Biological and translational implications of enteric nerves in colorectal cancer

SUMMARY

Colorectal cancer (CRC) is one of the most common and lethal cancers in the world with over 1.8 million newly diagnosed patients and approximately 900,000 deaths in 2018. Current predictions by the International Agency for Research on Cancer estimate a considerable increase in incidence and mortality in the upcoming decade in particular due to the aging population and the growing number of countries with a high human development index. Early detection and a better understanding of the biology of the disease will be instrumental in managing the growing burden of CRC.

Currently, colonoscopy and the fecal immunochemical test (FIT) are being used as tools for early detection of CRC. Colonoscopy is considered the gold standard for CRC detection, although the costs and the invasive nature of the procedure are considered as noteworthy limitations. Therefore, non-invasive tests such as the FIT and the Cologuard® have been developed, however, at present, both tests still have a suboptimal sensitivity (79-91% for FIT and 92.3% for Cologuard) when compared to the colonoscopy (92-99% sensitivity). For prognostic purposes, the tumor-lymph node-metastasis (TNM) classification is currently regarded as the gold standard for CRC. Nonetheless, TNM classification alone is insufficient in predicting prognosis as the clinical outcome can differ significantly among patients with a similar TNM stage.

Previously, CRC carcinogenesis was considered to be predominantly caused by the accumulation of genetic alterations and/or epigenetically induced changes in driver genes which are able to initiate the transformation of epithelial cells towards malignant cells. In the last decade, it has been recognized that tumor cells are able to reciprocally interact with a wide variety of other cell types such as endothelial cells, pericytes, fibroblasts, immune cells and nerve cells, the so-called tumor-microenvironment (TME). This interaction between CRC cells and the TME has major functional implications allowing the tumor to progress. Though some cells of the TME, such as immune cells, have been extensively studied and are promising targets for cancer treatment, many questions remain on the biological roles of other cell types in the CRC TME.

In an attempt to improve the current CRC early detection assays and the prognostic classification system for CRC, research continues to focus on the identification of novel biomarkers. Previously we identified *NDRG4* DNA methylation as an early detection marker for colorectal cancer, which thereafter has been implemented in an FDA-approved stool DNA test (Cologuard®). Moreover, we found that NDRG4 is specifically expressed in the (enteric) nervous system (ENS). So far, no role for the ENS has been described in CRC development and/or progression.

The **aim of the research described in this thesis** was 1) to clarify the role of *NDRG4* and the (enteric) neurons in CRC carcinogenesis and 2) to improve current CRC early detection and prognosis assessment by identifying novel diagnostic and prognostic biomarkers.

In **chapter 2**, we extensively reviewed the current knowledge on the role of (enteric) neurons in CRC development/progression. Based on the reviewed studies, we concluded that perineural invasion (PNI) and (neo)neurogenesis are indicators for tumor aggressiveness and can act as independent prognostic factors for 5-year disease-free, cancer-specific and overall survival in CRC. In addition, it has been shown that neuronal innervation can promote cancer development in multiple cancer types (prostate, pancreatic, skin and gastric) while denervation can attenuate progression. Similar observations were made for the ENS specifically, where the loss of enteric neurons was associated with a reduced risk for CRC development indicating a pivotal role of ENS in CRC. Moreover, different neuromodulators can either promote or inhibit CRC carcinogenesis by regulating cell proliferation, migration, angiogenesis and metastasis. Thus, multiples lines of evidence indicate a role for neurons in CRC carcinogenesis.

We further investigated the role of the enteric neuronal protein NDRG4 in CRC in **chapter 3**. Studying a genetic model (*APC*$^{Min/+}$) and a chemical model (azoxymethane), we observed that absence of *NDRG4* did not alter the number of adenomas but promoted the development of larger intestinal adenomas with a more aggressive phenotype. Through a co-culture model, in which intestinal epithelial organoids were subjected to conditioned medium from *NDRG4*$^{+/+}$ or *NDRG4*$^{-/-}$ primary ENS cultures, we observed that loss of *NDRG4* enhanced the relative growth and the formation of crypt buds of the intestinal epithelial organoids. Two secreted factors, which also enhance CRC cell migration *in vitro*, were significantly enriched within the medium of *NDRG4*$^{-/-}$ ENS cultures as compared to *NDRG4*$^{+/+}$ ENS cultures: nidogen-1 and fibulin-2.

To improve the early detection of CRC, novel biomarkers that complement the current diagnostic tools are required. Therefore, in **chapter 4**, we applied an *in silico* biomarker discovery analysis using publicly available data, to identify novel diagnostic DNA methylation markers. 221 genes were identified as potential diagnostic biomarkers which, after confirmation in CRC tissue samples, resulted in the selection of 5 genes for further validation using fecal DNA. In stool (n = 50 normal, n = 43 carcinoma), the sensitivity of the 5 genes ranged from 31.8-46.4% at 98.0% specificity with the combination of *gene 1*/*gene 4* acting as the best biomarker panel (sensitivity: 48.8% at 98.0% specificity). Though these biomarkers by themselves do not outperform the current standards for CRC early detection, the diagnostic value of these biomarkers in combination with these standard assays (FIT, Cologuard®) is yet to be explored. To study the pathways that exhibited promoter methylation, we performed gene ontology enrichment and pathway analyses on the identified gene set (n = 221). We found that 37.0-62.5% of all enriched biological process and 52.2-80.0% of all enriched cellular component

ontologies were linked with the nervous system, which was significantly higher than expected based on random distribution. Furthermore pathway analyses revealed that 35.0-90.9% of all affected pathways were associated with neurotransmitter signaling, synaptic vesicle cycle or axon guidance. These findings indicate that tumor cells might share characteristics with neuronal cells implicating an unexplored role of neuronal genes in epithelial/tumor cells and that these features can be exploited for improving existing early detection tests for colorectal cancer.

Next we explored whether the nervous system is associated with CRC prognosis by studying the expression of the neuronal markers neurofilament (NF) and protein gene product 9.5 (PGP9.5) in CRC in **chapter 5**. CRC tissues (n = 204) were immunohistochemically stained for either NF or PGP9.5 and were digitally evaluated, either via random sampling or whole slide evaluation. Both markers were expressed in large as well as small nerve fibers located within the stroma of colorectal tumors. NF-positive staining was associated with a higher number of CRC deaths (multivariate HR_{NF+} = 1.93; 95%-CI: 0.92-4.06 based on whole slide evaluation). Similarly, PGP9.5-positivity was associated with poorer survival (multivariate $HR_{PGP9.5}$ = 1.75; 95%-CI: 0.56-5.47 based on whole slide evaluation). The combination of NF and PGP9.5 did not result in any statistically significant association, neither when both markers were expressed ($p_{NF+/PGP9.5+}$ = 0.233) nor when only one of the two markers was positive ($p_{NF\ or\ PGP9.5}$ = 0.318). Nonetheless, multivariate analysis suggested a decreased survival in patients positive for one or two of the markers ($HR_{NF+/PGP9.5+}$ = 2.26; 95%-CI: 0.59-8.69), and $HR_{NF\ or\ PGP9.5}$ = 2.01; 95%-CI: 0.51-7.95). No statistically significant differences were observed between the evaluation methods.

In **chapter 6**, the significance and the underlying association between the obtained results in this thesis were explored and discussed. Our findings and the growing amount of literature suggest that the (enteric) nervous system could play, a currently underestimated, pivotal role in the development and progression of cancer and CRC in particular. We have shown that disruptions in neuronal vesicle trafficking might alter the intercellular crosstalk between neuronal cells and tumor cells, either via changes in release of neurotransmitters but also due to altered secretion of other soluble factors like ECM-related proteins. Evidence collected in this thesis illustrates that neuronal innervation is not only limited to a biological role in CRC but that expression of neuronal proteins or methylation of neuronal genes are possible candidates for diagnostic or prognostic marker development. If both the biological and translational role of neuronal innervation in CRC is further explored, applications towards therapeutic options need to be considered for the future.