpn expression in normal kidney epithelium is not associated with TAP down-regulation. Additional investigations are required to understand the impaired tpn expression in normal kidney tissue. Although tpn forms a bridge between TAP and MHC class I heterodimers (7), it is not absolutely essential for MHC class I surface expression. tpn influences both the quality and quantity of peptide loading onto MHC class I molecules: absence of tpn results in the presence of an altered peptide repertoire presented by MHC class I molecules rather than a complete lack of peptides (25, 40).

As far as abnormalities in HLA class I heavy chain expression in RCC lesions is concerned, this study differs from those published by others. The frequency of HLA class I downregulation found in the 51 RCC lesions analyzed is lower than that reported previously (Table 2; Refs. 14, 15, 41). Moreover, no correlation was detected between RCC subtypes and abnormalities in HLA class I antigen expression (14). In addition, TAP1 and HLA class I down-regulation was independent of the histopathological characteristics of RCC lesions. This is in contrast to melanoma, small cell lung carcinoma, cervical cancer, and breast carcinoma in which TAP1 and HLA class I antigen deficiencies were associated with tumor grading, tumor staging, and/or decreased patients’ survival (13, 30, 31, 42). The results obtained in surgically removed RCC lesions compared with other malignancies may at least partially be explained by the tissue specificity and by the differences in the immune status during pathogenesis and clinical course in various diseases. In view of the implementation of T-cell-based immunotherapies for the treatment of RCC patients, it is noteworthy that abnormalities associated with the expression of components of the HLA class I antigen processing pathway, especially of TAP1, may have a negative effect on the outcome of this type of therapy. Indeed, the importance of various APM components on the tumorigenicity and proper immune response directed against tumors has been shown in both human tumors and murine tumor systems, clearly demonstrating that APM deficiencies result in the lack of T-cell-based immune recognition and tumor outgrowth (42–46). Gene transfer of TAP1 into TAP-deficient cells restores T-cell-mediated lysis, reduced or even abrogates tumor growth and can additionally protect animals against challenge with TAP-deficient cells (42, 45, 46). Thus, analysis of APM component expression in RCC lesions may contribute to optimize the design of T-cell-based immunotherapeutic strategies as well as the selection of patients for such a treatment modality. In addition, TAP gene transfer has to be considered for inclusion in cancer therapies and might provide a method for increasing immune response against tumors.

ACKNOWLEDGMENTS

We thank Petra Böhmer for excellent technical assistance and Inge Schmidt for excellent secretarial assistance.

REFERENCES

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