Integrating translational research and clinical practice to refine risk stratification in renal cell carcinoma

Summary

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Renal Cell Carcinoma (RCC) is the most prevalent malignancy of the kidney, accounting for >90%kidney tumors in adults and 2-3% of all adult malignancies. The incidence of RCC varies across different geographic regions and demographic groups. The prevalence of RCC has been steadily rising over the past few decades, attributed to enhanced diagnostic capabilities, increased awareness, and risk factors such as smoking, obesity, and hypertension. Most RCC cases are diagnosed between the ages of 50 and 70, with a predominance in males compared to females. Although the incidence rate of RCC has been increasing, recent literature showed that survival rates of RCC have improved significantly over the past three decades. RCC survival is highly dependent on the stage at diagnosis, early cases (TNM stage I) have a favorable prognosis with a 5-year survival of >90% while patients with metastatic disease have 5- year survival rates of approximately 12%. As RCC rarely presents with clinical symptoms, 20% of the patients are diagnosed with an advanced disease stage and over one third will develop metastasis after complete resection of primary tumor. Surgical resection remains the primary treatment for localized disease, with nephron-sparing approaches increasingly favored to preserve renal function whenever feasible. For advanced or metastatic RCC, therapeutic strategies have evolved significantly in recent years, driven by the emergence of molecular-targeted therapies, immunotherapy, and combination regimens. Targeted agents such as tyrosine kinase inhibitors and immune checkpoint inhibitors have demonstrated efficacy in improving progression-free survival and overall survival outcomes for specific patient cohorts.

The prognosis of RCC is influenced by several histopathological and clinical factors. Clear cell renal cell carcinoma (ccRCC), the most common histological subtype, typically carries a less favorable prognosis compared to papillary or chromophobe subtypes. Additionally, tumor size, lymph node involvement, distant metastasis, and tumor invasion significantly impact the prognosis of RCC patients. Several prognostic models have been developed to stratify patients with RCC based on prognostic factors. The most widely used models include the TNM staging system developed by the American Joint Committee on Cancer (AJCC), the Memorial Sloan Kettering Cancer Center (MSKCC) risk model, The UCLA integrated staging system (UISS) and The tumor stage, size, grade, and necrosis (SSIGN) score. However, these models are still suboptimal and have difficulties predicting individual patient outcomes. Molecular markers in general, and DNA methylation markers in particular, hold promise in refining these risk stratification models, although validation and standardization remain ongoing challenges in the field. Therefore, we aimed in this thesis to improve and validate prognostic models for risk stratification in RCC.

In **chapter 2**, we performed a head-to-head comparison of previously published DNA methylation markers and proposed a potential prognostic model for non-metastatic ccRCC. DNA methylation of several genes was evaluated (i.e. *PCDH8*, *BNC1*, *SCUBE3*, *GREM1*, *LAD1*, *NEFH*, *RASSF1A*, *GATA5*, *SFRP1*, *CDO1*, and *NEURL*) using nested methylation-specific PCR. We relied on a previously published approach by our group (Koch *et al*) to identify the most clinically relevant methylated regions of the candidate genes using data from The Cancer Genome Atlas (TCGA) to guide primer design. Methylation of *GREM1*, *GATA5*, *LAD1*, *NEFH*, *NEURL* and *SFRP1* was associated with poor ccRCC-specific survival, independent of established prognostic clinicopathological factors such as age, sex, tumor size, TNM stage or tumor grade. A prognostic biomarker model incorporating methylation of *GREM1*, *GATA5*, *LAD1*, *NEFH*, and *NEURL* along with the clinicopathological characteristics outperformed a model with solely clinicopathological factors in both the population-based series of the Netherlands Cohort Study on Diet and Cancer (NLCS) and a validation population derived from the University Hospital of Leuven. However, the addition of the methylation markers to the model including only standard prognostic standards provided limited incremental prognostic value in the TCGA series This study highlights the potential prognostic value of methylation markers in ccRCC and emphasizes the importance of optimizing primer design for accurate DNA methylation measurement.

In **chapter 3** of this thesis, we expanded upon two prior systematic reviews on prognostic DNA methylation markers for RCC. We performed a systematic literature search from March 2017 to December 2021, including PubMed, EMBASE and Web of Science, thereby including the most recent research advancements since the two prior systematic reviews. We evaluated 58 studies using standardized data extraction, the Reporting Recommendations for Tumour Marker Prognostic Studies (REMARK) criteria and the level of evidence (LOE). We identified eleven promising single DNA methylation markers, three multimarker panels, and five DNA methylation signatures. However, despite clear recommendations in the previous systematic reviews, we only found a limited number of validation studies for previously recommended markers, highlighting the persistent lack of adequate validation in this field.

Several methods are used to detect DNA methylation, each method with its specific characteristics and limitations. However, the type of assay used can impact the detection, quantification and interpretation of DNA methylation marker results and can affect comparability and reproducibility of findings between studies. Therefore, selecting the most appropriate assay type for the specific research question is a crucial step in the biomarker development process. In **chapter 4**, we emphasize this importance and investigated how three commonly used PCR variants (i.e. nested MSP, quantitative MSP

(qMSP), and nested quantitative MSP (nested qMSP)) would influence the prognostic performance of the five DNA methylation markers (*NEFH*, *GATA5*, *GREM1*, *NEURL* and *LAD1*) from our previous model using fresh frozen (FF) and formalin-fixed paraffin-embedded (FFPE) tissue samples from RCC patients derived from Radboud University. Our results indicated that methylation percentages differed between the MSP variants, with nested MSP yielding the highest percentages for most genes. Sample type also influenced methylation percentages, with more discrepancies observed in FFPE samples compared to FF tissue. Moreover, the choice of assay influenced the association with survival, with all markers showing an association with overall survival when qMSP was used, while none showed this association with nested MSP. On the other hand, with the nested qMSP assay, conclusions on prognostic value based on the Kaplan-Meier analyses were mixed, as *NEURL* and *LAD1* were associated with overall survival. This study demonstrates the critical role of MSP variant selection in DNA methylation biomarker research. Furthermore, it highlights the importance of carefully considering assay choice upfront, considering the research question and sample type, to ensure valid conclusions regarding prognostic biomarker potential.

In light of our growing understanding of RCC and the recent changes in the RCC landscape, as addressed in the 2022 ISUP (International Society of Urological Pathology) consensus for RCC, we re-evaluated in **chapter 5** 457 RCC cases from the NLCS series according to the 2022 ISUP standards and World Health Organization (WHO) classifications to identify newly recognized subtypes of RCC and assess several new prognostic factors such as lymphovascular invasion and sarcomatoid features. The NLCS cases were initially evaluated according to the 2004 WHO classification, the Fuhrman grading system, and the TNM 3rd version. Here, we graded the NLCS cases according to the latest ISUP grading and the Tumor Node Metastasis (TNM) 8th edition. For this, we used data from the Netherlands Cancer Registry and the Pathologisch Anatomisch Landelijk Geautomatiseerd Archief, digital slides from these RCC cases were reviewed by two independent urogenital pathologists. After re-evaluation, we could not identify any of the new subtypes proposed in the ISUP consensus and there were no discrepancies with previous diagnoses. The comparison of WHO/ISUP grading with the original Fuhrman grading showed similarities in almost 50% of the cases. The staging according to the TNM classification 8th edition led to restaging in part of the cases (65.5%). and we further revealed the presence of lymphovascular invasion, tumor necrosis, sarcomatoid differentiation, and rhabdoid differentiation. Even though no new RCC entities were identified in our population, the study demonstrated the feasibility of assessing new prognostic features in an older sample collection. Besides, the study highlights the importance of updating existing data to facilitate up to date comparisons between biomarker studies. This is, to the best of our knowledge, the first study that reports such

a re-evaluation on a large, unselected population-based series of RCCs with extensive clinical data.

With the updated data from chapter 5 and the prognostic model from **chapter 2**, we updated our developed prognostic model for ccRCC in **chapter 6** by incorporating the new ISUP/ WHO standards, TNM 8th edition and the novel prognostic factors. All evaluated prognostic factors, i.e. ISUP grading, TNM 8th edition staging, necrosis, LVI, sarcomatoid differentiation, and rhabdoid differentiation, were statistically significantly associated with cause-specific survival. The clinical model with the ISUP and TNM 8th edition performed similar compared to the model using the Fuhrman grading and the 3rd TNM version. Adding the five DNA methylation markers to the clinical model with ISUP and TNM 8th edition slightly improved the model thereby highlighting the potential importance of molecular markers. The addition of necrosis and LVI did not further improve the results.

The main findings of the studies performed are summarized and discussed in **chapter 7**. We identified in this thesis methylation markers that could potentially enhance the prognostic performance of current clinical models. We also provided a comprehensive overview of the latest evidence in this field and touched upon multiple reasons why promising biomarkers could not be validated in independent studies and translated into clinical practice. Moreover, we discussed the value of updating and using existing data as it could lead to more comprehensive insights that are needed in the case of biological complex diseases such as RCC. Lastly, we emphasized the value of strong collaboration between clinicians and researchers, as this ensures that research questions adequately address emerging clinical complexities, and that scientific research matches with the evolving needs of patients and healthcare practitioners.