

# Histologic and Immunochemical Assessment of Colorectal Cancers

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## LEARNING OBJECTIVES

1. Define the grade and stage system used to classify colorectal cancers.
2. Summarize how staging is useful in prognosis and treatment.
3. List and describe the most common markers used in the diagnosis and prognosis of colorectal cancer.
4. Describe Lynch syndrome and the role of mismatch repair proteins.

## ABSTRACT

Traditional assessment of colorectal cancer includes gross anatomy, routine histology, special stains, and immuno-histochemistry. Newer methods, including molecular techniques, can better predict recurrence potential and directed treatments. Distinction of benign versus malignant neoplasms leads to a series of additional tests that are useful in guiding diagnosis, prognosis, and treatment. In this article, we focus on the histological assessment of grade and stage of the tumor, utilizing the College of American Pathologists and the American Society for Clinical Oncology guidelines. We then discuss the definition and utility of cancer staging in the determination of treatment. The 5-year median survival rate estimate is based on these staging principles; thus, practitioners utilize this system to develop a precise treatment plan taking individual patient variables into consideration. Screening of the patient for specific tumor markers from the serum or on the excised neoplasm further helps elucidate cancer subtype and therapy. Markers, such as carcinoembryonic antigen C-reactive protein in the serum as well as numerous immunohistochemical markers, are utilized for this purpose. Finally, we examine Lynch syndrome, mismatch repair proteins, and microsatellite instability as additional markers and potential treatment targets.

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**ABBREVIATIONS:** APC - adenomatous polyposis coli, CA - carbohydrate antigen, CAP - College of American Pathologists, CEA - carcinoembryonic antigen, CK - cytokeratin, CRC - colorectal cancer, FFPE - formalin-fixed and paraffin-embedded, H&E - hematoxylin and eosin, IHC - immuno-histochemistry, MMR - mismatch repair, PAS - periodic acid-Schiff.

**INDEX TERMS:** histopathology, colorectal cancer staging, immunohistochemistry, tumor markers.

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## INITIAL HISTOLOGICAL ASSESSMENT

Traditional histological assessment of colorectal cancer (CRC) includes size of the polyp, histologic type, tumor grade, stage, and lymph or vascular invasion.<sup>1</sup> Newer methods, including molecular techniques, can better predict recurrence potential and directed treatments. Polyps removed during routine colonoscopy are first sent to the anatomic pathology gross room for examination and preliminary assessment. Appearance of the tumor on examination of the patient and in the gross room gives the laboratorian an idea of the type of tumor and cancerous process. Benign tumors tend to be encapsulated, with an expansile growth pattern (similar to blowing up a balloon).<sup>2</sup> Gross room bisection of the benign tumor reveals a homogenous cut surface, typically lacking a necrotic core. By contrast, growth of malignant tumors tends to be invasive rather than expansive. Necrotic cores and a nonhomogenous surface are commonly seen as well as invasion into the lymphatic system and surrounding blood vessels. The grossing pathologist sampling the tissue can submit the bisected polyp in toto, meaning the entire polyp is placed into a cassette for processing and histological examination. Invasive CRCs that arrive in the gross room with sentinel lymph nodes and underlying tissue require sampling of various components of the tissues and multiple cassettes submitted for histology processing.

Initial microscopic evaluation of the tumor involves examination of the hematoxylin and eosin (H&E)-stained section to assess preliminary grading of the tumor for recapitulation of normal features (Table 1). Pathologists grade the specific tumor type according to well-defined grading criteria utilizing the College of American Pathologists (CAP) and the American Society for Clinical Oncology

**Table 1.** Histological morphology of tumors with H&E staining**Histologic Morphology**

Grade 1 (low)	Well differentiated: tumor mimics surrounding tissue morphology, gland formation, mucous production
Grade 2 (low)	Moderate differentiation: gland formation decreased (50%–75%), some recapitulation of normal features
Grade 3 (high)	Poorly differentiated: glands present but disorganized, decreased greatly; difficult to ascertain normal architecture
Grade 4 (high)	Undifferentiated: parent tissue morphology no longer recognizable

Preliminary observation of the tumor using H&E staining may be useful in the designation of low- or high-grade tumor. Additional investigation, special stains, and immunohistochemistry are required for an in-depth examination of the tumor.

guidelines.<sup>3,4</sup> Well-differentiated colorectal tumors that recapitulate normal colon epithelial histology with greater than 95% gland formation are designated grade 1. Mucus production can also be a component assigning grade, as grade 1 tumors tend to have near-normal mucus-producing cells.<sup>5</sup> Mucin can be assessed on the H&E slide, or additional special stains, such as the periodic acid–Schiff (PAS) or mucin stain, may be performed.<sup>6</sup> Grade 2 and grade 3 tumors reveal less differentiation and gland formation between 50% and 95% (grade 2) or less than 50% (grade 3).<sup>1</sup> Mucus may or may not be present, dependent on the specific subtype of the tumor, as described previously. Grade 4 tumors lack normal colon epithelial appearance, with no gland formation or mucin production. The cancer grade is an important component of the overall staging of cancers because it provides the tumor characteristics that are a component of the cancer staging. Low-grade tumors generally have a better prognosis and respond better to treatment than do high-grade neoplasms.

## CANCER STAGE

Cancer staging includes the examination of the microscopic tumor characteristics in addition to the degree of spread and migration to distant sites. The stage (I–IV) is assigned at the time of diagnosis of CRC but may be updated as the tumor progresses or the patient responds to treatment. Staging gives the physician and patient an idea of the severity of the disease and guides the treatment plan. Low-stage cancers may often be treated with surgical removal alone, whereas higher staging scores may require a much more aggressive treatment plan. Cancer staging involves 3 main components known as the TNM score. In this scoring system, the T stands for tumor characteristics, the N for lymph node involvement, and the M for metastasis. According to the American Joint Committee on Cancer Staging guide, CRC T scores vary from Tis (in situ dysplasia) to T4 (invasion into visceral peritoneum or adjacent organs).<sup>1</sup> The N score ranges from no lymph node invasion (N0) to migration in 4 or more regional lymph nodes (N2). When the lymph nodes cannot be assessed, the score is assigned as NX. The final component is distant metastasis, which is either absent (M0), present (M1), or unable to assess (MX). The scores for

the 3 categories are then used to assign the stage, and any metastasis is automatically categorized as stage IV.<sup>7–9</sup> The 5-year median survival rate is based on reports of patient mortality after being initially assigned a specific stage. Patients assigned to a higher stage may elect for more aggressive or experimental treatment methods. Early detection is key to survival because tumors identified as stage I have a mean 5-year survivability of 74%.<sup>1</sup> This drops as the stage increases, with a 5-year survival rate for stage IV CRCs with multiple metastases of less than 15%.<sup>10,11</sup> Although it is important to include the staging in any discussion with the patient regarding prognosis, it is also essential to include patient characteristics in the treatment scheme. Variables such as age, sex, nutritional status, race, and comorbidities have been shown to have a significant effect on outcomes,<sup>10,12–14</sup> but perhaps even more important is the patient's quality of life and risk versus reward stratification in the decision-making process. Recommendations from the National Cancer Institute's Physician Data Query for adults with CRC are shown in Table 2.<sup>15</sup> As can be seen from this table, stage 0–II CRCs are typically treated with surgery alone. A stage III diagnosis will involve adjuvant chemotherapy, usually with a 5-fluorouracil derivative or platinum-based chemotherapy. Stage IV CRC (with distant metastasis) requires more aggressive treatment, often dependent on the organ of metastasis. Treatment options for these patients include (in addition to surgery) chemotherapy, ablation of the metastatic site, and novel therapies.<sup>16–18</sup>

## CRC MARKERS

Screening of the patient for specific tumor markers may be performed from the serum or the excised polyp (See Table 3). Carcinoembryonic antigen (CEA) is a protein that is normally expressed in fetal tissues as a cell adhesion marker. Increased expression of CEA in the serum or colon polyp is a nonspecific marker for carcinomas in general<sup>15,19,20</sup>; however, it should not be used as a general screening tool for CRC because it may be elevated in several nonneoplastic conditions. Increasing levels of CEA in the serum have been correlated with increased levels of metastases in CRCs. CEA is also useful for monitoring response to treatment because, following surgical

**Table 2.** CRC staging**Standard Treatment for CRC Based on Stage**

Stage	Description	Treatment
Stage 0	Carcinoma in situ	Surgery for removal (if margins on polyp not clear)
Stage I	Low grade, no nodal involvement	Surgery (as above)
Stage II	High grade, no nodal involvement	Surgery (as above)
Stage III	Any grade, invasion of lymph nodes	Surgery and adjuvant chemotherapy
Stage IV	Metastatic	Surgery, chemotherapy, radiation

The CRC stage is determined at the time of diagnosis and involves the tumor characteristics (T), regional or distant lymph node involvement (N), and metastasis (M). Any metastatic tumor is automatically stage IV regardless of the T and N scoring. Treatment becomes more aggressive as the stage increases and may include other therapies beyond the traditional surgery, chemotherapy, and radiation. (Adapted from the PDQ Adult Treatment Editorial Board colon cancer treatment guidelines.<sup>15</sup>)

resection of the carcinoma, CEA should return to normal levels in the serum. The CEA test is an immunoassay performed on serum, and normal values are typically less than 3 ng/mL.<sup>21</sup> Another nonspecific marker in the serum in CRC patients is C-reactive protein.<sup>22</sup> This marker is increased dramatically in cases of inflammation and therefore may indicate any number of possible disorders, including inflammatory bowel disease (such as Crohn's disease or irritable bowel syndrome).<sup>23</sup> Additional serum markers that have been used in monitoring patients with advanced disease include carbohydrate antigen (CA) 19-9, tissue inhibitor of metalloproteinases type 1, CA 242, interleukin-6, and soluble CD40 ligand.<sup>19,22,24</sup>

The major uses for immunohistochemistry (IHC) staining in CRC include diagnosis of neoplasms, prognostication, choice of pharmacotherapy, and monitoring response to therapy.<sup>6,15,25</sup> IHC staining may be performed on the same tissue submitted for routine processing or may come from separate sections that have been frozen instead of going through formalin fixation. These sections are ideal due to the retention of immunogenicity in the tissue but may not always be possible because some IHC stains are requested after the tissue has been submitted. In addition, IHC staining that is performed on duplicate formalin-fixed and paraffin-embedded (FFPE) sections as the H&E stain provides a comparison between 2 nearly identical sites in the case of serial sections.<sup>6</sup> If working with FFPE sections, CAP guidelines recommend some form of antigen retrieval method to expose the antigenic proteins within the tissue.<sup>26</sup> Heat or enzymatic methods of antigen retrieval accomplish this task with minimal disruption to normal tissue architecture. Regardless of the specific method, it should be first developed and validated according to the laboratory's procedures and CAP's guidelines. Maintaining a tissue bank of known positive and negative biopsies is useful in validation, which may be retained on site or requested from tissue banks.

In the case of a poorly differentiated tumor, an entire battery of IHC stains is generally indicated in order to differentiate the many different types of tumors. Cytokeratins (CKs) are a group of intermediate filaments

numbered 1–20 found in epithelial cells,<sup>27</sup> and a pancytokeratin stain that looks for multiple CKs simultaneously can be used to verify a carcinoma, especially one that has invaded lymph nodes or metastasized to distant sites.<sup>28</sup> The most common pancytokeratin marker is AE1/AE3.<sup>29</sup> However, by examining specific CKs, certain gastrointestinal cancers can be differentiated more accurately. For example, colorectal adenocarcinomas are generally positive for CK20 and negative for CK7.<sup>28,30</sup> Epithelial mucins can be detected in mucinous tumors with special stains, such as the PAS stain,<sup>6</sup> or by utilizing antibodies for specific mucin phenotypes, such as MUC2, MUC5AC, and MUC6.<sup>5</sup> Increased MUC2 is also seen with microsatellite instability and may be useful in predicting resistance to platinum-based chemotherapy drugs.<sup>31</sup> Additional IHC markers that have been referenced in the literature for diagnosis and/or prognosis of CRC include  $\alpha$ -methyacyl-CoA racemase, villin, homeobox protein CDX-2, beta-catenin, and cadherin-17.<sup>30,32</sup>

Markers that are not specific for CRC can also be useful in examining the phenotype of the tumor cells in order to obtain a clearer picture of disease progression and ongoing mutations as the tumor undergoes uncontrolled proliferation. Cells undergoing rapid division can be examined using the proliferation markers Ki-67 and proliferating cell nuclear antigen because slower tumor growth leads to an improved prognosis. Tumor suppressor proteins that may become mutated during cancer progression include adenomatous polyposis coli (APC) and p53. These proteins are tasked with arresting the cell cycle when there is damage to the DNA or uncontrolled proliferation, and mutations in these genes lead to a worse prognosis. An estimated 60% of CRCs exhibit mutations in p53,<sup>33</sup> which is one of the most commonly mutated genes in all cancer types. Tumor suppressors can still function at normal levels when only 1 of the 2 genes encoding tumor suppressor proteins (APC, p53) is mutated. This is referred to as the "2-hit" hypothesis. Proto-oncogene mutations result in the production of oncogenes producing aberrant proteins that only require 1 gene to be mutated to result in

**Table 3.** Common markers used in screening, diagnosis, and prognosis of CRC**Common Markers for CRC**

Marker	Description	Notes
CEA	Carcinoembryonic antigen	Serum or IHC (tumor), nonspecific. Monitor response to therapy
CRP	C-reactive protein	Serum, nonspecific inflammation
AE1/AE3	Pancytokeratin	IHC, tumor of epithelial origin (carcinoma)
CK7	Cytokeratin 7	IHC, CRC negative (differentiation from other tumor types)
CK20	Cytokeratin 20	IHC, CRC positive (differentiation from other tumor types)
MUC2	Mucin 2	IHC, mucinous CRC
AMACR	$\alpha$ -Methacyl-CoA racemase	IHC, increased in some CRC
Ki-67	Ki-67 nuclear protein	IHC, marker of proliferation
APC	Adenomatous polyposis coli	IHC, tumor suppressor protein
p53	Phosphoprotein p53	IHC, tumor suppressor protein
B-Raf	B-Raf protein kinase	Serum, IHC, genetic screening (proto-oncogene)
K-Ras	K-Ras GTPase	Serum, IHC, genetic screening (proto-oncogene)
MMR	Mismatch repair proteins	Serum, IHC, genetic screening ( <i>MLH1</i> , <i>MSH2</i> , <i>MSH6</i> , <i>PMS2</i> )

a cancerous phenotype. Proto-oncogenes that may be mutated in CRC include *BRAF* and *KRAS*.<sup>34,35</sup>

## DNA REPAIR AND LYNCH SYNDROME

Lynch syndrome, historically referred to as hereditary non-polyposis CRC, is an inherited condition that increases the risk of developing CRC and other cancer types throughout the life of the individual. In “nonpolyposis” CRC, the cancer can develop when there are very few polyps and in some cases without the development of polyps at all. Lynch syndrome accounts for 2%–3% of all CRCs but is the most common form of hereditary colon cancer.<sup>36</sup> Individuals with Lynch syndrome inherit 1 or more germline mutations from a heterogenous group of genes with a similar function: DNA repair. These genes produce proteins that arrest cell cycle progression when base pair mismatches in DNA are detected during replication and activate mismatch repair (MMR) mechanisms that correct the aberrant sequence back to the original functional gene. During normal DNA replication, the enzyme DNA polymerase pairs the nitrogenous bases adenine and guanine to thymine and cytosine, respectively. Because DNA replication is semiconservative, a parent strand of DNA is used as a template for the developing daughter strand. If a mismatch occurs (adenine to cytosine or guanine to thymine), the repair proteins remove an entire segment of the daughter strand (identified through lack of methylation) and allow DNA polymerase to correct the error.<sup>37</sup>

The MMR system genes in the human include *MLH1*, *MSH2*, *MSH6*, and *PMS2*,<sup>38</sup> and mutation in any of these will increase the likelihood of developing CRC.<sup>39</sup> Inherited mutations of these MMR genes (Lynch syndrome) occur in every cell of the body, consequently increasing the

individual’s likelihood of developing multiple different cancer types. Because it is in every cell, the screening test for Lynch syndrome can be done from virtually any sample, including blood or saliva. An important point to consider is that testing for Lynch syndrome can be conducted on an individual who does not present with CRC and can be used to predict their risk for the development of multiple different cancer types. Nonhereditary mutations in the MMR genes are also possible as a CRC develops new mutations during uncontrolled proliferation, and, therefore, MMR mutation screening is also utilized for identified CRCs. This screening can be done directly on the tumor biopsy using IHC for the 4 previously mentioned MMR proteins, polymerase chain reaction–based microsatellite instability assay, or validated next-generation sequencing techniques.<sup>40</sup> Patients with Lynch syndrome or neoplastic loss of MMR proteins and microsatellite instability may also benefit from a new class of antibody therapies called immune checkpoint inhibitors (pembrolizumab, nivolumab, etc.).<sup>41</sup>

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