DNA methylation markers for early detection of colorectal cancer

SUMMARY

Colorectal cancer (CRC) is the third most common form of cancer in the world and ranks as the second leading cause of cancer in western countries, with 1.8 million new cases of CRC diagnosed and nearly 861,000 deaths attributed to this disease in 2018. The prognosis of a CRC patient is strongly associated with the disease stage at initial diagnosis. The 5-year survival rate in patients with early stage CRC is 90%, however detection of CRC at an early stage can be challenging, because the initial phase of the disease is often asymptomatic. Therefore, secondary prevention is essential to reducing its incidence, morbidity and mortality. CRC screening, using endoscopy-based imaging techniques (e.g. colonoscopy and flexible sigmoidoscopy) and fecal blood tests (e.g. Guaiac-based fecal occult blood testing (gFOBT) and fecal immunochemical test (FIT)), has been shown to decrease CRC incidence and mortality.

Colonoscopy, which is regarded as the gold standard for CRC screening, shows high sensitivity and specificity for detecting adenomas and CRC. However, this invasive method is associated with generally low patient participation rates and needs to be performed by well-trained endoscopists. Fecal blood tests, although less sensitive compared to colonoscopy, are non-invasive, easy to implement, consumer-friendly and cost-effective. Although testing the presence of hemoglobin in stool is a cost-effective strategy to screen for CRC in a population-based setting, we hypothesized that the test performance of a non-invasive test for early detection of CRC can be improved by adding molecular markers. Cancer DNA methylation markers are interesting markers to study because they occur frequently in all disease stages in CRC and can be detected easily using PCR- or sequencing based technology.

In this thesis, we aimed to 1) assess the problems in CRC DNA methylation biomarker research; 2) to identify and evaluate novel DNA methylation markers for non-invasive detection of CRC; and 3) to investigate novel human DNA extraction methods from stool samples to improve the analytical sensitivity of DNA methylation markers.

Prior to performing a new DNA methylation marker identification study, we conducted a systematic literature search on CRC DNA methylation markers and investigated the translational efficiency of CRC DNA methylation markers (**Chapter 2**). We observed that only three of 389 markers (0.8%) have been successfully translated into a clinically used test. We identified multiple issues (e.g. methodological and technical heterogeneity and lack of validation or clinical translation) that hinder the identification and validation of DNA methylation biomarkers for CRC diagnosis. To improve the situation, we summarized major requirements for the development of clinically relevant diagnostic CRC DNA methylation markers: 1) to enhance translational value of biomarker development in CRC diagnosis by improving the LoE of a candidate biomarker; 2) to ensure clinical relevance of biomarker research in CRC by defining the intended use of the biomarker and comparing the candidate marker with existing diagnostic measures (e.g. FIT); and 3) to improve reporting quality

for DNA methylation biomarkers in CRC by adhering to existing guidelines such as STARD. Nevertheless, to improve the reporting quality of biomarker studies, existing guidelines might need some adaptations to specifically address requirements essential for biomarker studies. In the end of the study, we provided recommendations for planning future clinical research to avoid research waste.

Aside from the technical and methodological problems mentioned in **Chapter 2**, genomic location of DNA methylation also plays an important role in optimal biomarker development and the clinical translatability of cancer biomarkers. In **Chapter 3**, we investigated the role of the genomic location of various published cancer DNA methylation biomarkers. Our study demonstrates that when developing promoter CpG island methylation markers with clinical application, information on gene expression can be a guide for identification of the optimal genomic region. Using data from the publicly available The Cancer Genome Atlas (TCGA) database, we validated this hypothesis by assessing the genomic regions of 14 DNA methylation biomarkers that are used in eight commercialized cancer tests. We confirmed that changes in DNA methylation in the promoter region of these markers are correlated with gene expression alterations in the tumor tissues of the cancer patients, indicating that our strategy could contribute to the discovery of more clinically relevant DNA methylation markers for cancer. Furthermore, our study emphasized the value of publicly available databases in facilitating cancer biomarker development. Finally, we provided recommendations for design of future DNA methylation marker studies, which may further enhance the clinical translation of DNA methylation markers for cancer diagnosis.

Based on the findings and recommendations of **Chapter 2** and **3**, we set out to identify novel DNA methylation markers for early detection of CRC. We identified novel *NDRG4* complementary DNA methylation markers by conducting an *in silico* marker discovery (**Chapter 4**). *In silico* comparison of publicly available TCGA data was performed between tumor and normal tissues. Genes that were frequently hypermethylated and down-regulated in tumors, in particular in samples that lacked *NDRG4* methylation were selected. In total, five DNA methylation markers were identified by our *in silico* approach. The methylation status of these marker candidates was examined in CRC and matching non-tumor tissue from the same patients (*ADHFE1*, *UNC5C*, *GFRA1*, *LONRF2* and *ITGA4*). The qMSP results showed that at 96% specificity, a three-marker panel (*NDRG4*, *ITGA4* and *LONRF2*) achieved 60.47% sensitivity in stool samples from patients with CRC or healthy controls. Combining this three-marker panel with FIT50 suggested that these DNA methylation markers might add some diagnostic value to FIT (sensitivity 86.05% and specificity 94%). Even though our current study is small, these results warrant a prospective study combining this DNA methylation marker panel and FIT50 to further study the potential value of these markers in the early detection of CRC.

A prerequisite for optimal detection of biomarkers in stool is efficient isolation of the minute amounts of human DNA from stool samples. To address this issue, we evaluated whether the methylation-on-beads (MOB) technique that has been developed to increase the genomic DNA yield from human plasma and sputum is applicable on stool samples (**Chapter 5**). The reagents of two stool DNA isolation kits that are commercially available were used for the development of the stool MOB assay. In addition, we introduced a pre-cleaning step prior to the isolation procedure in order to decrease the co-extraction of PCR inhibitors from the stool samples. Next, we compared the performances of these two MOB methods with three commercialized stool DNA isolation kits using the stools of patients with CRC or adenoma and healthy subjects. Unfortunately, neither of the two developed MOB approaches was able to increase the yield of human DNA from stool samples, probably because of the non-specific binding of the PCR inhibitors and bacterial DNA to the silica superparamagnetic beads used as DNA carriers in the MOB protocol. However, the added pre-cleaning step increased the total DNA purity, human DNA yield and the PCR efficiency compared to uncleaned stool samples. These results indicate that the yield of human DNA from stool samples can be increased by incorporating a pre-cleaning step in the isolation procedure, and thereby contributing by improving the analytical sensitivity of DNA methylation markers.

Next to the methodological, technical and clinical issues raised in **Chapters 2**, **3**, **4** and **5**, in **Chapter 6** additional issues that are also essential for an eventual translation of biomarkers into clinical practice have been discussed. As the performance of biomarker studies is time-consuming and costly, the performance of meta-research can be very valuable in this field, since meta-analysis may provide a more precise estimation of the performance of a biomarker. Such analysis combines multiple studies on the same biomarker and therefore can offer a more feasible alternative to improve the LoE. Moreover, obstacles lying in transferring academic knowledge/intellectual property to commercial partners for further biomarker development also have a negative impact on overall biomarker development. Although it is undeniably important for scientists to be always academically independent, lack of collaboration between academic researchers and commercial partners will prevent efficient development of new biomarkers and hinder translation to the clinic. We herein posit a transparent collaboration between university and industry can promote academic contribution to open science. Altogether, this thesis debates about the pitfalls and difficulties in the current CRC biomarker discovery and translation and proposes suggestions that may accelerate clinical translation of DNA methylation marker for CRC detection.