Molecular Diagnostics in Colorectal Carcinoma

Advances and Applications for 2018

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KEYWORDS

• Colorectal carcinoma • Serrated polyp pathway • KRAS • BRAF
• CpG island methylator phenotype

KEY POINTS

- The molecular pathogenesis and classification of colorectal carcinoma are based on the traditional adenoma–carcinoma sequence in the Vogelstein model, serrated polyp pathway, and MSI.
- The genetic basis for hereditary nonpolyposis colorectal cancer is based on detection of mutations in the MLH1, MSH2, MSH6, PMS2, and EPCAM genes.
- Genetic testing for the Lynch syndrome includes MSI testing, methylator phenotype testing, BRAF mutation testing, and molecular testing for germline mutations in mismatch repair genes.
- Molecular makers with predictive and prognostic implications include quantitative multigene reverse transcriptase polymerase chain reaction assay and KRAS and BRAF mutation analysis.
- Mismatch repair-deficient tumors have higher rates of programmed death-ligand 1 expression.

INTRODUCTION

The pathogenesis of colorectal carcinoma is heterogeneous and involves complex multistep molecular pathways initiated by genetic and epigenetic events. The molecular classification of colorectal carcinoma provides the basis for evaluation of prognostic, predictive, and theranostic markers. The goal is precise, efficient, and accurate application of molecular tests for patient management.[1–3]

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EPIDEMIOLOGY

Constitutional (endogenous) as well as environmental (exogenous) factors are associated with the development of colorectal carcinoma. Multiple risk factors have been linked to colorectal carcinoma. Colorectal carcinoma is more common in late-middle-aged and elderly individuals. Men are at a higher risk for developing this malignancy. There is a strong association with a Western type of diet consisting of high-calorie food, rich in animal fat.[4]

Clinical Features

Clinical presentation includes change in bowel habit, constipation, abdominal distension, hematochezia, tenesmus, weight loss, malaise, fever, and anemia. Regarding screening, the American Gastroenterological Association, American Medical Association, and American Cancer Society recommend endoscopy with biopsy as the standard screening approach. Radiologic evaluation by computed tomography scan and MRI are used to assess locoregional spread and distant metastases.[4–9]

PATHOPHYSIOLOGY AND MOLECULAR GENETICS

The various molecular alterations described in colorectal carcinoma are enlisted in **Box 1**.[10] The diagrams depict the adenoma–carcinoma sequence and serrated polyp pathway arising from a complex interplay of genetic alterations (**Figs. 1–4**).[1]

TRADITIONAL VOGELSTEIN MODEL AND APC GENE PATHWAY

The traditional model of Vogelstein describes the classic adenoma–carcinoma sequence and accounts for approximately 80% of sporadic colon tumors. The pathogenesis involves mutation of the APC gene early in the neoplastic process.[2]

APC Gene

A tumor suppressor gene located on the long (q) arm of chromosome 5 between positions 21 and 22 plays a key role in regulating cell division cycle and regulates the WNT/β-catenin signaling pathway. With loss of APC function, β-catenin accumulates and activates the transcription of MYC and cyclin D1 genes, resulting in enhanced proliferation of cells. More than 700 mutations in the APC gene have been identified in familial adenomatous polyposis (FAP), both classic and attenuated types. In this regard,

Box 1
Common genetic and epigenetic alterations in colorectal cancer

Tumor Suppressor Genes	Proto-Oncogenes	Other Molecular Alterations
• APC	• BRAF	• Chromosome instability
• ARID1A	• ERBB2	• CpG island methylator phenotype
• CTNNB1	• GNAS	• Microsatellite instability
• DCC	• IGF2	• Mismatch-repair genes
• FAM123B	• KRAS	• SEPT9
• FBXW7	• MYC	• VIM, NDRG4, BMP3
• PTEN	• NRAS	• POLE/POLD1
• RET	• PIK3CA	
• SMAD4	• RSPO2/RSPO3	
• TGFBR2	• SOX9	
• TP53	• TCF7L2	

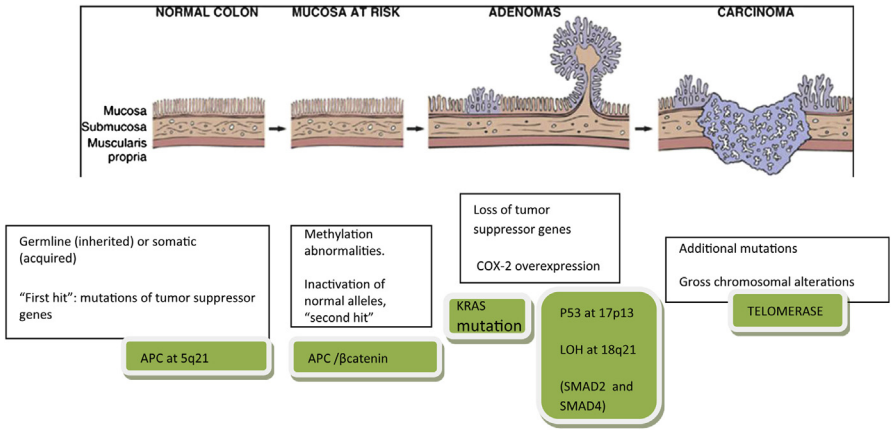

Fig. 1. Adenoma-carcinoma sequence. COX-2, cyclooxygenase-2; LOH, loss of heterozygosity. (*Modified from* Turner JR. The gastrointestinal tract. In: Kumar V, Abbas AK, Fausto N, et al, editors. Robbins and Cotran pathologic basis of disease. 8th edition. Philadelphia: Elsevier; 2010. p. 823; with permission.)

FAP is a syndrome with an inherited truncating APC mutation, leading to the production of an abnormally short, nonfunctional version of the protein that cannot suppress the cellular overgrowth and leads to the formation of polyps and subsequent progression to carcinoma. Both copies of the APC gene must be functionally inactivated, either by mutation or by the epigenetic events for development of adenomas; the second allele in adenomas harbors a loss or similar mutation, whereas homozygous deletions of APC are rare or absent. In sporadic colorectal tumors, the mutation may be in a mutation cluster region in the APC gene with allelic loss, or mutations may be outside this region with a tendency to harbor truncating mutations (Fig. 5).[2,3,11]

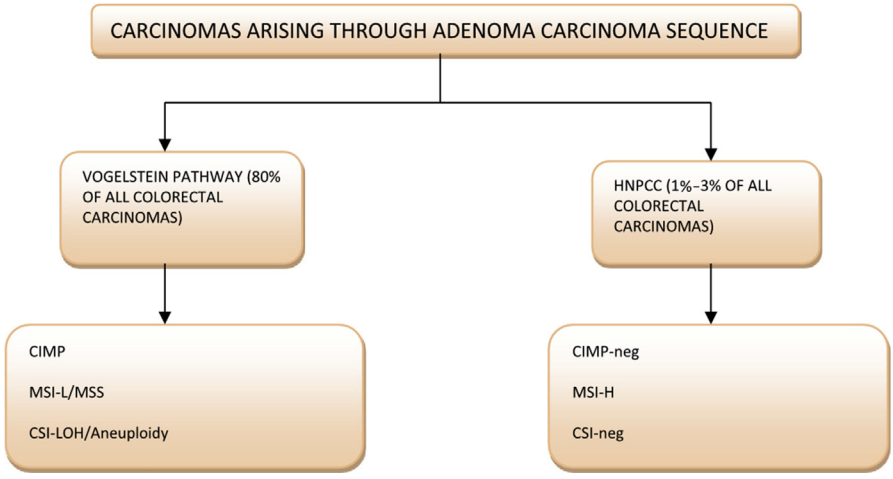

Fig. 2. Subtypes of carcinoma arising through the adenoma-carcinoma sequence. CIMP, CpG island methylator phenotype; LOH, loss of heterozygosity; MSI, microsatellite instability; MSS, microsatellite stable.

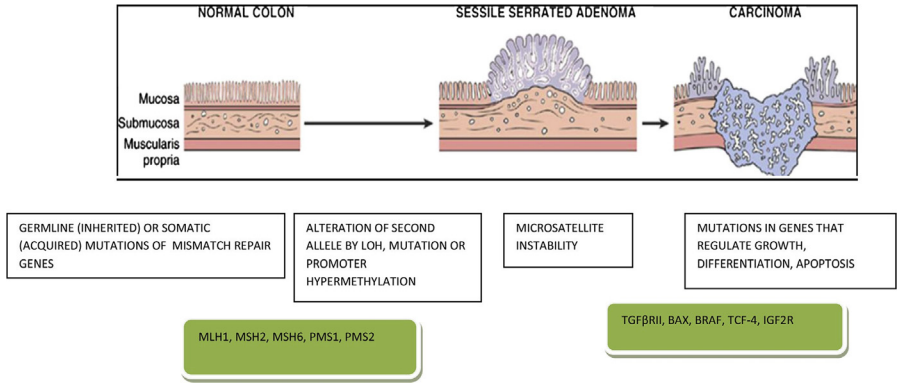

Fig. 3. Serrated polyp pathway. IGF, insulin-like growth factor; LOH, loss of heterozygosity; TGF, transforming growth factor. (*Modified from* Turner JR. The gastrointestinal tract. In: Kumar V, Abbas AK, Fausto N, et al, editors. Robbins and Cotran pathologic basis of disease. 8th edition. Philadelphia: Elsevier; 2010. p. 824; with permission.)

Neoplastic progression is associated with additional mutations and chromosomal instability, with involvement of the following.

- KRAS, an oncogene that enhances growth and prevents apoptosis;
- SMAD2 and SMAD4 (DPC4), tumor suppressor genes that are effectors of transforming growth factor-β signaling and allows unrestrained cell growth;
- DCC, a tumor suppressor gene located at 18q2.3;
- p53, which are tumor suppressor genes and are mutated in 70% to 80% of colon cancers; and
- Telomerase, which increases as lesions become more advanced.

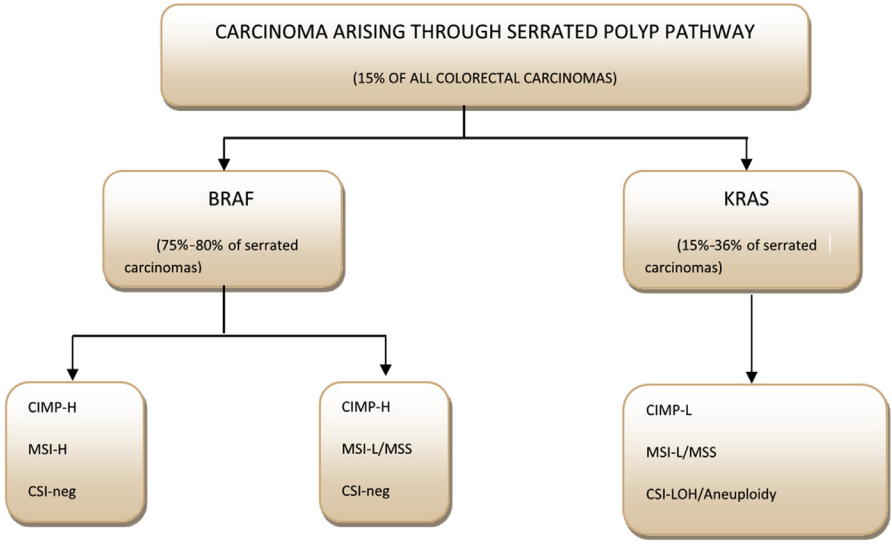

Fig. 4. Subtypes of carcinoma arising through serrated polyp pathway. CIMP, CpG island methylator phenotype; LOH, loss of heterozygosity; MSI, microsatellite instability; MSS, microsatellite stable.

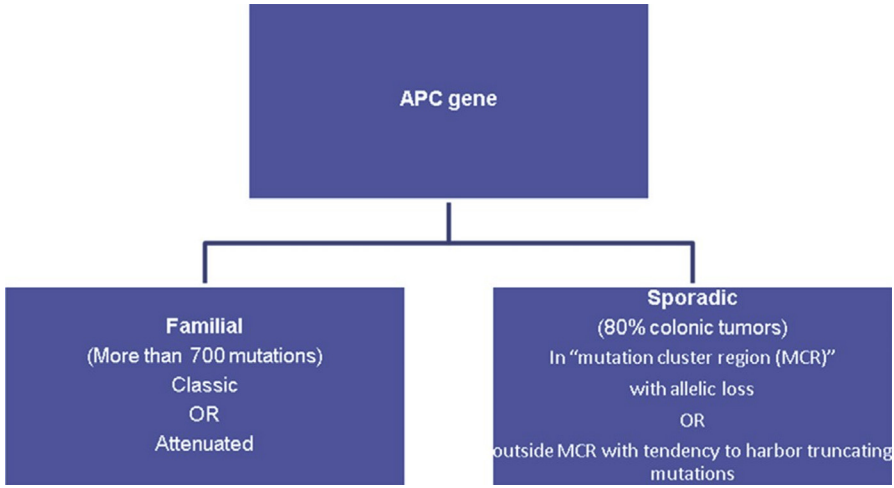

Fig. 5. APC gene mutations.

Other causes of chromosomal instability may be heterogeneous and include mutations in genes encoding mitosis checkpoint proteins such as BUB1 and BUB1B, abnormal centrosome number, amplification of aurora kinase A (AURKA, STK 15/BTAK), mutations of FBXW7, CHFR.1,2,11 Alternatively, tumor suppressor genes may also be silenced by methylation of a CpG-rich zone or CpG island (**Fig. 6**).[8] GNAS mutations have been reported in villous adenomas.[12]

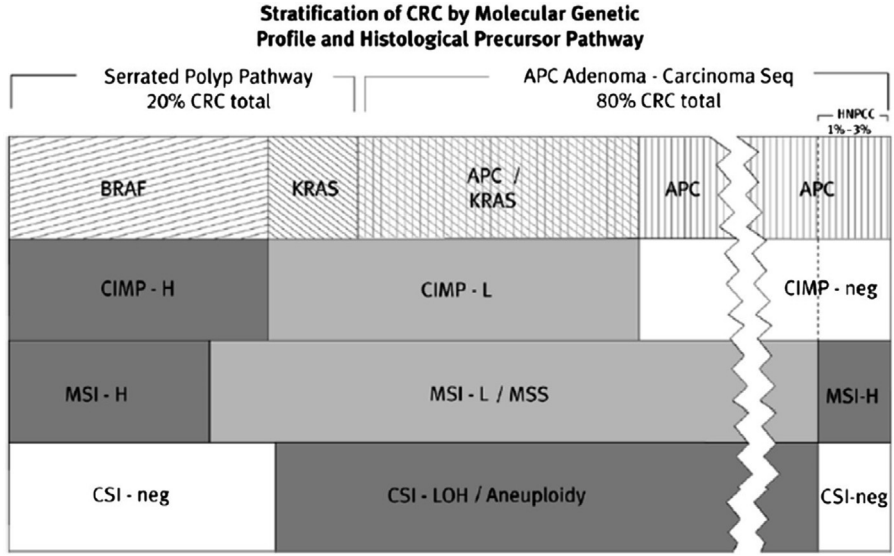

Fig. 6. Molecular genetic profiles of colorectal carcinoma. CIMP, CpG island methylator phenotype; CRC, colorectal cancer; HPNCC, hereditary nonpolyposis colorectal cancer; LOH, loss of heterozygosity; MSI, microsatellite instability; MSI-H, high-frequency microsatellite instability; MSS, microsatellite stable. (*Modified from* O'Brien MJ, Yang S, Huang CS, et al. The serrated polyp pathway to colorectal carcinoma. Mini-symposium: pathology of the large bowel. Diagn Histopathol 2008;14(2):90; with permission.)

Serrated polyp pathway

The serrated polyp pathway comprises a group of colorectal neoplasms with distinct morphologic and molecular characteristics. There are 20% to 30% of colorectal carcinomas that are thought to develop from serrated precursors.[13] Aberrant crypt focus and hyperplastic polyps comprise the earliest lesions. The other serrated polypoid lesions include sessile serrated adenoma/polyp (SSA/P), and traditional serrated adenoma (TSA).[14] BRAF mutation has been described as an early event seen in microvesicular hyperplastic polyp, which might progress to serrated adenoma/polyp (**Fig. 7**). SSA/P and hyperplastic polyps have different cancer risks, different recommended surveillances, and overlapping histologic features.[13,15] Hes1 is a downstream target of Notch signaling pathway and is found to be ubiquitously expressed in the nuclei of normal colonic epithelial cells. The complete loss or weak expression of Hes1 is observed in majority of SSA/P compared with normal expression of Hes1 in hyperplastic polyps. The dysplastic areas in sessile serrated adenomas, however, reveal the cytoplasmic staining of Hes1. Tubular adenoma and TSA show variable mixed positive and negative staining patterns.[16]

TSA are a heterogenous group of polyps with mutually exclusive KRAS and BRAF mutations. Molecular analysis of TSAs shows highly variable frequencies of KRAS, BRAF, and GNAS mutations in 10% to 46%, 29% to 90%, and 8% of tumors, respectively. Unlike SSA/P, TSA rarely reveal diffuse expression of ANXA10. BRAF-mutated TSA reveal more widespread methylation of a 5 marker CpG island panel compared with KRAS-mutated polyps.[12] In general, TSA may be CpG island methylator phenotype (CIMP) high, CIMP low, or CIMP negative. The CIMP-high tumors exclusively reveal methylation of RUNX3 and SOCS1, and are associated with BRAF mutations. The CIMP-low tumors are associated with KRAS mutations. They reveal restricted methylation pattern confined to NEUROG1. A strong association between KRAS mutations and high-grade dysplasia has been reported in a patient cohort from Korea.[17] Contiguous serrated lesions resembling SSA/P or hyperplastic polyps, when present, share the same mutations as the TSA. Tumors arising from TSA are predominantly left sided. Wnt/CTNNB1 alterations, and KRAS and p53 mutations are common genetic events in the traditional adenoma pathway of colorectal carcinoma. The characteristic genetic alteration PTPRK-RSPO3 fusion is also reported in a study. The TSA with the aforementioned fusion reveal distal localization,

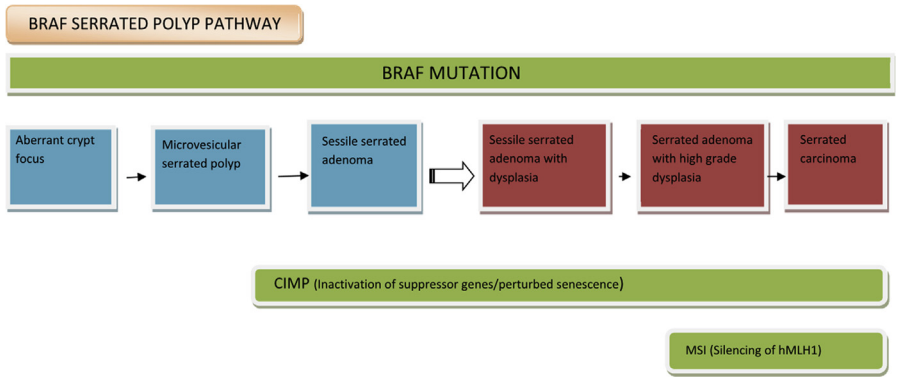

Fig. 7. BRAF serrated polyp pathway. CIMP, CpG island methylator phenotype; MSI, microsatellite instability. (*Modified from* O'Brien MJ, Yang S, Huang CS, et al. The serrated polyp pathway to colorectal carcinoma. Mini-symposium: pathology of the large bowel. Diagn Histopathol 2008;14(2):79; with permission.)

larger size, prominent ectopic crypt foci, association with high-grade component, progression to carcinoma, and the presence of KRAS mutations. Slitlike serrations are less prominent and associations with hyperplastic polyps and SSA/P are rare. RSPO overexpression is mutually exclusive with Wnt pathway gene mutation, but is involved in its activation[18] ANXA10 protein is highly expressed in the majority of SSA/P, but not in the TSA or contiguous precursor polyps associated with them.[19] The progress of nondysplastic serrated polyps to more advanced neoplasms is associated with increasing levels of CpG island methylation, leading to inactivation of tumor suppressor genes.

Carcinomas arising in the serrated pathway The carcinomas of this pathway frequently show MSI as a result of epigenetic silencing of hMLH 1.[14] Some studies have shown that transition to high-grade dysplasia and carcinoma is facilitated by methylation-induced silencing of p16 and escape from activation-induced senescence (**Fig. 8**).[20] The other major pathway of pathogenesis of serrated carcinomas arises after KRAS mutations. The carcinomas are microsatellite stable (MSS) and CIMP low, but show chromosomal instability and loss of heterozygosity of tumor suppressor genes. It is one of the earliest genetic mutations in colon carcinogenesis, detected in approximately 40% of the tumors. Along with BRAF mutations, it has been found in the earliest detectable lesions with a serrated morphology. They have been reported in 18% of aberrant crypt foci, 4% to 37% of hyperplastic polyps, 60% of admixed polyps, 80% of TSAs, and up to 10% of sessile serrated adenomas. KRAS mutation has been observed to be associated with a right-sided tumor location. The mutation has significant association with usual tumor histology (vs mucinous, signet ring, medullary), extramural tumor extension, peritumoral lymphocytic host response, presence of distant metastases, and absence of lymphovascular invasion at the time of diagnosis.[21–28] SSA/P with dysplasia are frequently associated with loss of MLH1 expression, which is critical to progression. The patterns of dysplasia have been classified in a recent study as minimal deviation, serrated, adenomatous, and not otherwise specified. The loss of immunostaining may help in supporting

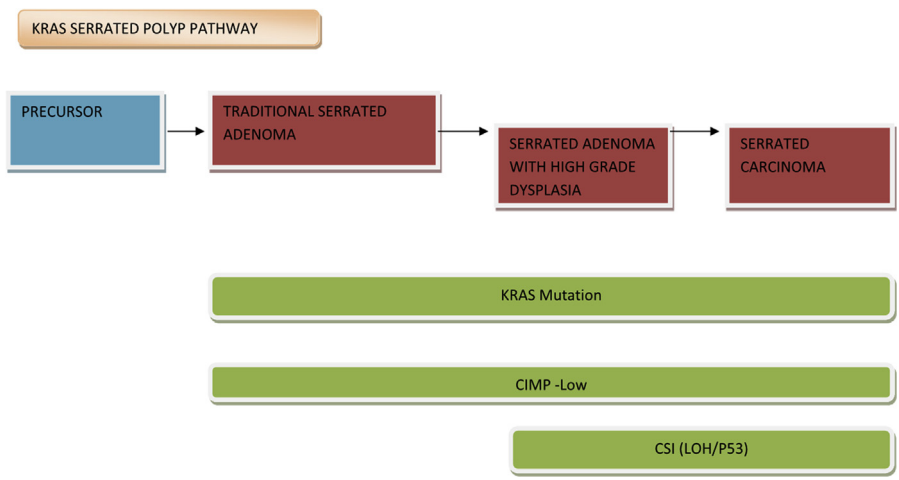

Fig. 8. KRAS serrated polyp pathway. CIMP, CpG island methylator phenotype; LOH, loss of heterozygosity. (*Modified from* O'Brien MJ, Yang S, Huang CS, et al. The serrated polyp pathway to colorectal carcinoma. Mini-symposium: pathology of the large bowel. Diagn Histopathol 2008;14(2):79; with permission.)

minimal deviation pattern of dysplasia, resolving the equivocal nature of atypical lesions, and differentiating sporadic adenomas from fragments of dysplasia associated with SSA/P. Tumors arising from SSA/P are predominantly right sided, microsatellite unstable, BRAF mutated, and hypermethylated at CpG islands (CIMP high).[19] They commonly present as interval colorectal carcinomas, because of missed precursor lesions, incomplete resection and rapid progression.[28]

Serrated polyposis Serrated polyposis is a clinically defined syndrome characterized by occurrence of multiple serrated polyps in the large intestine. A significant number of patients may have synchronous or metachronous tumors. Small numbers of conventional adenomas may also be present. Individuals with serrated polyps and their relatives are at increased risk of colorectal carcinoma. Mutations in BRAF along with CpG island mutator phenotype is the molecular marker for serrated neoplasia, with serrated polyps being precursor lesions.[29] The reported incidences of colorectal cancer (CRC) in serrated polyposis patients vary from 14% to 54%. They have diverse molecular alterations encompassing features of at least of the serrated neoplasia pathway and traditional adenoma pathway. The concomitant presence of conventional adenomas also increases the risk of development of carcinoma. However, the carcinomas contiguous to conventional adenoma are not associated with BRAF mutation.[29] Colorectal carcinoma with adjacent TSA or sessile serrated adenoma demonstrate more frequent mucinous differentiation, BRAF mutation, and mismatch repair (MMR) deficiency. Serrated colorectal carcinomas in distal colon are usually BRAF/KRAS wild type and MMR proficient. Activation of β-catenin was found in CRC with or without BRAF mutation. A large proportion of CRC from patients with serrated polyps do not develop CRC through the serrated neoplasia pathway and show various molecular phenotypes, including the traditional adenoma pathway. A few tumors show KRAS mutation along with low levels of CIMP, MSI, downregulation of MGMT by methylation, and frequent KRAS mutation. Atypical conventional adenomas in individuals who have at least 1 sessile serrated adenoma share some morphologic characteristics with serrated polyps and are all BRAF/KRAS wild type. This polyp may possibly be a precursor lesion of a large number of CRCs. Up to 95% of sessile serrated adenomas harbor a BRAF mutation and are the likely precursor lesions of CRC. Tumors with residual contiguous serrated polyps harbor V600E mutation. CRC in patients with serrated polyps may develop from nonserrated polyps through a derivative of the traditional adenoma pathway. Serrated polyps may be considered a disorder associated with hypermature mucosa secondary to alteration in DNA methylation with a propensity to develop early onset multiple serrated polyps. These patients are at increased risk of developing metachronous carcinoma when compared with the general population. In patients with a high-risk CRC syndrome, when patients with serrated polyps present with CRC more extensive colonic resection should be considered for both subsequent risk of metachronous cancer and future control of polyps. A high proportion of interval CRCs, diagnosed within 5 years of a complete colonoscopy, are serrated neoplasia pathway CRC. It is attributed to lower polyp detection rate for right-sided polyps or rapid progression of serrated lesions to dysplasia and carcinoma even for polyps less than 1 cm.[13,19,29–31]

Sporadic high-frequency microsatellite instability colorectal carcinoma MSI is prevalent in 10% to 15% of all sporadic colorectal carcinomas. Biallelic transcriptional silencing of MLH1 gene secondary to promoter hypermethylation leads to loss of normal MMR function in sporadic CRCs. The malignancy develops through the

serrated pathway, with sessile serrated adenoma as the precursor lesion. The molecular abnormality includes methylation of multiple regions of C-G dinucleotide or CpG islands within the promoter region of genes and subsequent downregulation of these genes. It is known as CIMP and is associated with BRAF mutation in 40% to 50% cases.[32] Genetic instability may operate at the chromosomal level (chromosomal instability), affecting the whole chromosome or parts of chromosomes or at a more subtle level affecting DNA sequences resulting from replication errors (high-frequency MSI). These forms of instability are mutually exclusive, so that CRCs with chromosomal instability are MSS (**Table 1**).[33–37] Appropriate caution must be exercised when correlating single molecular events with patient outcomes. The molecule examined might be associated with global genomic or epigenetic aberrations, and improved or adverse outcomes might be associated with alterations in other molecules. A positive correlation has been reported between BRAF mutated colorectal carcinoma, female sex, proximal tumor location, mucinous or serrated adenocarcinoma histologic type, and the presence of tumor infiltrating lymphocytes (**Tables 2** and **3**).[29] Metaanalyses of MSI status and survival of patients with colorectal carcinoma showed that high-frequency MSI tumors were associated with a better prognosis compared with MSS tumors. High-frequency MSI tumors show no benefit from adjuvant 5-fluorouracil. Patients with CIMP-positive tumors experience a significant survival benefit from chemotherapy in contrast with those with CIMP-negative tumors. CIMP in non–high-frequency MSI tumors predicts worse survival.[20]

Hereditary nonpolyposis colon cancer/the Lynch syndrome The Lynch syndrome is a hereditary autosomal-dominant syndrome with high penetrance. Its associated tumors show MSI owing to mutations in MMR proteins. The 4 DNA MMR genes are involved in the repair of mismatches resulting from misincorporation, or slippage events, during replication. Hereditary defects in 1 of the 4 MMR genes accounts for 80% to 90% of cases of hereditary nonpolyposis colon cancer (HNPCC).[38–41] Clinically, the Lynch syndrome is defined by applying either the Amsterdam I or Amsterdam II criteria and represents about 2% to 3% of all CRCs. An additional 2% to 3% of patients with CRC harbor similar MMR gene defects, but do not fulfill the criteria. Contrarily, some patients with attenuated FAP-associated and MUTYH- associated polyposis might fulfill Amsterdam criteria for having HNPCC. Revised Bethesda criteria show a higher sensitivity in the detection of new patients with the Lynch syndrome.[42–46] The subset of HNPCC cases caused by MMR gene defects is referred to as hereditary MMR-deficiency syndrome. The MMR-deficient cancers arise after loss of DNA MMR in tumor cells, leading to an increase in the rate of frameshift mutations in microsatellites. The frequency of mutations in short repetitive sequences located in coding regions of genes, such as transforming growth factor-BR2, is also increased. Germline mutations in 4 MMR proteins (MLH1, MSH2, MSH6, and PMS2) account for majority of the cases. Of the cases reported, 80% are attributed to mutations in MLH1 and MSH2.[19] Most of the genetic defects are a result of point mutations, insertions, and deletions.[47] Deletion in the EPCAM gene may cause epigenetic inactivation of MSH2. There are 20% to 25% of the cases that are suspected of having a mutation in MSH2, but without germline mutations, may be accounted for by germline deletions in EPCAM/TACSTD1. They account for about 1% of patients with the Lynch syndrome.

EPCAM is a calcium-independent cell adhesion membrane protein and is not involved in the physiologic functions of MMR. The EPCAM gene is located on the short (p) arm of chromosome 2 at position 21. Large germline deletions and rearrangement

Table 1
Molecular pathologic classification of colorectal cancer

Group Number	CIMP Status	MLH1 Status	MSI Status	Chromosomal Status	Precursor	Proportion (%)
1	CIMP high	Full methylation	MSI-H	Stable (diploid)	Serrated polyp	12
2	CIMP high	Partial methylation	MSS/MSI-L Associated with BRAF mutation	Stable (diploid)	Serrated polyp	8
3	CIMP low	No methylation	MSS/MSI-L Associated with KRAS mutation	Unstable (aneuploid)	Adenoma/serrated polyp	20
4	CIMP negative	No methylation	MSS Associated with KRAS mutation	Unstable (aneuploid)	Adenoma	57
5	CIMP negative	Germline MLH1 or other mutation	MSI-H	Stable (diploid)	Adenoma	3

Abbreviations: CIMP, CpG island methylator phenotype; MSI-L, low-frequency microsatellite instability; MSS, microsatellite stable.

Modified from Jass JR. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. Histopathology 2007;50:119, with permission; and *Adapted from* Redston M. Epithelial neoplasms of the large intestine. In: Odze RD, Goldblum JR, editors. Surgical pathology of the GI tract, liver, biliary tract and pancreas. 2nd edition. Philadelphia: Elsevier; 2009. p. 597–637, with permission.

Table 2
Clinical presentation of microsatellite instability and BRAF mutation in colorectal carcinoma

BRAF v600E	MMR/MSI	CIMP	Clinical Presentation	%
–	Unstable/high	Low/negative	Lynch syndrome/other	2–3
–	Unstable/high	High	Sporadic	35–55
+	Unstable/high	High	Sporadic	…
+	Stable/negative	Low/negative	Aggressive phenotype/serrated carcinoma	Rare

Abbreviations: CIMP, CpG island methylator phenotype; MMR, mismatch repair; MSI, microsatellite instability.

encompassing EPCAM-*MSH2* have been characterized from the 30 end region of EPCAM to the 50 initial sequences of the MSH (**Tables 4** and **5**).[39–41,47] The tumors do not, however, reveal expression of annexin 10.[19] A few unclassified variants inclusive of mutations and missense-type nucleotide substitutions have been reported in literature and have unknown clinical significance. Many variants are associated with defects in RNA splicing. The prevalence of variants has been reported to be up to 34% among the HNPCC cohorts.[47] The Lynch syndrome type I is confined to patients presenting only with CRC. The Lynch syndrome type II is associated with additional extracolonic cancers. The DNA MMR system is closely associated with tumor response to radiation. It has a critical role in the repair process of DNA structural damage caused by radiation. The MSI attributes to altered radiosensitivity.[48] Patients with the Lynch syndrome/HNPCC harbor a similar number of adenomatous polyps to the

Table 3
Site-specific variation in molecular phenotypes of colorectal carcinoma

	Predominant Tumor Type	Other Common Tumors		
Cecum	BRAF mutated MMR deficient	BRAF/KRAS wild type, MMR deficient		
Ascending colon	BRAF mutated MMR deficient	BRAF/KRAS wild type, MMR deficient	BRAF/KRAS wild type, MMR proficient	BRAF mutated MMR proficient
Transverse colon	BRAF mutated MMR deficient	BRAF mutated MMR proficient	BRAF/KRAS wild type, MMR deficient	KRAS mutated MMR proficient
Descending colon	BRAF/KRAS wild type MMR proficient			
Sigmoid colon	BRAF/KRAS wild type MMR proficient	BRAF mutated MMR deficient		
Rectum	BRAF/KRAS wild type MMR proficient	KRAS mutated MMR proficient		

Abbreviation: MMR, mismatch repair.
Data from Rosty C, Walsh MD, Walters RJ, et al. Multiplicity and molecular heterogeneity of colorectal carcinoma in individuals with serrated polyposis. Am J Surg Pathol 2013;37:434–42.

Table 4
Molecular testing for Lynch syndrome

Serial Number	Tests
1	Evaluation of tumor tissue for MSI: immunohistochemistry for 4 MMR proteins followed by MMR gene mutation testing by PCR
2	Molecular testing of the tumor for methylation abnormalities to rule out sporadic cases
3	Molecular testing of the tumor for BRAF mutations to rule out sporadic cases
4	Molecular genetic testing of the MMR genes to identify germline mutations when findings are consistent with Lynch syndrome

Abbreviations: MMR, mismatch repair; MSI, microsatellite instability; PCR, polymerase chain reaction.

Data from Redston M. Epithelial neoplasms of the large intestine. In: Odze RD, Goldblum JR, editors. Surgical pathology of the GI tract, liver, biliary tract and pancreas. 2nd edition. Philadelphia: Elsevier; 2009.

general population. The polyps are indistinguishable from conventional adenomas. Therefore, the detection of index cases is challenging and requires the use of specific testing (**Table 6**).[42–46]

KRAS serrated pathway

KRAS-mutated TSA progresses to a mixed tubulovillous adenomatous phenotype and acquires high-grade dysplasia. The interface of high-grade dysplasia and infiltrating carcinoma is associated with a p53 mutation.[1,14] TSA associated with high-grade dysplasia or malignancy is associated with high rates of MLH1 methylation. CIMP high and CIMP low tumors are reported with variable frequency (**Fig. 9**). An unequivocal diagnosis of serrated carcinoma is made when 6 of the 7 histologic criteria listed in **Box 2** are fulfilled.

Limitations of molecular classification and correlates

- Lack of gold standard and uniform methods, definition, and criteria.
- False-positive and false-negative results.
- Sampling bias.
- Markers used for studies on MSI are not uniform.
- Nonuniform methods of detection of methylation markers.
- Lack of standardized definition of chromosomal instability.[3]

Table 5
Genetic basis of HNPCC

High-Frequency MSI (%)	Gene Mutation (%)
Yes (80–90)	MLH1 (39) MSH2 (38) MSH6 (11) PMS2 (7) EPCAM (1) Unknown (5)
No (10)	Yes (as above; 10) Unknown (90)

Abbreviations: HNPCC, hereditary nonpolyposis colon cancer; MSI, microsatellite instability.

Data from Refs.[4,9,15]

Table 6
Differences in molecular phenotype of sporadic colorectal carcinoma and the Lynch syndrome

	Sporadic	Lynch Syndrome
MMR	MLH1 loss	Loss of any MMR protein
MSI high	+ (approximately 75%)	+
MLH1 promoter methylation	+	Majority negative; rare cases with germline defects
Mutations in MMR	–	+
BRAF	±	–
Annexin 10 IHC	Focal ±	Majority negative Rare cases with germline defects
Precursor lesions	SSA/P	Tubular and tubulovillous adenomas

Abbreviations: IHC, immunohistochemistry; MSI, microsatellite instability; SSA/P, sessile serrated adenoma/polyp.

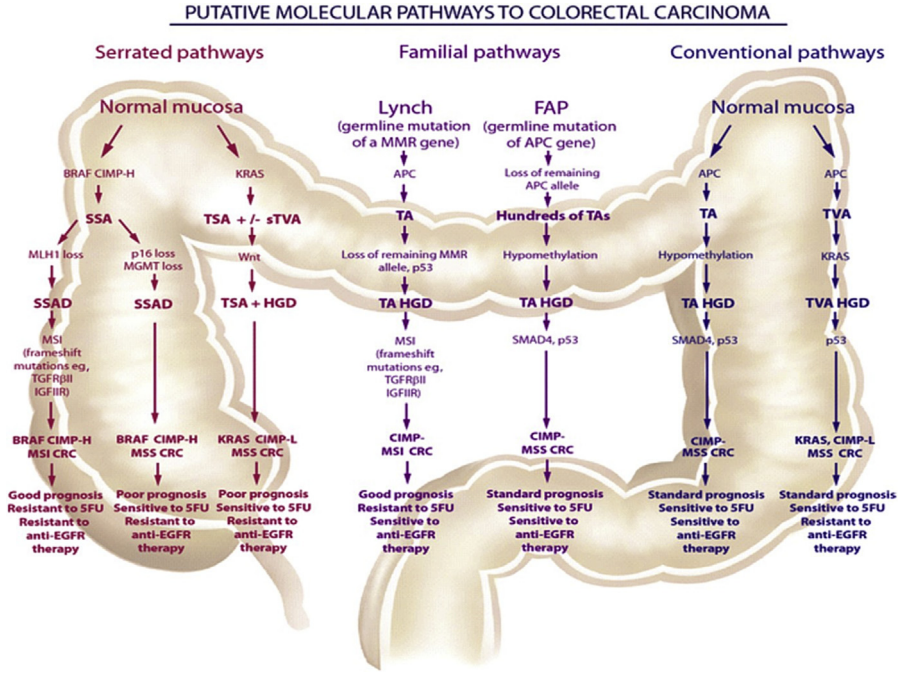

Fig. 9. Molecular pathogenesis of colorectal carcinoma (CRC). CIMP, CpG island methylator phenotype; EGFR, epidermal growth factor receptor; FAP, familial adenomatous polyposis; 5-FU, 5-fluoricail; HGD, high-grade dysplasia; MMR, mismatch repair; MSI, microsatellite instability; MSS, microsatellite stable; SSA, sessile serrated adenoma; SSAD, sessile serrated adenoma with dysplasia; TGF, transforming growth factor; TSA, traditional serrated adenoma. (*Adapted from* Bettington M, Walker N, Clouston A, et al. The serrated pathway to colorectal carcinoma: current concepts and challenges. Histopathology 2013;62:380; with permission.)

Box 2
Histomorphologic features of serrated carcinomas

Epithelial serrations

Eosinophilic or clear cytoplasm

Abundant cytoplasm

Vesicular nuclei with peripheral chromatin condensation and a single prominent nucleolus

Distinct nucleoli

Absence of necrosis (or <10% necrosis)

Intracellular and extracellular mucin

Cell balls and papillary rods

Adapted from Bettington M, Walker N, Clouston A, et al. The serrated pathway to colorectal carcinoma: current concepts and challenges. Histopathology 2013;62:382; with permission.

PATHOLOGIC FEATURES OF COLORECTAL CARCINOMA WITH HIGH-FREQUENCY MICROSATELLITE INSTABILITY

Shared by Both Inherited and Sporadic Tumors

- Tendency to occur on right side of colon.
- Medullary carcinoma phenotype.
- Presence of mucinous or signet ring component.
- Presence of tumor infiltrating and peritumoral lymphocytes.
- Crohnlike inflammatory response.
- Pushing tumor borders (**Fig. 10**; see **Tables 4** and **5**).[32]

Clinical correlation of specific subtypes of colorectal carcinoma

Medullary carcinoma Tumors with " medullary-type" are high-frequency MSI and generally have better prognosis and lower rates of locoregional nodal involvement and

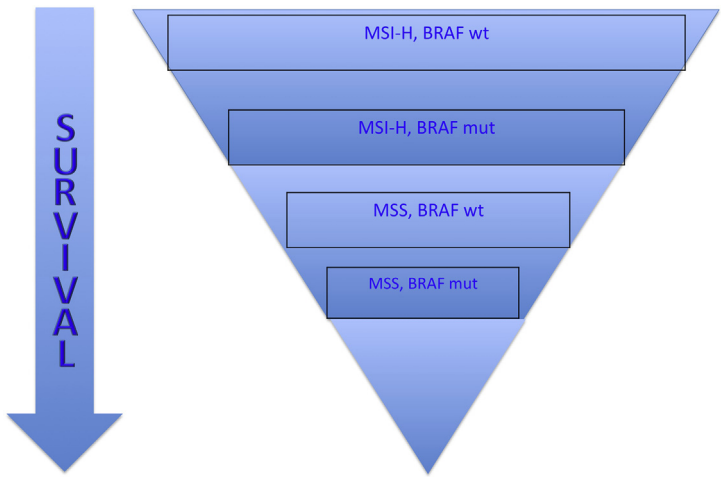

Fig. 10. Effect of microsatellite instability (MSI) and BRAF mutation on survival in colorectal carcinoma. mut, mutation; wt, wild type.

distant metastasis. On comparison of medullary carcinoma with MSS and MSI tumors, significant upregulation of several immunoregulatory genes induced by Interferon gamma including are identified. The specific genes include IDO-1, WARS (tRNA(trp)), GBP1, GBP4, GBP5, PD-1, and programmed death ligand 1 (PD-L1). The tumor reveals higher mean $CD8^+$ and PD-L1 tumor infiltrating lymphocytes compared with other tumors. The CD8 T lymphocytes are presumed to be activated upon presentation of neoantigens from the tumor cells. The lymphocytes promote a strong interferon response.[49]

Early onset colorectal carcinoma These tumors manifest in patients less than 40 years of age without underlying HNPCC, adenomatous polyposis, or inflammatory bowel disease. The tumor shows pathologic features associated with aggressive behavior. The adenomas and carcinomas reveal increased expression of AMACAR. miR-21, miR-20a, miR-106a, miR-181b, and miR-203 are increased compared with normal tissues. miR-21 was associated with poor clinical outcome.[50]

Adenoma-like adenocarcinoma Adenoma-like adenocarcinoma is an uncommon variant of CRC with a low rate of metastasis and good prognosis. The predominant mutation reported is KRAS in codons 12 or 13. Other mutations included PIK3CA and BRAF V600E.[51]

Micropapillary colorectal carcinoma CRC with micropapillary features have a high likelihood of locoregional and distant metastases. They show significant increase in tp53 mutation and frequent mutations in KRAS and BRAF. Increased expression of stem cell markers SOX2 and NOTCH3 has also been reported.[52,53]

Mucinous tumors Mucinous CRC, which are MMR deficient have similar outcomes as low-grade nonmucinous tumors on survival analysis. Mucinous MMR-proficient CRCs behave slightly better than nonmucinous high-grade tumors but worse than mucinous low-grade nonmucinous tumors.[54]

Sporadic microsatellite unstable colorectal carcinoma The underlying pathogenesis of sporadic microsatellite unstable colorectal carcinoma is attributed to MLH1 promoter hypermethylation, subsequently leading to silencing of gene transcription. A BRAF V600E mutation is highly specific for these tumors, but not sensitive. Focal annexin A10 expression has been reported in both BRAF mutated and wild-type subcategories.[19]

Braf-mutated microsatellite stable adenocarcinoma of the proximal colon Tumors demonstrate adverse histologic features inclusive of lymphatic invasion, lymph node metastasis, perineural invasion, perineural invasion, tumor budding, and mucinous and signet ring histology. It is associated with significantly poor overall and disease-free survivals.[19]

Undifferentiated/rhabdoid carcinomas of the gastrointestinal tract The switch sucrose nonfermenting chromatin remodeling complex components have been reported to reveal loss of expression in the undifferentiated tumors with variable rhabdoid features, pleomorphic giant cells, and spindle cells. The most common components showing loss include SMARCB1(INI1), SMARCA2, SMARCA4, and ARID1A. Concurrent loss of MMR proteins (MLH1/PMS2) has also been reported. Some tumors belong to well-defined molecular subtypes and sustain additional loss of the remodeling complex components.[55]

Synchronous and metachronous cancers Similar genetic changes have been reported in sporadic contiguous tumors. In tumors separated by 1 or more segments,

there was less consistency in genetic changes. Metachronous tumors did show variation, which was decreased when the subsequent tumor was located near the first tumor.[56]

Molecular biomarkers for the evaluation of colorectal cancer Guideline statements were established by the American Society for Clinical Pathology, the College of American Pathologists and Laboratory Quality Center, the Association for Molecular Pathology, and the American Society of Clinical Oncology to create standard molecular biomarker testing and guide therapies for patients with colorectal carcinoma. The guidelines follow well-established methods used in their development as well as for regular updates, so that new advances can be integrated in a timely manner in future. The biomarker guideline expert panel strongly recommends that laboratories must incorporate colorectal carcinoma molecular biomarker testing methods into their overall laboratory quality improvement program, establishing appropriate quality improvement monitors as needed to ensure consistent performance in all steps of the testing and reporting process. Laboratories performing the biomarker testing must participate in proficiency testing programs or alternative proficiency assurance activity. Anti-epidermal growth factor receptor (EGFR) monoclonal antibodies have been the main targeted therapies for CRC and require knowledge of mutational status of genes in the pathway as predictive biomarkers of response to therapies. The monoclonal antibodies target the EGFR extracellular domain and block the pathway.[55] Polymerase chain reaction (PCR)–based techniques and Sanger sequencing are mostly used for diagnosis; however, other sequencing techniques, including deep sequencing and hybridization-induced bead aggregation technology, are under evaluation.[57]

KRAS Patients carrying activating mutations of KRAS affecting exon 2 codons 12 and 13 do not benefit from anti-EGFR therapy, such as cetuximab and panitumumab. The expert panel on colorectal biomarker guideline recommends patients with colorectal carcinoma being considered for anti-EGFR therapy must undergo RAS mutational testing. Mutational analysis should include KRAS and NRAS codons 12 and 13 of exons 2, 59, and 61 of exon 3, and codons 117 and 146 of exon 4 ("expanded" or "extended" RAS).[57]

BRAF The expert panel on colorectal biomarker guideline recommends patients with colorectal carcinoma should receive BRAF p. V600 [BRAF c. 1799 (p. V600)] mutational analysis for prognostic stratification. In addition, BRAF p. V600 mutational analysis should be performed in deficient MMR tumors with loss of MLH1 to evaluate for risk of the Lynch syndrome. The presence of BRAF mutation strongly favors a sporadic pathogenesis.[31] Mutations in BRAF and KRAS are mutually exclusive. BRAF mutated stages III and IV CRCs are associated with worse prognosis, including survival after tumor recurrence. BRAF V600E mutation blocks the effect of anti-EGFR antibodies on disease progression in stage IV colorectal carcinoma. The effect of MSI and BRAF mutations on survival in colorectal carcinoma is shown in **Fig. 10**.[19,29–31,57]

Prognostic biomarkers for management of patients with colorectal carcinoma

POLE mutations Colorectal carcinoma with POLE (exonuclease domain of polymerase epsilon) proofreading domain mutations are more immunogenic and portend a better prognosis in stages II and III CRC. The presence of POLE mutations results in better recurrence-free survival and disease-free survival relative to MSI-proficient tumors.[58]

MASPIN MASPIN has been reported to be negative in normal colonic mucosa. Cytoplasmic and nuclear positivity in superficial and deep parts of the tumor have

been noted. The staining correlated positively with a right-sided location and a high tumor grade. Increased nuclear grade correlated with more than 4 positive lymph nodes. The tumors belonging to both conventional pathway and MSI pathway reveal MAPSIN expression.[59]

SATB1 SATB1 shows nuclear positivity in normal colonic mucosa and colorectal carcinoma. It has been reported that approximately 22% CRC show loss of expression, which is associated with worse overall survival predominantly in right-sided colon cancers. The loss is associated with younger age, mucinous or signet ring histology, poor differentiation, and less favorable response to chemotherapy. It correlates with CIMP-high phenotype.[60]

Histone deacetylases Global nuclear expression of histone modifications and histone deacetylases correlates with clinical outcomes in CRC. The deacetylases cause epigenetic changes and have been reported to have clinical prognostic value as individual markers and in combination when used for multimarker analysis. The specific deacetylases significantly reported to be dysregulated in CRC include SIRT1 (decreased nuclear expression), HDAC2 (increased nuclear expression), and H4K16Ac (decreased nuclear expression). It may correlate with long interspersed nuclear element-1 hypomethylation.[61]

RSPO fusions CRCs with RSPO fusions are sensitive to repression of WNT pathway signaling with anti-RSPO antibody and PORCN inhibitors.[16]

Phospholipase The expression of PLA2G2A, a phospholipase, is associated with an aggressive phenotype, low survival, and poor therapeutic response in patients receiving concurrent chemoradiotherapy.[62]

Exosomes ALG-2 interacting protein X, an exosome involved in transporting bioactive molecules, potentially mediates epithelial stromal interactions and reveals reduced expression in adenoma and colorectal carcinoma.[63,64]

Mismatch Repair Testing

Scientific rationale

The MMR gene MSH2 binds with MSH3 and MSH6, forming a functional molecular complex that facilitates the recognition of the DNA mismatch. Subsequently, the complex recruits MLH1, its binding partner PMS2, and other enzymes, leading to excision, repolymerization, and ligation of the repaired strand of DNA. Patients with HNPCC and 15% of sporadic tumors have defective DNA MMR and are high-frequency MSI.

Clinical rationale

Molecular testing is recommended in patients with CRC to evaluate for possible Lynch syndrome. It is used in patients less than 70 years of age, with high-grade right-sided colon cancer, mucinous histology, and Crohn's disease–like peritumoral lymphoid infiltrate. Lynch syndrome–associated colorectal adenomas have also been reported to have abnormal MSI or immunohistochemical (IHC) testing results. Initial screening is accomplished by MSI testing using PCR or immunohistochemistry for MMR proteins.[34] Definitive diagnosis of the disorder and presymptomatic detection of carriers in at-risk individuals is possible by follow-up germline testing, with the potential for a reduction in morbidity and mortality. MSI is also a good prognostic marker for patients without lymph node metastases after undergoing neoadjuvant radiotherapy. Guidelines for reporting MMR as a predictive biomarker of response to PD-L1 therapy are

in the pipeline. The information is used for the selection of patients for immunotherapy.[34,48,65–68]

Best method

MSI testing is generally performed with at least 5 microsatellite markers, generally mononucleotide or dinucleotide repeat markers. In 1998, a National Institutes of Health consensus panel proposed that laboratories use a 5-marker panel comprising 3 dinucleotide and 3 mononucleotide repeats for MSI testing. Because mononucleotide markers have a higher sensitivity and specificity, many commercially available kits use 5 mononucleotide markers.

QUALITY ASSURANCE

The detection of MSI in a tumor by microsatellite analysis requires that the DNA used for the analysis be extracted from a portion of the tumor that contains approximately 40% or more tumor cells. Thus, pathologists should help to identify areas of the tumor for DNA isolation that have at least this minimum content of tumor cells. MSI testing is frequently performed in conjunction with IHC testing for MMR protein expression (ie, MLH1, MSH2, MSH6, and PMS expression). If the results of MMR IHC and MSI testing are discordant (eg, high-frequency MSI phenotype with normal IHC or abnormal IHC with MSS phenotype), then the laboratory should ensure that the same sample was used for MSI and IHC testing and that there was no sample mix up. External proficiency testing surveys are available through the College of American Pathologists Molecular Oncology resource committee and other organizations. These surveys are invaluable tools to ensure that the laboratory assays are working as expected.

PITFALLS

- During IHC evaluation of MSI proteins, an intact expression of all 4 proteins indicates that the tested MMR enzymes are intact.
- It is common for intact staining to be patchy.
- Positive IHC reaction for all 4 proteins does not exclude the Lynch syndrome, because approximately 5% of families may have a missense mutation (especially in MLH1), which can lead to a nonfunctional protein with retained antigenicity.
- Defects in lesser known MMR enzymes may also lead to a similar result, but this situation is rare.
- Loss of expression of MLH1 may be caused by the Lynch syndrome or methylation of the promoter region (as occurs in sporadic MSI colorectal carcinoma). BRAF mutation testing can help in differentiating the cases, although definitive interpretation is possible by genetic testing.[65–68]

Recommendations

The National Comprehensive Cancer Network guidelines recommend MMR protein testing to be performed for all patients younger than 50 years of age with colon cancer based on an increased likelihood of the Lynch syndrome in the US population. The testing should also be considered for all patients with stage II disease, because patients with stage II high-frequency MSI may have a good prognosis and do not benefit from 5-fluorouracil adjuvant therapy.

Mismatch repair immunohistochemistry

The DNA MMR proteins are ubiquitously expressed in normal human tissues. HNPCC or the Lynch syndrome results in instability of the truncated messenger RNA transcript

and the protein product and results in complete loss of ICH-detectable MMR protein in tumors. Mutation of MLH1 results in its loss from the DNA MMR complex, subsequently leading to loss of PMS2 from the repair protein complex. Therefore, mutation and loss of the MLH1 protein is also usually accompanied by loss of PMS2 expression. The same mechanism holds true for MSH2 and its binding partner, MSH6. These IHC results are summarized in **Table 7**. The specificity of loss of protein expression for an underlying MMR defect is virtually 100%, although up to 10% of these tumors are MSS on MSI testing. The staining pattern of the tumor tissue is compared with the normal-appearing control tissue of the same patient to prevent misinterpretation caused by polymorphisms.[21–25]

Reporting guidelines (College of American Pathologists)

- The results of DNA MMR IHC and MSI testing should be incorporated into the surgical pathology report for the CRC case and an interpretation of the clinical significance of these findings provided.
- If DNA MMR IHC has not been performed, this testing should be recommended for any cases that show a high-frequency MSI phenotype, because this information helps to identify the gene that is most likely to have a germline mutation.
- Examination of expression of MLH1, MSH2, MSH6, and PMS2 is the most common IHC testing method used for suspected high-frequency MSI cases; antibodies to these MMR proteins are available commercially.
- Any positive reaction in the nuclei of tumor cells is considered as intact expression (normal).
- Loss of MSH2 expression essentially always implies the Lynch syndrome.[65–68]

MICROSATELLITE INSTABILITY TESTING

Frameshift mutations in microsatellites are identified by the amplification of selected microsatellites by PCR and analysis of fragment size by gel electrophoresis or an automated sequencer after extraction of DNA from both normal and tumor tissue (usually formalin-fixed, paraffin-embedded tissue). The sensitivity of the revised panel of MSI testing is at least 90% (**Table 8**).[4–7]

Various fluorescent multiplex PCR-based panels (eg, Promega panel) are used for detection of MSI loci. The prototype Promega panel uses fluorescently labeled primers

Table 7
Interpretation of DNA MMR IHC

MLH1	PMS2	MSH2	MSH6	Interpretation
1	1	1	1	Intact DNA MMR; or rare germline point mutation with intact IHC; or other gene
—	—	1	1	MLH1 methylation silencing or MLH1 germline mutation (HNPCC)
1	1	—	—	MSH2 germline mutation (HNPCC)
1	—	1	1	PMS2 germline mutation (HNPCC); rare MLH1 mutation may also have this pattern
1	1	1	—	MSH6 germline mutation (HNPCC)

Abbreviations: HNPCC, hereditary nonpolyposis colon cancer; IHC, immunohistochemistry; MMR, mismatch repair.

Adapted from Redston M. Epithelial neoplasms of the large intestine. In: Odze RD, Goldblum JR, editors. Surgical pathology of the GI tract, liver, biliary tract and pancreas. 2nd edition. Philadelphia: Elsevier; 2009. p. 631; with permission.

Table 8
Bethesda criteria for MSI

Loci with MSI (%)	Classification
40	MSI-H
10–30	MSI-L
0	MSS

Abbreviations: MSI, microsatellite instability; MSI-H, high-frequency microsatellite instability; MSI-L, low-frequency microsatellite instability; MSS, microsatellite stable.

Adapted from Redston M. Epithelial neoplasms of the large intestine. In: Odze RD, Goldblum JR, editors. Surgical pathology of the GI tract, liver, biliary tract and pancreas. 2nd edition. Philadelphia: Elsevier; 2009. p. 629; with permission.

for the coamplification of 7 markers for analysis of the high-frequency MSI phenotype, including 5 nearly monomorphic mononucleotide repeat markers (BAT-25, BAT-26, MONO-27, NR-21, and NR-24) and 2 highly polymorphic pentanucleotide repeat markers (Penta C and Penta D). Amplified fragments are detected using special spectral genetic analyzers.[20,21,36,37]

BRAF Mutation Testing

BRAF mutations in colorectal carcinoma neoplasms are activating point mutation at V600E, which may be detected in 6% to 10% of CRCs. This mutation constitutively stimulates other enzymes to promote continuous cell growth. This stimulation abrogates the ability of EGFR inhibitors to block cell proliferation and growth and confers resistance to anti-EGFR antibodies. The test is performed on formalin-fixed paraffin-embedded tumor tissue by sequencing-based technologies or allele-specific PCR. In addition, laboratory developed tests that involve standard genotyping or next-generation sequencing may be used to measure the level of this mutation. BRAF mutation testing is performed for prognostic stratification. It confers a worse clinical outcome and need for adjuvant therapy. Mutations are associated with reduced overall survival, and shorter progression-free survival. The poor prognosis is attributed to the genetic pathway in which it occurs. The adverse effects are negated in CIMP-positive tumors; it is also performed in MMR-deficient tumors to evaluate for the Lynch syndrome. There are insufficient data to guide the use of anti-EGFR therapy in the first-line setting with active chemotherapy based on BRAF V600E mutation status. IHC for mutated BRAFV600 E is not recommended for use in colorectal carcinoma because it is not as sensitive and concordant with genomic sequencing. However, it may be used for screening for the Lynch syndrome in conjunction with molecular genetic testing. Testing should be performed only in laboratories that are certified under the Clinical Laboratory Improvement Amendments of 1988 as qualified to perform high-complexity clinical laboratory (molecular pathology) testing.[19,30,69–72]

CpG Island Methylation Analysis Testing

A subset of CRCs (about 25%) have widespread aberrations in DNA methylation, including promoter silencing of genes. Referred to as CIMP, this subset includes most sporadic high-frequency MSI cancers with methylation silencing of MLH1. CIMP testing is a method to detect abnormal DNA methylation by using a panel of markers/loci and has been used in some studies to differentiate sporadic from hereditary MLH1-deficient cancers. Although there has not yet been an international consensus on the correct choice of markers for CIMP testing, several loci have begun

to emerge as the most sensitive and specific for this type of application. The CIMP genes commonly analyzed include CACNA1G, SOCS1, NEUROG1, RUNX3, and IGF2. COL2A repeats serves as normalization control. Methylation-specific PCR is widely used for analysis, although there is lack of standardization. Some high-frequency MSI tumors are CIMP high, but negative for BRAF mutations. Therefore, CIMP testing is not a surrogate for BRAF mutation testing and has additional significance. Sporadic MSI-high colon cancers rarely reveal IHC evidence of Wnt signaling activation.[73] Based on conventional pathway DNA methylation, MSS and CIMP-negative colorectal carcinomas comprise 47% to 55% of CRC, are mostly located in distal colon, and are presumed to arise from conventional adenomas. Distinct methylation patterns involving genes not included in the traditional CIMP assessment panels have been reported in the conventional pathway of CRC. The reported clusters included 30 CpG loci associated with homeobox genes, intestinal transcription factor CDX-2, and the prostate cancer susceptibility genes PRAC1 and PRAC2.[68–72,74]

KRAS MUTATION TESTING

Mutations in codons 12 and 13 in exon 2 of the coding region of the KRAS gene predict a lack of response to therapy with antibodies targeted to EGFR. The presence of the KRAS gene mutation has been shown to be associated with a lack of a clinical response to therapies targeted at EGFR, such as cetuximab and panitumumab. Although clinical guidelines for KRAS mutational analysis are evolving, provisional recommendations from the American Society for Clinical Oncology are that all patients with stage IV colorectal carcinoma who are candidates for anti-EGFR antibody therapy should have their tumor tested for KRAS mutations (available from: http://www.asco.org/CRC-markers-guideline, updated 2017). Testing for mutations in codons 12 and 13 should be performed only in laboratories that are certified under the Clinical Laboratory Improvement Amendments of 1988 as qualified to perform high-complexity clinical laboratory (molecular pathology) testing. The testing can be performed on formalin-fixed paraffin-embedded tissue, on primary or metastatic cancer. Sequencing (Sanger/pyrosequencing) and PCR-based technologies are commonly used. Hybridization-induced aggregation technology and deep sequencing techniques are in the pipeline.[14,75–79]

GERMLINE TESTING

Hereditary Nonpolyposis Colon Cancer

The goal of a genetic workup of families with HNPCC is to identify the underlying germline mutation. Confirmation of the germline mutation allows for the most accurate treatment and follow-up recommendations for the patient, and allows predictive testing to be undertaken in interested family members. The initial approach by most laboratories is to analyze the complete coding sequence of the relevant gene or genes (depending on IHC results), as well as a portion of the intronic regions important to exon splicing. Some laboratories use a variety of rapid screening approaches to find mutations, whereas others undertake a complete sequence analysis.[7,33]

APC Gene

Ninety-eight percent of alterations in FAP include frameshift, nonsense, splice site mutations, large deletions, and duplications of the APC gene. Testing is performed by mutation screening (Sanger sequencing, conformation sensitive gel electrophoresis, and protein truncation testing) with reflex conformation sequencing. Gene deletion or duplication analysis may be performed by multiplex ligation-dependent probe

amplification. False-negative results can occur because of deep intronic mutations, allele dropout, somatic mosaicism, and locus heterogeneity for the phenotype. Negative results may be followed by MUTYH targeted mutation testing.[22]

ALGORITHMIC STRATEGIES FOR MANAGEMENT OF MISMATCH REPAIR COLORECTAL CARCINOMA

There is no definitive standardized practice for the triage of colorectal carcinoma for molecular testing. Almost all microsatellite-instable colorectal carcinomas are detected by a combination of MSI and IHC testing. In the presence of deficient MMR, additional loss of protein expression of MSH2/MSH6, MSH6 alone, or PMS2 increases likelihood of the Lynch syndrome. Concomitant incidence of defective MMR, CIMP high, and MLH1 supports the diagnosis of sporadic defective MMR CRC. Detection of a BRAF c.1799T>A mutation serves to exclude diagnosis of the Lynch syndrome. Funkhauser and colleagues have critically analyzed the various recommendations and have advocated a screening algorithm to include MSI testing, BRAF c. 1799T>A mutation, and IHC for the 4 MMR proteins. **Fig. 11** shows MMR subgroup assignment for approximately 94% of colorectal carcinoma cases. Only the high-frequency MSI, MLH1 lost, and BRAF wild-type cases remain unassigned. The

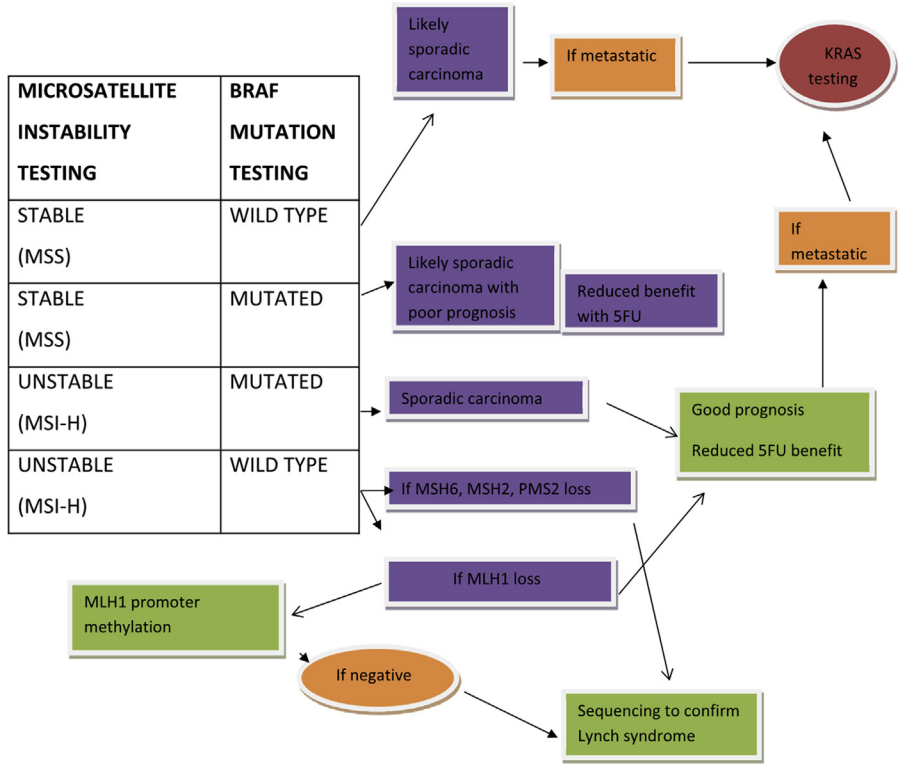

Fig. 11. Algorithmic strategies for prognosis and prediction of therapeutic response. 5-FU, 5-fluoracil; MSI-H, high-frequency microsatellite instability. (*Modified from* Funkhouser WK Jr, Lubin IM, Monzon FA, et al. Relevance, pathogenesis, and testing algorithm for mismatch repair–defective colorectal carcinomas. A report of the Association for Molecular Pathology. J Mol Diagn 2012;14(2):97; with permission.)

group recommends triage of unassigned 6% cases to referral laboratories performing high volumes of hypermethylation, sequencing, and deletion testing for resolution of subgroup assignment. An additional subgroup, comprising 1.7% of the cases (those assigned to the Lynch syndrome subgroup), would also be referred to define the germline mutation/deletion involved. The recommendation is based on the expectation that cost of testing is less than the cost of delayed diagnosis and absent surveillance of Lynch carriers.[32] The National Comprehensive Cancer Network (available from: www.nccn.org) recommends the use of the Amsterdam or revised Bethesda criteria as the initial screening step. This approach would miss the diagnosis of 5% to 58% of new cases of the Lynch syndrome, as well as most sporadic defective MMR CRC cases.

The Evaluation of Genomic Applications in Practice and Prevention model estimated detection rates and costs of testing using 4 different testing strategies:

a. MMR gene sequencing/deletion testing on all probands;
b. MSI testing, followed by MMR gene sequencing/deletion testing on all high-frequency MSI cases;
c. IHC testing, with protein loss guiding targeted MMR gene sequencing/deletion testing; and
d. IHC, with BRAF c.1799T>A testing of cases with MLH1 protein loss. Each of these would fail to detect all defective MMR CRC.

A similar comparison of 4 strategies, each starting with a single test, was recently published by the US Centers for Disease Control and Prevention, with similar limitations to the Evaluation of Genomic Applications in Practice and Prevention model. The IHC sequencing strategy and IHC_/_ BRAF c.1799T>A sequencing strategy were more cost effective for the diagnosis of Lynch syndrome probands and carriers. However, 11% to 12% of cases of the Lynch syndrome would not be diagnosed because of the absence of MSI testing to identify high-frequency MSI tumors with normal IHC in patients with the Lynch syndrome. Published recommendations by other investigators and clinical groups also exist. The aim of molecular subgrouping is improved diagnostic accuracy and appropriate therapy, genetic counseling for patients with germline MMR mutations, and appropriate counseling and screening of unaffected family members of patients with the Lynch syndrome.[32,33]

MOLECULAR INVESTIGATION OF LYMPH NODES IN PATIENTS WITH COLON CANCER USING ONE-STEP NUCLEIC ACID AMPLIFICATION

A diagnostic system called one-step nucleic acid amplification, has recently been designed to detect cytokeratin 19 messenger RNA as a surrogate for lymph node metastases. In a study by Güler and colleagues,[28] analysis of lymph nodes reported negative after standard examination with hematoxylin and eosin resulted in upstaging 2 of 13 patients (15.3%). Compared with histopathology, one-step nucleic acid amplification had a 94.5% sensitivity, 97.6% specificity, and a concordance rate of 97.1%. However, insufficient data are available for routine use in standard clinical practice.[80,81]

MOLECULAR STAGING INDIVIDUALIZING CANCER MANAGEMENT

GUCY2C is a member of a family of enzyme receptors synthesizing guanosine 3′5′ cyclic monophosphate from guanosine-5′-triphosphate, which is expressed on intestinal epithelial cells but not in extraintestinal tissues. The expression is amplified in colorectal carcinoma compared with normal intestinal tissues. It is identified in all

colorectal human tumors independent of anatomic location or grade, but not in extragastrointestinal malignancies. Therefore, it has a potential application in identifying occult metastases in the lymph nodes of patients undergoing staging for CRC. However, there are insufficient data to support its use in standard clinical practice.[82]

NOVEL MOLECULAR SCREENING APPROACHES IN COLORECTAL CANCER

Stool DNA potentially offers improved sensitivity, specificity, and cancer prevention by the detection of adenomas. The basis for stool DNA screening is the identification of genetic alterations in the initiation of a sequenced progression from adenoma to carcinoma, such as mutations in APC, KRAS, DCC, and p53. Key genetic alterations seen in many hereditary forms of CRC correspond with genetic alterations in sporadic CRC, indicating that the somatic occurrence of these genetic alterations leads to the initiation and progression of CRC and supports the targeting of these genes for generalized population screening. DNA methylation of CpG islands of known CRC markers has been shown in DNA samples from serum and stool samples of patients with CRC. SFRP2 methylation in fecal DNA was evaluated for detection of hyperplastic and adenomatous colorectal polyps. SFRP methylation was not found in healthy controls.[83]

PREDICTIVE AND PROGNOSTIC MARKERS

Quantitative Multigene Reverse Transcriptase Polymerase Chain Reaction Assay

Quantitative gene expression assays to assess recurrence risk and benefits from chemotherapy in patients with stages II and III colon cancer have been evaluated and are commercially available. The test provides information on the likelihood of disease recurrence in colon cancer (prognosis) and the likelihood of tumor response to standard chemotherapy regimens (prediction). The Oncotype D_x colon cancer assay evaluates a 12-gene panel consisting of 7 cancer genes and 5 reference genes to determine the recurrence score. This score was validated in the QUASAR (Quick and Simple and Reliable) study. The score improves the ability to discriminate high-risk from low-risk patients who have stage II colon cancer beyond known prognostic factors even in the cohort of apparently low-risk patients. Similar proportional reductions in recurrence risks with 5-fluorouracil/leucovorin chemotherapy were observed across the range of recurrence scores. Another Oncotype D_x score was validated in the National Surgical Adjuvant Breast and Bowel Project C-07 study, which differentiated risk of recurrence for patients with stage III disease and in the context of oxaliplatin-containing adjuvant therapy.[25,65,83–85]

Future trends

Other gene mutations associated with resistance to anti-EGFR therapy

- KRAS mutations at codons 61 and 146,
- PIK3CA exon 20 mutation, and
- PTEN protein inactivation.[33]

MicroRNAs Upregulated microRNAs in colorectal carcinoma include miR-96 oncogenic microRNAs involved in key signaling pathways. miR-96, miR-21, miR-135, and miR17 to 92 potentially target CHES1 (transcription factor involved in apoptosis inhibition). miR-21 correlates with the downregulation of tumor suppressor protein PDCD4. It may target PTEN, a tumor suppressor gene. miR-135a and miR135b correlate with reduced expression of the APC gene. Overexpression of miR17-92 results in the suppression of the antiangiogenic factors Tsp1 and connective tissue growth factor. It also mediates myc-dependent tumor growth promoting.

Downregulated microRNAs The downregulated microRNAs include 143 and 145, 31, 96, 133b, 145, and 183. microRNA-133 targets kras, which is known to be involved in signaling pathway for cell proliferation. Expression level of microRNA-31 correlates with development and stage of colorectal carcinoma. Experimentally mediated overexpression of microRNA-34a subsequent effects associated with actions of p53, such as cell cycle arrest and apoptosis, could be phenocopied. There is potential for early detection and staging. It detects precancerous adenomas. It relies on real-time qualitative PCR, which yields results within a 24- to 48-hour period.[85] hsa-miR-663b, hsa-miR-4539, hsa-miR-17-5p, hsa-miR-20a-5p, hsa-miR-21-5p, hsa-miR-4506, hsa-miR-92a-3p, hsa-miR-93-5p, hsa-miR-145-5p, hsa-miR-3651, hsa-miR-378a-3p, and hsa-miR-378 have been reported to be differentially expressed in colorectal carcinoma versus normal colonic mucosa. On comparison of MSI and MSS tumors, the majority of differentially expressed microRNAs were downregulated. The microRNAs most significantly associated with survival include miR-196b-5p, miR-31-5p, miR-99b-5p, miR-636, and miR-192-3p. Higher levels of expression increase the risk of dying from colon cancer, but improve survival if diagnosed in rectal cancer. miR-196a-5p and miR-196b-5p were downregulated in both CIMP-high and BRAF-mutated tumors. Tp53 mutated tumors revealed significant difference in expression of miR-224, miR-17, miR-1226, miR-532-5p, miR-17, miR-574-5p, miR-424, and miR-16. KRAS-mutated tumors revealed significant downregulation in expression of microRNA-204-3p, upregulation in miR-4255, and miR-518e-5p.[85–88]

EPIGENETIC INACTIVATION OF ENDOTHELIN 2 AND ENDOTHELIN 3 IN COLON CANCER

Therapeutic strategies target overexpressed members of the endothelin axis via small molecule inhibitors and receptor antagonists, but this work supports a complementary approach based on the reexpression of endothelin 2 and endothelin 3 as natural antagonists of endothelin 1 in colon cancer.[89]

Role of Programmed Death Ligand 1 Expression in Colorectal Carcinoma

At the time of detection of a tumor, the balance of power between the immune system and the cancer has shifted in favor of the growing tumor, and a state of immune tolerance has been established. The goal of cancer immunotherapy is to reestablish a targeted antitumor immune response. Blockade of inhibitory immune check point molecules enhances immune response to tumors. Immune checkpoint blockade targeting the programmed death-1 (PD-1) pathway has shown efficacy in several types of cancers, including MMR-deficient colorectal carcinoma. PD-L1 expression detected by immunohistochemistry has shown usefulness as a predictive marker for response to anti–PD-1 therapies. Most colorectal carcinomas with significantly increased lymphocytes fall into the MMR-deficient subset. The tumors also possess clinicopathologic parameters associated with MMR deficiency. The features included medullary morphology, a right side location, younger age, higher tumor infiltrating lymphocyte score, and peritumoral lymphocyte aggregates. In a study characterizing PD-L1–positive colorectal tumors, the significant features included poor differentiation, MMR deficiency, “stemlike” immunophenotype defined by loss or weak expression of CDX-2, and stem cell marker ALCAM positivity. Eighty-eight percent of the tumors also revealed the BRAF V 600E mutation. These features are associated with tumors arising via the serrated neoplasia pathway. In 1 study, it was found that 5% of colorectal carcinomas exhibited high tumor PD-L1 expression and 19% had increased PD-1–positive tumor-infiltrating lymphocytes. MMR-deficient tumors had significantly higher rates of PD-1 and PD-L1 expression and a stronger intensity of staining when

compared with MMR-proficient tumors. Tumors with proficient MMR function (96%) are less likely to respond to anti-PD1 therapy. Further, PD-1/PD-L1 expression stratified recurrence-free survival in an interdependent manner. Patients whose tumors had both PD-1–positive tumor infiltrating lymphocytes and high PD-L1 expression had a significantly worse recurrence-free survival rate. Tumors with high PD-1–positive tumor-infiltrating lymphocytes and low-level PD-L1 expression revealed improved recurrence-free survival rates.[90,91]

Cell-free nucleic acid analysis

Although solid tissue based analysis has been the mainstay of CRC diagnosis, interrogation of cell-free DNA (*cf*DNA) in fluids including serum, plasma, urine, spinal fluid has proven beneficial for diagnosis and prognosis of these disease states.[92–97] Studies by Pereira and colleagues[94] showed that in 78% of the samples tested (100/128), there were detectable somatic genomic alteration in studies comparing formalin-fixed, paraffin-embedded tissue from prior resections or biopsies with *cf*DNA obtained from peripheral blood samples in certain cases. In addition, 50% of *cf*DNA cases had potentially actionable alterations, and physicians reported that the cfDNA testing improved the quality of care they could provide in 73% of the cases. Furthermore, 89% of patients reported greater satisfaction with the efforts to personalize experimental therapeutic agents. Studies by Zhuang and colleagues[95] showed in a meta analysis that KRAS mutation in *cf*DNA obtained from plasma or serum was associated with a poorer survival in patients with cancer for overall survival in patients with CRC and that ethnicity did not seem to influence the prognostic value of this mutation. Similarly, other metaanalyses[97] have suggested that the *cf*DNA of both KRAS and BRAF mutations can serve as poor prognostic biomarkers associated with worse survival outcomes in patients undergoing hepatic resection as a result of CRC-related liver metastasis. Hypomethylation of long interspersed nuclear element-1 in plasma *cf* DNA obtained from patients with CRC with large tumors ($\geq$6.0 cm), advanced N stage ($\geq$2), and distant metastasis (M1) had statistically significantly higher *cf*DNA long interspersed nuclear element-1 hypomethylation index than other patients with CRC.[96] Furthermore, patients with early stages I and II CRC as well as patients with advanced stages III and IV CRC had significantly higher *cf*DNA long interspersed nuclear element-1 hypomethylation index than healthy donors, suggesting *cf*DNA long interspersed nuclear element-1 hypomethylation index as a disease progression biomarker for CRC.[96] *cf*DNA analysis in CRC may provide timely information on potentially actionable mutations and amplifications, thereby facilitating clinical trial enrollment, personalized treatment and improving the overall quality of care.

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