DNA methylation markers for early detection of colorectal cancer

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Summary
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Colorectal cancer (CRC) is one of the most preventable cancers in the world, with over 1.2 million newly diagnosed patients annually. Secondary prevention of CRC, by screening for early disease and precursor lesions, reduces incidence, mortality, morbidity and treatment costs. Over the past two decades, many countries have implemented screening for CRC. Imaging techniques such as flexible sigmoidoscopy, colonoscopy, and CT-colonography and non-invasive stool tests such as guaiac fecal occult blood test (gFOBT) and fecal immunochemical test (FIT) are being used as screening modalities for CRC. Although the sensitivity and specificity of imaging techniques is high, drawbacks are the high costs, complication risks, patient discomfort and requirement of experienced endoscopists. While gFOBT/FIT tests are noninvasive, cheap and easy to use, sensitivity rates for detecting CRCs and advanced lesions are inferior compared to colonoscopy. Additionally, these tests have been shown to obtain high false positive rates, together underscoring the need of sensitive and specific molecular marker tests for early detection of CRC as described in chapter 1.

The aim of this thesis was to evaluate novel, sensitive and specific promoter methylation markers for noninvasive early detection of CRC and explore the biological function of the identified biomarkers in CRC carcinogenesis.

In chapter 2, we analyzed 102 CRCs and 230 noncancerous controls for GATA4 and GATA5 promoter methylation which was detected in 70% and 79% of the CRC tissues, respectively. The frequency of GATA4 and GATA5 methylation in normal colon mucosa tissue was 6 and 13% respectively. Adenoma tissues collected from CRC patients and noncancerous individuals exhibited methylation frequencies of 33-50% and 41-62%, for GATA4 and GATA5 respectively, suggesting that GATA4/5 methylation is an early event in CRC carcinogenesis. To explore the biomarker potential of the most specific GATA marker, GATA4 was further analyzed in two series of stool samples collected from two independent series of CRC patients (n=28 and n=47) and colonoscopy negative controls (n=28 and n=30). Using quantitative MSP, a sensitivity of 51-71% and specificity of 84-93% for CRC detection was obtained. In addition, we demonstrated that GATA4 and GATA5 proteins exhibit tumor suppressor characteristics. Transfection of GATA4 and GATA5 in CRC cell lines significantly suppressed proliferation, migration, invasion, and anchorage-independent growth of CRC cells. A large amount of literature describing the role of GATA transcription factors is available. In chapter 3, these studies are summarized in a comprehensive review discussing the function of GATA transcription factors in development and disease.

Another candidate gene which we identified using epigenome-wide screening approaches was the N-myc downstream regulated gene 4 (NDRG4). We examined promoter methylation of NDRG4 in CRC and adenoma tissues, as described in chapter 4. Promoter methylation was detected in two independent series of CRC tissue
with a sensitivity of 86% (n=83) and 70% (n=184) and a specificity of 96%. As described for GATA4 and GATA5 methylation, NDRG4 methylation was also detected in 55% of adenomas from 62 CRC patients and 66% of adenomas from 22 noncancerous controls. Quantitative MSP was used to study promoter methylation of NDRG4 in stool DNA of CRC patients and healthy, colonoscopy negative controls of 50 years or older in two independent series. The first series (CRCs: n=28, controls: n=45) showed a sensitivity of 71% and specificity of 84% for CRC detection and validation to confirm the test performance in a second series (CRCs: n=47, controls: n=30) yielded a sensitivity of 51% and specificity of 93%. Overexpression of NDRG4 in human CRC cell lines revealed several tumor suppressive features compared to control transfectants, i.e. reduced colony formation, proliferation and invasion.

Since data about expression and function of NDRG4 is limited, we explored the expression profile in human and mice by immunohistochemistry, in situ mRNA hybridization and Western blotting in chapter 5. The NDRG4 gene is a member of the N-myc downregulated gene family and expression was reported in the central nervous system and heart, which was confirmed in this study. However, in the heart, NDRG4 expression was not observed in the cardiomyocytes, but was restricted to specialized subendocardial Purkinje fibers. Furthermore, NDRG4 protein expression was shown in the peripheral nervous system throughout the murine body. Surprisingly in the gastrointestinal tract, no NDRG4 expression was shown in the epithelial cells, but NDRG4 expression was confined to the enteric nervous system (ENS), including the submucosal (Meissner’s) plexus and myenteric (Auerbach’s) plexus. In situ hybridization confirmed NDRG4 expression in the ENS and immunofluorescence assays showed restriction of NDRG4 to neurons, as NDRG4 co-localized with the pan-neuronal marker HuC/D but never co-localized with the glia marker GFAP.

To explore the performance of candidate methylation markers in blood, NDRG4, GATA5, SYNE1 and FOXE1 methylation was analyzed in plasma of 154 CRC patients and 444 endoscopy negative controls in chapter 6. GATA5 and NDRG4 methylation was detected in 18% and 27% of CRC patients, respectively. SYNE1 and FOXE1 methylation frequencies of 47% and 46% were observed with specificities of 96% and 93%, respectively. When SYNE1 and FOXE1 are combined, the sensitivity increased to 58% with a specificity of 91%. Analysis of SYNE1 and FOXE1 in a panel showed a clear stage dependency with sensitivities ranging from 37% in stage I to 100% in stage IV CRC. Functional assays have been performed in order to investigate the biological function of SYNE1 and FOXE1 proteins using transfected CRC cell lines. No significant effect on migration or invasion was observed upon SYNE1 transfection in CRC cell lines. Surprisingly, SYNE1 transfectants showed an increased proliferation compared to control transfectants. Potential suppressive features of FOXE1 were shown, as upregulation in CRC cell lines inhibits colony formation.
Finally, in chapter 7, the significance of the results obtained in this thesis is discussed. Our findings, as well as the available literature, suggest a better performance of stool-based DNA tests compared to blood-based tests. Partly, this can be explained by the dependency of blood-borne spread of tumor cells predominantly occurring in a late CRC stage in which patients already have a worse prognosis. The data in this thesis also indicate that NDRG4 performs best of the analyzed stool-based epigenetic markers in CRC detection, which is confirmed by others. Finally, further developments to improve the clinical and analytical sensitivity, such as the identification of novel and/or complementary markers and implementation of cutting-edge techniques in order to increase the sensitivity for CRC detection, are discussed.