Biological and translational implications of enteric nerves in colorectal cancer

VALORIZATION

Colorectal cancer (CRC) is the third most common type of cancer and the second-leading cause of cancer-related death worldwide [1]. Worldwide in 2018, incidence was over 1.8 million cases and approximately 900,000 deaths occurred due to CRC [1]. Due to the aging population and the increasing adaptation of a 'westernized lifestyle' in countries located in Asia, Eastern Europe and Latin America, it is expected that both CRC incidence and mortality will rise in the upcoming decade [2, 3]. Due to the heavy patient and societal burden of CRC, many countries implemented CRC screening programs to improve CRC early detection, but societal and economic implications of CRC screening and treatment are substantial as well. In the United States alone, the national costs for CRC care were estimated at $14.1 billion in 2010, with projections estimating an increase towards $17.4 billion in 2020 [4]. Assuming that the United States represents 25-40% of the global costs of cancer health care, worldwide CRC costs can be estimated at $35-56 billion in 2020, which would imply a major increase compared to the estimated $14-22 billion in 2003 [5].

In the Netherlands, in 2017, €597 million were spent on health care of CRC patients, an increase of €109 million compared to 2011, which can be partly attributed to the implementation of the Dutch colorectal cancer screening program in 2014 [6]. As an increasing number of people are invited each year, costs for the screening program have risen to €20 million for the first-line screening (FIT) and €50 million for the follow-up (colonoscopy) [7]. Other costs for CRC care are predominantly associated with treatment of advanced stage CRC, including hospitalization, chemotherapy, radiotherapy and treatment of side-effects [5, 8].

Within the scientific and medical community, it is generally agreed upon that early detection of CRC and its precursor lesions is the most effective strategy for CRC management as early stage disease can be treated more effectively and is therefore associated with better survival. In **chapter 1**, an overview was provided of the currently applied diagnostic and screening methodologies including invasive techniques like colonoscopy, and non-invasive screening assays including FIT and the Cologuard®, a multitarget stool DNA test [9-13]. Colonoscopy is considered the gold standard for CRC screening [14]. However, its invasiveness and complication risk result in suboptimal participation rates [14]. Therefore, screening programs have been developed in multiple countries using FIT as a first-line screening and subsequent colonoscopy for FIT positive screenees [9-12, 14, 15]. Predictions suggest that by the year 2044 the Dutch national screening program can lead to a decrease of CRC incidence of 31.0-35.0% compared to 2014 while the mortality is projected to reduce by 45.0-47.0% [16]. Similar effects have been described in other European countries and in the United States ranging from an 8.0-16.0% decrease in mortality based on guaiac-based fecal occult blood testing (gFOBT) screening whereas screening with fecal immunochemical test (FIT) reduced mortality with

36.0%-52.4% [17, 18]. Nonetheless, the sensitivity of FIT can still be improved and therefore molecular marker tests such as Cologuard® have been developed and shown to improve the detection of CRC and advanced adenomas [13]. Despite the increased detection of CRC and advanced adenomas, these molecular marker tests are not cost-effective for implementation into population-based screening programs compared to the current screening standards unless considerable changes in e.g. screening uptake, sensitivity for adenomas, ability to distinguish progressive and non-progressive adenomas could be achieved [19-22]. Therefore, novel biomarkers that increase the sensitivity of FIT or other existing stool assays which can be detected in small units of stool can even more improve early detection and decrease CRC incidence and mortality.

In **chapter 4**, an *in silico* DNA methylation marker discovery analysis utilizing the publicly available data from the Cancer Genome Atlas (TCGA) identified five DNA methylation markers: '*Gene 1', 'Gene 2', 'Gene 3', 'Gene 4*' and '*Gene 5'*. The sensitivity of these markers in fecal DNA ranged between 32.6%-46.5% at 98.0% specificity with the best marker panel (*'Gene 1'* and *'Gene 4')* reaching 48.8% sensitivity and 98.0% specificity. By themselves, these markers thus lack the diagnostic potential for incorporation in the clinical setting. Studying their incremental value to FIT and increasing the analytical sensitivity by applying sensitive methylation detection techniques (e.g. Discrimination of Rare EpiAlleles by Melt [23]) could lead to major improvements to the detection rate. Moreover, before new biomarkers can be incorporated into either new or established screening assays, extensive (prospective) validation studies are needed to confirm the diagnostic value of the biomarkers and further evaluation of e.g. test characteristics, impact on mortality and cost-effectiveness (costs of analysis, organizational costs, expected compliance), are needed to ensure that the clinical application of novel biomarkers benefits both patients and the health care system [19, 24-26].

Not only the development of screenings programs, but also the accurate assessment of prognosis will lead to benefits for both patients and the health care system as this could help to optimize treatment strategies. In **chapter 1**, we shortly described the current measures of CRC prognosis revealing that the tumor-lymph node-metastasis (TNM) staging classification is considered as the golden standard for CRC classification and prognosis as for each TNM stage, a mean 5-year overall survival has been estimated which ranges from 90% at stage 0 and 1 to less than 10% for stage 4 CRC patients [27-29]. Other features that are currently being investigated in the context of CRC prognosis are 'Immunoscore', tumor budding and the consensus molecular subtypes [30-32]. Overall, the aim of new prognostic markers is to enable a more accurate prognostic assessment which is beneficial in determining the optimal treatment strategy as it has been shown that the prognosis based on the TNM staging system varies considerably between patients with similar staging [28, 29]. In **chapter 5**, we investigated whether the intratumoral presence of nerve fibers, using two different immunohistochemical

stainings for neurofilament (NF) and protein gene product 9.5 (PGP9.5), to learn more about the prognostic value of neuronal proteins for CRC. We observed that NF and PGP9.5 expressing nerve fibers are located within colorectal tumors. Interestingly, nerve fibers were not detected in all tumors which subsequently was studied in a prognostic context. We observed that presence of NF and/or PGP9.5 positive nerve fibers was associated with a poorer prognosis in CRC, independent of other established prognostic factors. Research on prognostic biomarkers has been recommended to follow a multistep approach [33, 34]. This approach is comprised out of three phases i.e. 1) early exploratory analyses for hypothesis generation and identification of potential markers, 2) exploratory analyses to assess association between marker and prognosis, and 3) large, protocol-driven validation studies set to test previously established hypotheses which should provide strong evidence for the potential clinical implementation [33]. Our study performed in chapter 5 can be classified as both a phase I and II study, while further validation (phase III) is still required. However, before a phase III validation can be initiated, set standardized protocols, which includes sample selection, inclusion criteria, exclusion criteria, standardized assays and statistical analyses, need to be established hereby reducing potential bias and increasing the reproducibility of the study [33, 34]. Thus a large and independent validation cohort is required to validate the findings of our study. Due to the limited sample size of the initial study, a large independent cohort will also assist in establishing other potential prognostic effects in specific subgroups and could provide first evidence of the additional role of these markers to currently established prognostic markers.

Finally, in **chapter 1**, we addressed the current treatment strategies and recent developments of CRC therapeutics. In patients with stage II disease or higher, different variants of (adjuvant) chemotherapy are applied although promising advances have been made due to the development of targeted therapies, e.g. immune therapy, which are more specific in their effect compared to chemotherapy [35-42]. The application of targeted therapy opens new avenues also considering the findings in **chapter 2** and **chapter 3**. In **chapter 2**, we briefly described the therapeutic potential of targeting neurons in cancer, as in prostate and gastric cancer (surgical or pharmacological) denervation of tumors resulted in the attenuation of carcinogenesis [43, 44]. Furthermore, antineurogenic strategies targeting neurotrophic factors like nerve growth factor have been suggested in inhibiting nerve infiltration and consequent cancer-promoting processes [45, 46]. Considering that neurotransmitters and other neuromodulators also have considerable impact on the development and the progression of CRC, as previously described in **chapter 2**, active targeting of neuromodulators and/or their receptors, resulting in either the increase of tumor-inhibitory or the reduction of tumor-stimulating neuromodulators, could be an interesting novel approach for the development of future therapeutic strategies. However, the field of cancer neuroscience is still at its infancy and though recently new initiatives have started to take aim at cancer-neuronal crosstalk, major efforts are required to gain a better understanding of neuronal signaling in carcinogenesis and to translate this potential into viable treatment strategies [47].

Additionally, the data obtained in chapter 3, also provided novel insights in the importance of neuronal signaling in CRC biology as until recently, the contribution of the nervous system and in particular the ENS in CRC was practically unstudied. We described that the absence of *NDRG4* increased intestinal tumorigenesis potentially due to the increased release of the extracellular matrix proteins: nidogen-1 and fibulin-2. Although further studies are necessary to gain more insight in the function of both ECM proteins and in particular the underlying mechanism through which nidogen-1 and fibulin-2 induce their effects, both proteins could be considered as potential new targets in counteracting tumorigenesis. Although not applied in clinical practice, targeting of ECM-related proteins as treatment opportunity for CRC has been discussed in previous studies. The increased expression of lysyl oxidase, an ECM-modifying enzyme, and microfibrial-associated glycoprotein 2, a crucial regulator for signal transduction between different types of cells and their ECM, both resulted in remodeling and stiffening of the tumor ECM which promotes the progression of CRC [48-50]. Interestingly, in several other studies, ECM molecules have been correlated with an altered response of tumor cells to chemotherapy, indicating that the ECM proteins can also act as predictive markers for therapy response [50-52].

All in all, the data in this thesis provides strong evidence that the nervous system could not only act as a major novel player in CRC biology but can also facilitate the discovery of novel diagnostic markers, prognostic markers or treatment strategies in the battle against CRC.

LITERATURE

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