

### A Review of the Most Promising Biomarkers in Colorectal Cancer: One Step Closer to Targeted Therapy

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#### ABSTRACT

Rapidly growing insights into the molecular biology of colorectal cancer (CRC) and recent developments in gene sequencing and molecular diagnostics have led to high expectations for the identification of molecular markers to be used in optimized and tailored treatment regimens. However, many of the published data on molecular biomarkers are contradictory in their findings and the current reality is that no molecular marker, other than the *KRAS* gene in the case of epidermal growth factor receptor (EGFR)-targeted therapy for metastatic disease, has made it into clinical practice. Many markers investigated suffer from technical shortcomings, resulting from lack of quantitative techniques to capture the impact of

the molecular alteration. This understanding has recently led to the more comprehensive approaches of global gene expression profiling or genome-wide analysis to determine prognostic and predictive signatures in tumors. In this review, an update of the most recent data on promising biological prognostic and/or predictive markers, including microsatellite instability, epidermal growth factor receptor, *KRAS*, *BRAF*, CpG island methylator phenotype, cytotoxic T lymphocytes, forkhead box P3-positive T cells, receptor for hyaluronic acid-mediated motility, phosphatase and tensin homolog, and T-cell originated protein kinase, in patients with CRC is provided. *The Oncologist* 2010;15:699–731

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#### INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed type of cancer in men and women worldwide. An estimated 148,810 new cases of colon/rectum cancer were forecast to be diagnosed in the U.S. in 2008 [1], while 376,400 cases were forecast to be diagnosed in Europe in

2004 [2]. In addition, CRC continues to be one of the most common fatal types of cancer.

CRC develops slowly over several years and progresses through cytologically distinct benign and malignant stages of growth ranging from single crypt lesions through adenoma to malignant carcinoma with the potential for inva-

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sion and metastasis [3, 4]. Colorectal carcinogenesis is characterized by the successive accumulation of mutations in genes controlling epithelial cell growth and differentiation leading to genomic instability whereby widespread loss of DNA integrity is perpetuated [5]. The development of genomic instability is an important event in the multistep progression of colorectal carcinogenesis. Two apparently independent pathways of genomic instability have been identified. The suppressor pathway is found in the majority of cancers (85%) and these tumors have a molecular profile characterized by specific chromosomal amplifications and transformations, aneuploidy, and loss of heterozygosity (LOH). Cancers originating from the mutator pathway have a defective DNA mismatch repair mechanism (MMR), which allows mutations to be accumulated at many times the normal rate. This inability to repair DNA mismatches can easily be demonstrated because it results in cell-to-cell variability in the length of DNA microsatellites, called microsatellite instability (MSI) [6]. Colorectal malignancies demonstrating MSI have a very heterogeneous histological appearance, better prognosis, and altered response to chemotherapy and radiotherapy; see further below [7, 8]. In addition, in recent years, it has become apparent that promoter methylation is as important in shutting down tumor suppressor genes, as are the various mechanisms of somatic mutation. More than half of the tumor suppressor genes that are involved in familial cancer syndromes, because of germline mutation, have been found to be silenced in sporadic colorectal cancer by promoter hypermethylation. Careful characterization of the epigenetic factors, particularly promoter sequence methylation, has led to the definition of CpG island methylator phenotype (CIMP) cancer, which is proposed as a novel, third pathway [9].

Treatment of CRC consists of complete surgical removal of the primary tumor and the regional lymph nodes [10]. At the time of resection, approximately 30%–40% of patients with CRC are diagnosed with stage II disease [11]. Despite improvements in surgical techniques and the dosing and scheduling of adjuvant and neoadjuvant therapy, the 5-year survival rate for patients with early-stage CRC, that is, without invasion or lymph node metastasis, is about 90%, but this falls to 65% for tumors with regional spread and to 10% for late-stage disease in which the cancer has metastasized to distant sites [12]. Prognostication relies on the stage or anatomic extent of disease based on the International Union Against Cancer tumor–node–metastasis (TNM) and American Joint Committee on Cancer staging classifications. The function of TNM staging has expanded from predicting prognosis to aiding in the choice of treatment [10, 12]. Accordingly, all node-positive cases (T3/4N<sub>pos</sub>M0, stage III) receive adjuvant therapy while,

despite several large randomized trials (Intergroup trials, Multicenter International Study of Oxaliplatin/5-Fluorouracil/Leucovorin in the Adjuvant Treatment of Colorectal Cancer; Immediate Preoperative Arimidex®, Tamoxifen, or Combined with Tamoxifen; Surveillance, Epidemiology, and End Results; and National Surgical Adjuvant Breast and Bowel Project trials) conducted over the past decades, the value of adjuvant therapy for node-negative cases (T3/4N0M0, stage II) is controversial [10, 13]. There may well be a subgroup of patients with stage II CRC who would benefit from adjuvant chemotherapy [14]. Patients with stage IIB (T4N0M0) CRC have a worse prognosis than patients with stage IIIA (T1–2N1M0) CRC, although the latter are usually treated with adjuvant therapy. The predictive value of TNM staging is limited because even the outcome within each stage group is not homogeneous.

Indeed, CRC should be regarded as a heterogeneous, multipathway disease, an observation sustained by the fact that histologically identical tumors may have neither a similar prognosis nor a similar response to therapy [12, 15]. Therefore, particularly in stage II CRC, there is a need for markers capable of selecting those patients with aggressive disease that might benefit from adjuvant chemotherapy [16].

With recent developments in gene sequencing, molecular diagnostics, and targeted therapies, cancer treatment is beginning to move from the traditional “trial and error” approach to a position involving a personalized approach. There is clearly a need for strong and independent prognostic markers that can reliably differentiate patients into subgroups for which different treatment options, including the possibility of no adjuvant treatment, are appropriate. In addition, markers to prospectively predict response or resistance to specific therapies and markers to identify patients who are likely to develop severe toxic side effects from these specific treatments are warranted [16]. However, in practice, the distinction between prognostic and predictive factors is not straightforward, and many factors are a mixture of the two [17].

Consequently, in recent years a huge amount of research has been devoted to the study of new biological prognostic/predictive markers (Table 1). Several criteria must be met to ensure that a biomarker is clinically useful. In addition, evidence needs to be derived from multiple independent studies, which ideally should include a prospective trial. Most importantly, the marker concentration or status must be able to affect patient management [16]. Although hundreds of these markers have been proposed in the last two to three decades, the current reality is that no molecular marker, other than the *KRAS* gene in the case of epidermal

<b>Table 1.</b> Summary of tissue-based prognostic/predictive markers most studied in colorectal cancer	
Histological	TNM staging Histological grade Margins Histological type Immune response Number of affected lymph nodes Lymphovascular invasion
Tissue-based markers	
Oncogenes	Kirsten rat sarcoma gene ( <i>KRAS</i> ) Epidermal growth factor receptor ( <i>EGFR</i> ) <i>c-myc</i> Transforming growth factor $\beta$ ( <i>TGF<math>\beta</math></i> ) v-raf murine sarcoma viral oncogene homolog B1 ( <i>BRAF</i> )
Tumor suppressor genes/LOH	<i>p53</i> <i>p27</i> <i>p21</i> Mutated in colorectal cancer ( <i>MCC</i> ) 18q LOH deleted in colorectal cancer ( <i>DCC</i> ) SMAD family member 2 ( <i>SMAD2</i> ) SMAD family member 4 ( <i>SMAD4</i> ) 1p LOH 8p LOH 9p LOH 14p LOH
Proliferating indices	Proliferating cell nuclear antigen Ki-67 MIB-1
Genetic instability	Microsatellite instability Chromosomal instability CpG island methylator phenotype Thymidylate synthase
Apoptosis	Bcl-2 BAX Antioxidants Fas/CD95
Angiogenesis	Phosphatidylinositol 3 kinase, catalytic, $\alpha$ polypeptide Vascular endothelial growth factor Microvessel density Thymidine phosphorylase Cell adhesion molecules
Metastasis and invasion	Thrombospondin-1 Matrix metalloproteinases Urokinase type plasminogen activator Plasminogen inhibitor CD44 E-cadherin Nonmetastatic protein 23
Abbreviations: LOH, loss of heterozygosity; TNM, tumor–node–metastasis.	

growth factor receptor (EGFR)-targeted therapy for metastatic disease, has made it into clinical practice [16, 17].

The aim of this review is to provide an update of the most recent data on biological prognostic and or predictive markers in patients with CRC that show promise in the clinic.

## TISSUE-BASED BIOMARKERS

### MSI

As mentioned above, MSI reflects the presence of a defective mismatch repair (MMR) mechanism and is characterized by somatic alterations in the size of simple repeat microsatellite nucleotide sequences common throughout the genome [18, 19]. As a consequence, genes containing simple repeat sequences, such as *TGF $\beta$ R2*, *EGFR*, or *BAX*, are often mutated in these tumors [20]. While 10%–15% of sporadic CRC cases display MSI, predominantly caused by epigenetic hypermethylation of the *MLH1* mismatch repair gene, the majority of hereditary nonpolyposis colorectal cancer (HNPCC) tumors are characterized by this MSI phenotype. Individuals with a high-frequency MSI (MSI-H) tumor have phenotypically distinct features that are substantially different from those following the chromosomal instability pathway, though not all characteristics have consistently been demonstrated in all studies [21, 22]. MSI-H tumors tend to be more proximal, poorly differentiated, and mucinous, and show marked lymphocytic infiltration [23]. In addition, these tumors tend to retain the native diploid state [24]. These distinct features might be useful as diagnostic, predictive, or prognostic markers. In addition, MSI-H CRCs have different behavior patterns and responses to chemotherapy, and possibly different outcomes [25]. Therefore, MSI is considered one of the most promising markers studied to date.

### *MSI as a Prognostic Factor*

Although several studies indicated that MSI is an independent prognostic factor in CRC, uncertainty remains. Controversial data have been reported (Table 2), leaving an area of uncertainty on the usefulness of this molecular marker to indicate a requirement for adjuvant therapy in clinical practice (as reviewed in [18, 20, 24, 26–34]). Graziano and Cascinu [18] focused on the role of MSI in early-stage sporadic CRC. Among the 26 selected studies, 17 retrospective investigations showed a significant association between MSI-H or abrogated hMLH1 and/or hMSH2 expression and a superior prognosis. However, available data were insufficient to support the prognostic role of MSI in Dukes' stage B CRC patients [18]. A recently published systematic review of MSI and CRC prognosis, pooling data from 32

studies and a total of 7,642 unselected patients, demonstrated a significant survival advantage for patients with MSI tumors, compared with patients with microsatellite stable (MSS) CRC [24]. The findings of that systematic review indicate that MSI status has the potential to identify patients who might be treated with surgery alone, particularly in those patients who already show indications of a favorable prognosis.

However, before MSI status can be routinely used to influence patient management, validation in the context of a prospective clinical trial is required. The relatively low frequency of MSI in sporadic CRC cases is a major limitation for planning such large prospective trials with MSI-based identification of high-risk patients [18]. Nevertheless, currently the impact of MSI and 18q LOH is being tested prospectively in stage II CRC patients (Intergroup E5202 trial, see below) [23]. In contrast, in the metastatic setting, MSI-H is not a common feature and does not seem to play a role in stratifying good versus poor prognosis in these patients [35].

### *MSI and Adjuvant Therapy: Predictive Potential*

MSI-H has been associated with a favorable prognosis in most studies; however, it is not clear whether this is because MSI-H tumors are inherently less aggressive or because they are more sensitive to chemotherapy [36].

Since the early 1990s, 5-fluorouracil (5-FU) has been the mainstay of chemotherapeutic treatment for patients with CRC in the adjuvant setting. Nowadays, almost all adjuvant chemotherapy regimens involve the use of 5-FU, typically in combination with leucovorin and more recently with oxaliplatin [21]. Warusavitarne and Schnitzler [25] recently reviewed the role of chemotherapy in MSI-H CRC. In several studies, the impact of 5-FU chemotherapy in CRC patients led to a survival advantage for MSI<sup>+</sup> disease [37–39]. However, contrary to this, most studies found that adjuvant chemotherapy with 5-FU itself did not benefit patients with tumors exhibiting MSI (as reviewed in [5, 31, 34, 36]) [40–42]. In addition, an overall lesser benefit from adjuvant therapy in MSI<sup>+</sup> CRC patients could not be demonstrated in recent systematic reviews and a meta-analysis [24, 32, 36, 43]. There is evidence to suggest that different chemosensitivity exists in MSI-H tumors and MSS CRCs. The most likely mechanisms by which 5-FU resistance is conferred are possibly reduced thymidilate synthase activity and inability of the MMR genes to bind 5-FU–modified DNA [25]. Although the clinical evidence is conflicting, the in vitro data suggest a strong association between MSI-H and resistance to 5-FU. However, closer analysis of the clinical data appears to suggest no benefit from 5-FU treatment in patients with MSI-H CRC, which is supported by in

**Table 2.** The prognostic relevance of MSI in CRC studied in the last 5 years with PCR analysis

Study	n	Stage	MSI-H	Prognostic significance	Remarks
Lim et al. (2004) [44]	248 CRC	I–IV	9.3%	Yes	
Emterling et al. (2004) [45]	438 CRC	I–IV	13%	No	
Benatti et al. (2005) [21]	1,263 CRC	I–IV	20.3%	Yes	The type of genomic instability could influence the prognosis of CRC, particularly in stage II and stage III cases
Storojeva et al. (2005) [46]	160 CRC	I–IV	13%	No	MSI-H phenotype did not predict a larger benefit of adjuvant chemotherapy
Vidaurreta et al. (2005) [47]	106 CRC	I–IV	9.4%	Yes	
Chang et al. (2006) [48]	140 CRC	I–IV	31%	Trend	Stage III MSI-H patients had a trend toward a longer disease-free survival interval
Chang et al. (2006) [49]	213 CRC	I–IV	8.9%	Yes	
Meng et al. (2007) [50]	128 rectal cancers	II–III	9.3%	No	
Lamberti et al. (2007) [51]	416 CRC	I–IV	13%	No	This prospective study found no clear influence of MSI status on overall survival and response to 5-FU therapy
Malesci et al. (2007) [52]	893 CRC	I–IV	10%	Yes	MSI is a stage-dependent predictor of survival in CRC patients; MSI was strongly associated with a lower likelihood of metastasis
Müller et al. (2008) [53]	108 mCRC	IV	4%	No	There was a correlation with a lower rate of disease control
Deschoolmeester et al. (2008) [54]	241 colon, 90 rectal cancers	I–III	12.4%	No	
Ogino et al. (2009) [55]	649 colon cancers	I–IV	18.6%	Trend	
Kumar et al. (2009) [56]	167 colon cancers		20/1%	Yes	The MSI and lymphocytic infiltration status shows promise as a combined prognostic marker and can prove particularly useful in selecting patients with stage II disease for adjuvant therapy
Barrasa et al. (2009) [57]	92 CRC	I–IV	15.4%	No	No impact of MSI on global or disease-free survival could be found
Banerjea et al. (2009) [58]	91 R0 resections	I–III	17.6%	Yes	Stratification of CRC by MSI and ploidy status may have prognostic value in patients undergoing curative surgery; MSI-H cancers also display enhanced immunogenic properties
Sanchez et al. (2009) [59]	391 CRC	I–IV	21%	Yes	
Ogino et al. (2009) [55]	649 CRC	I–IV	19%	Yes	

Older studies were reviewed by Popat et al. and others (see text).  
Abbreviations: 5-FU, 5-fluorouracil; CRC, colorectal cancer; IHC, immunohistochemistry; MSI, microsatellite instability; MSI-H, high-frequency MSI; PCR, polymerase chain reaction.

vitro studies. The studies that showed a benefit from 5-FU chemotherapy in MSI-H CRCs relied on retrospective re-

views of consecutive stage III CRC cases, for which no randomization to chemotherapy occurred. Because patients



with MSI-H tumors have a better prognosis than those with MSS CRCs, analysis of nonselected patients results in a selection bias in the absence of appropriate controls [25].

Because the role of chemotherapy in patients with MSI-H tumors has not been well determined in the literature, more randomized controlled trials are required to evaluate its predictive potential in the adjuvant setting [25].

## EGFR

### *EGFR Pathway*

EGFR is a member of the transmembrane glycoprotein receptor tyrosine kinase family known as the ErbB or HER receptor family [60]. It is composed of an extracellular ligand-binding domain, a hydrophobic transmembrane region, and an intracellular tyrosine kinase domain. When activated, EGFR phosphorylates and activates other intracellular proteins that affect cell signaling pathways, cellular proliferation, and control of apoptosis and angiogenesis [60, 61]. A major downstream signaling route of the ErbB family is via the RAS–RAF–mitogen-activated protein kinase (MAPK) pathway. Another important target in EGFR signaling is phosphatidylinositol 3-kinase (PI3K) and the downstream protein serine/threonine kinase Akt [62].

EGFR has been implicated in colorectal tumorigenesis, tumor progression, and metastasis, as reviewed in Lockhart and Berlin [63, 64]. Overexpression of EGFR has been described in up to 65%–70% of human colon tumors and has been associated with advanced stage disease [64]. In fact, the highest EGFR reactivity by immunohistochemistry (IHC) is seen in the deepest, most invasive regions of the tumor [65]. Differences in EGFR expression are also seen between stage II and stage IV tumors, implying that the pathway is potentially implicated in the progression of CRC to a more advanced stage [64]. Therefore, EGFR not only represents a possible prognostic marker in the adjuvant setting of primary tumors but primarily a rational molecular target for a new class of anticancer agents, especially in the setting of metastatic CRC (mCRC) [66–68].

### *EGFR as a Prognostic Factor*

Based on the importance of EGFR in many aspects of CRC pathogenesis, a multitude of studies has evaluated the prognostic relevance of EGFR in primary tumors [60, 61, 68–74], but the impact of its expression on survival remains controversial, and a convincing link to poorer survival has not been demonstrated to date [75]. As reviewed by Spano et al. [62], some studies showed evidence of correlation between EGFR expression and advanced stage, worse histological grade, and lymphovascular invasion [76–78]. In contrast, other evaluations, including a recent large-scale

study examining 249 CRC cases, have indicated no relation between EGFR status and histological type, tumor grade, stage, or survival [65, 68, 73, 74, 79]. In addition, Deng et al. [80] and Goldstein et al. [81] showed a trend toward shorter survival for patients with EGFR expression in metastatic lymph nodes in stage III and stage IV patients, suggesting that EGFR expression in metastatic lymph nodes may be more accurate in predicting survival than its expression in primary tissues or metastases at distant sites. The heterogeneity of the results may partly be attributed to non-comparable study populations, variability in laboratory protocols, fixation of tissue samples, choice of antibodies, and the lack of a uniform scoring system [82]. Additionally, the lack of a clear relationship between EGFR expression and prognosis is 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 greater receptor expression [62].

Despite the lack of a definitive association between EGFR dysregulation and clinical outcome in the adjuvant setting, pharmacological inhibitors of EGFR have resulted in significant benefit in certain CRC patient populations [64].

### *EGFR-Targeting Strategies*

In preclinical models, it was found that the inhibition of EGFRs had antitumor activity, and available data suggest synergy with both chemotherapy and radiotherapy [69]. EGFR inhibition can therefore be considered an attractive approach for cancer treatment. EGFR signaling can be targeted by either monoclonal antibodies (mAbs) (such as cetuximab and panitumumab), which interfere directly with receptor signaling, or tyrosine kinase inhibitors (TKIs) (such as gefitinib), which interfere with the catalytic activity of the cytoplasmic domain and alter downstream signal propagation [62, 68, 69]. Nowadays, EGFR-targeted therapy is undergoing extensive clinical evaluation [83] and while TKIs overall generated disappointing results in CRC, mAbs have reached the clinic for mCRC. Cetuximab, a mouse chimeric IgG<sub>1</sub> mAb, and panitumumab, a fully human IgG<sub>2</sub> mAb, are clearly active agents and have been investigated in several clinical trials as single agents and in combination with chemotherapy for the treatment of recurrent or first-line mCRC as well as in the adjuvant setting [62, 83–85]. Results of these studies have demonstrated a manageable and acceptable toxicity profile and a promising level of activity [67, 83]. It is believed that the cellular mechanism of action of cetuximab is related to inhibition of cell cycle progression, increased apoptosis, inhibition of angiogenesis, and possibly amplification of antineoplastic cytotoxic effects of chemotherapy [86]. At present, the an-

tibodies cetuximab and panitumumab are the only anti-EGFR therapies the U.S. Food and Drug Administration has approved for the treatment of patients with EGFR-expressing mCRC in combination with chemotherapy or as a single agent in patients who have failed oxaliplatin- and irinotecan-based therapy and who are intolerant to irinotecan [62, 83]. The European Medicine Agency (EMA) has restricted the use of cetuximab and panitumumab to patients with EGFR-expressing, wild-type *KRAS* mCRC (see below).

## ***KRAS***

### ***Biology of RAS***

The *RAS* gene family encodes low molecular weight (21 kDa) membrane-associated, guanine nucleotide-binding proteins (p21) that function as binary molecular switches that control intracellular signaling networks and are involved in the control of cellular proliferation and differentiation by transduction of extracellular mitogenic signals [18]. Similar to other guanine-binding proteins, the *RAS* proteins switch between an active guanosine triphosphate-bound form and an inactive, guanosine diphosphate-bound form. The activation of *RAS* proteins is dependent on post-translational modification via prenylation, a critical step mediated by farnesyl transferase, which allows for *RAS* protein docking in the plasma membrane [87]. The action of the *RAS* proto-oncogene lies in the *RAS*–*RAF*–*MAPK* pathway downstream of *EGFR* and is a major pathway for tumor cell proliferation in CRC [88]. Oncogenic mutations of the *RAS* gene are prevalent in a large array of human cancers and are correlated with tumor type, although absolute specificity is not seen. The most commonly seen *KRAS* mutations occur at hot spots that are critical for *KRAS* regulation (codons 12, 13, and 62). Mutations in the *KRAS* oncogene result in constitutive activation, even in the absence of growth factor receptor–ligand binding, which is associated with unregulated proliferation and impaired differentiation [89, 90]. Overexpression of *KRAS* also plays a role in the ability of cells to metastasize, in part by increased production of proteases that degrade the extracellular matrix and promote angiogenesis [88]. *KRAS* mutations are believed to be early events in colorectal tumorigenesis and are noted in 40%–50% of CRC cases [30]. The functions of *KRAS* support its putative prognostic role in the adjuvant CRC setting, and several studies have been performed in recent years in this setting [18].

### ***KRAS* as a Prognostic Marker**

The prognostic significance of both *KRAS* mutations (by polymerase chain reaction [PCR]) and p21 staining (by

IHC) has been assessed in a multitude of studies, with conflicting results (as reviewed in [19, 27–29, 91–93]). The majority of reported studies show *KRAS* mutation as an adverse prognostic indicator, indicating the need for adjuvant therapy, but these studies have wide variability in their specific results [19, 27, 91, 94]. As reviewed in [19, 27, 92, 95], some investigations have shown that *KRAS* mutation is prognostic only in some stages of the disease or only when associated with specific mutation types (transition or transversion, specific codons) [26], or when related to specific types of recurrence or in combination with other molecular abnormalities (*p53* mutation) (Table 3). In contrast, the study of Conlin et al. [96] indicated that the presence of *KRAS* mutations predicts poor patient prognosis in CRC, independently of tumor stage. Elnatan et al. [97], Samowitz et al. [98], and Pajkos et al. [99] described tumor site-dependent (proximal versus distal) prognostic relevance of *KRAS* mutation. In contrast, a substantial number of reported studies has found no association between *KRAS* gene mutation and survival, either in isolation or in combination with other tumor suppressor genes [19, 27, 28, 94] (Table 4).

However, as described by Klump et al. [28], if one considers only the five largest studies, including the RASCAL studies and the study by Samowitz et al. [98], prognostic relevance is reported by four of them. In the collaborative RASCAL studies [100, 101], researchers worldwide were invited by the Kirsten Ras in Colorectal Cancer Collaborative Group to share data on the clinical, histological, and outcome parameters of their patients with CRC in whom the *KRAS* status was known. This resulted in a collaborative database called “RASCAL.” In the first RASCAL study, the mutational status of the *KRAS* gene was analyzed in 2,721 patients collected from 22 centers from 13 different nations. In that study, the authors showed that mutations in the *KRAS* gene are important for the progression and outcome of established CRC, although some specific *KRAS* mutations (glycine–valine at codon 12) seem to have a more important prognostic role than others. The results of this collaborative study showed conclusively, for the first time, that different gene mutations have different impacts on outcome, even when the mutation occurs at the same site in the genome [100].

To explore the effect of *KRAS* mutations at different CRC stages, more patients were recruited to the database. The RASCAL II study analyzed data regarding 3,439 cases of CRC collected from 35 centers from 19 different nations, with a mean follow-up of 55 months. Thirty-five percent of the cases analyzed showed mutations in the *KRAS* gene, 26% of which were in codon 12 and 9% of which were in codon 13. About 9% of the overall mutations brought about

**Table 3.** *KRAS* mutation as a negative prognostic factor

Study	<i>n</i>	Design	Stage	Method	<i>KRAS</i> mutation	Remarks
Stage dependent						
Halter et al. (1992) [152]	71	R	I–IV	DS	54%	Poor prognosis in stage IV disease
Benhattar et al. (1993) [153]	99	R	II–III	PCR	25% nonrecurrent, 71% recurrent	Prognostic in advanced disease
Lee et al. (1996) [154]	64	R	I–IV	PCR/DS	20%	Only prognostic in early-stage CRC (stages I and II)
Ahnen et al. (1998) [155]	229	R	II–III	SSCP/DS	40%	Prognostic relevance only in stage II disease
Pajkos et al. (2000) [99]	88	R	II–IV	PCR	61.3%	Poor prognosis in Dukes' stage B and C
Belly et al. (2001) [156]	106	R	II	REMS-PCR	43.3%	<i>KRAS</i> mutation positivity in histologically negative lymph nodes in patients with stage II CRC might identify a subgroup more likely to develop recurrent and/or metastatic disease
Font et al. (2001) [157]	114	R	I–III	SSCP	29%	More aggressive disease, mainly in stage II CRC
Geido et al. (2002) [158]	135	R	I–IV	Mutation spectrum analysis	40%	Predictive of worse outcome, in particular, in stage II/III patients
Richman et al. (2009) [159]	711	R	aCRC	Pyrosequencing	43%	Mutation in <i>KRAS</i> was a poor prognostic factor for overall survival but had limited impact on progression-free survival
Mutation type						
Finkelstein et al. (1993) [160]	247 primary, 166 mCRC	R	I–IV			Tumor recurrence patterns seem to be correlated with <i>KRAS</i> mutation pattern
Moerkerk et al. (1994) [161]	73	R	II–III	SSCP/DS	55% (stage II) 52% (stage III)	G–T and G–C transversions are associated with metastatic behavior in CRC; type and number of <i>KRAS</i> mutations affect biological behavior of CRC

(continued)



**Table 3.** (Continued)

Study	n	Design	Stage	Method	KRAS mutation	Remarks
Span et al. (1996) [162]	35 adenoma, 177 CRC	R	I-IV	SSCP		Occurrence and type, but not location, of KRAS point mutation are prognostic
Andreyev et al. (1998) [100]	2,721	RASCAL	I-IV	RFLP/DS/hybridization		Codon 12 valine-to-glycine mutation had a significant impact on survival
Cerottini et al. (1998) [163]	989	P	I-IV	AS-PCR/SSCP	29.6%	A limited number of defined mutations (codons 12 and 13) in KRAS act as prognostic factors in CRC
Pajkos et al. (2000) [99]	88	R	II-IV	PCR	61.3%	Mutation in codon 13 appeared more frequently in cases of local recurrence
Samowitz et al. (2000) [98]	1,413	R	I-IV	DS	31.8%	KRAS mutation significantly more common in right than left CRC; codon 13 G-A mutation portends worse prognosis (borderline)
Andreyev et al. (2001) [101]	3,439	RASCAL II	I-IV			Glycine-to-valine mutation on codon 12 had statistically significant impact on disease-free and overall survival and the impact was greater in Dukes' C tumors
Bazan et al. (2002) [164]	160	P		PCR/SSCP	46%	Codon 12 KRAS mutation may have a role in mucinous differentiation pathway; codon 13 KRAS mutations may have biological relevance in terms of outcome
Bazan et al. (2005) [165]	160	P		PCR/SSCP	46%	Combination of KRAS mutation in codon 13 and p53 mutations in L3 domain alone are associated with worse prognosis

(continued)

**Table 3.** (Continued)

Study	<i>n</i>	Design	Stage	Method	<i>KRAS</i> mutation	Remarks
Poehlmann et al. (2007) [166]	65	R	I–IV	Pyrosequencing	29%	<i>KRAS</i> codons 12 and 13 were most frequently affected; codon 12 mutations related to worse prognosis
Abubaker et al. (2009) [167]	285	R	I–IV	DS/TMA/qRTPCR	28.1%	<i>KRAS</i> mutations were associated with poor overall survival, which remained significant in a multivariate analysis; in particular, the presence of codon 12 mutations was associated with a poor outcome
Patterns of recurrence						
Suchy et al. (1992) [168]	109	R			21%	Twice as many <i>KRAS</i> mutations were seen in metastatic than in nonmetastatic CRCs
Belly et al. (2001) [156]	106	R	II	REMS-PCR	43.3%	<i>KRAS</i> mutation status of lymph nodes in patients with stage II CRC might identify a subgroup more likely to develop recurrent and/or metastatic disease
Linnemann et al. (2004) [169]	121	R	IV	PCR/RFLP	44.6%	Patients with a <i>KRAS</i> mutation in their primary tumor had a significantly lower probability of survival and higher risk for harboring a synchronous second CRC
Nash et al. (2010) [170]	188	R	IV	PCR/LDR	27%	<i>KRAS</i> mutation is associated with more rapid and aggressive metastatic behavior of colorectal liver metastases; <i>KRAS</i> mutation was associated with shorter disease-specific survival after colon or complete resection

(continued)

**Table 3.** (Continued)

Study	<i>n</i>	Design	Stage	Method	<i>KRAS</i> mutation	Remarks
Combinations of <i>KRAS</i> mutation with other molecular abnormalities						
Bell et al. (1993) [171]	100		II–III	SSCP	24%	Only mutations in codon 12 were analyzed; combination of <i>KRAS</i> and <i>p53</i> mutation was correlated with worse prognosis
Hardingham et al. (1998) [172]	100	P	I–IV	SSCP/DS	35%	Patients positive for <i>KRAS</i> and <i>p53</i> mutation had worse prognosis than patients harboring only a <i>p53</i> mutation
Liang et al. (1999) [173]	166		Dukes' B2	RFLP	43%	Combination of <i>p53</i> and <i>KRAS</i> mutation is a stronger negative predictor than <i>p53</i> mutation alone; only rectal carcinomas evaluated
Tortola et al. (1999) [174]	140	P	I–IV	RFLP	41%	Survival was strongly correlated with <i>p53</i> mutation alone or in combination with <i>KRAS</i> mutation but not with <i>KRAS</i> mutation alone
Esteller et al. (2001) [175]	115	P	I–IV	SSCP	38%	Combination of <i>KRAS</i> mutation and <i>p16</i> mutation is correlated with worse survival
Font et al. (2001) [158]	114	R		PCR/allele-specific oligonucleotide hybridization	29%	<i>KRAS</i> mutation and 18q LOH are two genetic markers that may identify patients with tumors with more aggressive behavior, mainly stage II tumors
González-Aguilera et al. (2004) [176]	77	R	I–IV	RFLP/PCR	35%	Patients with mutation in <i>KRAS</i> but not <i>p53</i> had worse survival rates than those with mutations in <i>p53</i> and no mutations in <i>KRAS</i> ; mutations in both <i>KRAS</i> and <i>p53</i> showed worst outcome

(continued)

**Table 3.** (Continued)

Study	<i>n</i>	Design	Stage	Method	<i>KRAS</i> mutation	Remarks
Janssen et al. (2005) [177]		P		Compound transgenic mouse model		Synchronous activation of Wnt signaling pathway ( <i>APC</i> mutation) and <i>KRAS</i> signaling pathway is associated with poor prognosis and tumor multiplicity
Lee et al. (2008) [178]	134	R			33.6%	CIMP-H CRC with poor clinical outcome associated with <i>KRAS/BRAF</i> mutations
Nash et al. (2010) [179]	532	R	I–IV	PCR/LDR	36%	Strong evidence that likelihood of metastatic progression can be estimated on biomarkers present in primary tumor; <i>KRAS</i> mutations and MSI status provided excellent stratification of prognosis in stage I and II patients

Abbreviations: aCRC, advanced colorectal cancer; AS-PCR, allele specific PCR; CIMP, CpG island methylator phenotype; CIMP-H, high-frequency CIMP; CRC, colorectal cancer; DS, direct sequencing; DHPLC, denaturing high-performance chromatography; FISH, fluorescent in situ hybridization; LOH, loss of heterozygosity; P, prospective; qRT-PCR, quantitative real-time polymerase chain reaction; R, retrospective; REMS-PCR, restriction endonuclease-mediated specific PCR; RFLP, restriction fragment length polymorphism; SSCP, single-strand conformation polymorphism; TMA, tissue microarray.

the replacement of the amino acid glycine with valine in codon 12. No association was found between *KRAS* mutations and other clinicopathological variables. Multivariate analysis showed that advanced Dukes' stage, age, and the codon 12 glycine-to-valine mutation were significantly associated with a poorer prognosis. A separate analysis of the effects of codon 12 glycine–valine mutation in patients with Dukes' stage B or stage C tumors showed that the mutation brought about a significantly shorter disease-free interval and lower survival rate only in patients with Dukes' stage C tumors [100–102]. This collaborative study suggested that the presence of a codon 12 glycine-to-valine mutation is not only important for cancer progression but it may also predispose to more aggressive biological behavior in patients with advanced CRC.

Samowitz et al. [98] performed the first population-based study and largest nonmeta-analysis (1,413 individuals) of *KRAS* mutation in colon cancer patients. They

showed that *KRAS* mutation, in general, was not associated with a higher cancer-related mortality rate. However, mutations in codon 13 were associated with a 40% greater likelihood of dying, although this difference was of borderline statistical significance. In addition, a small but statistically significant relationship between codon 12 *KRAS* mutation and tumor stage, proximal location, and male gender was demonstrated.

Interpretation of the various studies published on the prognostic role of *KRAS* might be difficult because of the different mutations of the *KRAS* gene that are being investigated, differences in data collection among the different studies, staging techniques, and different methodologies used to detect *KRAS* mutations [91]. Therefore, future studies on the prognostic role of *KRAS* mutations in CRC should be conducted prospectively with standardized assays to define those patients in whom routine mutation analysis could be of benefit for determining adjuvant treatment options

**Table 4.** Studies in which *KRAS* mutation was not associated with survival

Study	<i>n</i>	Design	Stage	Method	<i>KRAS</i> mutation	Remarks
Kern et al. (1989) [180]	83	R				No prognostic significance
Laurent-Puig et al. (1992) [181]	109	R	I–IV	RFLP/cytometry		No correlation
Rochlitz et al. (1993) [182]	24	R	IV	Hybridization	46%	There is a position specificity of <i>KRAS</i> oncogene mutation in advanced CRC; however, these mutations do not seem to play a role in tumor progression
Morrin et al. (1994) [183]	52	R	I–IV	IHC/FISH	36%	No correlation
Dix et al. (1994) [184]	100	R	II	SSCP		No significant correlation
Markowitz et al. (1995) [185]	37	R	IV	Dideoxysequencing	51.3%	Indistinguishable in overall survival and equally likely to respond to 5-FU
Kastrinakis et al. (1995) [186]	26	R	IV	SSCP	36.8%	Neither the presence of a <i>KRAS</i> mutation nor the precise change is predictive of less aggressive tumor behavior
Pricolo et al. (1996) [187]	70	R	III	DS	NA	No significant correlation
Wadler et al. (1997) [188]	72	R	IV	Dot blot analysis	35%	No difference in response to chemotherapy and no difference in overall survival
Andersen et al. (1997) [189]	100	R	I–IV	RFLP/DS	40%	No correlation
Kressner et al. (1998) [190]	191	R	I–IV	TTGE	32%	CRC with <i>KRAS</i> mutation marginally shorter survival time but not statistically significant
Liang et al. (1999) [191]	166	R	II rectal	RFLP	43%	Only rectal tumors evaluated
Bouzourene et al. (2000) [192]	122	R	II	SSCP	28%	No correlation to prognosis in stage II CRC
Bleeker et al. (2001, 2000) [193, 194]	55	R	III	DHPLC	27%	<i>KRAS</i> mutation is not prognostic in stage III CRC patients who received adjuvant 5-FU; <i>KRAS</i> mutation predominantly in the right colon
Rosty et al. (2001) [195]	56	R	IV	SSCP	30%	
Petrowsky et al. (2001) [196]	41	R	IV	Microplate-based allele-specific hybridization	19%	<i>KRAS</i> mutation does not correlate with tumor proliferation status and patient survival
Gervaz et al. (2001) [197]	126	R	II	SSCP	38%	
Clarke et al. (2001) [198]	27	R		SSCP		No relationship to mortality
Okulczyk et al. (2003) [199]	36	R	I–IV	PCR/RFLP	41.7%	No statistically significant correlation
Losi et al. (2004) [200]	58 metastases	R			38%	<i>KRAS</i> cannot be used as a prognostic biomarker in CRC patients
Ogino et al. (2009) [201]	508	R	III	Pyrosequencing	35%	<i>KRAS</i> mutational status did not have any significant influence on disease-free or overall survival
Roth et al. (2010) [202]	1,404	P	II–III	Allele discrimination assay on RT-PCR	37%	<i>KRAS</i> mutation status does not have major prognostic value in stage II and III disease

Abbreviations: 5-FU, 5-fluorouracil; AS-PCR, allele-specific PCR; DHPLC: denaturing high performance chromatography; DS, direct sequencing; FISH, fluorescent in situ hybridization; IHC, immunohistochemistry; P, prospective; PCR, polymerase chain reaction; R, retrospective; REMS-PCR, restriction endonuclease mediated specific PCR; RFLP, restriction fragment length polymorphism; RT-PCR, real-time polymerase chain reaction; SSCP, single-strand conformation polymorphism; TTGE, temporal temperature gel electrophoresis.



[103]. Only a few studies have already been performed prospectively (Table 3).

Recently, the focus on *KRAS* has shifted from a prognostic to a predictive marker. Although *KRAS* mutations presently cannot be used to select patients who need adjuvant chemotherapy [104], they are strong predictive markers in mCRC patients treated with anti-EGFR mAbs [105].

***Predictive Markers in Anti-EGFR Therapy: EGFR Expression, EGFR Amplification, KRAS Mutations, and Skin Rash***

As mentioned above, cetuximab and panitumumab have been shown to be effective in mCRC treatment, either as monotherapy or in combination with cytotoxic agents [106]. Data (as reviewed in [62]) have shown no consistent association between tumor expression of the EGFR protein and response or survival following anti-EGFR-targeted therapy [107, 108]). The EGFR signaling pathway is complex, and it is possible that the level of expression of the 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 of treatment response than the total level of the receptor per se. A higher *EGFR* gene copy number, as determined by fluorescence in situ hybridization, has been described in mCRC as being associated with a better response to anti-EGFR antibody treatment [109–113]. However, reproducibility, methodological concerns, and uncertainty on the clinical cutoff values published thus far currently prevent its routine use in clinical practice [106, 108–110, 112–117].

Whereas tumor EGFR expression or gene copy number may not be a reliable predictor of response, the appearance of treatment-related rash and, particularly, the presence of *KRAS* mutation might be of significance [62, 84, 105, 106, 118, 119].

Several retrospective analyses (Table 5) of tumor samples from mCRC patients receiving anti-EGFR antibody treatment have shown that patients with mutated *KRAS* did not benefit from this therapy (as reviewed in [83, 108, 120–131]), independently of EGFR expression status [62, 132, 133]. The first large study to confirm the negative predictive value of *KRAS* mutation was the pivotal randomized phase III study of panitumumab monotherapy in the relapsed or refractory setting [134]. In addition, *KRAS* data from large randomized phase II–III cetuximab studies—Cetuximab Combined With Irinotecan in First-Line Therapy for Metastatic Colorectal Cancer [107], Oxaliplatin and Cetuximab in First-Line Treatment of mCRC [135], National Cancer Institute of Canada Clinical Trials Group [136], and Evaluation of Velcade® Employed as Retreat-

ment for Efficacy, Safety, and Tolerability [137]—have recently been published. The data from these studies strongly support that mutant *KRAS* is to be considered a predictive marker for anti-EGFR therapy resistance in mCRC. Lièvre et al. [106, 138] were the first to suggest that activating *KRAS* mutations could be responsible for acquired activation of the RAS–RAF–MAPK pathway independently of the ligand-induced activation of EGFR, leading to resistance to cetuximab treatment. This has been confirmed by others [134, 139], and the mutational status of *KRAS* is now routinely requested at the instigation of the EMEA to select mCRC patients who might benefit from the use of cetuximab and panitumumab [120]. This is also endorsed by the recent provisional opinion of the American Society of Clinical Oncology (ASCO), stating that if a *KRAS* mutation in codon 12 or codon 13 is detected then the patient should not receive anti-EGFR therapy as part of their treatment [130].

However, only half of wild-type *KRAS* patients benefit from treatment, suggesting the need to identify additional biomarkers for anti-EGFR mAb-based treatment efficacy. Alterations in other EGFR effectors, including members of the RAS–MAPK or PI3K pathways together with alternative *KRAS* mutations (in codons 61 and 146), could drive resistance to anti-EGFR therapy and are currently being investigated [140–148]. Furthermore, Sartore-Bianchi et al. [149] described that when expression of phosphatase and tensin homologue (PTEN) and mutation of *KRAS*, *BRAF*, and *PI3KCA* are concomitantly ascertained, up to 70% of mCRC patients who are unlikely to respond to anti-EGFR therapies can be identified.

Additionally, acneiform rash is a class effect of EGFR-targeted agents and occurs frequently and in a dose-dependent manner in treated patients. Skin toxicity has previously been shown to correlate with clinical benefit (response rate, progression, and overall survival) in patients with advanced CRC receiving anti-EGFR antibodies [23, 62, 117, 131]. Of note, patients not developing a skin reaction may still achieve a tumor response. The mechanism underlying the correlation between skin toxicity and tumor response is currently unclear. However, some research groups have hypothesized that the rash is a surrogate indicator of an adequate degree of receptor saturation by cetuximab. Another hypothesis to explain the predictive value of skin toxicity is germinal genetic polymorphisms among individuals [62].

***KRAS-Targeted Therapy***

Because of its pivotal role in oncogenesis, various strategies have been developed to target *KRAS* for the treatment of human cancers. These strategies have ranged from inhibiting protein expression via antisense oligonucleotides to

**Table 5.** The role of *KRAS* mutation as a predictive marker in anti-EGFR therapy with monoclonal antibodies

Study	<i>n</i>	Design	Stage	Method	<i>KRAS</i> mutation	Remarks
Moroni et al. (2005) [114]	31	R	mCRC	Mutation analysis/ FISH	32.3%	No significant correlation between response to cetuximab and <i>KRAS</i> mutation; however, a trend toward a higher response rate in CRC patients with wild-type <i>KRAS</i> was seen
Lièvre et al. (2006) [106]	30	R	mCRC	Mutation analysis/ FISH	43%	<i>KRAS</i> mutation is associated with resistance to cetuximab and shorter survival in EGFR <sup>+</sup> mCRC
Di Fiore et al. (2007) [203]	59		53 primary tumors 6 metastases	Sequencing/CR-LCR/ snapshot multiplex	37%	Presence of <i>KRAS</i> mutation is highly predictive of nonresponse to treatment based on cetuximab plus chemotherapy; <i>KRAS</i> mutation was identical between primary tumor and metastases
Benvenuti et al. (2007) [119]				Sequencing/CRIB assay/cell culture	33.3%	Mutations in <i>KRAS</i> and <i>BRAF</i> are associated with lack of response to anti-EGFR mAb treatment in mCRC; however, few <i>KRAS</i> mutant mCRCs are compatible with response; multitherapies with both anti-EGFR and anti-MAPK inhibitors should be considered
Frattini et al. (2007) [132]	27	R	mCRC	IHC/MSI/FISH and sequencing	37%	Patients with high polysomy or EGFR amplification, wild-type <i>KRAS</i> , or intact PTEN expression should be considered for cetuximab therapy
Khambata-Ford et al. (2007) [133]	110	P	mCRC	MA/ELISA/RT-PCR	38%	The data support a role for <i>KRAS</i> mutations in patients who do not respond to cetuximab; in addition, <i>AREG</i> and <i>EREG</i> gene are top sensitive markers for cetuximab therapy in mCRC
Finocchiaro et al. (2007) [204]	85	R	mCRC	NS	37.6%	<i>KRAS</i> mutation analysis identifies a group of patients with the lowest chance to benefit from therapy
De Roock et al. (2008) [205]	113	R	mCRC	Sequencing	40.7%	Wild-type <i>KRAS</i> in combination with early tumor size reduction are strong predictors of significantly longer PFS and OS in irinotecan-refractory patients

(continued)

**Table 5.** (Continued)

Study	<i>n</i>	Design	Stage	Method	<i>KRAS</i> mutation	Remarks
Lièvre et al. (2008) [138]	89; pooled with previous study, 144	R	mCRC	Allelic discrimination assay	27%	<i>KRAS</i> mutation showed a strong correlation with shorter OS and PFS and resistance to cetuximab; skin toxicity was only associated with OS
Amado et al. (2008) [134]	427	R	mCRC	Mutation kit	43%	Inhibition of the RAS–RAF–MAPK pathway is primarily responsible for the clinical activity of panitumumab in mCRC; <i>KRAS</i> status should be considered when selecting mCRC patients as candidates for this treatment
Cappuzzo et al. (2008) [111]	85	R	mCRC	Mutation analysis/FISH	52.5%	Presence of <i>KRAS</i> mutation represents the strongest predictor of cetuximab failure in EGFR FISH <sup>+</sup> CRC patients
Etienne-Grimaldi et al. (2008) [206]	93	R	IV (liver metastases)	PCR-RFLP	38.7%	Any predictive/prognostic value of <i>KRAS</i> mutation in treatments combining anti-EGFR monoclonal antibodies with 5-FU–based therapy should be exclusively linked to the presence of the anti-EGFR agent
Lurje et al. (2008) [207]	130	R	mCRC	Genotyping and mutation analysis	32.3%	<i>KRAS</i> mutation was significantly associated with lack of response to cetuximab; polymorphisms in <i>COX-2</i> , <i>EGF</i> , and <i>EGFR</i> were identified as potential molecular markers for clinical outcome in mCRC patients treated with single-agent cetuximab
Karapetis et al. (2008) [136]	394	R	mCRC	Sequencing	42.3%	Patients with a colorectal tumor bearing mutated <i>KRAS</i> did not benefit from cetuximab, whereas patients with a tumor bearing wild-type <i>KRAS</i> did benefit from cetuximab; mutation status of <i>KRAS</i> gene had no influence on survival among patients treated with best supportive care alone

(continued)

**Table 5.** (Continued)

Study	<i>n</i>	Design	Stage	Method	<i>KRAS</i> mutation	Remarks
Freeman et al. (2008) [208]	62	R	mCRC	Sequencing	38.7%	Patients with mCRC with activating <i>KRAS</i> mutations are less likely to respond to panitumumab alone
Tol et al. (2008) [209]	755	Clinical trial	mCRC			Mutation status of the <i>KRAS</i> gene was a predictor of outcome in the cetuximab group
Garm Spindler et al. (2009) [210]	71		mCRC	Sequencing and qPCR		The combined analysis of <i>KRAS</i> mutation and <i>EGFR</i> gene polymorphism is predictive for response to cetuximab
Sartore-Bianchi et al. (2009) [149]	132	R	mCRC	Sequencing and IHC	26.5%	When expression of PTEN and mutations of <i>KRAS</i> , <i>BRAF</i> , and <i>PI3KCA</i> are concomitantly ascertained, up to 70% of mCRC patients unlikely to respond to anti-EGFR therapies can be identified
Sohn et al. (2009) [211]	66	R	mCRC	DS/snapshot assay	40.9%	This study indicates the clinical relevance of <i>KRAS</i> mutation in predicting the efficacy of cetuximab- plus irinotecan-based chemotherapy in irinotecan-refractory Korean mCRC patients

Abbreviations: 5-FU, 5-fluorouracil; AREG, amphiregulin; COX-2, cyclo-oxygenase 2; CRC, colorectal cancer; DS, direct sequencing; ELISA, enzyme-linked immunosorbent assay; EGFR, epidermal growth factor receptor; EREG, epiregulin; FISH, fluorescent in situ hybridization; IHC, immunohistochemistry; MA, microassay; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; mCRC, metastatic colorectal cancer; MSI, microsatellite instability; OS, overall survival; P, prospective; PCR-LCR, polymerase chain reaction-ligase chain reaction; PCR-RFLP, polymerase chain reaction-restriction fraction length polymorphism; PFS, progression-free survival; PI3KCA, phosphatidylinositol 3-kinase  $\alpha$  catalytic subunit; PTEN, phosphatase and tensin homolog; qPCR, quantitative polymerase chain reaction; R, retrospective; RTPCR, reverse transcriptase polymerase chain reaction.

blocking post-translational modifications with farnesyl-transferase inhibitors (FTIs) to inhibiting downstream effectors [150, 151].

A number of reviews have described (pre)clinical data obtained with FTIs, and although an extensive body of literature supports preclinical efficacy, they still do not represent a meaningful strategy to target *KRAS* for cancer therapy as yet [151].

Alternatively, immunological approaches are being investigated. Mutated *KRAS* proteins can be considered tumor specific and could therefore represent a highly specific technique to target cytotoxic T cells to tumors. Vaccination with mutant RAS peptides can stimulate an immune re-

sponse that involves both CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes, in both cancer patients and normal individuals. Full activation of T cells requires multiple signals, and it seems that mutant *KRAS* is not highly immunogenic in the native tumor setting based on the low frequency of immunoreactions in nonvaccinated patients. Therefore, these peptides are often given in conjunction with an immunostimulatory adjuvant. In another technique, isolated antigen-presenting cells are loaded with RAS peptides *ex vivo* before reintroduction into the patient. An alternative immunological approach to targeting mutant *KRAS* uses whole yeast expressing mutant proteins. Because whole yeast cells are typically recognized as pathogens, they can elicit a cell-mediated immune

response directed against the recombinant protein. Yeast-based immunotherapy may represent an alternative to peptide-based vaccines directed at mutant *KRAS*, but the technique has not yet been tested in humans [151].

### ***BRAF***

*BRAF* is a member of the RAF kinase family that encodes kinases that are regulated by RAS and mediate cellular responses to growth factor signals [178]. Activating mutations in *BRAF* have been reported in 5%–15% of CRC cases, and >80% of all known mutations involve a thymine-to-adenine transversion in nucleotide 1799, which leads to a substitution of valine by glutamic acid at amino acid residue 600 (V600E), and this results in the upregulation of the RAS–RAF–MAPK pathway independently of *KRAS* mutation [178, 212, 213].

*BRAF* mutations are frequently found in MSI-H tumors, in which the MMR system is epigenetically inactivated [178]. Some groups speculate that the activation of *BRAF* is related to the inactivation of the MMR system [214]. However, alterations in the *BRAF* gene are not involved in the tumorigenesis of MSI-H tumors with germline mutations in *hMLH1* and *hMSH2* [215], and in addition they also occur in MSS tumors [216].

### ***BRAF as a Prognostic and Predictive Factor***

The presence of *BRAF* mutations in tumors has been characteristically associated with a worse clinical outcome, suggesting a need for adjuvant therapy in these circumstances [55, 142, 159, 217]. Whereas *BRAF* mutation is associated with worse survival in MSS tumors, the presence of high-grade CIMP (CIMP-H) appears to eliminate, at least in part, the adverse effect of *BRAF* mutation [55]. Ogino et al. [55] also found that the good prognosis associated with MSI-H tumors was abrogated in the presence of a *BRAF* mutation. In contrast, Samowitz et al. [218] and Roth et al. [202] found that *BRAF* mutations were associated with significantly shorter survival in patients with MSS tumors, but had no effect on the excellent prognosis of patients with MSI tumors. Therefore, it has been postulated that it is not the *BRAF* mutation itself that confers a poor prognosis but rather that the mutation has different effects depending on the type of genetic pathway in which it is produced [212]. In addition, patients whose tumors bear the *BRAF* V600E allele are not likely to experience significant benefit from either cetuximab or panitumumab treatment. Therefore, like *KRAS* mutation, *BRAF* mutation analysis presently cannot be used to select patients to receive adjuvant therapies but might be used as an additional predictive factor in the metastatic setting for the selection of patients who might ben-

efit from EGFR-targeted mAb therapies [122, 143, 149, 219].

Although *KRAS*- and *BRAF*-encoded proteins are mutated in many of the same types of cancer, concomitant mutations are extremely rare. Intuitively, this is because both genes undergo gain-of-function mutations and thus represent different mechanisms of activating the same pathway. More recently, however, the most active (V600E) *BRAF* mutation was described together with a *KRAS* mutation in advanced CRCs and their lymph node metastases [220]. It seems that concomitant *KRAS* and *BRAF* mutations also increase the progression of MSS tumors, suggesting that the activation of both genes is likely to harbor a synergistic effect [221]. All these findings need to be further elucidated by additional independent prospective studies that should consider a joint examination of CIMP, MSI, *KRAS* mutation, and *BRAF* mutation to decipher the role of these molecular features in the biological and clinical behavior of tumors [55].

### **Epigenetics: CIMP**

Epigenetics describes changes in phenotype or gene expression that do not involve DNA sequence changes. Among these, alteration in DNA methylation patterns is known to be a key component for the altered gene expression associated with human cancers [222]. Promoter CpG island hypermethylation acts as an important mechanism for inactivation of tumor suppressor genes and tumor-related genes [178]. Promoter CpG island hypermethylation and its associated histone modifications render the chromatin structure of a gene promoter into a closed compact structure inaccessible to transcription factors, which results in the inactivation of gene transcription [222].

Recent studies have demonstrated that promoter CpG island hypermethylation is more frequent than genetic changes in human CRCs, which suggests that promoter CpG island hypermethylation is a potential mechanism of colorectal carcinogenesis. In addition to two known molecular pathways in colorectal carcinogenesis, which involve chromosomal instability and MSI, a third epigenetic instability pathway, the promoter CIMP was proposed by the group of Dr. Issa [223]. CIMP<sup>+</sup> CRCs are characterized by widespread hypermethylation of promoter CpG island loci, which results in the inactivation of the involved genes. A growing number of studies has consistently demonstrated a close association between CIMP-H CRC and proximal colon location, MSI, and a high frequency of *BRAF* mutation, regardless of the methodology and CIMP marker panels used [178, 224]. In contrast, a phenotype with less widespread promoter methylation, CIMP-low (CIMP-L), has not been well characterized. Promoter hypermethylation



and silencing of the DNA repair gene O<sup>6</sup>-methylguanine-DNA-methyltransferase (MGMT) has been associated with G>A mutations and is a common event in colorectal tumorigenesis [225]. *MGMT* hypermethylation is associated with MSI-L tumors and *KRAS* mutations, and seems to be limited to CIMP-L tumors, supporting the suggestion that CIMP-H and CIMP-L might be different molecular phenotypes in CRC [226, 227].

### ***CIMP as a Prognostic and/or Predictive Factor***

Consequently, marked controversy exists over the prognostic implications of CIMP. Some studies suggest an adverse effect of CIMP on survival in CRC patients [55, 228–230], whereas other studies suggest little prognostic value for CIMP [231] or even a low cancer-specific mortality rate [55]. This issue is considerably complicated by the described associations between methylation and factors known to affect prognosis in CRC [59, 229].

Despite the close pathological similarity between MSI tumors and CIMP-H/MSS lesions, they have significantly different prognoses [59, 228]. Patients with CIMP-H/MSS tumors have a significantly worse outcome than patients with CIMP-0/MSS and MSI tumors [178, 227–229]. In addition, accumulating evidence suggests that the development of MSI may act as an antidote to the adverse prognostic effects of widespread methylation [55, 59, 178, 218, 228, 230, 232]. It is also controversial whether CIMP confers a survival benefit from chemotherapy in CRC patients. Van Rijnsoever et al. [233] provided evidence for the chemosensitivity of stage III tumors with aberrant DNA methylation. In contrast, Ogino et al. [227] and Shen et al. [229] suggested that CIMP may predict worse outcome among advanced CRC patients who receive 5-FU and irinotecan-based chemotherapy. Randomized trials are necessary to definitively assess treatment efficacy [34].

Lee et al. [178] and others [222] pointed out that the poor clinical outcome of patients with CIMP<sup>+</sup>/MSS tumors is closely associated with the presence of *KRAS/BRAF* mutation. Therefore, it is possible that the relationship between prognosis and CIMP-H tumors is actually a result of the relationship between prognosis and *KRAS/BRAF* mutation [34]. In addition, there was little prognostic value of CIMP in studies in which multivariate analyses were performed [55, 178, 218].

## **18q LOH/DCC**

### ***Biology of 18q***

Another promising prognostic marker is allelic loss of chromosome 18q, which is highly prevalent in CRC [92, 234, 235]. The long arm of chromosome 18 contains several

genes of potential importance in CRC pathogenesis and progression. Among the genes located on 18q are the *DCC* tumor suppressor gene, which codes for a neutrin-1 receptor important in cell adhesion and apoptosis; the *SMAD4* gene, which codes for a downstream signal transducer in transforming growth factor (TGF)- $\beta$ 1 signaling involved in tumor suppression; and the *SMAD22* gene, involved in endodermal differentiation [19, 92]. Most clinical studies on the *DCC* gene have examined 18q LOH by PCR amplification of polymorphic microsatellite markers at or near 18q21. Chromosomal loss in this region is thought to result in haploinsufficiency in *DCC*, and therefore reduced protein expression. However, the results of these studies are somewhat controversial [5].

### ***18q LOH as a Prognostic Factor***

In 1992, O'Connell et al. [236] suggested a possible relationship between 18q LOH in Duke's stage B and stage C tumors and poor survival. In 1994, Jen et al. [237] also showed that 18q LOH was associated with an adverse prognosis, suggesting a need for adjuvant therapy. Since then, several large series have accomplished the assessment of *DCC*/allelic variance of 18q and prognosis in resected CRC cases [238]. Although most subsequent studies have consistently agreed that 18q LOH and decreased *DCC* mRNA are associated with poor survival, estimates of the prognostic value have varied considerably among studies, as have the methods used (as reviewed in [18, 27–30, 92, 239, 240]).

An important meta-analysis of data concerning the prognostic significance of 18q LOH was performed by Popat et al. [239]. Those authors examined 27 studies (29 data sets) assessing survival by chromosome 18q allelic imbalance (AI) and *DCC* expression eligible for systematic review and meta-analysis. They found that, although different methods were used to assess chromosome 18q status, estimates of the frequency of AI were similar across all methods, including the rate of *DCC* loss of expression (LOE). Of the 27 studies, 17 provided suitable data to be included in the meta-analysis, that is, data from 2,189 patients for overall survival and from 683 patients for disease-free survival. Using these pooled estimates of outcome in CRC patients supported the notion that chromosome 18q AI and *DCC* LOE is a negative predictive factor for survival. Whereas CRC with chromosomal 18q AI/*DCC* LOE seem to be associated with a poorer prognosis, one caveat to this conclusion is that there was significant evidence for heterogeneity among studies because the optimal method and thresholds for assessing this phenotype are unclear at present. In addition, publication bias is a major concern in all forms of meta-analyses. Although there was some evidence of bias,

18q AI/DCC LOE still maintained its prognostic utility after correction. Popat et al. [239] concluded that their findings indicate that chromosome 18q AI/DCC status has the potential to define a group of patients who may benefit from adjuvant chemotherapy following potential curative surgery.

In addition, Locker et al. [19] performed a literature review on 18q LOH for the ASCO 2006 update of recommendations for the use of tumor markers in gastrointestinal cancer. Sixteen studies were considered to be of sufficient quality to evaluate. Although there was suggestive evidence of an association of 18q loss and survival, the small number of patients and the retrospective nature of the studies made it premature to use this marker as a prognosticator [19]. Moreover, the large prospective study of 555 patients with non-MSI-H CRC conducted by Ogino et al. [241] found no association between 18q LOH or AI and patient survival. Validation in the context of prospective clinical trials using consistent methodology is required before introducing assessment of this phenotype routinely into CRC patient management strategies.

### **Prospective Clinical Adjuvant Trial**

At this time, retrospective analysis of tumor biologic laboratory observations has been the dominant research methodology [39, 242]. In stage III colon cancer, a retrospective analysis by Watanabe et al. [39] and O'Connell et al. [242], both using formalin-fixed, paraffin-embedded material, suggested a statistically significant difference in survival favoring patients who retained the 18q allele and those who demonstrated MSI and a *TGF-βRII* mutation. These results and those from other investigators support the design of the current Gastrointestinal Intergroup stage II study (E5202 trial) coordinated by the Eastern Cooperative Oncology Group. The current E5202 stage II adjuvant colon cancer trial is a randomized study in which a total of 3,610 patients will be stratified according to stage and tumor biological characteristics (MSI and 18q LOH) to determine treatment strategy [243–245]. This is the first trial in colon cancer to use prognostic and predictive markers prospectively [19, 23, 235, 243, 245]. Stage II patients enrolled in the trial will have tumor block assessment for MSI and LOH on 18q to determine their risk for tumor recurrence and their likelihood of benefiting from adjuvant 5-FU. Patients at low risk—those with retention of 18q alleles or high levels of MSI—will be observed; on the other hand, patients at high risk—those with MSS or low levels of MSI with 18q LOH—will be assigned randomly to receive 5-FU, leucovorin, and oxaliplatin with or without bevacizumab.

Although this study is powered to compare the 3-year disease-free survival rates of stage II patients randomized

into two chemotherapy arms, one of the secondary objectives is to prospectively determine the impact of tumor biological characteristics on the survival of patients with stage II colon cancer (<http://clinicaltrials.gov/ct2/show/NCT00217737>) [235]. Although of interest, there are limitations to the study, that is, the addition of a control arm in which patients with an MSS/18q-stable tumor are stratified to receive postoperative 5-FU-based chemotherapy would be necessary to establish the prognostic value of MSI and 18q LOH [92]. First results are expected in April of 2011.

## **Host Lymphoid Response to Tumor**

### **Biology of Cytotoxic T lymphocytes in CRC**

Colorectal tumors become clinically malignant after cancer cells invade through the muscularis mucosa into the submucosa. With the invasion, various immune/inflammatory responses by competent immune effector cells, critically involved in the protection of the host organism against cancer cells, take place. These responses are more concentrated along the invasive margin [246, 247]. Tumor-associated antigens, expressed as a consequence of genetic alterations, are exposed on the tumor cells in association with human leukocyte antigen (HLA) class I proteins and can be recognized by cytotoxic T lymphocytes (CTLs) via T-cell receptors, finally resulting in a cellular immune response effective in limiting tumor growth and spread [86, 247]. Immune cells are present in the tumor stroma at the periphery of the tumor and occasionally invade cancer cell nests. CD8<sup>+</sup> CTLs and the CD4<sup>+</sup> T helper lymphocytes represent the adaptive, or specific, component of the immune response. CD4<sup>+</sup> T cells mainly produce cytokines, like interleukin (IL)-2 and interferon- $\alpha$ , which influence the type of immune response, whereas CD8<sup>+</sup> T cells produce perforin and granzyme B, which are cytotoxic to their target cells [248]. Consequently, CTLs are able to perform tumor-specific recognition and can mediate specific destruction of tumor cells [249, 250]. Koch et al. [251] were the first to show functional reactivity of tumor-infiltrating T cells against antigens in CRC patients. In addition, they demonstrated, for the first time, tumor-selective activation and cytotoxic activity in situ of tumor-infiltrating CD8<sup>+</sup> T cells and tumor-selective migration of CD4<sup>+</sup> T helper cells in CRC.

However, the induction of CTL responses takes time, leaving time for tumor cells to escape the immune system. Therefore, natural killer (NK) cells from the innate immune system, which are not HLA restricted, may also play an important role because these cells can lyse NK-sensitive tumor targets prior to antigen sensitization or clonal expansion. In addition, NK cells express several ligands of the tu-

mor necrosis factor family and can induce apoptosis of malignant cell targets that are phagocytosed by dendritic cells and macrophages and processed for subsequent presentation to T cells. Furthermore, NK cells constitutively express the IL-2 receptor and are able to respond to IL-2 stimulation that results in augmented cytotoxic activity [252]. Nevertheless, tumors are still capable of escaping immune recognition/destruction by several mechanisms [253–255]. The potential influence of these immune-cell infiltrates in CRC on the prognosis of patients has been investigated in several studies, but remains controversial. A greater understanding of the role of the host immune response in influencing the natural history of CRC might have important implications for risk stratification and the development of adjuvant immune-based therapies [248, 256].

### ***CTLs and Prognosis***

There is accumulating evidence showing a positive correlation between the number of tumor-infiltrating lymphocytes (TILs) and longer patient survival in CRC [257]. Jass et al. [258] pointed out that conspicuous infiltration along the invasive margin of rectal cancer is an independent prognostic factor for longer survival in a multivariate analysis. Ropponen et al. [259] confirmed the concept of a prognostic impact of TILs in CRC and showed an inverse correlation between the presence of TILs and tumor stage. Several subsequent studies also demonstrated that the infiltration of CD8<sup>+</sup> T cells around and within tumor stroma contribute to a better prognosis [260–268]. Galon et al. [269] showed that the type, density, and location of immune cells in CRC had a prognostic value that was superior to TNM classification. The assumption that not only the number of CD8<sup>+</sup> TILs but also their cytolytic capacity determines the effectiveness of immune system-mediated tumor control was further supported by Atreya et al. [270]. They showed that the expression of the T-box transcription factor eomesodermin, which is critically involved in controlling the cytolytic activity of CD8<sup>+</sup> CTLs, inversely correlates with the occurrence of lymph node metastasis in CRC patients [270].

Pronounced lymphocyte infiltration in CRC is more marked in MSI-H CRC and might explain the better clinical outcome for patients with these tumors [56]. It has been postulated that MSI-H CRCs are more immunogenic than MSS CRCs [271] because of the generation of a large number of abnormal peptides by frameshift mutations [272–276]. However, crosspriming of antigen-presenting cells by released intracellular antigens or the use of HLA class II machinery and T helper cell activity may provide an alternative pathway for immune stimulation [271, 272].

For a better prognostic assessment, Dolcetti et al. [277] suggested the combination of evaluation of both local lym-

phocytosis and MSI status. MSI in combination with a high content of intraepithelial lymphocytes was found to be related to longer overall survival in a group of patients with exclusively right-sided CRC. Consequently, it has been suggested that these lymphocytes may actually represent an immune response that contributes to longer survival in MSI-H CRC patients, and subsequent work confirmed a possible link [261, 271, 278–280]. Dolcetti et al. [277] and Michael-Robinson et al. [279] demonstrated that the greater frequency of TILs associated with MSI-H cancers are only weakly or moderately correlated with tumor apoptosis. However, although TILs might be expected to explain the higher apoptotic rate and better prognosis with MSI-H cancers, it is likely that MSI-H cancers are intrinsically more prone to apoptosis, independently of T cell attack [279]. In addition, Buckowitz et al. [281] suggested a protective role of functionally active lymphocytes directed against MSI-H CRCs that may prevent tumor cell dissemination and metastasis formation in distant organs. In contrast, Baker et al. [282] found TIL infiltration only to be of prognostic value in MMR-proficient CRCs. They also pointed out that perturbations in the TGF- $\beta$  signaling pathway play an important role in the recruitment and retention of TILs within CRC epithelium [282]. Although Prall et al. [283] also found a prognostic impact of high CD8<sup>+</sup> density in MSI-H CRCs, they showed that it was not solely restricted to this group. Therefore, they hypothesized that tumor infiltration by CD8<sup>+</sup> lymphocytes could reflect a general principle of antitumor immunity, irrespective of MSI status [283].

With regard to the explicit prognostic relevance of these CTLs and other components of the immune system in the setting of CRC, therapeutic tools that are able to influence these key immunological mediators present promising candidates for more successful clinical control of progression, metastasis, and recurrence of CRC. These new therapeutic approaches have already been successfully tested in various animal models of CRC or even in first clinical trials, demonstrating an encouraging tumor-suppressive capacity. Therefore, better integration of immunotherapy into clinically approved concepts of standardized treatment of CRC can be expected in the near future [247].

## **NEW PROMISING MARKERS**

### **Forkhead Box P3–Positive Regulatory T Cells**

Regulatory T cells (Tregs) were initially characterized by the CD4<sup>+</sup>CD25<sup>+</sup> phenotype and are thought to modulate the antitumor immune response. Adaptive Tregs contribute to an immunosuppressive microenvironment in CRC through a cyclooxygenase-2–prostaglandin-E2–dependent mechanism, direct cell–cell contact, or by the release of cy-

tokines, such as TGF- $\beta$ , thereby facilitating tumor growth [284, 285]. The most specific Treg marker identified to date is the nuclear transcription factor known as forkhead box P3 (FOXP3). Loddenkemper et al. [286] reported that Treg density was lower in node-positive disease but was not associated with survival. In contrast, Ling et al. [287] found no significant difference in Treg density between advanced and early-stage disease, but did not evaluate the association with patient survival. Salama et al. [288] showed that a high density of FOXP3<sup>+</sup> Tregs in CRC was associated with longer survival and had a stronger prognostic significance than CD8<sup>+</sup> and CD45RO<sup>+</sup> memory T lymphocytes in CRC. Furthermore, a high density of FOXP3<sup>+</sup> Tregs in normal colon tissue from CRC patients was associated with a worse prognosis. The worse outcome observed for these patients might be explained by the proposed role for these cells in suppressing antitumor immunity. However, the observation of better survival for patients with a high density of FOXP3<sup>+</sup> Tregs in their tumor tissue is counterintuitive and contrasts with what has been reported for other solid tumor types, for which Tregs are generally considered to be immunosuppressive. In contrast, Suzuki et al. [289] were not able to show a significant correlation between FOXP3<sup>+</sup> Tregs and survival, but they did find evidence that a balance of intratumoral Tregs and CD8<sup>+</sup> T cells is a more sensitive predictor of recurrence and survival than intratumoral Tregs or CD8<sup>+</sup> T cells alone. Functional studies of FOXP3<sup>+</sup> Tregs in cancer and normal tissue may shed more light on their role in the antitumor response and help to explain the observed associations with prognosis [288].

Although further studies are required before changes in clinical practice can be recommended, the results of Salama et al. [288] suggest that the assessment of FOXP3<sup>+</sup> Treg density in tumor and normal colorectal tissue in combination with vascular and perineural invasion could improve prognostication in early-stage CRC patients. Few studies analyzed the immune gene expression profile of FOXP3<sup>+</sup> Tregs according to MMR status. Le Gouvello et al. [290] observed higher expression levels of FOXP3 mRNA in MSS CRCs. In addition, Frey et al. [291] demonstrated that a high frequency of tumor-infiltrating FOXP3<sup>+</sup> Tregs is associated with early T stage and independently predicts longer disease-specific survival in MMR-proficient CRC patients but not in MMR-deficient patients. In contrast, Michel et al. [292] were the first to show that the density of FOXP3<sup>+</sup> Tregs infiltrating CRC was significantly higher in MSI-H tumors using IHC, paralleling the enhanced number of CD8<sup>+</sup> cells in these tumors. This discrepancy might be attributed to the different methodologies applied in these studies or the role of Tregs may differ according to the clinical stage and genetic background of tumors [291]. Further

research is recommended to elucidate the role of FOXP3<sup>+</sup> Tregs and MMR status in CRC.

## Members of the Extracellular Signal–Related Kinase–MAPK Signaling Pathway

### *Receptor for Hyaluronic Acid–Mediated Motility*

The receptor for hyaluronic acid–mediated motility (RHAMM) (intracellular hyaluronic acid binding protein, CD168) is a multifaceted protein with both intracellular and extracellular functions [293]. RHAMM binds hyaluronan [294], interacts with both microtubules and microfilaments [293, 295], localizes to the centrosome, and functions in maintaining spindle integrity [296], and is suggested to represent a member of the MAP family [297, 298]. In addition, RHAMM participates in cell motility, signaling, and oncogenic events [296]. The RAF–MAPK/ERK kinase–extracellular signal–related kinase (ERK) pathway belongs to the MAPK pathways and represents one of the best characterized RAS signaling pathways. The molecule ERK is activated by a cascade of phosphorylations downstream from the RAS proto-oncogene and plays a role in differentiation, secretion, and proliferation. RHAMM binds ERK and controls expression levels of ERK [298].

In addition to its role in cell migration, RHAMM expression, and overexpression, has been linked to RAS transformation, tumor progression, and metastasis [299] in different tumor types [298]. However, the prognostic significance of RHAMM in CRC is poorly understood.

**RHAMM as a Prognostic Factor.** Only a few studies, primarily from the group of Dr. Lugli, have investigated the prognostic significance RHAMM in CRC. The expression profile was assessed by means of tissue microarray IHC in CRC. Interactions between RHAMM and other potential prognostic and clinicopathological factors were also established.

In normal colonic mucosa, RHAMM was diffusely but weakly expressed in the cytoplasm of columnar cells in the crypts, but apparently not in the goblet cells. RHAMM was less strongly expressed and quantitatively less extensive in these normal tissues than in the cancer cell population [298].

Lugli et al. [298] demonstrated that higher RHAMM expression is needed to induce tumor progression and that expression of RHAMM is an independent adverse prognostic factor in MMR-proficient CRC and presumed HNPCC (MSH2<sup>−</sup> and/or MSH6<sup>−</sup> at any age or MLH1<sup>−</sup> and <55 years of age).

Zlobec et al. [300] evaluated the independent prognostic effect of a panel of IHC protein markers, including



RHAMM, in unselected CRC patients. They suggested that RHAMM should be considered a more important prognosticator than tumor grade, MMR status, tumor budding, and vascular invasion in CRC. In addition, diffuse RHAMM expression was second in terms of independent prognostic value behind N stage on multivariate analysis.

Moreover, the involvement of RHAMM within the RAS–MAPK pathway and its role as a receptor suggest that it might be a potential candidate for therapeutic intervention.

**Multimarker Analysis.** In a study of Lugli et al. [298], nuclear phosphorylated ERK (pERK) expression was correlated with RHAMM expression in MMR-proficient CRC and in presumed HNPCC (MSH2<sup>-</sup> and/or MSH6<sup>-</sup> at any age or MLH1<sup>-</sup> and <55 years of age), whereas an association was not found in MLH1<sup>-</sup> tumors. This leads to the hypothesis that pERK is involved in the mechanism of tumor progression in MMR-proficient CRC and HNPCC by interacting with the Wnt signaling pathway and RHAMM.

In a recent analysis including only MSI-H tumors, Zlobec et al. [300] found that the combination of p21 and RHAMM led to a highly adverse prognosis. This analysis suggested that node-negative patients overexpressing RHAMM but with loss of p21 may experience a potential benefit from postoperative treatment, whereas adjuvant chemotherapy should be reconsidered for node-positive RHAMM<sup>-</sup> tumors. Expression of p21 appears to act as a modifying factor in the survival time of patients with RHAMM<sup>+</sup> tumors.

These findings also outline the importance of evaluating multimarker phenotype combinations of IHC protein markers [300] and provide evidence for the inclusion of RHAMM as a new and promising prognostic factor in CRC. However, prospective studies are needed to further validate the prognostic role of RHAMM in CRC and its potential role in selecting patients for adjuvant therapies [301].

### **PI3KCA**

Class 1 PI3Ks are heterodimeric lipid kinases composed of a p85 regulatory subunit and one of several p110 catalytic subunits. Among several isoforms of the catalytic subunits, only the  $\alpha$ -type, PI3KCA, has been shown to harbor oncogenic mutations or amplifications in its gene in human malignancies [302, 303]. The regulatory subunit of PI3K can specifically bind protein factors including KRAS, integrate various signals from membrane receptors, and activate PI3KCA. Activated PI3KCA will phosphorylate phosphatidyl-inositol-4,5-bisphosphate to produce phosphatidyl-inositol-3,4,5-trisphosphate (PIP3), which localizes the

serine threonine kinase Akt to the cell membrane where it becomes activated. Activated Akt phosphorylates downstream protein effectors and amplifies the signaling cascade, enhancing cell proliferation and survival [302]. Components of this pathway, including PI3KCA, are often altered in human malignancies.

**PI3KCA as Prognostic and/or Predictive Factor.** The prognostic role of PI3KCA is still under investigation. Jehan et al. [302] reported PI3KCA amplification as an independent prognostic factor for longer survival and suggested that this might be one of the promising markers to define subsets of CRC patients who may maximally benefit from adjuvant therapy. Recent reports have highlighted the prognostic role of *PI3KCA* mutations as a marker of poor outcome in surgically resectable tumors [303, 304]; however, the study of Souglakos et al. [142] did not indicate PI3KCA as a useful predictor of current adjuvant treatment strategies. Although there have been reports suggesting that *PI3KCA* mutation (in exons 9 and 20) might be correlated with response to anti-EGFR inhibitors [140], large patient population studies are necessary to determine whether this factor will play an important role in determining response to anti-EGFR mAbs [113].

### **PTEN**

PTEN has been identified as a critical negative regulator of the cell-survival signaling pathway initiated by PI3K. The PTEN protein acts as a phospholipid phosphatase with PIP3 as a substrate. One of the most important downstream targets of PIP3, Akt, is activated by phosphorylation and inhibited by PTEN. PTEN protein is a negative regulator of the Akt signaling pathway, and thus inactivation of PTEN, which is a common event in human malignancies, facilitates cell proliferation and apoptosis [305, 306]. In CRC, it has been demonstrated that *PTEN* mutations correlate with advanced and metastatic tumors [305, 307]. Moreover, epigenetic silencing of *PTEN* by promoter hypermethylation has been observed in sporadic CRC cases, especially in MSI-H tumors [305, 308].

**PTEN as a Prognostic and/or Predictive Factor.** The prognostic role of PTEN in CRC is still under investigation, and inconclusive results have been reported. Although some groups report an association between loss of PTEN and a shorter progression-free survival interval [306, 146], others report an association with poor prognosis in stage II patients only [305] or, in contrast, in CRC patients with liver metastasis [307]. As mentioned above, PTEN shows promise as a predictive marker for wild-type *KRAS* patients treated with an anti-EGFR–based regimen [143, 146].



### T-Cell Originated Protein Kinase

In 2000, a new member of the ERK–MAPK pathway, T-cell originated protein kinase (TOPK), also known as PZD-binding kinase, was identified and described as a MAPKK (mitogen activated protein kinase kinase). TOPK is a serine/threonine kinase and has been shown to be involved in p38 and c-Jun N-terminal kinase signaling, and more recently in the ERK–MAPK pathway [309, 310]. TOPK seems to be overexpressed in a variety of tumors in vitro, whereas inhibition of TOPK has been shown to lead to apoptosis in breast and melanoma cell lines. Zhu et al. [310] systematically assessed this novel molecule in CRC and confirmed its oncogenic potential in vitro and in vivo. More importantly, they found that TOPK could promote malignant transformation by exerting a positive feedback loop on ERK-2 activity [310].

**TOPK as a Prognostic and/or Predictive Factor.** Zlobec et al. [309] were the first to assess the prognostic and predictive value of TOPK in CRC. Given its central involvement in ERK–MAPK signaling, TOPK overexpression was significantly related to *KRAS* and *BRAF* mutations. Their results showed that TOPK may be a valuable prognostic marker in patients with sporadic CRC with *KRAS* and *BRAF* gene mutations and in patients with metastatic disease with a proficient molecular profile for positive response to anti-EGFR therapies. If confirmed prospectively, the inhibition of TOPK may represent a novel avenue of investigation of targeted treatment in patients with CRC [309].

### CONCLUSION AND FUTURE PERSPECTIVES

For CRC patients who undergo successful surgery, additional treatment is recommended, especially for those patients with a high risk for relapse. Although CRC prognosis is stage and grade dependent, many tumors with similar histopathologic features show significantly different clinical outcomes [92]. The identification of molecular factors that have prognostic and/or predictive significance in CRC is essential to improve treatment and outcome [30]. Several biomarkers have been studied over the past decades; however, results of published studies have often been conflict-

ing and several drawbacks affect the reliability of conclusions [238]. Most published studies have used retrospective analyses of a single marker in a small series of patients. These study designs are unlikely to precisely predict progression of disease with sufficient resolution and reproducibility [92]. In spite of these problems, some biomarkers have shown promising results. Therefore, with the multitude of putative factors available for study, a combinatorial approach to molecular prognostics, similar to the prognostic profiles established for breast cancer patients, may have significance and be used in future patient management [30]. This understanding recently led to more comprehensive approaches of global gene expression profiling and genome-wide analysis to determine prognostic and predictive signatures in tumors. Many advances have been made in technologies for profiling and in decreasing the requirements of the input material, although data analysis and interpretation still remain challenging. Hence, although there are immense potential implications, clinicians are currently unable to use these data in clinical practice for decision making because of a lack of definition, adequate validation, and easy implementation [17]. Therefore, the current reality is that no molecular marker, other than the *KRAS* gene in the case of EGFR-targeted therapy for metastatic disease, has made it into clinical practice. Large prospective randomized trials have the potential to determine the role of various putative molecular markers. Unfortunately, biomarker-embedded clinical trials do not receive the same commercial attention as those for new chemotherapeutic compounds. Furthermore, standards need to be agreed upon for what determines the validity of a biomarker before any marker can be used in these clinical trials. The introduction of new therapeutic agents and the discovery and validation of prognostic and/or predictive markers along with new screening tools will enable oncologists to tailor patient-specific chemotherapy by maximizing drug efficacy and minimizing adverse and possibly severe side effects [92].

### AUTHOR CONTRIBUTIONS

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