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Monitoring disease with molecular markers in patients with colorectal adenocarcinoma

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1. Introduction

Colorectal cancer (CRC) is a collective term for tumorous changes affecting the colon and/or rectum, caused by neoplastic transformation of glandular epithelial cells. CRC is the third most diagnosed cancer and the second most common fatal malignant disease. The development of CRC is influenced by a variety of genetic and environmental factors.

RAS variants are the most common gene mutations associated with human malignancies. The RAS-mitogen-activated protein kinase pathway is a promising pharmacological target for developing effective anticancer agents in patients with contraindications for EGFR inhibition therapy. Targeting RAS enables personalized medicine by optimizing therapeutic outcomes and reducing toxicity. The development of genetic, genomic, and clinical biomarkers has created new opportunities for more effective targeting of RAS or key RAS effectors. These new drugs and approaches exhibit promising results in preclinical and clinical studies, but the complexity of RAS signaling and the potential for adaptive feedback continue to pose significant challenges. Therefore, a detailed understanding of the properties and dependencies of tumor RAS variants is necessary for the development of targeted therapies for RAS-driven malignancies.

CRCs provide an excellent platform for the exploration, tracking, and study of genetic changes involved in cancer development. Abundant clinical and histological data indicate that most, if not all, malignant colorectal carcinomas arise from pre-existing, fundamentally non-aggressive lesions known as adenomas. In contrast to many other common human tumor types, samples of tumors at various stages of development, ranging from very small adenomas to large metastatic carcinomas, can be obtained for CRCs. Furthermore, invasive techniques for diagnosis and monitoring have been replaced with non-invasive or minimally invasive methods. Liquid biopsies, for instance, have revolutionized the field of clinical oncology, facilitating continuous sampling, tracking temporal heterogeneity of tumors, devising personalized therapeutic strategies, and screening for therapy resistance.

Numerous tests are available to help select the most effective treatment based on the molecular characteristics of tumor tissue or other biological parameters of malignancies. Immunohistochemistry (IHC), which visualizes specific antigens within the tissue, has advanced personalized oncology. Polymerase chain reaction (PCR)-based techniques, on the

other hand, require relatively simple instruments and infrastructure, use only tiny amounts of biological material, and are highly compatible with clinical routines.

Access to Sanger sequencing was a significant breakthrough and is still considered the gold standard in molecular diagnostics today. Advances in this field led to second- and third-generation sequencing methods. Next-generation sequencing (NGS) technology has revolutionized the concept of nucleic acid sequencing. In NGS technology can be attributed to the ability to sequence millions of reads using very small amounts of nucleic acid, making NGS suitable for the rapid sequencing of complex genomes, which is highly efficient in terms of both time and cost.

Among environmental factors, the activation of adaptive mechanisms to circumvent hypoxic damage is a characteristic of aggressive, poor-prognosis cancer. As part of the adaptive process, tumor cells with anaerobic metabolism compensate for intracellular acidosis at the expense of extracellular pH, inducing functional changes in the hypoxic microenvironment, promoting resistance, and tumor progression. Carbonic anhydrase IX (CAIX) plays a significant role in the adaptive response to hypoxia, which is orchestrated by hypoxia-inducible factor-1 (HIF-1) in both normal and cancer cells. Furthermore, by contributing to microenvironmental acidosis, CAIX is involved in tumor-stroma and tumor-immune cell interactions and accelerates the degradation of the extracellular matrix to promote the invasion and proliferation of acid-resistant cells. Overexpression of CAIX due to hypoxic stress is an unfavorable prognostic feature in various tumors. Intratumoral changes associated with CAIX are complex resistance mechanisms that are not effectively neutralized by classic cancer drugs or biological therapies. Inhibition of CAIX can alter the proliferation, migration, invasion, and immunogenicity of tumor cells. In vivo studies show that metastatic growth can also be limited. The effectiveness of this inhibition is largely achieved through the regulation of pH in cancer cells. Recent research indicates that CAIX can interact with numerous other signaling pathways and mechanisms known to be active in cancer cells. These interactions may influence the response of cancer cells to radiation and chemotherapy. The sensitivity to radiation may be determined by additive or synergistic interactions between pH-dependent and independent mechanisms, suggesting that CAIX may have several important roles in tumor cells that could potentially be exploited therapeutically.

A detailed examination of the distribution and dynamic nature of CAIX and its relationship to the *KRAS* mutation status has not been conducted. We hypothesized that cancer therapies,

such as neoadjuvant therapy, have a fundamental impact on the metabolism and perfusion of malignant cells, which is reflected in measurable changes in the adaptation of cells, including CAIX expression. As a model system, we evaluated and compared CAIX expression and other available variables related to the disease in pre-neoadjuvant treatment (diagnostic biopsy) and post-treatment (surgical resection) samples from patients with rectal adenocarcinoma.

2. Objectives

2.1. Tracking changes in *KRAS* mutations in tissue and liquid biopsy samples at the University of Debrecen Pathology Institute

The determination of *KRAS* mutations from colorectal cancer (CRC) tissue samples is a daily routine task at the Clinical Center of the University of Debrecen Pathology practice. The frequent occurrence of KRAS mutations has provided an opportunity for the analysis and monitoring of different *KRAS* profiles found in multiplex and metastatic tumors. The aim of our prospective study was to examine all available primary and metastatic tumor samples in patients diagnosed with colorectal tumors, and, within another study, to analyze tumor and cell-free (cf) DNA samples treatment cycles with bevacizumab. Analysis of samples using reverse hybridization (StripAssay), Sanger sequencing, and next-generation sequencing (NGS) provided data to answer the following questions:

- What is the relationship between the DNA mutation status of histological samples and cfDNA abnormalities in the same patients?
- What are the changes in *KRAS* pathogenic variants during combined bevacizumab treatment?
- To what extent do cfDNA molecular abnormalities from peripheral blood (PB) of patients with *KRAS* mutated metastatic colorectal cancer correlate with the results from tumor tissue?

2.2. Relationship between hypoxia and mutant KRAS status in colorectal cancer

The aim of our retrospective study was to examine biopsy and surgical specimens from patients with rectal adenocarcinoma who received preoperative neoadjuvant treatment or patients with rectal adenocarcinoma who were untreated. We aimed to determine the extent of tumor hypoxia as indicated by the CAIX marker in treated and untreated cohorts. Thus, we aimed to assess the general pattern of CAIX expression in rectal adenocarcinoma, as follows:

- The CAIX expression in untreated (UT) patients and patients receiving preoperative neoadjuvant therapy (NAT) for rectal adenocarcinomas.
- The correlation between CAIX expression in pre-treatment biopsy samples and the treated surgical samples (control group).

- The correlation of CAIX expression with pathological and biological status, including cell proliferation, tumor regression grade, and *KRAS* mutation status.
- The impact of CAIX on patient survival.

3. Materials and Methods

3.1. Selection of Samples and study design

Archived histopathological and liquid biopsy samples from molecularly confirmed KRAS mutant colorectal adenocarcinoma cases diagnosed at the Department of Pathology at DE CC between 2015 and 2022 were included in the analysis. The study was conducted in accordance with the highest ethical standards and fell under the purview of national ethical approval (TUKEB reference numbers: 60355-2/2016/EKU, 4648-6/2018/EÜIG, and IV/8465-3/2021/EKU).

3.2. Sample processing

3.2.1. Tumor DNA extraction from FFPE tissue samples

FFPE tumor tissue was selected for molecular analysis if two independent pathologists detected > 10% tumor percentage on hematoxylin and eosin (H&E) stained slides. Genomic DNA was extracted using a QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). The DNA concentration was measured using a Qubit dsDNA HS Assay Kit and a Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

3.2.2. Cell-free DNA extraction from peripheral blood samples

Peripheral blood samples (liquid biopsy) were collected at three time points: pretreatment, before cycle two (range 14–21 days after starting therapy), and before cycle four (range 42–63 days). Blood samples (10 ± 0.1 ml) were collected in EDTA coagulation-inhibited blood collection tubes (Becton Dickinson and Company, Sparks Glencoe, MD, USA) and processed within 2 h. The isolation process started with two centrifugation steps. First, blood tubes were centrifuged at 1300 g for 10 min. The supernatant was then centrifuged a second time to remove any remaining cells and to eliminate debris at 16000 g for 10 min. The cfDNA was extracted from 5.0 ± 0.5 ml of plasma into 40 µl of elution buffer using QIAamp® Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany). The DNA concentration was measured using a Qubit dsDNA HS Assay Kit and a Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The fragment size and distribution of cfDNA were measured using a Bioanalyzer 2100 instrument (Agilent Technologies, Santa Clara, CA, USA).

3.2.3. Mutation status determination

Three methods were used to determine the mutation status of the target genes, including Sanger sequencing, reverse hybridization-based test, and next-generation sequencing.

3.2.3.1. Sanger sequencing

For Sanger-sequencing, exon 2 of the KRAS gene was amplified by PCR using the forward 5′-GGTACTGGTGGAGTATTTGATAGTG-3′ and reverse 5′-CGTCAAGGCACTCTTGCCTAC-3′ primers. The purified PCR products were sequenced using the BigDye Terminator v1.1 Cycle Sequencing kit. The samples were analyzed on the ABI PRISM 310 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). The variant allele frequency (VAF) of the Sanger sequencing electropherogram was calculated according to the following formula: mA% (proportion of mutant allele) = Hm (height of the mutant allele wave)/(Hm + Hwt (height of wild type allele wave))*100.

3.2.3.2. Reverse hybridization-based test (StripAssay)

Reverse hybridization was carried out using *KRAS* XL, *NRAS* XL, and *BRAF* 600/601 StripAssays according to the manufacturer's protocol (ViennaLab Diagnostics, Vienna, Austria). The assay covers 29 clinically relevant mutations in the *KRAS* gene, 22 mutations in the *NRAS* gene, and 9 alterations in the *BRAF* gene that are certified for human in vitro diagnostics (IVD). For interpretation, hybridization strips were aligned using the standardized layout supplied with the reagents, and positive bands were identified. The limit of detection of the reverse hybridization strip assays is 1% VAF.

3.2.3.3. Next-Generation Sequencing (NGS) from FFPE Tissue Samples

After the fragmentation of the genomic DNA, NGS libraries were created using the TruSight Tumor 15 Kit (Illumina, San Diego, CA, USA). This panel is a targeted sequencing assay that simultaneously detects and characterizes single-nucleotide variants (SNVs) and insertions and deletions (indels) in 15 genes associated with CRC tumors. The following 15 genes were included: *AKT1*, *BRAF*, *EGFR*, *ERBB2*, *FOXL2*, *GNA11*, *GNAQ*, *KIT*, *KRAS*, *KIT*, *NRAS*, *PDGFRA*, *PIK3CA*, *RET*, and *TP53*. The final libraries were quantified using the Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), diluted to a final concentration of 4 nM, and pooled by equal molarity. Sequencing was performed using the MiSeq Reagent kit (v3 2 × 300 cycles). All libraries were denatured by adding 0.2 nM NaOH and diluted to 40 pM with

hybridization buffer from Illumina (San Diego, CA, USA). The final loading concentration was 8 pM libraries and 1% PhiX. Sequencing was conducted according to the MiSeq instruction manual (Illumina, San Diego, CA, USA). The data were analyzed with the BaseSpase TruSight Tumor 15 Application for the presence of SNVs and indels (Illumina, San Diego, CA, USA). The sequence quality for each sample was assessed. The limit of detection was set to 2% variant allele frequency. A reliable variant detection required coverage of > 250 reads.

3.2.3.4. Next-generation sequencing (NGS) from liquid biopsy

NGS libraries were created using the Archer® Reveal ctDNA™ 28 Kit (ArcherDX, Boulder, CO, USA). This kit is a targeted sequencing assay that simultaneously detects and characterizes single nucleotide variants (SNVs), insertions, and deletions (indels) in 28 genes associated with solid tumors. The panel includes the following 28 genes: AKT1, ALK, AR, BRAF, CTNNB1, DDR2, EGFR, ERBB2, ESR1, FGFR1, HRAS, IDH1, IDH2, KIT, KRAS, MAP2K1, MAP2K2, MET, MTOR, NRAS, NTRK1, NTRK3, PDGFRA, PIK3CA, RET, ROS1, SMAD4, and TP53. The final libraries were quantified using a KAPA library quantification kit (Roche, Basel, Switzerland), diluted to a final concentration of 4 nM, and pooled by equal molarity. Sequencing was performed using a MiSeq System (MiSeq Reagent kit v3 600-cycle). All libraries were denatured by adding 0.2 nM NaOH and diluted to 40 pM with hybridization buffer from Illumina (San Diego, CA, USA). The final loading concentration of the libraries was 10 pM and 1% PhiX. Sequencing was conducted according to the MiSeq instruction manual. The data were analyzed with the Local Virtual Machine application of Archer DX Analysis software for the presence of SNVs and indels. The sequence quality for each sample was assessed. For the alignment, the human reference genome GRCh37 (equivalent UCSC version hg19) was built. The limit of detection for the assay was set at 1% variant allele frequency. A reliable variant detection requires minimum coverage of > 250 reads.

3.3. Immunohistochemical examinations

For standard tissue processing, the tumor samples were obtained from primary colonoscopic biopsies and post-surgical resections in PBS-buffered formaldehyde solution (4%). Formalin-fixed paraffin-embedded (FFPE) embedding and histopathological examination were performed at the Pathology Institute of the University of Debrecen. From the selected

blocks, 3 μm sections were prepared, and immunohistochemical examinations were conducted.

One part of the automated immunohistochemical staining was carried out on the Bond Max staining machine. We used the following primary antibodies for immunohistochemistry: anti-Ki-67, MIB-1 clone (Dako, Agilent Technologies), anti-p53, Do-07 clone (Dako, Agilent Technologies), anti-PMS2, EP51 clone (Dako, Agilent Technologies), and anti-MLH1, G168-728 clone (Cell Marque, Rocklin, CA, USA). The immunohistochemical reaction was detected using the Leica Bond detection kit (catalog number DS9800).

Further immunohistochemical reactions were performed on the BenchMark Ultra automated staining system. The primary antibodies used were as follows: carbonic anhydrase IX/CAIX with the EP161 clone (Cell Marque/Sigma-Aldrich, CA, USA), anti-MSH2 with the G219-1129 clone (Cell Marque, Rocklin, CA, USA), and anti-MSH6 with the 44 clone (Biocare Medical, Pacheco, CA, USA). The antigen-antibody binding was visualized using the OptiView DAB IHC Detection Kit (Ventana). The samples were subsequently counterstained with hematoxylin II and cover-slipped after dehydration.

The percentage of positive cells for Ki-67 cell proliferation index and the Histo-score for p53 intensity were determined following immunostaining in the microscope. The Histo-score included both the intensity of staining (graded as: 0, non-staining; 1, weak; 2, median; or 3, strong using adjacent normal mucosa as the median) and the percentage of positive cells following semi-quantitative assessment. An Histo-score was assigned using the following formula: [1 × (% cells 1+) + 2 × (% cells 2+) + 3 × (% cells 3+)]. The range of possible scores extends from 0 to 300.

The immunostainings were independently evaluated using light microscopy by two histopathologists in a blinded fashion. If conflicting values were obtained, the decision was made by mutual agreement following personal discussion. The expression of CAIX was quantified using a visual grading system based on the extent of staining (percentage of positive tumor cells: 0–100%). A median value was calculated from the obtained percentage expression values. Depending on the median value, CAIX low and CAIX high groups were formed for further analysis. The CAIX low group included all cases with CAIX values below the median, including negative staining results, and the CAIX high group represented cases with values equal to or above the median. CAIX low and CAIX high categories were separately evaluated within the NAT and UT groups. For comparison, we investigated the distribution of

CAIX and general features of CAIX low and CAIX high categories in both initial biopsy and surgical resection samples.

3.4. Statistical analysis and graphs

For the statistical analysis, we used the GraphPad Prism 8 statistical software (Dotmatics, Boston, MA, USA). We applied the Wilcoxon matched rank test, Spearman correlation test, and Mann-Whitney U test. We also used the VassarStats online software (http://vassarstats.net, accessed on January 4, 2023) to perform the Fisher exact test, which was used to assess the statistical relationships between protein expression and clinical and histopathological parameters. We considered only p-values less than 0.05 as significant. For creating figures in the literature review, I used the online platform www.biorender.com.

4. Results

4.1. Molecular pathological analysis of colorectal adenocarcinoma cases

At the Pathology Institute of the University of Debrecen Clinical Center, a total of 1339 colorectal tumor cases underwent molecular testing for the target genes *KRAS*, *NRAS*, and *BRAF*, which play a significant role in the MAPK pathway, between 2015 and 2022. In 1315 cases (98.2%), the actual determination of the target genes was conducted.

During the investigated time interval, more than half of the colorectal tumor cases in our institute were males (64.5%). The vast majority of colorectal tumors predominantly affected the left side. In male patients, the distribution of right/left-sided tumors was 12.97%/51.41%, while in female patients, it was 8.9%/26.09%. Tumors affecting both the right and left sides of the colon showed a nearly equal distribution between the sexes (female/male = 0.3%/0.23%).

We assessed the number of mutant and wild-type MAPK pathway colorectal tumor cases. Wild type MAPK pathways occurred in more than half of the cases. We also examined the prevalence of mutations in the MAPK pathway, with wild type confirmed in more than half of the cases, followed by mutant *KRAS*, *BRAF,* and *NRAS*. In mutant cases, we examined the percentage prevalence of each *KRAS* variant. The most prevalent variant was Gly12Asp (29.57%), followed by Gly12Val (23.25%), Gly13Asp (16.51%), Gly12Cys (9.08%), Gly12Ala (8.25%), and Gly12Ser (4.95%).

4.2. Examination of *KRAS* status and dynamics of multiplex colorectal adenocarcinoma-a case presentation

From 2015 to 2022, the Pathology Institute of the University of Debrecen Clinical Center conducted sequential examinations of secondary metastases in 25 cases of primary colorectal adenocarcinoma. In these cases, the goal was to determine and compare the *KRAS* status in primary and metastatic colorectal adenocarcinomas. Among these cases was a unique scenario involving multiple metastases causing colorectal cancer with four simultaneously existing, separate colorectal tumors. We tracked the clonality and genetic variation of this case retrospectively for four years. The aim of our study was to retrospectively identify individual colorectal adenocarcinoma foci using molecular genetic methods and to detect clonal relationships between primary tumors and distant metastases. To achieve this, we used

next-generation sequencing, employing a 15-gene solid tumor gene panel (Illumina MiSeq platform). Our NGS results were validated using reverse hybridization testing (StripAssay) and conventional Sanger sequencing.

A 57-year-old man with cachexia, loose stool, and hematochezia was directed to the Department of Gastroenterology, University of Debrecen. The ultrasound and abdominal computer tomography (CT) scan revealed significant intestinal wall thickness and a mass in the right colonic (flexura hepatica) region, moderate paraaortic lymphadenopathy, and a 3.2-cm lesion corresponding to metastasis in the hepatic VI–VIII segment. The preoperative colonoscopic histology revealed a tubulovillous adenoma with focal high-grade dysplasia. Due to the imminent occlusion by the tumor, a subtotal colectomy was performed. Metastasis in the hepatic VI–VIII segment detected by CT could not be approached, but two smaller metastatic foci found in the hepatic III segment were removed during surgery in the same session. Detailed pathological examination of the surgical sample identified (1) a 9.5-cm moderately differentiated adenocarcinoma of the cecum, with preexisting villous polyp; (2) a 4-cm poorly differentiated adenocarcinoma of the hepatic flexure, with preexisting villous polyp; (3) an 8-cm moderately differentiated adenocarcinoma of the colon transversum, with preexisting villous polyp; (4) a 5-cm poorly differentiated adenocarcinoma of the colon transversum, without preexisting polyp; and (5) two necrotic metastatic tumors (0.8 and 1.3 cm) from the hepatic III segment with cribriform architecture. The histological pattern was generally microglandular with limited masses of mucinous components. The pathologic stage was pT3, N0, M1. The preserved expression of the mismatch repair proteins MLH1, MSH2, MSH6, and PMS2 determined by IHC ruled out the major causative role of mismatch repair deficiency. Routine molecular testing of the surgical sample detected a *KRAS* mutation in the primary tumor (exon 2, codon 12 with genotype c.35G > T; p.Gly12Val). Following postoperative restaging, additional liver metastasis were detected and first-line combined chemo- and biotherapy (Folfiri and Bevacizumab) was decided on. Nearly one year later, regression determined by CT scan enabled liver exploration (M2). The patient received postoperative Xelox therapy, but the patient had severe intolerance (diarrhea and hand-foot syndrome) and the treatment was switched to Folfox. After seven months, progression was shown by CT imaging, and another liver metastasis was removed, followed by postoperative deGramount therapy (M3). Nine months later, a further three independent liver metastases were removed (M4). Thoracic CT showed lung metastases. Based on the *KRAS* wild-type status

determined by Sanger sequencing from the sample M3, second-line Vectibix + Folfiri treatment was started. However, further regression could not be achieved. The last surgical intervention was the decompressive resection of a symptomatic metastatic spinal tumor (M5), which was followed by the third-line Lonsurf therapy.

4.2.1. *KRAS* validation studies

Due to the complexity of the *KRAS* results obtained by NGS, we performed validation studies on KRAS variants at the single-gene level on two alternative platforms. The reverse-hybridization-based StripAssay constructed for IVD testing of clinically relevant *KRAS* alterations identified all variants previously detected by NGS, although quantification was not enabled. The results of classic bidirectional Sanger sequencing directed toward activating *KRAS* exon 2 alterations covering codon 12/13 were in full agreement with the NGS data, except for low-frequency alterations (below 10% VAF), which were not represented.

4.2.2. NGS-based mutation profiling

The 15-gene solid tumor panel analysis identified a series of mutations in the *KRAS* and *TP53* genes, while all other genes relevant in colon carcinoma (including *BRAF*, *EGFR,* and *NRAS*) appeared to be uninvolved. As the most critical genetic change, *KRAS* status was analyzed in every detail (*KRAS* Reference Sequence NM_004985.4.). In the preoperative colonoscopic biopsy (B), the *KRAS* variant c.35G > A; p.Gly12Asp (variant allele frequency—VAF: 10.2%) was detected, similar to the first primary tumor sample (T/1) (*KRAS* c.35G > A; p.Gly12Asp, VAF: 40.6%). In the second colon tumor sample (T/2), wild-type *KRAS* was detected. The third colon tumor (T/3) had the *KRAS* variant c.34G > T; p.Gly12Cys (VAF: 16.2%) and the fourth primary tumor (T/4) had the *KRAS* variant c.35G > T; p.Gly12Val (VAF: 15.1%). The hepatic metastases removed at the date of the bowel surgery had different genotypes, including wild-type *KRAS* in the first tumor (M1/1) and pathogenic *KRAS* variant (c.34G > T; p.Gly12Cys, VAF: 19.1%) in the second tumor (M1/2). The same *KRAS* variant was confirmed (VAF: 6.4%) in the second liver metastasis (M2) resected 11 months later. In the liver metastasis from the third time point (M3, 18 months), no *KRAS* variants could be detected. Metastatic samples from the last two time points (M4, 27 months and M5, 36 months after primary surgery) presented again with the *KRAS* c.34G > T; p.Gly12Cys variant, although at very different allele frequencies (VAF: 2.0 and 32.1, respectively).

4.2.3. Examination of *TP53* gene variations

In addition to *KRAS*, the 15-gene solid tumor NGS panel indicated pathogenic *TP53* gene variants with a highly variable pattern. Altogether, 16 *TP53* variants could be detected in the 13 samples analyzed, with VAFs ranging between 2.9 and 72.8%. The *TP53* gene variant c.820G > T; p.Val274Phe was detected in the T/3 primary tumor and was identified in all metastatic tumors. M2 and M3 samples presented with low allele frequencies (7.1 and 3.14, respectively). In the last two metastatic samples (M4 and M5), this sequence variant occurred at highly enriched allele frequencies (72.8 and 45.6% VAF, respectively). Retrospective analysis of the p53 protein by immunohistochemistry of all histological samples resulted in different, but generally measurable degrees of immunopositivity throughout the course of the disease. Except for the initial biopsy sample (B) and primary tumors T/1 and T/4, all samples presented with high H-scores referring to a mutant-type reaction concordant with the NGS results.

4.3. Monitoring tumor heterogeneity in the *KRAS* gene in histopathological and liquid biopsy samples

Tumor heterogeneity is one of the most critical characteristics of cancer, significantly contributing to disease progression and therapy resistance. As we showed earlier, residual and recurrent tumor foci may represent genetically distinct subclones that are not always identified, as repeated sampling is usually limited. The analysis of circulating cell-free DNA (cfDNA) from peripheral blood plasma (liquid biopsy, LB) is an effective tool for monitoring genetic variations in the tumor.

In our study, we also examined genetic variability observed in patients with metastatic *KRAS* mutant colorectal cancer. Samples were collected between September 2020 and August 2022 from confirmed primary cases who underwent primary sampling. In the molecular tumor pathology laboratory at the University of Debrecen Pathology Institute, a total of 211 out of 490 (43.06%) colorectal tumor tissue samples were examined during the specified time to determine the *KRAS*, *NRAS*, and *BRAF* statuses.

Out of the examined *KRAS* mutant cases, multiple *KRAS* variants were confirmed in 12 out of 211 (5.68%) samples. In these cases, histopathological samples (primary tumor and metastasis) were examined using both StripAssay and Sanger sequencing, and NGS and StripAssays were performed on all LB samples.

The criteria for patient monitoring were met in three cases (sample collection before treatment, before the second cycle, and before the fourth cycle), allowing for prospective peripheral blood sampling and cfDNA isolation. The patients received combined bevacizumab chemotherapy due to their mutant *KRAS* status. The mutational status of *KRAS*, *NRAS,* and *BRAF* in primary tumor samples was assessed using high-specificity reverse hybridization technology (StripAssay) on all available tissue samples from cases 1–3. Mutations identified in the primary tumor were also confirmed by Sanger sequencing.

The VAFs of Sanger electropherograms were determined in all histological samples (cut-off >5%). The VAF of *KRAS* pathogenic variants ranged from 5 to 50% (mean 19.3%). The cfDNA concentrations ranged from 0.6 to 55.0 ng/µl (mean 6.33 ng/µl), indicating a high variability of DNA content.

4.3.1. Monitoring *KRAS* status from LB samples (Case 1)

A 66-year-old man with abdominal symptoms, bleeding, typical right colon tumor, multiple liver foci, loco-regional, mesenterial, and hepatic portal lymph node metastases described by CT findings was admitted to the surgery department. A right hemicolectomy was performed and a Grade 2 adenocarcinoma with a pathological stage pT3, pN1a with 1/15 positive lymph nodes was diagnosed. The patient was treated with bevacizumab and xelox therapy due to the positive *KRAS* mutation status. The first LB plasma sample was collected 33 days after surgery.

The *KRAS* mutation profile of the primary tumor resection samples (R1-R5), metastasis (M), and the three PB cfDNA samples were analyzed. In the R3 and R5 samples, two *KRAS* variants were detected (c.34G > T; p.Gly12Cys and c.35G > T; p.Gly12Val). In sample R1, only the c.34G > T; p.Gly12Cys mutation was identified. In samples R2, R4, and M, the other genotype was found (c.35G > T; p.Gly12Val). In the first and second LB plasma samples (LB1, LB2), a *KRAS* c.34G > T; p.Gly12Cys aberration was identified. The third plasma cfDNA (LB3) was negative for KRAS alterations.

The StripAssay results from the liquid biopsies were further validated by NGS. The 28 gene ctDNA panel analysis identified *KRAS* mutation c.34G > T; p.Gly12Cys as the single pathogenic variant in samples LB1 and LB2 with VAF frequencies of 3.0% and 1.5%, in complete agreement with the reverse hybridization screening method. The last plasma sample (LB3), similar to the

result of the reverse hybridization assay, demonstrated none of the previously described mutations.

4.3.2. Monitoring *KRAS* status from LB samples (Case 2)

A 70-year-old man was examined by rectoscope due to a tumor mass next to the anus causing nearly complete stenosis. Whole-body imaging (CT and MRI) revealed a tumor mass in the rectum area with regional perirectal involvement, lymphadenomegaly, and multiplex hepatic metastasis. Pathological examination of the biopsy identified adenocarcinoma Grade G2. Based on these results, the patient was diagnosed and treated with first-line neoadjuvant radiochemotherapy. Lymph node and liver metastases were not resectable, so no surgery has been performed to date. Bevacizumab with xelox treatment was started 189 days after the initial diagnosis, and the first blood sample (LB1) was taken at this time.

Reverse hybridization and NGS-based sequencing of the primary tumor revealed a *KRAS* c.38G > A, p.Gly13Asp and an *NRAS* c.37G > C, p.Gly13Arg variant. In contrast, the first plasma sample after the first line neoadjuvant treatment (LB1) presented with two new *KRAS* mutations at c.35G > A; p.Gly12Asp and c.35G > T; p.Gly12Val. Interestingly, in the second cfDNA (LB2), only the c.35G > C; p.Gly12Ala variant appeared. In the third plasma sample (LB3), the c.35G > C; p.Gly12Ala variant appeared and the c.35G > A; p.Gly12Asp variant reappeared just like in sample LB1. However, no *NRAS* mutations were found in any of the plasma cfDNAs.

In addition to the RAS variants, the following gene mutations could be detected using NGS: *MET* c.2908C > T; p.Arg970Cys, *NTRK1* c.1702C > T; p.His568Ter and *NTRK1* c.1730G > T; p.Gly577Val, which were continuously present throughout the follow-up samples. Close to 50% of VAF suspected a germline origin of these alterations; therefore, a tumor-free liver biopsy sample was tested to confirm the germline origin.

The *MET* NM_000245.4:c.2908C > T (p.Arg970Cys) variant is also known as NM_001127500.3:c.2962C > T (p.Arg988Cys) and is listed 23 times in ClinVar, mostly as a benign mutation. The *NTRK1* NM_002529.4:c.1702C > T variant is annotated as p.Gln568Ter with uncertain significance. The *NTRK1* NM_002529.4:c.1730G > T (p.Gly577Val) variant is identified as c.1712G > T using NM_001012331.2.

4.3.3. Monitoring *KRAS* status from LB samples (Case 3)

A 77-year-old man underwent a colonoscopic examination in 2015 that revealed an occluding tumor of the transverse colon with a biopsy finding of adenocarcinoma. Left

hemicolectomy with a metastatic lymph node was performed revealing adenocarcinoma grade G3, pT3, pN2b, and mutant *KRAS* status. After surgery, the patient received first-line adjuvant chemotherapy (Folfox), distant metastases and residual tumor were not detected at that time. In 2020, four years and five months later, an abdominal CT examination revealed a solid lesion in the pancreas, which was subsequently sampled. The histology and immunophenotype of the pancreatic lesion confirmed a metastasis originating from the colon adenocarcinoma. Bevacizumab and xelox therapy were decided and the first peripheral blood sample (LB1) was collected before initiating treatment. Similar to the previous cases, cfDNA testing was repeated after two (LB2) and four (LB3) cycles of the therapy regimen.

The primary tumor and pancreatic metastasis presented with the same c.38G > A; p.Gly13Asp *KRAS* variant while the lymph node metastasis collected during the primary surgery carried the c.34G > T; p.Gly12Cys variant. The original c.38G > A; p.Gly13Asp aberration was identified only in the first PB plasma cfDNA (LB1). In the second plasma sample (LB2), two new variants, c.34G > T; p.Gly12Cys (VAF: 2%) and c.35G > A; p.Gly12Asp (VAF: 2%), were identified. Moreover, in the third cfDNA, only the c.34G > T; p.Gly12Cys (VAF: 1.5%) aberration was detected.

4.4. Investigation of hypoxia and adaptation in treated (NAT) and untreated (UT) rectal adenocarcinoma

We examined 55 matched initial biopsies and post-treatment surgical samples that were obtained from patients with rectal adenocarcinoma undergoing preoperative neoadjuvant chemo-radiotherapy (NAT group). In addition, 34 matched biopsies and surgical samples were included from untreated rectal adenocarcinoma patients (UT group). Patients undergoing NAT received the following treatments based on the prescribed protocols: capecitabine monotherapy protocol 2500 mg/m2; Mayo protocol: fluorouracil (5-FU) 425 mg/m2 and calcium folinate (FOL) 20 mg/m2 and fluorouracil (5-FU) monotherapy protocol 500 mg/m2. All patients received radiotherapy (total dose, 50.4 Gy, 1.8 Gy/day, 5 days/week) together with chemotherapy. Two cases received only radiotherapy.

The selection criteria included treatment of at least TNM Stage II and both the biopsy and the resection sample contained representative tumor tissue for accurate analysis of the CAIX expression. Basic clinical and histopathological parameters, including sex, tumor grade and

depth, presence/absence of metastasis, mucinous phenotype, KRAS status, type of neoadjuvant treatment, and tumor regression grade, were compared.

4.4.1. CAIX expression pattern in rectal adenocarcinoma samples

CAIX expression was highly variable in the rectal adenocarcinoma samples. In general, selective staining of tumor cells was observed, while the normal/unaffected rectal mucosa was negative for CAIX. Within the tumor areas, characteristic and selective cell membrane staining was observed with variable intensity. Regarding the distribution in individual cases, a strong association with necrotic foci was detected, including strong perinecrotic tumor cell labeling and a dynamic loss toward the more distant layers. The relationship with necrosis was closely evaluated in surgical resection samples: NAT resections presented with necrosis in 40 out of the 55 evaluated samples (72.72%). CAIX positivity was observed in 24 samples (60%) around the perinecrotic area and no CAIX expression was identified in 16 (40%) cases. In the 34 cases of UT resected samples, 29 out of the 34 samples (85.29%) with necrotic areas were identified, including 26 samples (89.65%) with CAIX positivity around the necrotic area; 3 (10.34%) samples remained negative for CAIX. Interestingly, CAIX expression in areas with severe dysplasia was also frequently identified, well separated from the normal epithelium or low-grade changes. Moderate to severe dysplasia with characteristic membrane CAIX expression was identified in 5/55 (9.09%) NAT surgical specimens and 5/34 (14.7%) UT surgical specimens.

Regarding the neoplastic cell clusters of glandular epithelial origin, CAIX expression within the tumor neostroma was also observed. Increased CAIX expression within the tumor stroma was observed in 24/55 (43.63%) NAT surgical samples while 31/55 (57.37%) samples were stroma CAIX-negative. In the 34 UT cases, only 9/34 (26.47%) of the surgical specimens showed stromal positivity for CAIX.

4.4.2. Expression dynamics of CAIX in neoadjuvant-treated (NAT) rectal adenocarcinomas (n = 55)

CAIX expression was quantified in all samples by defining the proportion of positive labeling in the percentage of the tumor area. CAIX labeling in the individual samples from before and after treatment was compared. In the statistical analysis of the NAT biopsies and the NAT surgical specimens, we found a significant increase in CAIX following treatment (mean 21.8 ± 24.9 SD vs. 39.4 ± 29.4 SD) (Wilcoxon matched rank test $p < 0.0001$). Furthermore, a positive

mathematical correlation between the biopsy and surgical samples was demonstrated (Spearman correlation test $p < 0.0001$, rho: 0.5654).

For further comparison, the 55 NAT and 34 UT rectal carcinomas were split exactly by the median CAIX percentage and classified as CAIX-low and CAIX-high carcinomas. Available clinicopathological data were evaluated to determine the potential differences associated with CAIX status. Most importantly, a significant correlation between CAIX expression and mutant *KRAS* status was established (biopsy $p < 0.0151$; surgical specimens $p < 0.0316$), but no correlation with any other clinicopathological parameters was found.

4.4.3. Immunohistochemical CAIX expression of untreated (UT) rectal adenocarcinoma (n = 34)

Untreated rectal adenocarcinoma samples were evaluated the same way as previously described. CAIX expression was highly variable with values ranging from 0 to 80%. In contrast to the NAT tumor group, statistical analysis of the UT biopsies and UT surgical specimens did not result in statistical differences regarding CAIX expression (mean 15.0 ± 21.3 SD vs. 20.0 ± 23.02 SD, Wilcoxon matched rank test $p < 0.073$) but the correlation between the biopsy and surgical samples was well established (Spearman correlation test $p < 0.0001$, rho: 0.8077).

Classification as CAIX-low and CAIX-high samples based on the median CAIX score in UT samples was followed by the analysis of related clinicopathological data. Similar to the NAT group of carcinomas, we detected a statistically significant correlation between CAIX expression and the *KRAS* status when biopsy CAIX values were considered (biopsy $p < 0.0454$; surgical specimens $p < 0.0921$). All other evaluated parameters were independent of the CAIX status.

4.4.4. Tumor Regression Grade and CAIX in NAT Biopsy and NAT Surgical Specimens (n = 55)

To investigate the effects of neoadjuvant treatment, we determined the tumor regression grade (TRG) according to the WHO recommendations in the NAT surgical specimens obtained after therapy. The resulting TRG correlated with CAIX expression in the same post-treatment sample, and also with the pretreatment biopsy scores for potential predictive features. When evaluating pretreatment biopsies, the majority of TRG2–3 cases were associated with the CAIX-low phenotype (64.3 and 61.9%), and TRG4–5 cases were associated with the CAIX-high phenotype (71.4 and 83.3%), indicating an increased potential of treatment failure in initially

CAIX-high tumors. In contrast, CAIX expression was generally increased following NAT, contributing to the elevated scores in residual tumors with low regression grades. Nevertheless, tumors with limited/no treatment response (TRG4–5) presented with the CAIX-high phenotype (71.4% and 100.0%).

4.4.5. *KRAS* Status and CAIX Expression in NAT and UT

The *KRAS* mutational profile was determined in all rectal adenocarcinoma samples that were included to determine the relationship with CAIX expression. The comparison of KRAS mutant and wild-type tumor groups confirmed significant differences; KRAS mutant samples presented with much higher CAIX scores and the correlation of CAIX expression was statistically significant in NAT biopsies, surgical samples, and UT biopsies, but not in UT surgical samples, according to the Fisher's exact test ($p < 0.05$).

The *KRAS* mutant rectal adenocarcinomas were significantly more represented in the CAIX-high group in both NAT and UT samples. Thus, we also performed a combined analysis using the exact CAIX scores related to the *KRAS* status in the unified rectal carcinoma cohort (n = 89, mutant n = 46, and wild type n = 43). As expected, the CAIX expression score was significantly higher in *KRAS* mutant cases (initial biopsies: wild-type mean 16.28 ± 24.34 vs. mutant mean 27.67 ± 24.60; Mann–Whitney test p = 0.0138; NAT & UT surgical samples: wild-type mean 25.51 ± 25.10 vs. 43.15 ± 27.62; Mann–Whitney test p = 0.002).

4.4.6. Patient survival and CAIX expression in NAT and UT samples

Next, we examined the correlation between CAIX expression in NAT biopsy and surgery samples with overall survival (OS) and progression-free survival (PFS). According to the statistical analysis (Kaplan–Meier curves), no significant differences in OS or PFS were detected between the CAIX-high and CAIX-low groups (OS biopsies p = 0.9769 vs. surgical samples p = 0.6585; PFS biopsies p = 0.2129 vs. 0.7382). Similarly, the evaluation of UT biopsy and surgery samples did not show a significant correlation between OS and PFS and the CAIX-high and CAIX-low expression groups (OS biopsies p = 0.1620 vs. surgical samples p = 0.7940; PFS biopsies p = 0.4830 vs. p = 0.1380).

4.4.7. Cell proliferation and CAIX expression

In addition to the examination of CAIX expression, we also focused on observing the expression pattern of proliferation activity before and after treatment. The proliferation

activity expression in untreated biopsies and surgical specimens (n = 34) showed a strong correlation (average 55.8 ± 16.54 SD vs. 57.79 ± 19.97 SD), which was statistically significant (Wilcoxon matched rank test p <0,1671,1671). Furthermore, a positive correlation (trend) was confirmed between the untreated biopsies and surgical samples (Spearman correlation test p < 0.0001, rho: 0.9371).

In contrast, the initial proliferative activity of untreated biopsies and treated surgical specimens was significantly reduced by treatment. A statistically significant difference was confirmed for Ki-67 expression (mean 56.82 ± 24.29 SD vs. 32.04 ± 23.62 SD Wilcoxon matched rank test p < 0.0001), but only a slight correlation was detected between untreated biopsies and treated surgical specimens (Spearman correlation test p < 0.404 rho: 0.2772).

Furthermore, during the examination of CAIX and Ki-67 expression patterns within rectal tumors, we observed that in most cases, higher Ki-67 expression values were associated with lower CAIX expression values in untreated cases. However, in treated cases, higher CAIX expression values were associated with lower Ki-67 expression activity. Ki-67 expression values were significantly higher in the biopsy samples compared to the CAIX expression pattern (initial biopsies: CAIX average 21.78 ± 24.97 SD vs. Ki-67 average 56.82 ± 24.29 SD; Mann-Whitney test p < 0.0001; NAT surgical samples: CAIX average 39.44 ± 29.47 vs. Ki-67 average 32.04 ± 23.62; Mann-Whitney test p = 0.2630).

5. Main findings and conclusions

In summary, the main findings of this Ph.D. work are as follows:

- Multiple primary tumors in the colon have different metastatic potential. In our study, a single tumor site served as the source of all metastases, emphasizing the importance of the molecular biology of the primary tumor.
- When examining multiple primary colorectal cancers and distant metastases within a single patient, it is advisable to evaluate each primary and metastatic focus separately because individual foci have unique genetic profiles. Different foci can be considered separate entities, and treatment options should be considered based on the foci's heterogeneity.
- Some tissue biopsies may be non-informative or clinically relevant genetic changes cannot be identified, and rebiopsy can be cumbersome and not always feasible as an invasive sampling method. Therefore, the diagnostic option of liquid biopsies is a preferred and increasingly realistic alternative.
- Liquid biopsies, the analysis of cfDNA in peripheral blood, allows continuous genetic monitoring of the tumor and identification of different predictive genes.
- A liquid biopsy, especially in metastatic CRCs, is indispensable for identifying resistance to anti-EGFR therapy and various drug resistance mechanisms, monitoring heterogeneity over time, and selecting personalized therapy.
- The emergence and disappearance of *KRAS* mutant subclones during the course of colorectal cancer shows dynamic variations more often than expected.
- CAIX showed highly specific cell membrane localization and heterogeneous expression in tumor cells in untreated and neoadjuvant-treated rectal tumors under hypoxic stress.
- Significant increases in CAIX expression were observed in post-treatment tissue samples, whereas no differences were observed in untreated samples.
- The presence of *KRAS* mutations was associated with high CAIX expression both before and after treatment. The adaptive mechanism signaled by CAIX is likely to play a prominent role in the regulation of metabolism and resistance of KRAS mutant rectal tumor cells.

- In pre-treatment samples, high cell proliferation values were accompanied by lower CAIX expression, whereas in post-treatment samples, hypoxic stress induced by therapy resulted in a significant decrease in proliferative activity, with CAIX expression increasing in a vast majority of cases.
- CAIX may be potentially useful for characterizing post-treatment tumors, but its predictive role remains controversial in CRC.

6. Publication list

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Candidate: Emese Sarolta Bádon
Doctoral School: Doctoral School of Clinical Medicine

List of publications related to the dissertation

1. **Bádon, E. S.**, Beke, L., Mokánszki, A., András, C., Méhes, G.: Carbonic Anhydrase IX Expression and Treatment Response Measured in Rectal Adenocarcinoma Following Neoadjuvant Chemo-Radiotherapy.
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2. **Bádon, E. S.**, Mokánszki, A., Mónus, A., András, C., Méhes, G.: Clonal diversity in KRAS mutant colorectal adenocarcinoma under treatment: Monitoring of cfDNA using reverse hybridization and DNA sequencing platforms.
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3. **Bádon, E. S.**, Mokánszki, A., Mónus, A., András, C., Damjanovich, L., Méhes, G.: Quadruplicate Synchronous Adenocarcinoma of the Colon With Distant Metastases-Long-Term Molecular Follow-Up by KRAS and TP53 Mutational Profiling.
   Diagnostics (Basel). 10 (6), 1-13, 2020.
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List of other publications

4. Matolay, O., **Bádon, E. S.**, Balázs, L., Juhász, P., Csonka, T., Méhes, G.: A szénsavanhidráz IX szerepe a malignus daganatok progressziójában - lehetséges terápiás célpont?
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6. Mokánszki, A., Chang Chien, Y. C., Mótyán, J. A., Juhász, P., **Bádon, E. S.**, Madar, L., Szegedi, I., Kiss, C., Méhes, G.: Novel RB1 and MET Gene Mutations in a Case with Bilateral Retinoblastoma Followed by Multiple Metastatic Osteosarcoma.
Diagnostics. 11 (1), 1-11, 2021.
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Diagnostics. 10 (1), 1-11, 2020.
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