The clinicopathological implication of biomarker expression diversity in specific regions of colorectal tumours and their corresponding metastases

Synopsis of PhD thesis

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1. INTRODUCTION

Colorectal cancer (CRC) is now regarded as a molecular biologically heterogeneous disease, characterised by genetic and epigenetic diversity. Advancements in molecular biology have expanded our understanding of tumorigenesis, growth, invasion, and migration during tumour progression. Besides the anatomical extent of the tumour and the degree of histological differentiation, molecular biological features also carry prognostic impact. In recent years, with the increasing opportunity to investigate gene defects, defective gene products, microenvironmental specificities, and immunological factors, the identification of colorectal cancer subtypes based on transcriptional compartmentalization and the growing knowledge of the functions of tumour, stroma, and immunological components have led to an increasing use of a multimolecular, combination and targeted chemotherapy strategy. The *KRAS* and *BRAF* mutations, microsatellite instability (MSI), phosphatase and tensin homolog deleted on chromosome 10 (PTEN), epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), tumour infiltrating immune cells (ICs), and immune checkpoint inhibitors (ICIs) are frequently studied biomarkers in CRC. Tumours exhibiting high levels of MSI (MSI-H) show distinct clinical, pathological, and molecular features compared to those with low microsatellite instability or microsatellite stability (MSI-L/MSS). Typically, MSI-H tumours are less aggressive, located proximal to the splenic flexure, characterized by poorly differentiated mucinous and

mixed histology, accompanied by peri- and intratumoural infiltration of lymphoid cells, and show better prognosis. The enhanced immune activity that is typical of MSI tumours has rendered them a subject of interest in scientific investigations involving immune checkpoint inhibitors. Studies have shown that the PI3K/AKT pathway can modulate immune cell activities, impacting the effectiveness of cancer immunotherapy. Loss of PTEN function leads to the activation of this signalling pathway, and can induce PD-L1 overexpression in various cancers, potentially leading to resistance indifferent therapies. Tumour-infiltrating immune cells and cytokines have been shown to be promising prognostic markers, and intratumoural T cell infiltration has been discovered to be an independent prognostic factor in CRC. Patients with high immunoscore (IS) demonstrate better clinical outcome, lower risk of recurrence, longer disease-free (DFS) and overall survival (OS). As negative regulators of the immune system, immune checkpoints are responsible for keeping the immune response under control. Therapeutic agents that interrupt these immune checkpoint-regulated processes (such as anti-CTLA-4, anti-PD-1 and anti-PD-L1), enhance the anti-tumour immune response and facilitate tumour regression. The use of remedies acting on these pathways alone or in combination therapy became a subject of many studies. The discovery and characterization of these biomarkers, has the potential to enable clinicians to select the most effective therapy for individual patients in the clinical practice.

2. OBJECTIVES

The study aimed to provide insights into the following questions:

1. What is the frequency, intratumoural heterogeneity of MSI in our colorectal cancer surgical specimens?
2. Is there a difference in the expression of MMR markers between primary tumour regions and their corresponding liver metastases?
3. What is the predictive value of the clinicopathological factors available in our cohort?
4. Is there a difference in the expression of MMR markers between distinct regions of primary tumours and lymph node metastases?
5. Is the MMR status prognostic for DFS and OS in our patients after surgical resection?
6. Is the MMR status predictive for the effectiveness of 5-FU-based chemotherapy regimens (including oxaliplatin or irinotecan) or chemotherapy regimens in combination with targeted biological therapy (bevacizumab or cetuximab, or panitumumab)?
7. Do stage II and stage III MSI colon tumours respond differently to 5-FU-based treatment or are the outcomes of stage II and III patients after 5-FU treatment unfavourable compared to MSS tumours of the same stage?
8. How is the PTEN expression and their intracellular staining pattern with Dako, CellSignaling and Neomarker antibodies?

9. What is the degree of variation in PTEN expression based on the utilisation of various scoring methodologies?
10. What is the expression pattern of the three antibodies in the chosen tumour regions and metastatic sites?
11. Are there any discernible variations in PTEN expression based on the anatomical location of the tumour within the colon?
12. What is the relationship between PTEN expression and clinicopathological characteristics?
13. How is the distribution of the different immune cells and checkpoint inhibitors in the main tumour mass and metastases?
14. Does tumour localisation in the large bowel shows any differences in the IC and ICI pattern?
15. How is the distribution of the immune markers with the lymph node status?
16. How is the distribution of the immune markers within the liver metastases?
17. How is the distribution of markers in different areas of CRC and in metastatic lymph nodes?
18. Which genes show different expression between the main tumour mass and the metastases and what is their prognostic significance?

3. METHODS

The tumour samples in this study consisted of primary CRC and lymph node metastases from 55 patients and metastatic CRC samples from 56 patients (including 33 samples of primary tumours and liver metastases from patients treated with Cetuximab, and 23 samples from patients treated with Bevacizumab). Patients were diagnosed between 1987 and 2011, randomly selected from Semmelweis University (SE)'s database. Surgical procedures were conducted at SE's Department of Surgery, Transplantation, and Gastroenterology, with postoperative oncological treatment at the United St. István and St. László Hospital. A control group of 34 patients from Uzsoki Teaching Hospital, surviving over five years without disease progression, was included. Ethical approval was obtained from SE's Committee of Science and Research Ethics (SE-TUKEB 207/2011).

Microsatellite instability analysis included 122 patients, PTEN study had 55 patients, and immune-based biomarkers investigation involved 137 individuals, with 11 paired samples for immune panel gene expression assay. Clinicopathological data and survival info were systematically organized. Tissue microarrays were generated from formalin-fixed, paraffin-embedded samples, and immunohistochemical reactions assessed biomarkers (MSI, PTEN, immune cells, immune checkpoint inhibitors). The cores with a diameter of 2 mm, were obtained from the normal colorectal mucosa (NORMAL), main tumour mass (MAIN), the tumour-normal interface (BORDER), deepest infiltrative area (FRONT),

lymph node metastasis (LN) and/or liver metastasis (MET). To analyse the biomarkers, 4 micrometre thick sections were cut from TMA blocks and mounted on adhesive glass slides (SuperFrost UltraPlus from Gerhard Menzel Ltd., Braunschweig, Germany). The tissue sections were processed for immunohistochemistry in an automated immunostainer (Ventana Benchmark XT, Roche, Tucson, AZ, USA), using the manufacturer's recommended solutions and settings. For the assessment of MSI, MLH1, MSH2, MSH6 and PMS2 immunohistochemical reactions were used and evaluated according to the following scheme: intensity (0-3), frequency (0-5). The analysis of PTEN expression patterns included the following dimensions: intracellular localization (nuclear, cytoplasmic, nuclear, and cytoplasmic), intensity (0: none, 1: weak, 2: intermediate, 3: strong expression), and proportion (0: none, 1: 0-1 %, 2: 2-10 %, 3: 11-33 %, 4: 34-66 %, 5: 67-100 % of respective cells stained). The scores obtained from duplicate areas were averaged, and raw data was used for statistical evaluation. Three different scoring systems were used to calculate the Histochemical scoring assessment (H-score) for a tumour region based on the intensity and frequency of staining. The H1-score multiplied the intensity and frequency resulting in a range of 0-15, the H2-score summed the intensity and frequency resulting in a range of 0-8, and the H3-score was weighted towards intensity resulting in a range of 0-15. Immunoreactions were assessed in lymphocytes for all reactions, and in tumour cells specifically for CTLA-4, PD-L1, and PD-1. The reactions were quantified and analysed using

computer-assisted image analysis with the QuantCenter digital analyzer. This method allowed for the calculation of the number of positive cells per annotation, where each annotation corresponded to the surface area of a core cylinder measuring 3.14 mm2. Digital images of the slides were obtained using a Pannoramic P250beta slide scanner (3DHistech Ltd., Budapest, Hungary). The evaluation of the images was performed using the Pannoramic Viewer software, which utilized the TMA and Histoquant modules for analysis (3DHistech). For gene expression of selected immune related genes 12 primary tumors and 12 liver metastases samples were investigated using NanoString method (NanoString, USA). The resulting gene expression profiles were digitalized using the nCounter Digital Analyzer and quantified using nSolver 4.0 Analysis Software (NanoString, USA).

Tumour staging was determined based on the American Joint Committee on Cancer (AJCC) grouping system, which utilized both histopathological examination of surgical specimens and imaging studies.

The statistical analysis was performed with SPSS 22, and R for Windows (v4.1.2), and all tests were two-sided with p-values of less than 0.05 accepted as statistically significant.

4. RESULTS

The study found that 11.5% of our cases exhibited microsatellite instability. There was no significant difference in mismatch repair *(MMR)* status observed between tumour regions and lymph nodes. In 14 cases, there was a discrepancy in MMR status between the primary tumour and liver metastases. In relapse-free survival (RFS), Dukes classification and clinical stage both showed significant predictive power ($p = 0.001$). The latter was prognostic to OS too ($p = 0.009$). Regarding the sidedness, it was found that there was only a trend in progression-free survival (PFS) ($p = 0.072$), with patients with left colon tumors having the most favorable outcome, followed by patients with rectal and right colon tumors. The MMR status was not found to be prognostic for RFS and OS, and the MMR status was also not predictive for RFS, PFS and OS, with either chemotherapeutic or biological therapies. Furthermore, the use of 5-fluoro-uracil showed no significant difference between MSS and MSI tumours for stage II and III colorectal cancer. The assessment of PTEN expression using the three antibodies found that the Neomarker showed nuclear, while Dako and CellSignaling antibodies showed both nuclear and cytoplasmic staining. Calculated H-scores (H1, H2, H3) were strongly correlated, and no significant difference was observed among them. Regarding the intratumoural distribution of PTEN, its expression decreased slightly as tumour progression advanced (not significantly). There was a significant decrease in PTEN expression in all tumour regions compared to normal colon (with Dako and CellSignaling

antibodies), but no significant difference was observed in staining within tumour regions or lymph nodes. The study also found no significant difference in PTEN expression across different colon areas and sidedness. Additionally, there was no relationship between PTEN and TNM or clinical stage, grade, Dukes, Dukes-MAC, or MSI phenotype in clinicopathological evaluations. However, there was a significant decrease in PTEN expression in *mKRAS* (exon13) with the Neomarker antibody. Furthermore, PTEN loss was not prognostic for RFS with any of the three antibodies. The number of CD56 was significantly higher in the MAIN than the MET samples, while CD23 and PD-1 exhibited a tendentious increase. Additionally, PD-1 expression was significantly higher in the right colon, while CD45 exhibited a tendentious increase. More advanced lymph node metastasis was associated with significantly lower CD20 expression, while CD3 and CD45 exhibited a tendentious increase. In the MET samples, CD56 expression was significantly lower in stage IV CRC, while CD45 expression was marginally higher. Regarding tumour regions, CD56 expression was higher in NORM, while CD3, CD8, CD20, CD23, CD45, CTLA-4, and PD-1 exhibited significantly higher expression in lymph nodes only. With the NanoString method we found 11 and 29 differentially expressed genes (DEGs) to be significantly and marginally different, respectively, between the two sample types. The analysis revealed that among the 11 DEGs, the genes complement C4B (*C4B*), complement factor I (*CFI*), defensin beta 1 (*DEFB1*), interleukin-1 receptor accessory protein

(IL1RAP), interleukin-27 (*IL27*), mannose binding lectin 2 (*MBL2*), and metallophosphoesterase domain containing 1 (*MPPED1*) were observed to be downregulated. On the other hand, the genes caspase recruitment domain family member 9 (*CARD9*), C-C motif chemokine receptor 7 (*CCR7*), lymphotoxin beta (*LTB*), and tumour necrosis factor (TNF) receptor superfamily member 8 (*TNFRSF8*) were observed to be upregulated. No significant difference was observed in the gene expression profiles between left-sided and right-sided tumours. In relation to the prognostic significance of DEGs, the diminished expression of genes associated with the innate immune response and pathways involving TNF superfamily members is observed in liver metastasis samples. A reduced expression of *TNFRSF8* correlates with a shorter duration of DFS, while a prolonged PFS is linked to elevated *DEFB1* counts. In the metastatic setting, a decreased PFS is connected to lower expression levels of *C4B, CF1, IL1RAP*, whereas higher *CARD9* counts are associated with the same outcome. However, a mixed effect Cox regression model showed that *C4B, MBL2, CARD9*, and *TNFRSF8* significantly impacted DSS, while *IL27* and *LTB* were prognostic of PFS. Moreover, *DEFB1* was found to significantly affect both DSS and PFS.

5. CONCLUSIONS

Despite numerous studies, personalized therapy for CRC continues to pose a significant challenge. The heterogeneity of CRC has been well-recognized and must be considered in clinical decision-making at multiple levels, including epigenetic, genetic, transcriptomic, proteomic, and microenvironmental levels (166). Based on the outcomes of our investigations, we deduce the subsequent conclusions:

- Our cohort MMR status did not have a prognostic nor predictive impact on RFS and OS with any of the provided treatment.
- There was no significant difference in the therapeutic response observed between MMS and MSI tumors in stage II and III CRC when treated with 5-FU.
- No significant difference was identified among the three scoring methods we employed concurrently to evaluate PTEN expression, considering three principal factors: intracellular localization, intensity, and frequency.
- PTEN expression did not show statistically significant variation in staining among tumour regions, lymph nodes, or localisations in the colon. Our observations were limited to a gradual decrease trend observed towards the invasive front.
- Neither of the administered PTEN antibodies demonstrated prognostic significance in any of the conditions tested.
- In our immune study we observed a higher quantity of NK cells and more mature B cells expressing PD-1 in the primary tumour

region compared to metastatic lymph nodes, where B cell counts were significantly lower and leukocyte counts were higher.

- 11 differentially expressed immune-related genes were identified between primary tumour and liver metastasis samples, highlighting significant alterations in the innate immune response and the TNF superfamily pathways. These changes in gene expression were linked to shorter survival times in CRC patients.

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