Systematic Comparative Protein Expression Profiling of Clear Cell Renal Cell Carcinoma

A PILOT STUDY BASED ON THE SEPARATION OF TISSUE SPECIMENS BY TWO-DIMENSIONAL GEL ELECTROPHORESIS

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Proteome-based technologies represent powerful tools for the analysis of protein expression profiles, including the identification of potential cancer candidate biomarkers. Thus, here we provide a comprehensive protein expression map for clear cell renal cell carcinoma established by systematic comparative two-dimensional gel electrophoresis-based protein expression profiling of 16 paired tissue systems comprising clear cell renal cell carcinoma lesions and corresponding tumor-adjacent renal epithelium using overlapping narrow pH gradients. This approach led to the mapping of 348 distinct spots corresponding to 248 different protein identities. By implementing restriction criteria concerning their detection frequency and overall regulation mode, 28 up- and 56 down-regulated single target spots were considered as potential candidate biomarkers. Based on their gene ontology information, these differentially expressed proteins were classified into distinct functional groups and according to their cellular distribution. Moreover, three representative members of this group, namely calbindin, gelsolin, and heart fatty acid-binding protein, were selected, and their expression pattern was analyzed by immunohistochemistry using tissue microarrays. Thus, this pilot study provides a significant update of the current renal cell carcinoma map and defines a number of differentially expressed proteins, but both their potential as candidate biomarkers and clinical relevance has to be further explored in tissues and for body fluids like serum and urine. Molecular & Cellular Proteomics 8: 2827–2842, 2009.

Human renal cell carcinoma (RCC) is a heterogeneous disease with increasing incidence over the past 20 years. It is classified into various subtypes according to morphological features of the tumors. The most common subtype, clear cell (cc) RCC, accounts for ~75% of all kidney cancers, whereas the papillary, chromophilic RCC (10–15%), the chromophobic RCC (5%), tumors of unclassified subtypes (<2%), collecting duct RCC (<1%), and the benign tumors of the oncocytic subtype (<1%) are less frequently represented (1). So far, surgery is the first line curative treatment option for organ-defined disease, but more than 30% of RCC patients have already developed an advanced disease or metastases at the time of presentation leading to a 5-year survival rate of less than 20% (2). Despite its radio- and chemotherapy resistance, 10–20% of RCC patients respond to immunotherapies (3, 4) and to targeted molecules, including inhibitors of growth factors, growth factor receptors, and signal transduction molecules (5–7).

During the last decade, classical molecular genetics, cytogenetics, and expression profiling have increased the knowledge about the biology and pathophysiology of the various histological subtypes of RCC (8–12). Results of cDNA profiling support the existence of an individual fingerprint of each RCC subtype regarding its biological features, clinical behavior, and unique sensitivity to therapy. The identification of a number of excellent diagnostic tools allows the differentiation between the respective RCC subtypes (8). These include for example the c-kit gene, which is up-regulated in chromophobic RCC (13), as a marker or genes of the carbonic anhydrase II for chromophobich RCC, GST for ccRCC, and α-methylacil racemase for chromophilic RCC (14).

However, sequential changes in cellular mechanisms defining the transformation process from a normal to a malignant phenotype not only occur at the transcriptional level but also involve posttranscriptional alterations, particularly protein heart fatty acid-binding protein; ID, identity; IHC, immunohistochemistry; mAb, monoclonal antibody; NDSB, non-detergent sulfobetaine (here dimethylbenzylammonium propane sulfonate); TMA, tissue microarray; 2DE, two-dimensional electrophoresis; 2D, two-dimension al; S/N, signal/noise ratio; PMF, peptide mass fingerprinting; PTM, posttranslational modification; ER, endoplasmic reticulum; L-FABP, liver fatty acid-binding protein; TNM, staging system for classification of malignant tumors.
modifications like phosphorylation and glycosylation, which are not detected by microarray analysis. Therefore, the characterization of the differential protein expression pattern in malignant versus normal cells/tissues is at least complementary to the cDNA microarrays to identify putative biomarkers involved in cancer-related processes.

Among the proteome technologies, two-dimensional electrophoresis (2DE) followed by mass spectrometric analysis is the most established method, resulting in the identification of differentially expressed proteins. Indeed, proteome studies have identified important protein sets that are involved in the molecular mechanisms underlying the malignant behavior of tumor cells. This might consequently lead to the definition of biomarkers and novel therapeutic targets. Using distinct proteome-based strategies, a number of differentially expressed proteins have recently been identified in RCC lesions and cell culture models that might significantly contribute to the understanding of the development of this disease (15–21). These candidate biomarkers belong to different protein families, including annexins, metabolic enzymes, proteins of signal transduction pathways, antigen processing and presentation components, growth factors, differentiation markers, tumor suppressor genes, cytoskeletal components, and stress proteins as well as proteins involved in chemotherapy resistance.

Despite these studies, the information existing for RCC is limited to a small number of differentially expressed proteins, which often lack proper verification by RT-PCR and/or immunohistochemistry on a large series of RCC tissue specimens. In addition, the overall RCC proteomes still remain to be elucidated. Thus, validated biomarkers used for diagnosis, prognosis, and treatment monitoring of this disease are still urgently needed. Therefore, in this pilot study, two virtual ccRCC maps highlighting 348 differentially expressed protein spots on either acidic (pH 4–7) and/or on basic (pH 6–11) 2DE consensus gels were established. Upon annotation of the identified spots, the map represents 248 distinct protein identities. Moreover, immunohistochemical stainings using a tissue microarray (TMA) comprising ccRCC but also other malignant and benign RCC subtypes as well as corresponding tumor-adjacent tissue sections were performed for three representative differentially expressed proteins selected based on their novelty. The data, although not sufficiently conclusive for any of the tested candidate targets, nevertheless demonstrate that each of the analyzed targets provides at least some hints and thus that this approach is indeed suited to uncover candidate biomarkers, which upon further verification and validation might indeed define novel diagnostic, prognostic, and/or therapeutic markers for this malignancy.

**EXPERIMENTAL PROCEDURES**

**Patients and Tissue Samples**—Tissue samples from 16 primary ccRCC lesions and autologous tumor-adjacent renal tissue were obtained from RCC patients who had undergone radical nephrectomy at the University Hospital of the Johannes Gutenberg University in Mainz, Germany. Informed consent was obtained from each of the patients. None of the patients had received chemotherapy or radiation in advance. Histopathological classification of each tumor was performed according to previously proposed criteria (22, 23), which are in line with the recent classification by the World Health Organization. The criteria include stage of disease and tumor invasion according to the TNM system as well as tumor grade as outlined in Table I and Ref. 24. The tumor samples used for analyses were either snap frozen (proteomics) or paraffin-embedded (IHC) immediately after resection and have been previously described in detail (25–27).

**2D Gel Electrophoresis of Tissue Specimen**—The set of tissue specimens was continuously collected over a time period of 3 years but directly subjected to 2DE upon receipt. For sample preparation, ~100 mg of frozen tissue material directly cryoconserved in liquid nitrogen upon organ resection were transferred into a sterile 6-well plate (Falcon, BD Biosciences), thawed, thoroughly sliced with a disposable scalpel (PMF AG, Cologne, Germany), and resuspended in 500 μl of PBS (Cambrex Bio Science, Vibiers, Belgium). The cell suspension was centrifuged for 2 min at 10,000 × g and washed twice with PBS. In case that the separation in the first dimension was performed in the pH 4–7 range, the resulting cell pellet was directly resuspended in 200 μl of lysis buffer (7 M urea (AppliChem Biochemica, Darmstadt, Germany), 2 M thiouria (Sigma-Aldrich), 0.2 M dimethylbenzylammonium propanesulfate (NDSB-256, ICN Bio-medicals, Eschwege, Germany), 1% DTT (AppliChem Biochemica), 4% v/v CHAPS (AppliChem Biochemica), 0.5% v/v Phall Excise (Amersham Biosciences), and a trace of the dye bromophenol blue (Serva, Heidelberg, Germany) and further processed using the PlusOne sample grinding kit (Amersham Biosciences) according to the manufacturer’s guidelines. Finally, the tissue lysate was subjected to sonication (2 × 5 cycles, 0.5 s at 100% power; Bandelin UW 2070 sonicator, MS 73 needle, Bandelin, Berlin, Germany) in the presence of the grinding resin. Isoelectric focusing, second dimension SDS-PAGE separation, and gel staining procedures were carried out as described previously (28). Prior to the protein separation in the pH 6–11 range, the lysates were additionally TCA/acetone-precipitated according to the method described by Gör et al. (29).

**Image Analysis**—Quantitative image analysis was carried out with the Proteomweaver software package (Versions 3.1 or 4.0; Bio-Rad GmbH) according to the manufacturer’s instructions. The mapping data reflect the mean of at least three to five technical replicates. The digitized spot pattern of each gel was first matched within the given group (ccRCC or tumor-adjacent kidney) and subsequently between the two experimental groups. The matching procedures of each step were visually controlled and reedited if necessary prior to the normalization process. The Proteomweaver software relies on a prematch and pair match-based normalization as recently described in more detail (30). Protein spots were considered as differentially expressed if a 2-fold altered expression level was observed. In addition, the spots of interest were statistically analyzed with the Student’s t test for each matched sample set and only those selected for mass spectrometric identification with a p value <0.05. In addition, spots displaying a protein expression pattern restricted to either the tumor or the corresponding tumor-adjacent renal epithelium were also selected for mass spectrometric identification. Furthermore, to gain information about the quality of the differentially expressed proteins as potential biomarkers and to exclude the individual genetic heterogeneity, the overall regulation frequency across the complete set of samples was compared.

**Microanalytical Characterization**—Protein identification was performed as described previously (31). Relevant spots were excised from the gel, in-gel digested, and then subjected to mass spectrometry. For spot excision and tryptic in-gel hydrolysis of 2D gel spots, 2D gels were colloidal Coomassie Blue-stained overnight (50% methanol, 10% acetic acid, and 0.05% colloidal Coomassie Blue R-250)
and destained for 8 h (5% methanol and 7% acetic acid). The gel fragments were briefly washed with 50 µl of 50 mM NH₄HCO₃ and then washed three times with 50 µl of 30% ACN and 50 mM NH₄HCO₃ before they were incubated twice for 20 min in 100 µl of ACN and subsequently air-dried. The resulting pellets were resuspended in 1–3 µl of 5 mM Tris buffer, pH 8.0 containing 0.03 µg/µl bovine trypsin (Roche Diagnostics), and then 5–15 µl of 5 mM Tris buffer, pH 8.0 (depending on the size of the gel piece) were added and incubated overnight at 37 °C.

MALDI target preparation was performed using the following procedure: 0.45 µl of the supernatant was loaded on the target and allowed to dry at room temperature before 0.45 µl of matrix solution (5 mg/ml a-cyano-4-hydroxycinnamic acid in 50% ACN and 0.1% TFA) was added and also dried at room temperature. If necessary the tryptic peptides were desalted with ZipTip C₁₈ reverse-phase tips according to the manufacturer’s instructions (Millipore,Schwalbach, Germany) and directly eluted on the MALDI target plate. MS and MS/MS spectra were acquired with 2500 laser shots (neodymium-doped yttrium aluminium garnet laser, 355 nm, N₂ collision in the MS/MS mode) using a 4700 MALDI-TOF-TOF mass spectrometer (Applied Biosystems, Foster City, CA). Peak lists were generated using a minimum S/N of 5 within the window of 200 m/z and cluster area S/N of 12 for PMF and an S/N of 5 within the window of 200 m/z and cluster area S/N of 8 for MS/MS.

The PMF data set was analyzed using the Mascot search engine with the following parameters: fixed modification, carbamidomethyl; variable modifications, oxidation (Met), pyro-Glu (Glu), cleavage enzyme, trypsin (*KR), maximum missed cleavages, 1; and mass tolerance (monoisotopic), 50.0 ppm. MS/MS spectra were searched as above using a peptide mass tolerance of 200 ppm and a fragment mass tolerance of 0.5 Da.

The confidence in terms of the target identification is based on the overall sequence coverage of matching peptide fragments following digestion with trypsin and/or the probability-based Mowse score with the web-based Mascot search engine searching the Swiss-Prot Knowledgebase Release (Versions 41.18–53.1) (Matrix Science Ltd., London, UK) and the probability score (web-based Profound Peptide Knowledgebase Release (Versions 41.18–53.1) (Matrix Science Ltd., London, UK) and the probability score (web-based Profound Peptide Knowledgebase Release (Versions 41.18–53.1) (Matrix Science Ltd., London, UK)).

Molecules were assigned as a self-made web-based tool, which was programmed in php combined with a mySQL database. To combine the information from the spots obtained by Proteinweaver analysis and the protein spots identified by mass spectrometry, the raw data were reorganized into the database. To eliminate redundancy, the heterogeneous data sets of the identified proteins were linked to the most recent Swiss-Prot identities (UniProt Knowledgebase Release 14.9), which served as the major source for additional information such as the chromosomal localization, and gene ontology, which provides information about gene function and cellular localization. Based on this information, the protein families and compartment localizations were assigned to each protein. In terms of the cellular function, the proteins were distributed into the categories cell adhesion, cell motility, expression control, metabolism, multiple, other (not further specified), proliferation, signal transduction, structural molecules, stress proteins, transport proteins, and unknown function. The categories for the cellular localization include the cytoplasm, cytoskeleton, endoplasmic reticulum, extracellular region, membrane, mitochondrion, multiple compartments, nucleus, other (not further specified), and unknown. Furthermore, a consensus gel for each pH gradient with all spots identified was generated for a comprehensive spot overview. To understand possible interactions of the various regulated proteins, these targets were further assigned if possible to known pathways.

**Immunohistochemistry**—Paraffin blocks of tumor and tumor-adjacent renal tissues were used for representative immunohistochemical stainings. The samples were selected and cut into 5-µm sections using a cryostat. For the preparation of TMAs, at least three tissue cylinders (each 4 mm in diameter) were punched from selected regions of the respective donor blocks and placed on 25 × 35-mm paraffin blocks. The panel of formalin-fixed and paraffin-embedded RCC lesions represented on the TMAs comprised primary RCCs, including up to 40 ccRCC lesions, 31 chromophobetic RCC lesions, 16 chromophobic RCC lesions, and nine renal adenomas of oncocytic subtype as well as corresponding tumor-adjacent renal tissue. For antigen retrieval, consecutive sections were incubated for 2–8 min in citrate buffer in a microwave oven followed by a washing procedure with Tris-buffered saline and an additional incubation with normal swine serum (dilution 1:5; Dako, Hamburg, Germany) for 10 min. All sections were stained with hematoxylin and eosin to identify tumor tissue. Immunohistochemistry was performed as described previously (27) by applying the mAbs directed against calbindin (1:100; ab9481, Abcam plc, Cambridge, UK) and gelsolin (1:50; 610412, BD Transduction Laboratories) or a polyclonal Ab directed against heart fatty acid-binding protein (H-FABP; 1:50; 18-003-42444, GenWay Biotech Inc., San Diego, CA) for 1 h at room temperature. Immunoreactivity was detected using the commercially available streptavidin-biotin labeled streptavidin-biotin technique)-peroxidase kit and 3-amino-9-ethylcarbazol (Dako). Negative controls were performed by omitting the primary antibodies. The extent of immunostaining was scored according to the following criteria: negative, <5% positive cells; weak positive, 5–25% positive tumor cells; intermediate positive, 26–50% positive tumor cells; and strong positive, >50% positive tumor cells.

**RESULTS**

Systematic Comparison of Protein Expression Profiles of ccRCC Lesions and Corresponding Tumor-adjacent Kidney Epithelium—To determine the differential protein expression pattern of RCC, total protein extracts obtained from 16 tissue systems consisting of primary RCC lesions of the ccRCC subtype and corresponding tumor-adjacent kidney epithelium were subjected to classical 2DE using overlapping pH gradients of pH 4–7 and pH 6–11. To strengthen the quality and reproducibility of the expression profiling, the systematic comparisons relied on three to five technical replica gels per sample. Because of the limited amount of sample, the systems 2894 and 2938 had to be excluded from the analysis in the alkaline pH range (Table I). The quality and reproducibility of the individual profiling is summarized in supplemental Fig. 1A, which provides information regarding the average spot count and its coefficient of variation within each of the completed gel series. On average, between 798 ± 80 and 484 ± 49 protein spots per gel were detected in the acidic and basic pH gradients, respectively.

In total, 348 spots displaying either a restricted expression pattern to tumor or tumor-adjacent tissue or a regula-
tion factor >2 were analyzed and mapped. These spots represent 248 distinct protein identities as verified by either conclusive PMF and/or MS/MS analysis from which 231 spots represent single target spots (containing one protein identity), 84 spots represent double target spots (containing two distinct proteins), and 33 spots represent multiple target spots (containing more than two proteins). Representative virtual 2DE maps of combined consensus protein expression profiles of ccRCC lesions/tumor-adjacent kidney epithelium are shown in Fig. 1. The mapped spots are marked by numbers. High resolution views of the representative gels used for the design of the consensus maps are provided in supplemental Fig. 1B.

In terms of the distribution pattern, 71% (n = 176) of the mapped proteins were identified in the pH 4–7 range, and 24% (n = 59) were identified in the pH 6–11 range. The remaining 5% (n = 13) of the identified proteins were found in both pH gradients (supplemental Fig. 2A). 151 of the proteins presented in this report are shared with the 461 proteins that have already been described using RCC-specific profilings. However, with 97 additional entries, the number of proteins identified is now increased to 558 (supplemental Fig. 2B) (17–21).

By applying restriction criteria such as a minimal detection frequency set at 5, requiring detection in at least five independent tissue pairs, and merely focusing on the group of single target spots, the number of candidate spots was reduced to 132 members. Nine of these 132 spots represent blood contaminants such as serum albumin (five spots), serotransferrin (two spots), Ig α-1 chain C region (one spot), and haptoglobin (one spot), which were further excluded from the data. Some key features for the remaining subset of 123 differentially expressed spots are summarized in Table II, which lists the virtual map ID next to the given Swiss-Prot ID, protein name, function, cellular localization, detection frequency, theoretical and experimental pI values, molecular weight, and the regulation mode. The respective data sets for less frequently detected single, double, and multiple target spots are provided in supplemental Table 1.

By additionally focusing on spots with a predominant regulation tendency (meaning that the overall distribution ratio of the regulated spots had to reach a ratio of 2:1), the list of potential candidate spots was further trimmed to 84 members, representing 28 up- and 56 down-regulated spots. The remaining 39 proteins within the group of single target spots showed a heterogeneous expression pattern.

However, it is noteworthy that although not considered as putative biomarker candidates all single target spots including those identified in <5 biopsy systems as well as the double and multiple target spots (supplemental Table 1) are represented in the updated 2DE maps (Fig. 1). Moreover, it is noteworthy that 105 proteins are represented by more than a single spot ID on the established 2DE maps (Fig. 1), suggesting the frequent occurrence of posttranslational modifications (PTMs) in renal tissues. Because the majority of these proteins were detected with co-migrating proteins, they are largely represented in the groups of double and/or multiple target spots (Table II and supplemental Table 1). The discrepancy between the total number of mapped protein spots and the number of identified proteins is due to the clustering of the spots into single, double, and multiple targets and the occur-

### Table I

| Characteristics of RCC lesions analyzed |
|----------------------------------------|
| The characteristics of the 16 RCC biopsy systems (RCC lesion/corresponding tumor-adjacent renal tissue) including TNM status, tumor grading, metastasis formation, the patients’ follow-up in months, and the survival of patients are summarized. In addition the number of technical replicates for each tissue type and pH gradient is given. All samples were obtained from the Department of Urology, University Hospital, Johannes Gutenberg University, Mainz, Germany. NN, tumor-adjacent renal epithelium; TU, tumor. |
| Biopsy system no. | TNM status | Grading | Metastasis | Follow-up | Survival | Replicates |
|-------------------|-------------|---------|------------|-----------|----------|-----------|
|                   |             |         |            | months   |          | pH 4–7    | pH 6–11    |
| 2884              | pT2, N0, Mx | G3      | Yes        | 10        | Yes      | 3         | 3         |
| 2891              | pT1, N0, Mx | G2–3    | No         | 25        | Yes      | 4         | 4         |
| 2894              | pT3, N0, Mx | G1–2    | No         | 24        | Yes      | 4         | 4         |
| 2907              | pT2, N0, Mx | G1      | No         | 17        | Yes      | 4         | 3         |
| 2908              | pT1, N0, Mx | G2      | No         | 0         | No*      | 4         | 3         |
| 2911              | pT2, N0, Mx | G2      | Yes        | 8         | Yes      | 4         | 3         |
| 2913              | pT2, N0, Mx | G2      | No         | 17        | Yes      | 3         | 4         |
| 2914              | pT1, N0, Mx | G2      | No         | 17        | Yes      | 5         | 3         |
| 2920              | pT1, N0, Mx | G2      | No         | 16        | Yes      | 4         | 3         |
| 2922              | pT3A, N0, Mx| G2      | No         | 16        | Yes      | 3         | 4         |
| 2923              | pT3A, N1, Mx| G3      | Not known  | 31        | Yes      | 4         | 4         |
| 2926              | pT1, N1, Mx | G1      | Not known  | 30        | Yes      | 3         | 5         |
| 2932              | pT2, N0, Mx | G2–3    | No         | 29        | Yes      | 5         | 5         |
| 2938              | pT2, N0, Mx | G2      | No         | 27        | Yes      | 4         | 4         |
| 2940              | pT1, N0, Mx | G2      | No         | 27        | Yes      | 5         | 5         |
| 2944              | pT1B, N0, Mx| G2      | Yes        | 24        | Yes      | 5         | 4         |

* Death not related to cancer.
rence of PTM or splice variants, all of which is in line with previously described 2DE analyses (16, 32).

Detailed analytical data for each of the mapped spots such as the sequence coverage, score, and number of matched and unmatched peptides for proteins identified by PMF analyses as well as the peptide mass and MS/MS score for identifications based on MS/MS analyses are summarized in supplemental Table 2. A representative MS spectrum leading to the identification of annexin 4 by PMF analysis is shown in supplemental Fig. 3.

Classification of Differentially Regulated Proteins—Based on existing gene ontology information, the group of differentially expressed proteins (Table II) was clustered according to their cellular function and localization (Fig. 2, A and B). They are involved in different biological processes that facilitate tumor-relevant adjustments concerning growth, invasion, apoptosis, and inflammation. A significant number of the differentially expressed proteins are associated with alterations in the cellular metabolism (40%), stress (11%), transport (10%), cell proliferation (7%), signal transduction (6%), multiple functions (6%), or cell motility (5%). Less frequently represented are proteins with known but not further categorized ontology information (4.5%); proteins associated with structural integrity (4.5%), gene/protein expression control (2.3%), and cellular adhesion (2.3%); and proteins with ion binding characteristics (1.1%). Thus, the categories exhibiting a frequency of <5% are summarized under “other” (Fig. 2A).

Concerning their cellular distribution, these proteins are localized in the mitochondrion (33%), cytoplasm (27%), multiple compartments (12%), cytoskeleton (10%), and the extracellular region (7%) or have unknown localization. The category other combines subcellular compartments with a cutoff frequency <5% such as proteins with as yet unknown localization (4.5%), the ER (3.4%), membrane (1.1%), other (1.1%), or nucleus (1.1%) (Fig. 2B and Table II). A similar distribution pattern was detected by clustering all mapped proteins (Table II and supplemental Table 1). Although the majority of proteins detected in both pH gradients are metabolic enzymes, proteins associated with stress, transport, cell motility, signal transduction processes, multiple functions, and structural integrity are more frequently represented in gels separated in the pH 4–7 range than in the corresponding pH 6–11 gradients, whereas proteins involved in gene/protein expression control and cell proliferation were more often detected in gels representing the basic pH gradient. The most frequent compartment localization in the acidic pH range is the mitochondrion followed by the cytoplasm and the cytoskeleton, although the number of mitochondrial proteins is even more pronounced in the alkaline pH range. In addition, a strong decrease of cytoskeletal proteins and proteins with as yet unspecified compartment localization was found in pH 6–11 gels. Moreover, the genes coding for the differentially expressed proteins were randomly distributed over a wide range of chromosomes (data not shown). Thus, the functional and

![Fig. 1. Consensus master maps of differentially expressed proteins in ccRCC lesions compared with tumor-adjacent renal epithelium. Representative 2DE gels displaying the consensus protein expression profiles characteristic for ccRCC using pH 4–7 (A) and pH 6–11 (B) gradients are shown. The numbers assigned to the mapped protein spots correspond to the proteins listed in Table II and supplemental Table 1.](image-url)
### TABLE II

Differentially expressed proteins identified in RCC biopsies compared with tumor-adjacent renal tissues

| Spot | Swiss-Prot ID | Protein | Cellular function | Cellular compartment | Theor. pI | Theor. mass, kDa | Exp. pI | Exp. mass, kDa | Dn | Up | NR/ND |
|------|---------------|---------|-------------------|---------------------|----------|-----------------|--------|----------------|----|----|--------|
| 2    | P12109        | Collagen α-1(VI) chain | Cell adhesion | Cytoskeleton | 5.26     | 108.5           | 5.14   | 115.3          | 3  | 6  | 7      |
| 4    | P38646        | Stress-70 protein | Stress protein | Multiple compartment | 5.87     | 73.68           | 5.37   | 73.16          | 5  | 1  | 10     |
| 8    | P05787        | Keratin, type II cytoskeletal 8 | Structural molecule | Cytoskeleton | 5.52     | 53.57           | 5.35   | 58.98          | 3  | 4  | 9      |
| 11   | P08670        | Vimentin | Cell motility | Cytoskeleton | 5.06     | 53.52           | 4.7    | 54.92          | 1  | 6  | 9      |
| 12   | P52566        | Rho GDP dissociation inhibitor 2 | Multiple | Cytoskeleton | 5.1      | 22.86           | 5.06   | 30.11          | 1  | 6  | 9      |
| 13   | P07858        | Cathepsin B | Other | Mitochondrion | 5.88     | 37.82           | 5.19   | 28.45          | 6  | 2  | 8      |
| 32   | P09525        | Annexin A4 | Signal transduction | Cytoskeleton | 5.85     | 35.75           | 5.85   | 38.76          | 4  | 3  | 9      |
| 33   | P30084        | Enoyl-CoA hydratase | Metabolism | Mitochondrion | 8.34     | 31.39           | 5.68   | 92.19          | 11 | 2  | 3      |
| 34   | P02753        | Plasma retinol-binding protein | Transport protein | Extracellular region | 5.76     | 23.01           | 5.3    | 24.93          | 5  | 1  | 10     |
| 35   | P22352        | Glutathione peroxidase 3 | Stress protein | Extracellular region | 8.2      | 25.51           | 4.81   | 21.08          | 11 | 2  | 3      |
| 36   | P22352        | Glutathione peroxidase 3 | Stress protein | Extracellular region | 8.2      | 25.51           | 4.87   | 20.96          | 7  | 1  | 8      |
| 39   | P07148        | Fatty acid-binding protein, liver (L-FABP) | Multiple | Cytoskeleton | 6.6      | 14.21           | 6.34   | 7.17           | 11 | 1  | 4      |
| 43   | P16083        | Ribosylhydridonicotinamide dehydrogenase (quinone) | Transport protein | Cytoskeleton | 5.88     | 25.82           | 6.06   | 28.73          | 6  | 2  | 8      |
| 50   | O00217        | NADH dehydrogenase (ubiquinone) iron-sulfur protein 8 | Stress protein | Mitochondrion | 6       | 23.71           | 5.09   | 26.59          | 7  | 2  | 7      |
| 53   | O75891        | 10-Formyltetrahydrofolate dehydrogenase | Metabolism | Cytoskeleton | 5.63     | 98.83           | 5.96   | 90.05          | 0  | 5  | 11     |
| 56   | P11021        | 78-kDa glucose-regulated protein | Stress protein | ER | 5.07     | 72.33           | 5.02   | 81.47          | 4  | 1  | 11     |
| 59   | P10809        | 60-kDa heat shock protein | Stress protein | Mitochondrion | 5.7      | 61.05           | 5.32   | 68.04          | 6  | 1  | 9      |
| 69   | O75947        | ATP synthase subunit d, mitochondrial | Transport protein | Mitochondrion | 5.22     | 18.36           | 5.2    | 24.68          | 8  | 2  | 6      |
| 78   | P19404        | NADH dehydrogenase (ubiquinone) flavoprotein 2 | Transport protein | Mitochondrion | 8.22     | 27.39           | 6.07   | 27.78          | 10 | 1  | 5      |
| 85   | P02753        | Plasma retinol-binding protein | Transport protein | Extracellular region | 5.76     | 23.01           | 5      | 21.66          | 10 | 1  | 5      |
| 87   | P05937        | Calbindin | Ion binding | Cytoskeleton | 4.7      | 29.89           | 4.47   | 29.83          | 6  | 0  | 10     |
| 90   | P52565        | Rho GDP dissociation inhibitor 1 | Cell motility | Cytoskeleton | 5.03     | 23.08           | 4.77   | 32.13          | 3  | 2  | 11     |
| 99   | P09104        | γ-Enolase | Metabolism | Cytoskeleton | 4.91     | 47.14           | 6.38   | 42.3           | 0  | 5  | 11     |
| 100  | Q13162        | Peroxiredoxin-4 | Other | Cytoskeleton | 5.86     | 30.54           | 5.76   | 32.13          | 5  | 4  | 7      |
| 102  | O15540        | Fatty acid-binding protein, brain | Multiple | Cytoskeleton | 5.41     | 14.76           | 5.24   | 8.99           | 2  | 4  | 10     |
| 103  | P51687        | Sulphite oxidase | Metabolism | Mitochondrion | 5.35     | 53.86           | 5.41   | 71.39          | 4  | 3  | 9      |
| 104  | P10809        | 60-kDa heat shock protein | Stress protein | Mitochondrion | 5.7      | 61.05           | 5.23   | 68.04          | 8  | 3  | 5      |
| 106  | P11142        | Heat shock cognate 71-kDa protein | Stress protein | Multiple compartment | 5.37     | 70.9            | 5      | 49.45          | 2  | 4  | 10     |
| 111  | P25705        | ATP synthase subunit α | Transport protein | Mitochondrion | 9.16     | 59.75           | 4.72   | 9.34           | 8  | 1  | 7      |
| 112  | P20674        | Cytochrome c oxidase subunit 5A | Transport protein | Mitochondrion | 6.3      | 16.77           | 4.72   | 9.61           | 9  | 3  | 4      |
| 113  | P10599        | Thioredoxin | Signal transduction | Cytoskeleton | 4.82     | 11.61           | 4.77   | 8.11           | 8  | 1  | 7      |
| 115  | P22352        | Glutathione peroxidase 3 | Stress protein | Extracellular region | 8.2      | 25.51           | 5.83   | 26.72          | 7  | 2  | 7      |
| Spot | Swiss-Prot ID | Protein | Cellular function | Cellular compartment | Theor. pI | Theor. mass | Exp. pI | Exp. mass | Dn | Up | NR/ND |
|------|--------------|---------|------------------|----------------------|---------|-----------|---------|-----------|-----|-----|-------|
| 116  | P50053       | Ketohexokinase | Metabolism | Cytoplasm | 5.64 | 32.73 | 6.16 | 36.28 | 8  | 2  | 6     |
| 120  | P05413       | Fatty acid-binding protein, heart | Proliferation | Cytoplasm | 6.34 | 14.73 | 6.34 | 9.43 | 7  | 3  | 6     |
| 121  | P14625       | Endoplasm | Stress protein | ER | 4.76 | 92.47 | 4.74 | 45.83 | 1  | 5  | 10    |
| 122  | Q16891       | Mitochondrial inner membrane protein | Proliferation | Mitochondrion | 6.08 | 83.68 | 6.18 | 87.01 | 1  | 6  | 9     |
| 123  | Q92597       | Protein NDRG1 | Proliferation | Multiple | 5.49 | 42.84 | 5.74 | 55.41 | 2  | 5  | 9     |
| 131  | P19971       | Thymidine phosphorylase | Multiple | Extracellular region | 5.36 | 49.96 | 5.24 | 52.33 | 0  | 6  | 10    |
| 138  | P18869       | Phosphoglycerate mutase 1 | Metabolism | Cytoplasm | 6.75 | 28.67 | 6.78 | 38.42 | 2  | 4  | 10    |
| 147  | P14625       | Endoplasmin | Stress protein | ER | 4.76 | 92.47 | 4.74 | 68.69 | 3  | 4  | 11    |
| 148  | Q99993       | Pyridoxine-5'-phosphate oxidase | Metabolism | Mitochondrion | 6.35 | 36.44 | 5.84 | 35.8 | 5  | 2  | 9     |
| 154  | P09960       | Leukotriene A-4 hydrolase | Stress protein | Cytoplasm | 5.8 | 69.15 | 6.17 | 72.45 | 4  | 3  | 9     |
| 163  | P07237       | Protein-disulfide isomerase | Multiple | Extracellular region | 4.76 | 57.12 | 4.73 | 69.68 | 1  | 5  | 10    |
| 169  | P42126       | 3,2-trans-Enoyl-CoA isomerase | Metabolism | Mitochondrion | 8.8 | 32.82 | 6.38 | 32.57 | 2  | 4  | 10    |
| 172  | P04792       | Heat shock protein β-1 | Stress protein | Mitochondrion | 5.98 | 22.78 | 6.25 | 31.98 | 3  | 4  | 9     |
| 181  | P10809       | 60-kDa heat shock protein | Stress protein | Mitochondrion | 5.7 | 61.05 | 6.01 | 56.15 | 5  | 2  | 9     |
| 182  | P30084       | Enoyl-CoA hydratase | Metabolism | Mitochondrion | 8.34 | 31.39 | 5.9 | 28.05 | 11 | 1  | 4     |
| 183  | P09525       | Annexin A4 | Signal transduction | Cytoplasm | 5.85 | 35.75 | 6.84 | 14.15 | 5  | 1  | 10    |
| 184  | P25705       | ATP synthase subunit α | Transport protein | Mitochondrion | 9.16 | 59.75 | 4.88 | 8.99 | 9  | 1  | 6     |
| 186  | P11021       | 78-kDa glucose-regulated protein | Stress protein | ER | 5.07 | 72.33 | 5.02 | 81.47 | 1  | 5  | 10    |
| 189  | P14550       | Alcohol dehydrogenase (NADP+) | Metabolism | Cytoplasm | 6.35 | 36.44 | 5.84 | 35.8 | 5  | 2  | 9     |
| 199  | Q9Y2T3       | Guanine deaminase | Metabolism | Other | 5.44 | 51 | 5.59 | 56.91 | 5  | 1  | 10    |
| 205  | Q9Y2T3       | Guanine deaminase | Metabolism | Other | 5.44 | 51 | 5.5 | 56.65 | 3  | 2  | 11    |
| 213  | P16825       | Endoplasm | Stress protein | Mitochondrion | 4.76 | 92.47 | 46.9 | 68.69 | 3 | 4 | 9     |
| 214  | P21281       | Vacuolar ATP synthase subunit B, brain isofrom | Transport protein | Cytoplasm | 5.57 | 56.5 | 5.54 | 65.83 | 2 | 3 | 11    |
| 216  | Q9PR27       | Succinyl-CoA ligase (ADP-forming) β chain | Metabolism | Mitochondrion | 7.05 | 50.32 | 6 | 52.79 | 8 | 1 | 7     |
| 223  | P06670       | Vimentin | Cell motility | Cytoskeleton | 5.06 | 53.52 | 5.09 | 67.71 | 2 | 4 | 10    |
| 224  | P06670       | Vimentin | Cell motility | Cytoskeleton | 5.06 | 53.52 | 4.94 | 63.72 | 5 | 2 | 9     |
| 233  | P02679       | Fibrinogen γ chain | Proliferation | Extracellular region | 5.37 | 51.51 | 5.69 | 61.15 | 3 | 5 | 8     |
| 239  | P07148       | Fatty acid-binding protein, liver (L-FABP) | Multiple | Cytoplasm | 6.6 | 14.21 | 6.34 | 7.17 | 4 | 3 | 9     |
| 241  | P06396       | Gelsolin | Structural molecule | Multiple | 5.9 | 85.7 | 5.96 | 90.05 | 0 | 5 | 11    |
| 242  | Q92597       | Protein NDRG1 | Proliferation | Multiple | 5.49 | 42.84 | 5.71 | 55.66 | 2 | 4 | 10    |
| 244  | P52565       | Rho GDP dissociation inhibitor 1 | Cell motility | Cytoskeleton | 5.03 | 23.08 | 4.93 | 30.96 | 4 | 2 | 10    |
| 246  | Q99497       | Protein DJ-1 | Signal transduction | Nucleus | 6.33 | 19.89 | 6.49 | 26.08 | 5 | 2 | 9     |
| 247  | P02766       | Transhyretin | Transport protein | Extracellular region | 5.52 | 15.89 | 5.6 | 12.48 | 5 | 2 | 9     |
| 249  | P20674       | Cytochrome c oxidase subunit 5A | Transport protein | Mitochondrion | 6.3 | 16.77 | 5.96 | 10.88 | 5 | 0 | 11    |
| 252  | P26038       | Moesin | Cell motility | Cytoskeleton | 6.09 | 67.69 | 6.69 | 81.47 | 3 | 3 | 10    |
| 253  | P17987       | T-complex protein 1 subunit α | Metabolism | Cytoplasm | 5.8 | 60.34 | 6.12 | 69.35 | 3 | 4 | 9     |
| 256  | Q14697       | Neutral α-glucosidase AB | Metabolism | ER | 5.74 | 106.9 | 6.02 | 95.61 | 2 | 4 | 10    |
| 257  | Q14697       | Neutral α-glucosidase AB | Metabolism | ER | 5.74 | 106.9 | 6.19 | 95.03 | 2 | 3 | 11    |
| 260  | P26038       | Moesin | Cell motility | Cytoskeleton | 6.09 | 67.69 | 6.57 | 81.47 | 2 | 3 | 11    |
| 266  | P67396       | Tropomyosin α-4 chain | Cell motility | Cytoskeleton | 4.67 | 28.39 | 4.53 | 47.55 | 3 | 3 | 10    |
| Spot | Swiss-Prot ID | Protein | Cellular function | Cellular compartment | Theor. pI | Theor. mass | Exp. pI | Exp. mass | Dn | Up | NR/ND |
|------|---------------|---------|-------------------|---------------------|-----------|------------|--------|----------|-----|----|-------|
| 267  | P07951        | Tropomyosin β chain | Structural molecule | Cytoskeleton       | 4.66      | 32.85      | 4.6    | 46.94    | 3   | 3  | 10   |
| 270  | O75891        | 10-Formyltetrahydrofolate dehydrogenase | Metabolism | Cytoplasm | 5.63 | 98.83 | 5.9 | 94.44 | 0 | 5 | 11 |
| 271  | Q9BSE5        | Agmatinase | Metabolism | Mitochondrion | 8.02 | 37.76 | 6.16 | 36.28 | 3 | 2 | 11 |
| 272  | P23526        | Adenosylhomocysteinase | Metabolism | Cytoplasm | 5.92 | 47.58 | 6.39 | 55.17 | 4 | 3 | 9 |
| 274  | P50440        | Glycine amidinotransferase | Metabolism | Mitochondrion | 8.26 | 48.46 | 6.57 | 57.16 | 4 | 1 | 11 |
| 275  | P16219        | Short-chain-specific acyl-CoA dehydrogenase | Metabolism | Mitochondrion | 8.13 | 44.3 | 6.66 | 37.09 | 6 | 2 | 8 |
| 278  | P14625        | Endoplasm | Stress protein | ER | 4.76 | 92.47 | 4.62 | 96.01 | 4 | 6 | 6 |
| 293  | P08670        | Vimentin | Cell motility | Cytoskeleton | 5.06 | 53.52 | 4.62 | 51.2 | 3 | 6 | 7 |
| 299  | P08670        | Vimentin | Cell motility | Cytoskeleton | 5.06 | 53.52 | 5.14 | 63.43 | 0 | 8 | 8 |
| 300  | P02879        | Fibrinogen γ chain | Proliferation | Extracellular region | 5.37 | 51.51 | 5.41 | 61.15 | 0 | 5 | 11 |
| 301  | P02879        | Fibrinogen γ chain | Proliferation | Extracellular region | 5.37 | 51.51 | 5.5 | 59 | 3 | 4 | 9 |
| 309  | P30837        | Aldehyde dehydrogenase X | Metabolism | Mitochondrion | 6.41 | 57.22 | 6.27 | 69.02 | 4 | 2 | 10 |
| 310  | P31040        | Succinate dehydrogenase (ubiquinone) flavoprotein subunit | Transport protein | Mitochondrion | 7.06 | 72.69 | 6.63 | 75.01 | 3 | 4 | 9 |
| 311  | P05165        | Propionyl-CoA carboxylase α chain | Metabolism | Mitochondrion | 6.63 | 73.35 | 6.75 | 74.63 | 4 | 6 | 6 |
| 312  | P13645        | Keratin, type I cytoskeletal 10 | Structural molecule | Cytoskeleton | 5.13 | 59.52 | 6.49 | 52.56 | 5 | 1 | 10 |
| 313  | Q14894        | μ-Crystallin homolog | Other | Cytoplasm | 5.06 | 33.78 | 4.87 | 43.98 | 4 | 1 | 11 |
| 323  | P21980        | Protein-glutamine γ-glutamyltransferase 2 | Cell adhesion | Extracellular region | 5.11 | 77.33 | 5.05 | 85.56 | 2 | 6 | 8 |
| 326  | P48637        | Glutathione synthetase | Metabolism | Unknown | 5.67 | 52.38 | 5.84 | 60.05 | 3 | 2 | 11 |
| 328  | P61981        | 14-3-3 protein γ | Signal transduction | Cytoplasm | 4.8 | 28.17 | 4.51 | 31.83 | 4 | 2 | 10 |
| 329  | P63104        | 14-3-3 protein γ/δ | Signal transduction | Multiple compartment | 4.73 | 27.75 | 4.42 | 32.13 | 4 | 4 | 8 |
| 331  | P09467        | Fructose-1,6-bisphosphatase 1 | Metabolism | Unknown | 6.61 | 36.68 | 6.92 | 48.59 | 5 | 1 | 10 |
| 332  | P50440        | Fructose-bisphosphate aldolase B | Metabolism | Mitochondrion | 8.26 | 48.46 | 6.88 | 56.91 | 4 | 2 | 10 |
| 333  | Q14764        | Major vault protein | Other | Multiple compartment | 5.34 | 99.2 | 5.41 | 107 | 3 | 4 | 9 |
| 334  | Q14764        | Major vault protein | Other | Multiple compartment | 5.34 | 99.2 | 5.33 | 106.2 | 2 | 5 | 9 |
| 335  | Q14764        | Major vault protein | Other | Multiple compartment | 5.34 | 99.2 | 5.33 | 106.2 | 2 | 3 | 11 |
| 341  | P09467        | Fructose-1,6-bisphosphatase 1 | Metabolism | Unknown | 6.61 | 36.68 | 6.4 | 43.48 | 7 | 0 | 7 |
| 343  | P05062        | Fructose-bisphosphatase aldolase B | Metabolism | Cytoplasm | 8.06 | 39.34 | 7.52 | 44.63 | 7 | 1 | 6 |
| 347  | Q99798        | Acotinate hydrolase | Metabolism | Mitochondrion | 7.36 | 85.43 | 7.13 | 78.53 | 4 | 3 | 7 |
| 360  | P25705        | ATP synthase subunit α | Transport protein | Mitochondrion | 9.16 | 59.75 | 7.16 | 58.87 | 7 | 2 | 5 |
| 367  | P09018        | Carbonic anhydrase 2 | Metabolism | Cytoplasm | 6.86 | 29.11 | 6.96 | 32.45 | 3 | 3 | 8 |
| 369  | P63261        | Actin, cytoplasmic 2 | Cell motility | Cytoskeleton | 5.31 | 41.79 | 7.16 | 58.87 | 6 | 2 | 6 |
| 373  | P25705        | ATP synthase subunit α | Transport protein | Mitochondrion | 9.16 | 59.75 | 8.01 | 63.3 | 2 | 3 | 9 |
| 388  | P06733        | α-Enolase | Expression control | Multiple compartment | 6.99 | 47.04 | 6.7 | 63.85 | 5 | 3 | 6 |
| 392  | P50991        | T-complex protein 1 subunit δ | Proliferation | Cytoplasm | 8.13 | 57.79 | 8.33 | 40.85 | 3 | 2 | 9 |
| 393  | Q99807        | Ubiquinone biosynthesis protein COQ7 homolog | Metabolism | Mitochondrion | 8.77 | 24.31 | 6.45 | 30.6 | 5 | 3 | 6 |

Targets identified on pH 6–11 2DE gels
### Table II—continued

| Spot | Swiss-Prot ID | Protein | Cellular function | Cellular compartment | pI  | mass | pI  | mass | Dn | Up | NR/ND |
|------|---------------|---------|-------------------|----------------------|-----|------|-----|------|----|----|-------|
| 85   | P30038        | 1-Pyrroline-5-carboxylate dehydrogenase | Metabolism | Mitochondrion | 8.25 | 61.72 | 6.72 | 63.85 | 5  | 1  | 8     |
| 93   | P04040        | Catalase | Stress protein | Mitochondrion | 6.95 | 59.62 | 7.51 | 50.37 | 6  | 0  | 8     |
| 100  | P11310        | Medium-chain-specific acyl-CoA dehydrogenase | Metabolism | Mitochondrion | 8.61 | 46.59 | 6.64 | 45.85 | 4  | 2  | 8     |
| 106  | P09651        | Heterogeneous nuclear ribonucleoprotein A1 | Expression control | Multiple | 9.26 | 38.71 | 8.44 | 29.83 | 7  | 0  | 8     |
| 114  | P25705        | ATP synthase subunit α | Transport protein | Mitochondrion | 8.93 | 34.28 | 8.06 | 39.34 | 6  | 0  | 8     |
| 121  | Q16822        | Phosphoenolpyruvate carboxykinase (GTP) | Metabolism | Mitochondrion | 7.56 | 70.64 | 6.61 | 45.13 | 6  | 2  | 6     |
| 137  | Q16836        | Hydroxyacyl-coenzyme A dehydrogenase | Metabolism | Mitochondrion | 8.88 | 44.48 | 8.62 | 48.22 | 3  | 3  | 9     |
| 143  | P07355        | Annexin A2 | Proliferation | Membrane | 8.72 | 57.84 | 7.51 | 51.9 | 3  | 3  | 8     |
| 153  | Q02722        | Methylmalonate-semialdehyde dehydrogenase (acylating) | Metabolism | Mitochondrion | 8.47 | 39.34 | 7.22 | 45.13 | 6  | 0  | 8     |
| 157  | Q07355        | H-FABP | Metabolism | Mitochondrion | 8.98 | 45.2 | 8.12 | 43.8 | 2  | 3  | 9     |

local distributions of the proteins identified are likely to represent specific characteristics of the ccRCC proteome in comparison with tumor-adjacent kidney epithelium.

**Verification of Selected Targets in RCC Lesions of Distinct Subtypes Using Immunohistochemistry**—An important step in the cancer biomarker identification is the verification of the putative diagnostic or prognostic biomarkers on a large series of tumors and/or body fluids like serum and urine. Here we selected three candidate biomarkers for immunohistochemical stainings based on commercially available antibodies and the novelty of the specific antigens in the context of ccRCC. These include (i) calbindin, representing a member of the single target spot cluster, which was selected because of its limited regulation pattern, (ii) gelsolin, one of the top scoring single target spots in terms of its prevalence, and (iii) H-FABP, a representative member of the FABP protein family, which has been shown in a previous report to be heterogeneously expressed in RCC (28). Moreover, H-FABP also seems to exhibit posttranslational modifications because it was detected in two single target spots on the consensus maps.

**Calbindin Immunostaining**—Immunohistochemical staining of tumor-adjacent renal tissues with a calbindin-specific antibody demonstrated a strong cytoplasmic staining for calbindin in mesangial cells of the glomeruli and the epithelium of the proximal and distal tubule system as well as in endothelial cells. However, calbindin is not ubiquitously expressed in the proximal and distal tubule system as well as in endothelial cells. Therefore, calbindin staining was seen in 74.2% of the analyzed lesions. For chromophilic RCC lesions (Fig. 4C) and chromophobic RCC (Fig. 4D). Thus, a predominant lack of calbindin expression might be associated with the malignant phenotype of the disease.
Gelsolin Immunostaining—Staining of the tumor-adjacent kidney tissue sections with a gelsolin-specific antibody showed that gelsolin is expressed in the epithelium of the distal tubule system and collecting ducts, whereas the epithelium of the proximal tubule system and the glomeruli revealed no gelsolin expression (Fig. 5A). This is in accordance with the staining pattern of RCC lesions demonstrating that 30% of ccRCCs (Fig. 5B) and about 42% of papillary RCCs both derived from the proximal tubules lack gelsolin expression (data not shown), whereas 75% of chromophobic RCC (Fig. 5C) and 55% of oncocytoma (Fig. 5D) originating from the distal tubule system/collecting ducts displayed a strong and intermediate staining for gelsolin, respectively.

The IHC data obtained from TMA analyses using the gelsolin-specific mAb revealed that ccRCC and chromophilic RCC displayed a strong positive cytoplasmic gelsolin staining with a frequency of 25 and 22.6%, respectively, and an intermediate staining for gelsolin in 15% of the ccRCC and 3.2% of the chromophilic RCC subtype. 27.5% of ccRCC and 32.3% of chromophilic RCC showed a weak staining, and 32.5% of the ccRCC and 41.9% of the chromophilic RCC subtype showed a lack of gelsolin expression (Fig. 6, A and B). 18.8% of chromophobic RCC lesions tested negative for gelsolin staining, 6.3% exhibited a weak staining, 18.8% exhibited an intermediate staining, and the remaining 56.3% exhibited a strong positive cytoplasmic staining (Fig. 6C). In contrast, renal cell adenomas of oncocytic type (Fig. 6D) displayed a strong positive staining for gelsolin in 11.1%, an intermediate staining in 44.4%, and a weak or negative staining in 22.2% of the cases analyzed. Thus, the results of the proteomics approach and IHC revealed a distinct regulation of gelsolin in the ccRCC lesions.

H-FABP Immunostaining—Using 2DE-based technology a distinct protein expression pattern for FABP family members in RCC has been described recently, but because of the lack of an IHC-suitable H-FABP-specific mAb this family member had not been yet been verified by IHC (28). As shown in Fig. 7, representative IHC stainings using a recently available H-FABP-specific polyclonal antibody confirmed the heterogeneous expression pattern for H-FABP in the various RCC subtypes and tumor-adjacent kidney epithelium, which might allow distinguishing among normal, benign, and malignant tissues. In tumor-adjacent renal kidney tissue, the cytoplasm of cells representing epithelium of the proximal and distal tubule system and mesangial cells were strongly stained with the H-FABP-specific Ab (Fig. 7A). Although ccRCC showed a rather heterogeneous staining pattern, for example showing a

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**Figure 2.** Classification of differentially expressed proteins according to their cellular function and their cellular localization. The pie charts display the classification of the subset of differentially expressed proteins listed in Table II into 12 functional categories (A) and into 10 distinct cellular compartments (B). The pool of proteins comprises 84 distinct protein identities defining the 123 spots, which were identified in at least five independent biopsy systems. The distribution frequencies in regard to the specified categories within the given chart pie are indicated in percentage of the total number of entries. For each pie chart a cutoff value was set to 5%, meaning that all of the cellular functions/compartment represented below this threshold were summarized under other.

**Figure 3.** Immunohistochemical analysis for calbindin expression in tumor-adjacent renal epithelium and distinct kidney tumor subtypes. Randomly selected sections of the TMA representing tumor-adjacent renal epithelium and various RCC subtypes were used to document the immunohistochemical staining with the anti-human calbindin-specific mAb. A shows the immunohistochemical analysis of calbindin in tumor-adjacent kidney tissue at 200× magnification; B refers to the staining pattern at a magnification of 400×. Calbindin is expressed in some but not all cells of the proximal (solid arrows) as well as the distal tubule system (open arrows) in non-tumorous kidney tissues. Yet both stainings show sporadic strong positive stainings of the epithelium of both the proximal and the distal tubule systems. In contrast, the ccRCCs (C; magnification, 400×) and RCC of the chromophobic type (D; magnification, 400×) lack calbindin staining.
strong positive cytoplasmic staining (Fig. 7B), chromophilic RCCs are defined by a weak to even negative staining pattern (Fig. 7C). In contrast, all oncocytomas analyzed showed a strong cytoplasmic staining pattern similar to that of the tumor-adjacent epithelium (Fig. 7D).

Staining of the TMA with the anti-H-FABP Ab confirmed an almost evenly distributed staining pattern of ccRCC with 23% of the analyzed tissue sections exhibiting either a strong, intermediate, or weak cytoplasmic H-FABP staining, whereas the remaining 31% of ccRCC lesions fully lacked H-FABP expression (Fig. 8A). The staining pattern of chromophilic RCC is defined by 13% strong, 10% intermediate, 13% weak, and 64% negative cytoplasmic stainings (Fig. 8B). In contrast, the pattern characteristics for chromophobic RCC exhibited a strong positive cytoplasmic staining in 68%, an intermediate staining in 13%, and a negative staining in 19% of RCC lesions analyzed (Fig. 8C). Moreover, all adenomas of oncocytic type demonstrated a strong positive cytoplasmic staining for H-FABP (Fig. 8D). Thus, the distinct staining pattern for H-FABP might be suited to distinguish between various RCC subtypes. Concerning the staining pattern for ccRCC, the heterogeneity of the staining is in line with the distinct regulation pattern observed in the proteomics profiling of different ccRCC lesions.

**DISCUSSION**

Proteomics profiling is now widely accepted as a tool for gaining insights into the dynamics of protein expression profiles reflecting complex cellular processes, including malig-
nant transformation. To date, different semiquantitative and quantitative proteome-based technologies have been implemented for the profiling of clinical samples like diseased or cancerous tissues, serum, and urine. These technologies allowed the identification of candidate biomarkers, which are particularly important for the development of early detection, prognosis, and diagnosis of diseases and therapeutic treatment modalities, including renal cell carcinoma (17, 19, 20, 33–37).

So far, the information available for RCC is restricted to a relatively small number of differentially expressed proteins defined in few selected RCC cell lines/lesions when compared with tumor-adjacent epithelium, emphasizing the need to identify novel putative biomarkers in a broader range of RCC specimens. Although Perroud et al. (21) recently described 105 proteins with a statistically significant grade-dependent change in regard to their protein expression levels in ccRCC, caused by their experimental approach, none of these candidate biomarkers could be assigned to a RCC map. Moreover, the most prominent RCC protein expression map hosted on the ExPASy Proteomics Server mainly relies on the data presented by Sarto et al. (38) more than 10 years ago, although a number of groups have contributed substantially more RCC-specific candidate markers as recently reviewed (17–19).

Thus, in this report, a systematic 2DE-based proteomics profiling of biopsy samples representing ccRCC in comparison with corresponding tumor-adjacent renal epithelium was performed. To the best of our knowledge, we provide here for the first time comprehensive ccRCC-specific protein expression maps using acidic and basic pH gradients in the first dimension to increase the spot resolution. However, despite the analysis of up to 16 pairs of tissue specimens, the presented consensus maps must still be regarded as preliminary. In line with the known disadvantages of 2DE-based proteome analysis, the identification of low abundant, hydrophobic, very basic, high and low molecular weight, and small proteins is still limited (17, 19, 34, 36, 39, 40). The majority of the differentially expressed proteins identified in the ccRCC/tumor-adjacent kidney biopsy systems analyzed in this study mainly reflect high abundant molecules, which have also been defined at least in part in other organ-specific protein expression maps. On the other hand, one could also speculate that the identification of high abundant proteins, which are altered in ccRCC when compared with tumor-adjacent epithelium, still might represent those with an impact on RCC-specific phenotypic changes.

Moreover, it is noteworthy that our consensus maps delineate 55 of the 105 proteins altered in a grade-dependent manner in ccRCC (21). 37 of the candidate biomarkers defined recently by Perroud et al. (21) are even represented as...
single target spots on our consensus maps, and the vast majority thereof showed a regulation pattern comparable with that obtained by the shotgun proteomics approach.

Overall, 348 protein spots, corresponding to 248 protein identities, were mapped. The plotting of the theoretical versus the experimental masses for each identified protein in a scatter diagram underscores the quality of the profiling (supplemental Fig. 4). Larger deviations of the experimental masses toward a higher molecular weight might be due to PTMs, whereas lower masses might reflect fragmentation events, but the characterization of such modifications was not the focus of this study.

Interestingly, the protein expression profiling using two overlapping pH gradients resulted not only in the mapping and verification of previously described members of the RCC proteome (17–21) but also led to the identification of 97 additional novel protein entries. Thus, a gain of 17% in the number of differentially expressed proteins in RCC was obtained, thereby now covering 558 protein identities (supplemental Fig. 2).

Furthermore, a database containing the complete 2DE-based profiling information of the 16 ccRCC lesions and corresponding tumor-adjacent kidney epithelium was generated, subsequently allowing not only the mapping of a large number of proteins and the establishment of the respective consensus maps for the RCC proteome but also the selection of a core subset of differentially expressed proteins prioritized and refined according to the detection frequency (>5) and their predominant regulation tendency (>2:1 ratio) (Table II). The group of differentially expressed proteins largely comprises enzymes involved in the energy metabolism and detoxification processes, cellular stress, transport, cell proliferation, and motility, which are in line with previous reports, but nevertheless some novel potential targets are highlighted.

Profiling of RCC versus tumor-adjacent renal epithelium using various "ome"-based profiling strategies such as classical 2DE-based proteomics, PROTEOMEX, and transcriptomics showed that each technology has its strengths and limitations (17, 41). In contrast to the expected significant overlap between the targets defined by gel-based approaches like proteomics and PROTEOMEX, the overlap of targets shared between transcriptomics and proteomics profiling is strikingly limited to about 10%, strongly arguing for the complementary implementation of transcriptomic and proteomic profilings. Moreover, it is evident that target validation is even more limited in cDNA microarray studies when compared with proteomics/PROTEOMEX profiling. Thus, the majority of the currently validated candidate markers are almost exclusively linked to proteome-based approaches. Some recent reviews highlight the contribution and impact of "ome"-based strategies for the discovery of candidate biomarkers in RCC and provide lists of partially validated markers (16–19, 41). Because the number of validated targets for RCC is still very limited, more verification/validation studies are urgently required to determine the diagnostic and prognostic value of candidate biomarkers and to design novel therapeutic approaches in this disease.

Consequently, three representative candidate markers identified in the context of the proteomics profiling of the 16 ccRCC biopsy systems analyzed in this report, namely calbindin, gelsolin, and H-FABP, were selected, and their protein expression pattern was independently analyzed by immunohistochemical staining performed on a TMA consisting of a large number of biopsies representing not only ccRCC but also chromophilic and chromophobic RCC subtypes and benign oncocytoma as well as corresponding tumor-adjacent epithelium.

Using this strategy, potentially interesting and novel differences in the protein expression profiles between tumor-adja-
cent kidney tissues and various RCC subtypes were found (Figs. 4–8). The TMA staining essentially confirmed the results obtained via the proteomics profiling with the exception of calbindin. Although only two of six regulated calbindin spots were only expressed in the tumor-adjacent renal tissues, the remaining four spots initially categorized as differentially spots displayed at least the same regulation tendency, a strong calbindin down-regulation. Nevertheless, based on the immunohistochemical staining pattern, calbindin might indeed be used for proper distinction of ccRCC from the other RCC subtypes because of its total lack of expression in ccRCC. Furthermore, this loss appears to be associated with the malignant phenotype of RCC because only 44% of the benign oncocytomas exhibited negative calbindin staining, whereas a strong cytoplasmic staining for calbindin was described in the tumor-adjacent epithelium. The functional role of calbindin is the partial stabilization of intracellular calcium levels, which can confer protection against different proapoptotic stimuli (42, 43). It has been further found to protect the embryonal kidney epithelium cell line HEK293 from parathyroid hormone-induced apoptosis (44). Although calcium signals can determine the fate of cancer cells (45), a role of calbindin expression in the development or progression of RCC has not been reported. However, calbindin is up-regulated in renal distal tubular cells following treatment with reverse transcriptase inhibitors, which further led to an enhanced immunogenicity of these cells associated with an improved induction of CD8+ effector memory cells (46). The lack of calbindin expression in ccRCC could be explained by its origin from the proximal tubule system because calbindin is mainly expressed in distal tubules. In discrepancy with the current literature, the IHC staining of the TMA clearly showed neither a ubiquitous nor a unique expression in the proximal or distal tubule system of the kidney (Fig. 3, A and B). Furthermore, the TMA staining is supported by the findings of Martignoni et al. (47) addressing the expression pattern of calcium-binding proteins in chromophobic RCC. The differential expression pattern of calbindin in benign versus malignant kidney tumors and its putative association with malignancy suggests that calbindin might serve as a candidate biomarker unlikely alone but at least in combination with other proteins to distinguish between the different renal tumor subtypes and their malignant potential.

Although gelsolin is known to be involved in cell motility, cancer progression, metastasis formation, signaling pathways, and apoptosis (48), a conclusive expression pattern of this protein has not been identified in solid tumors during carcinogenesis. For example, a decreased gelsolin expression was found in bladder, prostate, and breast cancers, thereby suggesting a tumor- and metastasis-suppressing role in these malignancies. In accordance with our study, decreased gelsolin expression in RCC of grade 2 tumors associated with a poor patient survival has been described previously (49). In contrast, an up-regulation of gelsolin was associated with poor prognosis of patients with non-small-cell lung cancer (50). These data argue for a tissue-specific activity of gelsolin, but the molecular mechanisms by which gelsolin exerts its biological function in cancer development have not yet been defined. Because of its heterogeneous expression pattern in ccRCC versus tumor-adjacent kidney epithelium, the implementation of gelsolin as a candidate biomarker for RCC is unlikely.

H-FABP is a member of the FABP family, which represents homologous intracellular proteins with a low molecular weight. FABPs are expressed in a variety of tissues, playing role in the fatty acid metabolism (51), and are therefore involved in a number of biological processes such as cell differentiation, cell growth, and apoptosis. Although H-FABP has been primarily identified to be expressed in heart and striated muscle, low H-FABP expression levels were also detected in kidney, lung, cerebral cortex, and placenta tissues. Intriguingly, H-FABP overexpression, which was associated with disease progression, tumor aggressiveness, and poor patient survival, has been described in a subset of human gastric carcinomas (52). These data correlate with the role of other FABP members, which might serve as prognostic markers to predict the outcome of patients, including RCC patients. Recently, the importance of L-FABP and brain FABP expression in RCC has been described (28). The proteomics profiling-based results as well as the verification data obtained from the immunohistochemical staining of the TMA demonstrated a heterogeneous but unique H-FABP expression pattern in the different RCC subtypes, suggesting the use of H-FABP for the classification of different RCC subtypes. In contrast to previously described IHC staining patterns on renal tissues (53), the H-FABP expression was not restricted to the distal tubule system but also was detectable in some cells of the proximal tubules as well as endothelial cells. However, the clinical significance of H-FABP in RCC has still to be proven.

In conclusion, the established ccRCC maps using pH 4–7 and pH 6–11 gradients provide a largely extended and improved resource for the tracking and identification of ccRCC-specific proteins. This pilot study resulted in a list of frequently dysregulated proteins (Table II), although the verification of these putative candidate proteins still has to be performed in clinical settings on a large series of RCC biopsies. Nevertheless, it seems reasonable that at least some members of these candidate lists might represent the basis for the design and development of diagnostic tools such as the design of multiparameter protein arrays, which might be suited not only for the discrimination of the various RCC subtypes from tumor-adjacent tissue but also, in combination with clinical data, for the monitoring of treatment regimens as well as for an improved prediction of risk and progression of RCC. In addition, the knowledge gained by these differentially expressed proteins will also contribute to a better understanding of the pathophysiology of RCC and likely provide starting points for functional experiments.
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37. Sarto, C., Valsecchi, C., Magni, F., Tremolada, L., Arizzi, C., Cordani, N., Caselli, S., Doro, G., Favini, P., Perego, R. A., Raimondo, F., Ferrero, S., Mocarelli, P., and Galli-Kienle, M. (2004) Expression of heat shock protein 27 in human renal cell carcinoma. *Proteomics* 4, 2252–2260.

38. Sarto, C., Marocchi, A., Sanchez, J. C., Giannone, D., Frutiger, S., Golaz, O., Wilkins, M. R., Doro, G., Cappellano, F., Hughes, G., Hochstrasser, D. F., and Mocarelli, P. (1997) Renal cell carcinoma and normal kidney protein expression. *Electrophoresis* 18, 599–604.

39. Shi, T., Dong, F., Liou, L. S., Duan, Z. H., Novick, A. C., and DiDonato, J. A. (2004) Differential protein profiling in renal-cell carcinoma. *Mol. Carcinog.* 40, 47–61.

40. Fountoulakis, M. (2001) Proteomics: current technologies and applications in neurological disorders and toxicology. *Amino Acids* 21, 363–381.

41. Seliger, B., Dressler, S. P., Wang, E., Kellner, R., Recktenwald, C. V., Lottspeich, F., Marincola, F. M., Baumga¨ rtner, M., Atkins, D., and Lich-tenfels, R. (2009) Combined analysis of transcriptome and proteome data as a tool for the identification of candidate biomarkers in renal cell carcinoma. *Proteomics* 9, 1567–1581.

42. Liu, H., Bowes, R. C., 3rd, van de Water, B., Sillence, C., Nagelkerke, J. F., and Stevens, J. L. (1997) Endoplasmic reticulum chaperones GRP78 and calreticulin prevent oxidative stress, Ca2+/H11001 disturbances, and cell death in renal epithelial cells. *J. Biol. Chem.* 272, 21751–21759.

43. Bellido, T., Huening, M., Raval-Pandya, M., Manolagas, S. C., and Christakos, S. (2000) Calbindin-D28k is expressed in osteoblastic cells and suppresses their apoptosis by inhibiting caspase-3 activity. *J. Biol. Chem.* 275, 26328–26332.

44. Christakos, S., and Liu, Y. (2004) Biological actions and mechanism of action of calbindin in the process of apoptosis. *J. Steroid Biochem. Mol. Biol.* 89–90, 401–404.

45. Sergeev, I. N. (2005) Calcium signaling in cancer and vitamin D. *J. Steroid Biochem. Mol. Biol.* 97, 145–151.

46. Landriscina, M., Altamura, S. A., Roca, L., Gigante, M., Piscazzi, A., Cavalcanti, E., Costantino, E., Barone, C., Cignarelli, M., Gesualdo, L., and Ranieri, E. (2008) Reverse transcriptase inhibitors induce cell differentiation and enhance the immunogenic phenotype in human renal clear-cell carcinoma. *Int. J. Cancer* 122, 2842–2850.

47. Martignoni, G., Pea, M., Chilosi, M., Brunelli, M., Scarpa, A., Colato, C., Tardanico, R., Zamboni, G., and Bonetti, F. (2001) Parvalbumin is constantly expressed in chromophobe renal carcinoma. *Mod. Pathol.* 14, 760–767.

48. Kim, H. L., Seligson, D., Liu, X., Janzen, N., Bui, M. H., Yu, H., Shi, T., Figlin, R. A., Horvath, S., and Belldegrun, A. S. (2004) Using protein expressions to predict survival in clear cell renal carcinoma. *Clin. Cancer Res.* 10, 5464–5471.

49. Visapää, H., Bui, M., Huang, Y., Seligson, D., Tsai, H., Pantuck, A., Figlin, R., Rao, J. Y., Belldegrun, A., Horvath, S., and Palotie, A. (2003) Correlation of Ki-67 and gelsolin expression to clinical outcome in renal clear cell carcinoma. *Urology* 61, 845–850.

50. Osman, I., Bajorin, D. F., Sun, T. T., Zhong, H., Douglas, D., Scattergood, J., Zheng, R., Han, M., Marshall, K. W., and Liew, C. C. (2006) Novel blood biomarkers of human urinary bladder cancer. *Clin. Cancer Res.* 12, 3374–3380.

51. Storch, J., and Thumser, A. E. (2000) The fatty acid transport function of fatty acid-binding proteins. *Biochim. Biophys. Acta* 1486, 28–44.

52. Hashimoto, T., Kusakabe, T., Sugino, T., Fukuda, T., Watanabe, K., Sato, Y., Nishimoto, A., Honma, K., Kimura, H., Fujii, H., and Suzuki, T. (2004) Expression of heart-type fatty acid-binding protein in human gastric carcinoma and its association with tumor aggressiveness, metastasis and poor prognosis. *Pathobiology* 71, 267–273.

53. Kimura, H., Fujii, H., Suzuki, S., Ono, T., Arakawa, M., and Gejyo, F. (1999) Lipid-binding proteins in rat and human kidney. *Kidney Int. Suppl.* 71, S159–S162.