Cancer cell metabolism and colorectal cancer survival

SUMMARY

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide. Despite advances in the early detection and treatment of CRC, it remains the second leading cause of cancer-related mortality worldwide, accounting for more than 900,000 deaths in 2020. To date, the tumor-node-metastasis (TNM) staging system remains the most important clinically used factor to predict patient prognosis and guide treatment decisions in CRC. However, even patients within the same TNM stage can have a significantly different prognosis and response to adjuvant therapy, most likely due to heterogeneity in patient and tumor characteristics.

The *PI3K/AKT/mTOR* signaling pathway is one of the most frequently activated molecular pathways in CRC. It has been proposed that this signaling pathway rewires cancer cell metabolism from oxidative phosphorylation towards aerobic glycolysis, a phenomenon known as the "Warburg-effect". Previous research suggests that the Warburg-effect increases the malignant potential of tumor cells and may even contribute to therapy resistance. However, evidence to date is scarce and results remain inconsistent.

In this thesis, we therefore aimed to investigate whether Warburg-subtyping, based on the estimated presence of the Warburg-effect, has prognostic value and is able to predict benefit from adjuvant therapy in CRC patients. Furthermore, using a molecular pathological epidemiology (MPE) approach, we investigated (i) the potential additional prognostic value of Warburg-subtyping in subgroups based on mutations in oncogenes and tumor suppressor genes that have been associated with the presence of the Warburg-effect (i.e., *RAS (KRAS, NRAS, HRAS), PIK3CA,* and *BRAF* mutations) as well as mismatch repair (MMR) status, and (ii) whether associations between long-term energy balance-related factors and survival in CRC differed according to Warburg-subtype. To this end, CRC patients were classified as having Warburg-low (i.e., low probability of the presence of the Warburg-effect), Warburg-moderate, or Warburg-high CRC using a pathway-based sum score based on the protein expression levels of six glycolytic proteins and transcriptional regulators indicative of the Warburg-effect (i.e., LDHA, GLUT1, MCT4, PKM2, p53, PTEN).

All results presented in this thesis were based on observational data from the Netherlands Cohort Study (NLCS) on diet and cancer. This large, population-based prospective cohort study was initiated in 1986, and included 120,852 men and women aged 55-69 at baseline. Information with regard to, but not limited to, long-term energy balance-related factors were collected at baseline through a mailed, self-administered questionnaire. Follow-up for cancer incidence was established by annual record linkage with the Netherlands Cancer Registry and PALGA, the nationwide Dutch Pathology Registry, covering 20.3 years of follow up (September 17, 1986 until January 1, 2007). After this follow-up period of

20.3 years, 4,597 incident CRC cases had occurred. Follow-up for vital status was carried out through linkage with the Central Bureau of Genealogy and the municipal population registries, until December 31, 2012, and causes of death were retrieved from Statistic Netherlands.

In 2012, the Rainbow-TMA project was initiated, aiming to enrich cohorts with Tissue MicroArrays (TMAs) and DNA. Tumor and normal formalin-fixed paraffin-embedded (FFPE) tissue blocks from CRC patients were retrieved from pathology laboratories throughout the Netherlands. For TMA construction, pathologists reviewed Hematoxylin & Eosin (H&E)-stained sections and marked areas with the highest tumor density. From these areas, three 0.6 mm diameter tumor cores and three normal tissue cores were sampled and assembled in TMA blocks.

For the current thesis, serial TMA sections were subjected to immunohistochemistry (IHC) for Warburg-related proteins (i.e., LDHA, GLUT1, MCT4, PKM2, p53, PTEN), as well as mismatch repair (MMR)-related proteins (MLH1, MSH2). Stained sections were scored by three trained non-pathologist assessors and a random 10% was additionally scored by an experienced pathologist. Expression levels of the Warburg-related proteins were combined into a pathway-based sum score and, based on this sum score, patients were categorized into three Warburg-subtypes (Warburg-low, -moderate, and -high). In addition, available tumor DNA from CRC patients was screened for *RAS (KRAS, NRAS, HRAS)*, *PIK3CA*, *BRAF*, and *MET* mutations. Patients were then classified into eight mutually exclusive mutational subgroups, based on observed mutation (mut) frequencies and MMR status (i.e., all-wild-type + $MMR_{proficient}$, $KRAS_{mut}$ + $MMR_{proficient}$, $KRAS_{mut}$ + $PIK3CA_{mut}$ + $MMR_{proficient}$, $PIK3CA_{mut}$ + $MMR_{proficient}$, $BRAF_{mut}$ + $MMR_{proficient}$, $BRAF_{mut}$ + $MMR_{deficient}$, other + $MMR_{proficient}$, and other + $MMR_{deficient}$).

After excluding patients who did not pass IHC quality control, 2,394 CRC patients with complete IHC expression data for Warburg-subtyping (**Chapter 3 and 5**) and 2,344 patients with complete data for mutational subgroups were available for analyses (**Chapter 4**). In addition, the relationship between adjuvant therapy and survival could be analyzed for 1,793 CRC patients (**Chapter 6**). Kaplan-Meier curves and Cox regression models were used to investigate associations with survival for Warburg-subtypes alone, and in combination with mutational subgroups, long-term energy balance-related factors and adjuvant therapy.

In **Chapter 2**, we investigated whether non-pathologists can generate valid and reproducible IHC scoring results. This was done by assessing inter-observer agreement between trained non-pathologists and an experienced histopathologist for three IHC markers with different subcellular localization (i.e., nucleus, membrane, cytoplasm). In addition, intra-observer

agreement among trained non-pathologists was assessed. Our results indicated that adequately trained non-pathologists were able to produce similar IHC scoring results as an experienced histopathologists. Combining the scores of at least two non-pathologists yielded the most optimal results.

In **Chapter 3**, we studied whether Warburg-subtyping has prognostic value in CRC patients. We found that patients with Warburg-high CRC had a worse survival compared to patients with Warburg-low CRC, independent of known prognostic factors such as TNM stage.

In **Chapter 4**, we investigated whether mutational subgroups based on somatic mutations in *RAS, BRAF, PIK3CA* and *MET*, as well as MMR status, hold prognostic value in CRC. Moreover, we investigated whether Warburg-subtyping had additional prognostic value within these mutational subgroups. We found that compared to patients with all-wild-type + $MMR_{proficient}$ CRC, patients with $KRAS_{mut}$ + $MMR_{proficient}$, $KRAS_{mut}$ + $PIK3CA_{mut}$ + $MMR_{proficient}$, $BRAF_{mut}$ + $MMR_{proficient}$, or other + $MMR_{proficient}$ CRC had a worse survival. Patients with $BRAF_{mut}$ + $MMR_{proficient}$ CRC had the worst survival, while patients with other + $MMR_{deficient}$ CRC had the most favorable survival. Furthermore, we found that $BRAF_{mut}$, $KRAS_{mut}$ + $MMR_{proficient}$ and other + $MMR_{deficient}$ CRC may be related to the Warburg-high subtype. No statistically significant survival differences were observed across Warburg-subtypes within mutational subgroups.

In **Chapter 5**, we investigated the association between long-term energy balance-related factors (i.e., adult BMI, non-occupational physical activity, weight change since age 20 years, adult-attained height, and exposure to energy restriction during childhood and adolescence) and survival in CRC. Moreover, we evaluated whether associations between long-term energy balance-related factors and survival differed according to Warburg-subtype. We found that of all studied long-term energy balance-related factors, only increased adult (pre-diagnostic) BMI was associated with a worse survival in the total series of CRC patients. In stratified analyses, we found that associations with survival for increased adult BMI, weight gain since age 20 years, energy restriction during childhood and adolescence and potentially increased adult-attained height differed according to Warburg-subtype. Weight gain since age 20 years and adult-attained height were associated with a worse overall survival only in patients with Warburg-high CRC. Increased adult BMI was associated with a worse survival only in patients with Warburg-moderate CRC. Associations between energy restriction proxies (i.e., place of residence during World War II or the Dutch Hunger winter, employment of father during the Dutch Economic Depression) and survival did not show consistent patterns when stratified on Warburg-subtype

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In **Chapter 6**, we explored whether Warburg-subtypes can predict survival benefit from adjuvant therapy in patients with CRC. We found that while in general patients with TNM stage II-IV CRC who received adjuvant (chemo)therapy had a significantly favorable survival compared to patients who received surgery only, this survival benefit was limited to patients with Warburg-moderate and potentially Warburg-high CRC. No survival benefit from adjuvant (chemo)therapy was found for patients with Warburg-low CRC.

In **Chapter 7**, this thesis was concluded by a summary of the main findings, interpretation of the study results, a discussion of methodological considerations, and recommendations for future research. All in all, the results presented in this thesis suggest that Warburg-subtyping has prognostic value in CRC and may be used to predict survival benefit from adjuvant (chemo)therapy.