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Patogeneze kolorektálního karcinomu

Pathogenesis of colorectal cancer

Disertační práce

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Contents

I. Summary

Despite significant advancements in the diagnosis and treatment of colorectal cancer, the statistics regarding its incidence and mortality remain unsatisfactory. Initially, we focused on the incidence and pathogenesis of colorectal carcinoma (CRC) after liver transplantation, using the unique advantage, that IKEM clinic is the facility with the highest number of liver transplants in the Czech Republic, enabling us to utilize our own data. In addition, we concentrated on colorectal precancerous lesions, specifically colorectal adenomas. The central idea was to understand the progression of colorectal adenomas to subsequent stages and to identify sufficiently sensitive marker that would detect adenomas, not just carcinomas.

Hypotheses: **I**. The liver transplant recipients exhibit an increased risk of developing CRC compared to the general population. **II**. Mutation profiles of colorectal low-grade adenomas, high-grade adenomas, and carcinoma in situ are mutually different. **III**. Chromosomal instability (CIN) is already present in colorectal adenomas before they progress to carcinoma stages. **IV**. The long non-coding RNA Metastasis Associated Lung Adenocarcinoma Transcript 1 (MALAT1) is a diagnostic and prognostic biomarker in colorectal adenomas and carcinomas.

Aims: **I.** Evaluate the incidence of CRC in liver transplant patients based on literature data and supplement it with experience from our transplant center in IKEM. **II**. Evaluate the differences and similarities in mutation profiles of low-grade adenomas, high-grade adenomas, and carcinoma in situ. **III**. Detect and characterize structural and numerical chromosomal aberrations in colorectal adenomas in comparison to the adjacent mucosa of the same patient. **IV**. Determine the genetic expression profile of MALAT1 in colorectal adenomas, carcinomas, healthy colon mucosa and plasma of the same patient.

Results: **I**. The incidence of CRC in liver transplant patients is equal or slightly higher than in the general population. The risk is significantly higher in liver transplant patients with primary sclerosing cholangitis (PSC), both with and without ulcerative colitis (UC). **II**. In the precancerous lesions the mutation frequencies of APC, KRAS, and TP53 genes increase from low-grade to high-grade adenomas towards in situ carcinomas. **III**. We documented a chromosomal gain of locus for MALAT1. **IV**. Our work indicates that MALAT1 could serve as a non-invasive (liquid-biopsy) biomarker for detection of colorectal adenomas and carcinomas and also as a predictor of chemotherapeutic response to oxaliplatin.[1-4]

II. Summary in Czech language:

Navzdory významným pokrokům v diagnostice a léčbě kolorektálního karcinomu (CRC) zůstávají statistiky týkající se jeho výskytu a úmrtnosti neuspokojivé. Tato disertační práce byla vypracována v průběhu pěti let. Zpočátku jsme se zaměřili na výskyt a patogenezi CRC po transplantaci jater, využívajíc jedinečnou výhodu, že IKEM je zařízením s nejvyšším počtem transplantací jater v České republice, což nám umožnilo využít naše vlastní data. Dále jsme se soustředili na prekancerózní léze tlustého střeva a konečníku, konkrétně na adenomy a in situ karcinomy. Cílem bylo porovnat mutační profily „low-grade“, „high-grade“ adenomů a karcinomů in situ, u genů spojených s rozvojem CRC. Hlavním cílem bylo pochopit progresi adenomů do dalších stadií a pokusit se o identifikaci markeru, který by byl dostatečně citlivý pro časnou detekci adenomů, ne až karcinomů

Hypotézy: I. Pacienti po transplantaci jater mají zvýšené riziko vzniku CRC ve srovnání s běžnou populací. **II**. Mutační profily low-grade, high-grade adenomů a karcinomu in situ se liší. **III.** Chromozomální nestabilita (CIN) je přítomna v adenomech již před jejich progresí do karcinomu. **IV**. Dlouhá nekódující RNA Metastasis Associated Lung Adenocarcinoma Transcript 1 (MALAT1) je diagnostickým a prognostickým biomarkerem u kolorektálních adenomů a karcinomů.

Cíle: I. Vyhodnotit výskyt CRC u pacientů po transplantaci jater na základě literárních dat a doplnit je o zkušenosti našeho transplantačního centra v IKEM. **II**. Vyhodnotit rozdíly a podobnosti v mutačních profilech low-grade, high-grade adenomů a karcinomu in situ. **III**. Detekovat a charakterizovat strukturální a numerické chromozomální aberace v adenomech tlustého střeva a konečníku a ve srovnání se zdravou sliznicí střeva téhož pacienta. **IV**. Stanovit genetickou expresi MALAT1 v adenomech, karcinomech, ve zdravé sliznici tlustého střeva i v plazmě téhož pacienta.

Závěr a aktuální výsledky: I. Výskyt CRC u pacientů po transplantaci jater je srovnatelný nebo mírně vyšší než u běžné populace. Riziko je výrazně vyšší u pacientů s primární sklerozující cholangitidou (PSC), a to jak s přítomností ulcerózní kolitidy (UC), tak bez ní. **II**.U prekancerózních lézí se frekvence mutací genů APC, KRAS a TP53 zvyšují od low-grade, přes high-grade adenomy až ke carcinoma in situ. **III**. Zdokumentovali jsme chromozomální amplifikaci a zisk lokusu pro MALAT1. **IV**. Naše práce dále naznačuje, že MALAT1 by mohla sloužit jako neinvazivní biomarker (liquid-biopsy) pro detekci kolorektálních adenomů a karcinomů a také jako prediktor odpovědi na oxaliplatinu.[1-4]

III. State of art

Epidemiology

Cancer is still one of the main causes of death at the beginning of the 21st century. Around 18 million people became newly diagnosed with cancer worldwide in 2018. There were 9.5 million cancer-related Deaths registered. Colorectal cancer (colorectal cancer, CRC) is the fourth most commonly diagnosed and third most deadly cancer according to International Agency for research on Cancer. Almost 1,9 million new cases were diagnosed in 2018 and almost nine hundred thousand patients died because of this cancer. In Czech Republic is the incidence by men 42/100 000 and 25/100 000 by women, which causes the CRC to become the second most diagnosed cancer in both sexes. Although the CRC is one of the best investigated malignant diseases in humans and there is a favorable course of disease if diagnosed and treated early, the mortality lies by 17/100 000 by men and 9/100 000 by women. In addition to genetic and environmental predisposition factors, colon cancer is above all a lifestyle disease that is increasingly occurring in countries with a high development index. Lack of exercise, obesity, alcohol and consumption of red and processed meat are suspected of provoking inflammatory bowel diseases, modulating the microbiome and thus favoring the development of colon cancer, with the risk of illness increasing with age. This western style of life consequently leads to an increase in annual numbers of new cases.

Etiology

Seventy to ninety% of colorectal neoplasms arise sporadically. This is due to multifactorial factors. The lifestyle of those affected plays an important role here. Nutrition in particular is essential for the disease. Depending on its composition, it can promote the development of the disease or counteract it. A large number of studies have shown that high fat and red meat intake is a risk factor for the development of intestinal neoplasms. Particularly a regular consumption of red meat is associated with the development of intestinal carcinomas. Statistical analysis of several independent studies showed an increased risk of 35% for colorectal neoplasia with high consumption of red meat. The consumption of plenty of fiber, fruits and vegetables, however, has a protective effect [5]. Also another metabolic diseases such as diabetes, obesity, hyperinsulinemia and insulin resistance caused by problematic nutritional behavior are also associated with an increased risk of colorectal neoplasia [6]. Long-term nicotine abuse (at least

16 years) was also identified in a study as a risk factor for the development of neoplasia. In contrast, in a study that examined the relationship between moderate alcohol consumption (max. 30 g / d) and increased risk of neoplasia, no significant increase in risk was found compared to the abstainer control group after lifestyle and dietary factors of the two groups had been adjusted [7]. In addition to lifestyle, long-standing inflammatory bowel diseases such as ulcerative colitis and Crohn's disease are important risk factors for the development of colorectal cancer. According to estimates, ≤ 2% of colorectal cancers develop on their soil annually [8]. The family disposition represents a small proportion of colorectal neoplasias. Here, ≤ 1% of cases are related to familial adenomatous polyposis and 2-3% to hereditary nonpolyposis colorectal cancer syndrome (Lynch syndrome I, II)

Symptoms

Colorectal cancer grows usually slowly and remains symptom-free for a long time. Typical complaints often only appear in advanced stages. In addition to B symptoms (weight loss, night sweats, fever) and non-specific general symptoms (weakness, fatigue), changes in bowel habits are typical. The symptoms depend on the type, location and extent of the tumor. Fatigue and weakness due to anemia based on occult bleeding may be the person's only symptoms. A tumor in the left part of the colon leads more often to an obstruction because of the smaller diameter than on the other side. Also, the stool is already semi-solid on the left side. Most colon tumors bleed, usually slowly. The stool may be streaked or mixed with blood, but often the blood is not visible. In rectal cancer, hematochezia is often the first symptom. Even if the patient has a diagnosis of hemorrhoids or diverticulosis, if there is bleeding from the rectum, differential diagnosis of a cancer has to be set. Pain by bowel movements and the feeling that the bowel has not been completely emptied can be further symptoms of rectal cancer. Other symptoms include cramping, diarrhea, paradoxical diarrhea, obstipation, changing of diarrhea and obstipation, ileus and paraneoplastic syndromes. Due to metastases also other symptoms can be seen, such as icterus and liver failure with advanced liver metastasis, tussis and dyspnoe with pulmonary or pleural metastasis, bone pain with skeletal metastases or neurological symptoms with cerebral metastasis.

Prevention

The recommendations for the prevention of colorectal cancer relate to the identified risk factors so far. One of the most important is the removal of adenomas. Removal of adenomas is a preventive measure by removing precursor stage of the carcinoma. This intervention is carried out as part of the endoscopic early detection measures. The change of lifestyle habits is recommended with measures such as weight loss in overweight people, regular physical exercise, minimal alcohol consumption or no smoking. Consumption of fiber (30 g/day), fruits and vegetables, calcium and vitamin B6 and avoiding red meat is also recommended.

Acetylsalicylic acid

The most extensive data for drug prevention are available for acetylsalicylic acid (ASA). With regular users of ASA at a dose of ≥75 mg / day, the rate of colorectal cancer is about half lower than in comparison groups [20]. These and numerous other studies on the association of colorectal cancer and certain forms or components of the diet, micronutrients, electrolytes or on medications such as low-dose ASA or COX-2 inhibitors have so far not been sufficiently validated for a specific positive recommendation for prevention, so that acetylsalicylic acid should not be used for primary prevention of colorectal cancer in the asymptomatic population [9].

Vitamin D

In addition to influencing bone metabolism, long-term positive effect of higher blood levels of vitamin D in the prevention of colorectal cancer was expected. The assumed mechanism of action includes inhibition of the Wnt/β-catenin pathway in tumor cells or modulation of cancer-associated fibroblasts (CAFs). However, the results of recent prospective studies of this fat-soluble vitamin showed often dissimilar results. These discrepancies could be caused by insufficient population size, duration of supplementation or noncompliance. The results of a study by the American Cancer Society further reveal the role of vitamin D[10]. Volunteers were measured 25-hydroxyvitamin D blood-levels. These data were obtained prior to diagnosis of colorectal cancer and only one standardized laboratory and identical assay were used to measure concentrations. The results were related to the concentration of 25-hydroxyvitamin D in the

blood (50-62.5 nmol/l) which are considered to be correct for bone metabolism. Subsequently, a total of 5,700 cases of colorectal cancer and more than 7,000 controls were studied, with a follow-up of 5.5 years. The study showed that concentrations below 30 nmol/l were associated with a 31% increase in the risk of developing colorectal cancer, while concentrations above 75 nmol/l reduced this risk by 19%, concentrations above 87.5 nmol/l reduced the risk even by 27%. The results of this study suggest that the blood vitamin D concentration sufficient for a properly functioning bone metabolism may not be sufficient to reduce the risk of colorectal cancer.

Types of colorectal carcinoma

From a genetic perspective , CRC is a heterogeneous disease with two main types. The primary representative is the so-called "sporadic" CRC, accounting for 70-90% of all CRC cases. Sporadic CRC results from the accumulation of multiple genetic and epigenetic changes caused by somatic mutations and chromosomal instability, which themselves may be an indirect consequence of environmental factors. This process can take years and is characterized by the development of colorectal adenoma initially, with subsequent mutations leading to colorectal carcinoma. This phenomenon of stepwise mutation accumulation is known as the "adenoma-carcinoma sequence" and was described as early as the 1990s by Vogelstein and Fearon. [11] This pathway accounts for the vast majority of CRC cases.

The second representative is the so-called "hereditary" CRC, where mutations are inherited from parents to offspring. This form occurs in 5-30% of cases in the population. [12, 13] Representatives of hereditary CRC include Lynch syndrome, also known as HNPCC (hereditary non-polyposis colorectal cancer), familial adenomatous polyposis (FAP), MUTYH-associated polyposis, and other polyposis syndromes.

The basis of the hereditary non-polyposis form of CRC, or Lynch syndrome, involves mutations in mismatch repair genes (MMR) responsible for recognizing and repairing mismatched base pairs. This leads to the accumulation of replication errors and the development of CRC typically at a younger age without the occurrence of preceding adenomas. Lynch syndrome represents the most common hereditary form of CRC, accounting for 1-3% of all CRC cases [14].

Familial adenomatous polyposis (FAP) is characterized by the presence of a large number of colorectal adenomas with a high likelihood of malignant transformation later on. It is

accompanied by so-called extraintestinal symptoms in the form of osteomas and skin lesions and is associated with an increased risk of other malignancies. It arises due to mutations in the APC gene and is inherited in an autosomal dominant pattern. FAP represents about 1% of all CRC cases. [15] There is also a clinically milder form of FAP known as attenuated FAP (aFAP), where fewer polyps develop later in life, along with less frequent extraintestinal symptoms.

Similarly, MUTYH-associated polyposis is very similar. Patients typically have dozens of adenomas, but the likelihood of malignant transformation is lower. The disease has an autosomal recessive pattern of inheritance. It is caused by mutations in the MUTYH gene, which is part of the mismatch repair (MMR) system. The resulting MUTYH protein is responsible for recognizing and correcting mismatched guanine and adenine pairs. [16, 17]

Histology and grading

More than 90% of colorectal carcinomas are adenocarcinomas originating from epithelial cells of the colorectal mucosa. In World Health Organization classification are other rare types of colorectal carcinomas included, such as NET, NEC, Mesenchymal tumors, hamartomas, Lymphomas and GISTs.

Adenocarcinoma

Conventional adenocarcinoma is characterized by the formation of glands, which forms the basis for the histological classification of tumors. The former grading system which included 4 types (well differentiated adenocarcinoma >95% gland formation, moderately differentiated adenocarcinoma 50-95% gland formation, poorly differentiated adenocarcinoma with <50% gland formation and Undifferentiated, where no gland or mucin formation can be found) was reduced to 2-tiered grading system so that today we distinguish low-grade adenocarcinoma, where >50% of the tumor forms glands and high-grade adenocarcinoma with a gland formation of <50%. Tumor grade is considered as stage-independent prognostic factor, but the data shows, that a high grade lesion is associated with poor patient survival [18]. This conclusion of histologic grading should only be used on conventional adenocarcinoma. Other histological variants may have high grade morphology, but behave like low grade tumors, for example, because of their MSI status. Invasive carcinoma typically penetrates the submucosa through the muscularis mucosae and can be often seen near to submucosal blood vessels. Other features which can be seen by the invasion, is fibrous proliferation that surrounds tumor cells or

characteristic necrotic deposits in the glandular lumen. These special signs can be useful by suggesting the primary tumor source when metastasis of unknown origin occurs. In contrary to other parts of the gastrointestinal tract (esophagus, stomach and small intestine) where already the mucosal invasion is sufficient for the diagnosis of invasive carcinoma, in the colorectum a submucosal invasion is necessary for the diagnosis of a pT1 tumor. The reasons for another biological behaviour are not completely clear. One of the explanations is the relative lack of lymphatic vessels, so that there is no risk of nodal or distant metastasis in an invasion restricted to the lamina propria and muscularis mucosae. As a prevention of unnecessary surgical intervention, the pathologists call the intramucosal carcinoma also as a high-grade dysplasia.

Mucinous adenocarcinoma

Mucinous adenocarcinomas are the second most common histological subtype of colorectal cancer (about 5%) and are diagnosed when mucus component occurs in > 50 % of the overall tumor volume. The mucinous subtypes have a worse prognosis than non-mucinous carcinomas. One of the reasons may be facilitation of the penetration of a tumor through the colon wall due to extracellular mucin [19]. The intermingling of the mucous component is not rare also in other types of adenocarcinomas. Mucous areas can be found in the tumor tissue in around 30% of patients with colorectal cancer [20]. Mucinous carcinomas have a predilection for the right side of the colon. They also have a poor responsiveness to neoadjuvant and adjuvant chemotherapy [21, 22].

Other types

Other types of adenocarcinomas such as signet ring cell, medullary, micropapillary, serrated, cribriform comedo-type are infrequent. The significance of these morphologic variants lies in the prognosis. As an example, signet ring cell carcinoma is an aggressive subtype with a very poor prognosis. On the other hand, the medullary adenocarcinoma has a relatively favorable prognosis.

Diagnostics

FOBT - fecal occult blood test

Examination of the fecal occult blood test using the guaiac test (gFOBT) lowers cancer-specific mortality [9, 23]. Immunochemical tests for occult blood (iFOBT) are more sensitive. From the beginning the principle of FOBT was based on the detection of hemoglobin, therefore it was necessary to eliminate the possibility of false positive results, which were caused by the

presence of hemoglobin contained in the diet (e.g. in some vegetables, etc.). Modern iFOBT tests can detect this difference.

gFOBT

The guaiac test (gFOBT) is based on a peroxidase reaction, which is burdened with false positives (e.g. after eating red meat) and false negative results (e.g. after taking a higher dose of antioxidant like vitamin C). Therefore, a diet with a restriction on certain foods must precede the examination. In large randomized trials, gFOBT showed a reduction in CRC mortality of 15-21% at 2 years and 33% at one-year intervals [23].

iFOBT/FIT – fecal immunochemical test

These tests are based on the direct detection of human hemoglobin so that patients do not need to be restricted in their diet. Immunochemical tests have higher sensitivity and specificity compared to gFOBT, their measurement can be automatised and cut off can be set. They are therefore considered for a method of choice.

Faecal DNA-based tests

Faecal DNA tests detect mutant or abnormal DNA from neoplastic colorectal lesions which are excreted in the stool. Until now, no single gene has been identified that is present in all neoplastic colorectal lesions. Because of that a whole panel of DNA markers is being used. This panel usually include mutant K-ras, mutant APC, mutant p53, BAT-26 and long DNA. [24, 25]

A large amount of studies had been carried out to compare the use of a faecal DNA tests with use of FOBT for detecting CRC. In one, overall 5486 subjects were enrolled with 4404 completing the study. Of the 31 invasive cancers found, the DNA panel detected 16, whereas FOBT detected only 4 ($p = 0.003$). Of the 71 invasive cancers and adenomas diagnosed with high grade dysplasia, the DNA panel detected 29 while FOBT detected only 10 ($p < 0.001$). In subjects with negative findings on colonoscopy, the DNA panel had a specificity of 94.4% with FOBT giving a specificity of 95.2%. This study documented that neither technique detects the majority of neoplastic lesions. But it has to be emphasized that the faecal DNA test displayed a higher sensitivity than FOBT without reduced specificity [26, 27].

Endoscopic method

Colonoscopy

Colonoscopy is considered the gold standard of colorectal cancer screening methods. The advantage lies in its ability to examine the entire colon and at the same time the possibility of removal of incidental polyps. In a systematic review and meta-analysis of 25 diagnostic studies providing data on 9223 patients with a cumulative CRC prevalence of 3.6 percent (414 cancers), the sensitivity of optical colonoscopy for detection of CRC was 94.7 percent (178 of 188, 95%) [28]. Thus, the missing rate was 5.3 percent. Colonoscopy can be used as a single screening method (so-called primary screening colonoscopy, PSC) or it can follow the positive primary screening result (so-called FOBT+ colonoscopy / screening colonoscopy). Colonoscopy itself is preceded by cleansing the intestine with laxatives. For most patients the preparation with polyethylene glycol (PEG) in high-volume or low-volume form is recommended for its good effect while minimizing side effects. Studies comparing the use of PEG-electrolyte solutions (PEG-ELS) in high volume (4-L) PEG-ELS form with low-volume (2-L) PEG-ELS preparation showed similar efficacy [29, 30]. Poor intestinal preparation significantly increases the risk of colorectal neoplasia being missed and the need to repeat the procedure. Unfortunately, up to 25% in U.S. population undergoing colonoscopy have inadequately prepared colon [31]. Screening colonoscopy with poor or very poor intestinal preparation cannot be considered conclusive.

Rectoscopy

In rectal cancer, rigid rectoscopy with the height of the lower margin of the tumor should be an integral part of preoperative diagnostics [9].

Endosonography

For local staging of rectal cancer, an endosonography should be carried out together with MRI. In the case of a T1 carcinoma the endosonography has the highest sensitivity and specificity in comparison with other imaging methods. On the other hand, it is not technically possible in cases of high-grade stenoses or if the tumor is localized in the proximal rectum. CT is not suitable for T1 carcinomas [9].

Cystoscopy

Cystoscopy can be carried out if bladder infiltration is suspected.

Imaging methods

Computed Tomography (CT)

A meanwhile established technique for preoperative staging and preparation for surgery is the computer tomography. It should be performed with both intravenous and oral contrast [32] and preferably in the range of thorax, abdomen and pelvis. The thorax CT can identify pulmonary metastases. At the time of primary diagnosis, they occur in approximately 4% to 9% of patients [33, 34]. On the basis of a total of 13 studies, the sensitivity for detection of tumor invasion beyond the bowel wall (T3–T4) was 83–95%, specificity 62–75%. For nodal involvement (N+) was the sensitivity 59–81% and specificity 46–83% [35]. In addition to FOBT, so-called virtual colonoscopy using computer tomography (CT colonography) is an alternative method to endoscopy as part of cancer screening examinations. The main disadvantage is that neither biopsy nor polypectomy can be performed and the sensitivity is lower (polypous lesions are visible from a size of 6 mm) than by standard colonoscopy [36]. This method is not standard, however, in indicated cases it is a valuable tool for precise location of the tumor and in detecting synchronous lesions or polyps.

Magnetic Resonance Imaging (MRI)

Abdominal CT or MRI is indicated for staging of CRC. The advantage of the MRI is its sensitivity of 80-88% and a specificity of 93-97% by detecting liver metastases [37]. A better outcome in detecting liver metastases has only PET or PET/CT.

An irreplaceable place has MRI for local staging of rectal cancer. The statement about the distance to the mesorectal fascia has important prognostic relevance. If the mesorectal fascia is infiltrated or the tumor reaches up to 1mm from the fascia, the risk of local recurrence is significantly increased [38]. The role of Endosonography by rectal cancer was discussed earlier.

PET, PET/CT

Positron emission tomography (PET) is a beneficial method for the detection of distant metastases in colorectal cancer. It is not recommended as a staging method by default. If, however, abnormalities appear on CT or MRI that are considered suspicious, but not specific to metastasis, PET or PET/CT examinations may be considered as another diagnostic option. PET/CT should not be performed within 4 weeks of systemic chemotherapy or antibody therapy because the sensitivity is significantly reduced [9].

Sonography

For the diagnosis of colorectal liver metastases, native and especially contrast-enhanced ultrasound is becoming increasingly important. Abdomen sonography has a sensitivity between 63 and 86% and a specificity of 98% [39]. Contrast-enhanced ultrasound of the liver reaches sensitivity between 83 and 86%. The specificity ranges between 94 and 98%, but requires the appropriate technical equipment and a high degree of experience [40].

Tumor markers

Tumor markers are defined as predominantly proteinaceous molecules that are present in the body as a result of development of a malignant process. Their occurrence in cancer tissue and body fluids is related to tumor growth. They are produced by either the tumor itself or other tissues in response to a malignant process in the body. The classification of tumor markers can be performed from different perspectives. Most often they are classified by tissue origin or function. According to the tissue origin we can divide the tumor markers into oncofetal antigens, tissue and organ specific antigens and non-specific antigens, enzymes, and hormones [41].

Oncofetal antigens are substances produced in the fetal period. They are also produced by the placenta after birth. In adulthood they are no longer formed and their production is associated with the appearance of cancer. Antigens appear early during ontogenesis and postnatally are typical of malignant tumors.

Tissue and organ specific antigens are commonly present in healthy tissue or organ and they can be detected only in very little concentrations. At the time of tumor growth, inflammation or other pathological phenomenon, these substances are released more intensively.

Non-specific antigens, enzymes, and hormones are produced by tumors from organs that do not normally produce them.

Carcinoembryonic antigen (CEA)

It was discovered in 1965 and is one of the longest established tumor markers. It is a mixture of oncofetal glycoproteins (55% carbohydrates and 45% protein) with a molecular weight of 150–300kDa. The high heterogeneity of the molecule is due to the heterogeneity of the carbohydrate component, the protein component being constant. Physiologically, CEA is produced in the developing embryo. It is detectable from the 8th week of pregnancy with the highest production around the 22nd week. In adulthood, under physiological conditions, it is

synthesized by epithelial cells of the intestinal mucosa, stomach and bronchi, but only in small quantities[41]. CEA plays a role in a various biological processes, especially in cell adhesion and apoptosis. Because of its involvement in cell adhesion, there is a correlation between CEA and cancer metastasis. Evidence for this were demonstrated by injection of CEA into mice enhanced experimental metastasis. Other work has shown enhanced metastatic potential following the transfection of CEA into colorectal cancer cells [42].

CEA as a marker for screening

The original study describing that elevated CEA can be found in almost all patients with CRC, but rarely in healthy patients, failed to be confirmed. For example, using a cut-off point of 2.5 mg/l, the sensitivity of CEA for early CRC (UICC I and II) was only 30–40% [43]. At this cut-off point, the specificity using healthy subjects was reported to be 87%. Based on a prevalence of 1 in 1000 cases of CRC in a healthy population, a sensitivity of 40% (UICC I and II) and a specificity of 90%, Fletcher calculated, that there would be 250 false-positive tests for every patient with cancer. Furthermore, 60% of the cancers would not be detected. Those are the reasons why CEA should not be used for screening for early CRC [9, 41].

CEA in postoperative surveillance

The main use of CEA is in surveillance following curative resection for primary cancer [44, 45]. In the guidelines from the American Society of Clinical Oncology (ASCO) it is concluded that for UICC stages II and III, CEA should be measured every 2–3 months for at least 3 years. That applies not only for patients who are suitable candidates for liver resection, but also for patients who are candidates for receiving systemic therapy [46]. In the German Evidenced-based Guideline for Colorectal Cancer, the frequency of CEA measurement is lower, with a period of 6 months in the first 2 years and then once a year, under the same criteria [9].

Summary of European Group on Tumour Markers (EGTM, 2003) guideline on the use of serum markers in CRC:

Lack of sensitivity and specificity precludes the use of CEA and all other existing serum markers for the early detection of CRC
Preoperative levels of CEA provide a baseline value for subsequent serial determinations and may also provide independent prognostic information
For patients with stages II and III disease that may be candidates for liver resection, CEA should be assayed every 2–3 months for at least 3 years after diagnosis
For monitoring therapy in advanced CRC, CEA should be measured every 2–3 months
Insufficient evidence exists at present to recommend routine use of other serum markers such as CA 19-9, CA 242, TPA, TPS or TIMP-1 in the management of patients with CRC

CA 19-9

CA 19-9, also known as carbohydrate antigen 19-9 or GICA (gastrointestinal cancer antigen), is a mucin and a glycolipid with a molecular weight of 36 kDa. It is also the Lewis a blood group determinant and part of many mucosal cells. Therefore, it cannot be expressed by Lewis negative subjects (3–7% of the European population) at all. CA 19-9 is excreted purely biliary and is contained in the fetal tissue of the stomach, small and large intestine, liver and pancreas. Although CA 19-9 is the best available marker for pancreatic adenocarcinoma, it is less sensitive than CEA for the detection of CRC. CA 19-9 does not increase the informative value with regard of a relapse of CRC in comparison to a separate CEA value [9].

CA 242

Likewise CEA and CA 19-9, CA 242 cannot be used for the detection of early stage CRC. But there is evidence, that in surveillance following curative resection for colorectal cancer, the CA 242, especially in combination with CEA, may be more sensitive than separate CEA measurement [47]. In other study CEA and CA 242 was compared in the surveillance of 149 patients who had undergone apparent curative resection for CRC. For the detection of recurrent disease, CEA alone had a sensitivity of 76% and a specificity of 86%. The sensitivity for CA 242 was 60% and specificity 87%. Combination of the 2 markers increased the sensitivity to

88%, but reduced specificity to 78% [48]. Other studies also reported that CA 242 can complement CEA in the surveillance of patients with diagnosed CRC [49]. Also, there are reports, that CA 242 is superior to CEA in detecting lung metastases, but CEA is more sensitive in diagnosing liver metastases [50]. These findings must now be confirmed in large prospective trials.

TPA (Tissue Polypeptide Antigen) and TPS (Tissue Polypeptide Specific antigen)

TPA is a circulating complex of polypeptide fragments of cytokeratins 8, 18 and 19. It was first demonstrated in 1957 as an antigen of carcinoma epithelial cells. It is produced by both normal and tumor cells. Physiologically, TPA is produced by the placental trophoblast, followed by the liver, lungs, intestine and kidneys of the developing fetus. TPA is a non-specific tumor marker that allows to detect malignant growth in various organs. Elevation in serum levels occurs when cell proliferative activity is significantly increased. It has been proven in most cancers. A healthy tissue does not contain it. However, its occurrence is associated with proliferation of epithelial cells of the fetus, eg increases during pregnancy (then returns to baseline values within 5 days after partus), and is also increased in inflammation. Elevated levels of these antigens can be found in malignant tumors of the uterus, the thyroid, the breast, the ovaries, the prostate, the lungs, the bladder, and others[51].

TPS is an antigen very similar to the TPA antigen. The antibody used for its detection is directed against the M3 epitope of the TPA molecule, which is the major epitope of the cytokeratin fragment 18.

Due to a lack of sensitivity and specificity, neither TPA nor TPS can be recommended for the detection of early stage CRC [9].

Summary of European Group on Tumour Markers (EGTM, 2007) guidelines for the clinical use of markers in CRC together with their level of evidence (LOE)

Marker	Proposed use/uses	EGTM guideline	LOE
Serum			
CEA	Determining prognosis	May be used in combination with standard prognostic factors	III
	Surveillance following curative resection	Should be used for stages II and III patients who may be candidates for liver resection or systemic treatment, should recurrence develop	Ia
	Monitoring therapy in advanced disease	Should be used, especially in patients with nonevaluable disease using standard criteria. Should be measured prior to start of treatment and at 2–3 monthly intervals during therapy b). Ideally, should be used in combination with radiology.	III
CA19.9	Determining prognosis	Not recommended	III
	Surveillance following curative resection	Not recommended	IV
CA 242	Determining prognosis	Not recommended	III
TIMP-1	Determining prognosis	Not recommended	III
Tissue			
TS	Determining prognosis	Not recommended	I
	Predicting response to chemotherapy	Not recommended	III
MSI	Determining prognosis	Not recommended	I
	Predicting response to chemotherapy	Not recommended	III

DCC	Determining prognosis	Not recommended	I
Ras	Determining prognosis	Not recommended	I
P53	Determining prognosis	Not recommended	I
Faecal			
FOBT	Screening for early CRC	Yes, for screening subjects 50 years or older	I
DNA-Based	Screening for early CRC	Not recommended at present	III/IVc (for most studies)
Tests for genetic susceptibility to CRC			
APC	For identifying subjects at high risk of developing FAP	Yes, should be used	
MSI/MMRE	IHC Prescreen for HNPCC	Yes, should be used	
MLH1/MSH2/MSH6	For identifying subjects at high risk of developing HNPCC	Yes, should be used	

Secondary prevention- Screening

The usually long-lasting period between the appearance of polyps and their malignant transformation offers the opportunity for early detection and prevention. CRC screening has a long tradition in the Czech Republic and is undergoing continuous development [52, 53]. Already in 2000, as s second country in the world, the Czech Republic launched a national program of CRC screening in asymptomatic individuals. It is based on the premise that age is one of the main risk factors of sporadic CRC. Asymptomatic individuals over 50 years of age were offered a guaiac FOBT followed by colonoscopy in case of positivity. At the beginning of 2009, the program was radically modified by introducing screening colonoscopy and immunochemical tests for faecal occult bleeding (iFOBT/FIT). Asymptomatic individuals aged 50 to 54 years are offered FOBT at one-year intervals. If this test is positive, screening colonoscopy is indicated. From the age of 55, every citizen can choose between repeated FOBT at a two-year interval and primary screening colonoscopy at a ten-year interval. Since 2013, in

line with the latest knowledge, the gFOBT has been replaced by iFOBT/FIT. The introduction of iFOBT/FIT, primary screening colonoscopy and the involvement of ambulatory gynecologists increased the coverage of the target population, which is 3.8 million individuals in the Czech Republic, to 25%. However, 50% to 65% of the target population is required to significantly change the incidence and mortality rate of this cancer. Therefore, in January 2014, personalized invitation was added to the program , organized by the state. Citizens younger than 70 years of age, who do not participate in long-term prevention programs, are encouraged by their health insurance companies to participate in colorectal cancer screening. The invitation program is coordinated by the Ministry of Health of the Czech Republic in cooperation with insurance companies. The screening program in Germany is equal.

CRC screening in Czech Republic and Germany in asymptomatic individuals	
Digital rectal examination	annually from the age of 50
iFOBT/FIT	annually between the 50th and 54th year, every 2 years from the age of 55 as an alternative to colonoscopy
Total colonoscopy	from the age of 55, Repeat after 10 years in case of no pathological finding

Liquid biopsy

As mentioned above, the standard diagnostic procedure includes taking a medical history with a particular focus on family history, screening for occult blood in the stool, and most importantly, colonoscopic examination. The indispensability of colonoscopy lies in its high sensitivity and specificity, the ability to immediately remove premalignant lesions, and in the case of detecting CRC, the collection of biopsies for subsequent histological examination. No other diagnostic method is capable of encompassing all these aspects. However, colonoscopy is uncomfortable for patients, and alternative methods are being sought to detect the presence of malignant and premalignant stages of CRC by different means. An alternative could be so-called liquid biopsy, where samples of body fluids, mainly blood, are collected for the analysis of specific biomarkers, which can detect the presence of adenomas or carcinoma and, last but not least, monitor treatment success.

One of the most compelling advantages of liquid biopsy in CRC is its non-invasive nature. Unlike traditional tissue biopsies that require invasive procedures, liquid biopsies can be

performed using a simple blood draw. This approach not only minimizes patient discomfort but also allows for repeated sampling, which enables real-time monitoring of disease progression and treatment response. The next advantage is the possible early detection. By detecting circulating tumor cells (cTCs), micro-RNA (miRNA), long non-coding RNA (lnc-RNA), cell-free DNA (cfDNA), and other biomarkers shed by tumors into the bloodstream, liquid biopsy holds promise for identifying CRC at earlier stages when treatment outcomes are better. Moreover, liquid biopsy offers a comprehensive view of tumor biology. CRC is known for its heterogeneity, where tumors exhibit diverse molecular profiles. Liquid biopsy can capture this heterogeneity by analyzing multiple biomarkers simultaneously. This holistic approach enables oncologists to decide about the treatment based on the specific molecular characteristics of an individual's tumor, thus moving towards personalized medicine. One of the advantages of this approach is seen, for example, in the therapy of MSI CRC when using immunotherapy. Accessibility and convenience are additional benefits of liquid biopsy. Blood-based tests are easily integrated into routine clinical work, making molecular profiling accessible to a broader population.

Naturally, this method also has its disadvantages. One notable concern is the sensitivity and specificity of biomarker detection. The amount of the biomarkers shed by tumors can vary, potentially leading to false positives or negatives. Technical limitations in isolating and analyzing these biomarkers also contribute to diagnostic uncertainties. Another critical challenge is the validation and standardization of liquid biopsy techniques. The clinical utility of liquid biopsy in CRC is still being evaluated. Standardized protocols, validation of biomarkers, and the establishment of clear clinical guidelines are essential for integrating liquid biopsy into routine practice in different healthcare settings. Also advanced molecular tests often come at a higher price point compared to conventional diagnostics. Furthermore, as fundamental disadvantage of liquid biopsy is the inherent inability to conduct histological analysis, which is essential for current diagnostics.

Micro-RNA (miRNA)

MiRNAs are small non-coding RNA molecules that post-transcriptionally regulate gene expression by binding to complementary sequences of target messenger RNAs (mRNAs), leading to mRNA degradation or translational repression. miRNAs play role in diverse cellular processes, including proliferation, differentiation and apoptosis through the, above mentioned

inhibition or degradation of target mRNA. Therefore, if miRNA affects the translation of tumor suppressor or oncogenic proteins, it becomes involved in the process of carcinogenesis.[54]

Numerous studies have demonstrated aberrant expression of miRNAs in CRC tissues compared to normal colonic mucosa. Some miRNAs act as oncogenes (OncomiRs) by promoting tumor initiation, progression, and metastasis, while others function as tumor suppressors by inhibiting oncogenic pathways. For instance, miR-21, miR-17-92 cluster, and miR-31 are frequently upregulated in CRC and associated with enhanced cell proliferation, invasion, and resistance to apoptosis. Conversely, miR-34 family members, miR-143, and miR-145 are downregulated in CRC and exert tumor-suppressive effects by targeting oncogenic signaling pathways.[55-58]

Intensive research is also underway exploring the potential use of miRNAs in CRC therapy. The strategies are to modulate miRNA expression for restoring tumor-suppressive miRNAs and anti-miRNA oligonucleotides (antimiRs) for inhibiting oncogenic miRNAs. [59] Despite the promising potential of miRNAs in CRC management, several challenges remain. Standardization of miRNA detection methods, validation of candidate biomarkers, and optimization of delivery systems for miRNA-based therapeutics are essential for translating miRNA research into clinical practice.

Long non-coding RNA (lncRNA)

Long non-coding RNAs have escaped attention in research for a long time. They were considered as “junk” RNA, and their significance began to be uncovered only recently. Primarily, miRNAs were investigated. LncRNAs belong, like miRNAs, to the group of non-coding RNAs, however, their length is much greater, typically ranging from 200 nucleotides upwards. After transcription, lncRNA remains in the nucleus, playing a role in regulating gene expression, both at the transcriptional and post-transcriptional levels, chromatin modification, and organization of nuclear structures. They can act as scaffolds, guides, decoys, or enhancers, interacting with proteins, RNA, and DNA to regulate cellular processes.[60]

There are numerous categories of lncRNA, and their classification is complex. The most commonly cited categorization includes: Intergenic lncRNAs (lincRNAs). These are lncRNAs that are located between protein-coding genes. Intronic lncRNAs. found within the introns of protein-coding genes. Sense lncRNAs overlap with the sense strand of protein-coding genes. Antisense lncRNAs, transcribed from the antisense strand and overlap with protein-coding

genes. Bidirectional lncRNAs, transcribed from the opposite strand of a protein-coding gene promoter. Enhancer RNAs (eRNAs), transcribed from enhancer regions of DNA and Pseudogene-derived lncRNAs, transcribed from pseudogenes, which are non-functional relatives of genes. However, the most comprehensive division is based on function, where lncRNAs are classified into: 1. Scaffolds, 2. Guides and signals, 3. Decoys.[61]

One of the first described lncRNA was the X-inactive specific transcript (XIST) that plays a critical role in X chromosome inactivation (XCI), a process that occurs in female to reduce the gene dosage in XX karyotype. It is an example of scaffold lncRNA and belongs to the category of intergenic non-coding RNAs (lincRNAs). XIST is transcribed from one of the two X chromosomes in female cells, which will undergo inactivation. It spreads along the length of the X chromosome from which it is transcribed. Once transcribed, XIST RNA coats the inactive X chromosome, covering it in a process similar to heterochromatinization. This coating is essential for the subsequent silencing of genes on the inactive X chromosome. The coating of XIST on the X chromosome recruits a complex of proteins known as Polycomb Repressive Complex 2 (PRC2) and other chromatin-modifying enzymes. These enzymes modify the chromatin structure of the inactive X chromosome, leading to gene silencing by adding repressive histone marks (such as H3K27me3) and compacting the chromatin structure.[62]

An example of a guide and signaling lncRNA is HOTAIR (HOX transcript antisense RNA). HOTAIR is involved in gene regulation and has been implicated in cancer progression. HOTAIR functions in histone-modifying complexes and chromatin remodelers, thereby regulating gene expression epigenetically. HOTAIR binds to Polycomb Repressive Complex 2 (PRC2), facilitating its recruitment to specific genomic loci. PRC2 catalyzes the trimethylation of lysine 27 on histone H3 (H3K27me3), leading to gene silencing. HOTAIR also interacts with the LSD1/CoREST/REST complex, which mediates histone demethylation. This complex removes activating histone marks, such as H3K4me2, resulting in transcriptional repression. By recruiting these chromatin-modifying complexes to specific genomic regions, HOTAIR regulates the expression of target genes involved in processes such as cell proliferation, invasion, and metastasis. Dysregulation of HOTAIR expression has been implicated in gastric, pancreatic and CRC, showing its role as a signaling lncRNA in cancer progression.[63, 64]

An example of a decoy lncRNA is the CCAT1 (Colon Cancer-Associated Transcript 1), which is involved in the pathogenesis of CRC. CCAT1 has been shown to play a significant role in the progression and metastasis of colorectal cancer. CCAT1 can act as a decoy by binding to

specific transcription factors, such as CTCF (CCCTC-binding factor), and modulating their activity. By sequestering CTCF, CCAT1 alters the transcriptional regulation of genes that are critical for cancer cell growth and metastasis. CCAT1 is also implicated in enhancing the activity of the Wnt signaling pathway, a key driver in colorectal cancer development. It does so by interacting with and stabilizing the β-catenin protein, thereby promoting the transcription of Wnt target genes which are essential for CRC progression. Additionally, CCAT1 can facilitate chromatin remodeling processes that lead to the transcriptional activation of oncogenes or the repression of tumor suppressor genes, further contributing to CRC pathogenesis. Through these mechanisms, CCAT1 supports the proliferation, survival, and metastatic potential of colorectal cancer cells, highlighting its role in the disease progression.[65]

In our study, which will be discussed later, we focused on the role of the lncRNA Metastasis Associated Lung Adenocarcinoma Transcript 1 (MALAT1) in the pathogenesis of CRC and identified it as a potential biomarker for colorectal adenomas as well as CRC.

Circulating cell-free DNA (cfDNA)

CfDNA are short fragments of nucleic acids, originates from various cell types, including tumor cells, shed into the bloodstream through apoptosis, necrosis, or active release mechanisms. In carcinoma, tumor-derived cfDNA carries genetic alterations characteristic of the malignancy, such as mutations, microsatellite instability and DNA methylation. The release of ctDNA into circulation offers a minimally invasive possibility to diagnose tumor's molecular profile and therefore represents another type of liquid biopsy-based diagnostics. There is a number of techniques for cfDNA analysis, such as PCR-based methods or next-generation sequencing (NGS). Polymerase chain reaction (PCR) assays enable the amplification and detection of specific genomic regions, facilitating the identification of known mutations like KRAS and BRAF mutations associated with CRC. NGS, on the other hand, offers genomic profiling, allowing for the detection of novel mutations and characterization of the tumor heterogeneity. Other emerging technologies, such as digital PCR and hybrid capture-based approaches, further enhance sensitivity and specificity in cfDNA analysis [66, 67].

CfDNA also presents several disadvantages and challenges. Firstly, the sensitivity and specificity of cfDNA-based assays may vary, potentially resulting in false-positive or false-negative results. Factors such as low levels of ctDNA and technical limitations of detection

methods can impact the accuracy of cfDNA analysis. Secondly, CRC tumors exhibit significant intra-tumoral and inter-tumoral heterogeneity, which may not be fully captured by cfDNA analysis, potentially leading to missed detections of important mutations or genetic alterations present in specific tumor subclones. Thirdly, the detection limits of cfDNA assays may pose challenges, particularly in early-stage CRC or cases with low tumor burden, where low levels of ctDNA in circulation may be difficult to detect. Additionally, pre-analytical variables such as sample collection, processing, and storage methods can influence the quality and integrity of cfDNA extracted from plasma or serum, affecting the reliability and reproducibility of results. Standardization of cfDNA-based assays is essential to ensure consistent results across different laboratories and platforms. [68]

Special preventive care for high-risk patients

HNPCC/Lynch syndrome

In genetically predisposed patients, early prophylactic measures are necessary. The Amsterdam II and revised Bethesda criteria are used to identify high-risk patients. The Revised Bethesda Guidelines have been reported as being more sensitive than the Amsterdam II Criteria in detecting individuals and families at risk of Lynch syndrome [61]. It has been proven, that the most sensitive criteria for Lynch syndrome are the Bethesda criteria, with a sensitivity of 94% (95% CI 88-100), the specificity of 25% (95% CI 14-36). Use of the first three criteria of the Bethesda guidelines only was associated with a sensitivity of 94% and a specificity of 49% (95% CI 34-64). The sensitivity of the Amsterdam II criteria was 72% (95% CI 58-86) and specificity of 78% (95% CI 64-92) [69].

Amsterdam II criteria: Each of the following criteria must be fulfilled:

- 3 or more family members with HNPCC-associated cancer (colon/rectum, endometrium, small intestine, urothelial (ureter/renal pelvis))
- 2 or more successive generations affected
- 1 first-degree family member affected
- 1 or more relatives diagnosed before the age of 50 years;
- Exclusion of a familial adenomatous polyposis
- Tumors should be verified by pathologic examination

Revised Bethesda criteria: Tumors from patients who fulfill one of the following criteria should be tested for microsatellite instability:

- CRC diagnosed in a patient who is less than 50 years old
- Patients with syn- or metachronic colorectal or other HNPCC-associated tumors (colon, rectum, endometrium, stomach, ovaries, pancreas, ureter, renal pelvis, biliary system, brain (especially glioblastoma), skin (sebaceous gland adenomas and cancer, ceratoacanthomas, small intestine)) independent of age at diagnosis.
- Patients with CRC before age 60 with typical histology of MSI-H- tumors (tumor-infiltrating lymphocytes, Crohn‘s like lesions, mucinous or signet ring cell differentiation, medular cancer).
- Patients with CRC who have a 1st degree relative with CRC or HNPCC-associated tumor before age 50.
- Patients with CRC (independent of age), who have at least two 1st or 2nd degree relatives who have been diagnosed with CRC or HNPCC-associated tumors (independent of age)

Recommendations for the process of molecular evaluation of patients identified as being at risk, based on meeting the Bethesda Guidelines [70]:

- The optimal approach to evaluation is microsatellite instability (MSI) or immunohistochemical (IHC) analysis of tumors, followed by germline MSH2/MLH1 testing in patients with MSI-H tumors or tumors with a loss of expression of one of the mismatch repair genes *[1]
- After the mutation is identified, at-risk relatives should be referred for genetic counseling and tested if they wish.
- An alternative approach, if tissue testing is not feasible, is to proceed directly to germline analysis of the MSH2/MLH1 genes.
- If no mismatch repair gene mutation is found in a proband with an MSI-H tumor and/or a clinical history of hereditary nonpolyposis colorectal cancer (HNPCC), the genetic test result is non-informative. The patients and the at-risk individuals (i.e., relatives) should be counseled as if HNPCC was confirmed and high-risk surveillance should be undertaken.
- There is a need to assure patients of confidentiality to allay fears related to discrimination based on genetic status.

[1] For families with a strong suspicion of HNPCC, germline testing should be considered, even when the MSI/IHC results indicate MSI-L, microsatellite stable, or normal expression. The likelihood of finding a germline mutation in the MLH1/MSH2 genes of patients with colorectal cancer tumors that are not MSI-H is expected to be low; however, this likelihood has not been thoroughly studied.

Patients at risk for HNPCC should undergo genetic counseling. If the diagnosis is made, the family members should be made aware of the possibility of predictive testing. HNPCC patients and high-risk individuals should usually be colonoscopically examined annually from the age of 25. A gastroscopy is recommended regularly from age 35. Because of the risk of endometrial and ovarian cancer, female patients should undergo annual gynecological examination with a transvaginal ultrasound, in addition an endometrial biopsy should be performed from age 35. Prophylactic hysterectomy and, if necessary, an ovariectomy at age 40 or five years before the earliest age of disease contraction in the family should be discussed. Drug prevention (eg ASS), or prophylactic colectomy should not be performed [9].

FAP – Familial Adenomatous Polyposis

Due to the autosomal dominant inheritance genetic counseling and predictive genetic testing is recommended for the relatives of FAP patients from age of 10. At this age also rectosigmoideoscopy should be done, in case of adenomas, a complete colonoscopy should follow. Patients with classic FAP should be prophylactically (whenever possible maintaining continence and if possible, no earlier than the end of puberty) proctocolectomized, regardless of the result of the molecular genetic testing. Gastroscopy is recommended from the age of 25. If therapy is indicated (symptoms, progression), first-line therapy of desmoids in FAP patients consists of a combination therapy using sulindac and tamoxifen [9].

Tumor classification and staging

Colorectal carcinomas are classified histopathologically according to the TNM system and the UICC system. If a category remained undetermined, the tumor is classified as UICC X. All adenocarcinomas of the colon, which oral margin is no more than 16 cm from linea anocutanea by examination with the rigid rectoscope, are classified as rectal cancer. Colon and rectal cancers are evaluated separately. This strict differentiation is essential because the rectum has differences compared to the colon: The rectal lymphatic drainage runs in the upper third along the inferior mesenteric vein to the para-aortic lymph nodes, in the middle third of the rectum along the two internal iliac veins pelvic wall and in the distal third of the rectum via the hemorrhoidal plexus into the groin region. The rectum is predominantly extraperitoneal and therefore has no serosal covering. Circumferentially, the rectum is encased by loose, lymphatic-rich adipose tissue - the mesorectum. These anatomical peculiarities as well as the challenging situs by operation are, the reason of local recidive problematic. The classification of colorectal

carcinomas by Dukes was introduced already in 1932. It was developed in order to determine accurately and objectively the spread, related treatment strategy and also the prognosis of colorectal cancer. The depth of the tumor but also the presence of lymph nodes metastases, have already been taken into account here. This classification, which was primarily based on the infiltration of defined anatomical structures, has been modified over time. Since 1987, the International Union of Contre le Cancre (UICC) and the American Joint Committee on Cancer (AICC) developed exist TNM classification with four staging systems.

The WHO TNM Classification:

Category	Description
Primary tumor (pT)	
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ, intramucosal carcinoma
T1	Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades through the muscularis propria into the pericolorectal tissues
T4a	Tumor penetrates to the surface of the visceral peritoneum
T4b	Tumor directly invades or adheres to other adjacent organs or structures
Regional lymph nodes (pN)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1 - 3 regional lymph nodes
N1a	Metastasis in 1 regional lymph node
N1b	Metastasis in 2 - 3 regional lymph nodes
N1c	No regional lymph nodes are positive but there are tumor deposits in the subserosa, mesentery, or nonperitonealized pericolic or perirectal/mesorectal tissues
N2	Metastasis in 4 or more regional lymph nodes
N2a	Metastasis in 4 - 6 regional lymph nodes
N2b	Metastasis in 7 or more regional lymph nodes

Distant metastasis (pM)	
M0	No distant metastasis by imaging; no evidence of tumor in other sites or organs
M1	Distant metastasis
M1a	Metastasis confined to 1 organ or site without peritoneal metastasis
M1b	Metastasis to 2 or more sites or organs without peritoneal metastasis
M1c	Metastasis to the peritoneal surface alone or with other site or organ metastases

Colon carcinomas can be classified according to UICC and Dukes. A stage classification (UICC stages) was formed based on the TNM classification. This was introduced by the "Union Internationale Contre le Cancer" (UICC) and is based on statistical studies that prove, for example, that the prognosis of the disease deteriorates from a certain size of a tumor. The classification of a tumor disease therefore allows prognostic statements and often also determines the further therapy. The former classification according Dukes is now less common and largely replaced by the UICC staging.

The TNM classification with UICC stages:

Stage	T	N	M
0	Tis	N0	M0
I	T1	N0	M0
	T2	N0	M0
IIA	T3	N0	M0
IIB	T4a	N0	M0
IIC	T4b	N0	M0
IIIA	T1-T2	N1/N1c	M0
	T1	N2a	M0
IIIB	T3-T4a	N1/N1c	M0
	T2-T3	N2a	M0
	T1-T2	N2b	M0
IIIC	T4a	N2a	M0

	T3-T4a	N2b	M0
	T4b	N1-N2	M0
IVA	Any T	Any N	M1a
IVB	Any T	Any N	M1b

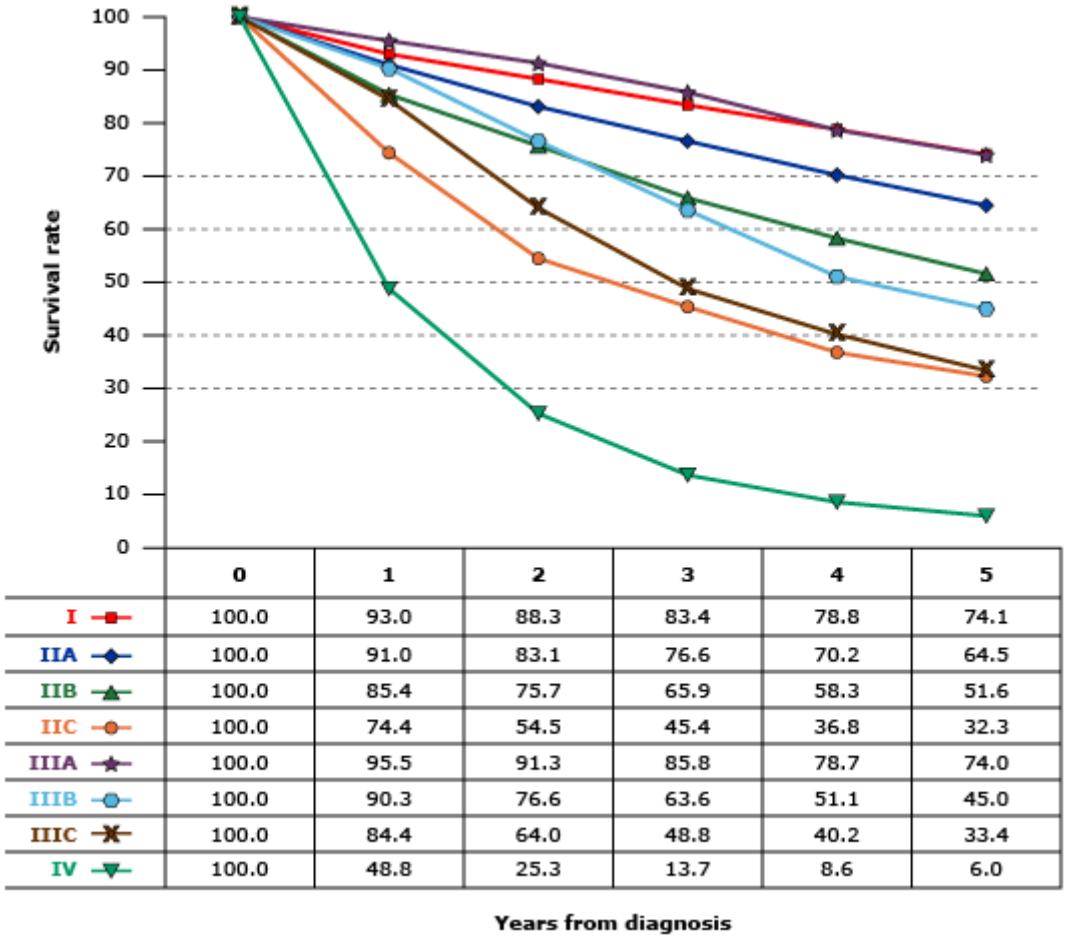
Source: AJCC 7th—Edge SB, Byrd DR, Compton CC, et al. AJCC Cancer Staging Manual. 7th ed. Chicago: Springer-Verlag; 2010. [71]

In approximately 5-15% of patients with colorectal carcinoma, the local tumor growth is so advanced at the time of diagnosis that adhesions or infiltrations of neighboring organ structures are already visible macroscopically [72]. This situation requires extended resection “en bloc”. If other affected organs are resected, then the term multiviseral resection is used and can usually be done with curative intent.

Prognosis

The most important indicator of outcome after resection of CRC is the pathologic stage [71]. Prognostic factors in addition to the TNM staging are the age, gender and tumor location. Prognosis worsen the male gender, age below 40 years, and tumor localization in the rectum or sigmoideum.

Survival rate according to the UICC stages:



	0	1	2	3	4	5
I	100.0	93.0	88.3	83.4	78.8	74.1
IIA	100.0	91.0	83.1	76.6	70.2	64.5
IIB	100.0	85.4	75.7	65.9	58.3	51.6
IIC	100.0	74.4	54.5	45.4	36.8	32.3
IIIA	100.0	95.5	91.3	85.8	78.7	74.0
IIIB	100.0	90.3	76.6	63.6	51.1	45.0
IIIC	100.0	84.4	64.0	48.8	40.2	33.4
IV	100.0	48.8	25.3	13.7	8.6	6.0

Source:AJCC Cancer Staging Manual, Seventh Edition (2010) published by Springer New York, Inc.

An important criterion for postoperative prognosis is the determination of the completeness of the resection – so-called R classification of the UICC. The UICC's Residual classification provides information about the absence or presence of detectable residual tumor after surgical therapy and is described with the symbol “R”. R-0 means "no detectable residual tumor", while R-1 denotes a microscopic and R-2 a macroscopically detectable residual tumor. According to the rules of the UICC, both a locoregional tumor and non-resectable distant metastases are considered residual tumors. Residual tumor classification should always be carried out for colon carcinoma, since primary palliative interventions (R1, R2 resections) can be distinguished from potentially curative (R0 resections). It should be emphasized that an R1 resection is only present if a surgical resection line runs through tumor tissue that is intact under the light microscope. The residual tumor classification is prognostically highly significant. In addition, the exact

residual tumor classification is a crucial starting point for decision about adjuvant tumor therapy. It should be emphasized that the residual tumor classification can only be carried out in close clinical-pathological cooperation. On the part of the pathologist, not only the aboral and oral surgical resection margin, but also the so-called lateral (deep mesenteric) surgical resection area must be examined histologically with regard to the residual tumor classification. The surgeon should mark the lateral surgical resection area with dye or with stitches.

The UICC Classification of the Completeness of Resection (R classification)	
Rx	cannot be assessed
R0	no residual tumor
R1	microscopic residual tumor
R2	macroscopic residual tumor

In the last years, there have been a number of other histopathological risk factors that have so far been less considered for the prognosis of colon cancer. These include lymphatic and venous invasion, submucosal invasion depth, tumor budding, the condition of the muscularis mucosae and perineural invasion. All of these histological risk factors independently represent negative predictive values with regard to the clinical outcome [73, 74].

Lymphatic and venous invasion

Lymphovascular invasion (LI) and Venous invasion (VI) in CRC are considered for a strong stage-independent prognostic factor and influences decisions regarding further therapy in different stages of CRC. The invasive tumor cells penetrate either the lymphatic system to be transported from there to regional lymph nodes or they penetrate the blood vessel system to get to other organs from there. Both histopathological factors correlate with the occurrence of lymph node metastases, the occurrence of tumor recurrence and overall survival [75]. Lymphatic invasion (L1) is defined in the literature as the detection of tumor cells in a preformed cavity lined with endothelium. This typically has no muscular wall layer and there are no erythrocytes in the lumen [76]. Vein infiltration (V1) is defined as the detection of tumor cells within a cavity lined with endothelium or smooth muscles, in which there are erythrocytes and / or fibrin clots.

Submucosal invasion

In healthy intestinal tissue, the lymphatic vessels extend to the lamina muscularis mucosae. A few lymphatic vessels pass through them into the basal crypt areas. A study [77] showed that the structure of the lymph vessels, both in adenomas and in invasive colorectal carcinomas, extends into much more superficial parts of the intestinal mucosa. However, these superficial vessels do not seem to communicate directly with deeper lymph nodes, since metastasis was only observed after the lamina muscularis mucosae (pT1) had broken through into the submucosa [77]. To determine the depth of invasion, the submucosa is divided into upper third (sm1), middle third (sm2), and lower third (sm3) and the depth of the infiltration is measured. The so-called early invasive forms (sm1 = submucosal invasion ≤1000 µm) have a low N + risk of 0-6% [78, 79]. In contrast, the risk of lymph node metastasis in sm3 carcinomas is about 20% [80].

Tumor cell budding

Tumor budding is the microscopic detection of fewer than five tumor cells or tumor cell groups on the invasion front of the carcinoma [81]. In numerous studies, tumor budding is said to have a negative impact on the occurrence of lymphatic and venous invasion, the detection of positive lymph nodes, the occurrence of local and distant recurrences and the occurrence of distant metastases [81-83]. In the case of positive tumor budding, some studies were able to demonstrate the occurrence of isolated tumor cells in patients with pN0 status [84]. This histopathological factor is not only accompanied by a poorer prognosis within the early invasive carcinomas, but also in all other tumor stages [85]. In order to be able to introduce and implement tumor budding as an independent histopathological risk factor, a suggestion from Japanese authors is now to be followed: Tumor budding is defined as histological detection of tumor cell clusters (five or fewer cells) of undifferentiated or isolated tumor cells at the invasion front. The invasion front should be microscoped at 200x magnification and the degree of budding should be determined as grade 1 with 0-4; grade 2 with 5-9 and grade 3 with> 9 buddings or tumor cell clusters. A grade 2 or 3 tumor budding on the invasion front were an additional parameter for an increased risk of lymph node metastases in several studies [86].

Condition of the muscularis mucosae

After multivariate analysis from 2004, a significant correlation between the condition of lamina muscularis mucosae and the more frequent occurrence of lymph node metastases could be determined [87]. Three hundred and twenty two patients were divided into three groups

according to the condition of the lamina muscularis mucosae. Group A: lamina muscularis mucosae clearly identified; Group B: muscularis mucosae incompletely disrupted with deformity; and Group C: completely disrupted muscularis mucosae. 11.8% showed lymph node metastasis, of which 0% in group A, 7.2% in group B and 17.3% in group C.

Perineural invasion

Perineural invasion is an additional independent prognostic factor for the occurrence of lymphatic and venous invasion and correlates with the detection of lymph node metastases in pT1 and pT2 carcinomas [88]. In the 7th edition of the TNM classification, the Pn classification is taken into account for the first time and can be used as an optional parameter within the tumor formula. In contrast, neural invasion is not an independent prognostic factor, but is associated with a poorer survival rate, whereby the extent of the infiltration must be taken into account [89, 90].

IV. Pathogenesis of colorectal cancer

There are three main mechanisms of colorectal cancer pathogenesis.

The most common pathway is chromosomal instability (CIN), present in up to 75% of CRC cases, which involves structural and numerical changes in chromosomes. This can result in amplifications, deletions, or loss of heterozygosity of various genes, typically affecting regions of oncogenes and tumor suppressor genes involved in CRC pathogenesis, such as APC, KRAS, or TP53 [91].

Another pathway is the epigenetic phenomenon of hypermethylation of gene promoter regions, known as CpG Island Methylator Phenotype (CIMP), which occurs in about 20% of CRC cases. This does not involve changes in the number or sequence of nucleotides in DNA but rather hypermethylation of cytosine in repetitive CpG sequences in promoter regions of tumor suppressor genes. This leads to silencing of gene expression, commonly affecting genes like APC, MLH1, MCC, MGMT, and others [92].

The third pathway is microsatellite instability (MSI), which represents damage to the mismatch repair system (MMR). This is discussed in more detail in other chapters, particularly regarding its critical importance in CRC therapy. MSI is found in approximately 15% of CRC cases.

Mutation in tumor suppressor genes

Adenomatous polyposis coli gen (APC gene)

The APC gene is a tumor suppressor gene, first identified in the hereditary syndrome known as familial adenomatous polyposis (FAP), which is an autosomal dominant pre-malignant condition that virtually progresses to malignancy in 100% of cases. The APC gene is located on the long arm of chromosome 5 and mutations in this gene contribute to the development of both sporadic CRC and hereditary forms of CRC, including FAP, attenuated FAP, Gardner syndrome, and Turcot syndrome. Inactivating mutations in the APC gene are found in up to 80% of all colorectal adenocarcinomas, and heterozygosity creates an autosomal dominant predisposition to colorectal adenocarcinomas.[93]

The APC gene is involved in cellular proliferation, adhesion, differentiation, migration, and plays an important role in apoptosis and cell cycle regulation, including microtubule

stabilization during mitosis. Loss of tumor suppressor function occurs, as with most tumor suppressor genes, only with mutations in both alleles, although point mutations are found in early tumor stages.

From a molecular genetic perspective, a critical function of the APC gene is the regulation of intracellular β-catenin concentration. β-catenin is involved in E-cadherin-mediated cell adhesion and the Wnt-1 signaling pathway. When the APC gene functions correctly, there is feedback to decrease β-catenin concentration. Specifically, the APC protein forms a complex with axin and facilitates the phosphorylation of β-catenin on serine-threonine residues. This marks the ubiquitination of the β-catenin complex by the β-transducin repeat-containing protein (β-TRCP), leading to subsequent proteasomal degradation. Phosphorylation of APC and axin by the GSK3-β protein promotes the binding of β-catenin to APC-axin complexes, thereby increasing β-catenin degradation. β-catenin is also involved in the Wnt-1 signaling pathway. Binding of Wnt-1 to its transmembrane receptor inhibits GSK3-β. Inhibition of GSK3-β suppresses β-catenin degradation, leading to accumulation of nuclear and cytoplasmic β-catenin and up-regulation of gene expression. In the nucleus, β-catenin activates the transcription of certain proto-oncogenes such as cyclin D1 or c-myc. By preventing β-catenin accumulation, APC controls the up-regulation of genes involved in cell cycle initiation and progression. The role of APC as a regulator that reduces β-catenin concentration is supported by experiments showing that nuclei of APC-negative colon carcinoma cells contain stable β-catenin complexes. Expression of APC protein subsequently reduces β-catenin concentration. [94, 95]

Tumor protein p53

The TP53 gene, also known as the "guardian of the genome," is another significant tumor suppressor gene. Its main function is to recognize DNA damage and initiate the transcription of genes that pause the cell cycle and genes involved in DNA repair. It plays a role in regulating cellular metabolism. In the event of DNA damage, TP53 coordinates its repair, and if repair fails, it induces apoptosis. Mutations in this gene are found in 35-55% of all colorectal adenocarcinomas.[91, 96]

The TP53 gene is located on the short arm of chromosome 17. Its transcript, the p53 protein, is normally inactive in the cytoplasm due to the enzyme MDM2. Upon DNA damage, p53 is released from MDM2, leading to its activation, which indirectly causes cell cycle arrest in the G1 phase within the cell nucleus. Subsequently, DNA repair may occur with cell cycle

continuation, or in the case of repair failure, apoptosis is induced. All these actions are mediated through genes whose transcriptional activity is regulated by the p53 protein. [97]

Clinically, the TP53 gene is significant in Li-Fraumeni syndrome, where its mutations play a key role in the pathogenesis of this hereditary cancer syndrome characterized by multiple soft tissue tumors. The frequency of TP53 mutations increases during the adenoma-carcinoma sequences and accumulates particularly in advanced stages of CRC. Due to the wide spectrum of malignancies associated with TP53 mutations, these gene alterations have little or no prognostic or screening value in relation to CRC. However, several in vitro, animal, and clinical studies have shown that normal p53 function is required for a good response of CRC to chemotherapy based on 5-fluorouracil. [98]

TGF-β Tumor suppressor cascade (TGFBR2 Gene and SMADs)

TGF-β (transforming growth factor β) is a family of cytokines with anti-proliferative activity that acts on many different types of cells. The TGF-β receptor complex consists of two subunits (RI and RII), and upon activation, it inhibits cell division. Specific somatic mutations in the RII subunit, involving polyadenine repeats, have been shown to be particularly characteristic of CRC and gastric carcinoma. These mutations, along with others affecting the TGF-β receptor complex, lead to the loss of TGF-β receptors on the cell surface and consequently to resistance of such cells to the anti-proliferative effect of TGF-β. Mutations in the TGFBR2 subunit are found in approximately 30% of all colorectal adenocarcinomas and are primarily associated with the transition from low-grade adenoma to high-grade epithelial dysplasia or carcinoma. Interestingly, mutations in the TGFB2 gene are present in more than 90% of CRC cases with microsatellite instability – indicating damage to mismatch repair genes. This might be explained by the fact that the functional region of the TGFB2 gene contains mononucleotide and dinucleotide repetitive DNA sequences similar to microsatellites. These sequences, like microsatellites, are not properly repaired by the mismatch repair system, leading to incorrect synthesis of the RII subunit. [91, 99]

In the TGF-β tumor suppressor cascade, a significant role is also played by the so-called SMAD proteins, which are the main signal transducers from TGF-β receptors to the nucleus. After translocation into the nucleus, SMADs regulate the transcription of target genes by directly binding to DNA. They also interact with other DNA-binding proteins. Mutations in SMAD genes naturally lead to faulty transmission of signals from TGF-β receptors and subsequently to resistance to the anti-proliferative effect of TGF-β. Mutations in SMAD genes, located on

the long arm of chromosome 18, are the basis for familial juvenile polyposis, characterized by the presence of multiple hamartomatous polyps in the digestive tract.[91, 100]

Mutations in oncogenes

KRAS, BRAF, PI3K

KRAS (kirsten rat sarcoma viral oncogene) is located on chromosome 12 and is a member of the RAS gene family, part of the RAS-RAF-MEK and PI3K-PKB signaling pathways. Mutations in RAS occur in 35-45% of colorectal carcinomas. The KRAS protein plays a crucial role in transmitting signals from the epidermal growth factor (EGF) receptor on the cell surface to the cell nucleus. KRAS is a GTPase, and for signal transmission, it requires binding to a GTP molecule. When KRAS is dephosphorylated to GDP, it becomes inactive and does not transmit signals. Mutations in KRAS lead to improper protein function, preventing dephosphorylation and resulting in continuous activation of the downstream pathway, regardless of EGF receptor activity, which KRAS precedes in the signaling cascade.[101]

KRAS protein is anchored in the membrane and activates BRAF, a serine/threonine kinase. BRAF then activates MEK (mitogen-activated protein kinase) through phosphorylation. The signal from the EGF receptor is thus transported to the cell nucleus, leading to cell cycle progression, growth, proliferation, and differentiation. Simultaneously, EGF receptor activation through KRAS also stimulates PI3K (phosphatidylinositol 4,5-bisphosphate kinase), which functions to inhibit apoptosis. [102, 103]

Mutations in BRAF have a similar effect to RAS mutations and are found in approximately 10% of CRC cases. The diagnosis of BRAF mutations has become increasingly important because it has been shown to be associated with very poor prognosis, early metastasis, and aggressive CRC growth. Up to 95% of BRAF mutations occur in codon 600 and are referred to as BRAFV600 mutations. They are found predominantly in tumors without RAS mutations but also result in continuous pathway activation independent of EGF receptor activity. [104]

Clinically, these findings are crucial for immunotherapy, as tumors with wild-type KRAS and BRAF respond well to treatment with monoclonal antibodies against the EGF receptor. However, mutations lead to pathway activation independent of the EGF receptor, rendering monotherapy with anti-EGFR antibodies ineffective. In the treatment of aggressive metastatic CRC with BRAF mutations, combinations of inhibitors targeting various levels of the signaling

pathway are used. These include direct BRAF inhibitors like Encorafenib, anti-EGFR antibodies like Cetuximab in combination with MEK inhibitors like Binimetinib. [91, 104-106]

Mutations and inactivation of DNA Mismatch Repair genes (MMR genes)

The DNA mismatch repair (MMR) system comprises a set of genes responsible for correcting incorrectly paired bases during DNA replication. This system is activated when the proofreading activity of DNA-dependent DNA polymerase fails. Its main functions include repairing mismatched nucleotide pairs, regulating the cell cycle, and initiating apoptosis when repair is not possible.

Approximately 12-17% of patients with colorectal cancer (CRC) exhibit dysfunction in the MMR system. This dysfunction can be either inherited, as in Lynch syndrome, or acquired due to epigenetic changes that reduce MMR gene transcription. Somatic mutations are another possible cause. Lynch syndrome, or hereditary non-polyposis colorectal cancer (HNPCC), is an autosomal dominant condition characterized by early development of gastrointestinal, gynecological, urologic, and neuroectodermal malignancies. The most common cancers associated with Lynch syndrome are colorectal cancer (1-3% of all CRC cases) and endometrial cancer (up to 2% of all endometrial cancers). Unlike other forms of CRC, Lynch syndrome does not begin with the growth of intestinal mucosal adenomas but directly progresses to CRC. Criteria based on family history were established to identify suspicious individuals, initially with the Amsterdam criteria and subsequently the Bethesda criteria, followed by genetic testing.[107]

The malfunction of the MMR system can be easily recognized by the associated genetic epiphenomenon known as microsatellite instability (MSI). Microsatellites are short repetitive DNA sequences scattered throughout the genome. These short DNA sequences are more prone to replication errors because DNA-dependent DNA polymerase often makes mistakes when incorporating the correct number of bases during replication of short repetitive DNA segments such as microsatellites. Slippage during replication of repetitive sequences leads to the formation of temporary insertion-deletion loops, which, like individual mismatched bases, are recognized and subsequently repaired by the MMR system. In the case of MMR system dysfunction, mismatches result in point mutations, and insertion-deletion loops lead to frame

shift mutations, nonsense mutations, and the production of non-functional proteins. Changes in microsatellite length are easily detectable and reciprocally indicate malfunction of the MMR system. Microsatellites are also present in some tumor suppressor and proto-oncogenes, so MMR mutations can directly contribute to CRC pathogenesis through this pathway. [108]

The MMR system itself comprises multiple genes with diverse functions but the same goal: repairing incorrectly paired bases. These genes include MutS protein homologue 2 (MSH2), MutS protein homologue 3 (MSH3), MutS protein homologue 6 (MSH6), Human mutL protein homologue 1 (MLH1), post-meiotic segregation increased homologue 1 (PMS1), and PMS1 homologue 2 (PMS2). These proteins function in common complexes, with some recognizing individual mismatched bases, others having higher affinity for insertion-deletion loops, and others initiating or enhancing the activity of exonucleases and endonucleases.[108-110]

The analysis of microsatellite instability involves testing a total of 5 loci according to international consensus, distinguishing 3 MSI phenotypes based on the results:

- MSI-high (MSI-H): mutations in at least 30%, or at least two, of the loci
- MSI-low (MSI-L): mutations in less than 30%, but in at least one, of the loci
- MSI-stable (MSS): no mutations detected in any of the tested loci

In MSI-H cases, mutations in the TGFβ receptor subunit II are present in up to 90% of cases, and BRAFV600 mutations are also common. Conversely, mutations in the tumor suppressor gene p53 and the proto-oncogene KRAS are not observed.[104] From a histopathological perspective, the most common findings include undifferentiated mucinous carcinoma and increased lymphocyte infiltration, likely due to increased mutagenicity and subsequent immunogenicity of MSI-H tumors. [111]

Determining microsatellite instability status is crucial for CRC therapy. Knowledge of MSI status is key to deciding whether to initiate adjuvant chemotherapy. For MSS tumors in UICC stages II and III, adjuvant therapy based on fluorouracil is recommended, whereas in the case of MMR system mutations (MSI-H), adjuvant therapy does not yield better outcomes. [112]

Complete remission of advanced rectal carcinoma with MSI-H during monotherapy with anti-PD-1 antibodies.

In MSI-H, there has been a significant benefit shown with anti-PD-1 antibody immunotherapy, especially in advanced stages of rectal carcinomas. The standard therapy for these tumors involves neoadjuvant radiochemotherapy, surgical resection with total mesorectal excision, and adjuvant chemotherapy, although this model is increasingly being replaced by total neoadjuvant therapy (TNT) for locally advanced rectal carcinomas (UICC II/III, T3/4, N0, or N+, M0), where subsequent surgical intervention occurs only in the case of TNT failure. [113-116]

Mainly, radiotherapy and surgery carry a high morbidity in terms of proctitis, continence disorders, sexual dysfunctions, and in a significant percentage of cases, the necessity of colostomy. However, in recent years, highly effective responses to anti-PD-1 antibodies have been demonstrated in MSI-H rectal tumors in advanced stages, leading to complete remission of carcinoma even with monotherapy with these antibodies, without the need for any additional chemotherapy or surgical intervention. There is a legitimate question about the duration of induced complete remission, as recent studies have only followed patients for up to 2 years after therapy. [117-120].

Anti-PD-1 antibodies belong to the group of so-called immune checkpoint inhibitors (ICI), which are antibodies that target key immune checkpoints. These key checkpoints can either suppress or stimulate the immune response. In the case of cancerous diseases, this process mainly occurs with the help of CD8+ T lymphocytes, which under normal conditions would destroy malignant cells, but cancer cells evade the immune system through various mechanisms. One possibility is the higher expression of the programmed cell death 1 ligand (PD-L1), which is a receptor located on the surface of cells. If this receptor comes into contact with the programmed cell death 1 protein (PD-1), localized on the surface of CD8+ T lymphocytes, and PD-L1 interacts with PD-1, the result is the inactivation of CD8+ T lymphocytes. With antibodies targeting either the tumor cell receptor or CD8+ T lymphocytes, lymphocyte inactivation does not occur, and the immune system destroys cancer cells. Further key interactions occur with antigen-presenting cells based on the interaction of the B7 receptor with CTLA-4 located on T lymphocytes. In this case as well, specific monoclonal antibodies can be used to block inhibitory signals. Overall, ICI are taking place for their effectiveness in MSI-H tumors in guidelines for both locally advanced and metastatic tumors. [120, 121]

V. List of Publications

Publication Nr.1:

Colorectal carcinoma after liver transplantation
J. Jungwirth, P. Mačinga, J. Král, P. Taimr, J. Froněk, J. Špičák, T.Hucl
Gastroenterologie a hepatologie 2022 Vol. 76 Pages 302-308
DOI: 10.48095/ccgh2022302
IF: 0,129

Publication Nr.2:

Mutational analysis of driver genes defines the colorectal adenoma: in situ carcinoma transition
J. Jungwirth, Marketa Urbanova, Arnoud Boot, Petr Hosek, Petra Bendova, Anna Siskova, Jiri Svec, Milan Kment, Daniela Tumova, Sandra Summerova, Zdenek Benes, Tomas Buchler, Pavel Kohout, Tomas Hucl, Radoslav Matej, Ludmila Vodickova, Tom van Wezel, Pavel Vodicka & Veronika Vymetalkova
Scientific Reports 2022 Vol. 12 Issue 1 Pages 2570
DOI: 10.1038/s41598-022-06498-9
IF: 4.6; 5-Year IF: 4.9

Publication Nr. 3:

Discovery of Long Non-Coding RNA MALAT1 Amplification in Precancerous Colorectal Lesions
Anna Siskova, Jan Kral, Jana Drabova, Klara Cervena, Kristyna Tomasova, **Jiri Jungwirth**, Tomas Hucl, Pavel Kohout, Sandra Summerova, Ludmila Vodickova, Pavel Vodicka, and Veronika Vymetalkova
Int J Mol Sci 2022 Vol. 23 Issue 14
DOI: 10.3390/ijms23147656
IF: 5.6; 5-Year IF: 6.2

Publication Nr. 4:

MALAT1 in Liquid Biopsy: The Diagnostic and Prognostic Promise for Colorectal Cancer and Adenomas?
Klara Cervena, Miroslav Levy, Anna Siskova, **Jiri Jungwirth**, Marin Volaric, Jan Kral, Pavel Kohout, and Veronika Vymetalkova
Int J Gen Med 2023 Vol. 16 Pages 3517-3531
DOI: 10.2147/IJGM.S420127
IF: 2.3 ; 5-Year IF: 2.4

VI. Hypotheses and Aims

Hypotheses:

I. The liver transplant recipients have an increased risk of developing CRC compared to the general population.

II. Mutation profiles of colorectal low-grade adenomas, high-grade adenomas, and carcinoma in situ differ.

III. Chromosomal instability (CIN) is already present in colorectal adenomas before they progress to carcinoma stages.

IV. The long non-coding RNA MALAT1 is a diagnostic and prognostic biomarker in colorectal adenomas and carcinomas.

Aims:

I. Evaluate the incidence of CRC in liver transplant patients based on literature data and supplement it with experience from our transplant center in IKEM.

II. Evaluate the differences and similarities in mutation profiles of low-grade adenomas, high-grade adenomas, and carcinoma in situ.

III. Detect and characterize structural and numerical chromosomal aberrations in colorectal adenomas in comparison to the adjacent mucosa of the same patient.

IV. Determine the genetic expression profile of MALAT1 in colorectal adenomas, carcinomas, and healthy colon mucosa and plasma of the same patient.

VII. Material, Methods and published results

Colorectal Cancer After Liver Transplantation

Patients who undergo solid organ transplants have an increased risk of malignancies, particularly skin cancers and lymphoproliferative disorders. CRC is one of the most common cancers and its incidence is higher after the transplantation of certain organs. The risk of CRC after liver transplantation (LTx) is not definitively known.

Transplantation is a life-saving treatment for patients with organ failure. However, transplant recipients have a higher incidence of malignancies compared to the general population. These cancers can be de novo, recurrent, donor-derived, or pre-existing undetected tumors. Post-transplant patients commonly develop skin cancers and lymphoproliferative disorders. Immunosuppression, necessary after the transplantation, plays a critical role in the development of these malignancies, along with oncogenic viral infections. Increasing age and survival time of organ recipients also contribute to this risk.

Patients with inflammatory bowel disease (IBD) have an increased CRC risk due to the pro-neoplastic effects of chronic inflammation. However, the CRC incidence has declined over the past 30 years due to successful screening and better pharmacological control of inflammation. Risk factors for CRC in IBD include disease duration, extent, severity, presence of inflammatory pseudopolyps, coexisting PSC, and family history. Screening guidelines recommend more intensive CRC surveillance for IBD patients compared to the general population.

Aim and Methods

The aim of this study was to evaluate literature data on the incidence of CRC in liver transplant patients and supplement it with our experience from our transplant center in IKEM. We conducted a retrospective review of medical records and disease progression in patients diagnosed with CRC after LTx at our center.

Results

Literature data shows that the risk of CRC in liver transplant patients ranges from comparable to nearly five times higher than the general population. A proven risk factor for CRC is primary sclerosing cholangitis with ulcerative colitis. Studies evaluating liver transplant recipients with

and without PSC/UC consistently show a high risk of CRC in those with PSC/UC and usually no risk in those without.

Studies Showing Comparable CRC Incidence Post-Transplantation:

Silva MA et al. analyzed data from various transplant registries, including the Israel Penn International Transplant Tumor Registry and the Australian Combined Liver Transplant Registry, reviewing 6,476 post-LTx patients, 42 of whom developed CRC. The standardized incidence ratio (SIR) was 1.01, indicating no significant increase in CRC incidence.

Aigner et al. studied 3,595 organ transplant recipients in Austria and found a CRC incidence of 0.25%, comparable to the general population. Merchea et al. retrospectively analyzed 3,946 organ transplant recipients at Mayo Clinic, identifying 20 patients with CRC (incidence 0.5%). The median time from transplant to CRC diagnosis was 8.7 years. Most patients had kidney transplants (n=8), followed by liver transplants (n=3). CRC was most commonly found in the right colon and often at stage IV.

A British multicenter study by Rompianesi G., et al. involving 8,115 adult liver transplant recipients found no increased CRC incidence compared to the general population (SIR 0.92).

Studies documenting comparable incidence of CRC Post-Organ Transplantation:

Author	Year of Publication	Number of Transplant Patients	Transplanted Organ	Number of CRC Cases	Institution	SIR or Incidence Rate
Aigner et al.	2007	3595	Liver (757), Kidney, Heart, Lung, Pancreas, Small Intestine	9	Innsbruck Medical University, Innsbruck, Austria	0.25% (0.01–3.9% normal)
Merchea et al.	2014	3946	Kidney, Liver (6), Heart, Lung	20	Mayo Clinic	0.5%

Rompianesi G. et al.	2018	8115	Liver (8115)	52	Institute for Liver and Digestive Health, Royal Free Hospital, London, UK	SIR 0.92
Silva M.A. et al.	2005	6476	Liver	42	University Hospital Birmingham	SIR 1.01

Studies Showing Increased CRC Incidence Post-Transplantation:

Engels et al. reviewed data involving 175,732 solid organ transplant recipients from 13 registries found that the overall cancer risk post-transplant was double that of the general population (SIR 2.1). Liver transplant recipients constituted 22% of this cohort. The risk was elevated for 32 different malignancies, notably non-Hodgkin lymphoma, lung, liver, and kidney cancers. CRC risk was slightly increased (SIR 1.24).

A 2010 meta-analysis by Sint Nicolaas et al. reviewed 29 studies on CRC incidence post-LTx, finding a 2.6-fold increased risk. This risk decreased to 1.8 times after excluding PSC patients. Kang et al. reported an 8.4-fold higher CRC risk in liver transplant recipients in South Korea, with hepatitis B virus (HBV), hepatitis C virus (HCV), and alcohol being the main indications for transplantation.

A Swedish study by Adami et al. found a 4.0 SIR for neoplasms in transplant recipients and a 2.3 SIR for colorectal adenocarcinoma.

A recent epidemiological study Huo et al. evaluated 2,105,122 organ transplant recipients and found a 2.5-fold higher overall cancer risk and nearly double the CRC risk (SIR 1.89) compared to the general population.

Studies confirming increased incidence of CRC Post-Organ Transplantation:

Author	Year of Publication	Number of Transplant Patients	Transplanted Organ	Number of CRC Cases	Institution	SIR or Incidence Rate
Engels et al.	2011	175 732	Liver (37,958), Kidney, Heart, Lung	627	US Scientific Registry of Transplant Recipients	SIR 1.24 for CRC
Sint Nicolaas et al.	2010	18620	Liver	111	Erasmus MC University Medical Center, Rotterdam, Netherlands	SIR 1.8
Kang et al.	2018	348	Liver	17	Seoul National University School of Medicine, Seoul, Korea	SIR 8.4
Adami et al.	2003	5931	Liver (394), Kidney, Heart, Lung, Pancreas	25	Karolinska Institutet, Stockholm, Sweden	SIR 2.3 for CRC
Huo Z. et al.	2020	2105122	Kidney, Liver, Heart, Lung	Data available only as Standardized Incidence Ratio	Hospital of Guangzhou Medical University, Guangzhou, China	SIR 1.89 for CRC
Safaeian M. et al.	2016	224098	Liver (220), Kidney, Heart, Lung	790	Division of Cancer Epidemiology and Genetics, Bethesda, USA	SIR 1.34 for CRC

Haagsma et al.	2001	174	Liver	3	University Hospital Groningen, Netherlands	SIR 4.3 for all types of malignancies

Our Experience

From 1996 to May 2022, 2,223 transplants were performed at IKEM, involving 2,047 patients. Six patients (0.29%) were diagnosed with CRC post-transplantation, detected between 2 to 6 years post-transplant despite pre-transplant colonoscopy showing no neoplasia. Four cases (66.67%) were in patients without PSC/UC, all located in the right colon. One patient had regular colonoscopy screenings leading to early CRC detection and surgical resection. Three cases were incidental findings during other examinations. Two cases (33.33%) were in patients with PSC/UC, detected during regular colonoscopy 4- and 8-years post-transplant.

Characteristics of Patients with CRC Post-OLTx at IKEM:

Gender	Age at LTx	Indication	Time to CRC Onset from LTx (years)	Location	TNM	Treatment	Course	Immunosuppression
Female	58	Alcohol+HBV	4	Cecum	pT2pN0 Mx	Ileocecal resection	Alive	Tacrolimus
Male	56	HCV	6	Cecum	pT3pN0 pMx	Right hemicolectomy	Alive	Tacrolimus, Mycophenolate
Female	70	Cryptogenic	2	Hepatic flexure	pT2pN0 pM1b	Right hemicolectomy, adjuvant chemotherapy, palliative chemotherapy	Deceased 4 years after CRC diagnosis	Mycophenolate
Male	35	PSC/UC	8	Descending colon	Unknown	Left hemicolectomy, total colectomy for recurrence in anastomosis, palliative chemotherapy	Deceased 11 years after CRC diagnosis	Cyclosporine, Prednisone

Male	61	PSC/UC	4	Splenic flexure	pT4aN0 pMx	Proctocolectomy, adjuvant chemotherapy	Alive	Tacrolimus, Mycophenolate, Prednisone
Male	41	Biliary atresia, secondary biliary cirrhosis	9	Right transverse colon	pTxNxM1	Palliative chemotherapy	Deceased 0.5 years after CRC diagnosis	Tacrolimus, Mycophenolate

Conclusion

The incidence of CRC in liver transplant patients is comparable to or slightly higher than in the general population. The risk is significantly higher in liver transplant patients with PSC, both with and without the presence of UC. This supports the need for regular endoscopic surveillance in these patients. For patients transplanted for other indications, the CRC risk is minimally increased, suggesting standard screening protocols are sufficient. Pre-existing liver and bowel diseases are the main risk factors, with transplantation-associated malignancies playing a minor role. CRC progression can be faster, and advanced stages are more common, leading to worse prognosis.

Mutational analysis of driver genes defines the colorectal adenoma: in situ carcinoma transition

In this publication we determined some differences and similarities of mutation profile among low- and high-grade adenomas and in situ carcinoma and also transitions between them through mutational analysis of the key driver genes, which are known in the adenoma-carcinoma sequence. Utilizing a high-throughput genotyping technique, the study investigated the mutation spectrum of genes such as APC, KRAS, and TP53. The research aimed to define the genetic heterogeneity and mutation that characterize this progression, supporting the multistep model of carcinogenesis and providing insights into potential biomarkers for early detection and treatment strategies.

Objectives

The aim of the study was to determine the differences and similarities in the mutation profiles of well-known genes involved in CRC across low-grade adenomas, high-grade adenomas, and in situ carcinomas. By investigating the mutation spectrum of genes such as APC, BRAF,

EGFR, NRAS, KRAS, PIK3CA, POLE, POLD1, SMAD4, PTEN, and TP53 in a well-defined series of 96 colorectal adenomas and in situ carcinomas using a high-throughput genotyping technique, the study tried to explain the genetic progression of early stages of adenomas and in situ carcinomas. Additionally, the study aimed to analyze microsatellite instability and the promoter methylation status of APC and MLH1 to better understand their roles in the CRC progression.

Specific aims included:

1. Investigating the mutation spectrum of genes involved in the early stages of CRC (APC, BRAF, EGFR, NRAS, KRAS, PIK3CA, POLE, POLD1, SMAD4, PTEN, and TP53).
2. Assessing the frequency and types of mutations in these genes.
3. Exploring the association between mutation profiles and clinical-pathological characteristics.
4. Providing insights into the multistep model of CRC progression and identifying potential biomarkers for early diagnosis.

Methods

Tissue Samples:

Fresh frozen tissue samples were collected from colorectal adenomas and in situ carcinomas at three institutions in Prague, Czech Republic: Thomayer University Hospital, University Hospital Kralovske Vinohrady, and Mediconas. The samples were obtained during routine colonoscopy procedures. The cohort consisted of 96 patients, with adenomas classified into tubular, villous, and tubulo-villous histologies. Patients with hereditary CRC syndromes, inflammatory bowel disease, hyperplastic polyps, or previous malignancies were excluded from the study. Ethical approval was granted by the respective committees, and all participants provided informed consent.

DNA and RNA Isolation:

Total DNA was extracted using the AllPrep DNA/RNA Isolation kit (Qiagen, Germany) according to the manufacturer's instructions. The purity and quantity of DNA were assessed using a Nanodrop spectrophotometer, ensuring OD260/280 ratios between 1.8 and 2.0. The extracted DNA was stored at -80°C until further use. For methylation analysis, 200 ng of DNA

from each sample was treated with sodium bisulfite using the EpiTect Bisulfite Kit (Qiagen, Germany).

Mutational Analysis:

A custom multiplex PCR sequencing panel was employed to detect mutations in 11 key genes implicated in CRC: APC, BRAF, EGFR, NRAS, KRAS, PIK3CA, POLE, POLD1, SMAD4, PTEN, and TP53. The panel included M13-tailed primer pairs designed to amplify mutation hotspots in these genes. PCR amplification was performed using FastStart Hifi Enzyme Blend (Sigma-Aldrich, St. Louis, MO, USA) in two PCR pools. PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter Life Sciences, Brea, CA, USA). The Ion Torrent PGM sequencer (Thermo Fisher) was used for sequencing, with reads mapped against the human reference genome (GRCh37/hg19) using TMAP 5.0.7 software. Variants were called using VarScan with stringent coverage and variant allele frequency cut-off values.

MSI Status:

Microsatellite instability (MSI) status was determined using a pentaplex PCR assay targeting five mononucleotide repeat markers (BAT-25, BAT-26, NR-21, NR-24, NR-27). PCR products were analyzed using an ABI 3130 genetic analyzer (Applied Biosystems), and fragment analysis was conducted with GeneMapper v4.1 software. Samples were classified as MSI-H if two or more markers were unstable, MSI-L if one marker was unstable, and microsatellite stable (MSS) if no instability was observed.

Statistical Analysis:

Statistical analysis was performed using STATISTICA (version 11Cz; TIBCO Software Inc.), Matlab (version 2019b; The MathWorks, Inc.), SISA, and JVenn. Fisher's exact test and Mann–Whitney U test were used to assess the associations between mutations, clinical characteristics, and methylation status. Confidence intervals for mutation frequencies were calculated using the Agresti and Coull method. Statistical significance was set at $\alpha = 0.05$.

Results

Patient Characteristics

The study cohort consisted of 58 men and 38 women, with a mean age of 65.6 years. Adenomas were predominantly located in the colon, while in situ carcinomas were more frequently found in the rectum.

Patient's characteristics:

		All n = 96 (%)	Adenomas n = 74 (%)	In situ carcinomas n = 22 (%)	*p* value (test) for difference between adenomas and in situ carcinomas
Age (mean ± SD) years		65.6 ± 10.4	65.1 ± 10.6	67.0 ± 9.9	0.48 (*t* test)
Sex	Men	58 (60.4)	46 (62.2)	12 (54.5)	0.62 (Fisher's)
	Women	38 (39.6)	28 (37.8)	10 (45.5)	
Lesion site	Colon	54 (56.2)	46 (62.2)	8 (36.4)	0.05 (Fisher's)
	Rectum	42 (43.8)	28 (37.8	14 (63.6)	
Polyp type	Tubular	46 (47.9)	37 (50)	9 (40.9)	0.62 (Fisher's)
	Tubulo-villous	38 (39.6)	29 (39.2)	9 (40.9)	
	Villous	12 (12.5)	8 (10.8)	4 (18.2)	
Grade*	Low	–	49 (66.2)	–	–
	High	–	25 (33.8)	–	

Table 1. Patient´s clinical characteristics. *For adenoma patients only.

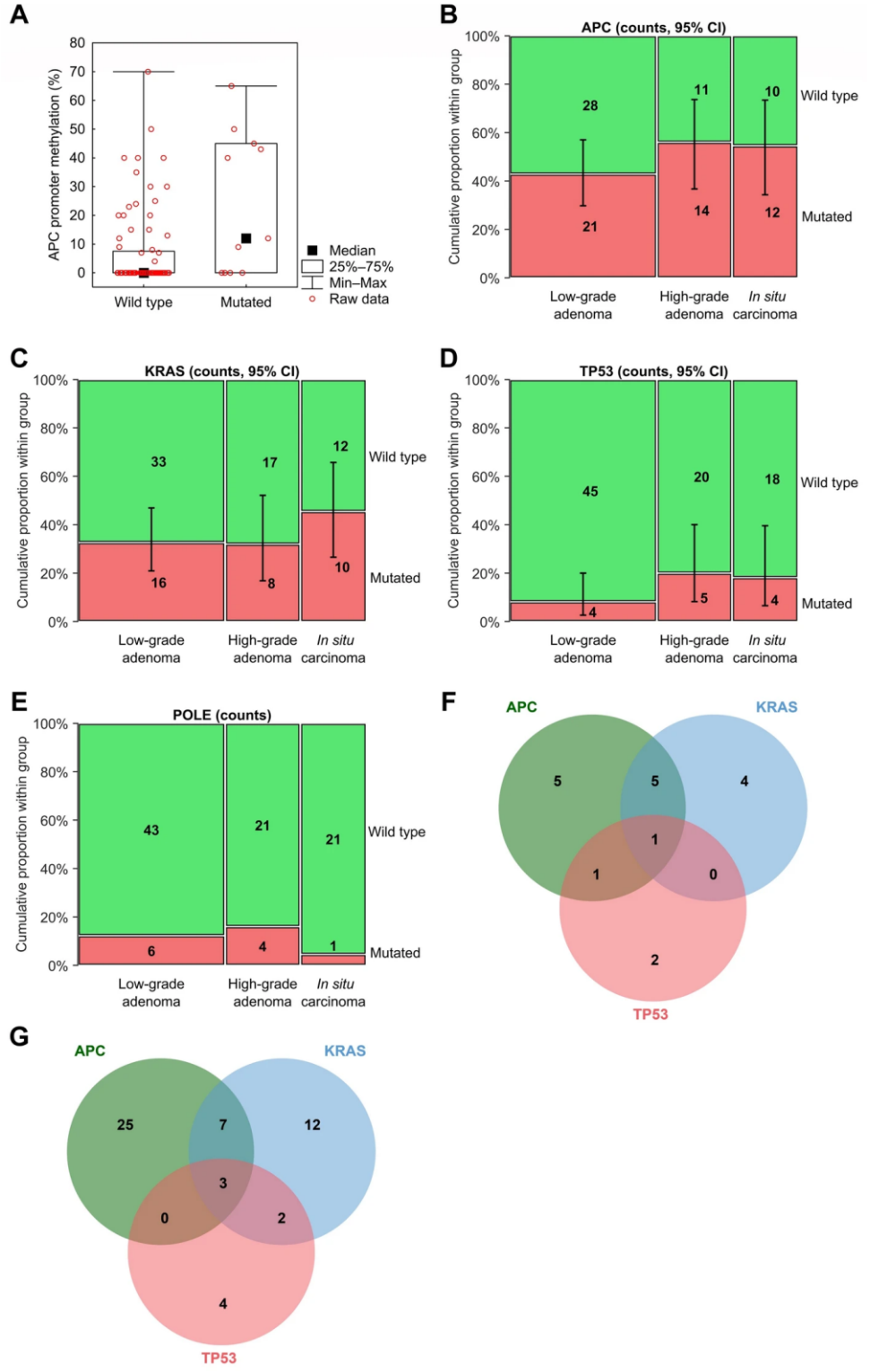
The studied set included 96 patients, out of which 74 were patients with adenomas and 22 with in situ carcinoma. The clinic-pathological characteristics are presented in the Table.

Mutation Spectrum and key findings:

In total, 96 adenomas and in situ carcinomas were analyzed for mutations in the selected genes.

- The APC, KRAS and TP53 mutation frequencies were slightly lower in adenoma samples than in in situ carcinoma samples.
    - APC gene 47.3% - 54.5%
    - KRAS gene 32.4% - 45.5%
    - TP53 gene 12.2% - 20%
- When we stratified mutation frequency based on the grade, the frequency distribution was as follows: low grade adenoma—high-grade adenomas—in situ carcinoma:
    - APC gene 42.9 – 56.0 – 54.5%;
    - KRAS gene 32.7 – 32.0 – 45.5%;
    - TP53 gene 8.2 – 20.0 – 18.2%.
- In our cohort, only 3 samples had microsatellite instability (2 of them with MSI-L and one with MSI-H status)

The mutated gene signature of colorectal adenomas and in situ carcinomas:



(A) The APC promoter methylation distribution with POLE genetic variations, (B) The mutation distribution of APC gene between low-, high-grade adenomas and in situ carcinomas, (C) The mutation distribution of KRAS gene between low-, high-grade adenomas and in situ carcinomas, (D) The mutation distribution of TP53 gene between low-, high-grade adenomas and in situ carcinomas, (E) The mutation distribution of POLE gene between low-, high-grade adenomas and in situ carcinomas, (F) The Venn diagram of mutations of APC, TP53, and KRAS genes in in situ carcinomas, (G) The Venn diagram of mutations of APC, TP53, KRAS, and POLE genes in adenomas.

APC Mutations

The APC gene, a critical component of the WNT signaling pathway, exhibited a variety of mutations across adenomas and in situ carcinomas. In adenomas, 36 deleterious mutations were identified, including deletions, insertions, nonsense, and missense mutations. In in situ carcinoma samples, 13 mutations were observed, including similar types of alterations. Notably, specific mutations (e.g., p.Q1226fs, p.E1379X) were found in both high-grade adenomas and in situ carcinomas, suggesting these mutations may play a significant role in the transition from adenoma to in situ carcinoma.

KRAS Mutations

KRAS mutations were detected in 34 individuals, with a higher frequency of mutations at codon 12, particularly the c.35G>A transition. These mutations were associated with villous histology, indicating a potential link between KRAS mutations and specific morphological features of adenomas. The presence of KRAS mutations in both adenomas and in situ carcinomas suggested their involvement in early and later stages of tumor progression.

TP53 Mutations

TP53, a tumor suppressor gene, showed mutations in 13 patients, including missense and insertion mutations. The frequency of TP53 mutations had an increasing tendency from low-grade towards in-situ carcinoma. The p.R43H mutation was recurrent in both high-grade adenomas and in situ carcinomas.

Co-mutations of APC, KRAS, and TP53

A subset of samples (4 out of 96) exhibited concurrent mutations in APC, KRAS, and TP53 genes. These co-mutations were predominantly found in high-grade dysplasia adenomas and in situ carcinomas, reinforcing the idea that the accumulation of multiple driver mutations is critical for the adenoma-carcinoma transition.

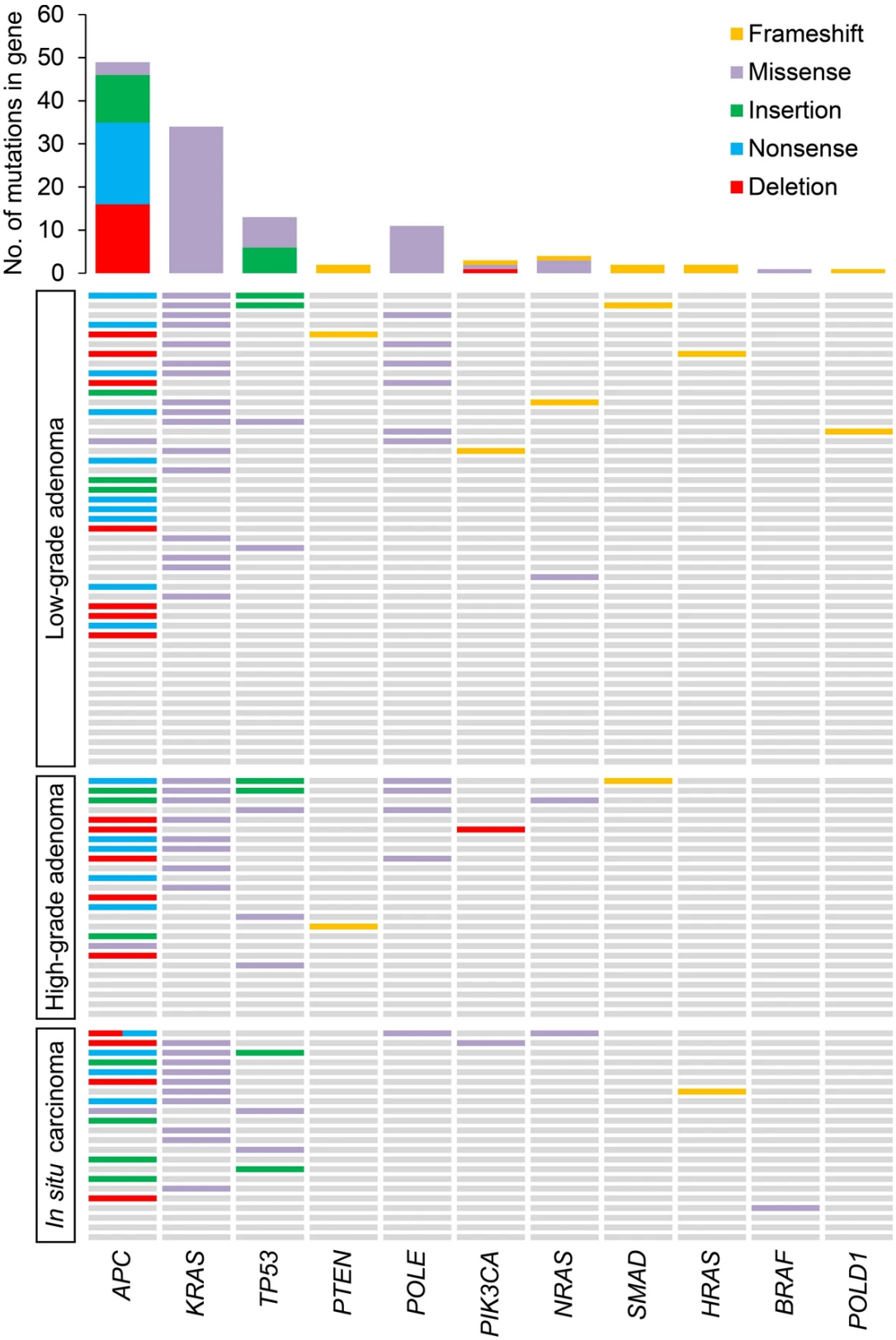
Other Mutations

Mutations in other genes, such as NRAS, BRAF, PIK3CA, SMAD4, and PTEN, were also identified but were less frequent. Notably, a BRAF mutation (c.1799T>A, p.V600E) was found in an MSI-H in situ carcinoma, consistent with the association between BRAF mutations and

MSI status in CRC. PIK3CA mutations were detected in both adenomas and in situ carcinomas, with a higher frequency in the latter, suggesting their role in later stages of tumor development.

Mutations in the POLD1 and POLE genes were also studied. A c.1263 dupG:p.L421fs mutation in the POLD1 gene was observed in low-grade dysplasia. In 12 samples (11 adenomas and 1 in situ carcinoma), a POLE (c.T1166C:p.F389S) mutation was identified. This particular POLE mutation was not recorded in major public databases (COSMIC, LOVD, or HGMD), which lead us to classify it as a variant of uncertain significance (VUS) rather than a pathogenic mutation.

The distribution of genetic alterations detected in low-grade, high-grade adenomas, and in situ carcinomas:



Each row represents a patient, and each column represents a gene. Different mutation types are indicated by different colors. The bar chart on the top shows the total number of the given gene's mutations observed in the sample.

MSI status

MSI status of all adenomas and in situ carcinomas was tested. In our set, only 3 samples had MSI instability: 2 of them with MSI-L and one with MSI-H status. The MSI-H status was observed in an in situ carcinoma located in the right colon while MSI-L was noticed in two low grade dysplasia samples located in the left colon.

APC and MLH1 promoter methylation

The study examined the promoter methylation of APC and MLH1 genes using MS-HRM. The mean promoter methylation in the APC gene was 9%, with 8% in in situ carcinoma and 8.4% in adenomas. There was a significant association between APC promoter methylation and POLE genetic variations ($p = 0.02$). The only hypermethylated MLH1 promoter was found in the single sample with MSI-H status.

Follow up

In the follow-up study of 70 out of 96 patients, 21 developed a subsequent adenoma. Among these, 17 had prior adenomas (7 high-grade, 10 low-grade), and 4 had in situ carcinomas. All patients were of microsatellite stable (MSS) status except one low-grade adenoma which was MSI-L. The follow-up adenoma tissue was not available for further analysis. Most patients had mutations in high-risk genes such as APC, KRAS, or TP53, but no specific mutation was linked to the occurrence of subsequent adenomas. Patients with mixed tubule-villous histology were less likely to develop new adenomas compared to those with villous or tubular histology alone ($p = 0.02$). Older patients were more likely to develop subsequent adenomas than younger ones ($p = 0.04$).

All in situ carcinoma patients had their tumors surgically removed and were monitored regularly. Of the 22 in situ carcinoma patients, 4 developed new adenomas, and 6 progressed to invasive carcinoma within a few years. The risk of developing invasive carcinoma was significantly higher for patients with initial in situ carcinoma (27.3%) compared to those with low-grade (4.1%) or high-grade adenomas (4.0%) ($p = 0.009$). In situ carcinoma patients who progressed to CRC often had new tumors in the same location within two years, while patients with adenomas that later developed CRC had new cancers in different colon segments.

Conclusion

The frequency of mutations in APC, TP53, and KRAS increased towards carcinoma in situ. These data support Vogelstein's multistep model of CRC development, with our cohort showing that adenomas and in situ carcinomas only rarely have MSI status.

Discovery of Long Non-Coding RNA MALAT1 Amplification in Precancerous Colorectal Lesions

The malignant progression of adenoma to carcinoma involves multiple factors, including chromosomal instability (CIN), microsatellite instability (MSI), epigenetic influences like CpG island methylation (CIMP), and mutations in driver genes. CIN, which occurs in 65-70% of sporadic colorectal cancer cases, is caused by losses or gains of loci on short or long arms of chromosomes or even losses or gains of whole chromosomes. Despite its prevalence, CIN is rarely studied in colorectal adenomas. This study aimed to investigate chromosomal aberrations in colorectal adenoma tissues with similar histological and clinical features, utilizing the array-based comparative genomic hybridization (aCGH) method over a wider number of precancerous colorectal stages.

Methods:

Sample Collection**:** The study included 16 individuals with tubular or tubulo-villous adenomas who underwent colonoscopy for preventive reasons or due to intestinal discomfort. Sample collection occurred from March 2017 to December 2020 in cooperation with Thomayer Hospital and the Institute for Clinical and Experimental Medicine in Prague. Ethical approval and informed consent were obtained. Adenoma and adjacent tissue biopsies were stored at -80°C, and histopathological examinations confirmed no malignancy.

DNA Extraction**:** Genomic DNA was extracted from adenomas and adjacent tissues using the AllPrep DNA/RNA Mini Kit. Tissue disruption was performed using MagNa Lyser Green Beads in a MagNaLyser Instrument. DNA concentration was measured with the Qubit™ dsDNA BR Assay Kit on a Qubit 3.0 fluorometer.

Comparative Genomic Hybridization Array Design**:** The study used SurePrint G3 Cancer CGH+ SNP Microarray Kits to cover cancer-associated genomic regions. DNA from adjacent

tissues served as reference DNA, labeled with Cy5, while adenoma DNA was labeled with Cy3. The average input DNA amount was 850 ng. Hybridization was conducted with an Oligo aCGH/ChIP-on-chip Hybridization kit.

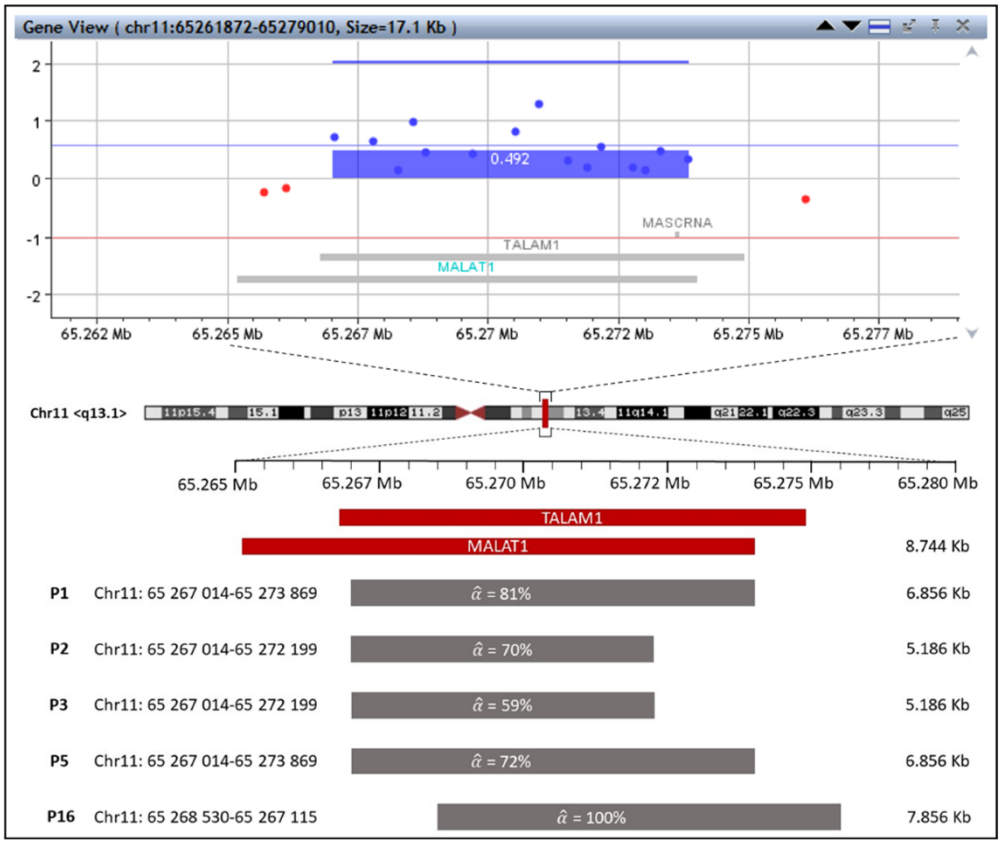
Array Processing and Bioinformatics Data Analysis: Arrays were scanned using the SureScan Microarray Scanner, and data were processed in Agilent CytoGenomics software. The presence of mosaicism was estimated using a modified formula based on observed fold changes in mean log ratio.

Results:

The aCGH method was successfully applied to 16 pairs of colorectal adenoma and adjacent mucosa samples, demonstrating varying degrees of CIN. The samples were categorized into four groups based on observed chromosomal aberrations:

Group 1: Patients P1, P2, P3, P5, and P16 exhibited gains on chromosome 11, specifically at 11q13.1, encoding long non-coding RNA (lncRNA) MALAT1 and its antisense transcript TALAM1.

The first Group of patients with MALAT1 and TALAM1 gain:



The representative example of gain in region encoding MALAT1, TALAM1, and MASCRNA is pictured as a blue rectangle in the region at chr11, q13.1, in adenoma of P1 at the top of the image. Thin horizontal red and blue lines represent values corresponding to the non-mosaic state of deletions (−1) or duplications (0.58). A comparison of this gained region between patients from the first group (P1, P2, P3, P5, and P16) is shown at the bottom of the image. Mosaicism of each patient is expressed by the symbol 'α'. [3]

Group 2: Patients P6, P7, and P10 showed numerous chromosomal microdeletions compared to adjacent tissue, without gains in regions encoding MALAT1 or TALAM1.

Group 3: Patients P4, P5, and P16 had disrupted karyotypes with many losses and gains. This group was characterized by relatively young patients.

Group 4: Patients P8, P9, P11, P12, P13, P14, and P15 showed no differences between adenoma and adjacent tissue.

Mosaicism was detected in all nine samples with aberrations, and the loss of the tumor suppressor gene TSC2 was notable in five patients. Some microdeletions were recurrent, including COL1A1, NOTCH1, MIR4673, and GNAS.

Conclusion:

We analyzed paired samples of colorectal adenomas and its adjacent mucosa from 16 patients with histologically similar samples. CIN was confirmed in 56% of the adenomas. A significant gain on chromosome 11q13.1, encoding the lncRNA MALAT1, was found in five patients. This study offers novel insights into chromosomal aberration in colorectal adenomas and identifies the lncRNA MALAT1 as a promising candidate biomarker for further investigation.

MALAT1 in Liquid Biopsy: The Diagnostic and Prognostic Promise for Colorectal Cancer and Adenomas

The long non-coding RNA Metastasis-Associated Lung Adenocarcinoma Transcript 1 (MALAT1) was first linked to carcinoma in 2003, when higher MALAT1 expression levels were found and associated with a poorer prognosis in metastatic non-small cell lung cancer. Since then, numerous studies have investigated MALAT1 in various cancers, revealing its aberrant expression in gastric, breast, and pancreatic cancers, among others.[4, 122]

Despite significant advancements in the diagnosis and treatment of CRC, early detection remains a critical challenge due to the invasiveness of current methods like colonoscopy. Research has increasingly focused on non-invasive alternatives, such as liquid biopsy, which involves analyzing cell-free nucleic acids (cfNAs) from body fluids. This approach offers a promising tool to detect early-stage adenomas and CRC.

In our previous study we identified MALAT1 as a potential biomarker for CRC and adenomas, by showing MALAT1 DNA amplification in adenoma tissues compared to healthy adjacent mucosa of the same patient. [3]

This study aimed to investigate the role of MALAT1 in CRC development. While MALAT1 expression levels have been identified as a prognostic biomarker in lung cancer, few studies have explored its expression in CRC and colorectal adenomas. The study focused on four objectives:

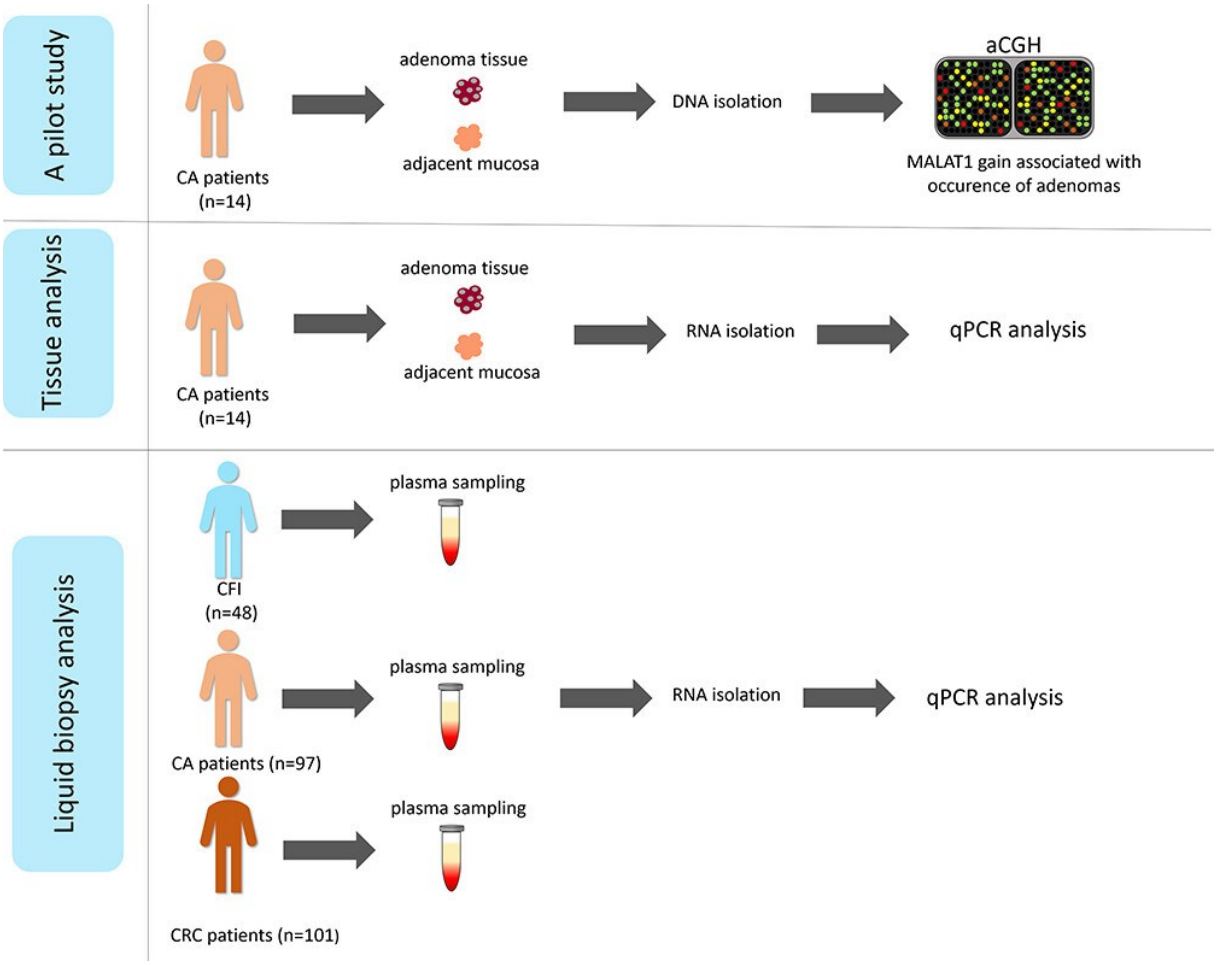
I. The amplification of MALAT1 might be reflected at the mRNA expression level
II. MALAT1 expression levels can be detected in plasma from CRC and CA patients
III. MALAT1 expression levels have a potential as a circulating diagnostic biomarker for CA, CRC, and cancer-free individuals (CFI)
IV. MALAT1 levels might be associated with the therapy response

Materials and Methods:

MALAT1 expression was measured in CA tissues and plasma samples from patients with CA, CRC, and cancer-free individuals (CFI) using real-time quantitative polymerase chain reaction. The study included a pilot cohort of 14 histologically confirmed CA patients, with biological samples collected during planned colonoscopies between March 2017 and May 2022. Additionally, CRC patients were recruited from 2007 to 2018 and followed until August 2021. Patient data, including demographics, cancer history, smoking habits, body mass index, and disease characteristics, were collected through structured questionnaires. Exclusion criteria were hereditary CRC syndromes, inflammatory bowel disease, hyperplastic polyps, and prior malignancies. RNA was extracted from tissue and plasma samples, reverse transcribed, and analyzed using RT-qPCR, with HPRT1 as the reference gene. Statistical analysis using the Wilcoxon rank-sum test evaluated differences in MALAT1 expression, and bioinformatics

tools identified overrepresented biological pathways affected by MALAT1. The study was approved by the Ethics Committees of the Institute for Clinical and Experimental Medicine.

Schematic workflow of the study:



RT-qPCR analysis of MALAT1 was performed in the tumor tissue and adjacent mucosa of CA patients (n=14) who were part of the pilot study and further in the plasma of CFI (n=48), CA patients (n=97) and CRC patients (n=101). Abbreviations: CFI, cancer-free individuals; CA patients, colorectal adenoma; CRC, colorectal cancer. [4]

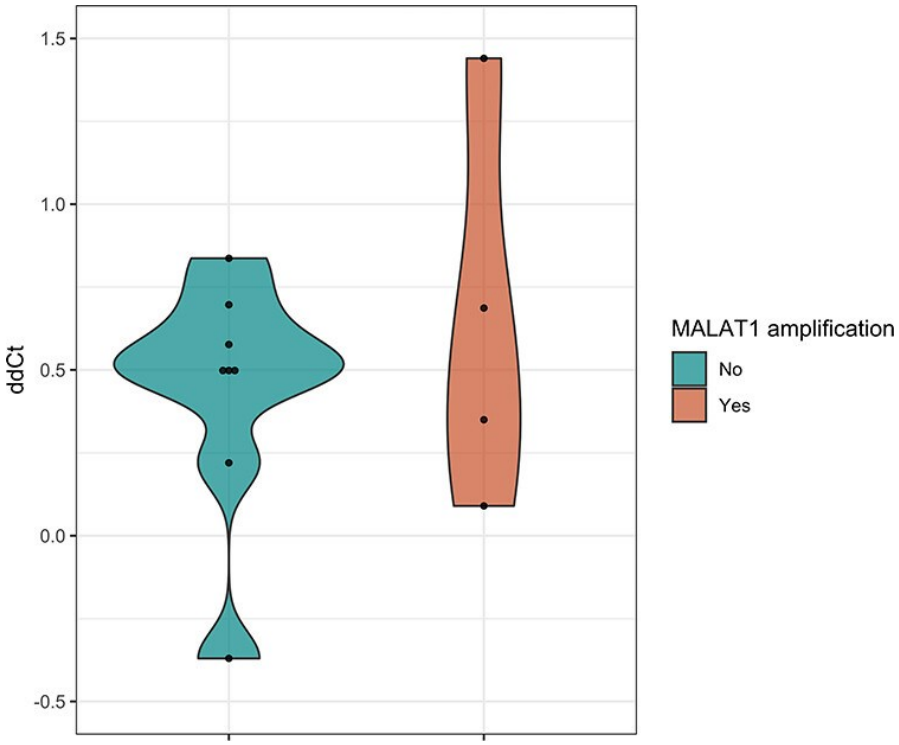
Results:

I. The amplification of MALAT1 might be reflected at the mRNA expression level

In the pilot study, chromosomal instability was investigated using array comparative genomic hybridization (aCGH) in adenoma tissue and adjacent mucosa from 16 patients who underwent planned colonoscopies. MALAT1 amplification was identified in 5 of these patients. This

finding prompted further analysis of MALAT1 expression levels in the same group of colorectal adenoma patients using real-time quantitative polymerase chain reaction. The analysis included 14 colorectal adenoma patients from the pilot phase, divided into groups based on the presence of MALAT1 amplification. The results showed no significant difference in MALAT1 expression levels between patients with and without MALAT1 amplification.

The expression levels of MALAT1 in the tissue of CA patients from the pilot phase:



No significant difference was observed between the groups with and without MALAT1 amplification (p=0.08, 2.08-fold-change) [4]

II. MALAT1 expression levels can be detected in plasma from CRC and CA patients

III. MALAT1 expression levels have a potential as a circulating diagnostic biomarker for CA, CRC, and cancer-free individuals (CFI)

In the plasma-based analysis, MALAT1 expression levels were examined as a potential non-invasive diagnostic biomarker through a liquid biopsy approach. Plasma samples from patients with colorectal adenomas (CA, n=97), colorectal cancer (CRC, n=101), and cancer-free individuals (CFI, n=48) were analyzed. MALAT1 levels were significantly elevated in CA (5.25-fold increase, $p<0.001$) and CRC patients (5.16-fold increase, $p<0.001$) compared to CFIs. (Figure 1.) However, MALAT1 could not distinguish between CA and CRC patients. When CA patients were categorized by adenoma histology, MALAT1 levels were higher in

tubulovillous/villous adenomas, which have a higher cancer risk, compared to hyperplastic adenomas (6.14-fold increase, p=0.002). (Figure 2).

The expression level of MALAT1 in plasma:

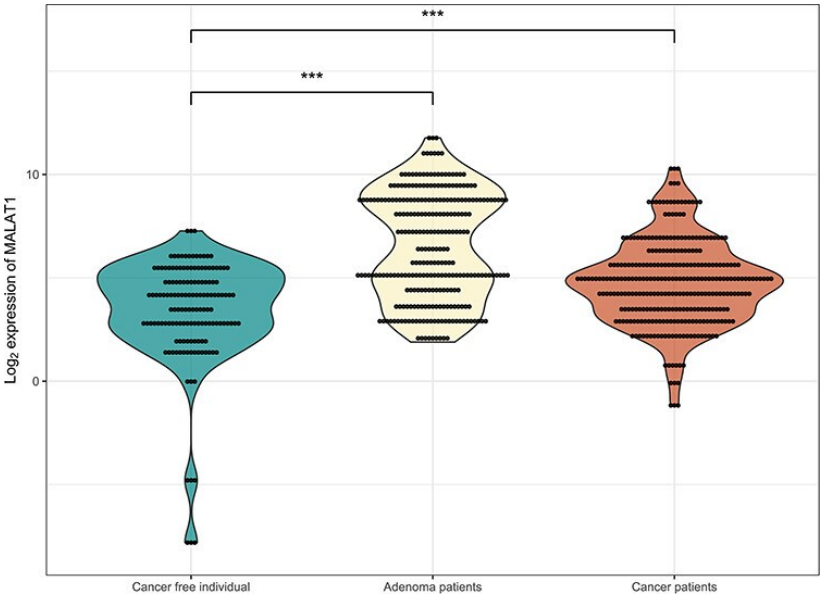


Figure 1 There are significant differences between CA and CRC patients compared to CFI (CA vs CFI – p<0.001, 5.25- fold change; CRC vs CFI – p<0.001, 5.16-fold change). The asterisk indicator (***) represents p ≤ 0.001.[4]

The expression level of MALAT1 in different types of adenomas:

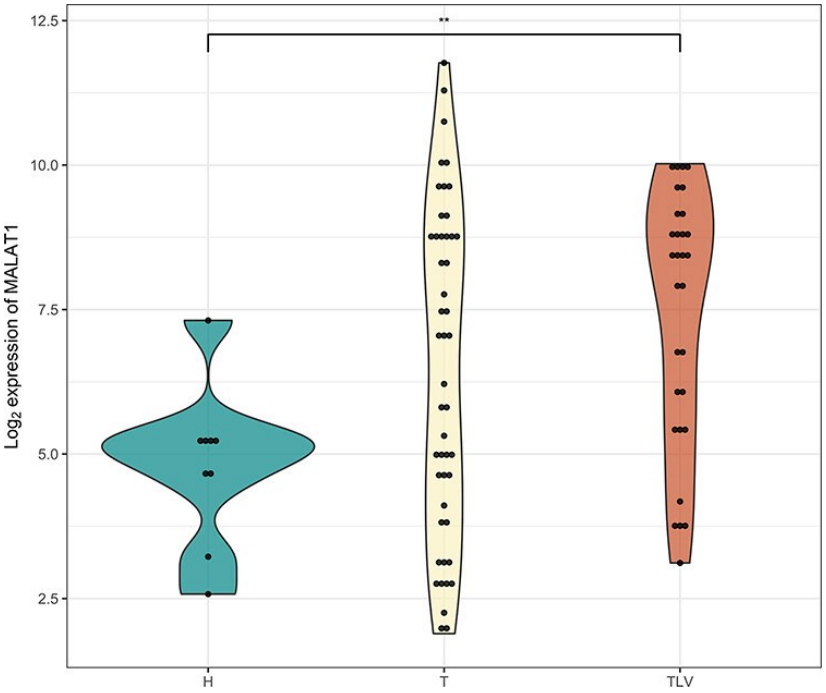


Figure 2. There was a significant difference between hyperplastic (H; n=9) and tubulovillous/villous (TLV; n=30) adenomas (p=0.002, 6.14-fold change). Tubular (T; n=50) adenomas did not show a significant difference in MALAT1 expression (p=0.15, 3.09-fold change). The asterisk indicator (**) represents p ≤ 0.01. [4]

IV. MALAT1 levels might be associated with the therapy response

Among CRC patients, those who responded poorly to therapy had significantly higher MALAT1 expression levels than good responders (1.86-fold increase, p=0.04). (Figure 3) No association was found between MALAT1 levels and other patient characteristics such as sex, tumor location, or CRC stage.

The expression level of MALAT1 in CRC patients divided according to therapy response:

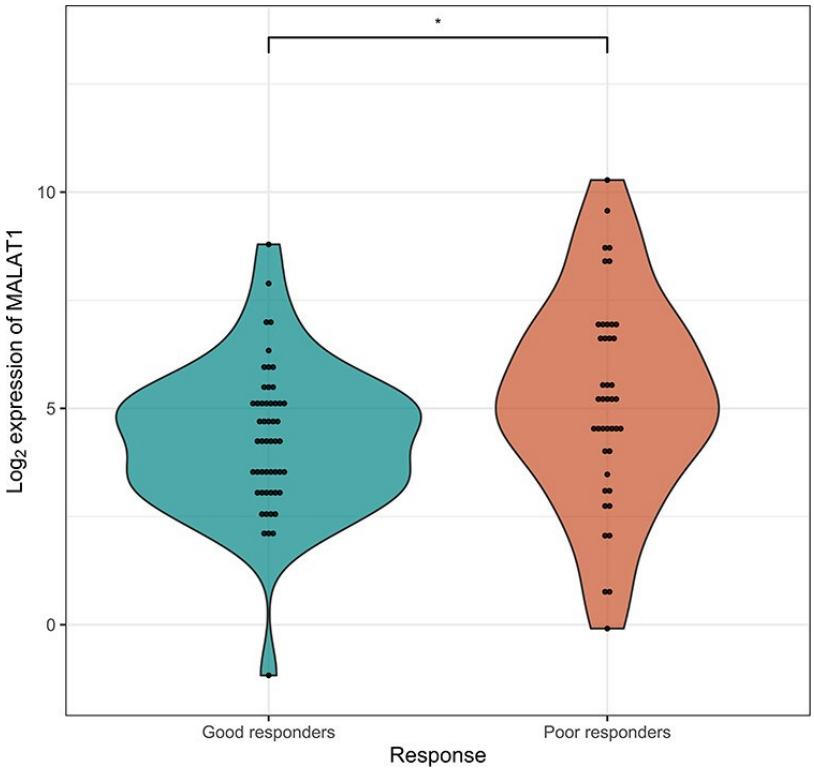


Figure 3.. There was a significant difference between good (n=52) and poor responders (n=42; p=0.04, 1.86-fold change). The asterisk indicator (*) represents p ≤ 0.05. [4]

Bioinformatic Analysis

In the study we conducted a Gene Ontology (GO) analysis of the validated and predicted MALAT1 targets. Several biological processes, molecular functions, and cellular component were identified. These included those related to the Major Histocompatibility Complex (MHC) class I protein complex and ribosome-related processes, highlighting MALAT1's involvement in immune response regulation and proteosynthesis. [4]

These findings suggest that MALAT1 may play a role in the adaptive immune response, potentially influencing antigen presentation and immune escape mechanisms in cancer cells

through various molecular mechanisms, acting primarily as a competing endogenous RNA (ceRNA) by sponging microRNAs that would otherwise regulate the expression of target genes involved in cancer cell proliferation, migration, and invasion. MALAT1 negatively correlates with miR-106b-5p, upregulating SLAIN2 and promoting cell invasiveness and CRC progression. By sponging miR-101, MALAT1 increases autophagy activation and cellular viability, contributing to irradiation resistance in CRC cells. MALAT1 overexpression and miR-145 downregulation accelerate cancer cell growth, invasion, and migration by upregulating SOX9. Additionally, MALAT1 influences EZH2 expression by sponging miR-363-3p, promoting CRC development through epigenetic modifications. MALAT1 indirectly affects RUNX2 by sponging members of the miR-15 family, leading to increased cell proliferation and tumor growth. High MALAT1 levels suppress miR-203, increasing DCP1A expression and promoting mRNA degradation and cancer progression. MALAT1's interaction with miR-324-3p affects drug resistance by regulating ADAM17, while upregulation of miR-20b decreases MALAT1, reducing cancer cell migration and invasion by downregulating Oct4. MALAT1 also sponges miR-26a/26b, upregulating FUT4 and SMAD1, enhancing CRC cell aggressiveness and metastasis. Furthermore, MALAT1 interacts with miR-508-5p, miR-619-5p, miR-663a, and miR-21, affecting various downstream targets like RAB14 and YAP1. Histone demethylase JMJD2C regulates MALAT1 expression, and MALAT1 interacts with non-histone protein HMGB1 through miR-129-5p sponging, impacting chromatin organization and transcription regulation. Overall, MALAT1's interaction with multiple miRNAs and its regulation of various target genes and proteins highlight its crucial role in CRC progression and its potential as a therapeutic target. [123]

Conclusion:

Our study did not confirm an association between MALAT1 amplification and its gene expression. However, plasma MALAT1 levels could distinguish carcinoma and especially adenoma patients from cancer-free individuals. This could lead to the earlier detection of precancerous lesions, their subsequent removal, and thus prevent the development of CRC. In addition, MALAT1 expression level might reflect the patient´s therapy response to oxaliplatin. In conclusion, MALAT1 expression levels could serve as a circulating biomarker for the early diagnosis of colorectal adenoma and carcinoma, as well as a predictor of therapy response in CRC patients.

VIII. Discussion

Colorectal Cancer After Liver Transplantation

The etiopathogenesis of post-transplant malignancies involves patient-related factors, transplantation itself, and accompanying immunosuppressive therapy. Patient-related factors include age, smoking, alcohol consumption, chronic inflammatory diseases, reactivation of oncogenic viruses (e.g., Epstein-Barr virus, cytomegalovirus, JC virus, human papillomavirus, hepatitis B and C viruses), or undiagnosed pre-existing malignancies. Tumor transmission from the donor, although rare, is possible, with melanomas and carcinomas of the lung, breast, colon, and kidney being most commonly reported.

Immunosuppression inhibits antitumor immune mechanisms and increases the incidence of virus-induced malignancies. Selgrad et al. demonstrated that the presence of JC virus (JCV) in colorectal mucosa combined with immunosuppression leads to viral reactivation and expression of the transforming T antigen (TAg), which inactivates tumor suppressor proteins p53 and pRb, disrupting cell cycle regulation and promoting tumorigenesis. [124]

One of the most significant risk factors for CRC is the IBD. Patients with PSC associated with UC have a tenfold increased risk of developing CRC, and those with PSC without UC have a fivefold increased risk compared to the general population. In this group, CRC can occur at an earlier age, in more advanced stages, and predominantly in the right colon. This relationship between PSC and UC with CRC is the strongest patient-side risk factor for post-transplant CRC, supported by numerous studies.

In 2013, Singh et al. highlighted the high risk of CRC post-liver transplantation in patients with PSC (IR 5.8/1,000 person-years, 95% CI 3.8–7.8) and PSC/UC (IR 13.5/1,000 person-years, 95% CI 8.7–18.2). This increased risk was subsequently confirmed in other studies. An analysis of American registries from 1987 to 2010 identified 790 CRC patients out of 224,098 post-transplant patients, with a SIR for CRC post-liver transplantation of 1.34 (95% CI 1.16–1.52). A more detailed analysis revealed that the risk significantly depends on the pre-transplant diagnosis. For all PSC patients, the SIR was 4.49 (95% CI 3.36–5.89); for PSC and UC patients, it was 5.69 (95% CI 3.98–7.88); for PSC without UC, it was 3.05 (95% CI 1.74–4.96); and for patients without PSC, it was 1.10 (95% CI 0.94–1.28). Similar results were observed in a national British study by Rompianesi et al., which did not show an increased risk of CRC in the overall population of liver transplant patients (SIR 0.92), but did show a significantly higher

risk in patients with PSC/UC (SIR 7.0). The knowledge of the high risk of CRC with often unfavorable disease progression supports the necessity for long-term endoscopic surveillance of patients, post-transplantation for PSC. At a minimum, monitoring should be comparable to recommendations for non-transplanted patients, with colonoscopy at one-year intervals. Some authors also discuss the role of prophylactic colectomy, partly because it has been shown that PSC patients who had a colectomy before transplantation have a lower risk of PSC recurrence. Most studies agree that CRC in liver transplant recipients is often detected in the right colon and at more advanced stages compared to the general population. Merchea et al. analyzed 63 CRC cases post-organ transplantation at Mayo Clinic, finding that CRC developed an average of 61 months post-transplant, with most cases in the right colon and a significant percentage at stage IV. The five-year survival rate was lower than in the general population, particularly for advanced-stage disease.

Mutational analysis of driver genes defines the colorectal adenoma: in situ carcinoma transition

The transition from adenoma to carcinoma in colorectal cancer (CRC) is a lengthy process, often spanning up to 20 years, and involves multiple genetic pathways including chromosomal instability, microsatellite instability, and CpG island methylator phenotype. This complex leads to genetic instability in adenomas, which can progress to malignant carcinomas. Despite extensive studies on advanced carcinomas, there is limited understanding of the mutation profiles in advanced adenomas and in situ carcinomas, and it is unclear if these stages share the same genetic background or if driver mutations are more prevalent in in situ carcinomas.

The present study aimed to investigate the mutation status of 11 genes implicated in CRC, as proposed by the Vogelstein model, in a series of adenomas and in situ carcinomas. These genes are involved in various signal pathways. Previous research indicated that mutations in APC, KRAS, and TP53 are common in CRC but rare in unaffected colonic crypts, suggesting these mutations play a key role in the transition from normal epithelium to adenoma and carcinoma. However, in this study, only 4 out of 96 individuals had concurrent mutations in APC, KRAS, and TP53, indicating that additional genetic alterations may occur during CRC progression.

Mutation frequencies in APC, KRAS, and TP53 were similar between adenomas and in situ carcinomas, though slightly higher in the later stages. Stratification by adenoma grade revealed

that APC and TP53 mutations increased in frequency towards in situ carcinoma, while KRAS mutations were lower in both low- and high-grade adenomas compared to in situ carcinomas. This suggests, that in situ carcinoma still carries the mutation profile of the adenoma and only during further progression the mutation frequencies changes, or alternatively vice versa, indicating that high-grade adenomas are already approaching in situ carcinomas with their mutation profile.

The study also highlighted the presence of POLE as variants of uncertain significance (VUS) in 12 samples, including 11 adenomas and 1 in situ carcinoma. The association of APC promoter methylation with POLE VUS suggests links between methylation, mutations, and DNA repair mechanisms, which are crucial in cancer genomics.

BRAF mutations were infrequent in adenomas and were observed in only one in situ carcinoma sample with MSI-H phenotype. The study also identified mutations in PIK3CA and PTEN genes, with PIK3CA mutations being less common in adenomas than in carcinoma in situ, supporting their later emergence in the adenoma-carcinoma transition.

The clinical follow-up of 70 patients revealed that several developed subsequent adenomas or CRC. Patients with tubulo-villous histology were less likely to develop further adenomas compared to those with tubular or villous histology. Older patients were more prone to subsequent adenomas, likely due to more frequent colonoscopies. Among the 22 patients with in situ carcinoma, 4 developed adenomas and 6 progressed to invasive carcinoma within a few years, highlighting the importance of monitoring and early intervention.

The study's limitations include a small patient cohort and incomplete follow-up of all patients, preventing comprehensive comparison of mutational profiles over time. Additionally, the analysis was restricted to adenoma tissue without adjacent unaffected tissue, limiting the ability to confirm somatic variants exclusively.

The findings emphasize the need for early cancer biomarkers that can differ healthy individuals from those with adenomas or early-stage CRC. Liquid biopsy, particularly circulating cell-free DNA (cfDNA), shows promise as a minimally invasive tool for detecting pathogenic mutations in genes like KRAS, BRAF, APC, and TP53. However, the sensitivity of cfDNA-based markers remains lower for early-stage disease compared to advanced stages, an issue that future studies aim to address by detecting pathogenic mutations in cfDNA from both plasma or stool samples of patients with adenomas and early carcinoma stages. In summary, the study provides valuable

insights into the mutation profiles of adenomas and in situ carcinomas, reinforcing the need for further research on early detection and monitoring strategies to improve patient outcomes.

Discovery of Long Non-Coding RNA MALAT1 Amplification in Precancerous Colorectal Lesions

Understanding the genetic changes in adenomas is crucial for early detection and prevention of CRC. This study focused on identifying structural and numerical chromosomal aberrations in colorectal adenomas, hypothesizing that chromosomal instability (CIN) is already present in colorectal adenomas before they progress to carcinoma stages. Using array comparative genomic hybridization (aCGH), we analyzed fresh frozen colorectal adenomas and their adjacent mucosa from 16 patients who underwent colonoscopy examinations.

The results revealed a wide variability in chromosomal instability among histologically similar colorectal adenomas. Based on the chromosomal profiles, patients were stratified into four different groups. The first group exhibited gains in the MALAT1 its antisense transcript TALAM1 long non-coding RNAs (lncRNAs) on chromosome 11. The second group consisted of patients with numerous chromosomal microdeletions without gains in the MALAT1 or TALAM1 regions. The third group showed significant karyotype disruption, with many chromosomal gains and losses, and included relatively younger patients. The fourth group had no detectable chromosomal instability in adenomas compared to adjacent tissues.

Frequent chromosomal losses were identified in genes such as TSC2, COL1A1, NOTCH1, MIR4673, and GNAS. The gain of MALAT1 in 5 out of the 16 patients possibly represented an event in adenoma development. MALAT1 affects cell proliferation by upregulating the Wnt/β-catenin signaling pathway, regulates transcription, and acts as a microRNA sponge, promoting cancer cell growth and migration. Therefore, we concluded that amplification of the region encoding MALAT1 and TALAM1 in 5 out of the 16 adenoma samples revealed cancer potential in these samples and we focused on this lncRNA in our later work.

Our findings also indicate that the presence of CIN in adenomas significantly contributes to the risk of CRC development. While most research has focused on CIN in carcinomas, this study demonstrated that CIN appears early in precancerous adenomas. In our cohort, 56% of the adenomas exhibited CIN, a slightly lower percentage than the 65-70% typically seen in CRC.

This difference aligns with our hypothesis that CIN is less prevalent in adenomas than in carcinomas.

Histological classification of adenomas did not correlate with the extent of CIN, highlighting the genetic diversity within adenomas with similar histological features. The study also revealed that young age might be a factor contributing to extensive karyotype variability in adenoma tissue cells, as seen in the third group of patients with severely disrupted karyotypes.

Identifying genetic markers such as MALAT1 in adenomas could enhance early detection and intervention strategies for CRC. Our findings raise doubt on the adequacy of histology-based classification alone and suggest that it should be complemented with genetic analysis to better predict adenoma progression to CRC.

Based on this study, where we discovered the gain of MALAT1, we decided to undertake further research to determine whether this gain also results in higher gene expression. Additionally, we aimed to focus on the function and potential use of MALAT1 in early diagnosis as follows.

MALAT1 in Liquid Biopsy: The Diagnostic and Prognostic Promise for Colorectal Cancer and Adenomas

MALAT1 plays roles in both transcriptional and posttranscriptional processes by interacting with various transcriptional and splicing factors. It acts as a competing endogenous RNA (ceRNA) that regulates microRNAs (miRNAs) with shared miRNA recognition elements (MREs), thereby inhibiting miRNA function and influencing gene expression. Key miRNAs associated with MALAT1 in CRC include the miR-15 family, miR-101, and miR-363-3p, which have significant clinical relevance.

Our previous study identified MALAT1 amplification in adenoma tissue, suggesting its role in CRC development. This study aimed to explore MALAT1 expression levels in adenoma tissues and plasma samples from patients with colorectal adenoma (CA), CRC patients, and cancer-free individuals (CFI). No significant difference was found in MALAT1 expression between patients with and without MALAT1 gain. We assume that CA patients with MALAT1 gain have this aberration in their adenoma tissue to compensate for the need of elevated MALAT1 levels in their tissue as a possible deviation from healthy tissue and possibly CRC development..

This opinion could be supported by the fact of increased MALAT1 plasma levels in CA patients as well as in CRC patients compared to CFI.

Bioinformatic analysis also linked MALAT1 to immune response pathways, including interactions with MHC proteins, inhibition of T-cell proliferation and sponging of miR-195 causing the upregulation of PD-L1. Additionally, MALAT1 regulates oncogenes like SOX9 and YAP1, promoting CRC progression through various molecular pathways such as Wnt/β-catenin, autophagy or PI3K/AKT.

Our study highlighted the diagnostic potential of MALAT1 in plasma, where MALAT1 levels were upregulated in CRC and CA patients compared to CFI. But MALAT1 levels were not able to distinguish patients with CA and CRC. In CA patients, MALAT1 expression levels were associated with the histological type of adenomas, with the highest levels observed in the types at the greatest risk of CRC development. While many studies analyze MALAT1 expression in CRC tumor tissue and adjacent mucosa, few focus on CA as we did.

Regarding the chemoresistance Li et al. categorized patients based on their response to oxaliplatin therapy, revealing that poor responders exhibited higher MALAT1 expression compared to good responders, a finding confirmed in both tumor tissue and serum. These results are in concordance with our results. Similarly, Fan et al. observed significant upregulation of MALAT1 in oxaliplatin-resistant patients. Li et al. proposed that high MALAT1 levels decrease CDH1 expression, a key protein for cell adhesion, leading to epithelial-mesenchymal transition (EMT), which promotes invasiveness and chemoresistance. This interaction is mediated through the EZH2 protein, with miR-218 also potentially involved. Another pathway involves the MALAT1-miR-324-3p-ADAM17 axis, contributing to drug resistance in CRC.[4, 125, 126]

While MALAT1's role in CRC is documented in tissue studies, this research uniquely emphasizes its plasma levels in colorectal adenomas patients, reinforcing its significance in non-invasive diagnostics and personalized cancer treatment strategies. The study's limitations include the small tissue sample size as well as the lack of patient tissues used in our liquid biopsy analysis.

IX. Conclusion

I. The incidence of CRC in liver transplant patients is comparable to or slightly higher than in the general population. The risk is significantly higher in liver transplant patients with primary sclerosing cholangitis (PSC), both with and without ulcerative colitis (UC) - which itself represents a precancerous condition. Standard screening protocols appear adequate for patients transplanted for other indications than PSC.

II. We examined mutational profiles of colorectal adenomas and in situ carcinomas, revealing that the mutation frequencies of APC, KRAS, and TP53 genes increase from low-grade to high-grade adenomas towards in situ carcinomas. These data support Vogelstein's multistage model of CRC development, with our cohort showing that adenomas and in situ carcinomas rarely have microsatellite instability status.

III. We provided novel insights into chromosomal instability in colorectal adenomas, highlighting the early occurrence of genomic alterations in precancerous lesions. Based on the analysis of paired samples of colorectal adenomas and its adjacent mucosa of the same patient, we identified significant chromosomal gain at 11q13.1, which encodes the long non-coding RNA MALAT1. This amplification suggests potential driver of adenoma progression. While some adenomas displayed widespread karyotypic disruption, others exhibited minimal genomic alterations, underscoring the heterogeneity of early neoplastic changes. Our findings support the hypothesis that chromosomal instability emerges at the adenoma stage.

IV. We explored the potential of the long non-coding RNA MALAT1 as a diagnostic and prognostic biomarker for colorectal adenomas and carcinomas. Our work indicates that MALAT1 could serve as a non-invasive (liquid-biopsy) biomarker for detection of colorectal adenomas and carcinomas and also as a predictor of chemotherapeutic response to oxaliplatin.

This dissertation has brought several new insights in the pathogenesis of CRC. Despite all the current knowledge, further independent studies, including clinical trials, are needed to validate

these results in a larger, independent cohort of patients and thereby facilitate the transition of these findings into clinical practice.

X. References

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XI. List of appendices

Appendix No. 1: **Colorectal carcinoma after liver transplantation**

J. Jungwirth, P. Mačinga, J. Král, P. Taimr, J. Froněk, J. Špičák, T.Hucl
Gastroenterologie a hepatologie 2022 Vol. 76 Pages 302-308
DOI: 10.48095/ccgh2022302
IF: 0,129

Appendix No. 2: **Mutational analysis of driver genes defines the colorectal adenoma: in situ carcinoma transition**

J. Jungwirth, Marketa Urbanova, Arnoud Boot, Petr Hosek, Petra Bendova, Anna Siskova, Jiri Svec, Milan Kment, Daniela Tumova, Sandra Summerova, Zdenek Benes, Tomas Buchler, Pavel Kohout, Tomas Hucl, Radoslav Matej, Ludmila Vodickova, Tom van Wezel, Pavel Vodicka & Veronika Vymetalkova
Scientific Reports 2022 Vol. 12 Issue 1 Pages 2570
DOI: 10.1038/s41598-022-06498-9
IF: 4.6; 5-Year IF: 4.9

Appendix No. 3: **Discovery of Long Non-Coding RNA MALAT1 Amplification in Precancerous Colorectal Lesions**

Anna Siskova, Jan Kral, Jana Drabova, Klara Cervena, Kristyna Tomasova, **Jiri Jungwirth**, Tomas Hucl, Pavel Kohout, Sandra Summerova, Ludmila Vodickova, Pavel Vodicka, and Veronika Vymetalkova
Int J Mol Sci 2022 Vol. 23 Issue 14
DOI: 10.3390/ijms23147656
IF: 5.6; 5-Year IF: 6.2

Appendix No. 4: **MALAT1 in Liquid Biopsy: The Diagnostic and Prognostic Promise for Colorectal Cancer and Adenomas?**

Klara Cervena, Miroslav Levy, Anna Siskova, **Jiri Jungwirth**, Marin Volaric, Jan Kral, Pavel Kohout, and Veronika Vymetalkova
Int J Gen Med 2023 Vol. 16 Pages 3517-3531
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