Identification of markers for the selection of patients undergoing renal cell carcinoma-specific immunotherapy

Renal cell carcinoma (RCC) represents the most common malignant tumor in the kidney and is resistant to conventional therapies. The diagnosis of RCC is often delayed leading to progression and metastatic spread of the disease. Thus, validated markers for the early detection of the disease as well as selection of patients undergoing specific therapy is urgently needed. Using treatment with the monoclonal antibody (mAb) G250 as a model, proteome-based strategies were implemented for the identification of markers which may allow the discrimination between responders and nonresponders prior to application of G250-mediated immunotherapy. Flow cytometry revealed G250 surface expression in approximately 40% of RCC cell lines, but not in the normal kidney epithelium cell lines. G250 expression levels significantly varied thereby distinguishing between low, medium and high G250 expressing cell lines. Comparisons of two-dimensional gel electrophoresis expression profiles of untreated RCC cell lines versus RCC cell lines treated with a mAb directed against G250 and the characterization of differentially expressed proteins by mass spectrometry and/or Edman sequencing led to the identification of proteins such as chaperones, antigen processing components, transporters, metabolic enzymes, cytoskeletal proteins and unknown proteins. Moreover, some of these differentially expressed proteins matched with immunoreactive proteins previously identified by proteome analysis combined with immunoblotting using sera from healthy donors and RCC patients, a technique called PROTEOMEX. Immunohistochemical analysis of a panel of surgically removed RCC lesions and corresponding normal kidney epithelium confirmed the heterogeneous expression pattern found by proteome-based technologies. In conclusion, conventional proteome analysis as well as PROTEOMEX could be successfully employed for the identification of markers which may allow the selection of patients prior to specific immunotherapy.

Keywords: G250 immunotherapy / PROTEOMEX / Renal cell carcinoma

1 Introduction

The renal cell carcinoma (RCC) represents the most common neoplasia of the kidney in Western countries. However, the molecular mechanisms leading to the initiation as well as to the progression of this disease are still not well defined. RCC is associated with the accumulation of different genetic alterations, such as the amplification of the epidermal growth factor receptor (EGF-R) [1, 2], mutations and/or loss of tumor suppressor genes, like the von Hippel Lindau gene [3]. So far, no validated molecular markers for the diagnosis, prognosis and for the monitoring of this disease during therapy are available. RCC represents a relatively therapy-resistant tumor and conventional treatment modalities like surgery, radio- and chemotherapy are of limited success [4]. The high spontaneous occurrence of partial or complete remissions, the existence of lymphocytic infiltrates in RCC lesions as well as the frequency of objective remission upon immunotherapy suggest that RCC represents an attractive target for different vaccine strategies. In contrast to melanoma, only a limited number of RCC-associated antigens have been identified which may be useful for this approach. These include for example the antigens RAGE, SART3, HER-2/neu and G250, also termed MN/CAIX [5–11]. G250 is a transmembrane protein with car-

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Abbreviations: HSP, heat shock protein; PROTEOMEX, a combination of PROTEOMics and serEX (serological expression cloning); RCC, renal cell carcinoma

* This work represents part of Matthias Menig’s MD thesis.
bion anhydrase activity. It is overexpressed in a variety of tumors of distinct histology, including cervical carcinoma and RCC of clear cell subtype [12–13]. Normal tissue expression is limited to large ductal bile epithelium and stomach mucosal cells [14]. Recently, G250-specific epitopes presented by either HLA-A2 or HLA-DR have been identified, which might be employed as targets for peptide based vaccines to induce both CD4+ and CD8+ specific T cell responses in RCC patients [15, 16]. Furthermore, a monoclonal chimeric antibody directed against G250 was developed and is currently used in clinical trials for the treatment of RCC patients [17–20]. In phase I/II clinical trials with the [131] labeled monoclonal antibody (mAb) G250, antitumor responses have been observed [21], while in a phase II trial of patients with metastatic RCC receiving G250 immunotherapy the median survival was 15 months and 25% of the patients had a clinical benefit (Press release WILEX, 2002). Tso and coworkers [22] described an induction of G250-targeted, T cell-mediated antitumor activity against RCC using a chimeric fusion protein of G250 and GM-CSF. Furthermore, antibody-mediated endocytosis of G250 antigen allows targeted gene transfer to RCC in vivo. Although the experimental and clinical results of G250 treatment are encouraging [14, 21, 23, 24], the efficacy of G250 therapy might be enhanced by the identification of markers which discriminate between therapy-responders and nonresponders. Therefore, proteomic approaches were implemented for the identification of such markers. Total cellular extracts from untreated as well as from RCC cell lines treated with a mAb directed against G250 were separated and differentially expressed proteins identified by mass spectrometric analysis. In addition, these differentially expressed proteins were compared to target structures previously defined by a technique named PROTEOMEX [25, 26] which is based on a combination of proteomics and Western blot analyses using sera from RCC patients and healthy donors. Since a number of mAb G250-regulated target structures match with those previously detected with PROTEOMEX the implementation of proteome-based techniques in the search for markers that might improve the selection of patients undergoing RCC-specific immunotherapy is proposed.

2 Materials and methods

2.1 Cell culture, treatment with the mAb G250 and immunosera

The human renal cell carcinoma (RCC) cell lines were derived from patients with primary RCC of clear cell type as previously described [27]. All RCC cell lines were maintained in DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/mL penicillin/100 µg/mL streptomycin, 1% MEM nonessential amino acids and 1% sodium pyruvate (all components purchased from Gibco/BRL, Life Technologies, Karlsruhe, Germany). The SV40LT-transformed normal kidney epithelium cell line MZ2733NN was expanded in the presence of 60 µg/mL Hygromycin B (Roche Diagnostics, Mannheim, Germany). Treatment of RCC cells with a mAb directed against G250 was performed for 24 h, addressing changes in the expression profiles at an early time point during treatment, or for 21–23 d, thereby focussing on effects of long term treatment. For mAb treatment, cell cultures were incubated in the presence of 6 µg/mL anti-G250 specific antibody (WX-G250) kindly provided by Wilex (Munich, Germany) for the length of time indicated. Serum samples employed for PROTEOMEX analyses were obtained from venous human peripheral blood of seven RCC patients and of seven healthy volunteers after informed consent was obtained from each individual.

2.2 Flow cytometric analysis

The FITC-conjugated humanized mAb WX-G250 directed against G250 as well as a FITC-conjugated goat-anti-mouse immunoglobulin (Beckman/Coulter, Krefeld, Germany) which served as a control were used for flow cytometry [28]. Harvesting by trypsinization prior to the mAb staining had no effect on the G250 surface expression levels (data not shown). For staining of G250 antigens, cells were incubated with a primary antibody for 30 min on ice, washed twice in PBS before cells were analyzed on a flow cytometer (Coulter EPiX XL MCL; Beckman/Coulter) as described earlier [26]. The experiments were performed twice (short term cultures) or three times (established RCC lines) and the data expressed as mean specific fluorescence intensity (MFI).

2.3 Two-dimensional gel electrophoresis

For sample preparation, 1 × 10^7–1 × 10^8 untreated and anti-G250 mAb-treated logarithmically growing cells were harvested, washed up to four times in PBS and stored as dry cell pellets in aliquots of 5 × 10^6 or 1 × 10^7 cells/tube in liquid nitrogen until further use as described previously [29]. Cell pellets were resuspended in lysis buffer (7 M urea; AppliChem Biochemica, Darmstadt, Germany, 2 M thiourea; Sigma-Aldrich, Deisenhofen, Germany), 0.2 M dimethylbenzy1-ammonium propane sulfonate (NDSB, ICN Biomedicals, Eschwege, Germany), 1% DTT (AppliChem), 4% CHAPS (Applichem), 0.5% Pharmalytes (Amersham Biosciences, Freiburg, Germany) and a trace of the dye bromophenol blue (Serva, Heidelberg, Germany). Lysates were processed as
recently described [29] and the protein concentration was
determined by a modified Bradford method. Samples
were then loaded onto either linear pH 4–7 or nonlinear
pH 3–10 immobilized gradient (IPG) strips (Immobiline
DryStrips, pH 4–7 or pH 3–10 NL; Amersham Biosciences)
using 300–500 μg total protein/strip. IEF was
performed on an IPGphor unit (Amersham Biosciences),
as previously described [29]. The samples were subse-
sequently separated on 13% SDS-PAGE gels. All samples
analyzed were run at least in triplicate, the majority in
sets of five.

2.4 Gel staining, documentation and analysis

Gels for mass spectrometric analysis and Edman degra-
dation were either stained with colloidal Coomassie Blue
(Molecular Probes, Leiden, The Netherlands) or with silver,
respectively. Coomassie and silver stained gels were
scanned either on a ScanJet 6100C (Hewlett-Packard,
 Palo Alto, CA, USA) or on an UMAX PowerLook III scanner
equipped with a UTA III Transparency Adaptor (UMAX Sys-
tems, Düsseldorf) at a resolution of 300 dpi and stored as
TIFF images. In contrast, SYPRO® Ruby stained gels were
scanned with a FLA-3000 fluorescence scanner (Raytest,
 Straubenhardt, Germany). Gel analyses were performed
either using PDQuest (Bio-Rad, Munich, Germany) or the
ProteomeWeaver (Definiens, Munich, Germany) software
packages, respectively. The calculation of the theoretical
molecular weight and pI values of identified protein spots
is based on algorithms included in the MacVector software
package (V 5.02; IBI, New Haven, USA).

Spot picking was performed using the PROTEINEER sp
(Bruker Daltronik, Bremen, Germany) automated spot
picker. A modified digital scanner served as the spot-
picking platform while the software included allowed for
manual selection of single spots by mouse-clicking on
the scanned gel image.

2.5 Immunoblotting

Immunoblotting of 2-D PAGE gels (Hoefer DALT-blotting
kit; Amersham Biosciences) was performed as recently
described [29]. Membranes were incubated overnight at
4°C with the respective sera samples (dilution 1:50). The
horseradish peroxidase (HRP)-conjugated antihuman
mAb PO214 (DAKO, Hamburg, Germany) served as the
secondary antibody and signal detection was achieved
using a chemiluminescence detection system (Lumi-Light
Western blotting substrate; Roche Molecular Biochem-
icals) according to the manufacturer’s instructions.

2.6 Protein identification

Protein analysis was performed as described [29]. Peptide
mass fingerprinting and post-source decay experi-
mements were obtained using a Voyager STR MALDI mass
spectrometer (Applied Biosystems, Foster City, CA, USA).
Edman degradation was performed using a Procise 494
system (Applied Biosystems). The peptide masses and
sequences obtained were employed for database
searches for protein identification using the Protein Pro-
spector software package [32].

2.7 Immunohistochemistry

For immunohistochemical analysis, surgically removed tis-
sue samples from RCC and corresponding normal kidney
epithelium were randomly obtained from patients who had
undergone radical nephrectomy. Histopathological classi-
fication of each tumor was performed according to the cri-
teria proposed by Thoenes and coworkers [33]. These data
include gender, stage of disease, tumor invasion, and
lymph node involvement according to the TNM system.
The standard panel of 64 primary renal tumors comprised
51 clear cell carcinoma and 13 chromophobic carcinomas
as well as 64 autologous normal kidney specimens, all col-
lected at resection. For the evaluation of RCC subtype spe-
cific marker expression, the RCC tissue panel for imunohis-
tochemical staining were expanded to 87 primary RCCs,
representing 40 clear cell carcinomas, 31 papillary carci-
nomas, 16 chromophobic carcinomas and 9 renal adenomas
of oncocytic subtype as well as corresponding normal
renal tissue using multi-tissue array technology. The tissue
samples were formalin-fixed and paraffin-embedded.

Immunohistochemical staining was performed with the
anti-vimentin mAb (clone V9, DAKO; dilution 1:40), the
anti-heat shock protein-(HSP)-27 mAb (MA3–015; Affinity
Bioreagents, Cambridge, UK; dilution 1:500) and anti-
statmin mAb (B37545; Calbiochem, La Jolla, USA; dilu-
tion 1:500). For antigen retrieval, consecutive sections
were incubated for 8 and 6 min in citrate buffer in a micro-
wave oven, respectively, followed by a washing proce-
dure with TBS and an additional incubation with normal
swine serum (dilution 1:10) for 10 min. Slides were incu-
bated with the primary antibodies for 1 h at room tem-
perature. Detection was performed using the LSAB
Labelled streptavidin biotin technique-peroxidase kit and
AEC (3-amino-9-ethylcarbazole) as recently described
(DAKO). Negative controls where performed by omitting
the primary antibody. The extent of immunostaining was
scored according to the following criteria: negative: < 5%
positive cells; weak positive: 5–25% positive tumor cells;
moderate positive: 26–50% positive tumor cells; strong
positive > 50% positive tumor cells.
3 Results

3.1 Frequency of G250 expression in RCC cell lines

Twenty-six established RCC cell lines, six short term RCC cultures with < 5 passages, one RCC metastasis as well as one representative normal epithelium cell line were analyzed for the surface expression of antigen G250/MN/CAIX using flow cytometry (Table 1). Twelve RCC cell lines, five short term cultures and the RCC derived lymph node metastasis cell line (MZ1851LN) showed constitutive G250 surface expression, but the level of G250 antigens significantly varied in the G250 expressing RCC cell lines (Fig. 1). In accordance with the literature, staining of the normal renal epithelium cell line was negative [4, 11]. Three distinct G250 phenotypes were identified in the RCC panel and classified as low, medium and high G250 expressing cell lines (Fig. 2). Although the frequency of G250 expressing cells and quality in terms of G250 expression levels appears to be distinct in short term RCC cultures when compared to established RCC cell lines, no final conclusions could be drawn due to the low number of short term RCC cultures analyzed. However, no linkage exists between G250 expression levels and the culture conditions currently applied, since a time-dependent monitoring of G250 expression levels over 15 passages in representative short term RCC cultures showed no significant alteration in G250 surface expression (data not shown).

Table 1. Panel of cell types analyzed for G250 surface expression by flow cytometric analysis

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Number of samples analyzed</th>
<th>G250 staining positive / negative</th>
<th>G250 subtype high / medium / low</th>
</tr>
</thead>
<tbody>
<tr>
<td>Established RCC cell lines</td>
<td>26</td>
<td>12 / 14</td>
<td>3 / 6 / 3</td>
</tr>
<tr>
<td>Short term RCC cultures</td>
<td>6</td>
<td>5 / 1</td>
<td>0 / 2 / 3</td>
</tr>
<tr>
<td>RCC metastasis</td>
<td>1</td>
<td>1 / 0</td>
<td>0 / 1 / 0</td>
</tr>
<tr>
<td>Normal renal epithelium cell lines</td>
<td>1</td>
<td>0 / 1</td>
<td>negative staining</td>
</tr>
</tbody>
</table>

The different cell lines were analyzed for G250 surface expression by flow cytometry as described in Section 2.2.

3.2 Protein expression profile of untreated and G250-treated RCC cell lines

In order to identify proteins which were regulated by mAb G250 three RCC cell lines expressing different levels of G250 surface antigens were selected for proteome analysis. These included the G250 expressing cell lines MZ2789RC (low level), MZ1846RC (medium level) and MZ1879RC (high level). The cells were either left untreated or treated with 6 μg/mL anti-G250 mAb for 24 h (short term treatment) or 21 to 23 d (long term treatment), respectively and subsequently subjected to proteome...
Marker identification in RCC

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Figure 2. Three distinct G250 phenotypes in RCC. Flow cytometry was performed to determine G250 surface expression in RCC cell lines as described in Section 2.2. Representative histograms are shown for low, medium and high G250 expressing cells. The x-axis represents log fluorescence intensity, whereas the y-axis represents the cell number.

Figure 3. Representative 2-D gel expression profile of a G250 long term treated MZ2789RC cells. 300 μg total protein/sample were subjected to 2-DE as described in Section 2.3 using Immobiline DryStrips pH 4–7. The gel is stained with Sypro Ruby. A representative selection of differentially expressed proteins, listed in Table 2, is marked by arrows and indicated by name. The section defined by the black frame is shown in more detail in Fig. 4.

analysis. 200–300 μg total protein/sample was employed for 2-DE using pH 3–10 NL or pH 4–7 gradients in the first dimension (analytical scale). A representative 2-DE spot pattern of long term anti-G250 mAb treated MZ2789RC cells is shown in Fig. 3, a detailed comparison of untreated and anti-G250 mAb-treated cells is represented in Fig. 4. In general, between 633 and 1118 protein spots were detected per average gel depending on the staining method. Based on a ratio of ± 1.5, approximately 30–60% of the proteins were differentially expressed in untreated versus G250-treated cells. In the G250 low expressing cell line MZ2789RC 12–17% of the proteins were up-regulated, whereas 10–23% were down-regulated in the presence of the G250 specific mAb. In the cell line MZ1846RC expressing G250 at a moderate level, the number of up-regulated proteins remains at a similar range of about 10%, but the number of down-regulated proteins increased to 34%. The G250 high expressing cell line MZ1879RC exhibited the highest number of regulated proteins with 20% up-regulated and approximately 40% down-regulated (data not shown). Individual regulation factors for some differentially expressed spots identified in MZ2789RC ranging between 0.3 (down-regulation) to 2.5 (up-regulation) are listed in Table 2.
Figure 4. Comparison of the spot pattern of untreated versus G250 treated M2789RC cells. The left panel represents the zoomed 2-DE segment indicated by the black frame in Fig. 3 of untreated cells, whereas the right panel represents the corresponding segment of G250 mAb treated cells. The arrows mark the location of the differentially expressed proteins annexin V, HSP-27, tubulin β and vimentin as indicated in Fig. 3. Sample preparation and gel staining were performed as described in Fig. 3.

Table 2. Characteristics of the proteins differentially expressed upon G250 treatment

<table>
<thead>
<tr>
<th>Protein name</th>
<th>SWISS-PROT/TrEMBL accession no.</th>
<th>M / pI</th>
<th>seq. coverage</th>
<th>Regulation factor</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat shock 27 kDa protein 1</td>
<td>P04792</td>
<td>22.8 / 6.0</td>
<td>41%</td>
<td>2.5 up</td>
<td>Involved in stress resistance and actin organization. Associates with alpha- and beta-tubulin and microtubules.</td>
</tr>
<tr>
<td>Ubiquitin-activating enzyme E1</td>
<td>P22314</td>
<td>118.9 / 5.6</td>
<td>14%</td>
<td>2.2 up</td>
<td>Activates ubiquitin by first adenylating with ATP its carboxy-terminal glycine residue and thereafter linking this residue to the side chain of a cysteine residue in E1, yielding an ubiquitin-E1 thiolester and free AMP.</td>
</tr>
<tr>
<td>(AL136382) dJ977L11.1(KIAA 1229 protein)</td>
<td>Q9HCZ9</td>
<td>79.0 / 6.2</td>
<td>16%</td>
<td>2.1 up</td>
<td>Hypothetical protein, function unknown</td>
</tr>
<tr>
<td>Vimentin</td>
<td>P08670</td>
<td>53.8 / 5.0</td>
<td>44%</td>
<td>2.1 up</td>
<td>Vimentins are class III intermediate filaments found in various non-epithelial cells, especially mesenchymal cells.</td>
</tr>
<tr>
<td>IQ motif containing GTPase activating protein 1</td>
<td>P46940</td>
<td>189.9 / 6.1</td>
<td>14%</td>
<td>2.0 up</td>
<td>Binds to activated CDC42 but does not stimulate its GTPase activity. It associates with calmodulin. Could serve as an assembly scaffold for the organization of a multimolecular complex that would interface incoming signals to the reorganization of the actin cytoskeleton at the plasma membrane.</td>
</tr>
<tr>
<td>FKS06-binding protein 4 (59 kDa)</td>
<td>Q02790</td>
<td>52.0 / 5.3</td>
<td>27%</td>
<td>1.8 up</td>
<td>Component of unactivated mammalian steroid receptor complexes that sediment at 8-10 S. May have a rotamase activity. May play a role in the intracellular trafficking of hetero-oligomeric forms of steroid hormone receptors.</td>
</tr>
</tbody>
</table>
Table 2. Continued

<table>
<thead>
<tr>
<th>Protein name</th>
<th>SWISS-PROT/TrEMBL access. no.</th>
<th>Mr / pl</th>
<th>seq. coverage</th>
<th>Regulation factor</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major vault protein</td>
<td>Q14764</td>
<td>99.6 / 5.3</td>
<td>30%</td>
<td>1.7 up</td>
<td>Function unknown, though MVP is required for normal vault structure. Vaults are multi-subunit structures that may be involved in nucleo-cytoplasmic transport.</td>
</tr>
<tr>
<td>Tubulin, beta, 2</td>
<td>P05217</td>
<td>50.3 / 4.8</td>
<td>23%</td>
<td>1.7 up</td>
<td>Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a nonexchangeable site on the alpha chain.</td>
</tr>
<tr>
<td>Chain A, annexin V</td>
<td>P08758</td>
<td>35.8 / 4.9</td>
<td>22%</td>
<td>1.7 up</td>
<td>This protein is an anticoagulant protein that acts as an indirect inhibitor of the thromboplastin-specific complex, which is involved in the blood coagulation cascade (indirect apoptosis marker).</td>
</tr>
<tr>
<td>Chloride intracellular channel 4</td>
<td>Q9Y696</td>
<td>29.0 / 5.5</td>
<td>36%</td>
<td>1.6 up</td>
<td>Chloride channel or a regulator or accessory subunit of other proteins that could provide the pore-forming function.</td>
</tr>
<tr>
<td>Valosin-containing protein</td>
<td>11265337 (Entrez-ID)</td>
<td>47.9 / 4.8</td>
<td>34%</td>
<td>1.6 up</td>
<td>Hypothetical protein DKFZp434Ko126.1</td>
</tr>
<tr>
<td>Aldehyde reductase</td>
<td>P14550</td>
<td>36.8 / 6.3</td>
<td>41%</td>
<td>3.3 down</td>
<td>Enzyme, catalytic activity: an alcohol + NADP+ = an aldehyde + NADPH. Interconverts GlcNAc-6-P and GlcNAc-1P, pathway: UDP-GlcNAc biosynthesis from Fru-6-P.</td>
</tr>
<tr>
<td>N-acetylglucosamine-phosphate mutase</td>
<td>O95394</td>
<td>60.3 / 5.8</td>
<td>27%</td>
<td>2.6 down</td>
<td>Hypothetical protein XP028726, function unknown, presumably component of the cytoskeleton.</td>
</tr>
<tr>
<td>Hypothetical protein MGC14580 (similar to alpha tubulin)</td>
<td>no entry (14761545)</td>
<td>46.6 / 5.0</td>
<td>51%</td>
<td>2.5 down</td>
<td>Hypothetical protein XP028726, function unknown, presumably component of the cytoskeleton.</td>
</tr>
<tr>
<td>Moesin</td>
<td>P26038</td>
<td>67.9 / 6.1</td>
<td>50%</td>
<td>2.2 down</td>
<td>Probably involved in connections of major cytoskeletal structures to the plasma membrane.</td>
</tr>
<tr>
<td>(BC005022) SH3 and PX domain-containing protein SH3PX 1</td>
<td>Q9YSX1</td>
<td>66.98 / 5.4</td>
<td>22%</td>
<td>2.1 down</td>
<td>May be involved in several stages of intracellular trafficking.</td>
</tr>
<tr>
<td>Enolase 2 (gamma, neuronal)</td>
<td>P09104</td>
<td>47.6 / 4.9</td>
<td>66%</td>
<td>2.0 down</td>
<td>Enzyme, catalytic activity: 2-phospho-o-glycerate = phosphoenolpyruvate + H₂O, pathway glycolysis.</td>
</tr>
<tr>
<td>Heat shock 90 kDa protein-1, beta</td>
<td>P08238</td>
<td>83.6 / 5.0</td>
<td>22%</td>
<td>1.5 down</td>
<td>Molecular chaperone, has ATPase activity.</td>
</tr>
</tbody>
</table>

3.3 Identification of differentially expressed proteins

The identity of differentially expressed proteins upon treatment with the G250 specific mAb was determined by MS. Identified proteins as well as their physical parameters, regulation factors and biological functions from the analysis of the low expressing cell line MZ2789RC are summarized in Table 2. Some of these targets, such as annexin V and tubulin-β derived from samples after short term incubation with anti-G250 specific mAb or HSP-27, moesin, N-acetylglucosamine-phosphate mutase and vimentin obtained from samples after long term anti-G250 specific mAb treatment are shown in Figs. 3 and 4. The categories of differentially expressed protein in the various phenotypes of G250 expressing cell lines analyzed belong to different protein families, such as meta-
bolic enzymes, chaperones, antigen processing components, transporters, cytoskeletal proteins, putative tumor-associated antigens, cell cycle regulators as well as some unknown proteins. The overlap of differentially expressed proteins between the three G250 subtypes is currently rather restricted, although some differentially expressed proteins are commonly regulated in both short term and long term incubations and are shared between various G250 subtypes.

3.4 Evaluation of the differentially expressed proteins

In order to analyze the frequency of differentially expressed proteins upon treatment with the G250-specific mAb and their use as markers of therapy responders and non-responders, immunohistochemical analysis of a selected number of proteins was performed on a panel of RCC lesions (Table 3). These include vimentin and HSP-27. Vimentin is a cytoskeletal protein of mesenchymal ori-

Table 3. Panel of tissue samples used for the evaluation of potential target structures by immunohistochemistry

<table>
<thead>
<tr>
<th>RCC subtype</th>
<th>Patient no.</th>
<th>Grade</th>
<th>T</th>
<th>N</th>
<th>M</th>
<th>Agea</th>
<th>Genderb</th>
</tr>
</thead>
<tbody>
<tr>
<td>clear cell</td>
<td>3144</td>
<td>1</td>
<td>1</td>
<td>x</td>
<td>x</td>
<td>65</td>
<td>f</td>
</tr>
<tr>
<td></td>
<td>3146</td>
<td>1</td>
<td>1</td>
<td>x</td>
<td>x</td>
<td>42</td>
<td>m</td>
</tr>
<tr>
<td></td>
<td>3210</td>
<td>1</td>
<td>1</td>
<td>x</td>
<td>x</td>
<td>56</td>
<td>m</td>
</tr>
<tr>
<td></td>
<td>3239</td>
<td>3a</td>
<td>x</td>
<td>x</td>
<td>70</td>
<td>f</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3240</td>
<td>1</td>
<td>1</td>
<td>x</td>
<td>x</td>
<td>76</td>
<td>f</td>
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<td>x</td>
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<td>f</td>
</tr>
<tr>
<td></td>
<td>3249</td>
<td>1</td>
<td>1</td>
<td>x</td>
<td>x</td>
<td>79</td>
<td>f</td>
</tr>
<tr>
<td></td>
<td>3323</td>
<td>1</td>
<td>1</td>
<td>x</td>
<td>x</td>
<td>75</td>
<td>m</td>
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<td>3384</td>
<td>1</td>
<td>1</td>
<td>x</td>
<td>x</td>
<td>76</td>
<td>f</td>
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<tr>
<td></td>
<td>3387</td>
<td>1</td>
<td>1</td>
<td>x</td>
<td>x</td>
<td>63</td>
<td>f</td>
</tr>
<tr>
<td></td>
<td>3082</td>
<td>1</td>
<td>1</td>
<td>x</td>
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| a) Age at time of surgery
| b) f, female; m, male

Tumor specimens and corresponding normal kidney tissues were obtained after radical nephrectomy from primary RCC of clear cell and chromophobic subtype. TNM staging and histopathological evaluation was performed according to the classification of Thoenes and coworkers [33]. Informed consent was received from all patients. RCC specimens and corresponding kidney epithelium were obtained after radical nephrectomy. Paraffin-embedded tissue samples were subjected to immunohistochemical analysis using antibodies specific to antigen processing machinery (APM) components and to HLA class I antigen submits.
gin which has been found in tumor cells expressing mesenchymal characteristics. It is often associated with the cellular differentiation, invasion, migration and metastatic potential of tumors [34]. The HSP-27 protein belongs to a large family of highly conserved ubiquitously expressed proteins that can be induced upon exposure to environmental stress including heat shock, oxidative stress, heavy metals or pathophysiological conditions. HSP-27 is a cytoplasmic chaperone participating in stress resistance, cell growth and differentiation, microfilament organization and assembly of polypeptides [35]. High levels of HSP-27 have been demonstrated in human cancers of distinct origin and appear of prognostic value at least in osteosarcoma [36–38]. As demonstrated in Fig. 5, a heterogeneous expression pattern of vimentin and HSP-27 was observed. Moreover, some of these proteins appear to be associated with the RCC subtype and/or disease progression as recently described [26, 39]. An example for the expression analysis via microarray technology representing all the four major subtypes of RCC is shown in Fig. 6 for stathmin.

3.5 Comparison of target structures identified by PROTEOMEX with those differentially expressed in ± G250 treated cell lines

To strengthen or even improve the identification of markers for the selection of patients undergoing G250-based immunotherapy, a matching of RCC target structures previously defined by PROTEOMEX to those differentially expressed upon G250 mAb treatment of RCC cell lines was performed. The concept of PROTEOMEX analysis is based on the identification of immunoreactive proteins by combining conventional proteomics with a serological screening using sera from RCC patients as well as from healthy donors. Employing this approach a number of immunoreactive proteins have been identified [25, 38, 39]. As shown in Table 4, several proteins, such as annexin V, cytokeratin 8, HSP-27, HSP-90, moesin, stathmin, thioredoxin, tropomyosin, tubulin-α, tubulin-β and vimentin were detected with both technologies. However, the use of these putative markers to differentiate between responders and nonresponders of G250 therapy has still to be further exploited.

4 Discussion

The long term aim of this study is the identification of markers to discriminate between therapy responders and nonresponders using the antigen G250 as a model. The prerequisite for this analysis was the determination of the frequency of G250 surface expression in human RCC cell lines. As shown in Figs. 1 and 2, the frequency of G250 expression detected was approximately 40% in established long term RCC lines which was lower than those described by other authors [14] and significant by higher in short term cultures (Fig. 1) Various authors have recently demonstrated antitumor response in clinical trials

![Figure 5](image_url)

**Figure 5.** Immunohistochemical analysis of normal kidney tissue and RCC of clear cell type (G1) for HSP-27 and vimentin. In normal kidney tissue the epithelium of the distal tubule system and collecting ducts show positive staining for HSP-27 (upper left panel, 200 x) and the RCC of clear cell type (G1) sample also demonstrated positive staining for vimentin (lower right panel, 200 x).
Figure 6. Immunohistochemical analysis for stathmin expression using the tissue microarray technology. RCC of the clear cell type (upper left panel) and the papillary type (upper right panel) displayed strong positive cytoplasmic staining at 5 and 19.4%, respectively. 15% of the RCC of the clear cell type and 12.9% of RCC of the papillary type display an intermediate positive reaction, whereas 80% of RCC of clear cell type and 67.7% of RCC of the papillary type show weak or negative staining for stathmin. In RCC of the chromophobic type (lower left panel), 81.3% tested negative and the remaining 18.7% of the tumors show only weak cytoplasmic staining using the stathmin antibody, whereas renal cell adenomas of oncocytic type (lower right panel) display strong positive staining for stathmin at 33.3% and weak or negative staining at 22.2% and 44.5%, respectively. The classification of the staining pattern indicated in each panel is based on the criteria defined in Section 2.4. Normal renal tissue (data not shown) showed strong cytoplasmic staining for stathmin in mesangial cells of the glomerula, the tubulus epithelium of the proximal and distal tubule system as well as endothelial cells.

Table 4. Matching of proteins identified in untreated and G250-treated RCC cells to those previously characterized by PROTEOMEX

<table>
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<tr>
<th>Protein</th>
<th>± G250</th>
<th>PROTEOMEX</th>
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<tr>
<td>annexin I</td>
<td>-</td>
<td>+</td>
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<tr>
<td>annexin V</td>
<td>+ (G250 low)</td>
<td>+</td>
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<tr>
<td>cytokeratin 8</td>
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<td>+</td>
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<td>enolase 1</td>
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<td>enolase 2</td>
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<td>-</td>
</tr>
<tr>
<td>HSP27</td>
<td>+ (G250 low)</td>
<td>+</td>
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<tr>
<td>HSP75</td>
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<td>HSP90</td>
<td>+ (G250 low)</td>
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<td>+</td>
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<td>tubulin b</td>
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<td>+</td>
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<tr>
<td>vimentin</td>
<td>+ (G250 low)</td>
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**Bold:** Proteins detected by both experimental approaches. The type of G250 surface expression level corresponding with the differentially expressed target is listed in brackets.

mod: moderate

using $^{131}$I-labeled mAb G250 [21]. In addition, G250 mAb was currently used alone or in combination with cytokines for the treatment of RCC patients. However, the efficacy of a certain treatment might be enhanced if one could select patients prior to undergoing vaccination strategies. Therefore markers distinguishing between non-responders and responders are urgently needed. Thus, proteome analysis of untreated as well as mAb G250-treated RCC cell lines was employed to identify differentially expressed protein spots. As shown in Table 2, a series of differentially expressed proteins was identified by proteome analysis. Some of these proteins were either up-regulated or down-regulated upon G250 treatment. A number of the differentially expressed proteins listed in Table 2, such as moesin, the IQ motif containing GTPase activating protein 1, SH3PX, FK506 binding protein 4, the chloride intracellular channel 4 or major vault protein (MVP) appears to be linked to G250.

The relevance of several differentially expressed proteins was analyzed on a larger series of surgically removed RCC lesions demonstrating a heterogeneous expression pattern of these proteins. One of these proteins is the MVP which appears to be an indicator for chemoresistance [40, 41]. Although the immunohistochemical staining pattern is not yet conclusive, MVP is thought to play a
role in the protection of RCC cells from the toxic impact of antineoplastic drugs. The relevance of these findings in the clinical situation has still to be determined and appears to be a multifactorial complex process.

The combination of conventional proteome analysis with serology, termed PROTEOMEX, resulted in the characterization of targets which induce antibody responses in RCC patients (Table 2) [25, 26, 38, 39]. Interestingly, some of the targets defined by PROTEOMEX were also affected by mAb G250 treatment of RCC cell lines. These represent mainly cytoskeletal components, metabolic enzymes and chaperones. As an example, a heterogeneous expression pattern of HSP-27 was previously found in RCC lesions when compared to normal renal epithelium cells. However, the role of HSP-27 overexpression in RCC has still to be determined. Since its physiological functions affect cell growth, differentiation as well as apoptosis, one might speculate that HSP overexpression is involved in the proliferation of RCC. This is supported by the fact that HSP-27 can be induced during mitogenic stimulation. Furthermore, overexpression of HSP-27 can be associated with the aggressive behavior of the tumor and patient survival in several types of malignancies [36]. In addition, HSP-27 overexpression implies the resistance of RCC to chemotherapeutic agents, which also supports the results of Österreicher et al. [42] demonstrating a correlation between growth and drug resistance and the level of HSP-27 expression in human breast carcinoma cell lines.

On the other hand it is noteworthy that tumor-derived HSP can chaperone peptides and can initiate protective and tumor-specific cytotoxic T lymphocyte responses. This is mainly attributable to cross-priming since HSPs are released from cells as a result of necrosis and mediate representation of antigens by antigen presenting cells [43]. The unusual efficiency of this process is explained by utilization of the CD91 receptor [44]. Besides the role of HSPs in mediating proper immune responses most of the other markers identified, such as moesin and tubulin, appear also to be involved in immune recognition. For example, moesin, a widely expressed phosphoprotein links transmembrane molecules to the actin cytoskeleton. It controls the membrane molecule distribution and membrane topology during the immunological synapse formation [45] which is critical for T cell activation. Although the formation of the immunological synapse is poorly understood, cytoskeletal-dependent processes have been proposed. Different cytoskeletal proteins are involved in the assembly of the immunological synapse including actin, myosin and tubulin [45]. Thus, the differentially expressed proteins detected by PROTEOMEX and G250 mAb treatment of RCC cell lines influence the immunological competence of the tumor, suggesting that both technologies could be successfully implemented for the identification of diagnostic and therapeutic markers in RCC.

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5 References