Diplomarbeit

MICROSATELLITE INSTABILITY IN COLORECTAL CANCER: CLINICOPATHOLOGICAL SIGNIFICANCE

A histological, immunohistochemical and molecular approach

eingereicht von

Lisa Setaffy

zur Erlangung des akademischen Grades

Doktor(in) der gesamten Heilkunde
(Dr. med. univ.)

an der

Medizinischen Universität Graz

ausgeführt am

Institut für Pathologie

unter der Anleitung von

Univ. Doz. Dr. Cord Langner

Graz, 31.10.2015

Declaration of Authorship

I declare this thesis and the work presented in it are my own and have been generated by me as the result of my own original research. Where I have quoted from the work others, the source is always given at their point of use.

Graz, 31.10.2015

Lisa Setaffy eh.

Acknowledgements

I would like to express my deepest gratitude to my supervisor, Univ. Doz. Dr. Cord Langner, whose profound knowledge, guidance and patience made this thesis possible.
I also would like to thank Brigitte Tessaro and Gerlinde Winter for explaining the techniques of immunohistochemistry and pyrosequencing to me.

Contents

Index of Figures

Index of Tables

Abbreviations

AJCC	American Joint Committee on Cancer
APC	Adenomatous Polyposis Coli
BRAF	B-Raf proto-oncogene
CIMP	CpG Island Methylator Phenotype
CIN	Chromosomal Instability
CK1	Casein Kinase 1
CRC	Colorectal Cancer
FAP	Familial Adenomatous Polyposis
GSK3	Glycogen Synthase Kinase 3
K20	Keratin 20
KRAS	Kirsten Rat Sarcoma viral oncogene homolog
LOH	Loss of Heterozygosity
MLH1	Mut L Homologue 1
MMR	Mismatch Repair
MSH2	Mut S Homologue 2
MSH 3	Mut S Homologue 3
MSH6	Mut S Homologue 6
MSI	Microsatellite Instability
MSI-H	High-Level Microsatellite Instability
MSI-L	Low-Level Microsatellite Instability
MSS	Microsatellite Stable
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
P53	Tumor suppressor p53

PCR	Polymerase Chain Reaction
PMS1	Post-meiotic Segregation S 1
PMS2	Post-meiotic Segregation S 2
SSA/P	Sessile Serrated Adenoma/Polyp
TCF	T-Cell Factor
TILs	Tumor-Infiltrating Lymphocytes
TSA	Traditional Serrated Adenoma
UICC	Union Internationale contre le Cancer
WHO	World Health Organization

ABSTRACT

Although often viewed as a single disease, colorectal cancer more accurately represents a constellation of heterogeneous subtypes that result from different combinations of genetic events and epigenetic alterations. Chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP) have been identified as the three major molecular characteristics which interact with other significant mutations, such as mutations in the *KRAS* and *BRAF* genes. High-level MSI (MSI-H) is of eminent clinical importance. It is the seminal molecular feature for the identification of individuals with Lynch syndrome, but it may also occur in sporadic cancers with CIMP phenotype, which arise from serrated precursor lesions. MSI-H status is a marker of favorable prognosis and may be used for outcome prediction, that is, molecular grading. Among others, mucinous and medullary histology, signet-ring cell differentiation, and marked anti-tumoral immune response are histological features suggesting MSI. Universal tumor testing is recommended and may be performed using immunohistochemistry (mismatch repair protein expression) or molecular analysis, as has recently been recommended by an international task force. In this review, I will refer in detail to the molecular pathogenesis of colorectal cancer, focusing on the diagnosis of MSI in both hereditary and sporadic tumors. Additionally, the analysis of the data of colorectal cancer patients who were operated within a three-year period aims to identify and summarize histopathological characteristics as well as the molecular background of colorectal cancer diagnoses. The prospective database of colorectal cancer patients was generated at the Institute of Pathology, Medical University Graz, in collaboration with the Department of Surgery (“Darmkrebszentrum”), Krankenhaus der Barmherzigen Brüder, St. Veit an der Glan. The results are in line with previous reports.

ZUSAMMENFASSUNG

Das kolorektale Karzinom (KRK) wurde lange Zeit als “eine” Krankheit betrachtet, doch dieses Bild hat sich gewandelt, seit die grundlegenden Mechanismen der Karzinogenese mittels molekularer Analyse geklärt wurden. Deshalb betrachtet man das KRK als Karzinom mit mehreren Subtypen, die nach ihrem Entstehungstyp klassifiziert werden. Ausschlaggebend dafür sind die Kombination von genetischen Events und epigenetischen Alterationen. Chromosomale Instabilität (CIN), Mikrosatelliten Instabilität (MSI) und CpG Island Methylator Phenotype (CIMP) sind als die drei großen Pathogenese-Wege identifiziert worden, die zusätzlich mit anderen wichtigen Genmutationen (*BRAF*, *KRAS*) interagieren. Speziell High-level MSI (MSI-H) ist von eminenter klinischer Bedeutung. MSI-H dient zum einen zur Identifikation von Patientinnen und Patienten mit Lynch Syndrom, zum anderen dient es auch als Marker für das sporadische KRK mit CIMP Phänotyp, welches aus serratierten Vorläuferläsionen entsteht. MSI-H Status ist außerdem ein prognostischer Marker und kann für das Abschätzen des Outcomes verwendet werden, sogenanntes „Molecular Grading“. Darunter fallen auch Charakteristika wie muzinöse und medulläre Histologie, Siegelringzell-Differenzierung und Immunantwort gegen den Tumor. Universales Vorgehen in der Detektion von MSI in Tumorpräparaten wird zunehmend empfohlen, und wird entweder durch Immunhistochemie (Mismatch Repair Protein Expression) oder molekulare Analyse bewerkstelligt. Dieses Procedere wurde auch durch eine internationale Task Force bestätigt. In diesem Review werden die molekularen Pathogenese-Wege von KRK erläutert und vor allem die Diagnose von MSI in erblichen bedingten und sporadischen Tumoren beleuchtet. Zusätzlich wurden MSI und *BRAF* Status in Tumorpräparaten von KRK Patientinnen und Patienten analysiert, die in einem Zeitraum von drei Jahren operiert wurden. Die Daten stammen aus einer prospektiven Datenbank, die vom Institut für Pathologie, Medizinische Universität Graz, in Zusammenarbeit mit der Abteilung für Chirurgie (“Darmkrebszentrum”), Krankenhaus der Barmherzigen Brüder, St. Veit/Glan angelegt wurde. Die Ergebnisse stimmen großteils mit vorangegangen Ergebnissen anderer Studien überein.

INTRODUCTION

Colorectal cancer (CRC) is still the third most common cancer and the third leading cause of cancer death in men and women in the United States. In 2014, an estimated 71,830 men and 65,000 women will be diagnosed with CRC and 26,270 men and 24,040 women will die of the disease [1]. However, the overall incidence rate decreased by approximately 3% per year during the past decade (2001 to 2010). Specifically, rates for tumors located in the distal colon decreased by more than 5%, while, in contrast, rates among adults younger than 50 years increased during this period [1]. In the EU in 2014, 168,400 deaths from CRC are predicted (92,900 men and 75,400 women), corresponding to standardized death rates of 16.5/100,000 men and 9.5/100,000 women, falling by 4% and 7%, respectively, since 2009 [2].

Although often viewed as a single disease, CRC more accurately represents a constellation of heterogeneous subtypes that result from different combinations of genetic events and epigenetic alterations [3]. Thus, a growing body of evidence supports the ability to separate CRC subtypes based upon combinations of genetic markers, such as microsatellite instability (MSI), CpG island methylator phenotype (CIMP), somatic *BRAF* mutation, and/or somatic *KRAS* mutation status [3]. It is of note that not only the combination, but also the timing of the molecular alterations is critical for neoplastic pathway determination [4]. Approximately 60% of all CRCs are believed to arise from conventional adenomas via the adenoma-carcinoma-sequence (suppressor pathway) and 35% from serrated precursor lesions via the serrated pathway, respectively [5]. Up to 5% of CRCs arise in the setting of well-defined inherited syndromes, including Lynch syndrome, familial adenomatous polyposis (FAP), MUTYH-associated polyposis, and certain hamartomatous polyposis conditions [6].

The risk factors for CRC are well-established. Positive family history of CRC, prior CRC or adenomatous polyps as well as chronic inflammatory bowel disease, that is, ulcerative colitis and Crohn’s disease play a central role, while obesity and Western lifestyle/diet have been recognized as additional factors [7, 8]. Interestingly, migrants who move from low-risk to high-risk countries adapt to the CRC incidence rates of the host country [7, 9]. Smoking also contributes to the risk of CRC, as has

been associated especially with the presence of large adenomas [7, 8]. Early recognition is important and screening tests have helped to reduce CRC-related mortality [10, 11].

In this diploma thesis, I will present the molecular pathogenesis of CRC, focusing on the diagnostic significance of MSI in both hereditary and sporadic tumors. The clinical relevance of MSI testing and the different tools for establishing the diagnosis in the routine evaluation of cancer specimens will be discussed in detail. Data for this review were compiled using MEDLINE/PubMed and Thomson Reuters Web of Science®, assessing articles published before September 2015. Search terms included colorectal cancer, Lynch syndrome, microsatellite instability, and molecular analysis. Only articles published in English were considered.

In addition, I will analyze the data of a database of CRC patients which was generated at the Institute of Pathology, Medical University Graz, in collaboration with the Department of Surgery (“Darmkrebszentrum”), Krankenhaus der Barmherzigen Brüder, St. Veit an der Glan. This prospective database aimed to summarize the histopathological and molecular diagnoses of all colorectal cancers operated within a three-year period.

MOLECULAR CLASSIFICATION OF COLORECTAL CANCER

The purpose of a molecular classification is to identify similar characteristics among individual tumors and then empirically predict the pathogenesis and biological behavior of a particular tumor. The most accepted way of creating a classification model is to identify and correlate single cellular events that have been statistically proven to play a role in tumorigenesis [12].

In CRC, chromosomal instability (CIN), MSI and CIMP have been identified as the three major molecular characteristics, which interact with other significant mutations, such as mutations in the *KRAS* and *BRAF* genes (Figure 1). CIN occurs in approximately two thirds of sporadic CRCs [13]. The term refers to an accelerated rate of gains and losses of whole or large portions of chromosomes. The consequence of CIN is an imbalance in chromosomal number (reflected by aneuploidy) and a higher frequency of loss of heterozygosity (LOH) [14].

CIN in conjunction with adenomatous polyposis coli (APC) mutation characterizes the “traditional pathway” according to Leggett and Whitehall [4], resulting in microsatellite stable (MSS), CIMP-negative, BRAF and KRAS wild type tumors. Conventional adenomas, i.e. tubular, tubulovillous and villous adenomas, are considered to be the precursor lesions of sporadic CRCs arising via the traditional pathway (adenoma-carcinoma sequence), but also the precursor lesions of hereditary cancers arising in Lynch syndrome and FAP [15, 16].

Approximately 15 to 20% of CRC are characterized by high-level MSI corresponding to a hypermutable phenotype that results from impaired DNA mismatch repair (MMR) and may be observed in both sporadic and Lynch syndrome-associated tumors [17]. Microsatellites are short repetitive DNA nucleotide sequences (1 to 6 base pair units) scattered throughout the genome which are prone to frame-shift mutations and base-repair substitutions during DNA replication due to their propensity to DNA strand slippage [12, 18].

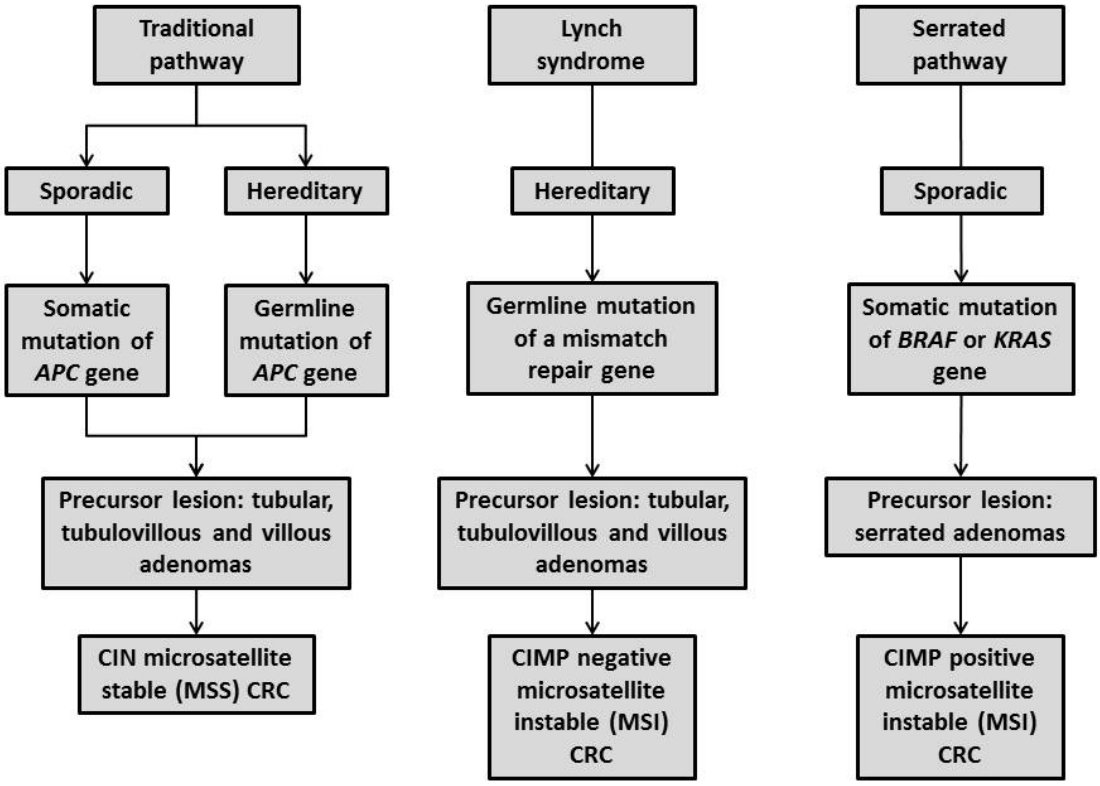

Figure 1 Chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP) have been identified as the three major molecular events in colorectal cancer which are involved in both sporadic and hereditary tumor development.

MSI is defined as a change of any length of repeating units, due to insertion or deletion [19]. Basically, MSI analysis is done by comparing allelic profiles of microsatellite markers generated by amplification of DNA from test (tumor) and matched unaffected (non-neoplastic) samples. Length variations in the test sample that are not found in the corresponding normal sample indicate MSI. Several panels of microsatellite markers have been used to diagnose MSI. In a first consensus meeting organized by the National Institutes of Health (Bethesda, MY, USA) a panel of five microsatellite markers (composed of two mononucleotide and three dinucleotide repeats) validated by a German consortium [20] was recommended as a

reference panel [19]. This panel requires that normal tissue is compared with tumor tissue. Alternative and more recently developed panels are based exclusively upon mononucleotide repeat markers, which can be amplified and analyzed in a single assay, i.e. without the evaluation of matched normal DNA [21, 22]. Tumors may be classified as follows: high-level MSI (MSI-H), if two or more of the five applied markers are altered and low-level MSI (MSI-L), if only one of the five markers is altered (Figure 2); MSS tumors do not show MSI [23].

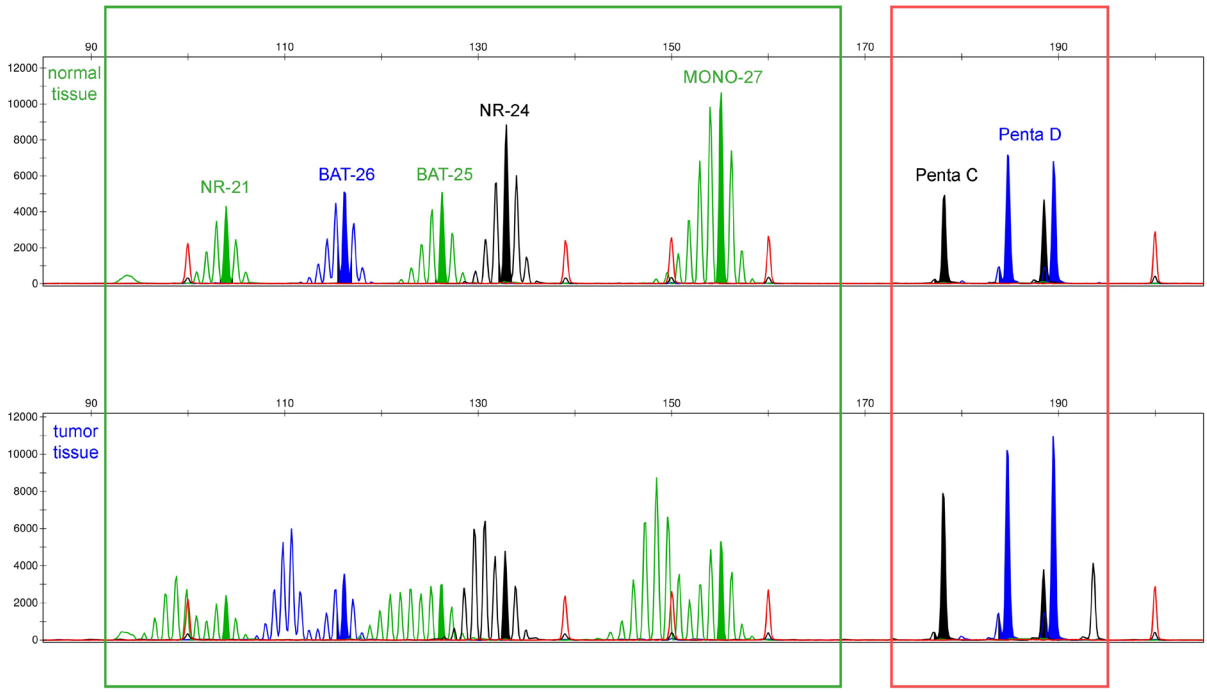

Figure 2 Representative example of a colorectal cancer with high-level microsatellite instability (MSI-H). The MSI profile assessed by a panel of five mononucleotide repeats (pentaplex panel) illustrates instability for all markers. Additional alleles (allelic shifts) are indicated (arrows).

About half of the genes in the human genome have promoters that are embedded in clusters of cytosine-guanosine residues called CpG islands. Aberrant hypermethylation in CpG-rich promoters has been recognized as a common feature of human neoplasia, associated with transcriptional inactivation of tumor suppressor

genes or other tumor-related genes [23]. Genome-wide studies of cancer epigenomes revealed 1 to 10% of CpG islands are aberrantly methylated, which suggests that thousands of gene promoters may be hypermethylated in average cancers [24].

Cancers can be classified according to their degree of methylation, and those cancers with high degrees of methylation (CIMP phenotype) represent a clinically and etiologically distinct group that is characterized by “epigenetic instability” [23]. In the colorectum, DNA hypermethylation in CpG-rich promoters defines a distinct tumor subgroup [25], which has been associated with MSI and *BRAF* mutation in sporadic tumors [26, 27]. This phenotype accounts for approximately 15 to 20% of CRC [24, 28]. It is of note that DNA hypermethylation in conjunction with *BRAF* mutation is not only seen in sporadic MSI-H CRC, but also frequently in sessile serrated adenomas/polyps (SSA/P), which have been identified as precursor lesions in the “serrated pathway” [16, 29].

Molecular analysis of CIMP including designation of methylation level is poorly standardized, since until now a precise definition of CIMP is lacking and no consensus recommendation available. In 2012, Hughes et al. [30] summarized the existing literature on CIMP in CRC, paying particular attention to the various methods and definitions used to classify a tumor as CIMP positive: Utilizing methylation-specific polymerase chain reaction (PCR) with or without quantification (quantitative real-time PCR), DNA methylation is usually measured in a panel of five [31] or eight [32] CIMP-related gene promoters. It is unclear whether CIMP should be reported in two categories (“CIMP” and “non-CIMP”) or three categories (“CIMP-high”, “CIMP-low”, “non-CIMP”) [30]. In a systematic study comparing panels with five and eight gene markers, Berg et al. [33] analyzed a total of 18 alternative combinations of scoring CIMP positivity on probe-, gene- and panel-levels and observed statistically significant variations in the frequency of CIMP depending on the cut-offs and genes included in the test panels, respectively.

In the following, I will refer to the different tumorigenic pathways, adenoma-carcinoma-sequence and CIN as well as MSI, associated with Lynch syndrome and in sporadic CRC, in detail.

CIN Pathway and Adenoma-Carcinoma-Sequence

In 1990, Fearon and Vogelstein [34] were the first to propose a multistep model of sequential genetic alterations, which are responsible for adenoma and ultimately carcinoma formation within the colorectum. The earliest step of tumorigenesis in this model leads to an activation of the Wnt signaling pathway, in the majority of cases resulting from a mutation in the *APC* gene. Adenoma growth and malignant transformation require additional activating mutations of proto-oncogenes, such as *KRAS* (or *NRAS*) as well as mutations in other genes, such as the tumor suppressor *TP53*, the "guardian of the genome", which is located on the short arm of chromosome 17 (17p13) [35, 36]. P53 dysfunction has been reported in 4 to 26% of adenomas, 50% of adenomas with invasive foci, and 50 to 75% of CRCs (Figure 3) [14, 37].

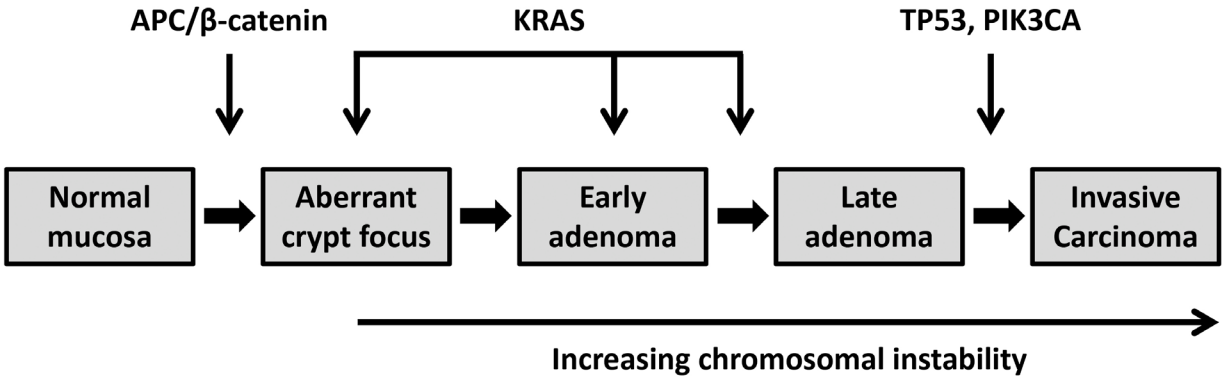

Figure 3 Multistep genetic model of colorectal carcinogenesis following the "traditional pathway" (adenoma-carcinoma sequence). Chromosomal instability is observed in benign (conventional) adenomas and increases with tumor progression (from [16] with permission).

Recognition of the central role of *APC* mutations in tumorigenesis has also improved our understanding of FAP, since this syndrome with hundreds to thousands

of neoplastic polyps distributed among the colorectum has been attributed to germline mutations in the *APC* gene [38]. FAP, however, accounts for only <1% of all CRCs. From the first occurrence of adenomatous polyps to carcinoma formation it may take ten or more years [39-41].

It is worth looking at *APC* in detail. *APC* is a tumor suppressor gene which has been localized to the long arm of chromosome 5 (5q21-q22) [42]. The *APC* gene is involved in cell adhesion and migration, organization of the cytoskeleton, spindle formation, and chromosome segregation [43]. Specifically, *APC* regulates the Wnt/β-catenin signaling pathway. This pathway plays a central role in stem cell function and processes involved in the development, differentiation and apoptosis of intestinal cells [44]. In the absence of activated Wnt/β-catenin signalling, cytosolic β-catenin is phosphorylated by a complex of proteins termed the “destruction complex”, containing the proteins APC, axin/conductin, GSK3 (glycogen synthase kinase), and CK1 (casein kinase 1). In this complex, β-catenin is phosphorylated by GSK3, enabling its ubiquitination and destruction by the proteasome. Activated Wnt/β-catenin signalling inhibits GSK3 activity, allowing β-catenin to escape from degradation, accumulate in the cytosol, and finally translocate to the nucleus, where it binds to members of the T-cell factor (TCF) family acting as a transcription factor [45, 46]. In the neoplastic cells, *APC* mutation leads to the formation of a truncated protein that can bind β-catenin, but cannot degrade and inactivate it and therefore leads to the accumulation of β-catenin in the nucleus and formation of constitutively active nuclear β-catenin/TCF complexes [16, 46]. Unfortunately, APC and β-catenin protein levels are impractical prognostic or predictive markers, as they are frequently overexpressed in CRCs arising from this pathway. Likewise the *APC* mutation status has not been helpful in this regard to date [47].

According to the presented model (Figure 3), *KRAS* mutation plays a critical role in the development from aberrant crypt foci to early adenoma [14]. Basically, *KRAS* mutation can be found in up to 40% of CRCs [47]. The majority of activating mutations in the *KRAS* gene are detected in codons 12 (82% to 87%) and 13 (13% to 18%), but also mutations in codons 61, 63 and 146 are reported, mostly as somatic mutations [48], leading to activation of the RAS–RAF–MAPK pathway, downstream of the epidermal growth factor receptor [49]. Genotype-phenotype correlation is

largely inconclusive. However, codon 12 mutations may preferentially display mucinous histology, whereas codon 13 mutations rather present as non-mucinous, but more aggressive tumours with a greater metastatic potential [48, 50]. Moreover, *KRAS* mutation status is generally accepted as predictive marker for response (or resistance) to epithelial growth factor inhibitors, such as cetuximab or panitumumab [48].

Basic therapy regimes for CRCs arising from the CIN pathway foresee immediate surgery (or local endoscopic therapy in early submucosal invasion) in patients with American Joint Committee on Cancer (AJCC)/Union Internationale contre le Cancer (UICC) stage I cancer, which will result in high rate of cure with surgery alone without adjuvant therapy. Patients with AJCC/UICC stage II/III disease profit from adjuvant chemotherapy, or in terms of rectal cancer, neoadjuvant chemoradiation [51, 52].

In conclusion, CIN is characterized by karyotypic abnormalities coupled with a set of mutations in tumor suppressor genes and oncogenes. It is still unclear, whether CIN creates the appropriate environment for these mutations or vice versa [14]. LOH is considered to be a hallmark feature of CIN-positive CRC. An average of 25 to 30% of alleles is lost in tumors, and chromosome segregation defects (mitotic nondisjunction) seem to play a central role [14, 53, 54]. Allelic loss at chromosome 18q is particularly common, occurring in as many as 70% of cases [34]. The prognostic significance of CIN is still under debate, but some data indicate that the CIN phenotype is associated with a less favorable outcome compared with MSI-H tumors [14, 55].

Microsatellite Instability in Hereditary Colorectal Cancer

The MMR system is necessary for maintaining genomic stability by correcting single-base mismatches and insertion-deletion loops that form during DNA replication [6]. Impaired MMR function leads to high-level MSI, which can be found in approximately 15 to 20% of CRC and may be observed in both sporadic and hereditary, i.e. Lynch syndrome-associated tumors.

When active, the MMR proteins form heterodimers. MLH1 builds a functional complex with PMS2 and MSH2 with its partner MSH6, respectively [56, 57]. It is of note that the MLH1 and MSH2 proteins are obligatory partners of their respective heterodimers. Mutations in the *MLH1* or *MSH2* gene result in proteolytic degradation of the respective dimer and consequent loss of both the obligatory and the secondary partner proteins [58]. The reverse, however, is not true: A mutation in one of the secondary genes, i.e. *PMS2* or *MSH6* does usually not lead to concurrent loss of the obligatory proteins (MLH1 or MSH2, respectively). Compensation of the function of the secondary partner protein by other proteins, such as MSH3, MLH3, and PMS1 is the most likely explanation for this observation. Consequently, mutations of *MLH1* or *MSH2* usually cause concurrent loss of PMS2 and MSH6 proteins, respectively, by immunohistochemistry, whereas mutations of *PMS2* or *MSH6* often cause loss of PMS2 or MSH6 proteins only [59].

Earlier studies focusing on MLH1 and MSH2 suggested that immunohistochemistry has a lower sensitivity (85%) than MSI testing (93%) in predicting germline mutation. Inclusion of PMS2 and MSH6 in analysis increases the sensitivity of immunohistochemistry significantly, more recent studies, which included these additional proteins, have demonstrated a predictive value for immunohistochemistry that is virtually equivalent to that of MSI testing [59].

Immunohistochemistry is reliable in screening for mutations that result in truncation or degradation of the protein [59]. However, not all pathogenetic mutations result in loss of protein expression. Hence, more than one third of *MLH1* mutations are missense mutations, which result in mutant proteins that are catalytically inactive, but antigenically intact [60, 61].

Compared with MSI testing, immunohistochemistry can help to identify the affected gene, whereas MSI testing can only demonstrate impaired function of one of the four MMR genes. It is of note that high-level MSI is not specific for Lynch syndrome: Of the 15 to 20% MSI-H CRC, 12 to 15% are caused by sporadic, acquired hypermethylation of the *MLH1* gene promoter, which occurs in tumors exhibiting CIMP, while only 3 to 5% are associated with Lynch syndrome [62].

Lynch syndrome is an autosomal dominant cancer predisposition syndrome that is caused by a germline mutation in one of the four DNA MMR genes, with *MLH1* and *MSH2* accounting for most cases (approximately 40% each) and *MSH6* and *PMS2* accounting for fewer cases (approximately 10% and 5%, respectively) [63, 64]. It is characterized by early-onset, frequently right-sided CRCs, often syn- and metachronous tumors, and also a higher risk for extracolonic tumors [18]. At a meeting in Amsterdam in 1990 a first set of clinical selection criteria for families with Lynch syndrome was established to provide a basis for collaborative studies [65]. In subsequent years, these criteria were expanded, now including also extracolonic tumor sites as diagnostic features (Table 1) [66]. While the Amsterdam Criteria were initially designed to serve for research, the purpose of the Bethesda Guidelines and later on the revised Bethesda Guidelines is to select CRC patients for MSI testing, that is, to limit molecular analysis to cancers with high likelihood for heredity (Table 2) [67-69].

Table 1 Amsterdam Criteria I and Amsterdam Criteria II for the diagnosis of Lynch syndrome [65, 66, 137-140].

Amsterdam Criteria I
1: Three or more relatives with histologically verified CRC, one of whom is a first-degree relative of the other two.
2: Two or more generations should be affected.
3: One or more patients with CRC should be diagnosed before the age of 50 years.
4: Familial adenomatous polyposis (FAP) should be excluded.
Amsterdam Criteria II
1: Three or more relatives with histologically verified Lynch-syndrome associated cancer (CRC, cancer of the endometrium, small bowel, ureter, or renal pelvis), one of whom is a first-degree relative of the other two.
2: Two or more generations should be affected.
3: One or more cancer patients should be diagnosed before the age of 50 years.
4: Familial adenomatous polyposis (FAP) should be excluded.

Table 2 According to the revised Bethesda Guidelines [68, 137-140] colorectal cancers (CRCs) should be tested for MSI in the following situations:

1: CRC diagnosed in a patient who is less than 50 years of age.
2: Presence of synchronous or metachronous CRC or other Lynch syndrome-associated tumor*, regardless of age.
3: CRC with MSI-H histology diagnosed in a patient who is less than 60 years of age.
4: Patient with CRC and CRC or Lynch syndrome-associated tumor* diagnosed in at least one first-degree relative less than 50 years of age.
5: Patient with CRC and CRC or Lynch syndrome-associated tumor* diagnosed in two first-degree or second-degree relatives, regardless of age.

* Lynch syndrome-associated tumors include cancers of the colorectum, endometrium, stomach, ovary, pancreas, biliary tract, small bowel, ureter, renal pelvis, and brain tumors (usually glioblastoma as seen in Turcot syndrome), as well as sebaceous gland adenomas and keratoacanthomas (in Muir-Torre syndrome).

The lifetime risk of CRC has been variably estimated and appears depending on sex and the mutated MMR gene (Table 3) [70-77]. It is of note, that *PMS2* mutation appears to be less common (probably it was not detectable due to technical difficulties earlier ago and the presence of pseudogenes), and also the lifetime risks of CRC, endometrial and other cancers associated with *MSH6* and *PMS2* mutation seem to be lower than those associated with *MLH1* and *MSH2* mutation [78]. In an analysis of 98 families with *PMS2* mutation, the cumulative risk of CRC for male mutation carriers by the age of 70 years was calculated with 19%, among female mutation carriers it was 11% for CRC and 12% for endometrial cancer. The mean at diagnosis of CRC was 52 years [79].

Table 3 Gene-specific cumulative risks of colorectal cancer in Lynch syndrome (modified after Giardiello) [137-140].

Site of gene mutation	**Cumulative risk at the age of 70 years**	**Mean age at diagnosis**
Sporadic cancer (risk general population)	5.5%	69 years
MLH1/MSH2	Male: 27-74% Female: 22-53%	27-46 years
MSH6	Male: 22% Female: 10% Male and female 18%	54-63 years
PMS2	Male: 20% Female: 15%	47-66 years

As already indicated above, patients with Lynch syndrome are at higher risk also for extracolonic tumors (Lynch syndrome-associated tumors), in particular endometrial and ovarian cancers, but also cancers of the renal pelvis/ureter, stomach, and other sites. The frequency of these tumors is summarized in Table 4 [80-82].

Table 4 Spectrum of extracolonic tumors and lifetime risks for patients with Lynch syndrome; general information for all MMR genes (data from the German HNPCC Consortium) [81].

Tumor	**Life time risk**
Endometrial cancer	39 to 50%
Ovarian cancer	7 to 8%
Stomach cancer	1 to 6%
Cancer of the renal pelvis/ureter	2 to 8%
Cancer of the bile ducts	1 to 4%
Cancer of the small bowel	1 to 4%
Pancreatic cancer	Approx. 4%
Brain tumors	Approx. 2%

It is of particular note that the lifetime risk of endometrial cancer is estimated to be up to 60%, equivalent to the lifetime risk of CRC in women [83]. Age at diagnosis of Lynch syndrome-associated endometrial cancer is at least one decade lower compared with women developing sporadic endometrial cancer [137-140]. Screening for CRC is standard in patients with Lynch syndrome, but screening for endometrial cancer is under debate. Recently published multi-society task force recommendations state that, to date, there is no evidence of survival benefit from endometrial surveillance, although the authors acknowledge that this is difficult to measure [137-140]. Uncertainty on the expert level seems to result in less awareness for screening endometrial cancer in affected women, who undergo screening for endometrial cancer less often than screening for CRC. However, undergone genetic

counseling increases the patients' willingness to participate in screening programs, increasing from 30% (before genetic counseling) to 54% (after genetic counseling) [84]. Several screening methods have been proposed (pelvic examination, transvaginal ultrasound, endometrial sampling, CA-125), but only the histological analysis of endometrial samples seems to be useful in asymptomatic patients.

MSH2 mutation has been associated with increased risk of brain tumors in general. Therkildsen et al. [85] identified 41 primary central nervous system tumors in 288 individuals suffering from Lynch syndrome. Glioblastoma was the most frequent (56%) histological subtype, followed by astrocytoma (22%) and oligodendroglioma (9%). The majority of the patients (68%) had mutations in *MSH2*, the remaining patients are distributed among *MLH1* (15%), *MSH6* (15%) and *PMS2* (2%) mutation carriers, respectively.

Regarding the colorectum, affected individuals present with only few or no adenomas, but may already have established CRC. The development of adenomas occurs at a rate similar to that of adenomas in the sporadic setting [86]. The rate of progression from adenoma to cancer, however, is believed to occur at an increased rate, since the germline inactivation of one of the MMR genes, coupled with somatic inactivation of the remaining allele in the initiated lesion, i.e. the conventional adenoma, greatly increases the mutation rate and, subsequently, cancer development [16, 86].

The suggested treatment for patients with Lynch syndrome with CRC or premalignant polyps, which cannot be removed by colonoscopy, is surgery [137-140]. Total colectomy, partial or subtotal colectomy, and hemicolectomy (with ileorectal anastomosis) are performed, depending on patient's needs. In segmental resection, the high risk for metachronous cancer must be considered [87]. Hemicolectomy may be an option in elderly patients, although the preferred treatment is total colectomy. Gained life expectancy performing total colectomy vs. hemicolectomy in Lynch syndrome patients at ages 27, 47, and 67 years by Markov modeling was 2.3, 1, and 0.3 years, respectively [88].

Microsatellite Instability in Sporadic Colorectal Cancer

As already stated above, the majority of MSI-H CRCs are non-hereditary tumors attributable to the CIMP or serrated pathway [25]. This pathway is characterized by *BRAF* V600E mutation and hypermethylation in CpG-rich gene promoters, thereby leading to transcriptional inactivation of a large number of genes, including the MMR gene *MLH1*. The silencing of this gene is responsible for the development of MSI [89, 90].

CIMP tumors share many features with Lynch syndrome-associated tumors, such as occurrence in the right colon and mucinous histology. However, CIMP tumors are diagnosed at an advanced age and with female preponderance [91, 92]. CIMP tumors originate from lesions that are characterized morphologically by a serrated (saw-toothed or stellate) architecture of the epithelial compartment. It is of note that DNA hypermethylation in conjunction with *BRAF* mutation is not only seen in established CIMP carcinomas, but also frequently in these precursor lesions (Figure 4) [16, 29].

In fact, aberrant methylation seems to play an early role in tumorigenesis. Chan et al. [93] reported CpG island hypermethylation in hyperplastic ("heteroplastic") aberrant crypt foci in grossly normal mucosa obtained from colectomy specimens of patients with sporadic CRC. In their integrative genomic and epigenetic approach, Yamamoto et al. [92] identified CIMP in 7 of 28 (25%) hyperplastic polyps and 27 of 29 (93%) SSA/P. Including mixed lesions, that is, lesions containing both precancerous and malignant components in analysis the authors were able to demonstrate that most aberrant methylation is acquired at the precursor stage, whereas copy number aberrations are acquired during the progression from precursor to malignant lesion. The early aberrant methylation goes along with early activating mutations in the *BRAF* gene [29].

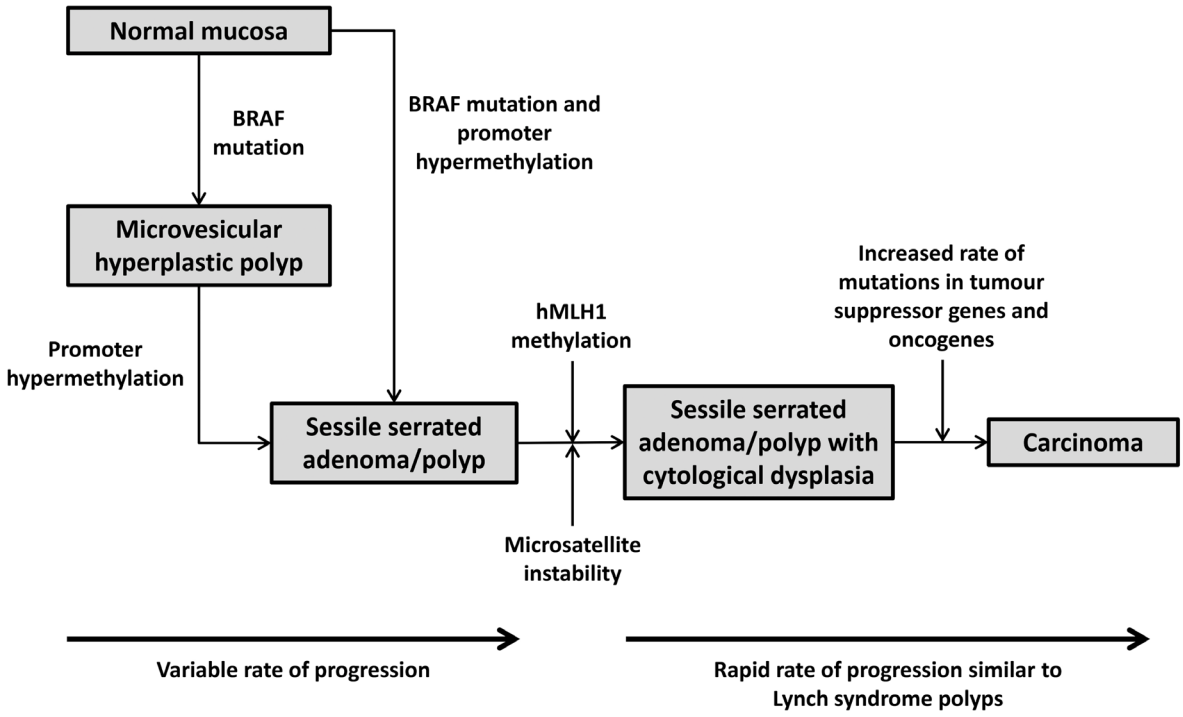

Figure 4 Colorectal carcinogenesis following the "serrated pathway". Sporadic colorectal adenocarcinomas with high-level microsatellite instability (MSI-H) develop from serrated precursor lesions due to epigenetic silencing of the MLH1 gene (from [16] with permission).

SSA/Ps have been identified as immediate precursors. They account for approximately 5 to 25% of all serrated lesions [15, 94, 95] and may develop preferably in the right colon from large microvesicular hyperplastic polyps or may arise *de novo* from normal colonic mucosa. The average size of SSA/Ps is larger than that of hyperplastic polyps. More than half of the lesions measure >5 mm and 15 to 20% of the lesions are >10 mm [96]. Histologically, they are characterized by distorted crypt architecture with dilated, mucus-filled, L- and T-shaped crypts with mature cells at the crypt bottom (Figure 5). This growth pattern results from an upward shift of the proliferative zone that is, moving away from its usual location at the base of the crypts to the mid-crypt region [5]. Cytological dysplasia is not present in uncomplicated SSA/P but develops with progression toward carcinoma (Figures 6,

7 and 8). In addition to conventional adenoma-like dysplasia, more cuboidal cells with eosinophilic cytoplasm and vesicular nucleoli with prominent nucleoli may occur – referred to as “serrated-type dysplasia” [16].

SSA/P has to be differentiated from so-called traditional serrated adenoma (TSA), which represents the least common type of adenoma occurring in the colorectum (less than 1% of all adenomas). Macroscopically, TSAs usually occur as polypoid lesions, preferentially in the left colon. But they may also be seen on the right side, here often in a more broad-based or sessile growth pattern. Histologically, they show a complex villiform growth pattern. For the diagnosis of TSA, polyps need to show at least two of the following three features: (i) typical cytology, that is columnar cells with marked cytoplasmatic eosinophilia and elongated “pencillate” nuclei, (ii) slit-like epithelial serration, or (iii) presence of ectopic crypt formation, that is short abnormal crypts with loss of orientation to the muscularis mucosae [15, 16, 97-99]. It is of note that TSAs often develop from precursor lesions, including both hyperplastic polyps and SSA/P [97, 99, 100]. On the molecular level, they may show *KRAS* mutation or *BRAF* mutation [97, 98, 100-102].

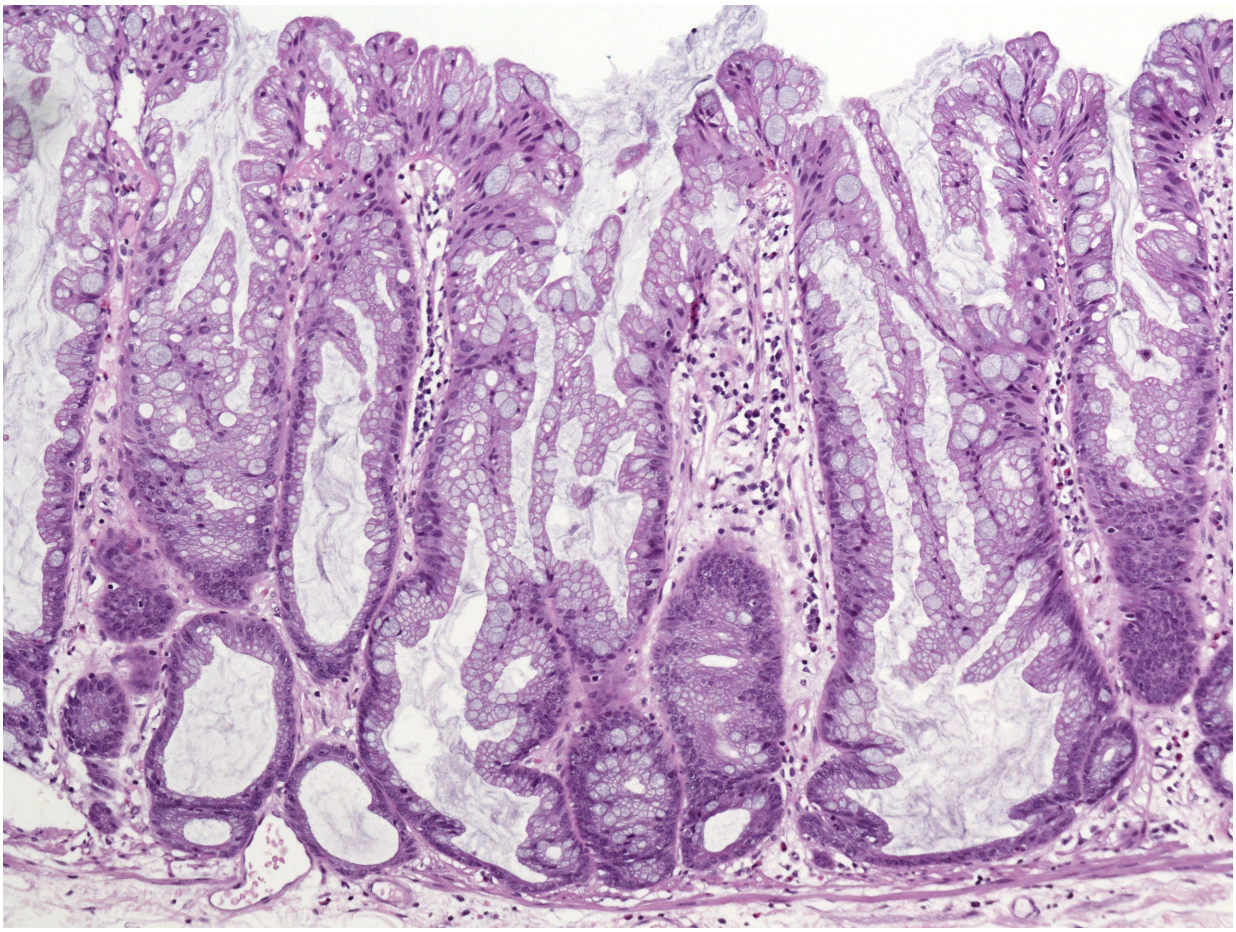

Figure 5 Sessile serrated adenoma/polyp (SSA/P) with marked serration, dilated, L-shaped (“boot”) and T-shaped (“anchor”) crypts and the presence of mature goblet cells above the muscularis mucosae.

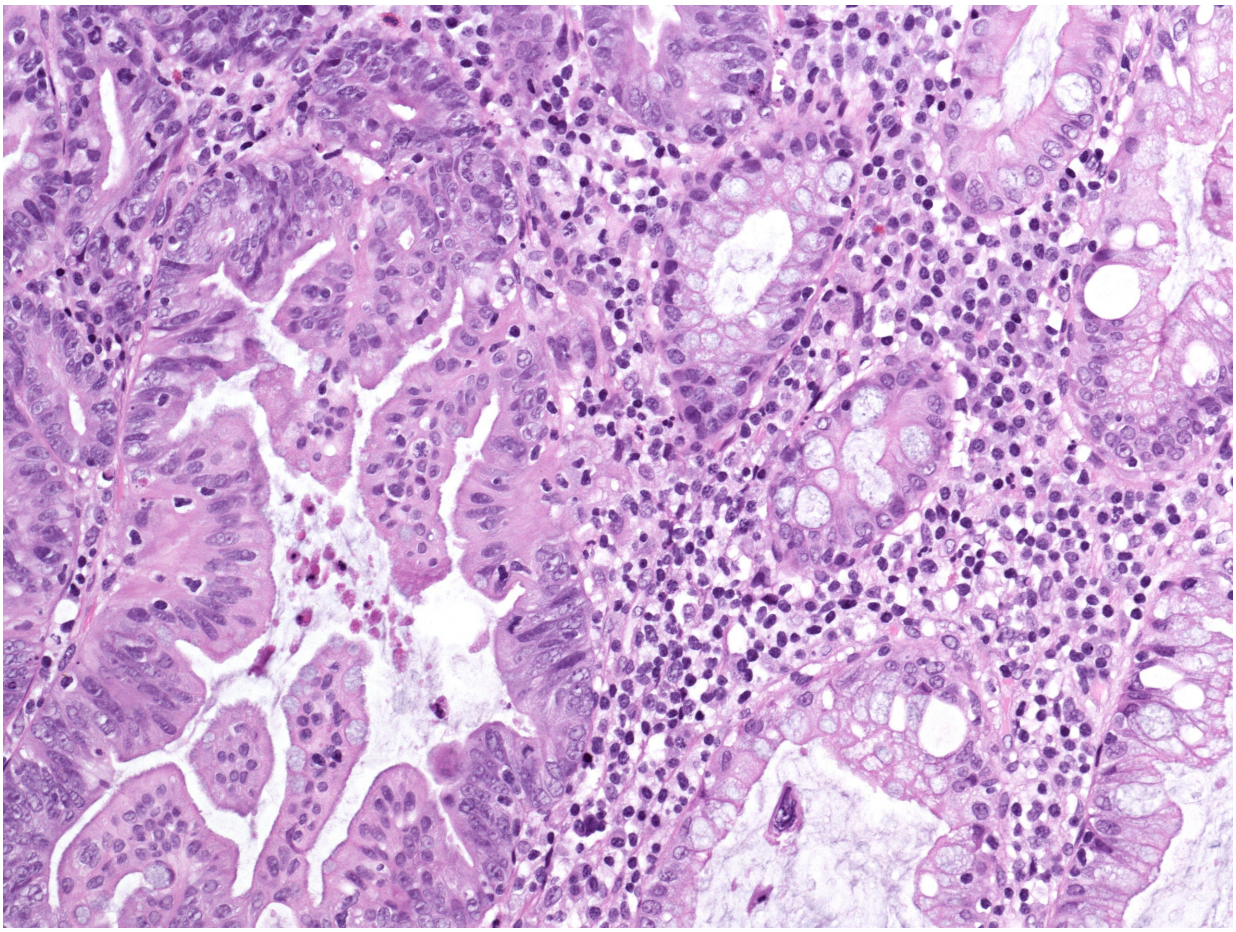

Figure 6 Sessile serrated adenoma/polyp (SSA/P), cytological dysplasia is not present in uncomplicated SSA/P, but develops with progression toward carcinoma.

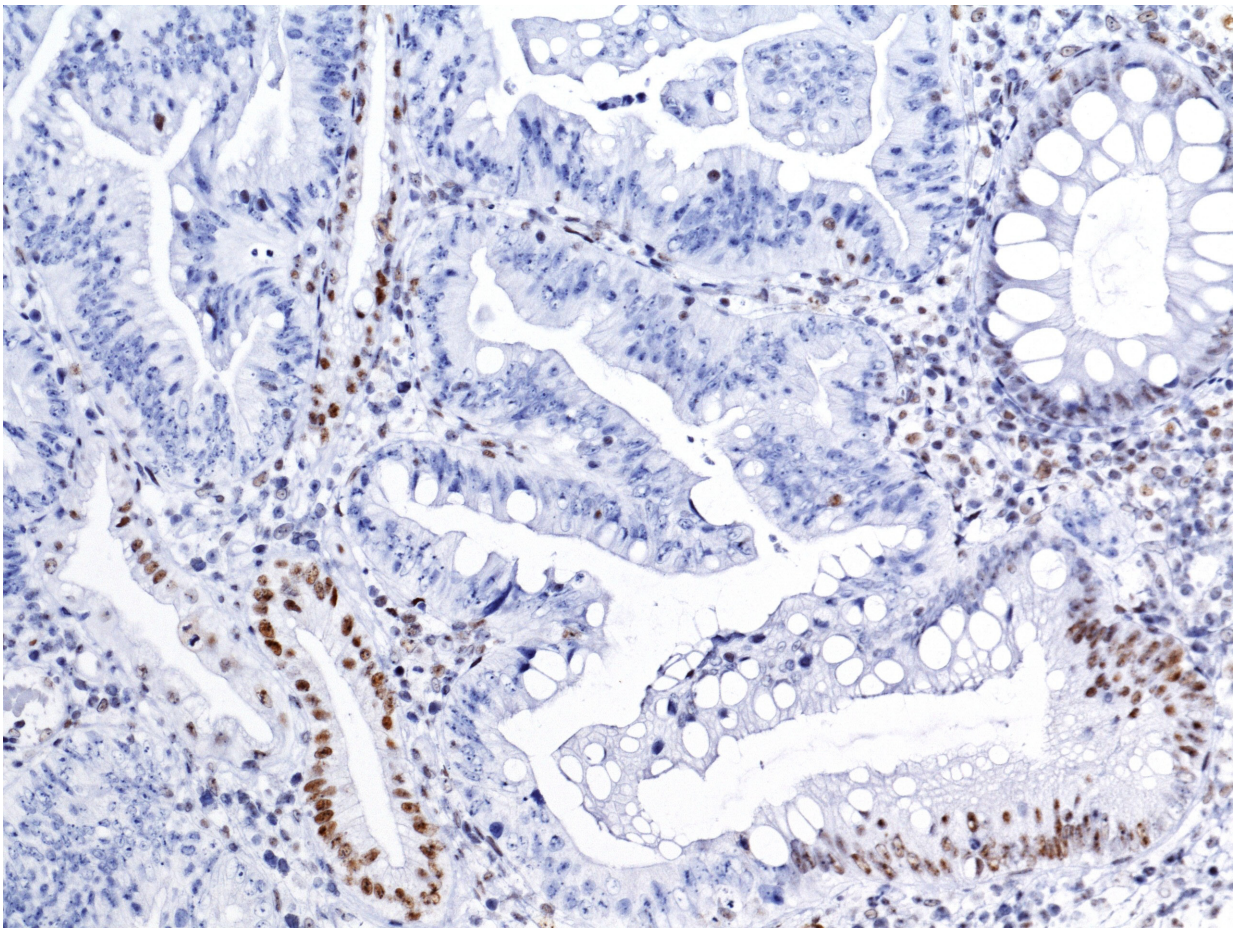

Figure 7 Sessile serrated adenoma/polyp (SSA/P), often in conjunction with epigenetic silencing (promoter hypermethylation) of the MLH1 gene, as shown by loss of nuclear MLH1 expression in the neoplastic cells.

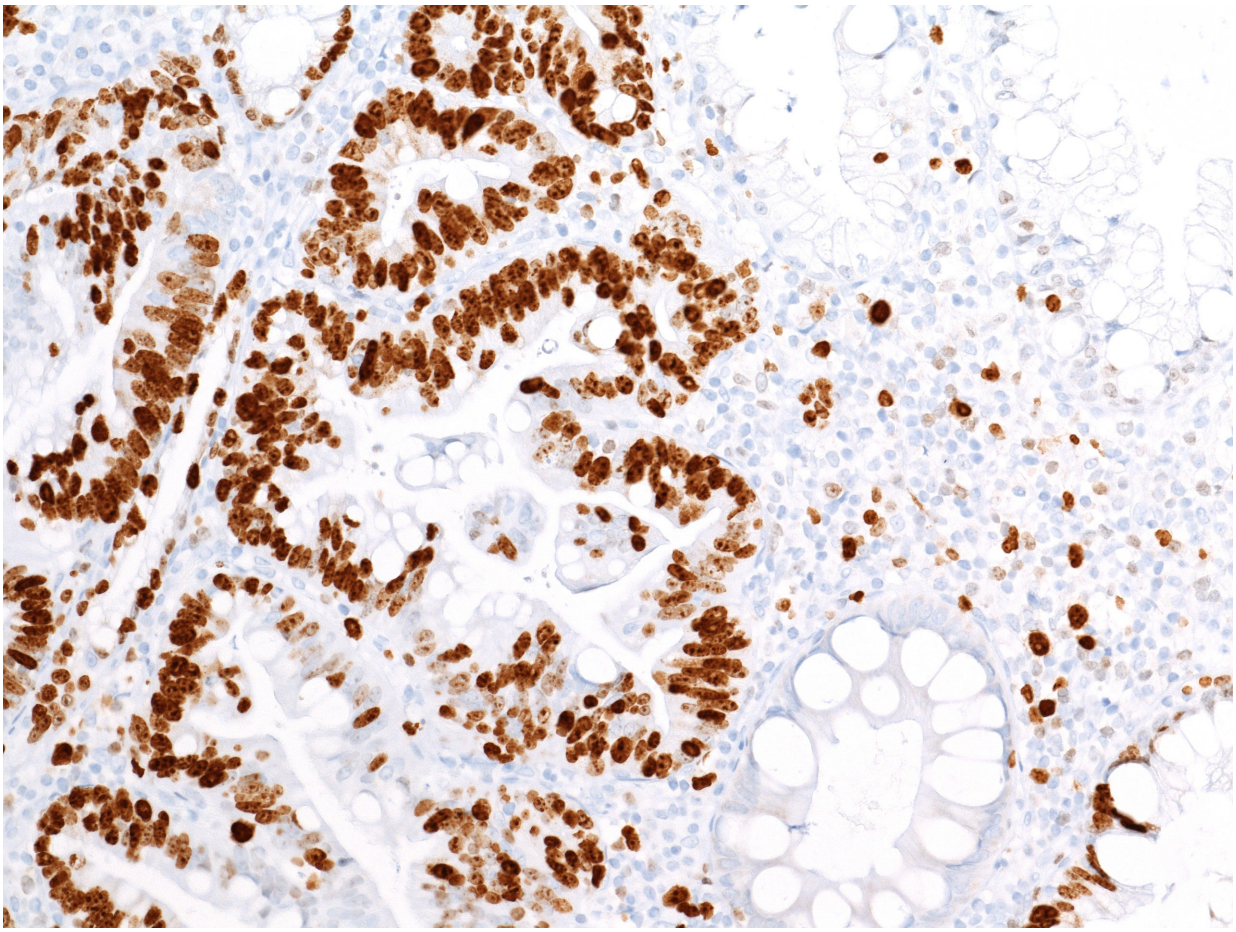

Figure 8 Sessile serrated adenoma/polyp (SSA/P), displaying increased proliferation rate (MIB-1) in the dysplastic glands.

It is of note that serrated lesions may also be associated with the familiar occurrence of CRC, in particular in serrated polyposis syndrome. In this syndrome, multiple and/or large serrated polyps occur throughout the colon, in particular proximal to the sigmoid colon [103, 104]. Individuals suffering from serrated polyposis syndrome are at an increased risk for CRC and need close endoscopic surveillance. In the study by Boparai et al. [105] the cumulative cancer risk was 7% at 5 years. To prevent malignant progression, adequate detection and removal of all polyps seems advisable. If this is not feasible, surgical resection should be considered [105]. On the molecular level, *BRAF* mutations can be found in 63% and *KRAS* mutations in 10% of lesions occurring in the serrated polyposis syndrome. 43% of lesions are CIMP-high. A per-patient analysis revealed that all patients had a *BRAF* or *KRAS* mutation in more than 25% of their polyps; 84.8% of patients had a mutation in *BRAF* or *KRAS* in more than 50% of their polyps [106].

The prognostic significance of CIMP and/or *BRAF* mutation status in established cancers is complex, in particular due to confounding factors, such as MSI and *KRAS* mutation status as well as different therapy regimens. Compared with the majority subtype (MSS/*BRAF* wild type), MSS/*BRAF* mutant, MSI-H/*BRAF* mutant, and MSI-H/*BRAF* wild type subtypes showed multivariable colorectal cancer-specific mortality hazard ratios [HR] of 1.60 (95% confidence interval [CI] = 1.12 to 2.28; p=0.009), 0.48 (95% CI = 0.27 to 0.87; p=0.02), and 0.25 (95% CI = 0.12 to 0.52; p<0.001), respectively [107].

Pai et al. [108] analyzed the histology of MSS/*BRAF* mutant CRCs of the proximal colon in comparison with MSS/*BRAF* wild type CRCs: *BRAF*-mutated tumors more frequently demonstrated adverse histologic features such as lymphatic invasion (16/20, 80% vs. 75/161, 47%; p=0.008), mean number of lymph node metastases (4.5 vs. 2.2; p=0.01), perineural invasion (8/20, 40% vs. 13/161, 8%; p=0.0004), and high tumor budding (16/20, 80% vs. 83/161, 52%; p=0.02). In addition, *BRAF*-mutated adenocarcinomas frequently contained areas with mucinous histology (p=0.0002) and signet-ring cell histology (p=0.03). Popovici et al. [109] likewise draw our attention to the fact that the prognostic value of *BRAF* mutation is context-dependent: In AJCC/UICC stage II/III CRCs *BRAF* mutation is a marker of poor survival only in subpopulations involving MSS and left-sided tumors, with higher

effects than in the whole population. There was no evidence for prognostic value in MSI or right-sided tumors. Data obtained from a recently published Australian community-based cohort (n = 375) indicate, that survival in AJCC/UICC stage II/III CRCs is independently predicted by CIN and MSI, but not by specific driver mutations, such as mutations in *KRAS* or *BRAF* [110].

Juo et al. [90] analyzed thirty-three studies reporting survival in 10,635 patients to determine the prognostic significance of CIMP status in CRC. Nineteen studies provide data suitable for meta-analysis. Pooled analysis shows that CIMP is significantly associated with shorter disease-free survival (pooled HR estimate 1.45; 95% CI = 1.07 to 1.97) and overall survival (pooled HR estimate 1.43; 95% CI 1.18 to 1.73) among CRC patients irrespective of MSI status. When subgroup analysis was performed, CIMP was found to be an indicator of poor prognosis only in MSS, but not in MSI tumors (comparable to *BRAF* mutation status). These data are well in the line with an earlier study by Bae et al. [111], who noted prognostic implication of CIMP status only in distal tumors.

Very recently, Phipps et al. [112] performed a large systematic analysis in which they related molecular phenotype to patients' survival, using the Seattle Colon Cancer Family Registry. Patients with MSS/MSI-L, CIMP-positive and *BRAF* mutant tumors had a higher disease-specific mortality (HR = 2.20, 95% CI = 1.47 to 3.31) compared with patients with MSS/MSI-L, CIMP-negative and *BRAF/KRAS* wild type tumors. In this study MSI-H, CIMP negative and *BRAF/KRAS* wild type tumors showed the lowest disease-specific mortality (HR = 0.30, 95% CI = 0.14 to 0.66).

HISTOLOGY OF MICROSATELLITE INSTABILITY-HIGH COLORECTAL CANCER

The clinical characteristics and predominant right-sided location of MSI-H CRCs are well established. However, the tumors also display distinct features on the histological level, which should raise suspicion of MSI and prompt further analysis. The following features are commonly seen: mucinous histology, signet-ring cell differentiation, medullary carcinoma, poor differentiation, host response characterized by intra- and peritumoral lymphocytes as well as “Crohn-like” reaction, tumor heterogeneity, lack of “dirty” necrosis, and a “pushing” tumor margin with no or low-level tumor budding (Table 5) [113-118]. It is worth looking at some of these features in greater detail.

Table 5 Histological features of colorectal cancers with high levels of microsatellite instability (MSI-H) [113-118].

Mucinous histology (“any mucin”)
Signet-ring cell differentiation
Medullary carcinoma
Marked anti-tumor host response (intra- and peritumoral lymphocytes as well as “Crohn-like” reaction)
Lack of “dirty” necrosis
“Pushing” tumor margin with no or low-level tumor budding
Poor differentiation
Tumor heterogeneity

According to WHO (World Health Organization) criteria [119] the designation of mucinous adenocarcinoma is used if >50% of the lesion is composed of pools of extracellular mucin that contain malignant epithelium as acinar structures, layers of tumor cells, or individual tumor cells including signet-ring cells (Figure 9). Carcinomas with mucinous areas of <50% are categorized as having a mucinous component. It is of note that already small amounts of mucin ("any mucin") may indicate MSI. In the study by Greenson et al. [114] 79 tumors were found to have focal mucinous differentiation, 23 (29.1%) of which were MSI-H. By comparison, 43 tumors had >50% mucinous differentiation, 12 (28.6%) of which were MSI-H. Multivariate analysis proved "any mucinous differentiation" as independent histological predictor of MSI-H status with an odds ratio of 2.69 (95% CI = 1.05 to 6.89; p=0.0393). This observation was confirmed in a subsequent publication by the same group, in which the authors concluded that the current WHO definition of mucinous adenocarcinoma may not be biologically relevant in the era of molecular testing [117].

Colorectal signet-ring cell carcinoma is an uncommon, but often highly aggressive malignancy, which is defined by the presence of >50% of tumor cells with prominent intracytoplasmic mucin, typically with displacement and molding of the nucleus (Figure 10) [119]. MSI-H status has been associated with signet-ring cell differentiation in several investigations with rates varying between 46 and 86% [113-115], but the significance of most studies is limited due to small sample size. In 2013, Hartman et al. [120] systematically analyzed 53 signet-ring cell carcinomas (composed of >50% signet-ring cells), which they classified as mucin-rich (n = 40; >50% extracellular mucin with signet-ring cells floating within pools of mucin) or mucin-poor (n = 13; diffusely infiltrating carcinomas with minimal to no extracellular mucin). Twenty-three of 53 (43%) signet-ring cell carcinomas were MSI-H. Twenty-two of 23 (96%) MSI-H signet-ring cell carcinomas were mucin-rich, whereas only one MSI-H signet-ring carcinoma was mucin-poor (p=0.0033). Mucin-poor signet-ring cell carcinoma had significantly reduced overall and recurrence-free survival compared with mucin-rich signet-ring cell carcinomas (p=0.0035 and p=0.0001, respectively), even when adjusted for tumor stage. It is of note that MSI-H and MSS

signet-ring cell carcinomas had similar overall and recurrence-free survival (p=0.2266 and p=0.1055, respectively), also when adjusted for tumor stage.

The anti-tumor host response characterized by intra- and peritumoral lymphocytic infiltration as well as Crohn-like reaction, that is, peritumoral lymphocytic aggregates has been identified in several studies as strong, if not the strongest predictor of MSI-status (Figure 11) [67, 113-115, 117, 121-123]. Tumor-infiltrating lymphocytes (TILs) refer to the lymphoid components intimately admixed with the tumor [18]. Specifically, TILs are intraepithelial lymphocytes, characterized by usually round, compact nuclei with dense chromatin pattern and perinuclear halo [67]. Various methods (and thresholds) for counting TILs have been reported, including evaluation of hematoxylin & eosin or CD3-immunostained slides, which mostly defined a positive result as >2 TILs per high power field (HPF) [18]. On the molecular level, TILs have been shown to consist largely of CD3/CD8 co-expressing cytotoxic T-cells. Their prominence has been discussed to represent (i) a response to abundant tumor neoantigen formation owing to the “mutator phenotype” of MSI-H tumors and (ii) a possible basis for the improved prognosis in MSI-H tumors [18]. It is of note that TILs are of particular help in identifying MSI-H cancers among non-mucinous tumors, and, consequently, they are regarded as the most important tissue biomarker for Lynch syndrome [67].

The Crohn-like reaction pattern is composed of prominent nodular lymphoid aggregates at the infiltrating edge of the tumor, typically identified at the junction of the muscularis propria and the fatty tissue. Their evaluation is poorly standardized. Hence, reported thresholds for a positive Crohn-like reaction include “2 or more large lymphoid aggregates in a section”, “a single 4x field of at least 3 nodular aggregates of lymphocytes”, “a minimum of 3 lymphoid aggregates per section”, and “at least 4 nodular aggregates in a low power field (4x)” [18].

Medullary carcinomas are characterized by syncytial sheets of malignant cells with vesicular nuclei, prominent nucleoli and abundant eosinophilic cytoplasm. The tumors show prominent infiltration by TILs and have well-defined peripheral margins, which may help to differentiate medullary carcinomas from undifferentiated carcinomas (Figure 12) [119, 124]. Frequently, medullary carcinomas arise in the proximal colon with an incidence increasing with age and a female predominance

[125]. Medullary differentiation is an indicator of favorable prognosis: Follow-up data showed 1- and 2- year survival rates of 92.7% and 73.8%, respectively [126]. On the molecular level, the majority of medullary carcinomas are MSI-H. Some may be associated with Epstein-Barr virus infection [124].

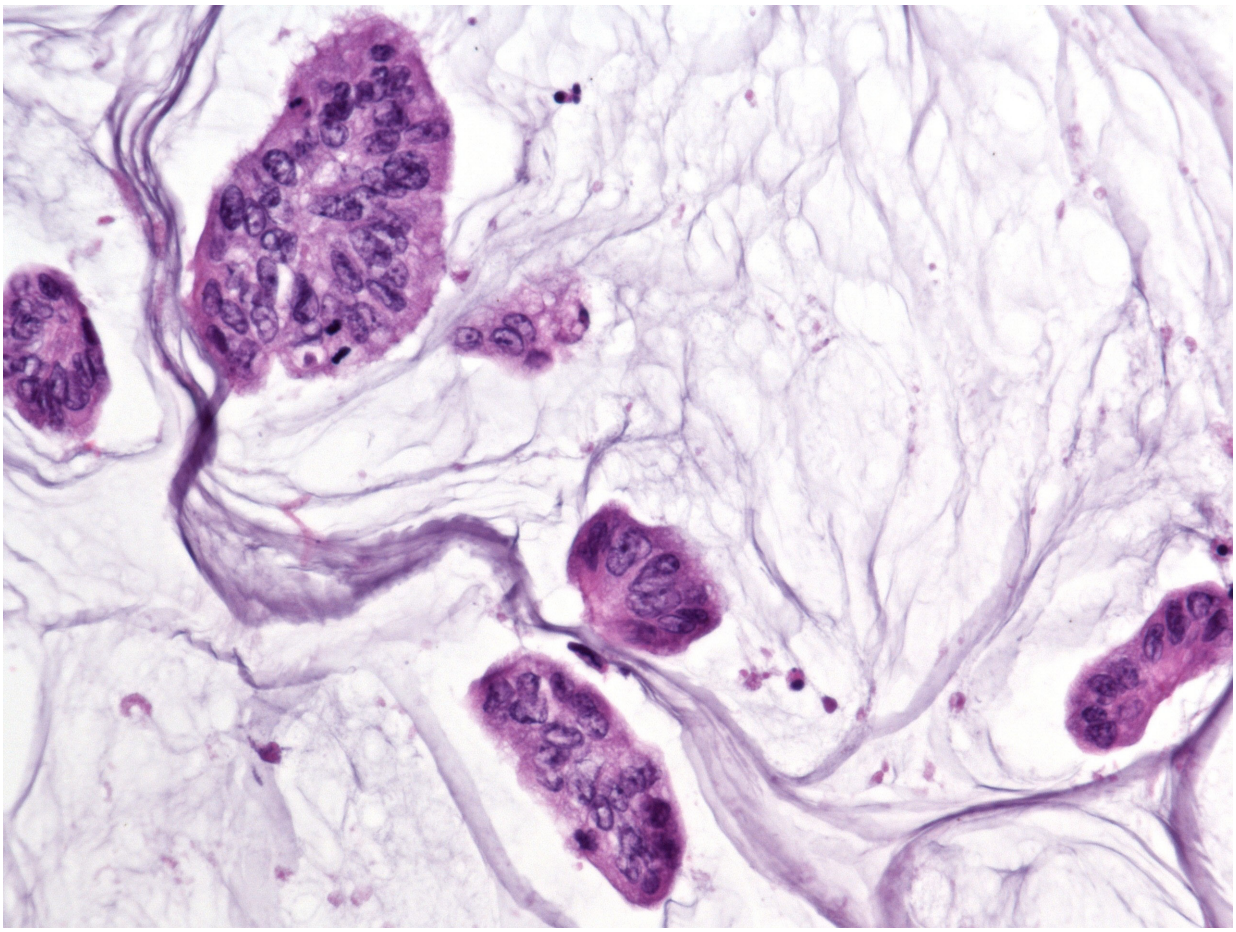

Figure 9 Histological features of colorectal cancer with high-level microsatellite instability (MSI-H): mucinous adenocarcinoma, >50% of the tumor is composed of pools of extracellular mucin.

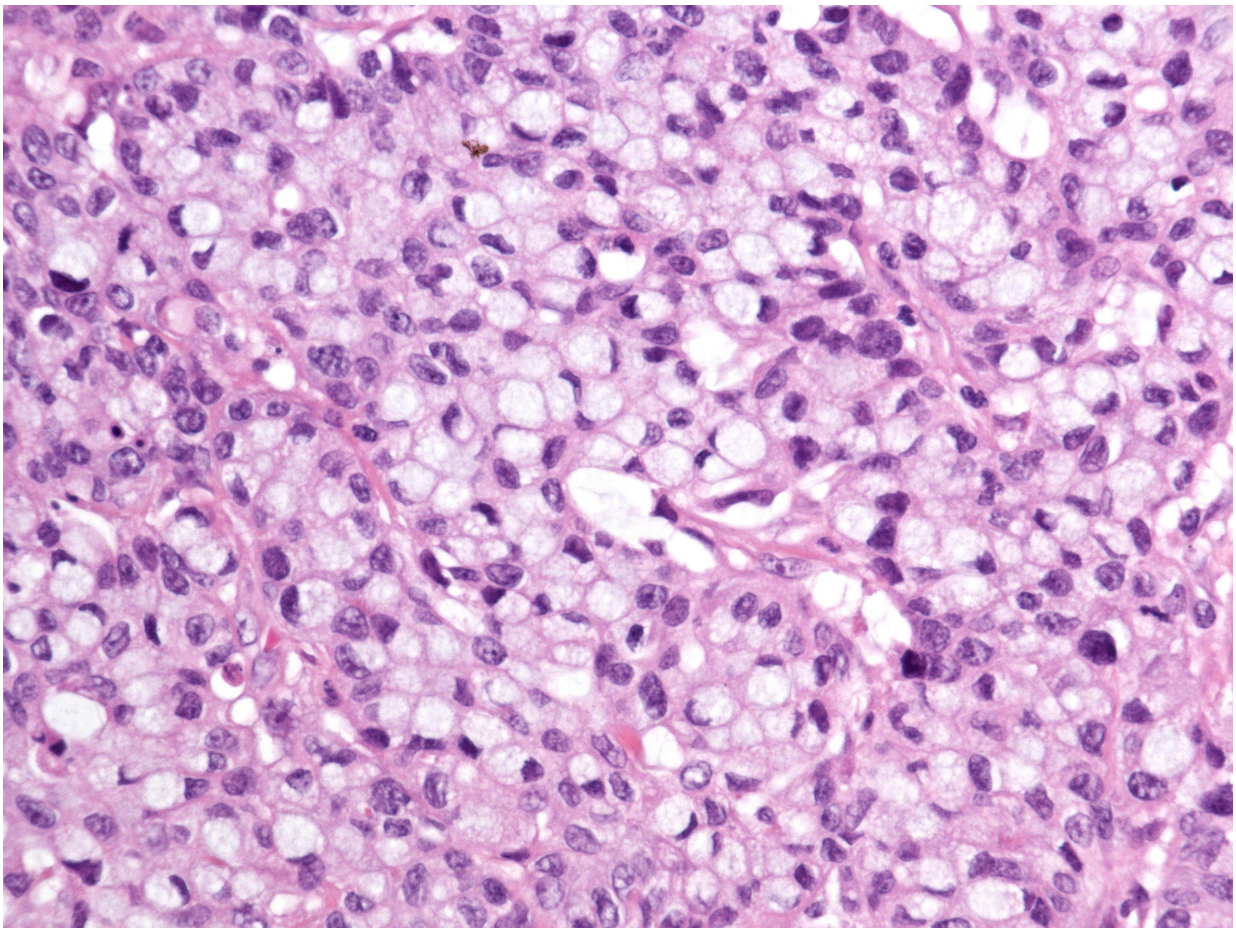

Figure 10 Histological features of colorectal cancer with high-level microsatellite instability (MSI-H): signet-ring cell carcinoma, >50% of the tumor cells show prominent intracytoplasmic mucin.

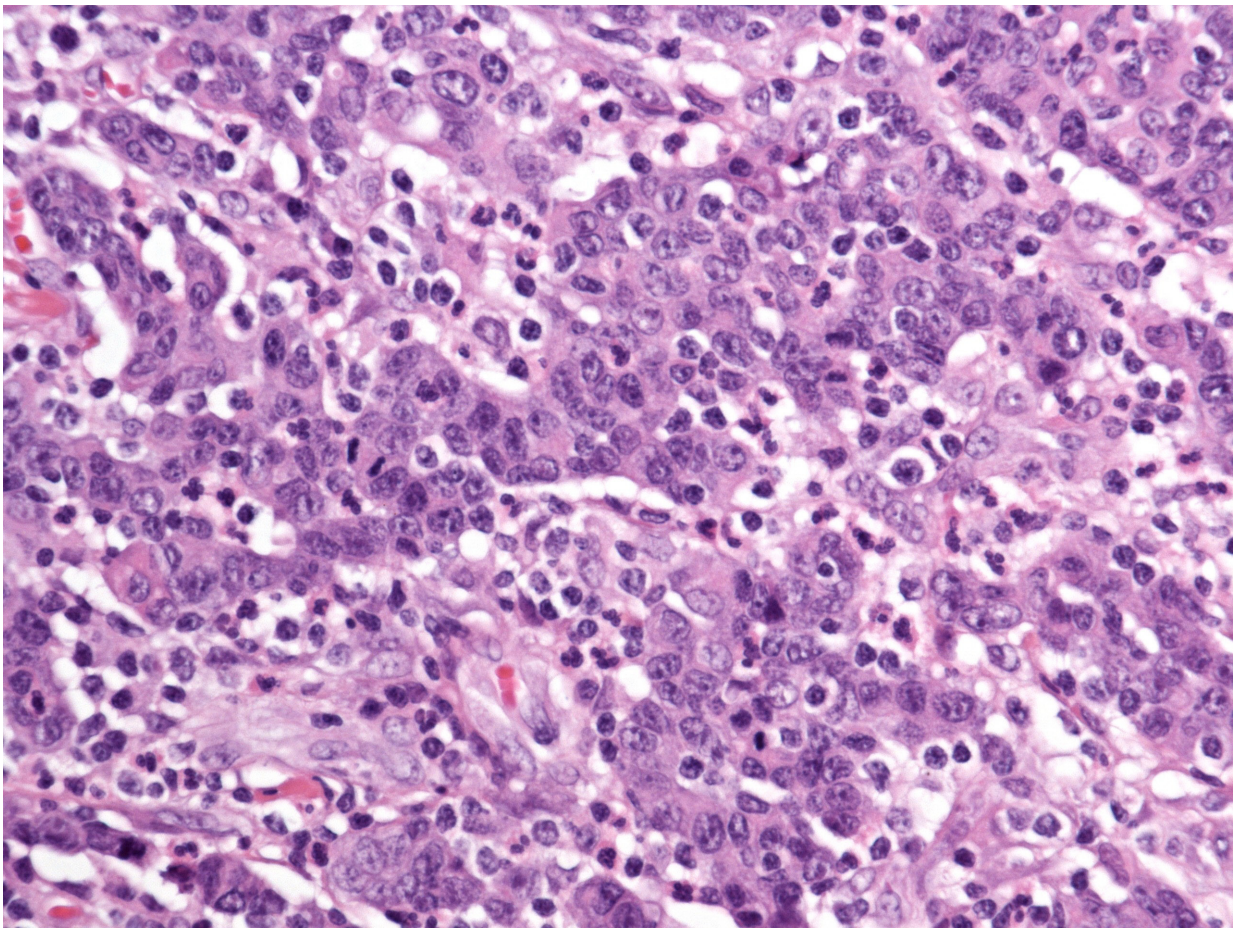

Figure 11 Histological features of colorectal cancer with high-level microsatellite instability (MSI-H): marked anti-tumor host responses, characterized by intra- and peritumoral lymphocytic infiltration.

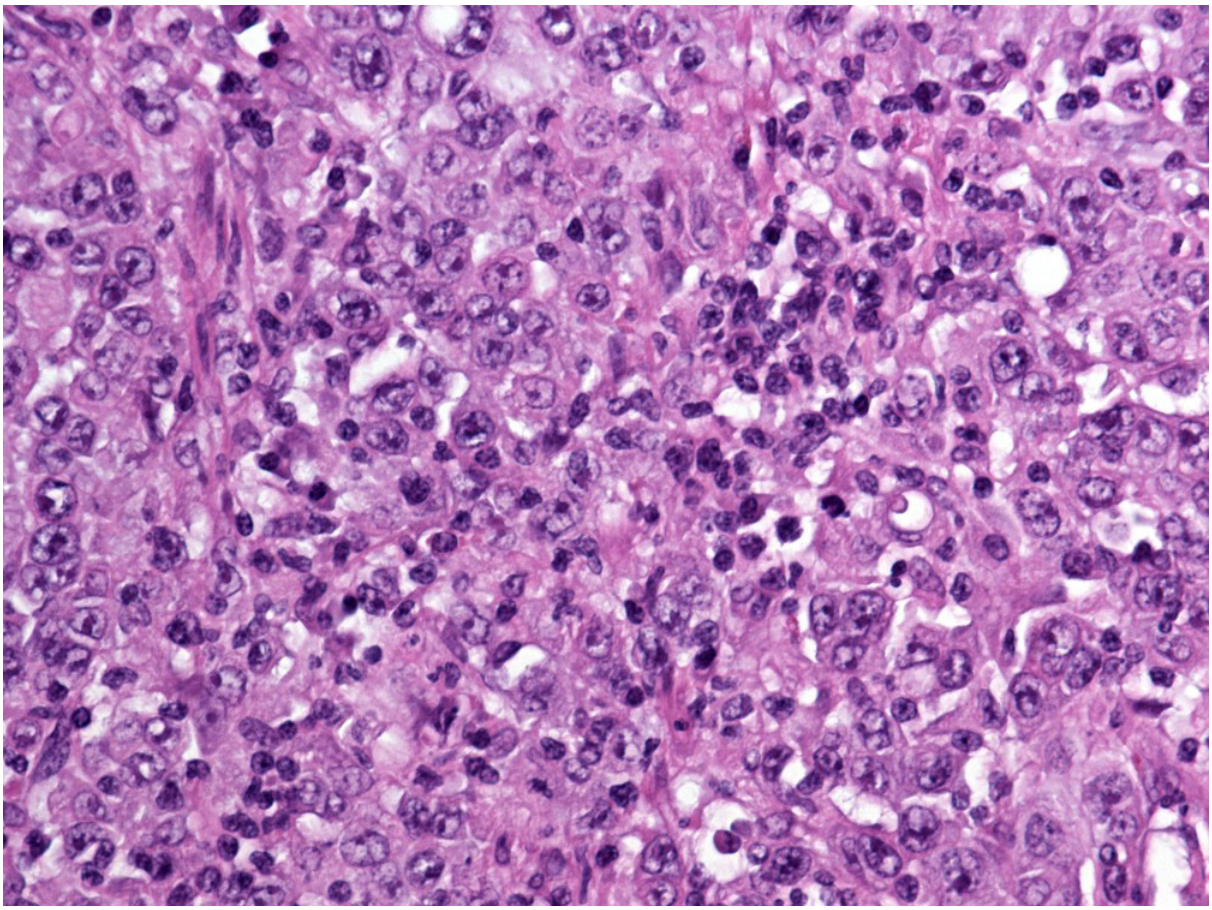

Figure 12 Histological features of colorectal cancer with high-level microsatellite instability (MSI-H): medullary-type carcinoma, characterized by syncytial sheets of malignant cells with vesicular nuclei, prominent nucleoli, and intratumoral lymphocytic infiltration.

Most histological features which serve as diagnostically useful markers of MSI-H status are apparent in both sporadic and hereditary, that is Lynch syndrome-associated MSI-H CRC. However, as demonstrated in detail above, the two principal subtypes of MSI-H CRC evolve through different pathways, and these differences in molecular pathogenesis translate into morphological distinctions, which deserve our attention. Hence, lymphocytic infiltration, tumor budding (de-differentiation), and co-existing adenomas are more evident in Lynch syndrome, while mucinous histology, poor differentiation, tumor heterogeneity and glandular serration with or without co-existing serrated polyps are more evident in sporadic MSI-H CRC [127]. Sporadic MSI-H CRC are also characterized by cytoplasmic eosinophilia and nuclei that are large, round, vesicular and contain a prominent nucleolus, while in Lynch syndrome the cytological features recapitulate the basophilia and nuclear characteristics of conventional adenomas [122, 128].

In 2009, Greenson et al. [117] presented two nearly equivalent logistic regression models that predict MSI-H status based on a review of 1649 CRCs from patients of all ages collected in a population-based case control study in northern Israel. In that cohort >2 TILs per high-powered field, lack of dirty necrosis, presence of a Crohn-like reaction, right-sided location, any mucinous differentiation, well or poor differentiation, and age less than 50 years were all independent predictors of MSI-H. The accuracy of both models was high, with an 85.4% vs. 85.0% probability of correctly classifying tumors as MSI-H. One year later, Hyde et al. [123] presented another histology-based model for predicting MSI-H status in CRC, termed Pathologic Role in Determination of Instability in Colorectal Tumors (PREDICT). In a population-based cohort of CRCs diagnosed in patients less than 75 years of age from Newfoundland (n = 710) the authors scored histological features, such as mucinous differentiation, peritumoral lymphocytes, TILs and Crohn-like reaction, but also the amount of stromal cells, and the presence, type, and grade of tumor subclones. The model identified MSI-H CRCs with a sensitivity of 92.1% and a specificity of 37.8%, whereas the Revised Bethesda Guidelines had a sensitivity of 81.3% and a specificity of 39.5%.

Finally, MSI-H CRCs appear to be associated with a distinct immunophenotype, unrelated to the lack of MMR protein expression. Thus, several

groups noted reduced expression of keratin 20 (K20) in MSI-H tumors. In the study by McGregor [129], which involved 44 CRC from 22 paired MSI-H and MSS cases matched for clinical-pathologic characteristics, the mean percentage of K20-positive tumor cells was 84% in MSS CRC but only 37% in MSI-H CRC ($p=0.0007$). Seven out of 22 (32%) of MSI-H CRC were K20-negative, as contrasted with 2 out of 22 (9%) of MSS CRC ($p=0.13$). In another study involving 371 CRC specimens K20 expression was significantly associated with tumor differentiation, tumor size, tumor location, histological subtype, lymphatic invasion, and MMR protein status: 16 (4.6%), 123 (35.3%), and 209 (60.1%) of 348 MMR proficient tumors were K20-negative or showed low or high K20 expression, respectively, as contrasted with 8 (34.8%), 12 (52.2%) and 3 (13%) of 23 MMR deficient tumors ($p<0.001$) [130]. It is of note, that the simultaneous loss of K20 and CDX-2 expression in tumor tissue has recently been associated with poor differentiation and CIMP in MSI-H CRC, serving as independent predictor of unfavorable prognosis in this tumor subset ($p=0.03$) [131].

MICROSATELLITE INSTABILITY TESTING IN THE ROUTINE SETTING

As shown in detail above, the identification of MSI-H CRCs is of eminent clinical importance. The MSI-H status is the central molecular tumor feature for the identification of individuals with Lynch syndrome, but it is also a marker of favorable outcome and, last but not least, a predictive marker of resistance to standard 5-fluorouracil-based adjuvant chemotherapy [123].

The selection of patients for MSI testing and the technical approach for this procedure are still under debate. Traditionally, the selection for testing is based upon the revised Bethesda Guidelines [68]. However, 12 to 28% of Lynch syndrome patients may be missed if testing is guided by these criteria and universal testing, that is testing of all CRC specimens has a greater sensitivity for the identification of Lynch syndrome patients compared with the Bethesda guidelines, but also compared with other selective strategies (e.g. tumor testing of patients with CRC <70 years of age or older patients meeting the Bethesda guidelines) [132-135]. It is of note that even 70% of Lynch syndrome patients may be missed when the selection is based upon the pathological Bethesda criteria only, that is, CRC in a patient aged less than 50 years, CRC with MSI-H phenotype in a patient aged less than 60 years, or meta-/synchronous CRC regardless of age [69]. In summary, the Bethesda guidelines or other selective strategies miss a considerable amount of individuals with Lynch syndrome, while there is growing evidence that universal testing for MSI starting with either immunohistochemistry or PCR-based molecular testing is cost-effective, sensitive, specific and is getting widely accepted [136].

Very recently, a multi-society task force, in collaboration with invited experts, developed “guidelines to assist health care providers with the appropriate provision of genetic testing and management of patients at risk for and affected with Lynch syndrome” [137-140]. According to these guidelines, testing for MMR deficiency of newly diagnosed CRCs should be performed as follows: (i) in all CRCs (provided an appropriate infrastructure is available) or (ii) in CRCs diagnosed at age 70 years or younger and in individuals older than 70 years, who have a positive family history regarding Lynch syndrome. Analysis can be done by routine tumor-based

immunohistochemistry for the MMR proteins MLH1, MSH2, MSH6, and PMS2 and/or testing for MSI.

In tumors with intact MMR protein expression additional molecular analysis is not generally recommended. However, in cases with equivocal staining or tumors with positive staining, yet high clinical suspicion for the presence of Lynch syndrome (e.g. the affected patient meets the revised Bethesda guidelines) additional molecular analysis should be performed, as very rarely tumors may show positive MMR protein staining despite MSI-H status [136-140]. Tumors that demonstrate loss of MLH1 (and PMS2) should undergo additional *BRAF* testing, which may serve as a surrogate marker for CIMP in order to exclude sporadic MMR deficiency. Individuals with tumors with loss of other MMR proteins should be referred to genetic counseling for germline testing, guided by immunohistochemical staining results (Figure 13) [137-140].

Similar recommendations have been made by a group of European experts. This group ("Mallorca Group") recommends investigation of all CRCs (or individuals with CRC <70 years) by immunohistochemistry of the four MMR proteins or by molecular testing. The tests should be accompanied by methods that identify *MLH1* promoter methylation, e.g. *BRAF* analysis. The authors stress that likewise the investigation of all endometrial cancers in individuals less than 70 years, by immunohistochemistry or molecular testing, can be considered to improve the identification of Lynch syndrome patients [141].

In mucinous and signet-ring cell carcinomas of the colon and rectum MMR immunohistochemistry can be used for prognostic stratification ("molecular grading"). That is, many mucinous adenocarcinomas are MSI-H and therefore low grade, whereas MSS or MSI-L cancers behave as high grade lesions. Likewise, signet-ring cell tumors that are MSI-H are regarded as low grade lesions, whereas those lacking MSI-H are usually highly aggressive [119]. Therefore, the concept of molecular grading should be expanded to poorly and undifferentiated cancers, as also in this subgroup the MSI-H status indicates favorable outcome [142-144]. Please note, molecular grading may be important also for patients with non-metastatic, that is AJCC/UICC stage II disease, who do usually not receive adjuvant therapy. Here, the combination of poor differentiation and MSS status (with or without other additional

risk factors, such as vascular or perineural invasion) may prompt the initiation of adjuvant treatment, e.g. in young patients.

The high sensitivity of immunohistochemistry supports the use of this tool as first step in the evaluation of the cancer specimen. However, for immunohistochemistry to be used as a first-line screening test requires that both pathologists and clinicians are aware of the fact that staining results may be considered as "genetic information," and that appropriate procedures are established to ensure patient understanding and consent [59]. Legal considerations, however, may vary from country to country.

Upon immunohistochemistry, the staining of MMR proteins should generally be interpreted as intact (positive, expressed) or lost (negative, not expressed). All four proteins are normally expressed in non-neoplastic tissue, and thus stroma, lymphocytes, and non-neoplastic crypts serve as critical internal controls [18]. A possible limiting factor is the quality of staining. In general, however, the presence of nuclear staining in the tumor cells, even when it is focal and weak, is good evidence of intact MMR protein, and additional molecular testing for MSI is not needed generally. In the rare situation where there is a lack of positive internal control in an otherwise negatively stained tumor repeating the stain in search for positive non-neoplastic stromal or inflammatory cells should be done [59].

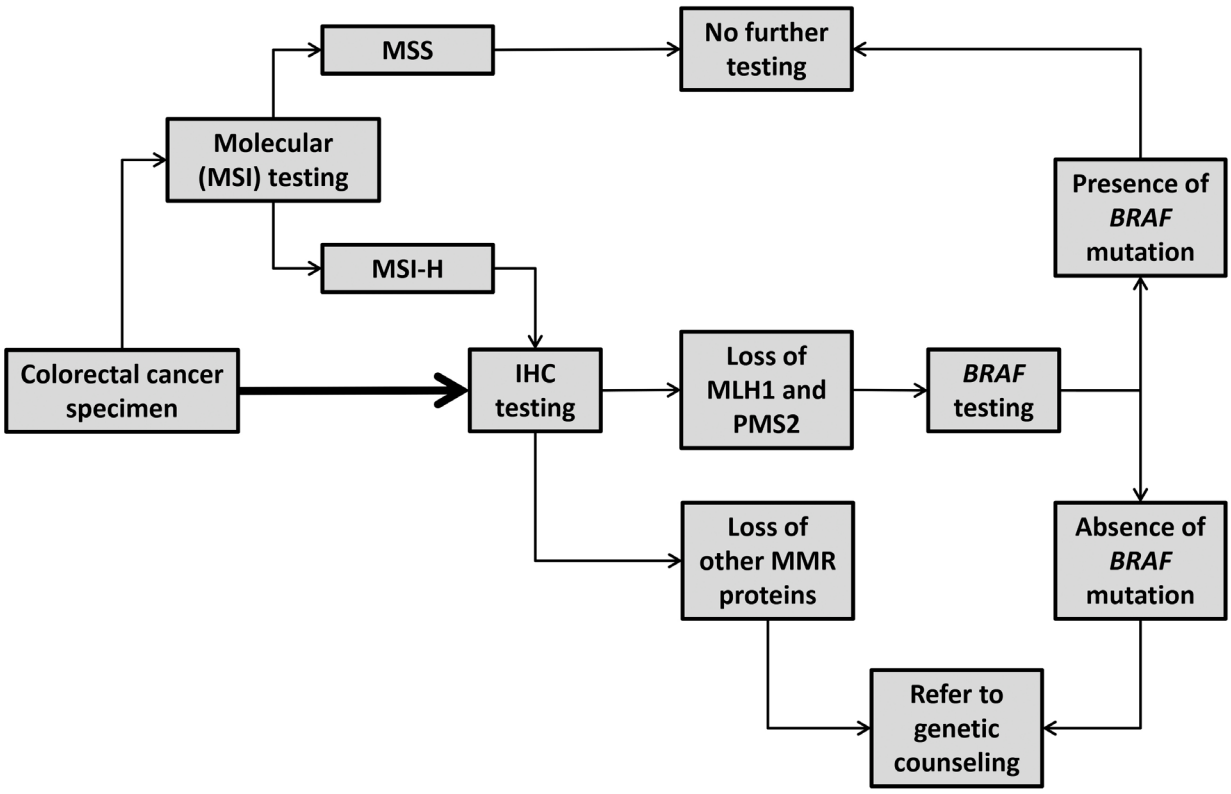

Figure 13 Screening for Lynch syndrome by tumor testing using immunohistochemistry, that is staining for mismatch repair (MMR) protein expression (MLH1, PMS2, MSH2, MSH6) or analysis of microsatellite instability (MSI), as has recently been recommended by a Multi-Society Task Force on Colorectal Cancer [137-140]. Tumors that demonstrate loss of MLH1 (and PMS2) should undergo additional *BRAF* testing to exclude sporadic MMR deficiency.

Basically, immunohistochemistry may render the following reaction patterns: (i) all four proteins intact, (ii) MLH1/PMS2 lost and MSH2/MSH6 intact, (iii) MSH2/MSH6 lost and MLH1/PMS2 intact, (iv) MSH6 lost and MLH1/PMS2/MSH2 intact, and (v) PMS2 lost and MLH1/MSH2/MSH6 intact. A typical MMR protein staining result is illustrated in Figures 14, 15, 16 and 17. The different staining patterns occur in varying frequency, implying different subsequent actions (Table 6).

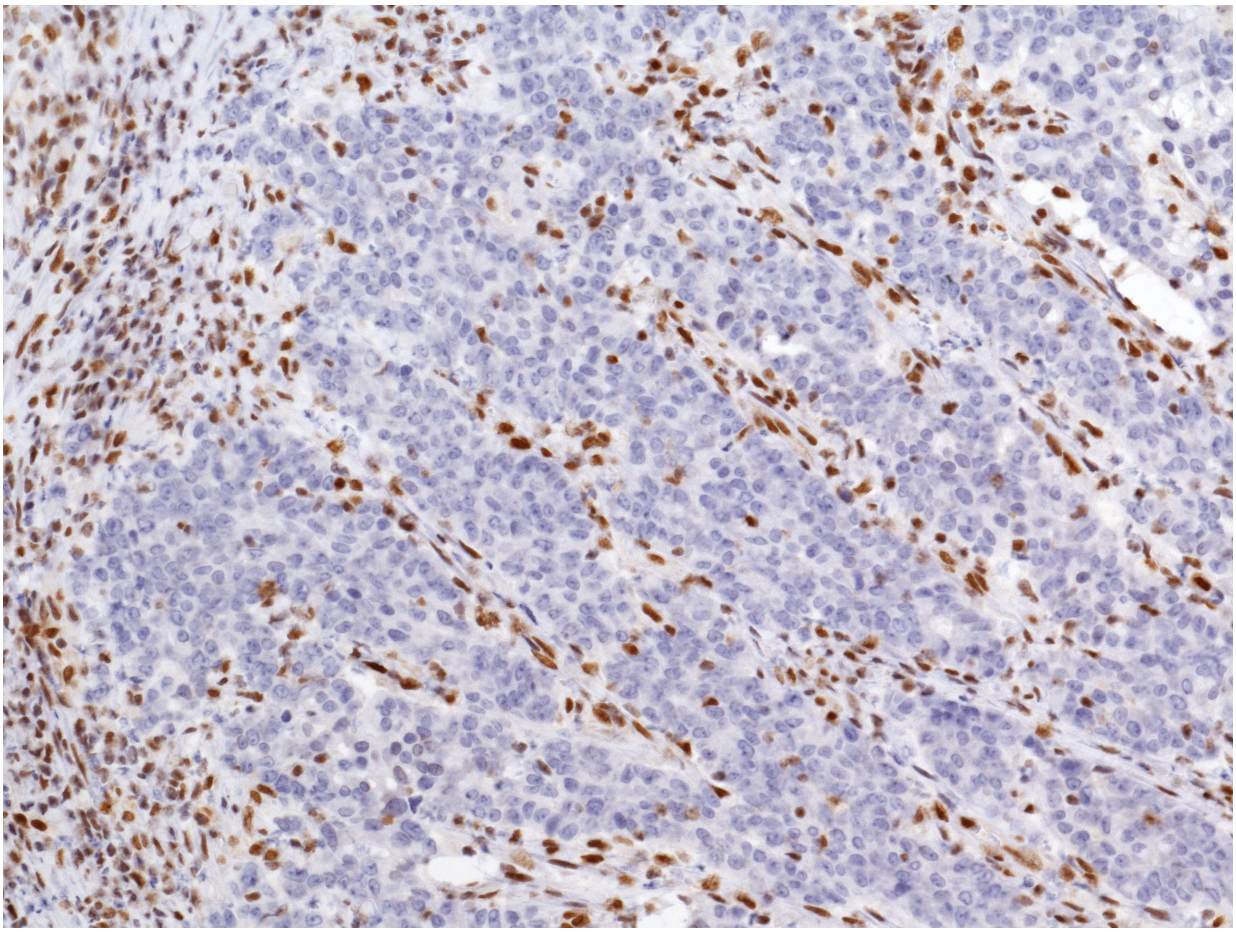

Figure 14 Example of lost mismatch repair (MMR) protein expression in a colorectal adenocarcinoma with high-level microsatellite instability (MSI- H): Loss of nuclear MLH1 staining in a right-sided tumor of a 75-year- old female. Non-neoplastic stromal tissue with inherent inflammatory cells serves as internal positive control.

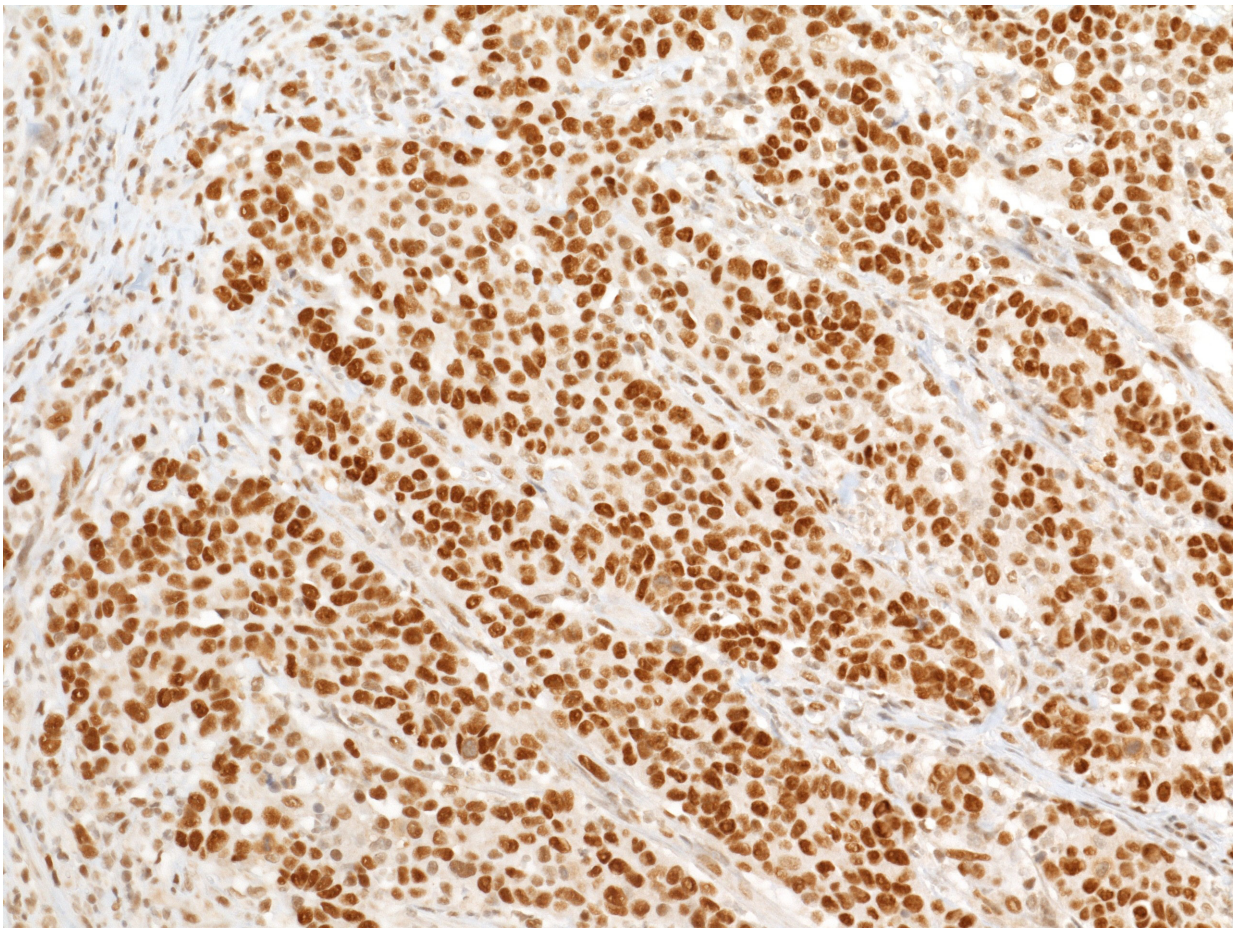

Figure 15 Example of lost mismatch repair (MMR) protein expression in a colorectal adenocarcinoma with high-level microsatellite instability (MSI- H): Intact nuclear MSH2 staining in a right-sided tumor of a 75-year-old female. Non-neoplastic stromal tissue with inherent inflammatory cells serves as internal positive control.

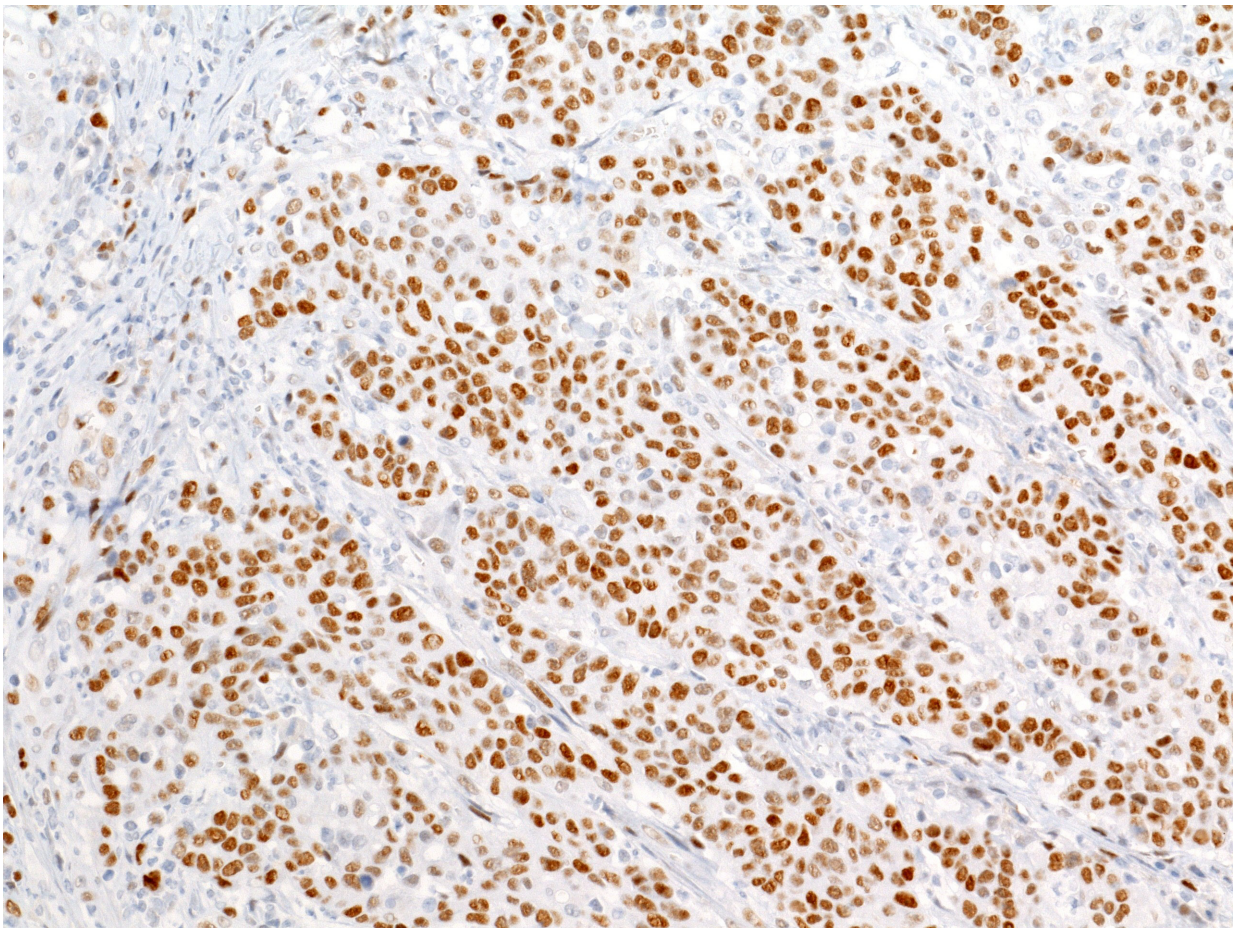

Figure 16 Example of lost mismatch repair (MMR) protein expression in a colorectal adenocarcinoma with high-level microsatellite instability (MSI- H): Intact nuclear MSH6 staining in a right-sided tumor of a 75-year-old female. Non-neoplastic stromal tissue with inherent inflammatory cells serves as internal positive control.

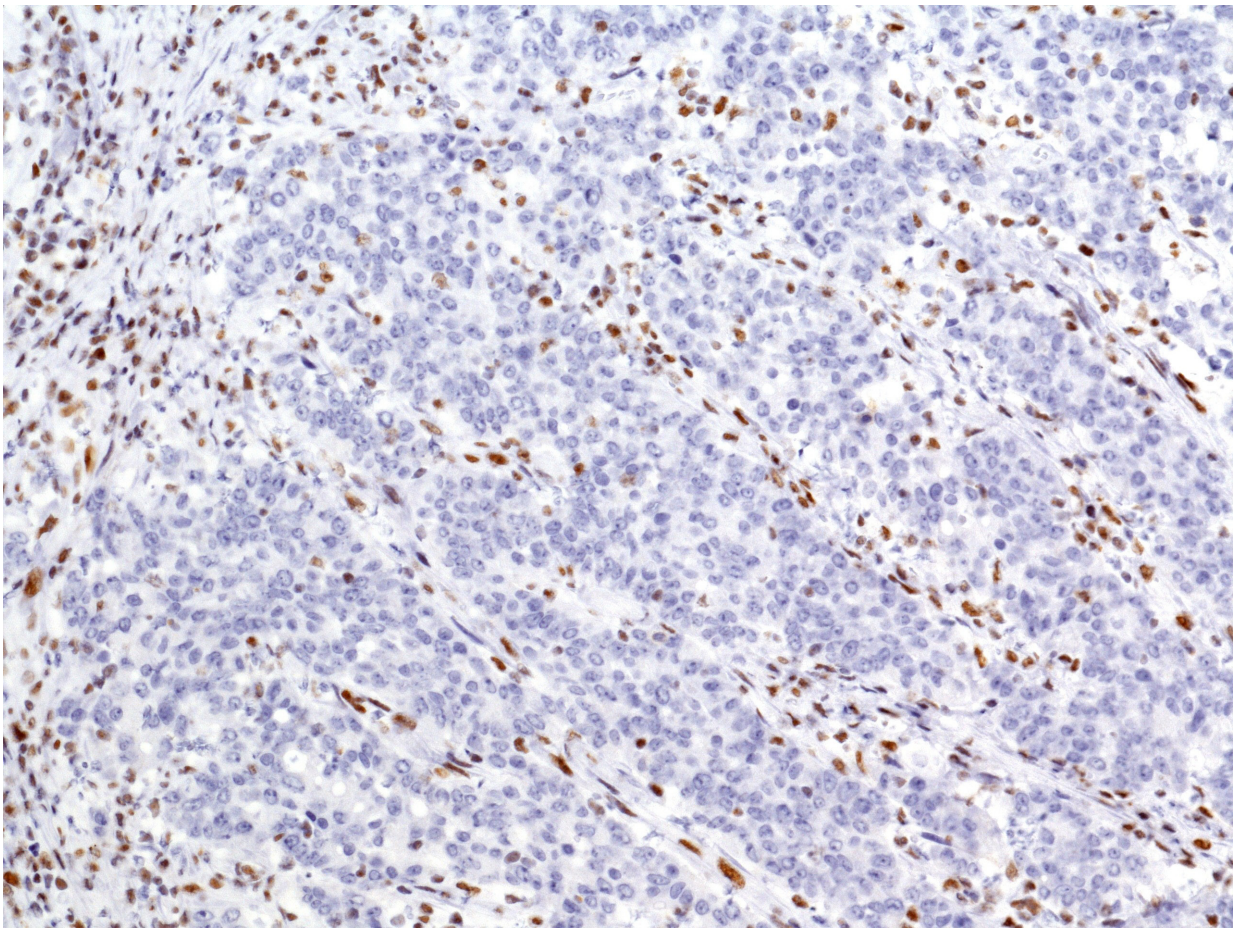

Figure 17 Example of lost mismatch repair (MMR) protein expression in a colorectal adenocarcinoma with high-level microsatellite instability (MSI- H): Loss of nuclear PMS2 staining in a right-sided tumor of a 75-year- old female. Non-neoplastic stromal tissue with inherent inflammatory cells serves as internal positive control.

Table 6 Mismatch repair (MMR) function testing in colorectal cancer (modified after Bellizzi [148]).

Immunohistochemistry	Frequency	Interpretation	Action(s)
All four proteins intact	80 to 85%	Normal MMR function (Lynch syndrome unlikely)	Consider additional MSI testing in cases with high clinical suspicion for the presence of Lynch syndrome
MLH1/PMS2 lost and MSH2/MSH6 intact	15%	Abnormal MMR function Likely sporadic MMR deficiency due to *MLH1* promoter methylation Less likely Lynch syndrome due to *MLH1* (usually) or *PMS2* (rarely) germline mutation	*BRAF* V600E and/or *MLH1* promoter methylation testing If the above are normal, refer to genetic counseling for *MLH1* germline testing (followed by *PMS2* if needed)
MSH2/MSH6 lost and MLH1/PMS2 intact	1 to 2%	Abnormal MMR function Likely Lynch syndrome due to *MSH2* (usually) or *MSH6* (rarely) germline mutation	Refer to genetic counseling for *MSH2* germline testing (followed by *MSH6* if needed)

MSH6 lost and MLH1/PMS2/MSH2 intact	Up to 0.5%	Abnormal MMR function Likely Lynch syndrome due to *MSH6* (usually) or *MSH2* (rarely) germline mutation	Refer to genetic counseling for *MSH6* germline testing (followed by *MSH2* if needed)
PMS2 lost and MLH1/MSH2/MSH6 intact	Up to 0.5%	Abnormal MMR function Likely Lynch syndrome due to *PMS2* (usually) or *MLH1* (rarely) germline mutation	Refer to genetic counseling for *PMS2* germline testing (followed by *MLH1* if needed)

It is of note that the intensity of staining for all four markers and, especially for MSH6, may be reduced due to neoadjuvant treatment, which is most evident in rectal cancers after neoadjuvant chemoradiation. In these cases, pre-treatment endoscopic biopsies rather than operative material may be used as the primary material for immunohistochemistry [145]. Please note, reduced expression of MSH6 due to neoadjuvant treatment [146, 147] has to be differentiated from loss of MSH6 expression due to secondary frameshift mutations in the *MSH6* gene in cancers with MLH1/PMS2 deficiency [147].

Patient Selection

During the period from January 1st, 2011 to December 31, 2013, a total of 180 CRC cases were prospectively collected from 176 consecutive patients, who underwent surgical resection at the Department of Surgery, Krankenhaus der Barmherzigen Brüder, St. Veit an der Glan.

Three patients had specific risk factors for CRC, such as ulcerative colitis (1 patient) and Crohn's disease (2 patients). There were 108 males (61%) and 68 females (39%), male-to-female ratio 1.6:1, with a median age of 69 (range 37 to 91) years. Of these, 97 (55%) patients were older than 70 years. In all, 69 tumors (39%) were identified on the right side (from caecum to transverse colon), 57 (32%) on the left side (descending to sigmoid colon), and 50 (28%) in the rectum.

Institutional review board approval was received from the Ethics Committee of the Medical University of Graz, Austria.

Macroscopy

The gross examination of the resection specimen was carried out by board-certified staff pathologists at the Institute of Pathology, Medical University of Graz, Austria according to international guidelines.

Immediately after surgery, the tumor specimens were submitted to neutral buffered formalin and were fixed for at least 24 hours. The depth of penetration into the bowel wall was recorded. Lymph nodes were manually dissected according to a standardized protocol.

Tumor staging was performed according to the 7th edition of the AJCC/UICC TNM classification [149]. Details are shown in Table 7.

Table 7 AJCC/UICC TNM classification of colorectal cancer [149].

Primary tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria
T1	Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades subserosa or into non-peritonealized pericolic or perirectal tissues
T4	Tumor directly invades other organs or structures and/or perforates visceral peritoneum
T4a	Tumor perforates visceral peritoneum
T4b	Tumor directly invades other organs or structures
Regional lymph nodes (N)	
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	Tumor deposit(s), i.e. satellites, in the subserosa, or in non-peritonealized pericolic or perirectal soft tissue *without* regional lymph node metastasis

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 (M)	
M0	No distant metastasis
M1	Distant metastasis
M1a	Metastasis confined to one organ (liver, lung, ovary, non-regional lymph node)
M1b	Metastases in more than one organ or the peritoneum

Histology

Histology was available for all 180 cancer specimens. Histological tumor typing was done using haematoxylin-and-eosin stained slides in accordance with WHO rules [119]. The designation mucinous carcinoma is used if >50% of the lesion is composed of pools of extracellular mucin that contain malignant epithelium, such as acinar structures, layers of tumor cells, or individual tumor cells including signet-ring cells. Carcinomas displaying mucinous areas of <50% are categorized as having a mucinous component [119].

Signet-ring cell carcinomas are defined by the presence of >50% of tumor cells with prominent intracytoplasmatic mucin, typically with displacement and molding of the nucleus. Carcinomas with signet-ring cell areas of <50% are categorized as adenocarcinoma with a signet-ring cell component [119].

The tumor-associated inflammation was classified as follows: diffuse intra- and peritumoural infiltration as well as nodular lymphoid peritumoural inflammation (Crohn-like reaction pattern). The severity of each type of inflammation was arbitrarily graded applying a three-tier grading system, respectively.

Immunohistochemistry of MMR Protein Expression

The following four antibodies were applied: anti-MLH1, anti-MSH2, anti-MSH6, and anti-PMS2. All antibodies were monoclonal mouse antibodies and commercially available: antibodies directed against MLH1 and MSH6 were purchased from Biocare Medical (Concord, CA, USA), the anti-MSH2 antibody was purchased from Ventana Medical Systems (Oro Valley, AZ, USA), and the anti- PMS2 antibodies was purchased from Cell Marque (Rocklin, CA, USA).

The staining protocol for anti-MLH1 and anti-PMS2 is the following:

Three-micrometer-thick tissue sections are dried for 1 hour at 70° C in the oven. Afterwards, tissue sections are deparaffinized with xylene and rehydrated in a graded series of alcohol (100% plus xylene, 100%, 90%, 80%, 70%, 50%) and rinsed twice with Phosphate-buffered saline (PBS), pH 7.3. Subsequently, tissue sections are submitted to the microwave for 40 minutes at 150 Watt and cooled down for 20 minutes. The protocol of the automated immunostainer (DAKO Autostainer Link 48, DAKO Glostrup, Denmark) involves 5 minutes of H_2O_2, 60 minutes for the primary antibodies (diluted 1:50, respectively), 30 minutes of DAKO EnVision detection kit, followed by 10 minutes of 3,3'-Diaminobenzidine (DAB) as chromogen. Afterwards the sections are counterstained with haematoxylin. Tissue sections then pass the graded alcohol line (80%, 90%, 100%), are fixed with the intermedium buthylacetat and covered by mounting medium (Entellan®, Merck Millipore Corporation, Darmstadt, Germany) and a cover slipper.

The protocol for anti-MSH2 and anti-MSH6 is the following:

Three-micrometer-thick tissue sections are dried for 1 hour at 70° C in the oven. Afterwards, the sections are incubated in the Ventana Benchmark "Cell Conditioning 1 mild" for 30 minutes. The protocol of the automated Ventana immunostainer (Ventana, Oro Valley, AZ, USA) involves 32 minutes for the primary antibodies (diluted 1:50, respectively), followed by the ultraView Universal DAB Detection Kit and haematoxylin. Afterwards, the sections are rinsed with H_2O and dishwashing detergent and pass the ascending alcohol line (80%, 90%, 100%). Finally, the slides are fixed with the intermedium buthylacetat and covered by mounting medium (Entellan®) and a cover slipper.

The staining was interpreted as intact (positive, expressed) or lost (negative, not expressed), with lost expression of at least MMR protein marker indicating MSI. As all four proteins are normally expressed in non-neoplastic tissues (compare above), stroma components as well as non-neoplastic crypts served as internal control.

BRAF Mutation Status

The *BRAF* gene was analyzed by pyrosequencing. This technique has been developed by Ronaghi et al. [150] in 1996 and is based on three enzymes, which convert the synthesis of a DNA molecule in a pattern of visible light signals. Emitted light signals are easily detected by a photodiode, photomultiplier tube, or a charge-coupled device (CCD) camera [151].

Template preparation for pyrosequencing is straightforward. After the DNA template is generated by PCR, the product needs to be purified before pyrosequencing. Subsequently, a biotin complex is attached to the PCR product, which is then captured onto streptavidin-coated magnetic beads. After sedimentation, pure double-stranded DNA is obtained by washing off the remaining components of PCR reaction. By alkali denaturation, single-stranded DNA is yielded. The immobilized biotinylated as well as the nonbiotinylated strands can be used as pyrosequencing templates [152, 153].

For DNA synthesis, one of the four nucleotides (A, C, G, and T) is added and therefore available for incorporation into the single strand template. DNA synthesis (elongation) is catalyzed by DNA polymerase. If the nucleotides are incorporated into the DNA strand template, this reaction is accompanied by the release of pyrophosphate. The generated pyrophosphate is converted to ATP by ATP sulfurylase, and in turn ATP powers oxidation of luciferin by luciferase. This reaction generates a light signal, which is detected by a camera. Because the order of the added nucleotides is known, the sequence of the template can be determined [151].

Regarding the analyzed CRC specimens, DNA from tumor tissue was extracted from formalin fixed paraffin-embedded tissue sections, containing at least 10% of tumor cells (cut-off for detection 5%). In brief, areas with tumor were marked on haematoxylin-and-eosin stained slides and the corresponding area was transferred to non-colored tissue sections and subsequently micro-dissected with sterile razor blades. DNA was isolated using a Maxwell® RSC DNA FFPE Kit (Promega, Madison, WI, USA) and the obtained amount of DNA was measured by a NanoDrop® spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Testing for *BRAF* mutation in codons 600 to 594 in exon 15 was performed using a ready-to-use Therascreen BRAF Pyrokit (Quiagen, Valencia, CA, USA) for PyroMark Q24 MDx (Quiagen, Valencia, CA, USA).

Statistical analysis

Categorical variables are presented as absolute and relative frequencies, numerical variables as means and medians as well as ranges.

Differences in categorical variables are examined using the χ^2 test or Fisher's exact test, as appropriate. All p-values are two-sided, and values <0.05 are considered statistically significant.

Statistical analysis was performed using IBM's SPSS Statistics 22.0 software (Armonk, New York, USA).

Results

Immunohistochemical and molecular data were available from 176 of 180 (97.7%) cases. Loss of at least one MMR protein was observed in 24 of 176 (13.6%) tumors (MSI tumors). Immunohistochemical profiles are summarized in Table 8.

Table 8 Mismatch repair protein expression in 176 colorectal cancers.

MLH1	
Present	157 (89.2%)
Lost	19 (10.8%)
PMS2	
Present	156 (88.6%)
Lost	20 (11.4%)
Combined loss of MLH1/PMS2	19 (10.8%)
Isolated loss of PMS2	1 (0.6%)
MSH2	
Present	173 (98.3%)
Lost	3 (1.7%)
MSH6	
Present	171 (97.2%)
Lost	5 (2.8%)
Combined loss of MSH2/MSH6	3 (1.7%)
Isolated loss of MSH6	1 (0.6%)
Loss of MSH6 in conjunction with loss of MLH1/PMS2	1 (0.6%)

The 24 cases with lost MMR protein expression (MSI tumors) were predominantly right-sided, compared to the 152 cases with intact MMR protein expression (MSS tumors), which were predominantly left sided ($p<0.001$). Mean and median age of patients with MSI tumors was 67.5 and 69 years (range 40 to 91), compared to 68 and 69 years (range 36 to 92) for patients with MSS tumors. The gender distribution was likewise unremarkable. Clinicopathological data are summarized in Table 9.

Table 9 Clinicopathological data of 176 colorectal cancers related to mismatch repair (MMR) protein expression (MSI/MSS) status.

	Intact MMR protein expression (MSS tumors; n = 152)	Lost MMR protein expression (MSI tumors; n = 24)	*P*-value
Gender			1
Male	96 (63.2%)	15 (62.5%)	
Female	56 (36.8%)	9 (37.5%)	
Tumor location			<0.001
Right	48 (31.6%)	20 (83.3%)	
Left	58 (38.2%)	2 (8.3%)	
Rectum	46 (30.1%)	2 (8.3%)	
Multiple tumors			0.36
Yes	2 (1.3%)	1 (4.2%)	
No	150 (98.7%)	23 (95.8%)	

Histological comparison showed the following results: in all, 32 (18.2%) tumors were mucinous adenocarcinomas, while only 3 (1.7%) were signet-ring cell carcinomas. As could be expected, cancers with lost MMR protein expression (MSI tumors) were more often mucinous, but statistical comparison lacked significance. It is of note that the 24 MSI tumors were characterized by prominent inflammation, that is, anti-tumor host response. This was proven with all three markers, i.e. intra- and peritumoral inflammation as well as Crohn's like inflammatory reaction ($p<0.001$, respectively). Data are summarized in Table 10.

Table 10 Histological data of 176 colorectal cancers related to mismatch repair (MMR) protein expression (MSI/MSS) status.

	Intact MMR protein expression (MSS tumors; n = 152)	Lost MMR protein expression (MSI tumors; n = 24)	*P*-value
Extracellular mucin			0.15
Absent	127 (83.6%)	17 (70.8%)	
Present	25 (16.4%)	7 (29.2%)	
Signet-ring cell differentiation			0.36
Absent	150 (98.6%)	23 (95.8%)	
Present	2 (1.4%)	1 (4.2%)	
Intratumoral inflammation			<0.001
0	46 (30.3%)	2 (8.3%)	
1	80 (52.6%)	7 (29.2%)	
2	20 (13.2%)	10 (41.6%)	
3	6 (3.9%)	5 (20.8%)	

Peritumoral inflammation			<0.001
0	8 (5.2%)	0	
1	81 (53.3%)	4 (16.6%)	
2	53 (34.9%)	13 (54.2%)	
3	10 (6.6%)	7 (29.2%)	
Crohn's like reaction (%)			<0.001
0	68 (44.7%)	3 (12.5%)	
1	60 (39.5%)	1 (4.2%)	
2	16 (10.5%)	7 (29.2%)	
3	8 (5.3%)	13 (54.2%)	

All tumors with lost MMR protein expression were subjected to *BRAF* mutational analysis. In all, 14 patients (58.3%) were *BRAF* wild type (mean age 63.1, range 40 to 87), suggesting cancer development within Lynch syndrome. These cases included the three patients with combined loss of MSH2/MSH6, the two patients with isolated loss of MSH6 and PMS2, respectively, as well as 9 of 19 (47.4%) patients with combined loss of MLH1/PMS2.

Mutation in the *BRAF* gene was identified in the remaining 10 (41.7%) patients (mean age 77.9, range 66 to 91). All these case were negative for MLH1 (and consequently PMS2), while the staining of MSH6 and MSH2 was intact. These data suggest that about half of MSI tumors with lost MLH1 (and PMS2) expression are sporadic, following the serrated route to cancer.

Discussion

The results of the immunohistochemical analysis of the 176 CRC cases show that universal testing for MSI by immunohistochemistry is feasible and can be easily done by trained pathologists.

Immunohistochemistry revealed that 13.6% of the tumors were MSI, which is defined as loss of at least one MMR protein. MSI tumors were predominantly located in the right colon, in comparison with MSS tumors. Concerning gender and age, no significant differences were found between MSI and MSS tumors. However, correlations between older age and MSI tumors, as well as female sex and MSI tumors have been reported [3, 91, 92]. The distribution among the four MMR proteins was as expected [63, 64], the majority of MSI cases showed MLH1 (10.8%) and PMS2 (11.4%) losses, followed by loss of MSH6 (2.8%) and loss of MSH2 (1.7%).

The proportion of MSI tumors displaying mucinous differentiation was 29.2% compared with 16.4% of MSS tumors with mucinous differentiation. Though it did not reach statistical significance, this result is in line with previous studies [114, 117], reporting that mucinous histology is one of the characteristic features of MSI tumors. Signet-ring cell carcinoma is a rare variant of CRC; this is also reflected by the result that only 3 out of all 176 cases were signet-ring cell carcinomas (1.7%).

One of the strongest markers for MSI CRCs is anti-tumor host response [67, 113-115, 117, 121-123]. The histological analysis of the cases mirrored this finding, as the 24 MSI tumors were characterized by prominent inflammation, and this was confirmed by all three markers of anti-tumor host response (intra- and peritumoral inflammation, as well as Crohn's like inflammatory reaction), this time reaching statistical significance ($p<0.001$).

For detection of somatic silencing (promoter hypermethylation) of the *MLH1* gene, all tumors with MMR deficiency were subjected to *BRAF* mutational analysis. Of the 24 MSI tumors, 58.3% were *BRAF* wild type, which indicates Lynch syndrome. Nonetheless, in these cases, germline mutation testing is recommended to confirm the diagnosis [154]. The 41.7% of the MSI tumors that were *BRAF* mutant showed loss of MLH1 and consecutively PMS2, while MSH2 and MSH6 were intact, suggesting sporadic cancer development via the serrated pathway.

Individuals in the MSI/*BRAF* wild type subgroup were younger than individuals in the MSI/*BRAF* mutant subgroup (mean age 63.1, range 40 to 87 in contrast to mean age 77.9, range 66 to 91, respectively). This finding contributes to the suspicion of Lynch syndrome in the MSI/*BRAF* wild type group.

The importance of pathology review for identifying MSI-H tumors [154] is also highlighted in the Bethesda guidelines. But histological features alone should not be used as a substitute method for establishing MSI-H status by immunohistochemistry or the molecular MSI analysis [115]. Also the Bethesda guidelines should not be used as a single detection tool in Lynch syndrome. Several reports [132-135] have proven that they lack sensitivity in detecting Lynch syndrome compared with immunohistochemistry and MSI analysis, and these data underline routine testing for MSI in all CRC specimens. In the 2009 Jerusalem Workshop, experts focused again on the identification of Lynch syndrome and on present and future challenges that come with Lynch syndrome and MSI tumors [155]. Initially, the suggestion was to test all CRCs regardless of age. Thus, the expert group tended to test all CRCs in patients younger than 70 years of age, which also became the final recommendation. Giardello et al. also proposed this recommendation in 2014 [137-140]. Boland et al. [155] point out that screening in all CRC patients younger than 70 years would miss 13.6% of Lynch syndrome cases, but exclude nearly half of all CRCs from analysis. This compromise limits the application of immunohistochemistry or MSI analysis, and possibly contributes to the cost-effectiveness of testing. In the analysis of the 176 CRCs, no evaluation of the costs was conducted, although positive results would further contribute to broader acceptance for universal testing or testing for all CRCs younger than 70 years, respectively.

The evaluation of anti-tumor host response is still rather subjective to date [156, 157]; also, a high interobserver variability was found in previous reports [113, 115]. Standardization of cut off points would decrease this ongoing lack of definition and is another future objective.

Histology and immunohistochemistry were reliable markers of MSI in the analysis of 176 CRCs, suggesting universal testing in all newly detected CRCs. Identifying the underlying pathway is crucial for clinical and prognostic implications as well as for the decision on therapeutic regimens [115].

CONCLUSION

The MSI-H phenotype of CRC is of eminent clinical importance. High-level MSI is the seminal molecular tumor feature for the identification of individuals with Lynch syndrome, but it is also a marker of favorable outcome and a predictive marker of resistance to standard 5-fluorouracil-based adjuvant chemotherapy. Among others, mucinous and medullary histology, signet-ring cell differentiation, and marked anti-tumoral immune response are histological features suggesting MSI. Universal tumor testing is recommended and may be performed using immunohistochemistry (staining for MMR protein expression) or molecular analysis, as has recently been recommended by an international task force.

Applying universal testing to a prospective cohort of 176 cancer cases operated within a three-year period proved the feasibility of the concept. We were able to identify a total of 14 patients with lost MMR protein expression, which were BRAF wild type, thereby suggesting cancer development within Lynch syndrome. This clinically relevant cohort comprised all cases with combined MSH2/MSH6 loss, isolated MSH6 and PMS2 loss as well as about every second patient with combined MLH1/PMS2 loss. The 14 patients were approximately 15 years younger than the 10 patients with presumed sporadic cancers. It is of note, however, that four patients were older than 70 years, thereby not fulfilling the revised Bethesda guidelines.

REFERENCES

1. Siegel R, Desantis C, Jemal A. Colorectal cancer statistics, 2014. CA Cancer J Clin 2014; 64: 104-117.
2. Malvezzi M, Bertuccio P, Levi F, et al. European cancer mortality predictions for the year 2014. Ann Oncol 2014; 25: 1650-1656.
3. Samadder NJ, Vierkant RA, Tillmans LS, et al. Associations between colorectal cancer molecular markers and pathways with clinicopathologic features in older women. Gastroenterology 2013; 145: 348-356.
4. Leggett B, Whitehall V: Role of the serrated pathway in colorectal cancer pathogenesis. Gastroenterology 2010; 138: 2088-2100.
5. Snover DC. Update on the serrated pathway to colorectal carcinoma. Hum Pathol 2011; 42: 1-10.
6. Jasperson KW, Tuohy TM, Neklason DW, et al. Hereditary and familial colon cancer. Gastroenterology 2010; 138: 2044-2058.
7. Labianca R, Nordlinger B, Beretta GD, et al. ESMO Guidelines Working Group. Primary colon cancer: ESMO Clinical Practice Guidelines for diagnosis, adjuvant treatment and follow-up. Ann Oncol 2010; 21: 70-77.
8. Wei EK, Giovannucci E, Wu K, et al. Comparison of risk factors for colon and rectal cancer. Int J Cancer 2004; 108: 433-442.
9. Flood DM, Weiss NS, Cook LS, et al. Colorectal cancer incidence in Asian migrants to the United States and their descendants. Cancer Causes Control 2000; 11: 403-411.
10. Brenner H, Kloor M, Pox CP. Colorectal cancer. Lancet 2014; 383: 1490-1502.
11. Coldman AJ, Phillips N, Brisson J, et al. Using the Cancer Risk Management Model to evaluate colorectal cancer screening options for Canada. Curr Oncol 2015; 22: 41-50.
12. Sideris M, Papagrigoriadis S. Molecular biomarkers and classification models in the evaluation of the prognosis of colorectal cancer. Anticancer Res 2014; 34: 2061-2068.

13. Jass JR. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. Histopathology 2007; 50: 113-130.
14. Pino MS, Chung DC. The chromosomal instability pathway in colon cancer. Gastroenterology 2010; 138: 2059-2072.
15. Bettington M, Walker N, Clouston A, et al. The serrated pathway to colorectal carcinoma: current concepts and challenges. Histopathology 2013; 62: 367-386.
16. Langner C. Serrated and Non-Serrated Precursor Lesions of Colorectal Cancer. Dig Dis 2015; 33: 28-37.
17. Boland CR, Koi M, Chang DK, et al. The biochemical basis of microsatellite instability and abnormal immunohistochemistry and clinical behavior in Lynch syndrome: from bench to bedside. Fam Cancer 2008; 7: 41-52.
18. Bellizzi AM, Frankel WL. Colorectal cancer due to deficiency in DNA mismatch repair function: a review. Adv Anat Pathol 2009; 16: 405-417.
19. Boland CR, Thibodeau SN, Hamilton SR, et al. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res 1998; 58: 5248-5257.
20. Dietmaier W, Wallinger S, Bocker T, et al. Diagnostic microsatellite instability: definition and correlation with mismatch repair expression. Cancer Res 1997; 57: 4749-4756.
21. Suraweera N, Duval A, Reperant M, et al. Evaluation of tumor microsatellite instability using five quasimonomorphic mononucleotide repeats and pentaplex PCR. Gastroenterology 2002; 123: 1804-1811.
22. Patil DT, Bronner MP, Portier BP, et al. A five-marker panel in a multiplex PCR accurately detects microsatellite instability-high colorectal tumors without control DNA. Diagn Mol Pathol 2012; 21: 127-133.
23. Issa JP. CpG island methylator phenotype in cancer. Nat Rev Cancer 2004; 4: 988-993.
24. Lao VV, Grady WM. Epigenetics and colorectal cancer. Nat Rev Gastroenterol Hepatol 2011; 8: 686-700.

25. Toyota M, Ahuja N, Ohe-Toyota M, et al. CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci USA 1999; 96: 8681-8686.
26. Hawkins N, Norrie M, Cheong K, et al. CpG island methylation in sporadic colorectal cancers and its relationship to microsatellite instability. Gastroenterology 2002; 122: 1376-1387.
27. Samowitz WS, Albertsen H, Herrick J, et al. Evaluation of a large, population-based sample supports a CpG island methylator phenotype in colon cancer. Gastroenterology 2005; 129: 837-845.
28. Dahlin AM, Palmqvist R, Henriksson ML, et al. The role of the CpG island methylator phenotype in colorectal cancer prognosis depends on microsatellite instability screening status. Clin Cancer Res 2010; 16: 1845-1855.
29. Kambara T, Simms LA, Whitehall VL, et al. BRAF mutation is associated with DNA methylation in serrated polyps and cancers of the colorectum. Gut 2004; 53: 1137-1144.
30. Hughes LA, Khalid-de Bakker CA, Smits KM, et al. The CpG island methylator phenotype in colorectal cancer: progress and problems. Biochim Biophys Acta 2012; 1825: 77-85.
31. Weisenberger DJ, Siegmund KD, Campan M, et al. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat Genet 2006; 38: 787-793.
32. Ogino S, Kawasaki T, Kirkner GJ, et al. Evaluation of markers for CpG island methylator phenotype (CIMP) in colorectal cancer by a large population-based sample. J Mol Diagn 2007; 9: 305-314.
33. Berg M, Hagland HR, Søreide K. Comparison of CpG island methylator phenotype (CIMP) frequency in colon cancer using different probe- and gene-specific scoring alternatives on recommended multi-gene panels. PLoS One 2014; 9: e86657.
34. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 1990; 61: 759-767.
35. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature 2000; 408: 307-310.

36. Smith G, Carey FA, Beattie J, et al. Mutations in APC, Kirsten-ras, and p53--alternative genetic pathways to colorectal cancer. Proc Natl Acad Sci USA 2002; 99: 9433-9438.
37. Leslie A, Carey FA, Pratt NR, et al. The colorectal adenoma-carcinoma sequence. Br J Surg 2002; 89: 845-860.
38. Claes K, Dahan K, Tejpar S, et al. The genetics of familial adenomatous polyposis (FAP) and MutYH-associated polyposis (MAP). Acta Gastroenterol Belg 2011; 74: 421-426.
39. Kartheuser A, West S, Walon C, et al. The genetic background of familial adenomatous polyposis. Linkage analysis, the APC gene identification and mutation screening. Acta Gastroenterol Belg 1995; 58: 433-451.
40. Half E, Bercovich D, Rozen P. Familial adenomatous polyposis. Orphanet J Rare Dis 2009; 4: 22.
41. Macrae F, du Sart D, Nasioulas S. Familial adenomatous polyposis. Best Pract Res Clin Gastroenterol 2009; 23: 197-207.
42. Cahill DP, Lengauer C, Yu J, et al. Mutations of mitotic checkpoint genes in human cancers. Nature 1998; 392: 300-303.
43. Minde DP, Anvarian Z, Rüdiger SG, et al. Messing up disorder: how do missense mutations in the tumor suppressor protein APC lead to cancer? Mol Cancer 2011; 10: 101.
44. Behrens J. The role of the Wnt signalling pathway in colorectal tumorigenesis. Biochem Soc Trans 2005; 33: 672-675.
45. MacDonald BT, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev Cell 2009; 17: 9-26.
46. White BD, Chien AJ, Dawson DW. Dysregulation of Wnt/β-catenin signaling in gastrointestinal cancers. Gastroenterology 2012; 142: 219-232.
47. Herzig DO, Tsikitis VL. Molecular markers for colon diagnosis, prognosis and targeted therapy. J Surg Oncol 2015; 111: 96-102.
48. Coppedè F, Lopomo A, Spisni R, et al. Genetic and epigenetic biomarkers for diagnosis, prognosis and treatment of colorectal cancer. World J Gastroenterol 2014; 20: 943-956.

49. Williams JP, Stewart T, Li B, et al. The retinoblastoma protein is required for Ras---induced oncogenic transformation. Mol Cell Biol 2006; 26: 1170–1182.
50. Bazan V, Agnese V, Corsale S, et al. Specific TP53 and/or Ki-ras mutations as independent predictors of clinical outcome in sporadic colorectal adenocarcinomas: results of a 5-year Gruppo Oncologico dell'Italia Meridionale (GOIM) prospective study. Ann Oncol 2005; 16 Suppl 4: 50-55.
51. Ahmed S, Johnson K, Ahmed O, et al. Advances in the management of colorectal cancer: from biology to treatment. Int J Colorectal Dis 2014; 29: 1031-1042.
52. Benson AB 3rd, Venook AP, Bekaii-Saab T, et al; National Comprehensive Cancer Network. Colon cancer, version 3.2014. J Natl Compr Canc Netw 2014; 12: 1028-1059.
53. Lengauer C, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. Nature 1998; 396: 643-649.
54. Thiagalingam S, Laken S, Willson JK, et al. Mechanisms underlying losses of heterozygosity in human colorectal cancers. Proc Natl Acad Sci USA 2001; 98: 2698-2702.
55. Walther A, Houlston R, Tomlinson I. Association between chromosomal instability and prognosis in colorectal cancer: a meta-analysis. Gut 2008; 57: 941-950.
56. Acharya S, Wilson T, Gradia S, et al. hMSH2 forms specific mispair-binding complexes with hMSH3 and hMSH6. Proc Natl Acad Sci USA 1996; 93: 13629–13634.
57. Kadyrov FA, Dzantiev L, Constantin N, et al. Endonucleolytic function of MutLalpha in human mismatch repair. Cell 2006; 126: 297–308.
58. Young J, Simms LA, Biden KG, et al. Features of colorectal cancers with high-level microsatellite instability occurring in familial and sporadic settings: parallel pathways of tumorigenesis. Am J Pathol 2001; 159: 2107-2116.
59. Shia J. Immunohistochemistry versus microsatellite instability testing for screening colorectal cancer patients at risk for hereditary nonpolyposis colorectal cancer syndrome. Part I. The utility of immunohistochemistry. J Mol Diagn 2008; 10: 293-300.

60. Salahshor S, Koelble K, Rubio C, et al. Microsatellite Instability and hMLH1 and hMSH2 expression analysis in familial and sporadic colorectal cancer. Lab Invest 2001; 81: 535-541.
61. Peltomäki P, Vasen H. Mutations associated with HNPCC predisposition -- Update of ICG-HNPCC/INSiGHT mutation database. Dis Markers 2004; 20: 269-276.
62. Zhang L. Immunohistochemistry versus microsatellite instability testing for screening colorectal cancer patients at risk for hereditary nonpolyposis colorectal cancer syndrome. Part II. The utility of microsatellite instability testing. J Mol Diagn 2008; 10: 301-307.
63. Cicek MS, Lindor NM, Gallinger S, et al. Quality assessment and correlation of microsatellite instability and immunohistochemical markers among population- and clinic-based colorectal tumors results from the Colon Cancer Family Registry. J Mol Diagn 2010; 13: 271-281.
64. Lynch HT, de la Chapelle A. Hereditary colorectal cancer. N Engl J Med 2003; 348: 919-932.
65. Vasen HFA, Mecklin J-P, Meera Khan P, et al. The International Collaborative Group on hereditary non-polyposis colorectal cancer (ICG-HNPCC). Dis Colon Rectum 1991; 34: 424–425.
66. Vasen HF, Watson P, Mecklin JP, et al. New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. Gastroenterology 1999; 116: 1453-1456.
67. Jass JR. Role of the pathologist in the diagnosis of hereditary non-polyposis colorectal cancer. Dis Markers 2004; 20: 215-224.
68. Umar A, Boland CR, Terdiman JP, et al. Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. J Natl Cancer Inst 2004; 96: 261-268.
69. Musulén E, Sanz C, Muñoz-Mármol AM, et al. Mismatch repair protein immunohistochemistry: a useful population screening strategy for Lynch syndrome. Hum Pathol 2014; 45: 1388-1396.

70. Dunlop MG, Farrington SM, Carothers AD, et al. Cancer risk associated with germline DNA mismatch repair gene mutations. Hum Mol Genet 1997; 6: 105-110.
71. Quehenberger F, Vasen HF, van Houwelingen HC. Risk of colorectal and endometrial cancer for carriers of mutations of the hMLH1 and hMSH2 gene: correction for ascertainment. J Med Genet 2005; 42: 491-496.
72. Jenkins MA, Baglietto L, Dowty JG, et al. Cancer risks for mismatch repair gene mutation carriers: a population-based early onset case-family study. Clin Gastroenterol Hepatol 2006; 4: 489-498.
73. Alarcon F, Lasset C, Carayol J, et al. Estimating cancer risk in HNPCC by the GRL method. Eur J Hum Genet 2007; 15: 831-836.
74. Senter L, Clendenning M, Sotamaa K, et al. The clinical phenotype of Lynch syndrome due to germ-line PMS2 mutations. Gastroenterology 2008; 135: 419-428.
75. Choi YH, Cotterchio M, McKeown-Eyssen G, et al. Penetrance of colorectal cancer among MLH1/MSH2 carriers participating in the colorectal cancer familial registry in Ontario. Hered Cancer Clin Pract 2009; 7: 14.
76. Baglietto L, Lindor NM, Dowty JG, et al. Risks of Lynch syndrome cancers for MSH6 mutation carriers. J Natl Cancer Inst 2010; 102: 193-201.
77. Bonadona V, Bonaïti B, Olschwang S, et al. Cancer risks associated with germline mutations in MLH1, MSH2, and MSH6 genes in Lynch syndrome. JAMA 2011; 305: 2304-2310.
78. Daniels MS, Lu KH. Clearer Picture of PMS2-Associated Lynch Syndrome Is Emerging. J Clin Oncol 2015; 33: 299-300.
79. Ten Broeke SW, Brohet RM, Tops CM, et al. Lynch Syndrome Caused by Germline PMS2 Mutations: Delineating the Cancer Risk. J Clin Oncol 2015; 33: 319-325.
80. Watson P, Lynch HT. Extracolonic cancer in hereditary nonpolyposis colorectal cancer. Cancer 1993; 71: 677-685.
81. Steinke V, Engel C, Büttner R, et al. Hereditary nonpolyposis colorectal cancer (HNPCC)/Lynch syndrome. Dtsch Arztebl Int 2013; 110: 32-38.

82. Lynch PM. When and how to perform genetic testing for inherited colorectal cancer syndromes. J Natl Compr Canc Netw 2013; 11: 1577-1583.
83. Ferguson SE, Aronson M, Pollett A, et al. Performance characteristics of screening strategies for Lynch syndrome in unselected women with newly diagnosed endometrial cancer who have undergone universal germline mutation testing. Cancer 2014; 120: 3932-3939.
84. Hadley DW, Jenkins JF, Steinberg SM, et al. Perceptions of cancer risks and predictors of colon and endometrial cancer screening in women undergoing genetic testing for Lynch syndrome. J Clin Oncol 2008; 26: 948-954.
85. Therkildsen C, Ladelund S, Rambech E, et al. Glioblastomas, astrocytomas and oligodendrogliomas linked to Lynch syndrome. Eur J Neurol 2015; 22: 717-724.
86. Fearon ER. Molecular genetics of colorectal cancer. Annu Rev Pathol 2011; 6: 479–507.
87. Schneider R, Schneider C, Kloor M, et al. Lynch syndrome: clinical, pathological, and genetic insights. Langenbecks Arch Surg 2012; 397: 513-525.
88. de Vos tot Nederveen Cappel WH, Nagengast FM, Griffioen G, et al. Surveillance for hereditary nonpolyposis colorectal cancer: a long-term study on 114 families. Dis Colon Rectum 2002; 45: 1588-1594.
89. Chirieac LR, Shen L, Catalano PJ, et al. Phenotype of microsatellite-stable colorectal carcinomas with CpG island methylation. Am J Surg Pathol 2005; 29: 429-436.
90. Juo YY, Johnston FM, Zhang DY, et al. Prognostic value of CpG island methylator phenotype among colorectal cancer patients: a systematic review and meta-analysis. Ann Oncol 2014; 25: 2314-2327.
91. Lee S, Cho NY, Choi M, et al. Clinicopathological features of CpG island methylator phenotype-positive colorectal cancer and its adverse prognosis in relation to KRAS/BRAF mutation. Pathol Int 2008; 58: 104-113.
92. Yamamoto E, Suzuki H, Yamano HO, et al. Molecular dissection of premalignant colorectal lesions reveals early onset of the CpG island methylator phenotype. Am J Pathol 2012; 181: 1847-1861.

93. Chan AO, Broaddus RR, Houlihan PS, et al. CpG island methylation in aberrant crypt foci of the colorectum. Am J Pathol 2002; 160: 1823-1830.
94. Rex DK, Ahnen DJ, Baron JA, et al. Serrated lesions of the colorectum: review and recommendations from an expert panel. Am J Gastroenterol 2012; 107: 1315-1329.
95. Fernando WC, Miranda MS, Worthley DL, et al. The CIMP Phenotype in BRAF Mutant Serrated Polyps from a Prospective Colonoscopy Patient Cohort. Gastroenterol Res Pract 2014; 2014: 374926.
96. Spring KJ, Zhao ZZ, Karamatic R, et al. High prevalence of sessile serrated adenomas with BRAF mutations: a prospective study of patients undergoing colonoscopy. Gastroenterology 2006; 131: 1400-1407.
97. Bettington ML, Chetty R. Traditional serrated adenoma: an update. Hum Pathol 2015; 46: 933-938.
98. Bettington ML, Walker NI, Rosty C, et al. A clinicopathological and molecular analysis of 200 traditional serrated adenomas. Mod Pathol 2015; 28: 414-427.
99. Wiland HO 4th, Shadrach B, Allende D, et al. Morphologic and molecular characterization of traditional serrated adenomas of the distal colon and rectum. Am J Surg Pathol 2014; 38: 1290-1297.
100. Kim M-J, Lee EJ, Suh JP, et al. Traditional serrated adenoma of the colorectum: clinicopathologic implications and endoscopic findings of the precursor lesions. Am J Clin Pathol 2013; 140: 898–911.
101. Fu B, Yachida S, Morgan R, et al. Clinicopathologic and genetic characterization of traditional serrated adenomas of the colon. Am J Clin Pathol 2012; 138: 356–366.
102. Kim K-M, Lee EJ, Kim Y-H, et al. KRAS mutations in traditional serrated adenomas from Korea herald an aggressive phenotype. Am J Surg Pathol 2010; 34: 667–675.
103. Chow E, Lipton L, Lynch E, et al. Hyperplastic polyposis syndrome: phenotypic presentations and the role of MBD4 and MYH. Gastroenterology 2006; 131: 30-39.
104. Snover DC, Ahnen DJ, Burt RW, Odze RD. Serrated polyps of the colon and rectum and serrated polyposis. In: Bosman FT, Carneiro F, Hruban RH,

Theise ND (eds). World Health Organization Classification of Tumours of the Digestive System. Lyon: IARC; 2010. pp 160-165.

105. Boparai KS, Mathus-Vliegen EM, Koornstra JJ, et al. Increased colorectal cancer risk during follow-up in patients with hyperplastic polyposis syndrome: a multicentre cohort study. Gut 2010; 59: 1094-1100.
106. Guarinos C, Sánchez-Fortún C, Rodríguez-Soler M, et al. Clinical subtypes and molecular characteristics of serrated polyposis syndrome. Clin Gastroenterol Hepatol 2013; 11: 705-711.
107. Lochhead P, Kuchiba A, Imamura Y, et al. Microsatellite instability and BRAF mutation testing in colorectal cancer prognostication. J Natl Cancer Inst 2013; 105: 1151-1156.
108. Pai RK, Jayachandran P, Koong AC, et al. BRAF-mutated, microsatellite-stable adenocarcinoma of the proximal colon: an aggressive adenocarcinoma with poor survival, mucinous differentiation, and adverse morphologic features. Am J Surg Pathol 2012; 36: 744-752.
109. Popovici V, Budinska E, Bosman FT, et al. Context-dependent interpretation of the prognostic value of BRAF and KRAS mutations in colorectal cancer. BMC Cancer 2013; 13: 439.
110. Mouradov D, Domingo E, Gibbs P, et al. Survival in stage II/III colorectal cancer is independently predicted by chromosomal and microsatellite instability, but not by specific driver mutations. Am J Gastroenterol 2013; 108: 1785-1793.
111. Bae JM, Kim JH, Cho NY, et al. Prognostic implication of the CpG island methylator phenotype in colorectal cancers depends on tumour location. Br J Cancer 2013; 109: 1004-1012.
112. Phipps AI, Limburg PJ, Baron JA, et al. Association between molecular subtypes of colorectal cancer and patient survival. Gastroenterology 2015; 148: 77-87.
113. Alexander J, Watanabe T, Wu TT, et al. Histopathological identification of colon cancer with microsatellite instability. Am J Pathol 2001; 158: 527-535.
114. Greenson JK, Bonner JD, Ben-Yzhak O, et al. Phenotype of microsatellite unstable colorectal carcinomas: Well-differentiated and focally mucinous

tumors and the absence of dirty necrosis correlate with microsatellite instability. Am J Surg Pathol 2003; 27: 563-570.

115. Shia J, Ellis NA, Paty PB, et al. Value of histopathology in predicting microsatellite instability in hereditary nonpolyposis colorectal cancer and sporadic colorectal cancer. Am J Surg Pathol 2003; 27: 1407-1417.
116. Yearsley M, Hampel H, Lehman A, et al. Histologic features distinguish microsatellite-high from microsatellite-low and microsatellite-stable colorectal carcinomas, but do not differentiate germline mutations from methylation of the MLH1 promoter. Hum Pathol 2006; 37: 831-838.
117. Greenson JK, Huang SC, Herron C, et al. Pathologic predictors of microsatellite instability in colorectal cancer. Am J Surg Pathol 2009; 33: 126-133.
118. Zlobec I, Bihl MP, Foerster A, et al. The impact of CpG island methylator phenotype and microsatellite instability on tumour budding in colorectal cancer. Histopathology 2012; 61: 777-787.
119. Hamilton SR, Bosman FT, Boffetta P et al. Carcinoma of the colon and rectum. In: Bosman FT, Carneiro F, Hruban RH, Theise ND (eds). World Health Organization Classification of Tumours of the Digestive System. Lyon: IARC; 2010. pp 134-146.
120. Hartman DJ, Nikiforova MN, Chang DT, et al. Signet ring cell colorectal carcinoma: a distinct subset of mucin-poor microsatellite-stable signet ring cell carcinoma associated with dismal prognosis. Am J Surg Pathol 2013; 37: 969-977.
121. Michael-Robinson JM, Biemer-Hüttmann A, Purdie DM, et al. Tumour infiltrating lymphocytes and apoptosis are independent features in colorectal cancer stratified according to microsatellite instability status. Gut 2001; 48: 360-366.
122. Jass JR. HNPCC and sporadic MSI-H colorectal cancer: a review of the morphological similarities and differences. Fam Cancer 2004; 3: 93-100.
123. Hyde A, Fontaine D, Stuckless S, et al. A histology-based model for predicting microsatellite instability in colorectal cancers. Am J Surg Pathol 2010; 34: 1820-1829.

124. Chetty R. Gastrointestinal cancers accompanied by a dense lymphoid component: an overview with special reference to gastric and colonic medullary and lymphoepithelioma-like carcinomas. J Clin Pathol 2012; 65: 1062-1065.
125. Schneider NI, Langner C. Prognostic stratification of colorectal cancer patients: current perspectives. Cancer Manag Res 2014; 6: 291-300.
126. Thirunavukarasu P, Sathaiah M, Singla S, et al. Medullary carcinoma of the large intestine: a population based analysis. Int J Oncol 2010; 37: 901-907.
127. Young J, Simms LA, Biden KG, et al. Features of colorectal cancers with high-level microsatellite instability occurring in familial and sporadic settings: parallel pathways of tumorigenesis. Am J Pathol 2001; 159: 2107-2116.
128. Jass JR, Walsh MD, Barker M, et al. Distinction between familial and sporadic forms of colorectal cancer showing DNA microsatellite instability. Eur J Cancer 2002; 38: 858-866.
129. McGregor DK, Wu TT, Rashid A, et al. Reduced expression of cytokeratin 20 in colorectal carcinomas with high levels of microsatellite instability. Am J Surg Pathol 2004; 28: 712-718.
130. Harbaum L, Pollheimer MJ, Kornprat P, et al. Keratin 20 - a diagnostic and prognostic marker in colorectal cancer? Histol Histopathol 2012; 27: 347-356.
131. Kim JH, Rhee YY, Bae JM, et al. Loss of CDX2/CK20 expression is associated with poorly differentiated carcinoma, the CpG island methylator phenotype, and adverse prognosis in microsatellite-unstable colorectal cancer. Am J Surg Pathol 2013; 37: 1532-1541.
132. Julié C, Trésallet C, Brouquet A, et al. Identification in daily practice of patients with Lynch syndrome (hereditary nonpolyposis colorectal cancer): revised Bethesda guidelines-based approach versus molecular screening. Am J Gastroenterol 2008; 103: 2825-2835.
133. van Lier MG, Leenen CH, Wagner A, et al. Yield of routine molecular analyses in colorectal cancer patients ≤70 years to detect underlying Lynch syndrome. J Pathol 2012; 226: 764-774.
134. Pérez-Carbonell L, Ruiz-Ponte C, Guarinos C, et al. Comparison between universal molecular screening for Lynch syndrome and revised Bethesda

guidelines in a large population-based cohort of patients with colorectal cancer. Gut 2012; 61: 865-872.

135. Moreira L, Balaguer F, Lindor N, et al. Identification of Lynch syndrome among patients with colorectal cancer. JAMA 2012; 308: 1555-1565.

136. Zhang X, Li J. Era of universal testing of microsatellite instability in colorectal cancer. World J Gastrointest Oncol 2013; 5: 12-19.

137. Giardiello FM, Allen JI, Axilbund JE, et al. Guidelines on genetic evaluation and management of Lynch syndrome: a consensus statement by the U.S. Multi-Society Task Force on Colorectal Cancer. Gastrointest Endosc 2014; 80: 197-220.

138. Giardiello FM, Allen JI, Axilbund JE, et al. Guidelines on genetic evaluation and management of Lynch syndrome: a consensus statement by the US Multi-Society Task Force on colorectal cancer. Gastroenterology 2014; 147: 502-526.

139. Giardiello FM, Allen JI, Axilbund JE, et al. Guidelines on genetic evaluation and management of Lynch syndrome: a consensus statement by the US Multi-Society Task Force on Colorectal Cancer. Dis Colon Rectum 2014; 57: 1025-1048.

140. Giardiello FM, Allen JI, Axilbund JE, et al. Guidelines on genetic evaluation and management of Lynch syndrome: a consensus statement by the US Multi-society Task Force on colorectal cancer. Am J Gastroenterol 2014; 109: 1159-1179.

141. Vasen HF, Blanco I, Aktan-Collan K, et al. Revised guidelines for the clinical management of Lynch syndrome (HNPCC): recommendations by a group of European experts. Gut 2013; 62: 812-823.

142. Kazama Y, Watanabe T, Kanazawa T, et al. Microsatellite instability in poorly differentiated adenocarcinomas of the colon and rectum: relationship to clinicopathological features. J Clin Pathol 2007; 60: 701-704.

143. Xiao H, Yoon YS, et al. Poorly differentiated colorectal cancers: correlation of microsatellite instability with clinicopathologic features and survival. Am J Clin Pathol 2013; 140: 341-347.

144. Imai Y. Poorly differentiated adenocarcinoma of the colon: subsite location and clinicopathologic features. Int J Colorectal Dis 2015; 30: 187-196.
145. Vilkin A, Halpern M, Morgenstern S, et al. How reliable is immunohistochemical staining for DNA mismatch repair proteins performed after neoadjuvant chemoradiation? Hum Pathol 2014; 45: 2029-2036.
146. Bao F, Panarelli NC, Rennert H, et al. Neoadjuvant therapy induces loss of MSH6 expression in colorectal carcinoma. Am J Surg Pathol 2010; 34: 1798-1804.
147. Shia J, Zhang L, Shike M, et al. Secondary mutation in a coding mononucleotide tract in MSH6 causes loss of immunoexpression of MSH6 in colorectal carcinomas with MLH1/PMS2 deficiency. Mod Pathol 2013; 26: 131-138.
148. Bellizzi AM. Immunohistochemistry in gastroenterohepatopancreatobiliary epithelial neoplasia - practical applications, pitfalls, and emerging markers. Surg Pathol 2013; 6: 567–609.
149. Sobin LH, Gospodarowicz M, Wittekind C, et al. (2009) TNM classification of malignant tumors (UICC), 7th edn. New York: Wiley-Blackwell: 100–106.
150. Ronaghi M, Karamohamed S, Pettersson B, et al. Real-time DNA sequencing using detection of pyrophosphate release. Anal Biochem 1996; 42: 84–89.
151. Ronaghi M. Pyrosequencing sheds light on DNA sequencing. Genome Res 2001; 11: 3-11.
152. Ronaghi M, Pettersson B, Uhlén M, et al. PCR-introduced loop structure as primer in DNA sequencing. Biotechniques 1998; 25: 876-884.
153. Ronaghi M, Nygren M, Lundeberg J, et al. Analyses of secondary structures in DNA by pyrosequencing. Anal Biochem 1999; 267: 65–71.
154. Jass JR. Hereditary Non-Polyposis Colorectal Cancer: the rise and fall of a confusing term. World J Gastroenterol 2006; 12: 4943-4950.
155. Boland CR, Shike M. Report from the Jerusalem workshop on Lynch syndrome-hereditary nonpolyposis colorectal cancer. Gastroenterology 2010; 138: 2197.

156. Jass JR, O'Brien MJ, Riddell RH et al. Recommendations for the reporting of surgically resected specimens of colorectal carcinoma. Hum Pathol 2007; 38: 537-545.
157. Jass JR, O'Brien MJ, Riddell RH, et al. Recommendations for the reporting of surgically resected specimens of colorectal carcinoma. Virchows Arch 2007; 450: 1-13.

Polish Journal of Pathology

Lisa Setaffy, Cord Langner. Microsatellite instability in colorectal cancer: clinicopathological significance. Pol J Pathol 2015; 66: 203-218.

Review paper

Microsatellite instability in colorectal cancer: clinicopathological significance

Lisa Setaffy, Cord Langner

Institute of Pathology, Medical University, Graz, Austria

Although often viewed as a single disease, colorectal cancer more accurately represents a constellation of heterogeneous subtypes that result from different combinations of genetic events and epigenetic alterations. Chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP) have been identified as the three major molecular characteristics, which interact with other significant mutations, such as mutations in the *KRAS* and *BRAF* genes. High-level MSI (MSI-H) is of eminent clinical importance. It is the seminal molecular feature for the identification of individuals with Lynch syndrome, but it may also occur in sporadic cancers with CIMP phenotype, which arise from serrated precursor lesions. MSI-H status is a marker of favorable prognosis and may be used for outcome prediction, that is, molecular grading. Among others, mucinous and medullary histology, signet-ring cell differentiation, and a marked anti-tumoral immune response are histological features suggesting MSI. Universal tumor testing is recommended and may be performed using immunohistochemistry (mismatch repair protein expression) or molecular analysis, as has recently been recommended by an international task force. In this review, we consider in detail the molecular pathogenesis of colorectal cancer, focusing on the diagnosis of MSI in both hereditary and sporadic tumors.

Key words: colorectal cancer, microsatellite instability, mismatch repair deficiency, Lynch syndrome, serrated pathway.

Introduction

Colorectal cancer (CRC) is still the third most common cancer and the third leading cause of cancer death in men and women in the United States. In 2014, an estimated 71,830 men and 65,000 women will be diagnosed with CRC and 26,270 men and 24,040 women will die of the disease [1]. However, the overall incidence rate decreased by approximately 3% per year during the past decade (2001-2010). Specifically, rates for tumors located in the distal colon decreased by more than 5%, while, in contrast, rates among adults younger than 50 years increased during this period [1]. In the EU in 2014, 168,400 deaths from CRC were predicted (92,900 men and 75,400 women), corresponding to standardized death rates of 16.5/100,000 men and 9.5/100,000 women, falling by 4% and 7%, respectively, since 2009 [2].

Although often viewed as a single disease, CRC more accurately represents a constellation of heterogeneous subtypes that result from different combinations of genetic events and epigenetic alterations [3]. Thus, a growing body of evidence supports the ability to separate CRC subtypes based upon combinations of genetic markers, such as microsatellite instability (MSI), CpG island methylator phenotype (CIMP), somatic *BRAF* mutation, and/or somatic *KRAS* mutation status [3]. It is of note that not only

the combination, but also the timing of the molecular alterations is critical for neoplastic pathway determination [4]. Approximately 60% of all CRCs are believed to arise from conventional adenomas via the adenoma-carcinoma-sequence (suppressor pathway) and 35% from serrated precursor lesions via the serrated pathway [5]. Up to 5% of CRCs arise in the setting of well-defined inherited syndromes, including Lynch syndrom, familial adenomatous polyposis (FAP), MUTYH-associated polyposis, and certain hamartomatous polyposis conditions [6].

In this review, we will refer to the molecular pathogenesis of CRC, focusing on the diagnosis of MSI in both hereditary and sporadic tumors. The clinical relevance of MSI testing and the different tools for establishing the diagnosis in the routine evaluation of cancer specimens will be discussed in detail. Data for this review were compiled using MEDLINE/PubMed and Thomson Reuters Web of Science, assessing articles published before November 2014. Search terms included colorectal cancer, Lynch syndrome, microsatellite instability, and molecular analysis. Only articles published in English were considered.

Molecular classification of colorectal cancer

The purpose of a molecular classification is to identify similar characteristics among individual tumors and then empirically predict the pathogenesis and biological behavior of a particular tumor. The most accepted way of creating a classification model is to identify and correlate single cellular events that have been statistically proven to play a role in tumorigenesis [7].

In CRC, chromosomal instability (CIN), MSI and CIMP have been identified as the three major molecular characteristics, which interact with other significant mutations, such as mutations in the *KRAS* and *BRAF* genes (Fig. 1). CIN occurs in approximately two thirds of sporadic CRCs [8]. The term refers to an accelerated rate of gains and losses of whole or large portions of chromosomes. The consequence of CIN is an imbalance in chromosomal number (reflected by aneuploidy) and a higher frequency of loss of heterozygosity (LOH) [9].

CIN, in conjunction with adenomatous polyposis coli (*APC*) mutation, characterizes the "traditional pathway" according to Leggett and Whitehall [4], resulting in microsatellite stable (MSS), CIMP-negative, *BRAF* and *KRAS* wild type tumors. Conventional adenomas, i.e. tubular, tubulovillous and villous adenomas, are considered to be the precursor lesions of sporadic CRCs arising via the traditional pathway (adenoma-carcinoma sequence), but also the precursor lesions of hereditary cancers arising in Lynch syndrome and FAP [10, 11].

Approximately 15 to 20% of CRC are characterized by high-level MSI, which corresponds to a hypermutable phenotype that results from impaired DNA mismatch repair (MMR) and may be observed in both sporadic and Lynch syndrome-associated tumors [12]. Microsatellites are short repetitive DNA nucleotide sequences (1 to 6 base pair units) scattered throughout the genome, which are prone to frameshift mutations and base-repair substitutions during DNA replication due to their propensity to DNA strand slippage [7, 13].

MSI is defined as a change of any length of repeating units, due to insertion or deletion [14]. Basically, MSI analysis is done by comparing allelic profiles of microsatellite markers generated by amplification of DNA from test (tumor) and matched unaffected

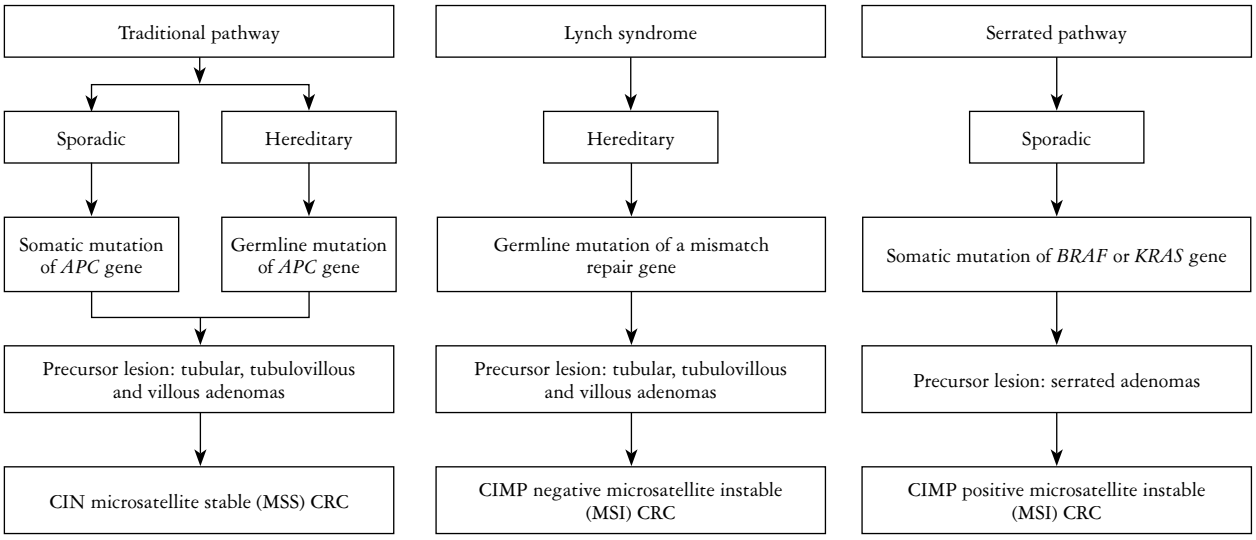

Fig. 1. Chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP) have been identified as the three major molecular events in colorectal cancer (CRC), which are involved in both sporadic and hereditary tumor development

(non-neoplastic) samples. Length variations in the test sample that are not found in the corresponding normal sample indicate MSI. Several panels of microsatellite markers have been used to diagnose MSI. In a first consensus meeting organized by the National Institutes of Health (Bethesda, MY, USA) a panel of five microsatellite markers (composed of two mononucleotide and three dinucleotide repeats) validated by a German consortium [15] was recommended as a reference panel [14]. This panel requires that normal tissue is compared with tumor tissue. Alternative and more recently developed panels are based exclusively upon mononucleotide repeat markers, which can be amplified and analyzed in a single assay, i.e. without the evaluation of matched normal DNA [16, 17]. Tumors may be classified as follows: high-level MSI (MSI-H), if two or more of the five applied markers are altered, and low-level MSI (MSI-L), if only one of the five markers is altered (Fig. 2); MSS tumors do not show MSI [18].

About half of the genes in the human genome have promoters that are embedded in clusters of cytosine-guanosine residues called CpG islands. Aberrant hypermethylation in CpG-rich promoters has been recognized as a common feature of human neoplasia, associated with transcriptional inactivation of tumor suppressor genes or other tumor-related genes [18]. Genome-wide studies of cancer epigenomes revealed that 1 to 10% of CpG islands are aberrantly methylated, which suggests that thousands of gene promoters may be hypermethylated in average cancers [19].

Cancers can be classified according to their degree of methylation, and those cancers with high degrees of methylation (CIMP phenotype) represent a clinically and etiologically distinct group that is characterized by "epigenetic instability" [18]. In the colorectum, DNA hypermethylation in CpG-rich promoters defines a distinct tumor subgroup [20], which has been associated with MSI and *BRAF* mutation in sporadic tumors [21, 22]. This phenotype accounts for approximately 15 to 20% of CRC [19, 23]. It is of note that DNA hypermethylation in conjunction with *BRAF* mutation is seen not only in sporadic MSI-H CRC, but also frequently in sessile serrated adenomas/polyps (SSA/P), which have been identified as precursor lesions in the "serrated pathway" [11, 24].

Molecular analysis of CIMP including designation of methylation level is poorly standardized, since until now a precise definition of CIMP is lacking and no consensus recommendation is available. In 2012, Hughes *et al.* [25] summarized the existing literature on CIMP in CRC, paying particular attention to the various methods and definitions used to classify a tumor as CIMP positive: Using methylation-specific polymerase chain reaction (PCR) with or without quantification (quantitative real-time PCR), DNA methylation is usually measured in a panel of five [26] or eight [27] CIMP-related gene promoters. It is

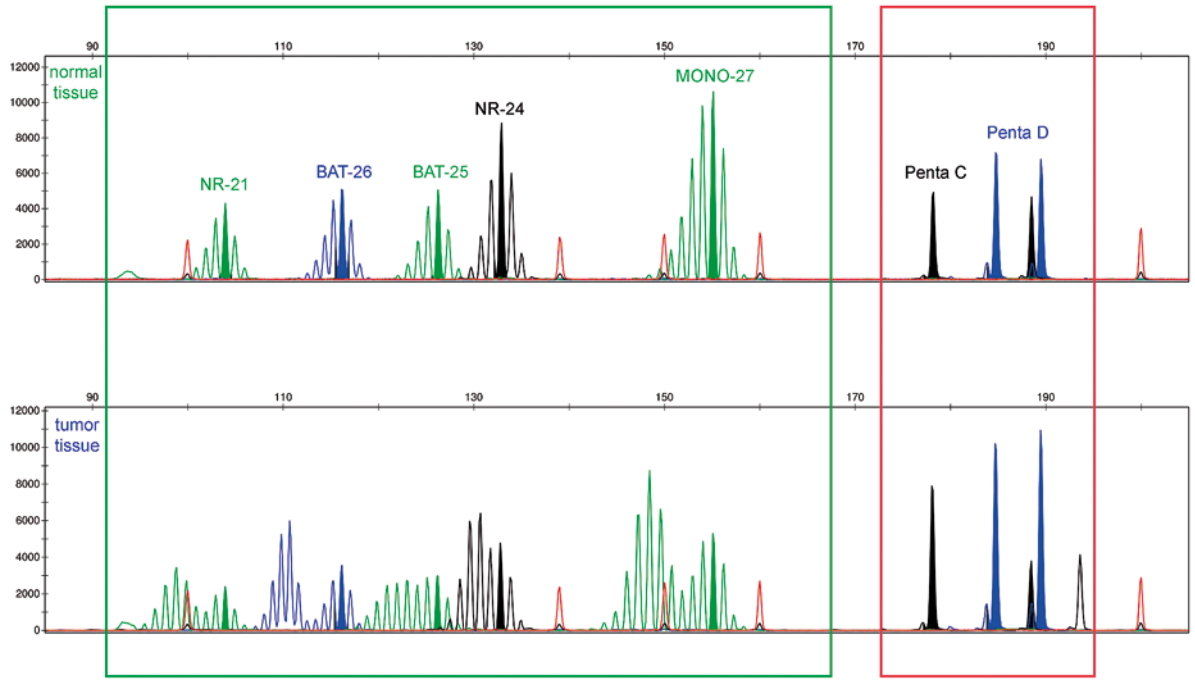

Fig. 2. Representative example of a colorectal cancer with high-level microsatellite instability (MSI-H). The MSI profile assessed by a panel of five nearly monomorphic mononucleotide repeats (pentaplex panel) illustrates instability for all markers, as shown by additional alleles (allelic shifts). Two polymorphic pentanucleotide repeats (Penta C and Penta D) are included for sample identification

unclear whether CIMP should be reported in two categories ("CIMP" and "non-CIMP") or three categories ("CIMP-high", "CIMP-low", "non-CIMP") [25]. In a systematic study comparing panels with five and eight gene markers, Berg *et al*. [28] analyzed a total of 18 alternative combinations of scoring CIMP positivity at probe, gene and panel levels and observed statistically significant variations in the frequency of CIMP depending on the cut-offs and genes included in the test panels, respectively.

The molecular pathology of CRC has recently been reviewed in this journal [29]. The authors of the review focused on molecular solutions to problems in the management of CRC, such as molecular screening, molecular prognostic tests, and molecular markers predictive of a response to chemotherapy and/or targeted therapy. In the following, we will add to the preceding review, focusing on MSI, occurring within Lynch syndrome or sporadically.

Microsatellite instability in hereditary colorectal cancer

The MMR system is necessary for maintaining genomic stability by correcting single-base mismatches and insertion-deletion loops that form during DNA replication [6]. Impaired MMR function leads to high-level MSI, which can be found in approximately 15 to 20% of CRC and may be observed in both sporadic and hereditary, i.e. Lynch syndrome-associated, tumors.

Table I. Amsterdam Criteria I and Amsterdam Criteria II for the diagnosis of Lynch syndrome [39, 40, 97, 98, 99, 100]

Amsterdam Criteria I
1. Three or more relatives with histologically verified CRC, one of whom is a first-degree relative of the other two
2. Two or more generations should be affected
3. One or more patients with CRC should be diagnosed before the age of 50 years
4. Familial adenomatous polyposis (FAP) should be excluded
Amsterdam Criteria II
1. Three or more relatives with histologically verified Lynch syndrome-associated cancer (CRC, cancer of the endometrium, small bowel, ureter, or renal pelvis), one of whom is a first-degree relative of the other two
2. Two or more generations should be affected
3. One or more cancer patients should be diagnosed before the age of 50 years
4. Familial adenomatous polyposis (FAP) should be excluded

When active, the MMR proteins form heterodimers. MLH1 builds a functional complex with PMS2 and MSH2 with its partner MSH6 [30, 31]. It is of note that the MLH1 and MSH2 proteins are obligatory partners of their respective heterodimers. Mutations in the *MLH1* or *MSH2* gene result in proteolytic degradation of the respective dimer and consequent loss of both the obligatory and the secondary partner proteins [32]. The reverse, however, is not true: A mutation in one of the secondary genes, i.e. *PMS2* or *MSH6*, does usually not lead to concurrent loss of the obligatory proteins (MLH1 or MSH2, respectively). Compensation of the function of the secondary partner protein by other proteins, such as MSH3, MLH3, and PMS1, is the most likely explanation for this observation. Consequently, mutations of *MLH1* or *MSH2* usually cause concurrent loss of PMS2 and MSH6 proteins, respectively, by immunohistochemistry, whereas mutations of *PMS2* or *MSH6* often cause loss of PMS2 or MSH6 proteins only [33].

Earlier studies focusing on MLH1 and MSH2 suggested that immunohistochemistry has a lower sensitivity (85%) than MSI testing (93%) in predicting germline mutation. Inclusion of PMS2 and MSH6 in analysis increases the sensitivity of immunohistochemistry significantly. More recent studies, which included these additional proteins, have demonstrated a predictive value for immunohistochemistry that is virtually equivalent to that of MSI testing [33].

Immunohistochemistry is reliable in screening for mutations that result in truncation or degradation of the protein [33]. However, not all pathogenetic mutations result in loss of protein expression. Hence, more than one third of *MLH1* mutations are missense mutations, which result in mutant proteins that are catalytically inactive, but antigenically intact [34, 35].

Compared with MSI testing, immunohistochemistry can help to identify the affected gene, whereas MSI testing can only demonstrate impaired function of one of the four MMR genes. It is of note that high-level MSI is not specific for Lynch syndrome: Of the 15 to 20% MSI-H CRC, 12 to 15% are caused by sporadic, acquired hypermethylation of the *MLH1* gene promoter, which occurs in tumors exhibiting CIMP, while only 3 to 5% are associated with Lynch syndrome [36].

Lynch syndrome is an autosomal dominant cancer predisposition syndrome that is caused by a germline mutation in one of the four DNA MMR genes, with *MLH1* and *MSH2* accounting for most cases (approximately 40% each) and MSH6 and PMS2 accounting for fewer cases (approximately 10% and 5%, respectively) [37, 38]. It is characterized by early-onset, frequently right-sided CRCs, often syn- and metachronous tumors, and also a higher risk for extracolonic tumors [13]. At a meeting in Amsterdam in 1990 a first set of clinical selection criteria for families with

Lynch syndrome was established to provide a basis for collaborative studies [39]. In subsequent years, these criteria were expanded, now including also extracolonic tumor sites as diagnostic features (Table I) [40]. While the Amsterdam Criteria were initially designed to serve for research, the purpose of the Bethesda Guidelines and later on the revised Bethesda Guidelines is to select CRC patients for MSI testing, that is, to limit molecular analysis to cancers with high likelihood for heredity (Table II) [41, 42, 43].

The lifetime risk of CRC has been variably estimated and appears depending on sex and the mutated MMR gene (Table III) [44, 45, 46, 47, 48, 49, 50, 51]. As already indicated above, patients with Lynch syndrome are at higher risk also for extracolonic tumors (Lynch syndrome-associated tumors), in particular endometrial and ovarian cancers, but also cancers of the renal pelvis/ureter, stomach, and other sites. The frequency of these tumors is summarized in Table IV [52, 53, 54].

Clinically, affected individuals present with only a few or no adenomas but may already have established CRC. The development of adenomas occurs at a rate similar to that of adenomas in the sporadic setting [55]. The rate of progression from adenoma to cancer, however, is believed to occur at an increased rate, since the germline inactivation of one of the MMR genes, coupled with somatic inactivation of the remaining allele in the initiated lesion, i.e. the conventional adenoma, greatly increases the mutation rate and, subsequently, cancer development [11, 55].

Microsatellite instability in sporadic colorectal cancer

As already stated above, the majority of MSI-H CRCs are non-hereditary tumors attributable to the CIMP or serrated pathway [20]. This pathway is characterized by *BRAF* V600E mutation and hypermethylation in CpG-rich gene promoters, thereby leading to transcriptional inactivation of a large number of genes, including the MMR gene *MLH1*. The silencing of this gene is responsible for the development of MSI [29, 56, 57].

CIMP tumors share many features with Lynch syndrome-associated tumors, such as occurrence in the right colon and mucinous histology. However, CIMP tumors are diagnosed at an advanced age and with female preponderance [58, 59]. CIMP tumors originate from lesions that are characterized morphologically by a serrated (saw-toothed or stellate) architecture of the epithelial compartment. It is of note that DNA hypermethylation in conjunction with *BRAF* mutation is not only seen in established CIMP carcinomas, but also frequently in these precursor lesions (Fig. 3) [11, 24].

Table III. Gene-specific cumulative risks of colorectal cancer in Lynch syndrome (modified after Girardiello) [97, 98, 99, 100]

Site of gene mutation	Cumulative risk at the age of 70 years	Mean age at diagnosis
Sporadic cancer (risk in general population)	5.5%	69 years
MLH1/MSH2	Male: 27-74% Female: 22-53%	27-46 years
MSH6	Male: 22% Female: 10% Male and female: 18%	54-63 years
PMS2	Male: 20% Female: 15%	47-66 years

Table II. The revised Bethesda Guidelines [42, 97, 98, 99, 100]. Colorectal cancers (CRCs) should be tested for MSI in the following situations

1. CRC diagnosed in a patient who is less than 50 years of age
2. Presence of synchronous or metachronous CRC or other Lynch syndrome-associated tumor*, regardless of age
3. CRC with MSI-H histology diagnosed in a patient who is less than 60 years of age
4. Patient with CRC and CRC or Lynch syndrome-associated tumor* diagnosed in at least one first-degree relative less than 50 years of age
5. Patient with CRC and CRC or Lynch syndrome-associated tumor* diagnosed in two first-degree or second-degree relatives, regardless of age

**Lynch syndrome-associated tumors include cancers of the colorectum, endometrium, stomach, ovary, pancreas, biliary tract, small bowel, ureter, renal pelvis, and brain tumors (usually glioblastoma as seen in Turcot syndrome), as well as sebaceous gland adenomas and keratoacanthomas (in Muir-Torre syndrome).*

Table IV. Spectrum of extracolonic tumors and lifetime risks for patients with Lynch syndrome; general information for all MMR genes (data from the German HNPCC Consortium) [53]

Tumor	Lifetime risk
Endometrial cancer	39-50%
Ovarian cancer	7-8%
Stomach cancer	1-6%
Cancer of the renal pelvis/ureter	2-8%
Cancer of the bile ducts	1-4%
Cancer of the small bowel	1-4%
Pancreatic cancer	Approx. 4%
Brain tumors	Approx. 2%

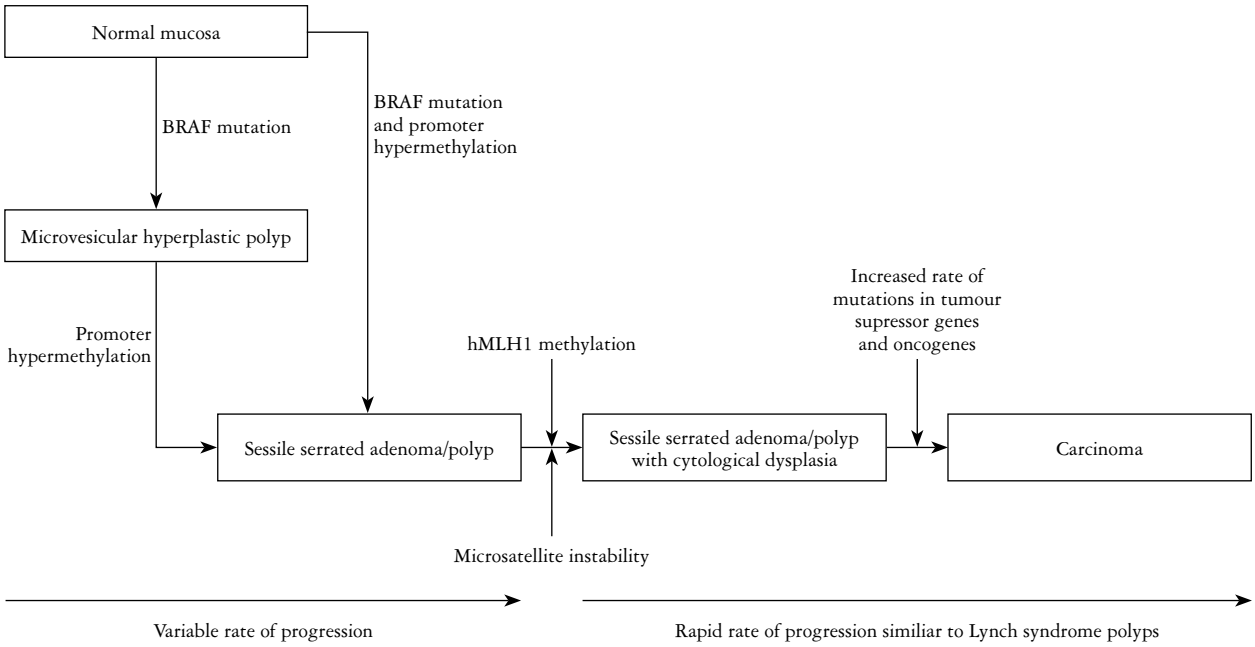

Fig. 3. Colorectal carcinogenesis following the "serrated pathway". Sporadic colorectal adenocarcinomas with high-level microsatellite instability (MSI-H) develop from serrated precursor lesions due to epigenetic silencing (promoter hypermethylation) of the *MLH1* gene (from [11] with permission)

In fact, aberrant methylation seems to play an early role in tumorigenesis. Chan *et al*. [60] reported CpG island hypermethylation in hyperplastic ("heteroplastic") aberrant crypt foci in grossly normal mucosa obtained from colectomy specimens of patients with sporadic CRC. In their integrative genomic and epigenetic approach, Yamamoto *et al*. [59] identified CIMP in 7 of 28 (25%) hyperplastic polyps and 27 of 29 (93%) SSA/P. Including mixed lesions, that is, lesions containing both precancerous and malignant components, in the analysis, the authors were able to demonstrate that most aberrant methylation is acquired at the precursor stage, whereas copy number aberrations are acquired during the progression from precursor to malignant lesion. The early aberrant methylation goes along with early activating mutations in the *BRAF* gene [24].

SSA/Ps have been identified as immediate precursors. They account for approximately 5 to 25% of all serrated lesions [10, 61, 62] and may develop preferably in the right colon from large microvesicular hyperplastic polyps or may arise *de novo* from normal colonic mucosa. The average size of SSA/Ps is larger than that of hyperplastic polyps. More than half of the lesions measure > 5 mm, and 15 to 20% of the lesions are > 10 mm [63]. Histologically, they are characterized by distorted crypt architecture with dilated, mucus-filled, L- and T-shaped crypts with mature cells at the crypt bottom (Fig. 4A). This growth pattern results from an upward shift of the proliferative zone, that is, moving away from its usual location at the base of the crypts to the mid-crypt region [5]. Cytological dysplasia is not present in uncomplicated SSA/P but develops with progression toward carcinoma (Fig. 4B-D). In addition to conventional adenoma-like dysplasia, more cuboidal cells with eosinophilic cytoplasm and vesicular nucleoli with prominent nucleoli may occur – referred to as "serrated-type dysplasia" [11].

It is of note that serrated lesions may also be associated with the familiar occurrence of CRC, in particular in serrated polyposis syndrome. In this syndrome, multiple and/or large serrated polyps occur throughout the colon, in particular proximal to the sigmoid colon [64, 65]. Individuals suffering from serrated polyposis syndrome are at an increased risk for CRC and need close endoscopic surveillance. In the study by Boparai *et al*. [66] the cumulative cancer risk was 7% at 5 years. To prevent malignant progression, adequate detection and removal of all polyps seems advisable. If this is not feasible, surgical resection should be considered [66]. At the molecular level, *BRAF* mutations can be found in 63% and *KRAS* mutations in 10% of lesions occurring in the serrated polyposis syndrome. 43% of lesions are CIMP-high. A per-patient analysis revealed that all patients had a *BRAF* or *KRAS* mutation in more than 25% of their polyps; 84.8% of patients had a mutation in *BRAF* or *KRAS* in more than 50% of their polyps [67].

The prognostic significance of CIMP and/or *BRAF* mutation status in established cancers is complex, in particular due to confounding factors, such as MSI and *KRAS* mutation status as well as different ther-

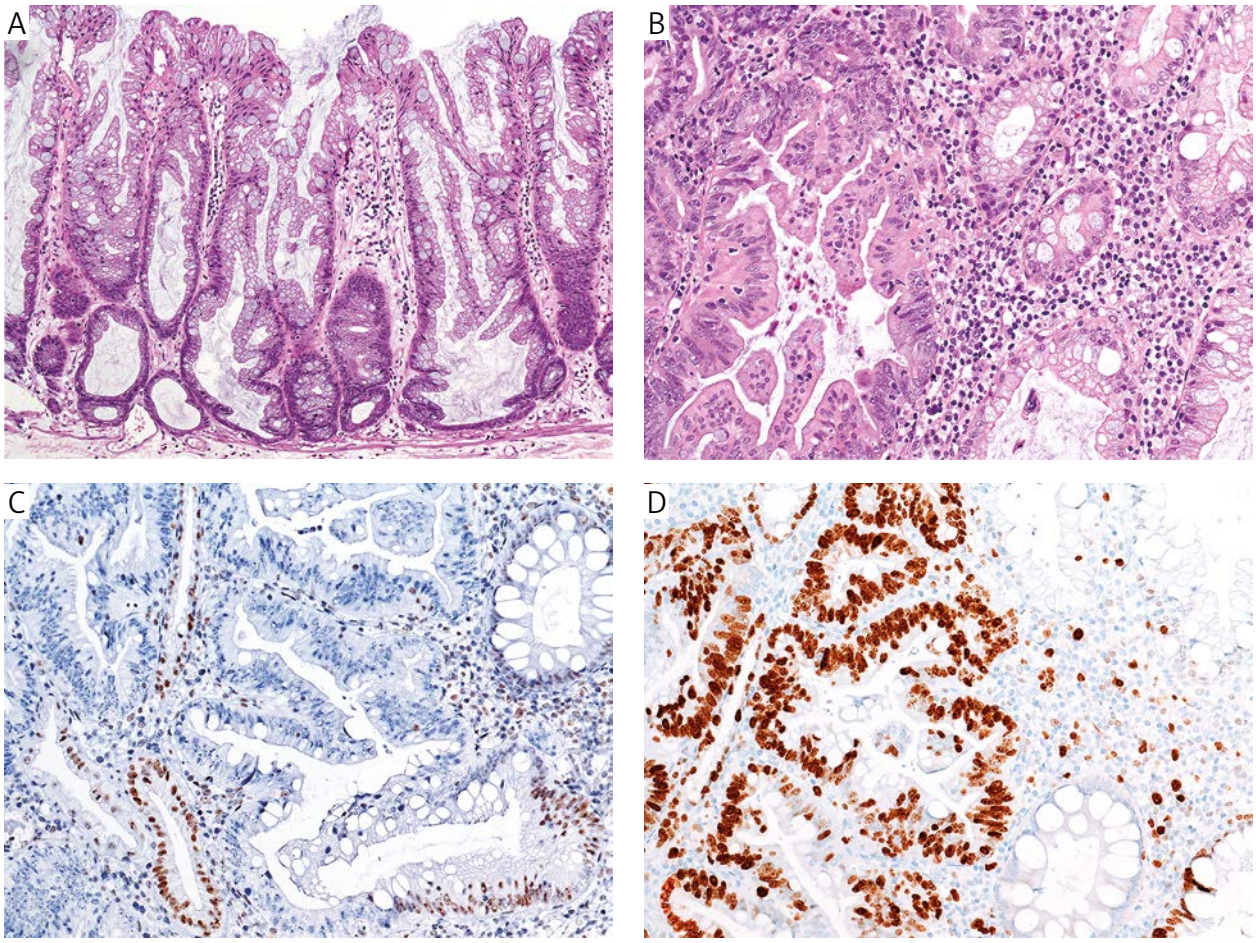

Fig. 4. Sessile serrated adenoma/polyp (SSA/P) with marked serration, dilated, mucus-filled, L-shaped ("boot") and T-shaped ("anchor") crypts and the presence of mature goblet cells above the muscularis mucosae (**A**). Cytological dysplasia is not present in uncomplicated SSA/P, but develops with progression toward carcinoma (**B**), often in conjunction with epigenetic silencing (promoter hypermethylation) of the *MLH1* gene, as shown by loss of nuclear MLH1 expression in the neoplastic cells (**C**). Note increased proliferation rate (MIB-1) in the dysplastic glands (**D**)

apy regimens. Compared with the majority subtype (MSS/*BRAF* wild type), MSS/*BRAF* mutant, MSI-H/*BRAF* mutant, and MSI-H/*BRAF* wild type subtypes showed multivariable colorectal cancer-specific mortality hazard ratios of 1.60 (95% confidence interval [CI]: 1.12-2.28; p = 0.009), 0.48 (95% CI: 0.27-0.87; p = 0.02), and 0.25 (95% CI: 0.12-0.52; p < 0.001), respectively [68].

Pai *et al*. [69] analyzed the histology of MSS/*BRAF* mutant CRCs of the proximal colon in comparison with MSS/*BRAF* wild type CRCs: *BRAF*-mutated tumors more frequently demonstrated adverse histologic features such as lymphatic invasion (16/20, 80% vs. 75/161, 47%; p = 0.008), mean number of lymph node metastases (4.5 vs. 2.2; p = 0.01), perineural invasion (8/20, 40% vs. 13/161, 8%; p = 0.0004), and high tumor budding (16/20, 80% vs. 83/161, 52%; p = 0.02). In addition, *BRAF*-mutated adenocarcinomas frequently contained areas with mucinous histology (p = 0.0002) and signet-ring cell histology (p = 0.03). Popovici *et al.* [70] likewise draw our attention to the fact that the prognostic value of *BRAF* mutation is context-dependent: In AJCC/UICC stage II/III CRCs *BRAF* mutation is a marker of poor survival only in subpopulations involving MSS and left-sided tumors, with higher effects than in the whole population. There was no evidence for prognostic value in MSI or right-sided tumors. Data obtained from a recently published Australian community-based cohort (n = 375) indicate that survival in AJCC/UICC stage II/III CRCs is independently predicted by CIN and MSI, but not by specific driver mutations, such as mutations in *KRAS* or *BRAF* [71].

Very recently, Juo *et al*. [57] analyzed thirty-three studies reporting survival in 10,635 patients to determine the prognostic significance of CIMP status in CRC. Nineteen studies provide data suitable for meta-analysis. Pooled analysis shows that CIMP is significantly associated with shorter disease-free survival (pooled HR estimate 1.45; 95% CI: 1.07-1.97) and overall survival (pooled HR estimate 1.43; 95% CI: 1.18-1.73) among CRC patients irrespective of MSI status. When subgroup analysis was performed, CIMP was found to be an indicator of poor prognosis

Table V. Histological features of colorectal cancers with high levels of microsatellite instability (MSI-H) [73, 74, 75, 76, 77, 78]

Mucinous histology ("any mucin")
Signet-ring cell differentiation
Medullary carcinoma
Marked anti-tumor host response (intra- and peritumoral lymphocytes as well as "Crohn-like" reaction)
Lack of "dirty" necrosis
"Pushing" tumor margin with no or low-level tumor budding
Poor differentiation
Tumor heterogeneity

only in MSS, and not in MSI tumors (comparable to *BRAF* mutation status). These data are well in the line with an earlier study by Bae *et al*. [72], who noted prognostic implications of CIMP status only in distal tumors.

Histology of high-level microsatellite instability colorectal cancer

The clinical characteristics and predominant right-sided location of MSI-H CRCs are well established. However, the tumors also display distinct features at the histological level, which should raise the suspicion of MSI and prompt further analysis. The following features are commonly seen: mucinous histology, signet-ring cell differentiation, medullary carcinoma, poor differentiation, host response characterized by intra- and peritumoral lymphocytes as well as "Crohn-like" reaction, tumor heterogeneity, lack of "dirty" necrosis, and a "pushing" tumor margin with no or low-level tumor budding (Table V) [73, 74, 75, 76, 77, 78]. We believe it is worth looking at some of these features in greater detail.

According to WHO criteria [79] the designation of mucinous adenocarcinoma is used if > 50% of the lesion is composed of pools of extracellular mucin that contain malignant epithelium as acinar structures, layers of tumor cells, or individual tumor cells including signet-ring cells (Fig. 5A). Carcino-

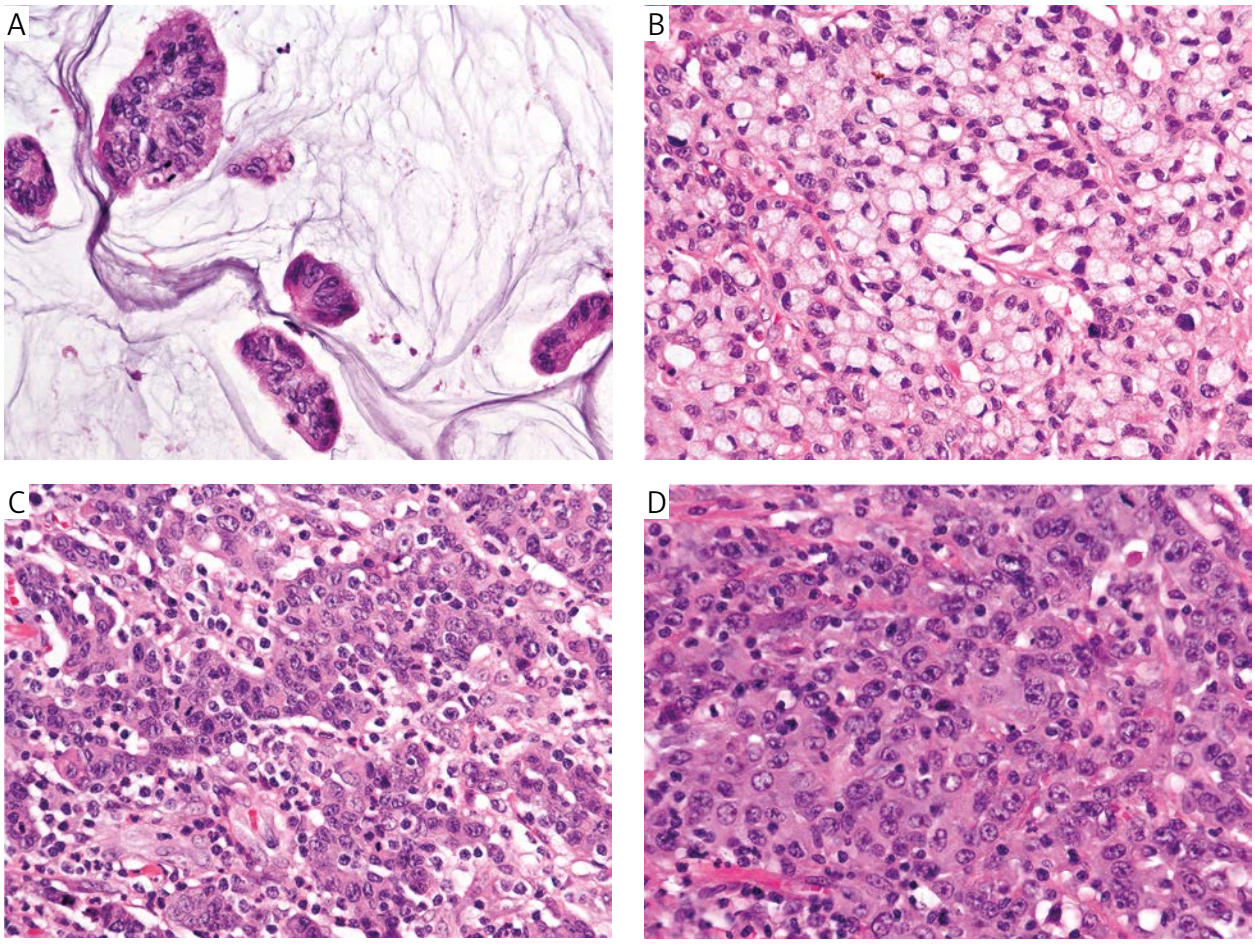

Fig. 5. Histological features of colorectal cancer with high-level microsatellite instability (MSI-H): mucinous adenocarcinoma, > 50% of the lesion is composed of pools of extracellular mucin (**A**); signet-ring cell carcinoma, > 50% of the tumor cells show prominent intracytoplasmic mucin (**B**); marked anti-tumor host response, characterized by intra- and peritumoral lymphocytic infiltration (**C**); medullary carcinoma, characterized by syncytial sheets of malignant cells with vesicular nuclei, prominent nucleoli, and intratumoral lymphocytic infiltration (**D**)

mas with mucinous areas of < 50% are categorized as having a mucinous component. It is of note that already small amounts of mucin ("any mucin") may indicate MSI. In the study by Greenson *et al*. [74] 79 tumors were found to have focal mucinous differentiation, 23 (29.1%) of which were MSI-H. By comparison, 43 tumors had > 50% mucinous differentiation, 12 (28.6%) of which were MSI-H. Multivariate analysis proved "any mucinous differentiation" as an independent histological predictor of MSI-H status with an odds ratio of 2.69 (95% CI: 1.05–6.89; p = 0.0393). This observation was confirmed in a subsequent publication by the same group, in which the authors concluded that the current WHO definition of mucinous adenocarcinoma may not be biologically relevant in the era of molecular testing [77].

Colorectal signet-ring cell carcinoma is an uncommon, but often highly aggressive malignancy, which is defined by the presence of > 50% of tumor cells with prominent intracytoplasmic mucin, typically with displacement and molding of the nucleus (Fig. 5B) [79]. MSI-H status has been associated with signet-ring cell differentiation in several investigations with rates varying between 46 and 86% [73, 74, 75], but the significance of most studies is limited due to small sample size. In 2013, Hartman *et al*. [80] systematically analyzed 53 signet-ring cell carcinomas (composed of > 50% signet-ring cells), which they classified as mucin-rich (n = 40; >50% extracellular mucin with signet-ring cells floating within pools of mucin) or mucin-poor (n = 13; diffusely infiltrating carcinomas with minimal to no extracellular mucin). Twenty-three of 53 (43%) signet-ring cell carcinomas were MSI-H. Twenty-two of 23 (96%) MSI-H signet-ring cell carcinomas were mucin-rich, whereas only one MSI-H signet-ring carcinoma was mucin-poor (p = 0.0033). Mucin-poor signet-ring cell carcinoma had significantly reduced overall and recurrence-free survival compared with mucin-rich signet-ring cell carcinomas (p = 0.0035 and p = 0.0001, respectively), even when adjusted for tumor stage. It is of note that MSI-H and MSS signet-ring cell carcinomas had similar overall and recurrence-free survival (p = 0.2266 and p = 0.1055, respectively), even when adjusted for tumor stage.

The anti-tumor host response characterized by intra- and peritumoral lymphocytic infiltration as well as Crohn-like reaction, that is, peritumoral lymphocytic aggregates, has been identified in several studies as a strong, if not the strongest, predictor of MSI status (Fig. 5C) [41, 73, 74, 75, 77, 81, 82, 83]. Tumor-infiltrating lymphocytes (TILs) constitute lymphoid components intimately admixed with the tumor [13]. Specifically, TILs are intraepithelial lymphocytes, characterized by usually round, compact nuclei with a dense chromatin pattern and perinuclear halo [41]. Various methods (and thresholds) for counting TILs have been reported, including evaluation of hematoxylin and eosin or CD3-immunostained slides, which mostly defined a positive result as > 2 TILs per high power field (HPF) [13]. On the molecular level, TILs have been shown to consist largely of CD3/CD8 co-expressing cytotoxic T-cells. Their prominence has been suggested to represent (i) a response to abundant tumor neoantigen formation owing to the "mutator phenotype" of MSI-H tumors and (ii) a possible basis for improved prognosis in MSI-H tumors [13]. It is of note that TILs are of particular help in identifying MSI-H cancers among non-mucinous tumors, and, consequently, they are regarded as the most important tissue biomarker for Lynch syndrome [41].

The Crohn-like reaction pattern is composed of prominent nodular lymphoid aggregates at the infiltrating edge of the tumor, typically identified at the junction of the muscularis propria and the fatty tissue. Their evaluation is poorly standardized. Hence, reported thresholds for a positive Crohn-like reaction include "2 or more large lymphoid aggregates in a section", "a single 4 × field of at least 3 nodular aggregates of lymphocytes", "a minimum of 3 lymphoid aggregates per section", and "at least 4 nodular aggregates in a low power field (4 ×)" [13].

Medullary carcinomas are characterized by syncytial sheets of malignant cells with vesicular nuclei, prominent nucleoli and abundant eosinophilic cytoplasm. The tumors show prominent infiltration by TILs and have well-defined peripheral margins, which may help to differentiate medullary carcinomas from undifferentiated carcinomas (Fig. 5D) [79, 84]. Frequently, medullary carcinomas arise in the proximal colon with an incidence increasing with age and a female predominance [85]. Medullary differentiation is an indicator of favorable prognosis: Follow-up data showed 1- and 2-year survival rates of 92.7% and 73.8%, respectively [86]. At the molecular level, the majority of medullary carcinomas are MSI-H. Some may be associated with Epstein-Barr virus infection [84].

Most histological features which serve as diagnostically useful markers of MSI-H status are apparent in both sporadic and hereditary, that is, Lynch-syndrome-associated, MSI-H CRC. However, as demonstrated in detail above, the two principal subtypes of MSI-H CRC evolve through different pathways, and these differences in molecular pathogenesis translate into morphological distinctions, which deserve our attention. Hence, lymphocytic infiltration, tumor budding (de-differentiation), and co-existing adenomas are more evident in Lynch syndrome, while mucinous histology, poor differentiation, tumor heterogeneity and glandular serration with or without co-existing serrated polyps are more evident in sporadic MSI-H CRC [87]. Sporadic MSI-H CRC is also characterized

by cytoplasmic eosinophilia and nuclei that are large, round, vesicular and contain a prominent nucleolus, while in Lynch syndrome the cytological features recapitulate the basophilia and nuclear characteristics of conventional adenomas [82, 88].

In 2009, Greenson *et al*. [77] presented two nearly equivalent logistic regression models that predict MSI-H status based on a review of 1649 CRCs from patients of all ages collected in a population-based case control study in northern Israel. In that cohort > 2 TILs per high-powered field, lack of dirty necrosis, presence of a Crohn-like reaction, right-sided location, any mucinous differentiation, well or poor differentiation, and age less than 50 years were all independent predictors of MSI-H. The accuracy of both models was high, with an 85.4% vs. 85.0% probability of correctly classifying tumors as MSI-H. One year later, Hyde *et al*. [83] presented another histology-based model for predicting MSI-H status in CRC, termed Pathologic Role in Determination of Instability in Colorectal Tumors (PREDICT). In a population-based cohort of CRCs diagnosed in patients less than 75 years of age from Newfoundland (n = 710) the authors scored histological features, such as mucinous differentiation, peritumoral lymphocytes, TILs and Crohn-like reaction, but also the amount of stromal cells, and the presence, type, and grade of tumor subclones. The model identified MSI-H CRCs with a sensitivity of 92.1% and a specificity of 37.8%, whereas the Revised Bethesda Guidelines had a sensitivity of 81.3% and a specificity of 39.5%.

Finally, MSI-H CRCs appear to be associated with a distinct immunophenotype, unrelated to the lack of MMR protein expression. Thus, several groups noted reduced expression of keratin 20 (K20) in MSI-H tumors. In the study by McGregor [99], which involved 44 CRCs from 22 paired MSI-H and MSS cases matched for clinical-pathologic characteristics, the mean percentage of K20-positive tumor cells was 84% in MSS CRC but only 37% in MSI-H CRC ($p = 0.0007$). Seven out of 22 (32%) MSI-H CRCs were K20-negative, as contrasted with 2 out of 22 (9%) MSS CRCs ($p = 0.13$). In our own study involving 371 CRC specimens, K20 expression was significantly associated with tumor differentiation, tumor size, tumor location, histological subtype, lymphatic invasion, and MMR protein status: 16 (4.6%), 123 (35.3%), and 209 (60.1%) 348 MMR proficient tumors were K20-negative or showed low or high K20 expression, respectively, as contrasted with 8 (34.8%), 12 (52.2%) and 3 (13%) 23 MMR deficient tumors ($p < 0.001$) [90]. It is of note that the simultaneous loss of K20 and CDX-2 expression in tumor tissue has recently been associated with poor differentiation and CIMP in MSI-H CRC, serving as an independent predictor of unfavorable prognosis in this tumor subset ($p = 0.03$) [91].

Microsatellite instability testing in the routine setting

As shown in detail above, the identification of MSI-H CRCs is of eminent clinical importance. The MSI-H status is the central molecular tumor feature for the identification of individuals with Lynch syndrome, but it is also a marker of favorable outcome and, last but not least, a predictive marker of resistance to standard 5-fluorouracil-based adjuvant chemotherapy [83].

The selection of patients for MSI testing and the technical approach for this procedure are still under debate. Traditionally, the selection for testing is based on the revised Bethesda Guidelines [42]. However, 12 to 28% of Lynch syndrome patients may be missed if testing is guided by these criteria and universal testing, that is, testing of all CRC specimens has a greater sensitivity for the identification of Lynch syndrome patients compared with the Bethesda Guidelines, but also compared with other selective strategies (e.g. tumor testing of patients with CRC < 70 years of age or older patients meeting the Bethesda Guidelines) [92, 93, 94, 95]. It is of note that even 70% of Lynch syndrome patients may be missed when the selection is based on the pathological Bethesda criteria only, that is, CRC in a patient aged less than 50 years, CRC with MSI-H phenotype in a patient aged less than 60 years, or meta-/synchronous CRC regardless of age [43]. In summary, the Bethesda Guidelines or other selective strategies miss a considerable amount of individuals with Lynch syndrome, while there is growing evidence that universal testing for MSI starting with either immunohistochemistry or PCR-based molecular testing is cost effective, sensitive, specific and is becoming widely accepted [96].

Very recently, a multi-society task force, in collaboration with invited experts, developed "guidelines to assist health care providers with the appropriate provision of genetic testing and management of patients at risk for and affected with Lynch syndrome" [97, 98, 99, 100]. According to these guidelines, testing for MMR deficiency of newly diagnosed CRCs should be performed as follows: (i) in all CRCs (provided appropriate infrastructure is available) or (ii) in CRCs diagnosed at age 70 years or younger and in individuals older than 70 years, who have a positive family history regarding Lynch syndrome. Analysis can be done by routine tumor-based immunohistochemistry for the MMR proteins MLH1, MSH2, MSH6, and PMS2 and/or testing for MSI.

In tumors with intact MMR protein expression, additional molecular analysis is not generally recommended. However, in cases with equivocal staining or tumors with positive staining, yet high clinical suspicion for the presence of Lynch syndrome (e.g. the affected patient meets the revised Bethesda

Guidelines), additional molecular analysis should be performed, as very rarely tumors may show positive MMR protein staining despite MSI-H status [96, 97, 98, 99, 100]. Tumors that demonstrate loss of MLH1 (and PMS2) should undergo additional *BRAF* testing, which may serve as a surrogate marker for CIMP in order to exclude sporadic MMR deficiency. Individuals with tumors with loss of other MMR proteins should be referred for genetic counseling for germline testing, guided by immunohistochemical staining results (Fig. 6) [97, 98, 99, 100].

Similar recommendations have been made by a group of European experts. This group (the "Mallorca Group") recommends investigation of all CRCs (or individuals with CRC < 70 years) by immunohistochemistry of the four MMR proteins or by molecular testing. The tests should be accompanied by methods that identify *MLH1* promoter methylation, e.g. *BRAF* analysis. The authors stress that likewise the investigation of all endometrial cancers in individuals less than 70 years, by immunohistochemistry or molecular testing, can be considered to improve the identification of Lynch syndrome patients [101].

In mucinous and signet-ring cell carcinomas of the colon and rectum, MMR immunohistochemistry can be used for prognostic stratification ("molecular grading"). That is, many mucinous adenocarcinomas are MSI-H and therefore low grade, whereas MSS or MSI-L cancers behave as high grade lesions. Likewise, signet-ring cell tumors that are MSI-H are regarded as low grade lesions, whereas those lacking MSI-H are usually highly aggressive [79]. We believe the concept of molecular grading should be expanded to poorly and undifferentiated cancers, as also in this subgroup the MSI-H status indicates favorable outcome [102, 103, 104]. Please note, molecular grading may be important also for patients with non-metastatic, that is AJCC / UICC stage II disease, who do usually not receive adjuvant therapy. Here, the combination of poor differentiation and MSS status (with or without other additional risk factors, such as vascular or perineural invasion) may prompt the initiation of adjuvant treatment, e.g. in young patients.

The high sensitivity of immunohistochemistry supports the use of this tool as a first step in the evaluation of the cancer specimen. However, for immunohistochemistry to be used as a first-line screening test, it is necessary that both pathologists and clinicians are aware of the fact that staining results may be considered as "genetic information," and that appropriate procedures be established to ensure patient understanding and consent [33]. Legal considerations, however, may vary from country to country.

Upon immunohistochemistry, the staining of MMR proteins should generally be interpreted as intact (positive, expressed) or lost (negative, not expressed). All four proteins are normally expressed in non-neoplastic tissue, and thus stroma, lymphocytes, and non-neoplastic crypts serve as critical internal controls [13]. A possible limiting factor is the quality of staining. In general, however, the presence of nuclear staining in the tumor cells, even when it is focal and weak, is good evidence of intact MMR protein, and additional molecular testing for MSI is not needed generally. In the rare situation where there is a lack of a positive internal control in an otherwise negatively stained tumor, repeating the stain in a search for positive non-neoplastic stromal or inflammatory cells should be done [33].

Basically, immunohistochemistry may render the following reaction patterns: (i) all four proteins in-

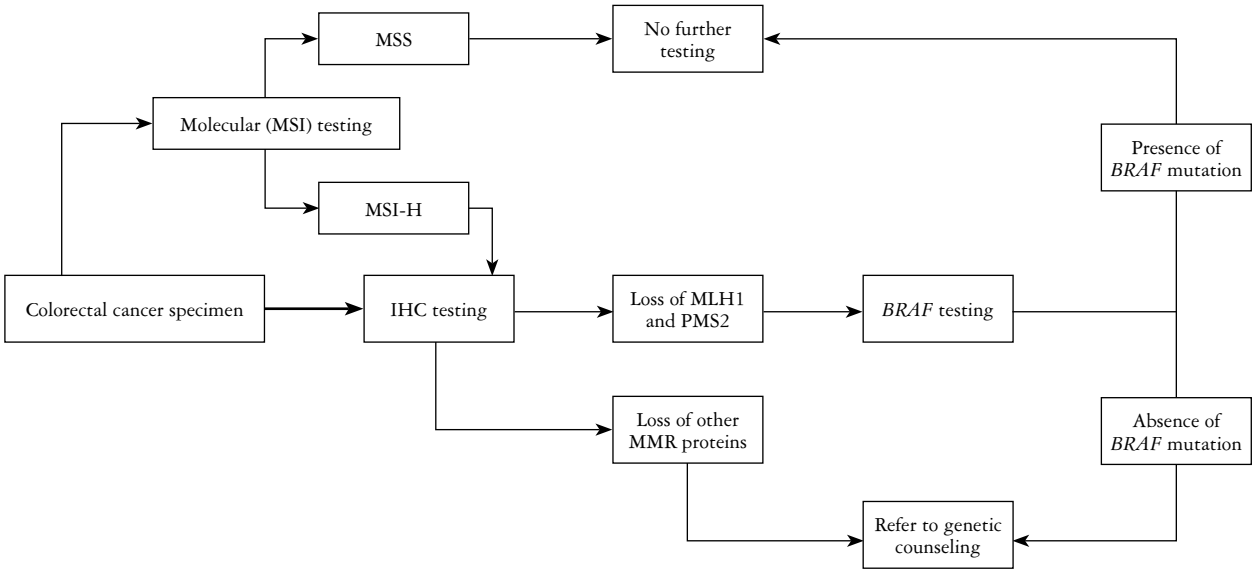

Fig. 6. Screening for Lynch syndrome by tumor testing using immunohistochemistry, that is staining for mismatch repair (MMR) protein expression (MLH1, PMS2, MSH2, MSH6) or analysis of microsatellite instability (MSI), as has recently been recommended by a Multi-Society Task Force on Colorectal Cancer [112, 113, 114, 115]. Tumors that demonstrate loss of MLH1 (and PMS2) should undergo additional BRAF testing to exclude sporadic MMR deficiency

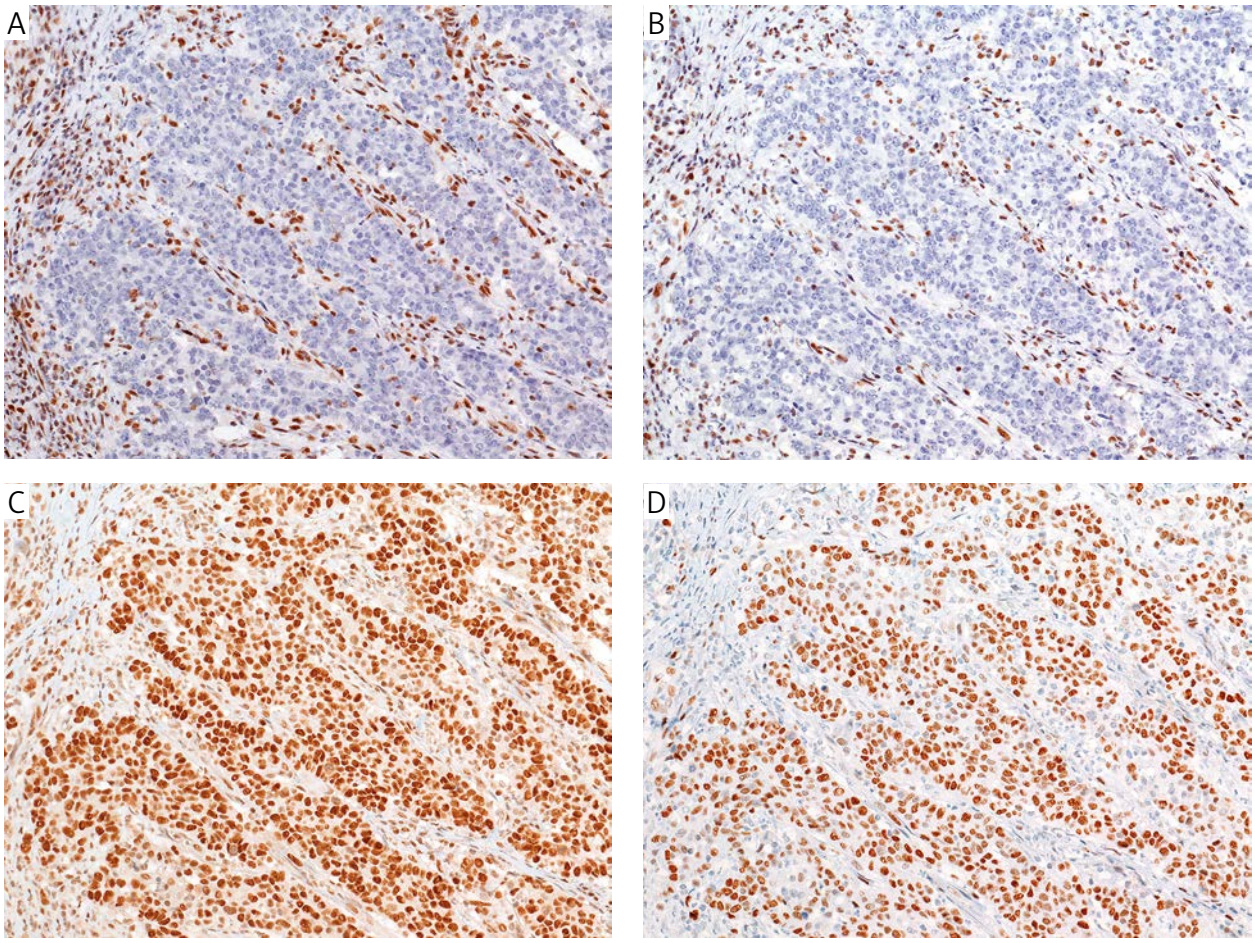

Fig. 7. Example of lost mismatch repair (MMR) protein expression in a colorectal adenocarcinoma with high-level microsatellite instability (MSI-H): Loss of nuclear MLH1 (**A**) and PMS2 (**B**) staining, but intact expression of MSH2 (**C**) and MSH6 (**D**) staining in a right-sided tumor of a 75-year-old woman; non-neoplastic stromal tissue with inherent inflammatory cells serves as an internal positive control (serial sections)

Table VI. Mismatch repair (MMR) function testing in colorectal cancer (modified after Bellizzi [108])

Immunohistochemistry	Frequency	Interpretation	Action(s)
All four proteins intact	80 to 85%	Normal MMR function (Lynch syndrome unlikely)	Consider additional MSI testing in cases with high clinical suspicion for the presence of Lynch syndrome
MLH1/PMS2 lost and MSH2/MSH6 intact	15%	Abnormal MMR function Likely sporadic MMR deficiency due to *MLH1* promoter methylation Less likely Lynch syndrome due to *MLH1* (usually) or *PMS2* (rarely) germline mutation	*BRAF* V600E and/or *MLH1* promoter methylation testing If the above are normal, refer to genetic counseling for *MLH1* germline testing (followed by *PMS2* if needed)
MSH2/MSH6 lost and MLH1/PMS2 intact	1 to 2%	Abnormal MMR function Likely Lynch syndrome due to *MSH2* (usually) or *MSH6* (rarely) germline mutation	Refer to genetic counseling for *MSH2* germline testing (followed by *MSH6* if needed)
MSH6 lost and MLH1/PMS2/MSH2 intact	Up to 0.5%	Abnormal MMR function Likely Lynch syndrome due to *MSH6* (usually) or *MSH2* (rarely) germline mutation	Refer to genetic counseling for *MSH6* germline testing (followed by *MSH2* if needed)
PMS2 lost and MLH1/MSH2/MSH6 intact	Up to 0.5%	Abnormal MMR function Likely Lynch syndrome due to *PMS2* (usually) or *MLH1* (rarely) germline mutation	Refer to genetic counseling for *PMS2* germline testing (followed by *MLH1* if needed)

tact, (ii) MLH1/PMS2 lost and MSH2/MSH6 intact, (iii) MSH2/MSH6 lost and MLH1/PMS2 intact, (iv) MSH6 lost and MLH1/PMS2/MSH2 intact, and (v) PMS2 lost and MLH1/MSH2/MSH6 intact. Typical MMR protein staining is illustrated in Fig. 7. The different staining patterns occur in varying frequencies, implying different subsequent actions (Table VI).

It is of note that the intensity of staining for all four markers, and especially for MSH6, may be reduced due to neoadjuvant treatment, which is most evident in rectal cancers after neoadjuvant chemoradiation. In these cases, pre-treatment endoscopic biopsies rather than operative material may be used as the primary material for immunohistochemistry [105]. Of note, reduced expression of MSH6 due to neoadjuvant treatment [106, 107] should be differentiated from loss of MSH6 expression due to secondary frameshift mutations in the *MSH6* gene in cancers with MLH1/PMS2 deficiency [107].

Conclusions

The MSI-H phenotype of CRC is of eminent clinical importance. High-level MSI is the seminal molecular tumor feature for the identification of individuals with Lynch syndrome, but it is also a marker of favorable outcome and a predictive marker of resistance to standard 5-fluorouracil-based adjuvant chemotherapy in patients with CRC. Among others, mucinous and medullary histology, signet-ring cell differentiation, and a marked anti-tumoral immune response are histological features suggesting MSI. Universal tumor testing is recommended and may be performed using immunohistochemistry (staining for MMR protein expression) or molecular analysis, as has recently been recommended by an international task force.

The authors declare no conflict of interest.

References

1. Siegel R, Desantis C, Jemal A. Colorectal cancer statistics, 2014. CA Cancer J Clin 2014; 64: 104-117.
2. Malvezzi M, Bertuccio P, Levi F, et al. European cancer mortality predictions for the year 2014. Ann Oncol 2014; 25: 1650-1656.
3. Samadder NJ, Vierkant RA, Tillmans LS, et al. Associations between colorectal cancer molecular markers and pathways with clinicopathologic features in older women. Gastroenterology 2013; 145: 348-356.
4. Leggett B, Whitehall V. Role of the serrated pathway in colorectal cancer pathogenesis. Gastroenterology 2010; 138: 2088-2100.
5. Snover DC. Update on the serrated pathway to colorectal carcinoma. Hum Pathol 2011; 42: 1-10.
6. Jasperson KW, Tuohy TM, Neklason DW, et al. Hereditary and familial colon cancer. Gastroenterology 2010; 138: 2044-2058.
7. Sideris M, Papagrigoriadis S. Molecular biomarkers and classification models in the evaluation of the prognosis of colorectal cancer. Anticancer Res 2014; 34: 2061-2068.
8. Jass JR. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. Histopathology 2007; 50: 113-130.
9. Pino MS, Chung DC. The chromosomal instability pathway in colon cancer. Gastroenterology 2010; 138: 2059-2072.
10. Bettington M, Walker N, Clouston A, et al. The serrated pathway to colorectal carcinoma: current concepts and challenges. Histopathology 2013; 62: 367-386.
11. Langner C. Serrated and Non-Serrated Precursor Lesions of Colorectal Cancer. Dig Dis 2015; 33: 28-37.
12. Boland CR, Koi M, Chang DK, et al. The biochemical basis of microsatellite instability and abnormal immunohistochemistry and clinical behavior in Lynch syndrome: from bench to bedside. Fam Cancer 2008; 7: 41-52.
13. Bellizzi AM, Frankel WL. Colorectal cancer due to deficiency in DNA mismatch repair function: a review. Adv Anat Pathol 2009; 16: 405-417.
14. Boland CR, Thibodeau SN, Hamilton SR, et al. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res 1998; 58: 5248-5257.
15. Dietmaier W, Wallinger S, Bocker T, et al. Diagnostic microsatellite instability: definition and correlation with mismatch repair expression. Cancer Res 1997; 57: 4749-4756.
16. Suraweera N, Duval A, Reperant M, et al. Evaluation of tumor microsatellite instability using five quasimonomorphic mononucleotide repeats and pentaplex PCR. Gastroenterology 2002; 123: 1804-1811.
17. Patil DT, Bronner MP, Portier BP, et al. A five-marker panel in a multiplex PCR accurately detects microsatellite instability-high colorectal tumors without control DNA. Diagn Mol Pathol 2012; 21: 127-133.
18. Issa JP. CpG island methylator phenotype in cancer. Nat Rev Cancer 2004; 4: 988-993.
19. Lao VV, Grady WM. Epigenetics and colorectal cancer. Nat Rev Gastroenterol Hepatol 2011; 8: 686-700.
20. Toyota M, Ahuja N, Ohe-Toyota M, et al. CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci U S A 1999; 96: 8681-8686.
21. Hawkins N, Norrie M, Cheong K, et al. CpG island methylation in sporadic colorectal cancers and its relationship to microsatellite instability. Gastroenterology 2002; 122: 1376-1387.
22. Samowitz WS, Albertsen H, Herrick J, et al. Evaluation of a large, population-based sample supports a CpG island methylator phenotype in colon cancer. Gastroenterology 2005; 129: 837-845.
23. Dahlin AM, Palmqvist R, Henriksson ML, et al. The role of the CpG island methylator phenotype in colorectal cancer prognosis depends on microsatellite instability screening status. Clin Cancer Res 2010; 16: 1845-1855.
24. Kambara T, Simms LA, Whitehall VL, et al. BRAF mutation is associated with DNA methylation in serrated polyps and cancers of the colorectum. Gut 2004; 53: 1137-1144.
25. Hughes LA, Khalid-de Bakker CA, Smits KM, et al. The CpG island methylator phenotype in colorectal cancer: progress and problems. Biochim Biophys Acta 2012; 1825: 77-85.
26. Weisenberger DJ, Siegmund KD, Campan M, et al. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat Genet 2006; 38: 787-793.
27. Ogino S, Kawasaki T, Kirkner GJ, et al. Evaluation of markers for CpG island methylator phenotype (CIMP) in colorectal cancer by a large population-based sample. J Mol Diagn 2007; 9: 305-314.

28. Berg M, Hagland HR, Søreide K. Comparison of CpG island methylator phenotype (CIMP) frequency in colon cancer using different probe- and gene-specific scoring alternatives on recommended multi-gene panels. PLoS One 2014; 9(1): e86657.
29. Bosman F, Yan P. Molecular pathology of colorectal cancer. Pol J Pathol 2014; 65: 257-266.
30. Acharya S, Wilson T, Gradia S, et al. hMSH2 forms specific mispair-binding complexes with hMSH3 and hMSH6. Proc Natl Acad Sci U S A 1996; 93: 13629-13634.
31. Kadyrov FA, Dzantiev L, Constantin N, et al. Endonucleolytic function of MutLalpha in human mismatch repair. Cell 2006; 126: 297-308.
32. Young J, Simms LA, Biden KG, et al. Features of colorectal cancers with high-level microsatellite instability occurring in familial and sporadic settings: parallel pathways of tumorigenesis. Am J Pathol 2001; 159: 2107-2116.
33. Shia J. Immunohistochemistry versus microsatellite instability testing for screening colorectal cancer patients at risk for hereditary nonpolyposis colorectal cancer syndrome. Part I. The utility of immunohistochemistry. J Mol Diagn 2008; 10: 293-300.
34. Salahshor S, Koelble K, Rubio C, et al. Microsatellite Instability and hMLH1 and hMSH2 expression analysis in familial and sporadic colorectal cancer. Lab Invest 2001; 81: 535-541.
35. Peltomäki P, Vasen H. Mutations associated with HNPCC predisposition -- Update of ICG-HNPCC/INSiGHT mutation database. Dis Markers 2004; 20: 269-276.
36. Zhang L. Immunohistochemistry versus microsatellite instability testing for screening colorectal cancer patients at risk for hereditary nonpolyposis colorectal cancer syndrome. Part II. The utility of microsatellite instability testing. J Mol Diagn 2008; 10: 301-307.
37. Cicek MS, Lindor NM, Gallinger S, et al. Quality assessment and correlation of microsatellite instability and immunohistochemical markers among population- and clinic-based colorectal tumors results from the Colon Cancer Family Registry. J Mol Diagn 2010; 13: 271-281.
38. Lynch HT, de la Chapelle A. Hereditary colorectal cancer. N Engl J Med 2003; 348: 919-932.
39. Vasen HFA, Mecklin J-P, Meera Khan P, et al. The International Collaborative Group on hereditary non-polyposis colorectal cancer (ICG-HNPCC). Dis Colon Rectum 1991; 34: 424-425.
40. Vasen HF, Watson P, Mecklin JP, et al. New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. Gastroenterology 1999; 116: 1453-1456.
41. Jass JR. Role of the pathologist in the diagnosis of hereditary non-polyposis colorectal cancer. Dis Markers 2004; 20: 215-224.
42. Umar A, Boland CR, Terdiman JP, et al. Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. J Natl Cancer Inst 2004; 96: 261-268.
43. Musulén E, Sanz C, Muñoz-Mármol AM, et al. Mismatch repair protein immunohistochemistry: a useful population screening strategy for Lynch syndrome. Hum Pathol 2014; 45: 1388-1396.
44. Dunlop MG, Farrington SM, Carothers AD, et al. Cancer risk associated with germline DNA mismatch repair gene mutations. Hum Mol Genet 1997; 6:105-110.
45. Quehenberger F, Vasen HF, van Houwelingen HC. Risk of colorectal and endometrial cancer for carriers of mutations of the hMLH1 and hMSH2 gene: correction for ascertainment. J Med Genet 2005; 42: 491-496.
46. Jenkins MA, Baglietto L, Dowty JG, et al. Cancer risks for mismatch repair gene mutation carriers: a population-based early onset case-family study. Clin Gastroenterol Hepatol 2006; 4: 489-498.
47. Alarcon F, Lasset C, Carayol J, et al. Estimating cancer risk in HNPCC by the GRL method. Eur J Hum Genet 2007; 15: 831-836.
48. Senter L, Clendenning M, Sotamaa K, et al. The clinical phenotype of Lynch syndrome due to germ-line PMS2 mutations. Gastroenterology 2008; 135: 419-428.
49. Choi YH, Cotterchio M, McKeown-Eyssen G, et al. Penetrance of colorectal cancer among MLH1/MSH2 carriers participating in the colorectal cancer familial registry in Ontario. Hered Cancer Clin Pract 2009; 7: 14.
50. Baglietto L, Lindor NM, Dowty JG, et al. Risks of Lynch syndrome cancers for MSH6 mutation carriers. J Natl Cancer Inst 2010; 102: 193-201.
51. Bonadona V, Bonaïti B, Olschwang S, et al. Cancer risks associated with germline mutations in MLH1, MSH2, and MSH6 genes in Lynch syndrome. JAMA 2011; 305: 2304-2310.
52. Watson P, Lynch HT. Extracolonic cancer in hereditary nonpolyposis colorectal cancer. Cancer 1993; 71: 677-685.
53. Steinke V, Engel C, Büttner R, et al. Hereditary nonpolyposis colorectal cancer (HNPCC)/Lynch syndrome. Dtsch Arztebl Int 2013; 110: 32-38.
54. Lynch PM. When and how to perform genetic testing for inherited colorectal cancer syndromes. J Natl Compr Canc Netw 2013; 11: 1577-1583.
55. Fearon ER. Molecular genetics of colorectal cancer. Annu Rev Pathol 2011; 6: 479-507.
56. Chirieac LR, Shen L, Catalano PJ, et al. Phenotype of microsatellite-stable colorectal carcinomas with CpG island methylation. Am J Surg Pathol 2005; 29: 429-436.
57. Juo YY, Johnston FM, Zhang DY, et al. Prognostic value of CpG island methylator phenotype among colorectal cancer patients: a systematic review and meta-analysis. Ann Oncol 2014; 25: 2314-2327.
58. Lee S, Cho NY, Choi M, et al. Clinicopathological features of CpG island methylator phenotype-positive colorectal cancer and its adverse prognosis in relation to KRAS/BRAF mutation. Pathol Int 2008; 58: 104-113.
59. Yamamoto E, Suzuki H, Yamano HO, et al. Molecular dissection of premalignant colorectal lesions reveals early onset of the CpG island methylator phenotype. Am J Pathol 2012; 181: 1847-1861.
60. Chan AO, Broaddus RR, Houlihan PS, et al. CpG island methylation in aberrant crypt foci of the colorectum. Am J Pathol 2002; 160: 1823-1830.
61. Rex DK, Ahnen DJ, Baron JA, et al. Serrated lesions of the colorectum: review and recommendations from an expert panel. Am J Gastroenterol 2012; 107: 1315-1329.
62. Fernando WC, Miranda MS, Worthley DL, et al. The CIMP Phenotype in BRAF Mutant Serrated Polyps from a Prospective Colonoscopy Patient Cohort. Gastroenterol Res Pract 2014; 2014: 374926.
63. Spring KJ, Zhao ZZ, Karamatic R, et al. High prevalence of sessile serrated adenomas with BRAF mutations: a prospective study of patients undergoing colonoscopy. Gastroenterology 2006; 131: 1400-1407.
64. Chow E, Lipton L, Lynch E, et al. Hyperplastic polyposis syndrome: phenotypic presentations and the role of MBD4 and MYH. Gastroenterology 2006; 131: 30-39.
65. Snover DC, Ahnen DJ, Burt RW, Odze RD. Serrated polyps of the colon and rectum and serrated polyposis. In: Bosman FT, Carneiro F, Hruban RH, Theise ND (eds). World Health Organization Classification of Tumours of the Digestive System. IARC, Lyon 2010; 160-165.
66. Boparai KS, Mathus-Vliegen EM, Koornstra JJ, et al. Increased colorectal cancer risk during follow-up in patients with hyperplastic polyposis syndrome: a multicentre cohort study. Gut 2010; 59: 1094-1100.

67. Guarinos C, Sánchez-Fortún C, Rodríguez-Soler M, et al. Clinical subtypes and molecular characteristics of serrated polyposis syndrome. Clin Gastroenterol Hepatol 2013; 11: 705-711.
68. Lochhead P, Kuchiba A, Imamura Y, et al. Microsatellite instability and BRAF mutation testing in colorectal cancer prognostication. J Natl Cancer Inst 2013; 105: 1151-1156.
69. Pai RK, Jayachandran P, Koong AC, et al. BRAF-mutated, microsatellite-stable adenocarcinoma of the proximal colon: an aggressive adenocarcinoma with poor survival, mucinous differentiation, and adverse morphologic features. Am J Surg Pathol 2012; 36: 744-752.
70. Popovici V, Budinska E, Bosman FT, et al. Context-dependent interpretation of the prognostic value of BRAF and KRAS mutations in colorectal cancer. BMC Cancer 2013; 13: 439.
71. Mouradov D, Domingo E, Gibbs P, et al. Survival in stage II/III colorectal cancer is independently predicted by chromosomal and microsatellite instability, but not by specific driver mutations. Am J Gastroenterol 2013; 108: 1785-1793.
72. Bae JM, Kim JH, Cho NY, et al. Prognostic implication of the CpG island methylator phenotype in colorectal cancers depends on tumour location. Br J Cancer 2013; 109: 1004-1012.
73. Alexander J, Watanabe T, Wu TT, et al. Histopathological identification of colon cancer with microsatellite instability. Am J Pathol 2001; 158: 527-535.
74. Greenson JK, Bonner JD, Ben-Yzhak O, et al. Phenotype of microsatellite unstable colorectal carcinomas: Well-differentiated and focally mucinous tumors and the absence of dirty necrosis correlate with microsatellite instability. Am J Surg Pathol 2003; 27: 563-570.
75. Shia J, Ellis NA, Paty PB, et al. Value of histopathology in predicting microsatellite instability in hereditary nonpolyposis colorectal cancer and sporadic colorectal cancer. Am J Surg Pathol 2003; 27: 1407-1417.
76. Yearsley M, Hampel H, Lehman A, et al. Histologic features distinguish microsatellite-high from microsatellite-low and microsatellite-stable colorectal carcinomas, but do not differentiate germline mutations from methylation of the MLH1 promoter. Hum Pathol 2006; 37: 831-838.
77. Greenson JK, Huang SC, Herron C, et al. Pathologic predictors of microsatellite instability in colorectal cancer. Am J Surg Pathol 2009; 33: 126-133.
78. Zlobec I, Bihl MP, Foerster A, et al. The impact of CpG island methylator phenotype and microsatellite instability on tumour budding in colorectal cancer. Histopathology 2012; 61: 777-787.
79. Hamilton SR, Bosman FT, Boffetta P et al. Carcinoma of the colon and rectum. In: Bosman FT, Carneiro F, Hruban RH, Theise ND (eds). World Health Organization Classification of Tumours of the Digestive System. Lyon: IARC; 2010; 134-146.
80. Hartman DJ, Nikiforova MN, Chang DT, et al. Signet ring cell colorectal carcinoma: a distinct subset of mucin-poor microsatellite-stable signet ring cell carcinoma associated with dismal prognosis. Am J Surg Pathol 2013; 37: 969-977.
81. Michael-Robinson JM, Biemer-Hüttmann A, Purdie DM, et al. Tumour infiltrating lymphocytes and apoptosis are independent features in colorectal cancer stratified according to microsatellite instability status. Gut 2001; 48: 360-366.
82. Jass JR. HNPCC and sporadic MSI-H colorectal cancer: a review of the morphological similarities and differences. Fam Cancer 2004; 3: 93-100.
83. Hyde A, Fontaine D, Stuckless S, et al. A histology-based model for predicting microsatellite instability in colorectal cancers. Am J Surg Pathol 2010; 34: 1820-1829.
84. Chetty R. Gastrointestinal cancers accompanied by a dense lymphoid component: an overview with special reference to gastric and colonic medullary and lymphoepithelioma-like carcinomas. J Clin Pathol 2012; 65: 1062-1065.
85. Schneider NI, Langner C. Prognostic stratification of colorectal cancer patients: current perspectives. Cancer Manag Res 2014; 6: 291-300.
86. Thirunavukarasu P, Sathaiah M, Singla S, et al. Medullary carcinoma of the large intestine: a population based analysis. Int J Oncol 2010; 37: 901-907.
87. Young J, Simms LA, Biden KG, et al. Features of colorectal cancers with high-level microsatellite instability occurring in familial and sporadic settings: parallel pathways of tumorigenesis. Am J Pathol 2001; 159: 2107-2116.
88. Jass JR, Walsh MD, Barker M, et al. Distinction between familial and sporadic forms of colorectal cancer showing DNA microsatellite instability. Eur J Cancer 2002; 38: 858-866.
89. McGregor DK, Wu TT, Rashid A, et al. Reduced expression of cytokeratin 20 in colorectal carcinomas with high levels of microsatellite instability. Am J Surg Pathol 2004; 28: 712-718.
90. Harbaum L, Pollheimer MJ, Kornprat P, et al. Keratin 20 - a diagnostic and prognostic marker in colorectal cancer? Histol Histopathol 2012; 27: 347-356.
91. Kim JH, Rhee YY, Bae JM, et al. Loss of CDX2/CK20 expression is associated with poorly differentiated carcinoma, the CpG island methylator phenotype, and adverse prognosis in microsatellite-unstable colorectal cancer. Am J Surg Pathol 2013; 37: 1532-1541.
92. Julié C, Trésallet C, Brouquet A, et al. Identification in daily practice of patients with Lynch syndrome (hereditary nonpolyposis colorectal cancer): revised Bethesda guidelines-based approach versus molecular screening. Am J Gastroenterol 2008; 103: 2825-2835.
93. van Lier MG, Leenen CH, Wagner A, et al. Yield of routine molecular analyses in colorectal cancer patients ≤70 years to detect underlying Lynch syndrome. J Pathol 2012; 226: 764-774.
94. Pérez-Carbonell L, Ruiz-Ponte C, Guarinos C, et al. Comparison between universal molecular screening for Lynch syndrome and revised Bethesda guidelines in a large population-based cohort of patients with colorectal cancer. Gut 2012; 61: 865-872.
95. Moreira L, Balaguer F, Lindor N, et al. Identification of Lynch syndrome among patients with colorectal cancer. JAMA 2012; 308: 1555-1565.
96. Zhang X, Li J. Era of universal testing of microsatellite instability in colorectal cancer. World J Gastrointest Oncol 2013; 5: 12-19.
97. Giardiello FM, Allen JI, Axilbund JE, et al. Guidelines on genetic evaluation and management of Lynch syndrome: a consensus statement by the U.S. Multi-Society Task Force on Colorectal Cancer. Gastrointest Endosc 2014; 80: 197-220.
98. Giardiello FM, Allen JI, Axilbund JE, et al. Guidelines on genetic evaluation and management of Lynch syndrome: a consensus statement by the US Multi-Society Task Force on colorectal cancer. Gastroenterology 2014; 147: 502-526.
99. Giardiello FM, Allen JI, Axilbund JE, et al. Guidelines on genetic evaluation and management of Lynch syndrome: a consensus statement by the US Multi-Society Task Force on Colorectal Cancer. Dis Colon Rectum 2014; 57: 1025-1048.
100. Giardiello FM, Allen JI, Axilbund JE, et al. Guidelines on genetic evaluation and management of Lynch syndrome: a consensus statement by the US Multi-society Task Force on colorectal cancer. Am J Gastroenterol 2014; 109: 1159-1179.
101. Vasen HF, Blanco I, Aktan-Collan K, et al. Revised guidelines for the clinical management of Lynch syndrome (HNPCC): recommendations by a group of European experts. Gut 2013; 62: 812-823.

102. Kazama Y, Watanabe T, Kanazawa T, et al. Microsatellite instability in poorly differentiated adenocarcinomas of the colon and rectum: relationship to clinicopathological features. J Clin Pathol 2007; 60: 701-704.
103. Xiao H, Yoon YS, Hong SM, et al. Poorly differentiated colorectal cancers: correlation of microsatellite instability with clinicopathologic features and survival. Am J Clin Pathol 2013; 140: 341-347.
104. Imai Y. Poorly differentiated adenocarcinoma of the colon: subsite location and clinicopathologic features. Int J Colorectal Dis 2015; 30: 187-196.
105. Vilkin A, Halpern M, Morgenstern S, et al. How reliable is immunohistochemical staining for DNA mismatch repair proteins performed after neoadjuvant chemoradiation? Hum Pathol 2014; 45: 2029-2036.
106. Bao F, Panarelli NC, Rennert H, et al. Neoadjuvant therapy induces loss of MSH6 expression in colorectal carcinoma. Am J Surg Pathol 2010; 34: 1798-1804.
107. Shia J, Zhang L, Shike M, et al. Secondary mutation in a coding mononucleotide tract in MSH6 causes loss of immunoexpression of MSH6 in colorectal carcinomas with MLH1/PMS2 deficiency. Mod Pathol 2013; 26: 131-138.
108. Bellizzi AM. Immunohistochemistry in gastroenterohepatopancreatobiliary epithelial neoplasia – practical applications, pitfalls, and emerging markers. Surg Pathol 2013; 6: 567-609.

Address for correspondence

Cord Langner MD
Medical University of Graz, Institute of Pathology
Auenbruggerplatz 25, 8036 Graz, Austria
tel. +43 (0)316 385 13665
fax +43 (0)316 385 13432
e-mail: cord.langner@medunigraz.at