Human Cancer Biology

Mining Tissue Microarray Data to Uncover Combinations of Biomarker Expression Patterns that Improve Intermediate Staging and Grading of Clear Cell Renal Cell Cancer

Corinne Dahinden1,2, Barbara Ingold3, Peter Wild3, Gunther Boysen3, Van-Duc Luu3, Matteo Montani3, Glen Kristiansen3, Tullio Sulser4, Peter Bühlmann1,2, Holger Moch2,3, and Peter Schraml3

Abstract

Purpose: Tumor stage and nuclear grade are the most important prognostic parameters of clear cell renal cell carcinoma (ccRCC). The progression risk of ccRCC remains difficult to predict particularly for tumors with organ-confined stage and intermediate differentiation grade. Elucidating molecular pathways deregulated in ccRCC may point to novel prognostic parameters that facilitate planning of therapeutic approaches.

Experimental Design: Using tissue microarrays, expression patterns of 15 different proteins were evaluated in over 800 ccRCC patients to analyze pathways reported to be physiologically controlled by the tumor suppressors von Hippel-Lindau protein and phosphatase and tensin homologue (PTEN). Tumor staging and grading were improved by performing variable selection using Cox regression and a recursive bootstrap elimination scheme.

Results: Patients with pT2 and pT3 tumors that were p27 and CAIX positive had a better outcome than those with all remaining marker combinations. A prolonged survival among patients with intermediate grade (grade 2) correlated with both nuclear p27 and cytoplasmic PTEN expression, as well as with inactive, nonphosphorylated ribosomal protein S6. By applying graphical log-linear modeling for over 700 ccRCC for which the molecular parameters were available, only a weak conditional dependence existed between the expression of p27, PTEN, CAIX, and p-S6, suggesting that the dysregulation of several independent pathways are crucial for tumor progression.

Conclusions: The use of recursive bootstrap elimination, as well as graphical log-linear modeling for comprehensive tissue microarray (TMA) data analysis allows the unraveling of complex molecular contexts and may improve predictive evaluations for patients with advanced renal cancer. Clin Cancer Res; 16(1); 88–98. ©2010 AACR.

The last decades have shown an incidental increase of patients diagnosed with renal cell carcinoma (RCC) with clear cell RCC (ccRCC) being the most frequent and aggressive subtype (1, 2). Patients with local tumors have a significantly better outcome as these cancers can be treated with radical or partial nephrectomy, whereas the 5-year survival rate of metastatic ccRCC remains poor. However, even in patients with organ-confined ccRCC, the 10-year cancer-specific death rates vary from 10% to 38% in pT1a and pT2 tumors, respectively (3).

To date, the best available predictor of the postoperative clinical course of localized RCCs is tumor stage at presentation (4, 5). Nevertheless, a significant difference of outcome exists within the same stage. Additional prognostic parameters are routinely used to refine prognosis in RCC patients. After tumor stage, the second most important prognostic parameter is the nuclear differentiation grade (6). Three- and four-tired grading systems are commonly applied for tumor grading. More than 50% of ccRCCs are classified as moderately differentiated, implying an intermediate risk of tumor recurrence (7), which is not informative for the clinician to stratify therapy. Therefore, many efforts have been made to uncover altered molecular markers and pathways with prognostic potential in patients with ccRCC.

Alteration of pathways regulated by the von Hippel-Lindau protein (VHL) due to mutation of its coding gene is the main characteristic molecular feature of most...
sporadic ccRCC. VHL is a multifunctional protein that acts as an adaptor for different molecular and subcellular complexes. The best-characterized function of VHL is its role as a substrate recognition component of the E3 ubiquitin ligase complex that targets the hypoxia inducible factor α (HIF-α) for proteolytic degradation. In ccRCC, loss of VHL leads to the upregulation of HIF-α-mediated transcriptional programs that favor metastatic processes (reviewed in ref. 8).

Studies using appropriate cell line and mouse models showed that VHL is also capable of positively influencing the expression and stability of other important tumor suppressors in a HIF-independent fashion. VHL increases p53 expression by suppressing proteolytic degradation and interacting with p53-activating proteins ATM and p300 (9). Because p53 mutations are relatively rare in ccRCC (10), p53 inactivation may rather be provoked by loss of VHL. Apart from p53, the expression of the cyclin-dependent kinase 2 inhibitor p27 (KIP1) seems to be dependent of VHL. Both VHL-mediated cell cycle arrest and cell cycle exit are accompanied by the accumulation of p27 (11, 12).

The presence of VHL is obviously essential for activating p27 expression, whereas the phosphatase and tensin homologue (PTEN) potentially influences the integrity of the function of p27. PTEN negatively regulates numerous growth factor receptor–mediated signal transductions by dephosphorylating phosphatidylinositol-3,4,5-triphosphate, which is a substrate of activated phosphatidylinositol-3-kinase (PI3K) enzymes (13). Loss of PTEN function leads to constitutive activation of PI3K downstream components including AKT and mammalian target of rapamy-

In this study, we used a tissue microarray platform to comprehensively analyze von Hippel-Lindau protein and phosphatase and tensin homologue (PTEN)–controlled pathways by examining the expression patterns of 15 proteins in over 800 clear cell renal cell carcinoma patients. By applying appropriate mathematical models for efficient and accurate data analysis of these renal cell carcinoma specimens, it was shown that the expression status of p27, PTEN, CAIX, and p-S6 is crucial for improving intermediate tumor staging and grading. In a second step, graphical log-linear modeling was used to identify conditional dependencies among the biomarkers. We deduced only a weak conditional dependence between von Hippel-Lindau protein and PTEN (de)regulated pathways. These observations suggest that the dysregulation of several independent pathways are important for tumor progression in clear cell renal cell carcinoma. The use of advanced mathematical models for comprehensive data analysis may improve predictive evaluations for patients with advanced renal cell cancer.

Translational Relevance

In this study, we used a tissue microarray platform to comprehensively analyze von Hippel-Lindau protein and phosphatase and tensin homologue (PTEN)–controlled pathways by examining the expression patterns of 15 proteins in over 800 clear cell renal cell carcinoma patients. By applying appropriate mathematical models for efficient and accurate data analysis of these renal cell carcinoma specimens, it was shown that the expression status of p27, PTEN, CAIX, and p-S6 is crucial for improving intermediate tumor staging and grading. In a second step, graphical log-linear modeling was used to identify conditional dependencies among the biomarkers. We deduced only a weak conditional dependence between von Hippel-Lindau protein and PTEN (de)regulated pathways. These observations suggest that the dysregulation of several independent pathways are important for tumor progression in clear cell renal cell carcinoma. The use of advanced mathematical models for comprehensive data analysis may improve predictive evaluations for patients with advanced renal cell cancer.

Materials and Methods

Tissue specimen and TMA construction. TMAs comprising 831 nephrectomy ccRCC collected at the University Hospital of Zurich (Zurich, Switzerland), the Kantonsspital St. Gallen (St. Gallen, Switzerland), and the University Hospital of Basel (Basel, Switzerland) were constructed as described (19). All ccRCC samples were histologically reviewed by one pathologist (H.M.). This study was approved by the local commission of ethics (ref. number SV 38-2005). Survival time was obtained for 519 patients. The mean age of patients was 62.7 y (15-88) and the mean follow-up of patients was 51.9 mo (0.1-1229). Tumors were graded according to the Thoenes grading system and histologically classified according to the WHO classification (20). There were 164 grade I (20%), 414 grade II (50%), 248 grade III (30%), 161 pT1 (20%), 223 pT2 (27%), 412 pT3 (50.5%), and 20 pT4 (2.5%) tumors. Fifteen tumors had no information about tumor stage and the tumor grade was missing for 5 tumors.

Immunohistochemistry. Fifteen antibodies that recognize proteins involved in the VHL- and PTEN-regulated pathways were selected for this study. CD10, which is overexpressed in the majority of ccRCC (21) and may therefore be regulated by the VHL/HIF-axis, was also included in our TMA analysis. TMA sections (2.5 μm) were transferred to glass slides and treated using Ventana Benchmark XT, Bond-maxX (Leica Microsystems) automated systems, and manual protocols. Antibodies and protocols are listed in Table 1. Besides nuclear expression, the

Phosphorylation by AKT impairs the nuclear import of p27, thus relieving CDK2 from p27-induced inhibition (15). Although PTEN mutations are rare in ccRCC, allele deletions and reduction of PTEN protein expression occur in ~25% of the tumors (16, 17), indicating its potential role in a considerable subset of tumors. Interestingly, a recent study supports the hypothesis that a cooperative interaction between functional PTEN and VHL is necessary to successfully suppress the formation of epididymal cystadenomas, which frequently occurs in human VHL patients (18). Based on these data, it is conceivable that in many ccRCC, the clinical outcome is mainly dependent on the integrity of pathways normally controlled by VHL and PTEN.

Here, tissue microarrays (TMA) with >800 ccRCC were used to analyze the expression patterns of 15 proteins known to be involved in pathways directly or indirectly regulated by VHL and PTEN. Information about the ccRCC patients such as tumor stage, nuclear grade, and survival was available. A schematic overview of these pathways is shown in Fig. 1. Cox regression with recursive bootstrap elimination was used to statistically depict survival time with the expression patterns of specific biomarker combinations in tumors with intermediate stage and grade. Furthermore, graphical log-linear modeling was applied to analyze the dependence structure among these biomarkers and pathways.
cytoplasmic staining of VHL, PTEN, and p27 was also analyzed, resulting in a total of 18 molecular parameters. To avoid false positivity and because only one core with a diameter of 0.6 mm was analyzed from each tumor nuclear, cytoplasmic and membranous expression of a protein was defined positive if at least 5% of tumor cells showed weak or strong staining. Additionally, the immunoreactivity of membrane proteins was scored semiquantitatively as follows: 0, <5% positive tumor cells; 1+, 5% to 70% positive tumor cells; 2+, >70% positive tumor cells.

**Statistical analysis.** Contingency table analysis and $\chi^2$ tests were used for the analysis of the associations between protein expression patterns, tumor stage, and grade. Five-year survival rates were determined according to the Kaplan-Meier method and were analyzed for statistical differences using a log-rank test. SPSS 15.0 software was used for univariate calculations.

**Data description and preprocessing.** Five hundred and twenty-seven of 831 ccRCC (63.4%) had no missing values. For 87 cases, one measurement was missing; 64 and 30 cases had two or three missing values, respectively. One hundred and twenty-three cases contained more than three missing values and were not used in the statistical analysis. For observations with one, two, or three missing values, multiple imputation was applied. Further details are in the Supplementary Material S1.

**Recursive bootstrap elimination of variables in Cox regression.** To find molecular parameters and specific combinations that significantly influence survival, a Cox proportional hazard model was used to estimate a regression coefficient $\beta$ corresponding to the full model with all molecular parameters and one or two clinical variables (tumor grade and/or stage). Each component of $\beta$ corresponded to a molecular parameter. To assess its significance, a procedure called bootstrapping (22) was used to calculate $P$ values for the components of $\beta$.

**Log-linear and graphical model.** We used log-linear and graphical models to quantify the association between the molecular markers in a graph and to uncover possible dependence structures. The observations were cross-classified in a contingency table, thus expanding the probability of an observation falling into a certain cell by a log-linear model. The log-linear model was translated into a graphical model that consists of nodes and edges. The nodes represent the molecular markers and the edges connecting these nodes represent dependencies between these markers. A detailed description of these models is given in the Supplemental Material.

**Results**

**Protein expression patterns, tumor phenotype, and survival.** The number of interpretable cases decreased only slightly with the number of the TMA sections cut. Only between 5% (CCND1) and 15% (GLUT-1) of the 15 immunohistochemistry analyses were noninformative because of the absence of tissue on the TMA, or lack of unequivocal tumor cells in the arrayed sample. There was a significant relationship between strong staining and high percentage of positive tumor cells among all individual markers. We tested the influence of different staining intensities for each marker in Kaplan-Meier survival plots.

![Fig. 1. Schematic illustration of the VHL/PTEN network according to cell line and mouse models published in the literature. Proteins in dotted circles are not analyzed.](image-url)
No significant differences were found between weak and strong staining intensity (data not shown). Therefore, any nuclear or cytoplasmic positivity in ≥5% of the tumor cells was regarded as positive and combined in all statistical analyses.

Examples of ccRCC with strong nuclear (PAX2), membranous (CAIX), and combined nuclear/cytoplasmic (p27) positivity are shown in Supplementary Fig. S2. Univariate analyses showed that the expression of p27 and PAX2 decreased strikingly with advanced pT category and higher differentiation grade. In contrast, epithelial-specific periostin and p-S6 increased highly significantly with tumor stage and grade. The expression patterns of all four proteins were also highly associated with overall survival. Combined nuclear and cytoplasmic VHL as well as p-mTOR expression correlated inversely with tumor stage and grade but not with survival. CAIX, CD10, p21, and stromal-specific periostin positivity was associated with higher differentiation grade. From these, only CAIX correlated with survival. No significant associations were found between CCND1, E-CDH, epidermal growth factor receptor (EGFR), GLUT1, p53 and PTEN expression, and grade, stage, and survival. All correlations between protein expression and tumor phenotype or 5-year survival are shown in Table 2.

**Recursive bootstrap elimination.** As displayed in Fig. 2A, cytoplasmic PTEN, nuclear p27, p-S6 expression, and tumor grade were found to be significant in the survival analysis using the recursive bootstrap elimination in Cox regression. For a variable with a positive β regression coefficient, such as for grade 3 and p-S6 expression, the risk for patients is increased, whereas for grade 1, nuclear p27 and cytoplasmic PTEN positivity the risk is decreased. The same procedure was repeated for the clinical variable stage (Fig. 2B), and stage and grade (Fig. 2C). As shown in Fig. 2B, a higher risk is predicted for patients with late tumor stage, absence of nuclear p27, EGFR, CAIX, and expression of p-S6. A similar result was obtained with stage and grade (Fig. 2C).

Using the molecular parameters nuclear p27, cytoplasmic PTEN, and p-S6 for the subgroup of intermediate grade 2 ccRCC, we were able to classify the Kaplan-Meyer survival curve into two subpopulations (Fig. 3B). Compared with the overall estimated percentage of grade 2 patients (54%), 71% of the patients with a favorable biomarker constellation (PTEN and p27 expressed, p-S6

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**Table 1. Antibodies, conditions, and criteria used for the TMA analysis**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Antibody no.</th>
<th>Species</th>
<th>Supplier</th>
<th>Dilution</th>
<th>Treatment</th>
<th>Compartment</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
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<td>CAIX</td>
<td>M75</td>
<td>Mouse</td>
<td>J. Zavada</td>
<td>1:200</td>
<td>Bond-maX (Vision Biosystems)</td>
<td>Membrane</td>
<td>N &lt;5%; 5-70%; &gt;70% N, wk, str</td>
</tr>
<tr>
<td>CCND1</td>
<td>P2D11F11</td>
<td>Mouse</td>
<td>Ventana</td>
<td>Prediluted</td>
<td>BenchMark XT (Ventana)</td>
<td>Nucleus</td>
<td>N &lt;5%; wk, str ≥5%</td>
</tr>
<tr>
<td>CD10</td>
<td>NCL-CD10-270</td>
<td>Mouse</td>
<td>Novocastra</td>
<td>1:30</td>
<td>BenchMark XT</td>
<td>Membrane</td>
<td>N &lt;5%; 5-70%; &gt;70% N, wk, str</td>
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<tr>
<td>E-CDH</td>
<td>ECH-6</td>
<td>Mouse</td>
<td>Cell Marque</td>
<td>1:10</td>
<td>BenchMark XT</td>
<td>Membrane</td>
<td>N &lt;5%; 5-70%; &gt;70% N, wk, str</td>
</tr>
<tr>
<td>EGFR</td>
<td>3C6</td>
<td>Mouse</td>
<td>Ventana</td>
<td>Prediluted</td>
<td>UVView DAB</td>
<td>Membrane</td>
<td>N &lt;5%; 5-70%; &gt;70% N, wk, str</td>
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<td>AB1341</td>
<td>Rabbit</td>
<td>Chemicon</td>
<td>1:1,000</td>
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<td>Membrane</td>
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<td>sc-397</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>1:50</td>
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<td>Nucleus</td>
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</tr>
<tr>
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<td>sc-528</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>1:30</td>
<td>BenchMark XT</td>
<td>Nucleus</td>
<td>N &lt;5%; wk, str ≥5%</td>
</tr>
<tr>
<td>p53</td>
<td>DO-7</td>
<td>Mouse</td>
<td>DAKO</td>
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<td>Nucleus</td>
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<td>PAX2</td>
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<td>Zymed</td>
<td>1:50</td>
<td>Bond-maX</td>
<td>Nucleus</td>
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<td>Rabbit</td>
<td>BioVendor</td>
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<td>Tumor cells</td>
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</tr>
<tr>
<td>p-mTOR</td>
<td>49F9</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
<td>1:50</td>
<td>BenchMark XT</td>
<td>Cytoplasm</td>
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<td>p-S6</td>
<td>2215</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
<td>1:50</td>
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<td>6H2.1</td>
<td>Mouse</td>
<td>Cascade</td>
<td>1:50</td>
<td>Citrate buffer; pH 6.4; 10°, 110°C</td>
<td>Nucleus</td>
<td>N &lt;5%; wk, str ≥5%</td>
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<tr>
<td>VHL</td>
<td></td>
<td>Rabbit</td>
<td>W. Krek</td>
<td>1:50</td>
<td>Bond-maX</td>
<td>Nucleus</td>
<td>N &lt;5%; wk, str ≥5%</td>
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**Abbreviations:** N, negative; wk, weak; str, strong.
Table 2. Protein expression, tumor phenotype, and 5-y survival

<table>
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<th>Protein</th>
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<td></td>
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<td>pT3/4</td>
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<td>771</td>
<td>73 (20.3)</td>
<td>74 (18)</td>
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<td>Pos.</td>
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<td>287 (79.7)</td>
<td>337 (82)</td>
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<tr>
<td>CCND1</td>
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<td>93 (25.1)</td>
<td>125 (29.8)</td>
<td>NS</td>
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<td>295 (70.2)</td>
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</tr>
<tr>
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<td>49 (14.3)</td>
<td>58 (15.4)</td>
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<td></td>
<td>Pos.</td>
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<td>293 (85.7)</td>
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<td>Pos.</td>
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<tr>
<td>EGFR</td>
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<td>49 (13)</td>
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<td>GLUT-1</td>
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<td>93 (27.8)</td>
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<td>37 (10.3)</td>
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<tr>
<td></td>
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<td>31 (8.6)</td>
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<td>163 (41)</td>
<td></td>
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<td>73 (20.4)</td>
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<td>73 (20.2)</td>
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<td></td>
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<td>11 (3)</td>
<td>9 (2.2)</td>
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<tr>
<td></td>
<td>Neg./pos.</td>
<td></td>
<td>216 (59.7)</td>
<td>268 (66.3)</td>
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<td>62 (17.1)</td>
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<td></td>
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</tr>
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</table>

Abbreviations: Pos, positive; NS, not significant; nuc./cyt, nucleus/cytoplasm; Tu, tumor cells; St, stromal cells.

not expressed), but only 49% of the patients with the remaining molecular parameter combinations, survive 5 years. As the same data were used for estimating and evaluating the model fit, the difference of 22% is not an unbiased estimate. A cross-validation study was conducted to obtain an unbiased estimate for the difference of 5-year survival from the favorable and the remaining grade 2 patients. For this purpose, the data set was randomly divided into two parts consisting of two thirds and one third of the observations. Two thirds were used to fit the model, i.e., the whole bootstrap backward elimination procedure was applied starting from the full model and ending in a final model including only significant variables. The remaining data were used to evaluate the model. This procedure was repeated 10 times. The cross-validation analysis revealed an unbiased estimated difference in 5-year survival of 15%.

The same procedure was repeated for tumor stages pT2, pT3, and all molecular markers (Fig. 3D). According to Fig. 2B, the relevant markers were nuclear p27, CAIX,
p-S6, and EGFR. With too few pT2 and pT3 tumors having the favorable combination (p27, CAIX, and EGFR positive, and p-S6 negative), no statistically significant subdivision into a favorable and a nonfavorable subgroup was obtained. However, the inclusion of only nuclear p27 and CAIX resulted in a balanced classification into a favorable and nonfavorable combination. Each of the groups of pT2 and pT3 tumors was divided into two curves. The upper curves belong to ccRCC with positive nuclear p27 and CAIX, and the lower curves belong to the remaining combinations. The 5-year survival difference was 73.3% versus 51.4% for pT2 tumors and 51.3% versus 39.0% for pT3 tumors for the favorable and the nonfavorable combination, respectively. The cross-validated difference was estimated to be 11% and 3% for pT2 and pT3 tumors, with the restriction that the favorable constellation has to consist of at least 20 cases.

**Graphical log-linear model.** The graphical model associated with the estimated main effects and interactions vector $\beta$ is displayed in Fig. 4. The edges colored in red and blue represent a negative and a positive association between the molecular parameters. The width of an edge between two nodes corresponds to the absolute value of the corresponding estimated log-linear interaction.
tion coefficient and can be interpreted as a measure of strength of dependence. This figure shows that the molecular parameters chosen for modeling survival times, nuclear p27, p-S6, cytoplasmic PTEN, and CAIX are not directly linked to each other. There is no short path with thick edges from one of these parameters to the other, implying that conditionally independent variables have been selected for the survival analysis. On the other hand, the most striking dependencies exist between cytoplasmic and nuclear p27, PAX2, and p21, as well as PAX2 and p-mTOR. Somewhat weaker dependencies are seen between cytoplasmic and nuclear PTEN, cytoplasmic PTEN and VHL, PAX2 and CCND1, PAX2 and nuclear PTEN, HIF targets CAIX and GLUT1, but also between GLUT1 and CD10, and CAIX and E-Cadherin (inverse). Notably, no obvious link is shown between VHL and HIF targets and only weak dependencies are yielded between PTEN, mTOR, and p-S6. Mathematically, we cannot directly conclude a causal relationship between the biomarkers, but we can deduce a dependence that might point to a relationship where one biomarker directly influences the other.

Discussion

In previous studies, the prognostic value of numerous tumor markers belonging to the VHL- and PTEN-dependent pathways was evaluated in ccRCC. In almost all of these studies, the expression of one or only a few proteins was analyzed in a rather limited number of cases. As a consequence, the possible interrelations of different molecular markers and pathways influenced by VHL and PTEN on clinicopathologic parameters have hardly been considered thus far. In an attempt to address this problem, we analyzed the expression data of 15 functionally well-characterized proteins on a large number of arrayed ccRCC specimens using advanced statistical modeling.

The expression frequencies and associations with clinicopathologic parameters obtained from our TMA analyses were predominantly concordant with data from the literature, a prerequisite to reliably perform mathematical modeling. Our TMA results provide further evidence that tiny amounts of tissue material are sufficient to evaluate the expression of immunostained proteins if high numbers of tumors are analyzed. Discrepant results were obtained only for EGFR,

Fig. 2. Recursive bootstrap procedures for grade (A), stage (B), grade/stage (C), and molecular markers. Markers with negative coefficients imply better prognosis; markers with positive coefficients indicate worse prognosis about survival. The lines correspond to the estimated 95% bootstrap confidence intervals for these coefficients (based on the model with the selected variables).
p-mTOR, and PAX2 for which contradictory data exists in the literature (23–26). This problem may be explained by the use of different antibodies, protocols, and/or the relative low numbers of tumors analyzed in previous studies. No published data were found for p21 expression in ccRCC. The frequency of p21-positive tumors was low (23%) and only slightly higher than that observed for p53-expressing ccRCC (20%). Compared with early-stage tumors, the fraction of p21-positive ccRCC decreased significantly in late-stage tumors, suggesting that loss of p21 expression is critical for aggressive tumor behavior. The finding that epithelial rather than stromal-specific expression of periostin correlated highly significantly with nuclear differentiation grade, tumor stage, and survival is novel and indicates the importance of this protein for metastatic processes as it has been described for breast and lung cancer (27, 28).

As an extension of a Cox proportional-hazard model, which is commonly used to analyze the effect of risk factors on survival, a recursive bootstrap approach was used for selecting important molecular parameters. Recursive bootstrapping with TMA expression data from 15 proteins enabled us to refine tumor grading and staging. ccRCC of

Fig. 3. Association of tumor grade and stage with the survival of patients in ccRCC (A and C, respectively). Separation of patients with intermediate grade and stage tumors into lower and higher risk subsets (B and D). The upper curve for grade 2 tumors represents patients with positive PTEN (cytoplasmic), positive p27 (nuclear) and negative p-S6 ccRCC (B). The upper curves for pT2 and pT3 represent patients with positive p27 (nuclear) and CAIX ccRCC. Dotted vertical line, the 5-y survival (B).
histologic grade 2 with nuclear p27, cytoplasmic PTEN, and inactive, nonphosphorylated S6 had an outcome that was nearly comparable with that of grade 1 tumors (80% versus 71%). In contrast, the 5-year survival rate for the remaining grade 2 tumors was below 50%. The association between favorable outcome and combined expression of p27 and PTEN suggests that the inactivation of both proteins is a critical step toward tumor progression.

The absence of p27 and PTEN was also described for most advanced prostate cancers (29, 30). In mice, it was shown that concomitant inactivation of PTEN and p27 are required to accelerate spontaneous neoplastic transformation (31). Therefore, it was concluded that the combined tumor-suppressive activity of PTEN and p27 is crucial for the control of cell cycle progression. Our data imply a mechanism for ccRCC progression that is similar to that described in prostate cancer. The identification of phosphorylated ribosomal protein S6, a downstream target of activated mTOR, as a potential risk factor is highly likely a consequence of PTEN and p27 loss and confirms the importance of an activated AKT/mTOR pathway in a considerable subset of ccRCC patients.

In contrast to prostate cancer in which p27 expression seems to be dependent on PTEN activity (31), our estimated graphical model shows only a weak association. Furthermore, the significant association between late-stage, high grade tumors and absence of nuclear p27 expression suggests that the downregulation of p27 rather than the loss of PTEN probably has more severe consequences for the patient’s survival. It also indicates that p27 expression

![Fig. 4. Estimated graphical log-linear model for 15 analyzed proteins including nuclear and cytoplasmic expression of PTEN, p27, and VHL (18 molecular parameters). Blue edge, positive association; red edge, negative association between molecular parameters.](image-url)
may only be partially dependent on the functional integrity of PTEN. Blocking the NOTCH1 receptor, a key player of the Notch signaling pathway in human ccRCC cell lines, was accompanied by elevated levels of p27 and p21 (32). Thus, for knocking down p27, the synergized activation of both the NOTCH1 and PI3K/AKT pathways may be necessary to enhance ccRCC progression.

As the cyclin-dependent kinase inhibitor p27 exhibits its function in the cell nucleus, recent immunochemical studies of ccRCC tissue have focused primarily on nuclear p27 expression (33–35). It is known that ubiquitylation and degradation (36) as well as sequestration (15) of p27 occurs mainly in the cytoplasm. Our graphical log-linear model shows a tight association between nuclear and cytoplasmic expression of p27. As a consequence, we speculated that both the nuclear and cytoplasmic presence is required for nuclear-cytoplasmic trafficking, thus ensuring proper functioning of p27. In fact, the number of tumors that were p27 negative in one or both cellular compartments markedly increased with tumor stage and grade (Table 2) and also had a worse outcome (data not shown). These results imply that alternation of subcellular p27 trafficking is of potential relevance for the biological behavior of ccRCC.

CAIX is upregulated in the vast majority of ccRCC (37–39). Notably, the expression of HIF targets CAIX and GLUT1 correlated well with CD10 positivity and suggests that CD10 might also be regulated by HIF. The role of CAIX as prognostic marker for ccRCC is contradictory. Here, we show that patients with positive CAIX tumors had a better outcome compared with those who were CAIX negative. Irrespective of the cutoff value used, our results were highly comparable with those in which large sections of 730 ccRCC were analyzed (37). Although loss of the VHL function considerably supports HIF-mediated transcription of CAIX, it is unclear why in metastasizing ccRCC CAIX is increasingly absent. Carbonic anhydrases, such as CAIX, catalyze intracellular carbon dioxide into carbonic acid outside the cells, which importantly contributes to the acidic microenvironment in tumors (40). Based on a previous study that showed considerable genetic heterogeneity between organ-confined and metastatic RCC (41), it is tempting to speculate that in advanced tumors, HIF-independent carbonic anhydrases other than CAIX become activated.

Strong associations were observed between PAX2, activated mTOR, p21, CCND1, and nuclear PTEN. PAX2 belongs to the PAX gene family of developmental transcription factors. PAX2 is absent in normal renal tubular epithelial cells but re-expressed in many ccRCC (42). A recent study showed that both loss of VHL and hypoxia are important for the upregulation of PAX2 in a significant subset of ccRCC (43). Interestingly, the strong link to the HIF-2α target CCND1 (44) in our graphical linear model may imply a similar regulatory mechanism for PAX2. In humans with kidney malformations, PAX2 is highly expressed in cystic and hyperproliferative epithelial cells (45). Cysts also occur frequently in hereditary and sporadic ccRCC, which are thought to be a result of uncontrolled cell proliferation and represent tumor precursor lesions (46). The ability to transcriptionally repress the tumor suppressor p53 (47) and its joint action with CCND1 and mTOR makes PAX2 a possibly important promoter of cyst formation in ccRCC. A potential interaction between PAX2 and nuclear PTEN still remains to be elucidated.

In summary, the use of recursive bootstrap elimination as well as graphical log-linear modeling is ideally suited for analyzing comprehensive TMA data. Here, we show that ccRCC progression is probably the result of a sequential switch-off of different tumor suppressive programs beginning with VHL and followed by TET and p27. Our data also indicate that in subsets of ccRCC HIF, PI3K-AKT-mTOR, and PAX2-dependent pathways are activated in a separate or concerted manner. This implies that components of these pathways should be considered as predictive parameters before treating patients with advanced ccRCC with kinase inhibitors sunitinib malate, sorafenib or temsirolimus or CAIX antibodies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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Mining Tissue Microarray Data to Uncover Combinations of Biomarker Expression Patterns that Improve Intermediate Staging and Grading of Clear Cell Renal Cell Cancer

Corinne Dahinden, Barbara Ingold, Peter Wild, et al.


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