Review paper

Microsatellite instability in colorectal cancer: clinicopathological significance

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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

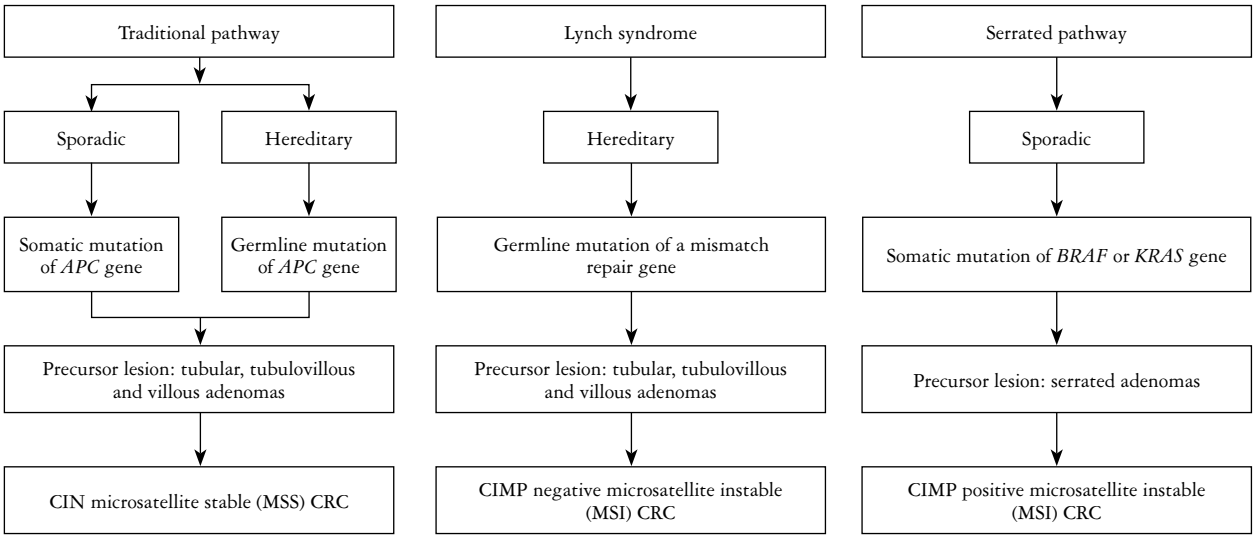

Fig. 1. Chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP) have been identified as the three major molecular events in colorectal cancer (CRC), which are involved in both sporadic and hereditary tumor development

(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

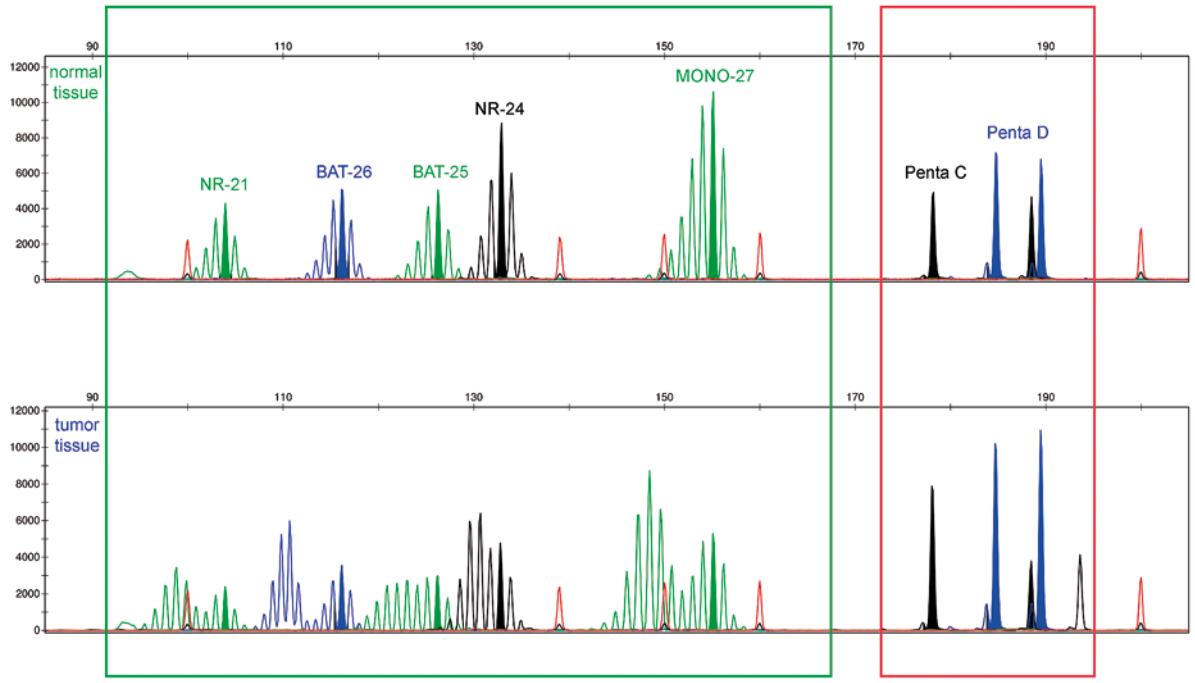

Fig. 2. Representative example of a colorectal cancer with high-level microsatellite instability (MSI-H). The MSI profile assessed by a panel of five nearly monomorphic mononucleotide repeats (pentaplex panel) illustrates instability for all markers, as shown by additional alleles (allelic shifts). Two 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%

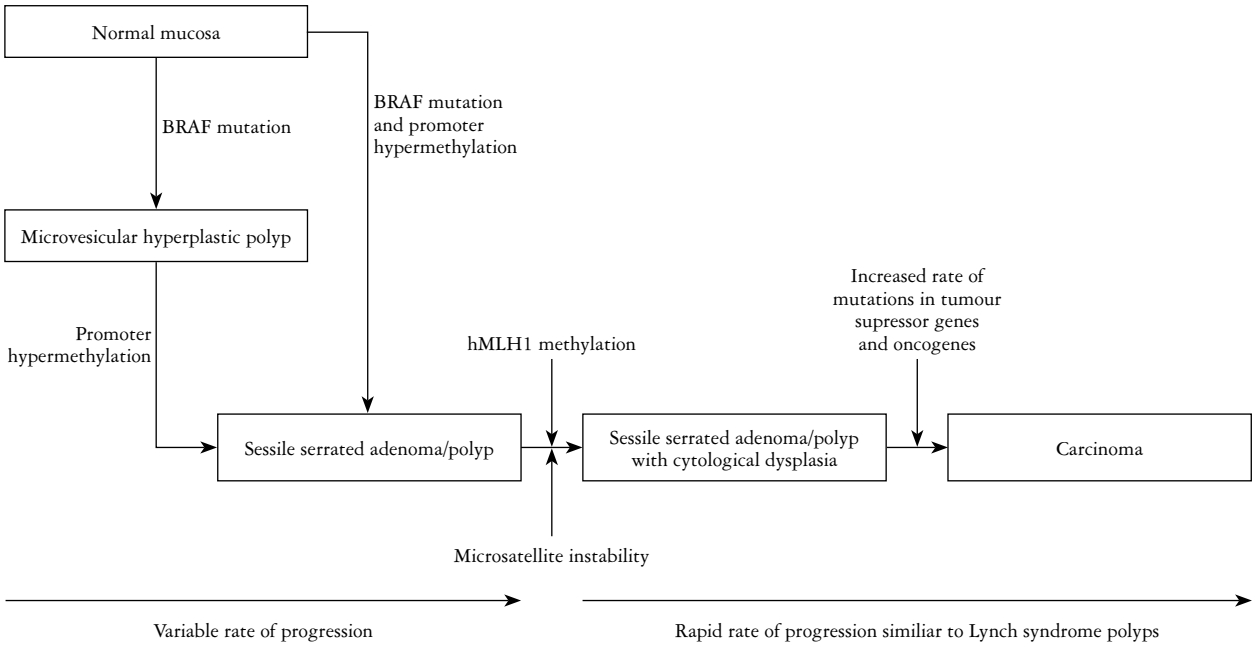

Fig. 3. Colorectal carcinogenesis following the "serrated pathway". Sporadic colorectal adenocarcinomas with high-level microsatellite instability (MSI-H) develop from serrated precursor lesions due to epigenetic silencing (promoter hypermethylation) of the *MLH1* gene (from [11] with permission)

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-

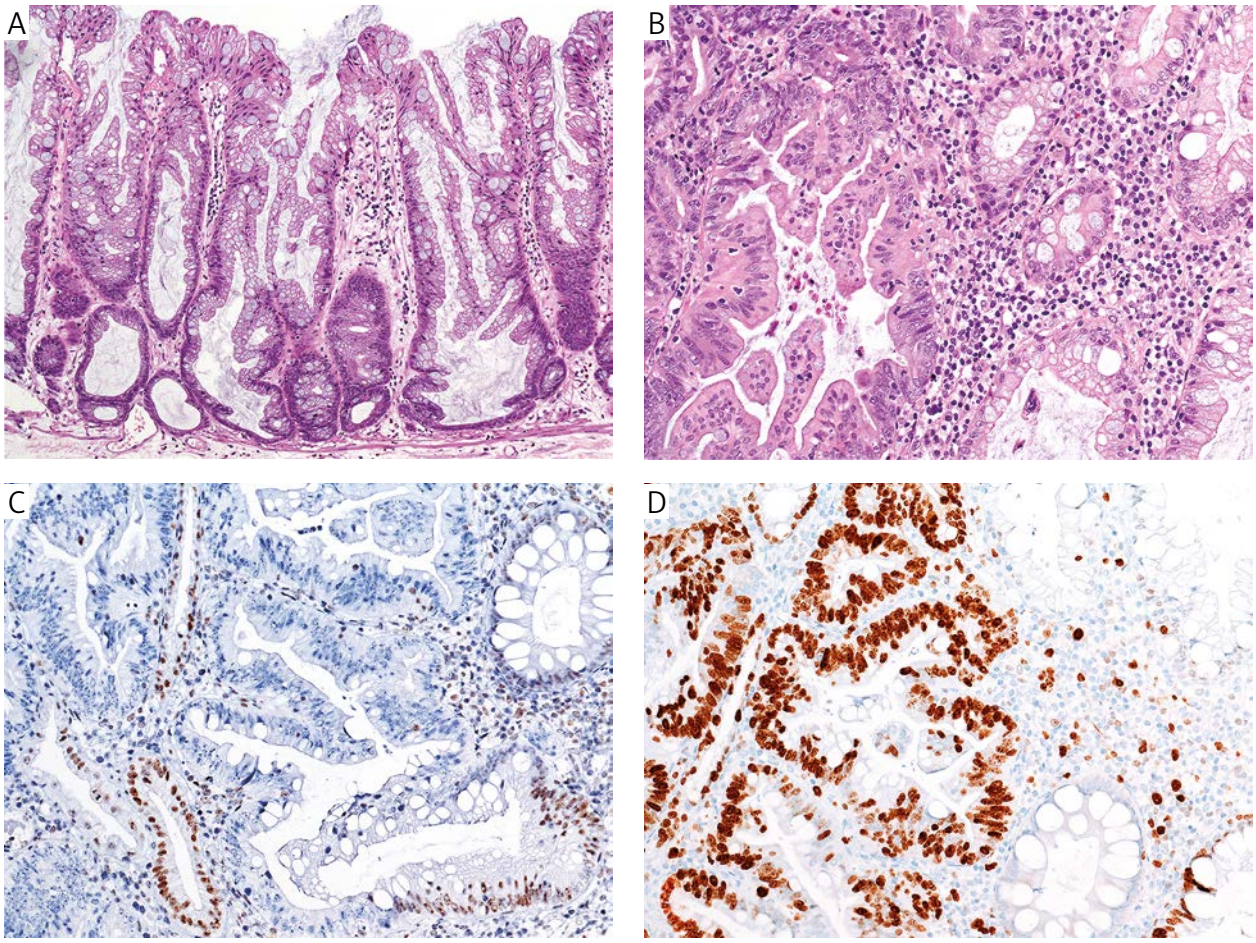

Fig. 4. Sessile serrated adenoma/polyp (SSA/P) with marked serration, dilated, mucus-filled, L-shaped ("boot") and T-shaped ("anchor") crypts and the presence of mature goblet cells above the muscularis mucosae (**A**). Cytological dysplasia is not present in uncomplicated SSA/P, but develops with progression 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-

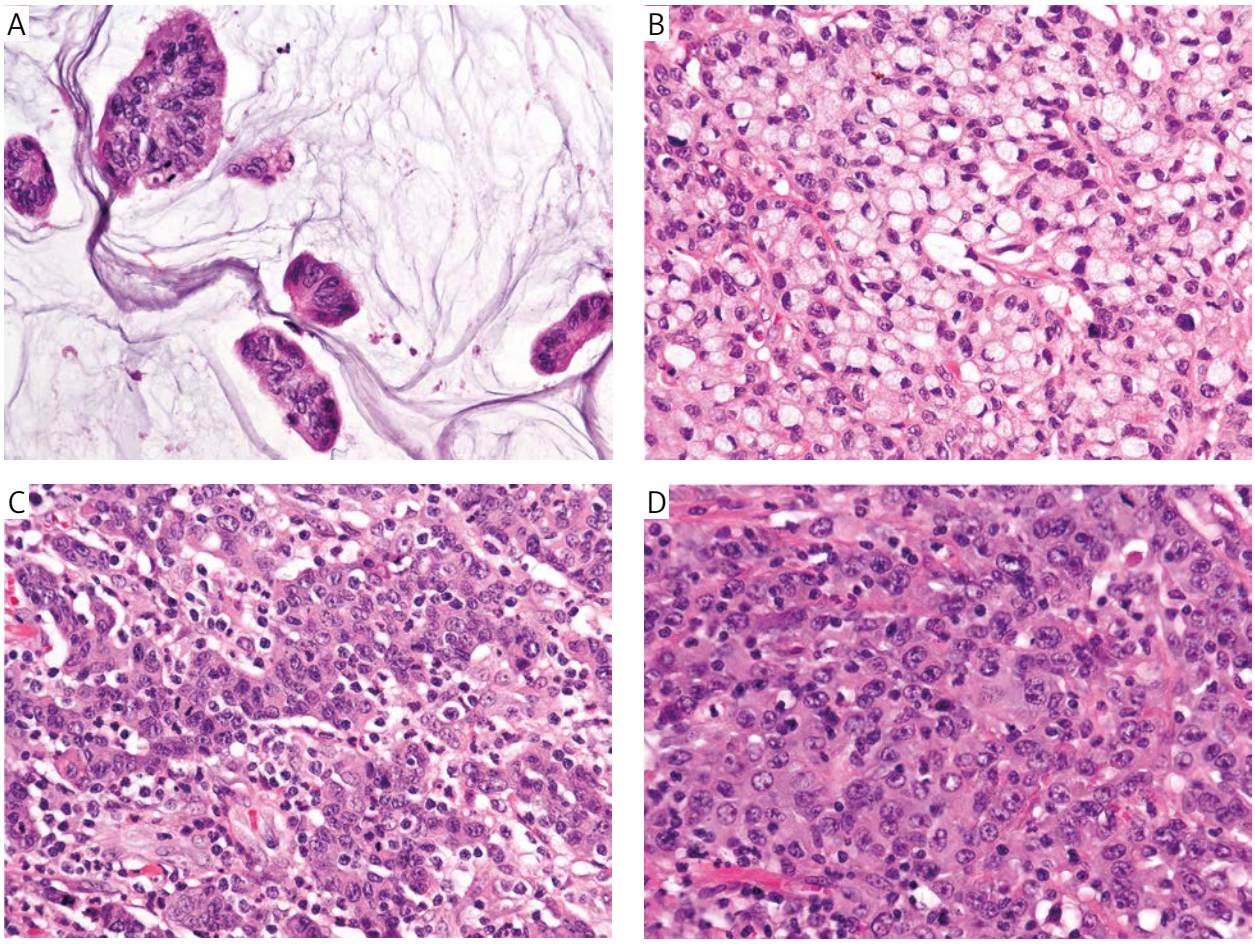

Fig. 5. Histological features of colorectal cancer with high-level microsatellite instability (MSI-H): mucinous adenocarcinoma, > 50% of the lesion is composed of pools of extracellular mucin (**A**); signet-ring cell carcinoma, > 50% of the tumor cells show prominent intracytoplasmic mucin (**B**); 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-

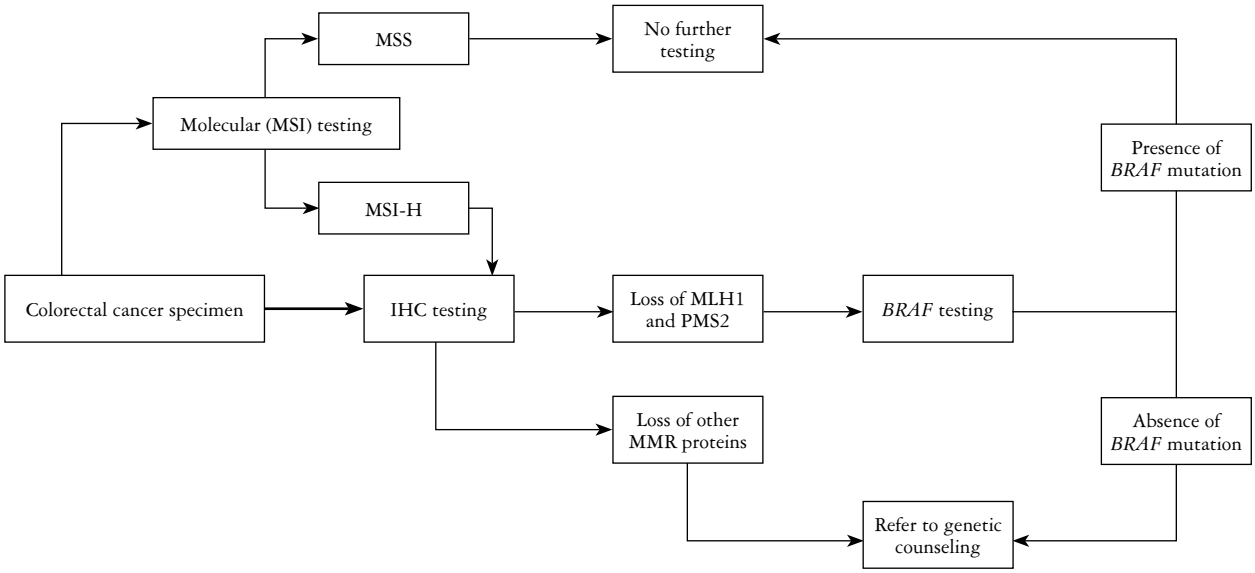

Fig. 6. Screening for Lynch syndrome by tumor testing using immunohistochemistry, that is staining for mismatch repair (MMR) protein expression (MLH1, PMS2, MSH2, MSH6) or analysis of microsatellite instability (MSI), as has recently been recommended by a Multi-Society Task Force on Colorectal Cancer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

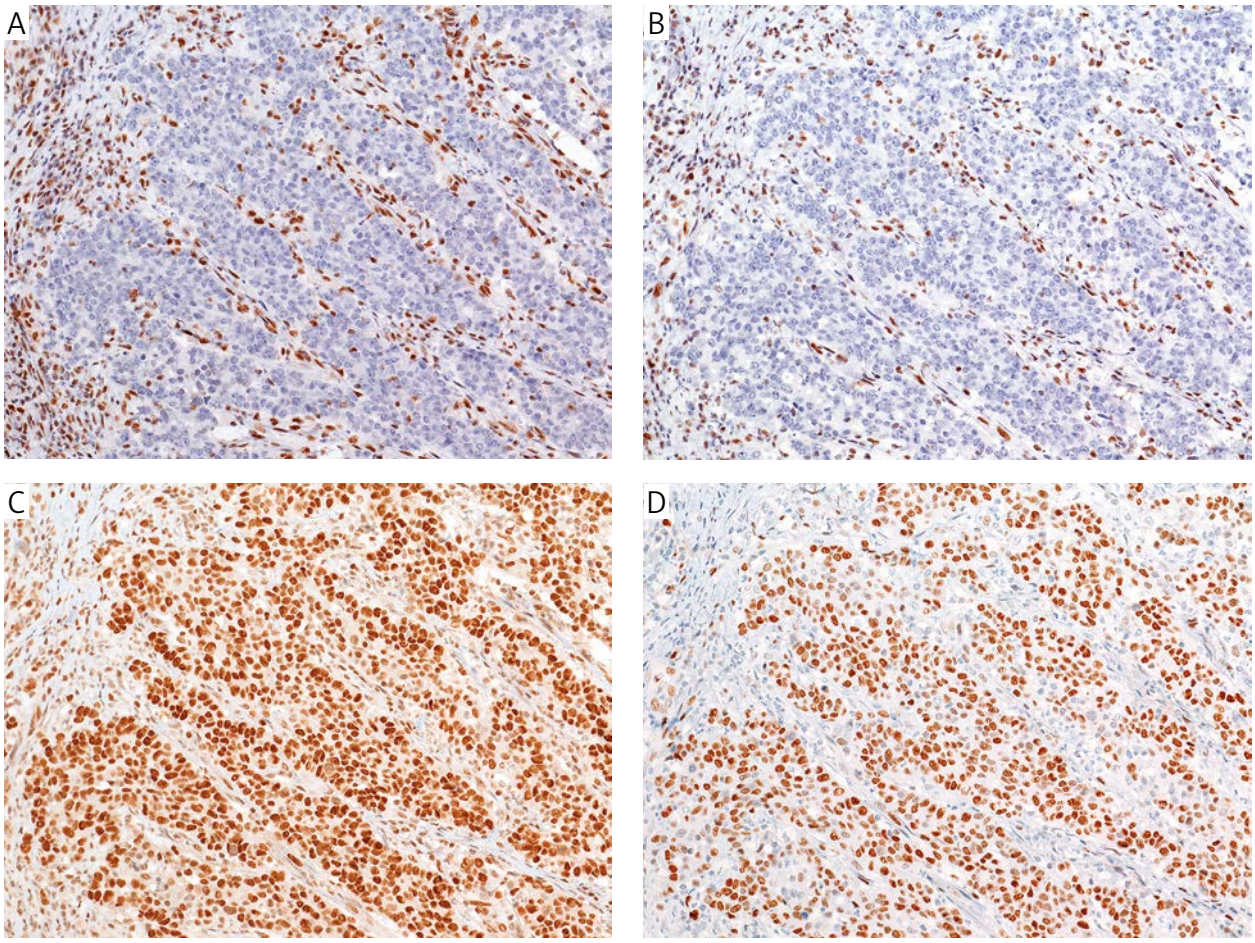

Fig. 7. Example of lost mismatch repair (MMR) protein expression in a colorectal adenocarcinoma with high-level microsatellite instability (MSI-H): Loss of nuclear MLH1 (**A**) and PMS2 (**B**) staining, but intact expression of MSH2 (**C**) and MSH6 (**D**) staining in a right-sided tumor of a 75-year-old 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.

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