Translation of DNA methylation markers for the early detection of renal cell cancer

SUMMARY

Renal cell carcinoma (RCC) is the most common type (90-95%) of kidney cancer, representing 2.2% of all diagnosed cancers globally. As early diagnosis is associated with favorable prognosis and low disease burden, it has become a main focus in cancer management. Because of the increasing use of cross-sectional imaging techniques, renal masses are also frequently detected coincidently during unrelated procedures; these masses are called incidentalomas and can be either benign or malignant. Partly due to this increase in incidental detection of renal masses, the incidence of RCC has been rising over the past decades. Large renal masses are often correctly diagnosed as being malignant RCCs, however only 50-70% of the small renal masses (SRM) <4 cm in diameter can be adequately diagnosed as being benign or malignant based on these imaging procedures. The increasing amount of detected SRMs and the challenge to diagnose these masses accurately based on imaging emphasizes the room for improvement in diagnosing early-stage RCC. Consequently, molecular markers for the early detection of RCC gained interest over the past years.

Despite the great interest in molecular markers, not a single molecular marker for diagnostic purposes has reached clinical care for RCC yet. Previously, it was described that less than 1% of all published biomarkers has successfully been implemented into clinical care, which also holds true for DNA methylation cancer biomarkers. The fact that the vast majority of research activities do not lead to clinical translation indicates that a large proportion of research investments are wasted.

The **aim** of this thesis was to identify and evaluate the utility of DNA methylation markers for the non-invasive early diagnosis of RCC. In addition, we evaluated reasons for the lack of clinical translation of diagnostic DNA methylation markers and discussed how to overcome these.

In **Chapter 2**, we systematically reviewed and summarized all literature regarding diagnostic DNA methylation biomarkers for RCC. We provided an overview of these biomarkers and summarized their current Level of Evidence (LoE). We found that 44 DNA methylation biomarkers and 11 multi-marker panels were described for diagnostic purposes in RCC; however, only 15 of these biomarkers were independently validated. None of the reported biomarkers exceeded LoE III, indicating that these biomarkers have limited clinical relevance at this moment. After initial publication, subsequent studies often do not considerably add to the LoE, indicating inadequate study design to facilitate validation. Additionally, we identified multiple issues that may hamper increasing LoE and thus clinical translation of these biomarkers, including methodological and technical heterogeneity between studies. Moreover, by evaluating The Standards for Reporting Diagnostic Accuracy criteria, we identified that study reproducibility and further development of these biomarkers is greatly hampered by inadequate reporting.

After identifying the issues described in **Chapter 2**, we further evaluated several technical considerations in PCR-based assay design for diagnostic DNA methylation markers in-depth in **Chapter 3**. As it was previously described that the exact genomic location of an assay could influence the biomarkers' diagnostic performance, we studied these genomic locations of all included biomarkers. We identified the optimal genomic locations of the studied biomarkers according to a previously proposed method utilizing TCGA data and compared that to the genomic locations used in the individual studies. The limited diagnostic performance of the included biomarkers might have partially been caused by the fact that the majority of the studied assays did not include an extracted optimal location. In addition, we assessed the primer and probe quality of all assays according to criteria based on both literature and expert opinion. Even though the most important criteria that allow discrimination of methylated from unmethylated DNA were covered in most assays, there is room for improvement in primer- and probe design. Therefore, we assembled a set of guidelines on how to adequately design PCR-based DNA methylation assays for diagnostic cancer biomarkers.

The availability of large study cohorts of appropriate samples, complemented by extensive and well-annotated clinical and pathological patient data is crucial for adequate and relatively fast validation of biomarkers. With increasing interest in liquid biopsies, efforts to establish large liquid biopsy biobanks are being made. Therefore, in **Chapter 4** we elaborated on considerations for establishing new biobanks, as well as for using existing biobanks, both in general and specific for certain specimen types, in order to develop optimal conditions for future validation of diagnostic cancer biomarkers in liquid biopsies.

Taking together all findings and recommendations made in **Chapter 2, 3,** and **4**, we used a novel *in silico* approach to identify diagnostic DNA methylation markers for RCC, and evaluated their diagnostic potential in both tissue and urine samples in **Chapter 5**. After evaluating nine DNA methylation markers in RCC and normal kidney tissue samples, the six most promising biomarkers were selected for further evaluation in urine samples. After evaluating these markers in RCC patients' and healthy persons urine, the final diagnostic model consisted of 4 biomarkers (*Gene 1, Gene 2, Gene 5* and *Gene 6*), sex and age, with an optimism-corrected AUC of 0.84. This DNA methylation marker panel for diagnosing RCC in urine showed to be a robust model in the sample set studied. Therefore, it serves as a promising starting point for further validation and extension by addition of other types of biomarkers, to further improve this model.

In addition to the previously discussed technical and methodological issues in biomarker research, the choice of appropriate control samples is an often overlooked factor. The fact that normally appearing tissues adjacent to the tumor might be molecularly predisposed to become malignant (called a field effect), emphasizes the importance of carefully selecting appropriate control tissues in biomarker studies. Nevertheless,

normally appearing tissue adjacent to the tumor is frequently used as control tissue in biomarker studies. In **Chapter 6**, we therefore evaluated DNA methylation alterations in ascending distances from the malignant RCCs, and demonstrated the impact of these alterations on biomarker identification and development. High methylation rates were present in RCC tissues (ranging from 34-56%) and matched adjacent normal (AN) tissues (ranging from 13-59%) as compared to NK (2% in all genes). All AN samples (100%) that were methylated for *Gene 4* corresponded to methylation in the RCC, whereas this holds true for only 0-55.6% of the other studied genes. A gradual decline in the percentage of methylated reference (%PMR) was found when moving from the tumor (41.4%) towards the area furthest away from the tumor (0.2%). Analyzing biomarker performance using AN rather than NK as control tissues yielded different cutoffs for test positivity. When using AN to determine the cutoff for test positivity, the sensitivity of the biomarker decreased due to presence of methylation in the AN tissue. The sensitivity increased when using NK tissue, as the absence of methylation in this tissue led to a different cutoff for test positivity. This indicates the importance of using appropriate control tissue when evaluating diagnostic performance of a biomarker. Although these different methods do not change the biomarkers' actual performance, using NK as control tissues might be most representative of the true biomarker performance.

In the general discussion of **Chapter 7**, the findings from this thesis were summarized and reflected upon. Along with discussing several reasons for the lack of clinical translation of DNA methylation biomarkers, we reflected on biomarker research in general. Several pitfalls have been identified and acknowledged in biomarkers research, and we here proposed recommendations to overcome these problems in DNA methylation based biomarkers. With plenty of research advocating a change in biomarker research in order to decrease research waste, and little impact so far, it is now time to constructively change the (biomarker) research environment and mentality. Co-operation of all involved parties to create a quality-based, rather than a quantity-based, research environment will eventually contribute to reducing research waste. Taken together, we have provided future perspectives and recommendations relevant to the development of clinically useful diagnostic DNA methylation biomarkers for cancer management.