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Introduction

Clinical applications of genomic medicine and molecular diagnostics based on testing of tumor tissues are becoming a reality in clinical practice, with significant impact on personalized therapies for cancer patients. Advances in targeted therapies for cancers of the gastrointestinal tract have recently emerged and are rapidly moving targets. In this chapter, we review the targeted therapies that are currently standard of practice in colorectal and gastric cancers, requiring specific molecular testing for selection of candidate patients for therapy.

Gastric and Gastroesophageal Cancers

EGFR Pathways

The epidermal growth factor receptor (EGFR) family of transmembrane receptor tyrosine kinases includes four members: HER1 (also known as the EGFR and ErbB-1), HER2 (p185, HER2/neu, ErbB-2), HER3 (ErbB-3), and HER4 (ErbB-4). The molecular structures of EGFRs comprise an extracellular ligand-binding domain, a short transmembrane

domain, and an intracellular domain with tyrosine kinase (TK) activity, except HER3. The binding of different ligands, including epidermal growth factor (EGF) and TGF- α to the extracellular domain, initiates a signal transduction cascade that elicits cell cycle progression, cell proliferation, anti-apoptotic signals, and survival, adhesion, migration, and differentiation.¹ Ligand binding to the EGFR extracellular domain induces EGFR homodimerization as well as heterodimerization with other types of HER proteins. HER2 does not bind to any known ligand, but it is the preferred heterodimerization partner for other members of the HER family. Ligand binding to EGFR followed by dimerization results in phosphorylation of the intracellular tyrosine kinase which triggers a series of intracellular signals including the activation of mitogen-activated kinase (MAPK): (KRAS/NRAS/RAF/MEK/ERK) or the phosphatidylinositol-3 kinase (PI3K) (PI3K/PTEN/AKT/mTOR) pathways (reviewed in²; Fig. 2.1).

Targeting HER2 Receptors

The human epidermal growth factor receptor 2 (Her2 or ErbB-2) was first described in gastric cancer in 1986.³ HER2 has no known ligand (orphan receptor), and preferentially heterodimerizes with HER3, which lacks intrinsic tyrosine kinase activity. The HER2 and the HER2/HER3 heterodimers are likely to be the most effective complex for activating downstream pathways.^{4,5}

Overexpression and amplification of HER2 have been described in 6–35% of gastric and gastroesophageal junction (GEJ) adenocarcinomas.^{6–9} Up to about a third of all GEJ adenocarcinomas and a quarter of non-GEJ gastric cancers have HER2 overexpression.

Importantly, as in breast cancer, HER2 overexpression has been linked to prognosis in gastric cancer. An early Japanese study showed 5-year survival rates of 11% and 50% for HER2-positive vs. HER2-negative gastric cancer, respectively.¹⁰ Another study showed that HER2 was an independent prognostic marker in resected gastric cancer, and overall survival was significantly associated with HER2

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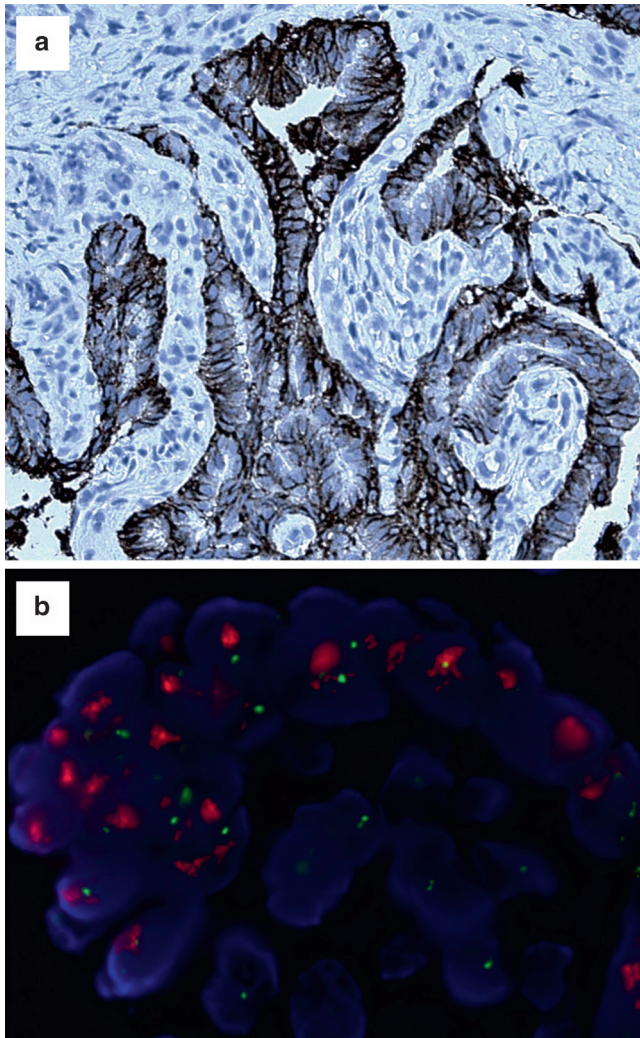


Fig. 2.1 Immunohistochemistry and FISH for HER2 in gastric adenocarcinoma. (a) Immunohistochemistry for HER2 shows a positive (3+) moderately differentiated adenocarcinoma. (b) FISH analysis reveals positive HER2 amplification by FISH (red dots). Courtesy of Dr. Paul Zhang MD, University of Pennsylvania

expression levels.⁸ HER2/neu-positivity rates have been reported to be more frequent in intestinal type gastric cancer (21.5%) than in diffuse gastric cancer (2%) or mixed types (5%).⁶ Overall, HER2/neu amplification in gastric carcinoma is associated with poor outcome^{6,11} and has been shown to be an independent prognostic factor.¹²

Trastuzumab is a monoclonal antibody that specifically targets HER2 protein by directly binding the extracellular domain of the receptor. Trastuzumab enhances survival rates in both primary and metastatic HER2-positive breast cancer patients. The efficacy of trastuzumab in breast cancer has led to investigation of its antitumor activity in patients with other

HER2-positive cancers, including gastric and GEJ adenocarcinomas. In preclinical studies, treatment with trastuzumab inhibited growth of gastric cancer cell lines.⁶ Based on the preclinical data in gastric cancer and clinical evidence in breast cancer, early phase trials of trastuzumab in metastatic gastric cancer wherein tumors overexpressed HER2 were conducted and the antibody therapy was shown to confer improved clinical outcomes. Thereafter, a large randomized phase 3 clinical trial (the ToGA trial) was concluded, definitively establishing the utility of targeting HER2 in advanced gastric cancer.¹³ In this study, metastatic gastric or gastroesophageal adenocarcinoma cases where the tumor overexpressed HER2 were randomized to receive standard chemotherapy (a fluoropyrimidine [5-fluorouracil or capecitabine] and platinum combination) with or without trastuzumab.¹³ Overall survival was improved in the trastuzumab arm, with little added toxicity. Median overall survival was 13.8 months in those assigned to trastuzumab plus chemotherapy compared with 11.1 months in those assigned to chemotherapy alone (HR 0.74, $p=0.0046$). Therefore, testing HER2 and adding trastuzumab to the chemotherapy regimen for HER2-positive tumors have become the standard of care for advanced gastric adenocarcinoma. In this trial, a HER2 scoring system modified from the protocol in breast cancer was used: a score of immunohistochemistry (IHC) 3+ and/or fluorescent in situ hybridization (FISH) positive (HER2:CEP17 ratio ≥ 2) was defined as HER2 positive. The study reported an overall HER2-positivity rate of 22.1% evaluated from 3,665 patients.¹³ An example of HER2-positive tumor by IHC and FISH is shown in Fig. 2.1.

Criteria for interpretation of HER2 modified for gastric and GEJ adenocarcinoma have been recently reviewed.^{14–19} Studies have shown good correlation of HER2 expression in primary vs. metastatic carcinoma lesions.²⁰ Notably, HER2 overexpression is already observed in early gastric cancers.²¹ Heterogeneity of HER2 expression occurs frequently in gastric and GEJ adenocarcinoma; however, testing is often done in biopsies when no resection specimen is available.¹⁶ The College of American Pathologists (CAP) reviewed current guidelines for interpretation of HER2 expression.^{13,14,19,22} Importantly, criteria for interpreting HER2 IHC on gastric and GEJ carcinomas differ significantly from the criteria used in breast cancer. First, gastric carcinoma interpretation criteria use 10% tumor cell staining as a cutoff to distinguish negative from 1+. In gastric carcinoma, the distinction between 1+, 2+, and 3+ depends on the intensity of staining presuming that more than 10% of tumor cells show HER2 expression (Table 2.1). Second, gastric cancers only show expression along the basolateral or lateral cell membranes, while apical membranes are negative. Therefore, the criteria for 2+ and 3+ staining in gastric cancer require only lateral

Table 2.1 Criteria for scoring and reporting HER2 expression in gastric and esophageal adenocarcinomas by immunohistochemistry

Staining pattern	Biopsy specimen	HER2 expression Interpretation
Resection specimen		
No reactivity or membranous reactivity in <10% of tumor cells	No reactivity in any tumor cell	Negative
Faint or barely detected membranous reactivity in ≥10% tumor cells	Tumor cell cluster of ≥5 cells with faint or barely detected membranous reactivity irrespective of percentage of tumor cells stained	Negative
Cells are reactive only in part of their membrane		
Weak to moderate complete, basolateral, or lateral membranous reactivity in ≥10% tumor cells	Tumor cell cluster of ≥5 cells with weak to moderate complete, basolateral, or lateral membranous reactivity irrespective of percentage of tumor cells stained	Equivocal
Strong complete, basolateral, or lateral membranous reactivity in 10% or more of tumor cells	Tumor cell cluster of ≥5 cells with strong complete, basolateral, or lateral membranous reactivity irrespective of percentage of tumor cells stained	Positive

FISH testing for *HER2* gene amplification should be performed when the IHC is equivocal (2+). Modified from the College of American Pathologists (CAP) web site⁷⁸ and based on studies reported by^{13,22}

or basolateral staining, in contrast to breast cancer criteria which require complete, circumferential staining. Third, the criteria for HER2 overexpression differ when interpreting biopsy and resection specimens due to heterogeneity of HER2 expression in gastric and gastroesophageal junction carcinomas (Table 2.1).

Colon Cancer

Molecular Testing of Colorectal Cancers for Targeted and Conventional Therapy

Molecular testing of colorectal cancer (CRC) tissues has important implications for treatment selection in these patients. We will discuss recently introduced therapy approaches that use information from molecular testing of CRC tumor tissues for selection of individualized therapy, representing the principles of personalized tumor diagnostics and targeted therapy. One application of tissue molecular testing discussed here considers the DNA mismatch repair status of CRC and takes into consideration the mutational status of the EGFR signaling pathway to select targeted therapy.

DNA Mismatch Repair Defects and Microsatellite Instability

Approximately 15% of all CRCs show underlying defects in DNA mismatch repair (dMMR) and the tumor tissues show microsatellite instability (MSI), discussed in detail in Chaps. 1, 7, and 8. In 3–5% of MSI-positive CRC patients harbor germline mutations related to the Lynch syndrome and the remaining 12% or so are sporadic-type CRC cases.²³ Microsatellites are short tandem repeats of nucleotides that occur throughout the genome. In cells with deficient

mismatch repair, errors in DNA replication accumulate and are detectable in these regions, identified as microsatellite instability (MSI).^{24–26} Therefore, MSI, particularly when a tumor is identified to have a high level of MSI (MSI-H), acts as a marker of deficient MMR.²⁴ MSI-positive status correlated with the tumors being in the proximal colon and with improved survival.²⁶ This was soon followed by the identification of the genes responsible for hereditary non-polyposis colorectal cancer (HNPCC) *MSH2*^{27,28} and *MLH1*.^{29,30} Subsequently, MSI has been shown to play a role in sporadic colorectal cancers also.³¹ In humans, at least six different genes (*MSH2*, *MLH1*, *PMS1*, *hPMS2*, *MSH6*, and *MLH3*) encode the mismatch repair system.³² In hereditary defects, recessive mutation of one allele followed by somatic inactivation of the other is the mechanism of gene silencing.³³ In sporadic CRC cases, the most prevalent mechanism of MMR gene inactivation is biallelic inactivation by methylation and transcriptional silencing of the *MLH1* promoter region.^{34–39} Assessment of MSI status can be done by immunohistochemistry to evaluate expression of DNA mismatch repair proteins or by PCR-based DNA testing for MSI to assess instability at microsatellite sequences.^{24,40} Combining testing for *BRAF* V600E activating mutation and CpG island methylation status of the promoter region of *MLH1* gene helps determine whether a MSI-positive tumor with loss of *MLH1* expression is likely to be an inherited Lynch syndrome/HNPCC tumor (*BRAF* mutation-negative and *MLH1* promoter methylation-negative) or sporadic-type CRC (*BRAF* mutation-positive in up to 70% of cases and *MLH1* promoter methylation-positive) (reviewed in Chap. 7).

An interesting aspect of MSI is its distinct relationship to colorectal cancer behavior. It was shown in a large series that MSI occurs in 17% of colon cancer cases in young (less than 50 years) individuals and this MSI was associated with a

lower likelihood of tumor metastasis to regional lymph nodes as well as distant organs, leading to an overall survival advantage, independent of stage of disease.⁴¹ Another study reported that MSI-H tumors were more likely than MSI-low level tumors to be in younger individuals, right-sided, poorly differentiated with mucin production, and with an overall better prognosis.⁴² In addition to being a prognostic marker, MSI has a predictive role also.⁴³ While earlier studies did not bear this out,⁴⁴ improved outcomes were seen with chemotherapy for advanced stage III colorectal cancers that were MSI-H.⁴⁵ However, another study showed that patients with stage II tumors characterized by deficient DNA mismatch repair and therefore an MSI-positive status receiving fluorouracil (5-FU) had no improvement in disease-free survival, and in fact, treatment was associated with reduced overall survival.⁴⁶ Larger trials are needed to determine with certainty the utility of these markers for treatment selection in routine patient care.⁴⁷ However, regimens with 5-FU alone should be avoided in patients with stage II CRC who may be candidates for chemotherapy.

Targeting EGFR Signaling Pathways in CRC

Aberrant activation of EGRF signaling pathways is frequent in CRC, and is primarily associated with activating mutations of genes in these pathways (MAPK and PI3K). Tyrosine kinase inhibitors targeting the intracellular domain of the EGFR, namely erlotinib and gefitinib, have not been shown to have meaningful clinical efficacy in this disease, given that activating mutations in the *EGFR* gene are not a feature of CRC.^{48,49} Based on data in the literature, the following are the proportions of cases harboring various mutations in EGFR pathway genes⁵⁰: MAPKinase pathway: *KRAS* (40–45%), *NRAS* (2.5%), *BRAF* (5–10%); PI3Kinase pathway: *PIK3CA* (15%), *PTEN* (10–20%), *AKT* (5%); and combined mutations: *KRAS/NRAS* and *PI3K* (10%). An interesting finding is that in CRC as in other tumors, *RAS* and *RAF* mutations are mutually exclusive.^{51,52} Therefore, together, *BRAF* and *KRAS* are mutated in about half of all CRC cases.

KRAS mutations are found in about 40–45% of all colorectal cancers and occur mostly at exon 2 [codon 12 (70–80%) or 13 (20–30%)], while there are rare mutations in codons 61 and 146.⁵³ *BRAF* mutations occur most commonly at exon 15 with thymine to adenine transversion at nucleotide position 1796, which leads to the substitution of valine for glutamate (a substitution mutation termed V600E), and are found in about 5–10% of all colorectal cancers⁵⁰. Importantly *BRAF* V600E mutation occurs in 4–12% of DNA mismatch repair proficient tumors (microsatellite stable) and in 40–74% of MSI-H sporadic CRC (MLH1-deficient), but is not found in MLH1-deficient MSI-H CRC in HNPCC/Lynch syndrome-associated CRC.^{51,54,55}

Mutations in the PI3K axis are seen in about 20% of all colorectal cancer cases.^{50,51} Interestingly, mutations across the two EGFR signaling axes are not mutually exclusive, and about 5% of tumors harbor mutations in genes from both arms of the pathway.⁵¹

The role of EGFR pathway gene mutations in the clinical management of colorectal cancer has been extensively studied. In terms of prognosis, *KRAS* mutations do not confer a poor prognosis by themselves, probably because they appear very early in cancer development.^{56–58} However, *BRAF* mutations confer a significantly poorer prognosis, as compared to wild-type *BRAF* tumors. It is still early to say if PI3K axis mutations play a prognostic role in CRC.^{56,57}

More importantly, the EGFR pathway has become an important therapeutic target. Cetuximab and panitumumab are anti-EGFR antibodies that target the extracellular domain of the receptor. They have been shown to improve progression-free, and in some cases, overall survival in metastatic colorectal cancer.⁵⁹ A landmark paper by Karapetis et al published in 2008 showed that in patients with wild-type *KRAS* tumors, treatment with cetuximab as compared with supportive care alone significantly improved overall survival (median, 9.5 vs. 4.8 months). In contrast, among patients with mutated *KRAS* tumors, there was no significant difference between those who were treated with cetuximab and those who were not. This study concluded that patients with a colorectal tumor bearing mutated *KRAS* did not benefit from cetuximab, whereas patients with wild-type *KRAS* CRC did benefit from cetuximab therapy.⁶⁰ *KRAS* mutations render these agents ineffective, because activated *KRAS* is downstream of EGFR and constitutive activation of the former leads to independence from the latter^{51,60} (Fig. 2.2). Therefore, given evidence from phase II and III clinical trials using monoclonal antibodies as monotherapy or in combination with chemotherapy for metastatic CRC (Stage IV: any T, any N, M1) that tumors with *KRAS* mutation in codons 12, 13, or 61 did not benefit from treatment with cetuximab or panitumumab,⁶¹ patients with metastatic CRC who are candidates for anti-EGFR antibody therapy should have their tumor tested for *KRAS* mutations in a CLIA-accredited laboratory.⁶¹ There is up to 40% response rate to anti-EGFR therapy in wild-type CRC while the remainder 60% wild-type tumors will not respond, presumably due to other gene/protein alterations in the EGFR or other signaling pathways.⁶¹

For CRC with an activated mutant *KRAS*, a number of drugs that may inhibit downstream signaling molecules (such as inhibitors of mTOR, RAF, and MEK) are under evaluation⁶² (Fig. 2.2). The predictive role of *BRAF* mutational studies in CRC is still unclear. While *BRAF* activating mutations should act similar to *KRAS* in terms of predicting

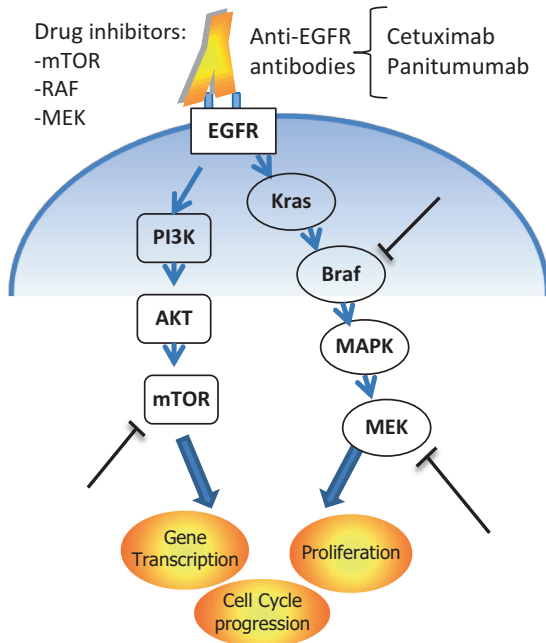


Fig. 2.2 EGFR signaling pathways: mutation targets and targeted therapies

response to EGFR antibody therapy, the markedly poor prognosis that a *BRAF* mutation confers, along with the relatively low proportion of cases with *BRAF* mutations in clinical trials, makes it difficult to assess this role clearly.⁵⁷ Although *BRAF* inhibitors have been tested in early studies, larger trials are needed to determine if colorectal cancer will respond to these agents in a manner similar to what is now seen in melanoma.⁶³ It has been demonstrated recently that colon cancer cells, in contrast to melanoma cells, are unresponsive to the *BRAF* inhibitor vemurafenib by employing rapid feedback activation of EGFR, which neutralizes the benefit of vemurafenib.⁶⁴ Therefore, a dual strategy of targeting *BRAF* and EGFR may be needed to effect clinical responses in *BRAF*-mutant CRC.

Preclinical data have shown that a *PIK3CA* or *PTEN* mutation (which leads to constitutive activation of the PI3K pathway) causes resistance of cancer cells to cetuximab.⁶⁵ Indirect evidence stems from preclinical work showing that *PIK3CA* mutation uncouples cell proliferation signaling from the *KRAS* pathway, leading to failure of inhibitors targeting the MAPK axis.⁶⁶ Initially, small studies showed conflicting roles of *PIK3CA* mutation in response to EGFR antibodies,^{67,68} but a recent large study has demonstrated that *PIK3CA* mutation is associated with poor response to cetuximab.⁵¹ Preclinical models indicate that in these tumors,

inhibition of the PI3K axis may be required to achieve cancer control. Blocking the PI3K pathway in cancer cells with activating *PIK3CA* mutations has been shown to inhibit cell growth and induce apoptosis.^{69,70} A study by de Roock et al found that *BRAF*, *NRAS*, and *PIK3CA* exon 20 mutations are significantly associated with a low response rate to cetuximab targeted therapy, in that objective response rates could be improved by stratifying patients by additional genotyping of *BRAF*, *NRAS*, and *PIK3CA* exon 20 mutations in a *KRAS* wild-type population.^{51,71}

In addition, when mutations in both EGFR pathway axes exist, dual inhibition with MEK and AKT/PI3K inhibitors is required to control cell growth.^{70,72} Thus, work is ongoing on various inhibitors of these signal transduction molecules to see if collective inhibition of some or all constitutively activated genes will achieve clinical benefit.

Molecular Testing for CRC Targeted Therapies

EGFR mutational testing is not indicated for CRC since activating *EGFR* mutations in CRC are rare and do not confer sensitivity to tyrosine kinase inhibitors or to cetuximab therapy.⁷³ Further, *EGFR* IHC is not warranted for selection or exclusion of patients for cetuximab therapy, as it was observed that cetuximab shows activity in CRC patients with tumors that do not express the epidermal growth factor receptor by IHC.⁷⁴ Currently, the standard of practice for selection of CRC patients with metastatic disease who are candidates for targeted therapies with anti-EGFR antibodies is primarily based on mutational status of *KRAS*.⁶¹ The mutational status of *BRAF*, *NRAS*, *PIK3CA*, and other genes downstream of EGFR may affect response to anti-EGFR targeted therapy.⁷¹ Therefore, testing for mutations in these genes may be indicated in candidate patients, particularly in the setting of clinical trials, at the present time.

Interestingly, in contrast to other activating mutations in *KRAS*, use of cetuximab among patients with chemotherapy-refractory colorectal cancer with the *KRAS* G13D mutation may be associated with longer overall and progression-free survival,⁵² although this remains a matter of debate.

Regarding the choice of tissue for DNA mutational analysis, since *KRAS* mutations occur early in colorectal carcinogenesis, most clinical trials tested the primary tumor site and published studies showed good correlation between *KRAS* mutation status in primary vs. metastatic colon cancer lesions with high average concordance of 93% (76–100%).^{75,76} Therefore testing tumor tissue from the primary site or from metastatic lesions is appropriate. Pathologists should select a block of formalin-fixed, paraffin-embedded (FFPE) tissue with the highest % of viable tumor and largest tumor area

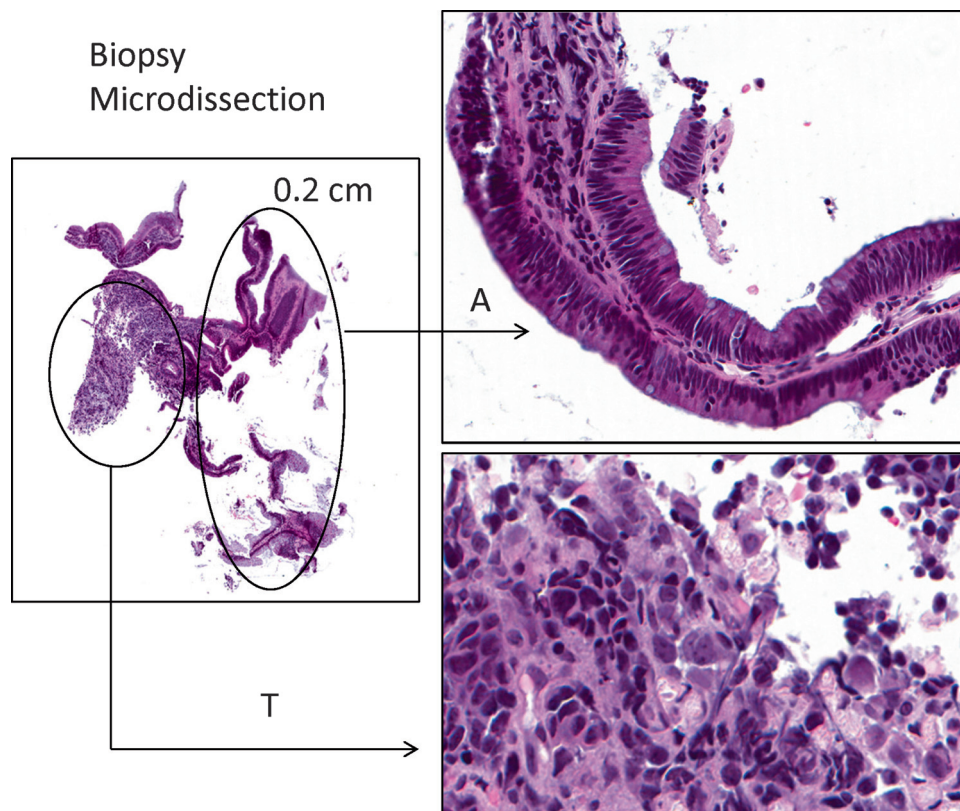


Fig. 2.3 Tissue microdissection from a small CRC biopsy sample (0.2 cm). Two tissue areas, *T* tumor, *A* adenoma, were separately marked to enrich the tumor area for DNA extraction. Microdissection was then

performed using unstained slides matching the H&E stained guide slide, shown in the figure

possible. Individual laboratories may have different requirements depending on the assay used. The technical approaches for mutational testing vary widely among laboratories and follow requirements for validation and interpretation, particularly for laboratory developed tests. Such methods include Sanger sequencing, allele-specific PCR, melt curve analysis, pyrosequencing, fluorescent bead detection assay, MassARRAY MALDI-TOF mass spectrometry, and newer next-generation deep sequencing approaches.

Adequate DNA amount can be obtained by pooling macro- or micro-dissected tissue from multiple tissue levels (Fig. 2.3). Importantly, a biopsy may be preferable to the resection specimen if the resection was done after neoadjuvant therapy (Fig. 2.4), and minimal numbers of

residual tumor cells persist, making the tissue inadequate for molecular testing.

In summary, *KRAS* mutational analysis of CRC tumor tissues is recommended as the standard of care in patients who are candidates for targeted anti-EGFR antibody therapy. Additional mutational testing of other EGFR pathway genes may be helpful to better select patients for targeted therapies with improved outcomes, as suggested by published studies, but a general consensus about which genes should be tested is not yet established. In large practice centers, the trend is to test all colorectal adenocarcinomas for *KRAS* codon 12–13 mutations, for *BRAF* V600E mutations, and for microsatellite instability, thus allowing for selection of patients for conventional therapy as well as targeted therapy.⁷⁷

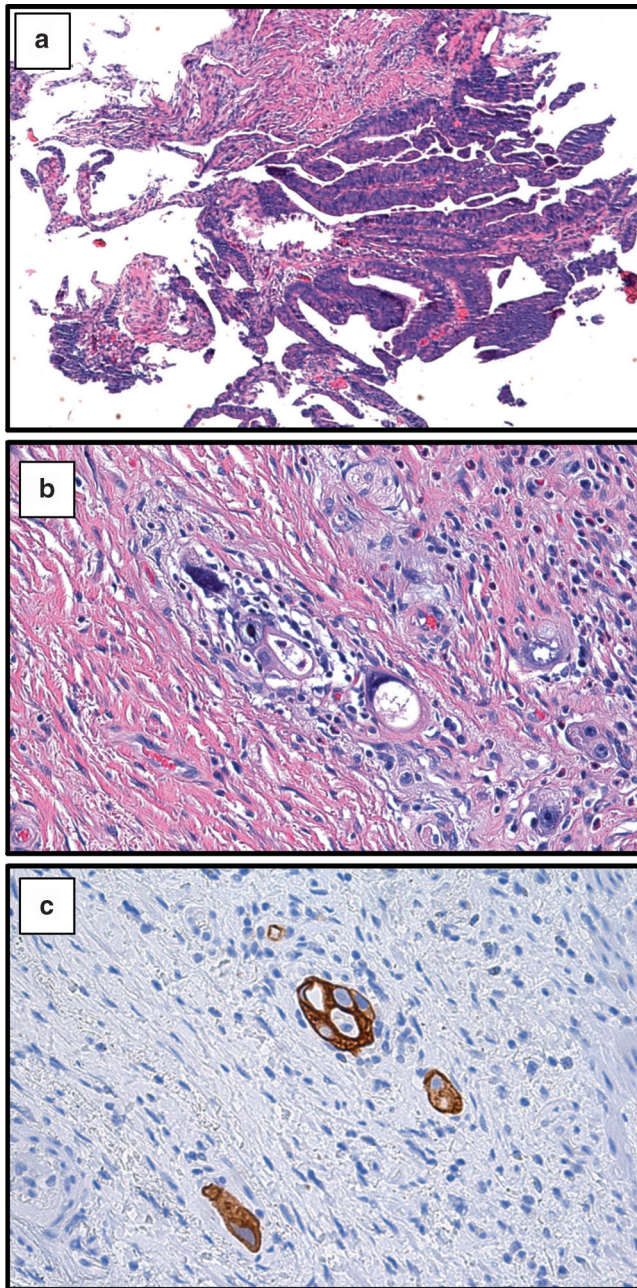


Fig. 2.4 Selection of CRC tissue specimen for DNA extraction and mutational analysis. A pretreatment small biopsy (a) with representative CRC was used for DNA testing in a patient with rectal cancer who received neoadjuvant therapy. Panels (b) and (c) represent rare residual tumor cells in the resection specimen. The limited number of tumor cells embedded in the background of fibrous tissue and muscularis rendered the resection specimen inadequate for molecular testing due to insufficient tumor cellularity

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