Chapter 5
Pathology of Rectal Cancer and Predictors of Response to Neoadjuvant Therapy

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Introduction

It has been reproducibly shown that rectal cancer patients managed by a multidisciplinary team of physicians yield better outcomes [1, 2]. The pathologist along with the surgeon, the medical oncologist and the radiation oncologist plays a key role in this team. The pathologist's role is important at all stages of patient treatment; namely: the preoperative stage confirming a diagnosis of malignancy on biopsy specimens, the intraoperative stage evaluating the distal margin of resection and the postoperative stage in the examination of the surgical specimen. The anatomical extent of the disease as determined by the pathological stage, the depth of tumor infiltration into the wall of the rectum and the status of the mesorectal lymph nodes have traditionally been the most important parameters guiding postoperative treatment.

In recent decades it has become apparent that the quality of the surgery, in part reflected by the integrity of the mesorectum excision has a significant impact on the incidence of both local and distant recurrences. Moreover, a shift from the distal to the circumferential margin of resection as the most influential factor in predicting local recurrence has become clearly evident. In this respect, the participation of the pathologist in the multidisciplinary team is well established in many centers in Europe, with a rather slow but general acceptance of this point across the United States. Regrettably, the pathologist's participation in the treatment of patients with rectal cancer worldwide still remains somewhat limited. One of the reasons for this absence is the historical tendency of pathologists to practice their work to a degree isolated from the rest of the health care providers involved in the management plan. In order to ensure comprehensive and effective care for this population of patients, this approach needs to be modified incorporating the pathologist as an integral part

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of the MDT. This labor can only be accomplished by full acceptance of this principle not only by the pathologist but also by clinicians and surgeons alike. It is therefore imperative to build educational programs that raise awareness around the fundamental role of the pathologist where this type of educational activity and approach have proven exceptionable value in centers in Europe. This chapter focuses in detail on the pathological evaluation of rectal cancer specimens, including gross and microscopic aspects as well as the basic concepts related to molecular characterization of these tumors.

Macroscopic Evaluation of the Rectal Cancer Surgical Specimen

Several studies have demonstrated that certain macroscopic and histological features have the capacity to reflect the quality of the surgery as delivered by the surgeon. These features can be readily recognized by most pathologists at the time of gross and microscopic examination and specifically include:

1. The integrity of the mesorectum.
2. The status of the resected margins with special emphasis on the circumferential margin of resection (the CRM) and
3. The number of dissected lymph nodes.

Mesorectal Quality/Plane of Surgery

The two major events responsible for the significant decrease in the episodes of pelvic recurrence in recent decades in patients with rectal cancer include the widespread acceptance of total mesorectal excision (TME) as a standard of operative care and the introduction of neoadjuvant chemoradiation [3, 4]. Local recurrence, although reduced still occurs and represents a major surgical challenge, in addition to significantly negatively impacting patient outcomes. In concert with the TME concept, it has become evident that the integrity of the mesorectum within the surgical specimen is one of the most critical prognostic factors for both local and systemic relapse [5–8]. The integrity of the mesorectum is directly related to the plane of surgical dissection and the separation of the rectum from the perirectal soft-tissues of the pelvis, based on the anatomical and embryological concepts advanced by Heald [9, 10]. In this widely accepted approach, the plane of surgery is identified at the mesorectum, within the mesorectum (intra-mesorectal) and at the muscularis propria (intramuscular). Based upon the latter anatomical view, the quality of the mesorectal excision (or the specimen mesorectum) can be classified as follows: [11, 12].

1. *Complete/mesorectal plane of surgery*: Intact mesorectum with minimal surface defects or defects in the mesorectal fat less <5 mm with no coning towards the distal margin of the specimen. The CRM is smooth on slicing (Fig. 5.1).

2. *Near complete/intramesorectal pla*ne: Tissue defects larger than 5 mm but without exposure of the muscularis propria (Fig. 5.2).
3. *Incomplete/muscularis propria plane*: Deep defects involving the mesorectal fat that lead to exposure of the muscularis propria. This latter situation carries the highest risk of recurrences as the remaining tissue in the pelvic cavity may contain residual cancer cells. There is little bulk to the mesorectum in these cases with a highly irregular CRM.

These categorizations are modified for abdominoperineal excisions such that:

Complete – the specimen shows a complete circumferential component of striated muscle at the levator insertion point

Near complete – there is no striated muscle with the resection margin formed by the muscularis propria

Incomplete – at the levator insertion point there is no muscularis propria and there may be perforations of the wall and in some cases evident surface tumor

Margins of Resection

Distal Margin

In 1908 Sir Ernest Miles published his landmark article in which he introduced the abdominoperineal resection operation to achieve cure in patients with rectal cancer [10, 13]. At the time the prevalent thought was that rectal cancer recurred mostly due

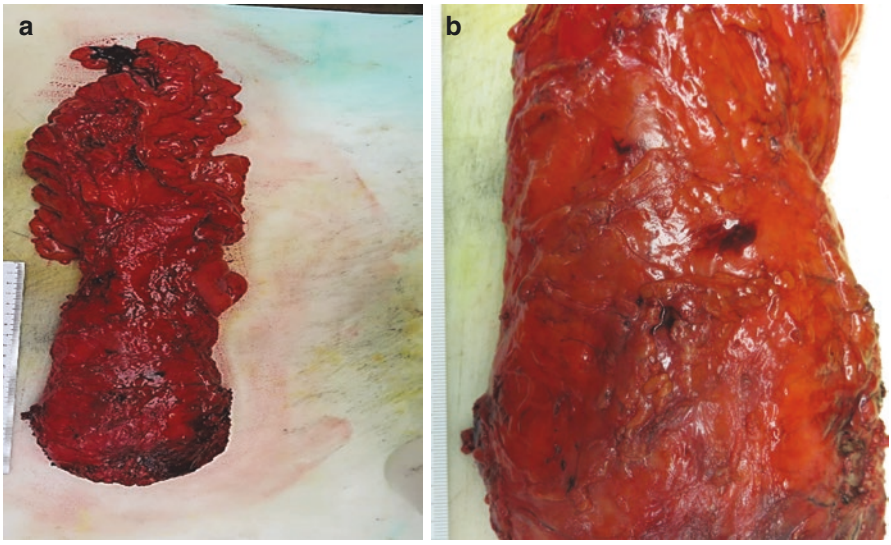


Fig. 5.1 (**a**) Complete mesorectum, anterior surface, no tears or defects are noted. (**b**) Complete mesorectum, posterior surface, bulky mesorectum with no exposure of the muscularis propria

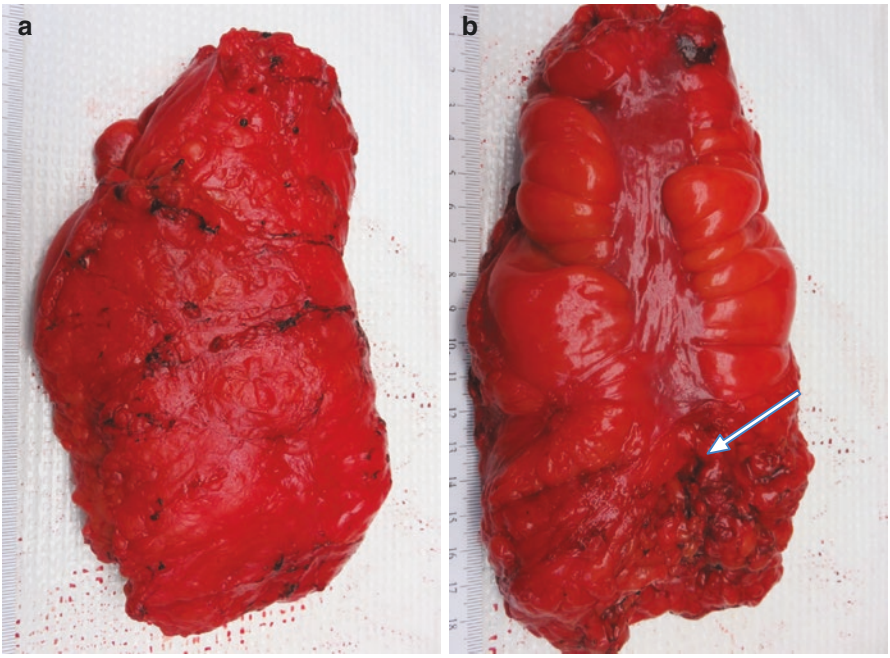


Fig. 5.2 (**a**) Complete mesorectum, posterior aspect, the mesorectal adipose tissue completely wraps the wall of the rectum. (**b**) Incomplete mesorectum, anterior aspect, although there is substantial bulk to the mesorectal envelope, deep defects that expose the muscularis propria are noted

to an inadequate distal margin of resection. For this reason, he proposed that curative rectal cancer surgery could only be achieved by removal of the sphincter complex. This belief spanned most of the first half of the last century. During that time, the distal resection margin was considered to be the only critical margin. This conjecture originated in the fact that cancerous cells distal to the primary mass were a frequent occurrence. This phenomenon is known as "distal tumor spread" (Fig. 5.3) and was first described in 1910 by Cole who identified nests of malignant cells extending up to 4 cm from the distal edge of the primary tumor [14]. The concept gave rise to the "5 cm" rule of distal clearance which held sway [15]. Several subsequent studies published in the 1950s demonstrated that, in actuality, the presence of tumor distal spread beyond 2 cm is a rare event. An important consideration is that in the majority of cases displaying far reaching distal tumor spread, other features of poor prognosis are exhibited, including vascular and perineural invasion as well as lymph node metastasis [16–18]. Subsequent to this realization, distal margins of 2 cm became generally accepted [19, 20]. More recently, with the introduction of surgical techniques such as double stapled anastomosis, as well as the more widespread use of neoadjuvant chemoradiation therapy and the standardization of the TME procedure, 1 cm or even sub-centimeter distal margins have gained greater acceptance [21].

How should we measure the distal margin of resection? And further, who should be doing the measuring? The surgeon of the pathologist? Moreover, should the mea-

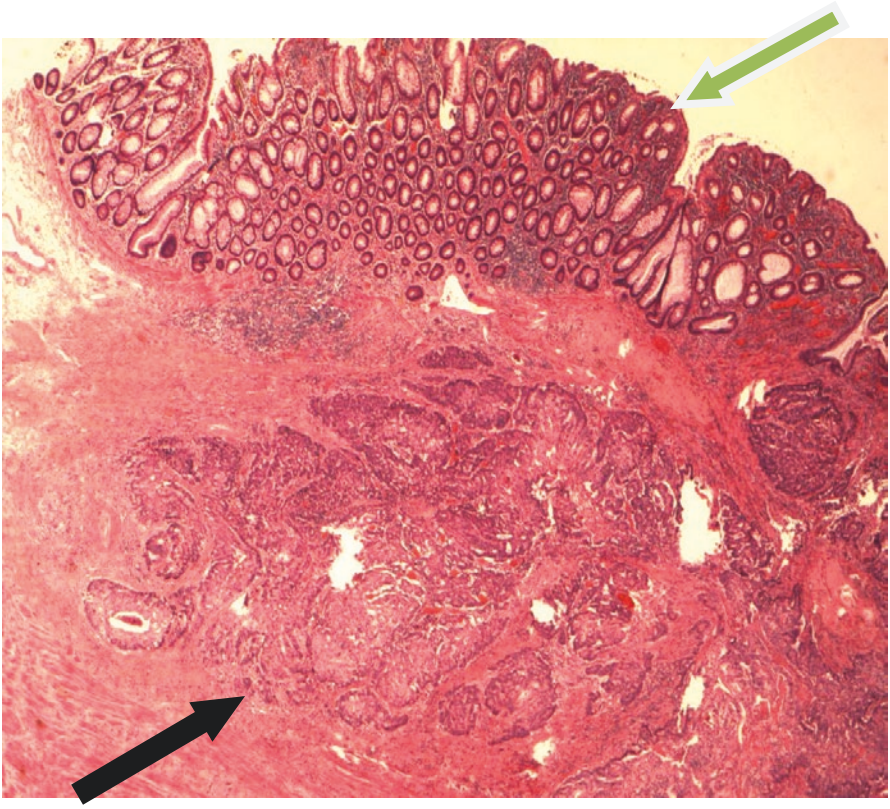


Fig. 5.3 Histological section of an hematoxyilin and eosin stain reveals a focus of discontinuous intramural tumor spread represented by malignant cells extending beyond the main tumor mass (black arrow). Note the benign mucosa overlying the tumor (green arrow)

surements be made *in vivo* or *ex vivo*? And should they be made on the fresh specimen or following formalin fixation? One could argue that the distal margin is better assessed *in vivo* in order to avoid the soft-tissue retraction that normally ensues after resection; an effect which could potentially cause a false decrease in the distance between the tumor and the resection margin [22]. Although the latter occurrence has been described in a few publications, the actual practical influence of this phenomenon is probably minimal [23]. Accurate evaluation of the distal margin is one of the common reasons offered by surgeons to justify the opening of the specimen in the operating room. Although this might be understandable, it is strongly advised, that if there is doubt that an intra-operative consultation is requested. Improper opening of the specimen through the tumor may result in retraction of the mesorectum which will then be difficult to evaluate as will be the circumferential margin of resection. Furthermore, the integrity of the mesorectum should be determined by the pathologist on an unopened specimen where the surface of the resection can be visualized in its entirety. Importantly, prior to any sectioning, the radial margin of resection needs to

be marked with ink, allowing for an accurate measurement on histology. Only after the aforementioned measures are taken should the surgical specimen be partially opened when the intention is to evaluate the distal resection margin specifically. For situations in which the tumor is very close to the distal margin, it is advisable to obtain perpendicular sections so as to include the tumor and the distal margin in one representative slide (Figs. 5.4 and 5.5). This technique will allow a more exact estimation of the distance between the tumor and the distal margin**.**

Circumferential Margin

It is important to remember that rectal cancer used to be associated with a worse prognosis when compared with colon cancer and that principally only obtaining ample distal margins did not significantly decrease the incidence of pelvic

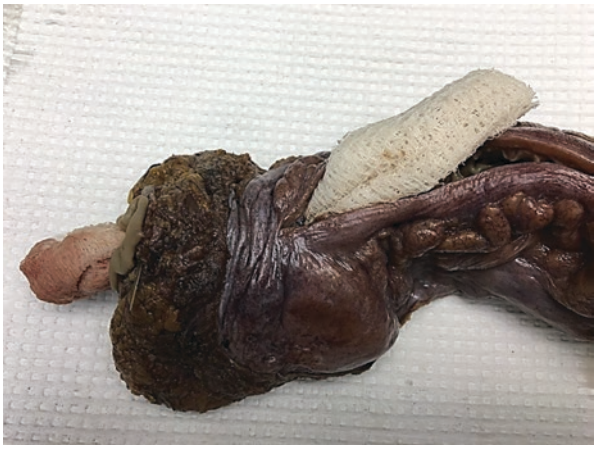


Fig. 5.4 The specimen is opened above and below the tumor and formalin-soaked gauze is introduced to obtain better fixation. This will allow for complete and thin coronal sections that will contain the tumor and surrounding mesorectum

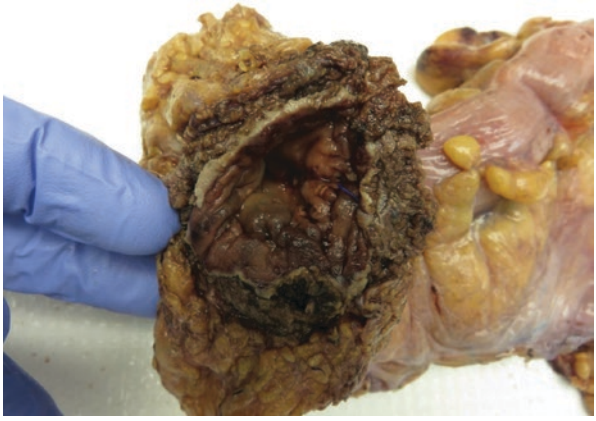


Fig. 5.5 In cases in which the tumor is close to the distal margin, perpendicular sections including the edge of the tumor and the distal margin in the same section allow a more accurate measurement

recurrences. In a landmark study published in 1986, Quirke et al. [24] were able to correlate the high incidence of local recurrence in patients with rectal cancer with the involvement of the circumferential (radial) margin of resection (CRM) rather than with the distal resection margin (Fig. 5.6). This finding was later supported by numerous studies where it has become evident that the presence of tumor ≤ 1 mm from the CRM is not only associated with local recurrence but also with distant metastasis [25–28]. Considering the influence of this parameter in the prognosis of patients with rectal cancer, it is of utmost importance that the pathologist is familiar and proficient with determining its status. Ideally, after recording the objective grading of the mesorectum and after careful palpation along the mesorectal surface to locate the tumoral mass, the entire soft-tissue surrounding the tumor should be ink marked. The purpose of this step is to readily recognize the CRM under the microscope. Although the processing of rectal cancer specimens varies between institutions, it is recommended to follow a specific protocol that will allow a radiology-pathology correlation. Typically the specimen is partially opened caudally and distally, leaving the tumor area itself unopened (Fig. 5.4). Adequate formalin fixation for at least 24 h will allow thin coronal sections (approximately 5 mm in thickness) that should include the tumor and the underlying mesorectum (Fig. 5.7). The number of histological sections varies according to the tumor volume and in also those cases where the patient has received preoperative chemoradiotherapy and where the intention is to assess the gross response of the tumor to treatment (tumor regression grade).

For large tumors, 3–5 sections including the area of the tumor closest to the CRM are usually sufficient. It is important to point out that the distance between the tumor and the CRM should be measured histologically in all cases. In this respect, on histology, involvement of the CRM is the result of 3 specific scenarios; namely:

1. Direct tumor extension
2. A focus of vascular/perineural invasion or tumor deposit and
3. A positive lymph node

Although there are currently no published series addressing the prognostic significance of each of these events, it would be intuitive that direct extension of the tumor into the CRM should carry a more ominous prognosis. In the era of neoadjuvant chemoradiotherapy, marked tumor response can lead to gross disappearance of the neoplasm with only an ulcer or a focus of fibrosis identified in the surgical specimen. In these cases, it is critical to submit the entire area for histological examination so as to capture any potential residual malignant cells and determine their association with the CRM. This approach requires considerable diligence in the era of "watch and wait" management when there has been a complete gross response by the tumor to neoadjuvant therapy. The likelihood of encountering a positive CRM increases with large and deep tumors, those with vascular and perineural invasion, cases of poor tumor differentiation, advanced age and where there are defects in the mesorectal quality [29, 30]. The latter feature has a profound influence on the status of the CRM where logically those specimens with an incomplete peri-tumoral mesorectum are at higher risk of presenting with positive CRMs.

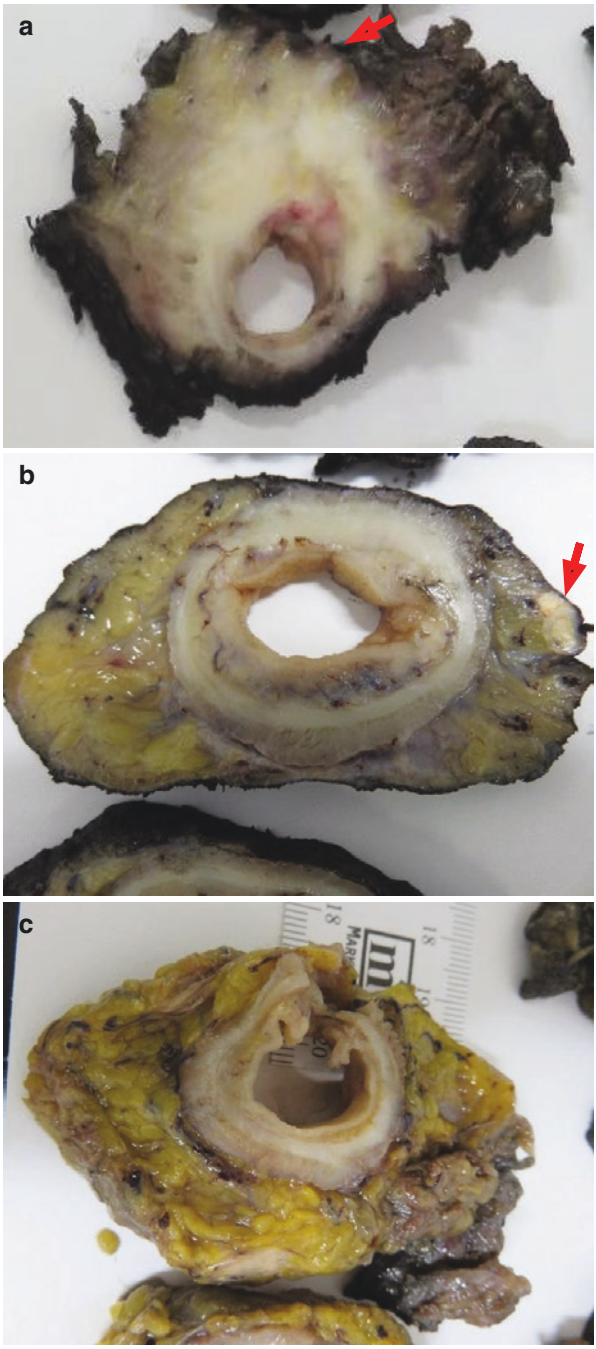


Fig. 5.6 Different patterns of CRM involvement. (**a**) Direct extension of the tumor (arrows). (**b**) Positive node is present at the CRM (arrow). (**c**) discontinuous focus of vascular invasion extends into the CRM (arrow)

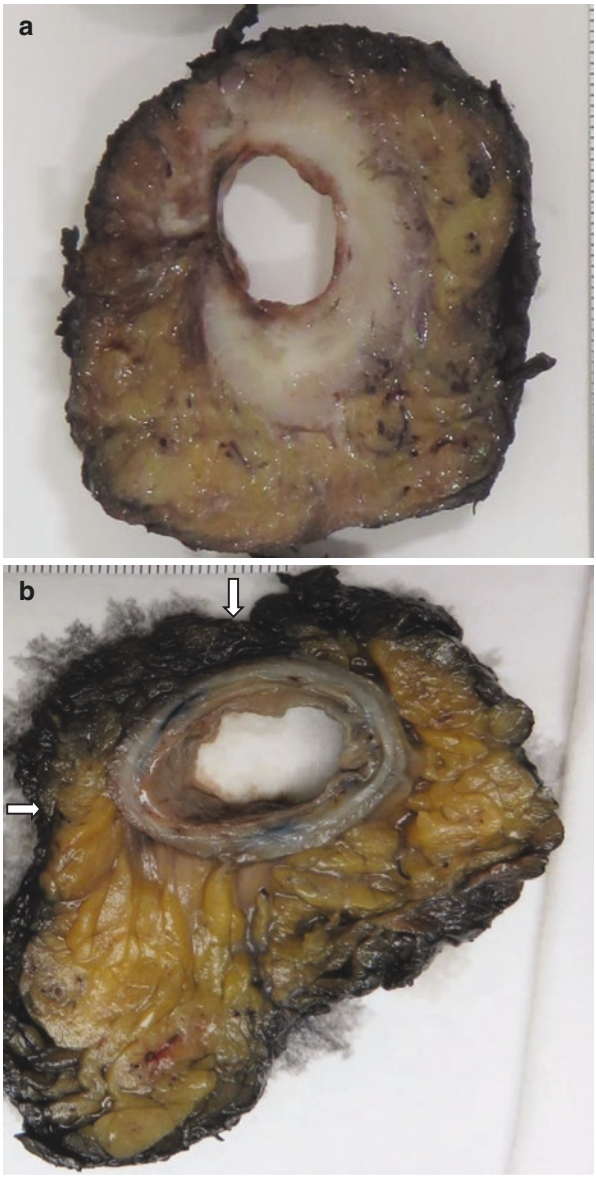
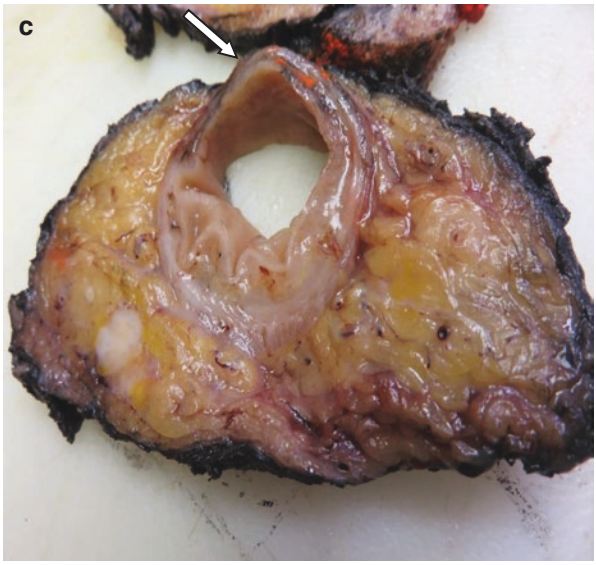


Fig. 5.7 (**a**) Coronal sections of a complete mesorectum shows the wall of the rectum concentrically surrounded by a mesorectal envelope with any defects or tears. (**b**) Cross sections of a near complete mesorectum showing minor defects on the anterior and lateral surfaces that do not expose the muscularis propria (arrows). (**c**) In this coronal section the muscularis propria on the anterior surface is exposed as the result of a large defect (arrow)

Fig. 5.7 (continued)



Lymph Node Evaluation

The number of lymph nodes dissected from colorectal specimens has been shown to correlate with patient outcome, regardless of the positive or negative involvement status [31–34]. This phenomenon appears to be multifactorial and is likely related not only to optimal staging but also to other factors including host age, gender, body habitus and immune response [35–40]. In principle, as the number of nodes retrieved increases so does the chance of the detection of positive nodes. In this regard, given the prognostic effect on survival of positive nodes, then those patients with fewer nodes retrieved are likely to have a worse cancer-specific overall survival. In an ideal world, if the minimum number of nodes required is met within the specimen, the overall hazard risk for death is similar regardless of how many nodes are retrieved. Despite remarkable progress in the field of molecular pathology, pathological stage remains the most important prognostic factor in rectal cancer. In addition lymph node status influences the post-surgical therapeutic decision-making. As the majority of patients with positive lymph nodes will be offered adjuvant chemotherapy, it is then critical that optimal lymph node dissection is carried out by the pathologist. It is important to point out that the rectum inherently contains fewer and smaller lymph nodes when compared with other segments of the intestinal tract [36].

It has been suggested that the number of lymph nodes detected in surgical specimens of rectal cancer reflects the quality of the surgery performed [35] as well as the diligence and effort of the dissecting pathologist. In those cases where there has been an optimal TME, the lymph node harvest depends entirely upon meticulous pathology work, however, in rectal cancer specimens where there is an incomplete

mesorectum, the number of nodes will be logically decreased somewhat regardless of the quality of the work by the pathologist. Currently, the number of lymph nodes considered as "optimal" has been set at 12. In the United States, respected medical organizations such as the American College of Surgeons and the College of American Pathologists have adopted this rather unpopular metric. The establishment of a fixed number of lymph nodes as a reflection of an adequate lymph node dissection has, not surprisingly, created enormous controversy. Furthermore, in rectal cancer patients treated with neoadjuvant therapy, there may be cases in which the number of harvested nodes would be well below this target. In this regard, it has been widely shown that radiation leads to a significant decrease in the number of retrievable mesorectal lymph nodes [41–44], although the relevance of this finding remains unclear. Whilst some studies have suggested that a low number of dissected nodes in rectal cancer specimens following preoperative radiation does not adversely impact patient outcomes, [45, 46] other authors have found a correlation between lymph node harvest post neoadjuvant radiation and survival [47]. A recent meta-analysis and systematic review of 31 articles addressing the number and status of mesorectal lymph nodes in patients with and without neoadjuvant chemoradiation, demonstrated that pre-operative chemoradiation resulted in a mean reduction of 3.9 lymph nodes as well as an average reduction of 0.7 in harvested positive lymph nodes [48]. Individuals who received neoadjuvant radiation only had, on average, 2.1 lymph node less (95% CI 1.7–2.5) resected compared with their counterparts who received no neoadjuvant treatment, show in review of six studies.

Classically, lymph node dissection from rectal cancer specimens is carried out by a combination of palpation and visualization of the mesorectal tissue. This technique results in disparate results as reflected by the extreme variability in lymph node yield noted amongst different centers around the world. In an attempt to increase lymph node harvest from colorectal specimens, several ancillary techniques have been developed. The majority of these are based upon dissolution of the mesenteric/mesorectal fat, a process known as "fat clearing". However, many of the solutions applied for this purpose contain xylene and other chemicals such as acetone, which have been proven to be highly toxic to the operator. Moreover, the majority of these techniques are relatively cumbersome and time consuming [49–51]. A simple and financially feasible alternative consists of the immersion of the mesorectum in pure alcohol for 24–48 h. This method hardens the fat allowing for ultrathin sectioning, while simultaneously whitening the lymph nodes to permit better nodal identification (Fig. 5.8) [47].

Tumor Response to Neoadjuvant Chemoradiotherapy

Over the last two decades, there has been a widespread use of pre-operative therapy to treat patients with advanced rectal cancer with debate concerning the differing radiation and chemotherapeutic scheduling [52–54]. The tumor response to chemoradiotherapy is reflected in volume reduction as well as with tumor downstaging, either in the pT or pN status or in both. Pathological reports of rectal cancer

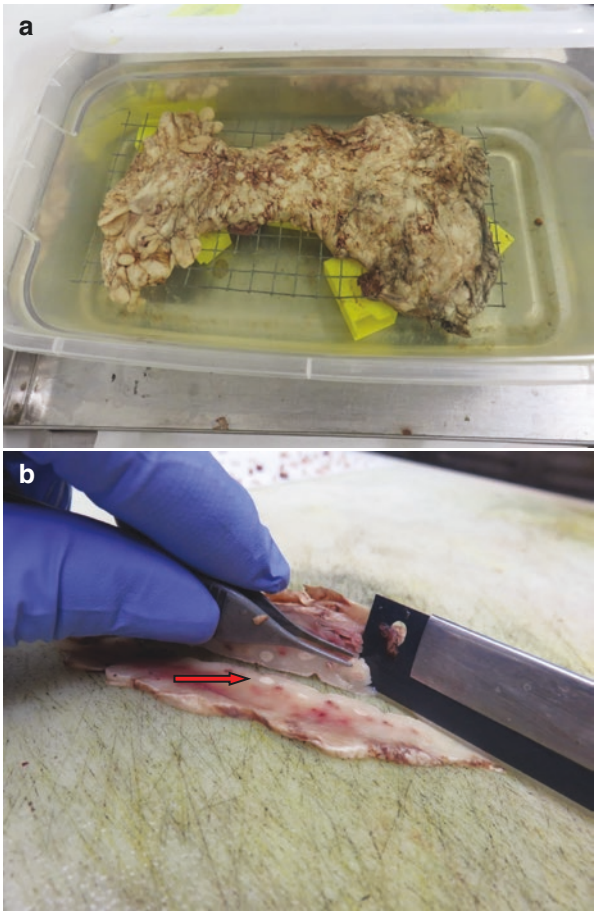


Fig. 5.8 (**a**) Gross picture of the mesorectum after 48 h of alcohol soaking. The adipose tissue becomes firm allowing thin cross sections. (**b**) After fixation in alcohol, the lymphoid tissue is easily recognized in the background of the mesorectal fat (arrows)

specimens following neoadjuvant therapy should always contain the letter "y" preceding the pathological stage (i.e. ypTN) to reflect this effect. Adherence to this nomenclature is critical as it universally communicates that the patient has received pre-operative therapy, even in cases in which the complete clinical history is not available. Tumor response to chemoradiotherapy is variable. Complete pathological response has been reported in up to 25% of cases [55–57]. Although several diagnostic methods have been proposed to evaluate the degree of tumor response to neoadjuvant therapy, it is important to emphasize that a definitive diagnosis of complete response can only be determined through thorough histological examination of the area where the tumor was located. Histological assessment of tumor response is estimated by applying what is known as tumor regression grades. These systems use numerical values that vary according to the degree of tumor volume reduction. The majority of these schemes record the different proportions of residual cancer cells and the surrounding fibrosis and inflammatory reaction and in accordance with which of these components predominates, a definitive number is then assigned (Fig. 5.9).

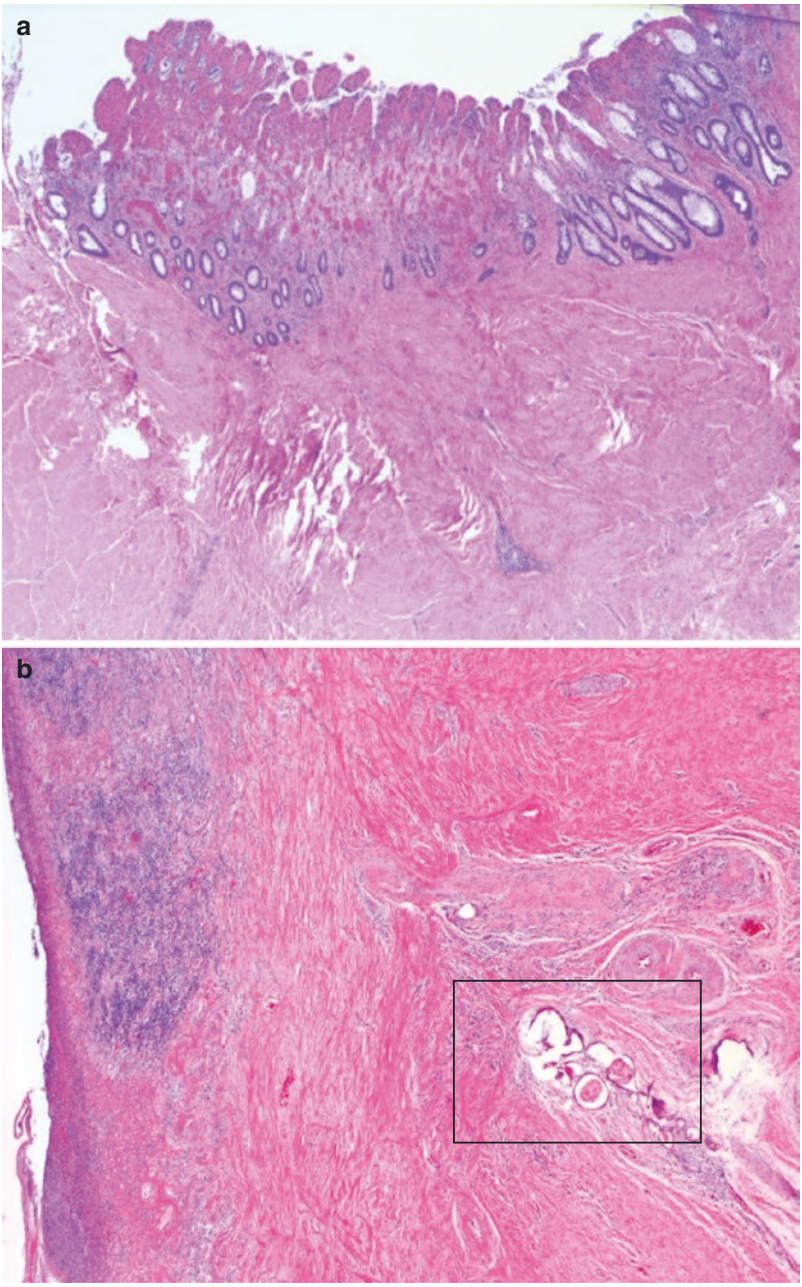


Fig. 5.9 (**a**) Complete pathological response (AJCC/CAP, grade 0) (H&E ×200). (**b**) Near complete response, only a single cluster of malignant glands remains in the specimen after pre-operative chemoradiation (rectangle), (AjCC/CAP grade 1), (H&E ×200). (**c**) Poor tumor response to pre-operative chemoradiation as shown by abundant aggregates of malignant glands, (AJCC/CAP grade 2), (H&E ×200)

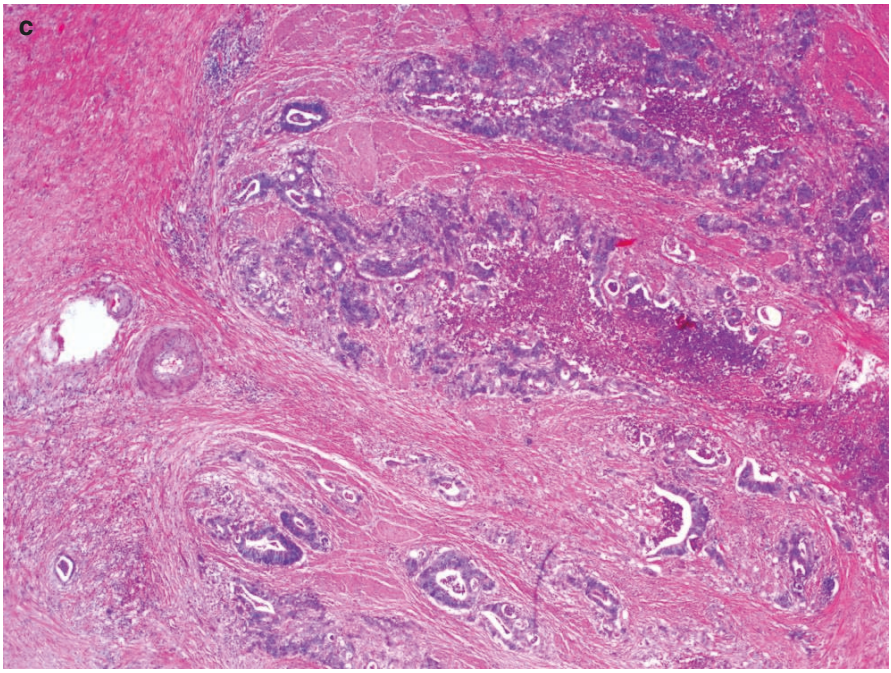


Fig. 5.9 (continued)

The Mandard regression grade system [58] was the first to be applied and was translated for use from esophageal cancer specimens (Table 5.1). Subsequently many other systems have been developed with the same goal including the Dworak method [59] which is widely used throughout Europe (Table 5.2). Following the same concept as these original systems, the College of American Pathologists more recently designed a regression grade [60] that has been proven to better correlate with outcomes (Table 5.3). Whereas the other TRG systems determine their scores based upon residual and tumor tissue, the 4-category AJCC/CAP system primarily focuses on scoring residual tumor. A limitation of such systems lies not in the ends of the spectrum of response or no response but rather in agreement concerning the assessment of intermediate groups as well as the general universality of adoption by pathologists of a standard assessment instrument [61, 62]. The other major drawback of these tumor regression classifications lies in the inter- and intra-observer variability amongst pathologists. This is related to the subjective nature attached to estimating the relative quantity of residual tumor and fibrosis. As expected, methods that use only 3 tiers demonstrate a better level of agreement amongst observers when compared with systems using 5 grades [63]. The ultimate relevance, however, of the degree of tumor regression recorded in response to chemoradiation is the impact of this parameter on patient cancer-specific outcome. In this regard, a near complete or a complete pathological response have both been shown to be associated with improved survival [64–67].

Table 5.1 Mandard tumor regression grade [58]

1. Complete regression (= fibrosis without detectable tissue of tumor)
2. Fibrosis with scattered tumor cells
3. Fibrosis and tumor cells with preponderance of fibrosis
4. Fibrosis and tumor cells with preponderance of tumor cells
5. Tissue of tumor without changes of regression

Table 5.2 Dworak tumor regression grade [59]

0. No regression
1. Predominantly tumor with significant fibrosis and/or vasculopathy
2. Predominantly fibrosis with scattered tumor cells (slightly recognizable histologically)
3. Only scattered tumor cells in the space of fibrosis with/without acellular mucin
4. No vital tumor cells detectable

Table 5.3 American Joint Committee on Cancer and College of American Pathologists Regression Grade AJCC/CAP [60]

Grade 0	Complete response – no viable cancer cells
Grade 1	Moderate response – single or small groups of cancer cells
Grade 2	Minimal response – residual cancer outgrown by fibrosis
Grade 3	Poor response – minimal or no tumor kill, extensive residual cancer

Neoadjuvant therapy frequently results in downstaging of the pT stage (the depth of invasion into the rectal wall). It is important, however, to clarify that T downstaging is not equivalent to tumor regression. The former specifically implies a decrease in the depth of tumoral invasion into the rectal wall, whereas the latter reflects a reduction in tumor volume. Concerning this point, significant tumor regression will often result in rare tumor cells still being identified within the mesorectal tissue (ypT3) without a modification of the tumor stage. Conversely, occasionally downstaging may occur from the pre-operative imaging staging (T3 to ypT2) without a marked decrease in tumor volume. The only circumstance in which tumor regression grades are analogous is when a complete pathological response is achieved, as disappearance of the tumor leads to declaration of a stage ypT0. Neoadjuvant CRT often leads to a discordance between the clinical (cPT) and the pathological stage (ypTN) and this poses the dilemma concerning which stage should be used in order to formulate the postoperative therapeutic plans. This questions the value of the TN staging system in cases where patients have received CRT.

Although the prognostic power of ypT (the depth of tumor invasion into the rectal wall) remains controversial, several studies have clearly shown that the presence of lymph node metastasis post-CRT portends an adverse prognosis. Furthermore, patients with positive nodes following neoadjuvant CRT fare worse than do patients with metastatic lymph nodes who have not received prior therapy. It is important to point out that the degree of tumor regression inversely correlates with the number of

dissected lymph nodes. As previously mentioned, the relationship between the number of lymph nodes harvested and prognosis in patients who received neoadjuvant CRT for rectal cancer remains unclear [35, 40, 44].

Microscopic Evaluation

The pathology report should point out not only the parameters used to define the pTN and ypTN, but also other histological findings that play a role in predicting the prognosis and the behavior of colorectal adenocarcinomas. One of these is in the determination of the variant of adenocarcinoma diagnosed since different types are associated with an increased risk of metastasis. For example, 27.6% of patients with a typical adenocarcinoma will develop metastasis whereas those with mucinous or signet ring cell morphology will have a metastasis in 33.9% and 61.2% of cases over time. These latter two variants also tend to metastasize to the peritoneum with the typical adenocarcinoma having more affinity for the liver [68]. It is equally important to determine the degree of differentiation as well as the presence or absence of perineural and vascular invasion [69–71] (Fig. 5.10). Unfortunately, reference to these findings may be

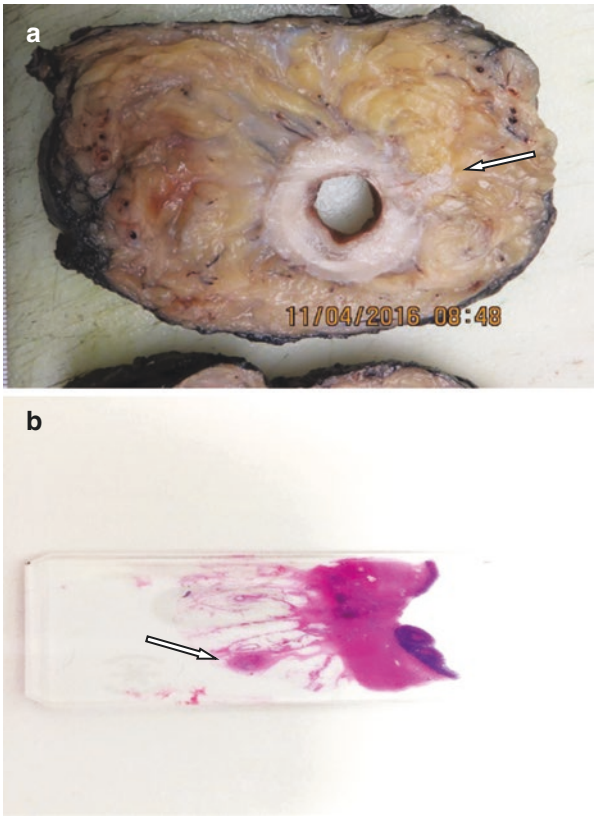


Fig. 5.10 (**a**) Cross section of a rectal cancer specimen showing a focus of extramural vascular invasion (arrow). (**b**) Scanning magnification of a hematoxilyn and eosin stain shows a vein outside the rectal wall filled by a tumor thrombus (arrow)

absent in up to 50% of pathology reports, however, the routine application of synoptic tumor summaries by pathologists at institutions where these have been used have shown an improvement in the quality of the reports [72, 73]. Even though synoptic reports are perceived by clinicians as better and more complete than narrative reports [72] the judgment of the histological findings may be subject to inter- and intra-observer variability hampering the overall accuracy of pathology reporting [74, 75].

Another histological feature that is being increasingly emphasized is the presence of "tumor budding," referring to the presence of individual carcinoma cells or small clusters of tumor cells coming-off the growth front of the main tumor mass (Fig. 5.11). Budding is a negative independent prognostic factor in patients with or without regional lymph metastasis [76–78]. The mechanism of this phenomenon is related to the mesenchymal-epithelial transition and the interaction with the environmental milieu involving complex molecular events controlling the *Wnt* pathway. Phenotypically, budding cells lose their expression of E-cadherin and acquire a mesenchymal characteristic producing and secreting metalloproteinases [79] and other enzymes which degrade the extracellular matrix and which as a consequence allow for the spread of the tumor cells [80]. Unfortunately, this finding may be omitted from the pathology reports or may also be subject to intra- and inter-observer variability. It is conceivable that in the near future tumor budding may be part of the staging system as its documentation, implications and clinical utility become more established.

There is considerable variability related to compliance in reporting of these factors. Although the depth of tumor invasion into the rectal wall and the status of the mesorectal lymph nodes is reported relatively consistently, few studies have shown information concerning the reporting of vascular and perineural invasion and even tumor differentiation is lacking in up to 50% of pathology reports. This fact is particular prevalent at institutions in which the pathologists are not gastrointestinal specialists. In this regard, as stated above, it has been shown by Lankshear et al. [72] in Ontario that the introduction of standardized synoptic pathology reviews which specifically mention each prognostic histopathological parameter in a separate and distinct field has significantly improved the quality of the reporting and the satisfaction of receiving clinicians (oncologists and pathologists). It is important to emphasize that a complete pathological report is not equivalent to an accurate pathological report. The majority of the aforementioned histopathological prognostic indicators suffer from both intra- and inter-observer variability, which diminishes their clinical value [73]. Nevertheless, it is imperative that pathologists worldwide make a concerted effort to become as familiar with and as proficient as possible in recognizing and reporting these important primary pathology elements.

Biologic and Molecular Markers in Colorectal Adenocarcinoma

The morphology and the staging of colorectal adenocarcinomas are not the whole story defining tumor behavior of therapeutic responsiveness. Knowledge obtained from the molecular biology of colorectal adenocarcinomas has expanded the

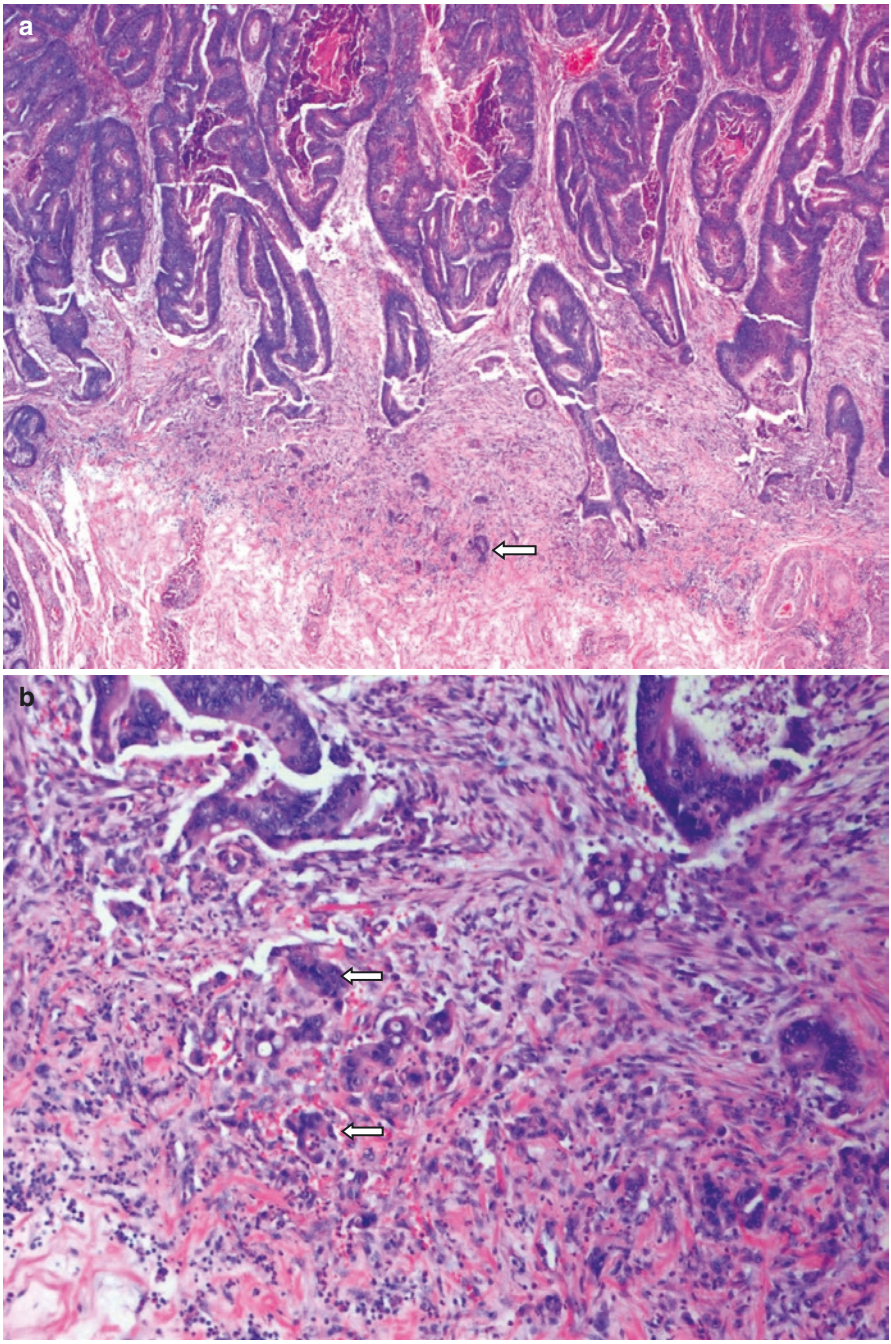


Fig. 5.11 (**a**) Example of "budding" effect. Small clusters of malignant cells are seen at the growing edge of the tumor (arrows), (H&E ×1000). (**b**) Higher power demonstrates in more detail the small nests and isolated malignant cells infiltrating the stroma at the tumor border (arrows), (H&E ×2000)

therapeutic approach. In this respect, there are three molecular pathways in the development of colorectal carcinoma. These include (1) Chromosomal instability, (2) DNA methylation [CpG island methylator phenotype (CIMP]) and (3). Microsatellite instability (MSI).

These systems are not mutually exclusive as a carcinoma may exhibit aberrations of multiple pathways [80, 81]. In the first pathway of chromosomal instability, the carcinomas arise due to an accumulation of chromosomal abnormalities including gains, losses and translocations in oncogenes and tumor suppressor genes that lead to aneuploidy. Aneuploidy is associated with approximately 70% of colorectal carcinomas and is implicated in the traditional adenoma-carcinoma sequence. Tumors that arise through this molecular route are more likely to display KRAS mutations and are more commonly located on the left side of the intestinal tract.

In the second mechanism, carcinomas result from aberrations within specific genes after the DNA duplication is completed. These are referred to as epigenetic changes, largely through methylation of certain genes such as MLH1, CDKN2A, p16 and MGMT. These are events which occur specifically in tumor suppressor genes. In daily practice, MLH1 testing is the most frequently utilized and when there is a defective MLH1 this is secondary to gene hypermethylation. This defective mismatch repair occurs mostly in sporadic carcinomas, with some of these cases related to Lynch syndrome. Such tumors will frequently demonstrate BRAF mutations and CpG island methylation. This CIM phenotype (CIMP) is particularly associated with recurrence following resection of stage III carcinomas of the proximal colon [81] and carries a poor prognosis with a resistance to 5-FU therapy.

The third pathway is based on the fact that DNA nucleotide microsatellite mismatches that occur during DNA duplication are repaired mainly by the genes MSH2, MSH6, MLH1 and PMS2 [82]. Mutations or epigenetic hypermethylation of these genes lead to abnormal proteins that produce microsatellite unstable/high tumors (MSI-H). MSI-H is observed in 15–20% of colorectal carcinomas, but most are sporadic and only 5% are seen in Lynch syndrome. It has long been known that hypermethylated colon cancers tend to occur in the right side of the colon and often display mucinous features as well as poor differentiation. Therefore, rectal cancer with MSI-H is not a common occurrence.

Molecular Testing

A recent consensus that gathered input from key pathology, oncology and molecular societies recommends the performance of testing for biomarkers that have clear prognostic value; most notably, BRAF and MMR along with KRAS and NRAS, which have both shown strong correlation as negative predictors to anti-EGFR therapy response [83]. Depending upon the institutionally-based accepted practices, it is expected that the pathologist will initiate testing in a timely fashion. Accordingly, the first line of molecular testing is usually the detection of mismatch repair protein (MMR) deficiency in carcinoma cells. This is an easy test to perform on formalin-fixed paraffin-embedded tissue using immunohistochemistry (IHC).

This will document the expression or the absence of MLH1, MSH2, MSH6 and PMS2. The tendency here is to perform IHC in all cases of colorectal carcinomas regardless of the patient age not only as a screening tool for possible cases of Lynch syndrome, but also as a prognostic marker and to help oncologists as they face a variety of clinical decisions. These might include whether or not to administer post-operative adjuvant therapy or might influence decision-making concerning the management of advanced disease. Universal testing is generally recommended because of these different goals [83]. MSI testing can also be performed readily with PCR methodology and both techniques (IHC and PCR) show similar sensitivity and specificity, [84] however, immunostaining is much cheaper and has a much faster turn-around time.

About 20% of patients have defects or mutations in one of the DNA repair genes. In about a quarter of those patients, the mutation is based in their germline; the underlying mechanism implicated in Lynch syndrome. Most of the defects are found in the MLH1 protein, but this finding is usually associated with sporadic carcinomas and a mutation for BRAF. The latter mutation essentially rules out familial cases. Recently, this has been shown to be an incrementally cost effective approach for Lynch screening using a combination of IHC, BRAF V600E and MLH1 promoter methylation testing [85]. Loss of the expression of any of the other 3 proteins is associated with Lynch syndrome and will direct further work-up for confirmation [84].

In either group of patients, (Lynch or the sporadic colorectal carcinomas), MMR mutations carry prognostic information. Those with a deficiency typically have a better outcome, regardless of the stage of their disease to the point that in a subset of stage II patients with an MMR defect, the use of postoperative adjuvant therapy may be avoided. Moreover, in more advanced cases, these patients will also not benefit from 5-fluorouracil adjuvant chemotherapy [86–88]. In addition, the value of the MSI-high marker in patients with advanced disease is that it predicts the response to immunotherapy with immune checkpoint-inhibiting drugs, specifically pembrolizumab [89].

Activation of epidermal growth factor receptors (EGFR) on the surface of the carcinoma cells triggers a cascade of signals and alters the role of KRAS, regulating cellular proliferation, angiogenesis and invasive/metastatic capabilities. Mutations in the KRAS gene result in permanent activation of the intracellular signals in the RAS/MAPK pathway. The mutations occur early in carcinogenesis and are observed in 20–50% of cases and when present patients will not benefit from anti-EGFR therapy. In this group the disease-free period tends to be short [89, 90].

Testing for NRAS mutations is thus advisable in order to identify patients who will not benefit from anti-EGFR therapy and it has been suggested that the use of TKIs in patients with RAS- mutated tumors may be detrimental [90]. The testing for KRAS and NRAS mutations can be performed on formalin-fixed paraffin-embedded tissues using PCR or next-generation sequencing techniques. BRAF gene mutations such as V600E transform the protein into its active form, leading to a constant activation of the MEK pathway independent of KRAS. The mutation may partly explain why 60% of patients with wild KRAS are unresponsive to anti-EGFR therapy, hence the importance of testing for BRAF mutation. BRAF mutations are consistently

associated with poor outcomes in patients with metastatic CRC, including those who relapse after adjuvant therapy [89–91]. The combined information rendered by MSI and BRAF testing may be more crucial than conventional staging, identifying specific prognostic subgroups and directing therapy. For patients with high levels of microsatellite instability and BRAF mutation the prognosis is more favorable. By contrast, patients with microsatellite stable or MSI-low tumors and BRAF mutation generally present with more advanced disease, displaying a far worse outcome even in stages I-II as well as seeming chemotherapeutic resistance.

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