Colorectal cancer (CRC) is an important global health problem. In 2002, it was the third most commonly occurring cancer with about 1 million new cases (9.4% of the world total). It was the fourth most frequently diagnosed cancer in men (after lung, prostate, and stomach cancer) and the third most common in women (after breast and cervical cancer). Age-standardized incidence rates varied 25-fold, with the highest frequencies in developed countries. Mortality in 2002 was about half the incidence rate. Five-year survival estimates in men were 65% in North America and 54% in Western Europe, but less elsewhere. This overall relatively good prognosis means that the prevalence of the disease is high: 2.8 million patients are still alive within 5 years of diagnosis, making it the second most prevalent cancer after breast cancer.  

Recent medical and surgical advances have broadened the array of therapeutic options available for CRC, with a definite impact on survival. Management plans must be constructed for individual patients on the basis of all the available evidence and the expertise of the various specialists confronted with the case. This implicates a need for multidisciplinary team meetings with participation of gastroenterologists, surgeons, radiologists, pathologists, oncologists, and radiotherapists. The pathologists’ role in such a team should not be underestimated, as tissue diagnosis and prognostication determine further treatment. Patients and clinicians deserve a consistent and informed approach to the reporting of CRC specimens.  

TRADITIONAL ROLE OF PATHOLOGISTS IN THE MULTIDISCIPLINARY TEAM  

Pathologists may encounter several types of specimens as the patient progresses along the CRC care pathway. These are the diagnostic biopsies, the local excision specimens (polypectomies, endoscopic mucosal resections, and local rectal resections), and the surgical specimens (various colectomy specimens, anterior resections, and abdominoperineal resections). Each type of specimen has its own limitations with associated diagnostic problems and a particular way of reporting.  

Diagnostic Biopsies  

These can be obtained at colonoscopy, by puncture (eg, lesions suggestive of liver metastases), or at laparoscopy. Tissue fragments obtained during endoscopy are taken from a polypoid, ulcerated, or flat lesion suggestive of malignancy. The aim is to confirm the diagnosis of malignancy, to classify its type (including primary versus secondary), and to assess tumor grade. In the colon and...
rectum, invasive adenocarcinoma can be diagnosed only when tumor cells are unequivocally present in the submucosa. This may be difficult, as biopsies from endoscopically malignant lesions may be superficial or consist of necrotic tissue. Also, not infrequently, only adenomatous tissue is present. A desmoplastic stromal reaction may then assist in the diagnosis of malignancy. Polypoid lesions should be properly sectioned before embedding to enable evaluation of the submucosa and section margin. In case of doubt, a repeated biopsy may be indicated. Biopsies taken from other sites than the colon should always be accompanied by sufficient clinical information. The histologic aspect may be suggestive of a metastasis of CRC. In most cases, however, assessment of classic morphology must be assisted by the evaluation of selected immunohistochemical stains.

**Local Excision Specimens**

In some circumstances, a biopsy diagnosis of adenocarcinoma may be followed by planned local excision. Lesions can be removed by endoscopic polypectomy, endoscopic mucosal resection, or, in the case of low rectal tumors, per anal excision after Parks procedure or transanal endoscopic microsurgery. Polypectomy specimens require the use of consistent terminology by pathologists and their clinical colleagues to avoid overtreatment or undertreatment. Intraepithelial neoplasia (previously called dysplasia) in adenomas was classically graded according to a 3-tiered system (mild, moderate, and severe) based upon tissue architecture, nuclear changes, and cytoplasmic differentiation. The mild and moderate categories are currently contracted into low-grade intraepithelial neoplasia as opposed to the high-grade variant. The term intramucosal adenocarcinoma can be used when the architectural and cytologic features are essentially those of malignancy (e.g., a cribriform growth pattern), but no invasion into the submucosa can be demonstrated. As this may be dependent on proper orientation of the specimen, carrying out multiple step sections and using selected immunohistochemical stains to evaluate the relation of the neoplastic glands with the muscularis mucosae may help to arrive at a correct diagnosis. When definite invasive adenocarcinoma has developed in an adenomatous poly, the pathology report should at least include a measure of the proximity of the tumor to the endoscopic resection margin, an assessment of its degree of differentiation and the presence of tumor budding, and a statement concerning the presence or absence of lymphatic or blood vessel invasion. A tumor-free margin less than 1 mm, a high-grade cancer, and presence of lymphovascular invasion all increase the risk for nodal metastases and will likely lead to a full resection depending on the clinical status of the patient. Local excisions of early rectal cancer may be an alternative to anterior and abdominoperineal resections for patients who are unfit for major abdominal surgery, but they are also increasingly used as a procedure with curative intention for those with early-stage rectal cancer. Each type of excision removes a full-thickness piece of rectal wall without regional lymph node sampling. The pathologist must ensure proper reporting of the depth of invasion, as this will determine subsequent case management. The fresh specimen should be pinned on a cork board to prevent distortion during formalin fixation. After macroscopic inspection, it should be thoroughly sampled and investigated microscopically.

**Surgical Specimens**

Colorectal resection specimens require careful handling and assessment to determine the key prognostic data reliably and accurately. The role of the pathologist varies with respect to each of the other members of the multidisciplinary team. We can help the surgeon in determining prognosis by gross dissection and microscopy, but we must also identify the plane of surgery that has been achieved and give feedback on the completeness of the excision. We may provide valuable information to radiologists by assessing the accuracy of the preoperative radiologic staging and by controlling their prediction of completeness of excision, particularly in rectal cancer. With regard to oncologists and radiotherapists, our function is to describe the effectiveness of neoadjuvant therapy, to identify those patients who need adjuvant therapy and, if possible, to suggest the most appropriate therapy by using particular molecular tests.

We must provide complete, consistent, and intelligible reports. This goal can be reached by developing and updating reporting standards based on continuing medical education, regular audit, and, importantly, feedback from our clinical colleagues. It has been demonstrated that the adoption of a minimal data set, such as the one developed by the United Kingdom Royal College of Pathologists, improves the completeness of pathology reports. Of course, such forms can be subject to international consensus guidelines. Given the lack of evidence for some critical changes in TNM6 relative to TNM5 (6th edition and 5th edition, respectively, of the TNM staging system of The American Joint Committee on Cancer/International Union Against Cancer), the British position has been to remain, for the time being, with TNM5 as far as standardized reporting is concerned. A minimal data set must contain some key features focused on treatment and prognosis. It has been amply demonstrated that patients with node-positive CRC have an improved survival when treated with adjuvant chemotherapy. Since the chance of detecting lymph node metastases depends partly on the number of retrieved nodes, pathologists must make an effort to find as many lymph nodes as possible. A failure of the pathologist to detect all lymph node metastases may come at a high cost for the patient. Some oncologists also offer adjuvant therapy to so-called high-risk stage II node-negative patients, because of the presence of pathologic features predictive of an increased risk of relapse (extensive extramural spread, extramural venous invasion, incomplete resection, peritoneal invasion, and tumor perforation). Pathologists assist in the selection of such patients by mentioning the presence or absence of these features in their report.

Prognosis is furthermore very much determined by the completeness of the excision. While pathologists have always investigated for the presence of tumor at the longitudinal margins, as this is a recognized though rare (1%–2%) poor prognostic feature, only recently have other surgical margins acquired attention. For example, tumor frequently involves the circumferential resection margin (CRM) in the rectum (5%–36%) and is then significantly associated with higher rates of local recurrence, metastasis, and a poorer overall survival. In contrast, in the rest of the colon, the surgeon usually creates a narrow mesocolic margin. Only in right hemicolectomies is there an area of
variable size on the posterior surface of the cecum and ascending colon, which is involved by tumor in about 7% to 10% of cases. The long-term consequences of a “positive De Hertogh & Geboes The appropriate number of blocks must be 23. It is therefore a good routine practice to 22. Practical and Molecular Evaluation of CRC—TOTAL MESORECTAL EXCISION AND ITS IMPLICATIONS

TOTAL MESORECTAL EXCISION AND ITS IMPLICATIONS

Total mesorectal excision (TME) is a fine illustration of a surgical principle that should be applied to all CRCs, namely, precise anatomic dissection of affected bowel with its mesentery, enclosed within an intact fascial plane. Total mesorectal excision may be a technically demanding and time-consuming procedure, but it has led to a demonstrable improvement of local cancer control with a decreasing number of complications. Its systematic use by well-trained surgeons in some locations has led to reductions of cancer recurrence rates from 20% to about 10%. The mesorectum is a fatty tissue layer of about 2 to 3 cm in thickness, with inlying blood vessels, nerves, lymphatics, and lymph nodes. It surrounds the rectum and is enveloped by a fascia. Mesorectal excision is the surgical removal of this soft tissue envelope under direct vision, ideally by dissecting between the visceral and parietal pelvic fascia. A mesorectal excision can be total (TME) or partial. Total mesorectal excision refers to excision down to the pelvic floor and is indicated for carcinoma of the middle and lower third of the rectum. In partial mesorectal excision, the mesorectum is transected perpendicular to the rectal wall at a distance of 5 cm beyond the gross distal border of the tumor. This is sufficient for surgical treatment of carcinomas of the upper third of the rectum. In general, a TME specimen with a smooth surface without incisions or tearing, and without coning, is an indicator of successful surgery. Coning is the result of the tendency of the surgeon to cut in the direction of the rectal wall rather than staying outside the visceral fascia, especially during distal dissection in a narrow pelvis. Mesorectal excision specimens offer an opportunity to study the CRM extensively. There is a nonperitonealized bare area both anteriorly and posteriorly to the rectum. While the anterior CRM is located only in the most distal part of the specimen, the posterior CRM has a triangular shape and extends to the mesosigmoid. A positive CRM is defined as continuous or discontinuous tumor extension or the presence of a positive lymph node within 1 mm of the radial, nonperitonealized soft tissue edge. It is the foremost predictor for local tumor recurrence. When the CRM is negative, the next most important factor for assessing recurrence risk is the quality of the mesorectal dissection. It is therefore a good routine practice to include an assessment of this feature in all reports on surgical specimens for rectal cancer. The assessment of the quality of mesorectal excision begins with an evaluation of the fresh specimen (complete, nearly complete, and incomplete mesorectum; Figure 1, A through C). Photographic documentation is recommended, especially when the mesorectum is incomplete. The specimen is then painted with ink, partially opened (leaving the bowel intact at a level just proximal and just distal to the tumor), and fixed for at least 48 hours. Afterwards, the unopened portion of the fixed specimen is sliced into 3- to 5-mm thick transverse sections. These are all laid out and inspected in detail to determine the relationship of the tumor to the CRM (Figure 2). A final grade can then be assigned to the completeness of the mesorectum. An appropriate number of blocks must be taken to ensure a proper microscopic evaluation of the tumor and its locoregional lymph node metastases and their relation to the CRM. Key factors to report are the location of the tumor within the mesorectum (anterior or posterior, proximal or distal), the effect of neoadjuvant radiotherapy on the lesion, the status of the distal margin and locoregional lymph nodes, and the distance of the tumor to the radial resection margin.

A proper evaluation of the mesorectum in TME specimens by the pathologist is thus important for 3 main reasons: first, it provides feedback on technical issues to the surgeon; second, assessment of the CRM is the most significant predictor of local recurrence; third, the quality of the excised mesorectum is a key factor affecting the risk of local recurrence in patients with a negative CRM. Thus far, North American policy regarding appropriate handling of TME specimens has lagged behind that of European policy. American guidelines, such as the year 2000’s Protocol for the Examination of Specimens from Patients with Carcinoma of the Colon and Rectum, do not yet include assessment of TME quality. Many centers in the United States and Canada continue to examine rectal cancer specimens that have been opened in the region of the tumor, fixed for 24 hours or less, and make little or no attempt to assess the completeness of the mesorectum.

TARGETED THERAPIES AND TISSUE BIOMARKERS

Although the mainstay of CRC care is surgery to remove the primary tumor, there are many potential therapies depending on the tumor itself, the condition of the patient, and previous application of particular therapeutic modalities. Systemic chemotherapy is currently used as an adjunct to surgery to improve survival in stage III and certain “high-risk” patients with stage II CRC. Appropriate chemotherapy in the setting of distant metastasis will also prolong survival and improve quality of life. During the last 20 years, there have been significant advances in the treatment of patients with CRC by chemotherapy. Early improvements occurred because of an increased understanding of the pharmacology of 5-fluorouracil and the discovery of modulators of its activity, for example, leucovorin. In more recent years, new and potent cytotoxic drugs such as oxaliplatin and irinotecan have become available.

The classic cytotoxic chemotherapeutic agents came into use after a drug development process that was based on the selection of molecules displaying a particular desirable functional effect. The corollary is that there are often notable side effects, which may at times render the patient intolerant to the treatment. Therefore, investigators have tried early on to develop drugs on the basis of a different principle, namely that of (1) selecting a particular molecular target and (2) designing and synthesizing a small molecule or producing a monoclonal antibody to affect specifically the chosen target. It was initially hoped that such “targeted” therapies would display no systemic toxicity.
However, because many targeted therapeutics entered clinical practice without an in-depth investigation of the anatomic distribution of the affected molecular pathways and their functional characteristics, they often have a much broader specificity than initially intended and may, at times, even lead to quite unexpected side effects.

A second problem is that targeted therapies are not effective in all patients. Ideally, patients should be selected for a particular therapy by assessment of their tumors for tissue biomarkers predictive of a successful treatment. The problem is that such biomarkers are often only identified when large patient groups are already being treated with the drug. Biomarker identification currently faces major logistic and biologic challenges. New technologies in the areas of genomics, methylomics, transcriptomics, proteomics, and metabolomics may overcome these limitations.25

**TARGETED THERAPIES AND COLORECTAL CANCER**

**Bevacizumab and CRC**

Bevacizumab is a humanized murine immunoglobulin (Ig) G1 monoclonal antibody against human vascular endothelial growth factor (VEGF). It is directly antiangiogenic but may also alter tumor vasculature, thus decreasing tissue pressure in tumors and possibly enhancing the delivery of classic chemotherapeutic agents. It has been approved by the US Food and Drug Administration in combination with chemotherapy in first- and second-line treatment for metastatic CRC. Apart from anti-VEGF treatment, targeted therapies in CRC aim to inhibit the function of the epidermal growth factor receptor (EGFR).

**EGFR in Normal Tissues and Cancers**

The epidermal growth factor receptor is a 170-kDa transmembrane tyrosine kinase receptor that is present in most epithelia and that plays an important role in cell growth and function. Extensive research during the last few years has broadened our knowledge about this receptor’s structural and functional characteristics. Epidermal growth factor receptor (also called HER1 or ErbB1) belongs to the HER or ErbB family, which also comprises HER2/neu, HER3, and HER4. All 4 receptors are topologically similar, comprising 3 domains: (1) an extracellular domain that binds specific ligands, such as epidermal growth factor (EGF), transforming growth

---

**Figure 1.** Assessment of total mesorectal excision quality and completeness on the fresh specimen. A, Complete (smooth intact mesorectum without defects or coning; regular circumferential resection margin). B, Nearly complete (irregular mesorectum with moderate bulk and moderate coning; no visible muscularis propria). C, Incomplete (irregular mesorectum with little bulk and moderate to marked coning; muscularis propria visible; see arrows).

**Figure 2.** Assessment of total mesorectal excision quality and completeness on a display of transverse sections.
factor α, and amphiregulin, which bind only to EGFR; (2) a hydrophobic transmembrane domain that is involved in interactions between cell surface receptors; and (3) an intracellular domain that shows tyrosine kinase activity. There are at least 2 exceptions to these general principles: HER2/neu binds no known growth factor ligand with high affinity, and HER3 shows virtually no tyrosine kinase activity. However, all receptors and their ligands interact to form an integrated system in which an initial signal can be amplified and diversified into multiple cellular responses. To activate the EGFR signaling system, 3 sequential steps are usually required. First, specific ligands bind to the extracellular domain of EGFR, resulting in a conformational change. Second, this structural change allows the receptor to form a dimer with another ligand-bound EGFR (homodimer) or with 1 of the other EGFR-related HER receptors (heterodimer). Finally, receptor dimerization causes autophosphorylation of tyrosine residues within the intracellular domain of the receptors, leading to the activation of particular downstream signal transduction pathways. Several such signaling cascades have been identified with regard to EGFR-mediated biologic effects. The 2 most important are (1) the mitogen-activated protein kinase (MAPK) pathway, with participation of KRAS, BRAF, and the extracellular signal-regulated kinase (ERK) proteins; (2) the phosphoinositide 3-kinase (PI3K)/phosphatase and tensin homolog (PTEN)/AKT pathway (Figure 3). In normal tissues, ligand binding by EGFR results in the activation of particular transcription programs. The ultimate consequences of these processes are cell proliferation on the one hand and survival and motility on the other hand, through the MAPK and PI3K/PTEN/AKT pathways, respectively. The pathways also include an autocrine loop with production and secretion of HER receptor ligands, which may then act in an autocrine, juxtacrine, or paracrine manner.

Tumor cells can use 2 basic strategies to rely less on growth signals and ultimately become self-sufficient. First, they may modulate the level of expression and the functional characteristics of their growth signal receptors. Second, they may activate the downstream signaling pathways without actually involving or modulating the cell surface receptors. Both strategies have been observed in colorectal carcinoma. This ultimately leads to the classic features of cancer: uncontrolled cell proliferation and tumor growth, suppression of apoptosis, stimulation of angiogenesis, and facilitated metastatic spread.

**EGFR Expression as a Prognostic Marker in CRC**

Since the EGFR axis is apparently involved in CRC tumorigenesis and progression, EGFR expression has been investigated as a possible prognostic marker in patients with CRC. To this end, several techniques at the DNA, RNA, and protein level can be applied. Commonly used methods include immunohistochemistry (IHC), Western blot, fluorescence in situ hybridization (FISH), and enzyme-linked immunoadsorption assays. Epidermal growth factor receptor protein expression detected by IHC in CRC has been reported to range between 16% in early cancer and 97% in advanced disease.

In most of these studies, any level of membranous EGFR expression above background level, irrespective of the percentage of positive cells or the completeness of the staining or its intensity, was considered positive. Actually, many tumors display a very heterogeneous staining pattern with usually between 1% and 10% positive cells. Staining is often accentuated along the invasive front of the tumor. Completeness of staining and the presence of (irrelevant) cytoplasmic staining generally parallel increasing staining intensity. About 10 studies have shown a correlation between EGFR expression and known prognostic indicators of CRC including tumor stage, grade, and lymphovascular invasion. In contrast, other evaluations could not demonstrate a relation between EGFR status and tumor type, grade, or stage. In a review of 200 studies involving more than 20,000 patients and 10 cancer types, Nicholson et al showed that increased EGFR
expression was associated with reduced recurrence-free or overall survival rates in 52% of studies. The EGFR status was considered by these authors to provide only a “modest prognostic indicator” in CRC. Overall, there is thus a disparity in the reports of EGFR expression as a prognostic factor. This may be due to the variable composition of the studied patient populations, tumor heterogeneity, and certainly also to the lack of a consistent method for evaluating EGFR expression as detected by immunohistochemistry. The lack of a clear relationship between EGFR expression and prognosis is perhaps to be expected given that its activity, and therefore, its influence on cancer cell survival can be amplified by a number of mechanisms other than increased receptor expression.

EGFR-Targeted Therapy in CRC

Given the evident role of EGFR in tumorigenesis and cancer progression, this receptor has been selected as a relevant and promising target for anticancer therapies. In vitro and in vivo preclinical studies have shown that blocking EGFR may lead to inhibition of cancer cell growth with good clinical effect. Two types of medications have been developed that interfere with either the ligand-binding domain of EGFR or its intracytoplasmic signal-transmitting domain. Thus, EGFR signaling can be targeted by (1) monoclonal antibodies that compete with the natural ligands for binding to the receptor; and (2) small-molecule tyrosine kinase inhibitors (TKIs) that compete with ATP for binding to the intracellular catalytic domain. Both approaches can suppress the downstream signaling pathways in tumors whose growth is still dependent on receptor modulation. Currently, monoclonal antibodies such as cetuximab and panitumumab (phase III trials) and low-molecular-weight TKIs such as gefitinib and erlotinib (phase II studies) are in the most advanced stages of clinical development in CRC. The activity of cetuximab (Erbitux, chimeric IgG1) and panitumumab (ABX-EGF or Abgenix, fully human IgG2), as single agents or in combination with chemotherapy for the treatment of recurrent and first-line metastatic CRC (mCRC), has been studied in phase III clinical trials. Cetuximab in combination with irinotecan was approved in 2004 by the US Food and Drug Administration for the treatment of irinotecan-refractory mCRC. The drug was also approved as a single agent for the treatment of patients with EGFR-expressing mCRC who are intolerant to irinotecan-based chemotherapy. Panitumumab has recently been approved in the United States and in Europe as a third-line treatment for mCRC. Compared with the anti-EGFR monoclonal antibodies, TKIs have not been extensively investigated in mCRC. It seems that gefitinib (Iressa, ZD1839) as a single agent is not active in chemorefractory CRC. When used in combination with the FOLFOX (folic acid [leucovorin], fluorouracil, oxaliplatin) regimen, it induced a high objective response rate (78% for chemotherapy-naive patients and 36% for recurrent patients), but toxicity was also present with grade 3/4 diarrhea for 49% of patients. The combination of erlotinib (Tarceva, OSI-774) with capecitabine and oxaliplatin is more feasible but less active. Finally, there are also less well-developed approaches including antisense nucleotides or ribozymes that target EGFR mRNA (messenger RNA) and block receptor translation or intracellular antibodies that prevent migration of the receptors to the cell surface.

EGFR Protein Expression as a Predictive Marker for Success of EGFR-Targeted Therapy in CRC

Both cetuximab and panitumumab have been shown to reduce the risk of tumor progression and to improve quality of life, progression-free survival, and overall survival in patients with refractory mCRC. Such were the results of clinical trials in which patients had been selected on the basis of a positive IHC staining for EGFR in the tumor. Because of prior experience with trastuzumab (a monoclonal antibody that blocks the HER2/neu receptor) in selected cases of breast cancer, it was expected that expression of EGFR in CRC tissues would be necessary for treatment efficacy. However, this does not seem to be the case. First, only a small proportion (8%–23%) of selected patients did achieve an objective response with either antibody. Second, no apparent correlation was observed between the extent of EGFR expression and the response of tumors to second-line cetuximab, panitumumab, or gefitinib therapy. Third, some patients with tumors in which absolutely no EGFR could be detected by IHC have still achieved an objective response to cetuximab.

The reasons why IHC detection of EGFR is a poor predictor of response to cetuximab may include a variety of biologic, technical, and analytic factors. A recent study by Francoual et al indicates that EGFR is a mixture of high- and low-affinity EGF binding sites. Only the high-affinity sites, sometimes underrepresented as compared to the low-affinity sites, would carry a meaningful biologic activity. It has been suggested that IHC is not the optimal method for quantification of the high-affinity EGF binding sites. Furthermore, the EGFR signaling pathway is complex, and it is possible that the level of expression of the endogenous receptor ligands, the level of tyrosine phosphorylation of the receptor, and the expression of other downstream molecules are critically involved in the action of cetuximab and therefore are more predictive for treatment response than the total level of the receptor per se. From a technical point of view, tissue processing and handling (type and duration of fixation, type of antibody and antigen retrieval) and prolonged storage time of tissue samples may influence detectability of protein expression. Finally, the positivity rate for EGFR staining in any study depends on the cutoff point applied, and this has varied in the literature. The correct cutoff point may actually depend upon the antibody being used.

Cetuximab and panitumumab therapy are both costly and may cause side effects. It was thus necessary to look for better predictive tissue biomarkers than EGFR protein expression. The currently identified possible markers are either related to EGFR itself or to its various downstream targets.

Tissue Biomarkers Related to EGFR Itself

EGFR Gene Mutations and Polymorphisms

In contrast to the situation in non–small cell lung cancer, only very infrequent mutations in the catalytic domain of EGFR, predictive for response to gefitinib, have been observed in CRC (1 of 293 cases). Nagahara et al detected no somatic mutations in 11 CRC cell lines, although 4 of 33 clinical tumors (12%) presented missense mutations clustered in the EGFR kinase domain in exons 19 and 20. Ogin et al confirmed the rarity of activating EGFR mutations in CRC. These studies suggest that for mCRC, the impact of treatment with a TKI is likely to be

Practical and Molecular Evaluation of CRC—De Hertogh & Geboes
limited. The type III mutated variant of EGFR (EGFR vIII), which is characterized by a deletion in the extracellular domain and has been identified in various human tumors, is rare in CRC. On the other hand, a recent study reported on a variant of exon 13 (R521K; arginine-to-lysine substitution at residue 521) detected in 12 of 32 investigated tumors, 11 of which achieved objective response or stable disease with cetuximab treatment. Exon 13 encodes a segment of the extracellular part of the receptor. The arginine-to-lysine amino acid substitution is located at the boundary between EGFR domain III, which represents the direct interaction site with cetuximab, and domain IV. While this genetic polymorphism is frequent in the general population (20% for the homozygous and 50% for the heterozygous variant), it has also been shown to significantly reduce transforming growth factor α binding and ligand-induced EGFR signalling. It has been speculated that the polymorphism-associated, attenuated EGFR signaling could be more sensitive to targeted receptor inhibition.

**Increased EGFR Gene Copy Number**

The presence of an increased EGFR gene copy number (GCN) in CRC, as detected by FISH, has been associated with a better response to anti-EGFR therapies. Moroni et al found that 8 of 9 patients with objective response after treatment with cetuximab or panitumumab had an increased EGFR GCN as opposed to only 1 of 21 nonresponders ($P < .001$). In a study by Frattini et al in which 27 patients with mCRC were treated with cetuximab, an increased EGFR GCN was observed in 6 of 10 responders compared with only 2 of 17 nonresponders ($P < .05$). On the other hand, in a phase II trial of cetuximab in refractory mCRC, Lenz et al showed that an increased EGFR GCN, assessed by polymerase chain reaction ($N = 34$), did not correlate with response or progression-free survival ($P = .03$). This is perhaps not unexpected because, during DNA extraction for polymerase chain reaction, the abnormal signal derived from the tumor cells may be diluted by the normal signal present in stromal cells. However, Italiano et al also reported that increased EGFR GCN detected by FISH did not select patients with CRC who might benefit from cetuximab therapy. This lack of consistency among reports may be due to the existence of various subpopulations of EGFRs with different levels of low- and high-affinity sites, the lack of standardized EGFR FISH testing methods, tumor heterogeneity, and a less than perfect correlation between EGFR DNA and protein levels (low-level polysomy and gene amplification and their relation with immunohistochemistry; Figure 4, A through C). Personeni et al concluded that for the time being, it may not be warranted to make clinical decisions on the basis of previously published cutoff points. It has also been suggested that the crucial finding in all these studies is that the nonincreased, rather than the increased, EGFR GCN status is the most accurate predictive element for clinical outcome.

![Figure 4](image-url)
with a low EGFR GCN are indeed unlikely to have tumors that respond to treatment and have a worse time to progression and overall survival than patients with an increased GCN. In those tumors with an increased GCN that do not respond to treatment, resistance to therapy may be due to constitutive activation of downstream signalling pathways by mutations of oncoproteins such as KRAS, BRAF, or PI3K or by loss of a tumor suppressor gene such as PTEN.81

**Increased Expression of EGFR Ligands**

Kambata-Ford et al.82 found that patients with high gene expression levels of the EGFR ligands epiregulin and amphiregulin in their metastatic tumor lesions were more likely to achieve disease control upon cetuximab treatment. Epiregulin binds more weakly to EGFR than EGF, but is much more potent and leads to a state of prolonged receptor activation.83 Elevated expression of epiregulin and/or amphiregulin may play an important role in tumor growth and survival by stimulating an autocrine loop through EGFR. This may characterize a tumor that is dependent on EGFR for growth factor signaling and therefore particularly sensitive to the ability of cetuximab to block ligand-receptor interaction.

**Activated EGFR**

Among a subgroup of 23 patients with EGFR-positive mCRC refractory to irinotecan who were treated in the BOND (Bowel Oncology With Cetuximab Antibody) study, expression of the activated/phosphorylated EGFR (pEGFR) appeared to be a useful predictor of response to cetuximab-based therapy. Disease control rates differed between patients with a pEGFR immunohistochemical score of at least 7 (7/7, 100%) and those with a score of less than 7 (7/13, 53.8%), showing a trend toward higher disease control in patients with high levels of pEGFR who were treated with cetuximab, with or without irinotecan (P = .05).84,85

**Tissue Biomarkers Related to EGFR:**

**Conclusion**

In contrast to HER2/neu expression and trastuzumab treatment in breast cancer, EGFR protein expression, as assessed by immunohistochemistry, has no predictive value for the efficacy of EGFR-targeted therapy in CRC. Other proposed tissue biomarkers related to EGFR are still under investigation. The most promising seem to be the evaluation by FISH of the EGFR gene copy number and the detection of an increased expression of EGFR ligands.

**TISSUE BIOMARKERS RELATED TO THE DOWNSTREAM SIGNALING PATHWAYS**

**KRAS Mutations**

The KRAS oncogene is mutated in more than 30% of CRCs.86 More than 3000 point mutations have been reported in CRC samples thus far. Somatic missense mutations in the KRAS gene lead to single amino acid substitutions and occur independently of EGFR mutations.87 The most frequent alterations are detected in codon 12 (Gly to Asp/Val/Ala/Cys/Ser/Arg, together about 82% of all reported KRAS mutations) and 13 (mainly Gly to Asp, about 17%) in exon 2 of the gene. Mutations in other positions, such as codons 61 and 146, have also been reported but are rare and of unknown significance.88 The KRAS gene encodes a G protein that functions downstream of EGFR in the MAPK pathway. RAS proteins normally cycle between active GTP-bound and inactive GDP-bound conformations. They are activated by guanine nucleotide exchange factors (GEFs) which are recruited to protein complexes at the intracellular domain of activated receptors. Signaling is terminated when GTP is hydrolyzed to GDP by the intrinsic hydrolase activity of the RAS protein, which is enhanced by GAPs. Mutations in genes that encode RAS proteins disrupt this balance. Particular KRAS mutations result in RAS proteins that remain permanently in the active state because of a defective intrinsic GTPase activity and resistance to GAPs. Unlike wild-type RAS proteins, which are inactivated after a short time, the aberrant proteins continuously activate signaling pathways, even in the absence of any upstream stimulation of HER receptors. In tumors carrying KRAS mutations, blocking EGF signaling at the receptor level will not ablate the transmission of a signal in the MAPK pathway. Tumor KRAS mutation status is thus expected to have a large impact on therapeutic decisions for patients with CRC. Indeed, several clinical studies have illustrated that KRAS mutations in codon 12 and 13 lead to resistance to anti-EGFR monoclonal antibodies.51,89,90 These mutations are associated with poor response to therapy, reduced progression-free survival, and shorter overall survival in patients with CRC who are treated with cetuximab, either alone or in combination with chemotherapy. Similarly, an analysis of KRAS mutations in tumor samples from 92% of the patients in a registration clinical trial of panitumumab for the treatment of mCRC predicted a lack of efficacy of panitumumab on progression-free and overall survival in patients with KRAS-mutant tumors.91 Taken together, these results indicate that KRAS mutation status is an important parameter for selecting patients for therapy: patients with mutant tumors will not benefit from EGFR-targeted therapies. On the basis of these data, the European Medicines Agency has approved the use of cetuximab and panitumumab for the treatment of mCRC in patients who carry a normal, wild-type KRAS gene.32 However, as only a fraction of patients with wild-type KRAS in colorectal tumors can achieve a clinical response with EGFR-targeted therapies, the search for additional predictive parameters must go on. The tissue chosen for KRAS mutation testing is usually the primary tumor as KRAS mutations develop early on in the carcinogenic sequence.94 The representativity of endoscopically obtained tumor samples versus biopsies taken from surgical specimens is still being investigated. Various user-developed and commercial techniques are currently applied for the detection of KRAS mutations in CRC. A European Quality Assurance Program has been proposed. This will be organized by the European Society of Pathology in close collaboration with existing regional and national quality assurance programs.95

**BRAF Mutations**

BRAF is the principal effector of KRAS. It has thus been hypothesized that in patients with wild-type KRAS, BRAF mutations could have a predictive value for response to cetuximab or panitumumab. Di Nicolantonio et al.96 retrospectively analyzed objective tumor responses, time
to progression, overall survival, and the mutational status of KRAS and BRAF in 113 tumors from patients with mCRC treated with cetuximab or panitumumab. The effect of the BRAF Val600Glu mutation (exon 15) on response was also assessed by using cellular models of CRC. KRAS mutations were present in 30% of the patients and were associated with resistance to cetuximab or panitumumab (P = .01). None of the KRAS-mutated samples carried a BRAF mutation, in accordance with previous findings on the mutual exclusivity of KRAS and BRAF mutations in CRC. The BRAF mutation was detected in 11 of 79 patients who had wild-type KRAS. None of the patients with this BRAF mutation responded to treatment, whereas none of the responders carried BRAF mutations (P = .03). Patients with BRAF mutation had significantly shorter progression-free survival (P = .01) and overall survival (P < .001) than patients with the wild-type gene. The introduction of a BRAF allele resulting in Val600Glu substitution in CRC cell lines impaired the therapeutic effect of cetuximab or panitumumab. On the other hand, treatment of mutant cell lines with the BRAF inhibitor sorafenib restored sensitivity to the EGFR inhibitors. A BRAF wild-type status thus seems to be required for tumor response and can potentially be used to select patients eligible for treatment. The authors also suggested to test double-hit therapies with simultaneous inhibition of EGFR and BRAF in patients with CRC who carry the Val600Glu mutation.

Malfunctions of the PI3K/PTEN/AKT Pathway

Preclinical studies on tumor cell lines (A431, MDA-468, and H157) have suggested that PTEN inactivation is a predictor for resistance to EGFR-family antagonists.\(^7\)\(^-\)\(^9\) PTEN is a lipid phosphatase and a tumor suppressor protein that regulates the PI3K/AKT signaling pathway. When PTEN function is lost, its major substrate, phos- phatidylinositol 3,4,5-trisphosphate, which is a second messenger of PI3K, accumulates in the cell membrane, where it binds and activates AKT. Thus, the loss of PTEN function results in constitutive overactivation of the PI3K/AKT pathway, increasing its antiapoptotic functions. PTEN loss has been associated with resistance to trastuzumab, which binds to the HER2/neu receptor.\(^10\) As HER2/neu and EGFR form heterodimers upon ligand binding and use identical downstream signaling pathways, it seemed reasonable to test PTEN as a potential predictive factor for cetuximab resistance. PTEN expression has been found to be decreased in approximately 40% of colorectal cancers, often with associated mutations, deletions, or promoter hypermethylation.\(^10\) Frattini et al\(^1\) showed that PTEN tumor expression was correlated with response to cetuximab for 27 patients with mCRC. All responder patients were found to express PTEN in their tumor (10/10) versus 35% of nonresponder patients (6/17) (P < .001). In this study, PTEN expression was detected by IHC staining. PTEN-negative tumors were defined as those with a dramatic reduction or absence of immunostaining in at least 50% of the tumor cells, as compared with the internal control (eg, nerves).

AKT was also identified in preclinical studies as a major resistance factor for anti-EGFR therapy.\(^7\)\(^-\)\(^9\) Perez-Soler et al\(^1\) showed that erlotinib-resistant clones of human squamous cancer cell lines had significantly higher levels of total and phosphorylated AKT, suggesting persistent downstream signaling even in the presence of EGFR inhibitors. These findings need to be confirmed in colorectal carcinomas.

Investigation of cetuximab response in colon cancer cell lines suggests that constitutive and simultaneous activation of the MAPK and PI3K/AKT pathways confers maximal resistance to this agent. A priori screening of colon tumors for PTEN expression status and PI3K and KRAS/ BRAF mutation status could help in stratifying patients who are likely to benefit from this therapy.\(^7\)

**Tissue Biomarkers Related to Signaling Pathways Downstream From EGFR: Conclusion**

The results on the investigation of KRAS mutation status as a marker for clinical benefit of anti-EGFR antibodies are so impressive that they have already had a major impact on treatment strategies. The European Medicines Agency has approved panitumumab for the treatment of patients with wild-type KRAS tumors only. This would be the first example of the approval of a drug therapy for solid tumors that is based on a genetic test. The 10 ongoing cetuximab studies sponsored by the National Cancer Institute in the United States are currently being amended to include KRAS testing. Further molecular analysis of tumor specimens banked for these and other studies may yield other markers that can identify patients who have a chance to benefit from EGFR-targeting antibodies.

**TAKE-HOME MESSAGES**

1. Management of CRC is now a team process. Pathologists play an important role in diagnosis, prognostication, and, increasingly, selection of patients likely to react to particular therapeutic regimens.

2. Tissue biomarkers indicative for response to target-ed therapies are often identified only when the medica-tion is already being used clinically. Pathologists can participate in this endeavor by their access to and knowledge of the structure of tumor tissues and the various techniques with which these can be investigated.

3. Several biomarkers have been investigated with regard to EGFR-related targeted therapies in CRC. The KRAS mutation status of the tumor has a proven predictive value and is currently determined for many patients in a metastatic setting. Pathologists can assist in this effort by ensuring investigation of the proper tissues with the optimal techniques.

**References**


(<1%) levels of epidermal growth factor receptor [abstract 3547]. J Clin Oncol. 2006;24(suppl):157s.
36. She QB, Solt D, Basso A, Moasser MM. Resistance to gefitinib in PTEN-null HER-overexpressing tumor cells can be overcome through restoration of PTEN function or pharmacologic modulation of constitutive phosphatidylinositol 3'-kinase/Akt pathway signaling. Clin Cancer Res. 2003;9(12):4340–4346.