What can molecular pathology offer for optimal decision making?

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In recent years, colorectal cancer (CRC), once a uniform disease with well-understood carcinogenesis, has been divided into at least five different subgroups with distinct precursor lesions, pathways of carcinogenesis, morphological, and molecular characteristics. Moreover, new therapeutic concepts with ‘targeted’ substances have added to the complexity of the management of CRC patients. The clinical value of biomarkers in advanced CRC is indisputable ever since activating mutations of the KRAS oncogene have been shown to predict resistance to anti-epidermal growth factor receptor antibodies. Prognostic biomarkers predicting patient outcomes and predictive biomarkers forecasting response to a certain therapy may help us to improve therapeutic agent selection and patient management with the ultimate goal of maximizing the benefit of treatment and minimizing toxicity. Biomarkers with known implications in advanced CRC will be discussed in this paper.

Key words: biomarker, colorectal, metastatic, predictive, prognostic

introduction

There is increasing evidence that colorectal cancer (CRC) is not a uniform disease arising through the well-described adenoma–carcinoma sequence [1], but rather a multi-pathway disease comprising at least five distinct molecular and morphological subtypes (Table 1) [2, 3]. This knowledge about different pathways of colorectal carcinogenesis including their key molecular findings not only helps to explain the observed heterogeneity of clinical outcomes but also may lead to a more selective and systematic approach to therapy. However, it also divides a common disease into multiple uncommon subentities, thus increasing the need for large multi-center trials to reach substantial evidence for the superiority of one treatment over the other.

The advent of targeted agents directed against distinct molecules and pathways such as the epidermal growth factor receptor (EGFR) pathway has added to the complexity of the therapeutic management of metastatic CRC (mCRC). With the knowledge that activating Kirsten rat sarcoma viral oncogene homolog (KRAS) mutations predict resistance to anti-EGFR antibodies (cetuximab and panitumumab) [4, 5], the clinical value of biomarkers became unquestionable. The advances of chemotherapy and targeted biological therapy will entail an important role not only in providing tissue but also in providing valid and accurate tests for these biomarkers.

This paper will give an overview of the current knowledge about biomarkers with known implications in CRC. Some of these biomarkers, such as defective mismatch repair (MMR) and KRAS, are thought to be useful as both prognostic and predictive markers while others are only known for their prognostic or their predictive value.

microsatellite instability/defective mismatch repair as a prognostic marker

The MMR system eliminates base mismatches and insertion/deletion loops resulting from DNA polymerase slippage during DNA synthesis [8]. Defective MMR can either occur through germline mutations in MMR genes in ‘hereditary nonpolyposis colorectal cancer’ (HNPPCC) or through promoter hypermethylation of—most commonly—hMLH1 in sporadic CRC. It results in microsatellite instability (MSI), an expansion or contraction of mono-, di-, tri-, or tetrănucleotide repeat sequences within the DNA. MSI can affect the coding regions of genes including regulators of cell proliferation, cell cycle, or apoptosis [9].

There is undeniable evidence for significantly better clinical outcomes in patients with MSI tumors; the hazard ratio (HR) for overall survival (OS) was reported 0.65 for MSI tumors in a large meta-analysis of 7642 CRC [9]. Other authors have corroborated these findings in retrospective analyses of large multicenter trials [10–12]. However, most evidence for MSI as a favorable prognostic marker originates from studies of locally advanced tumors (UICC stage II and III); this may be due to
the fact that the number of MSI tumors decreases with increasing Union International Contre le Cancer (UICC) stage [11, 13], suggesting that MSI tumors only rarely progress to distant metastatic disease. In mCRC (UICC stage IV), MSI is still reported as a favorable clinical marker, but statistical significance is not reached due to the relatively small numbers [12, 14].

Nevertheless, defective MMR should be routinely determined in CRC to identify patients with a more favorable prognosis. It can easily be tested by immunohistochemistry for the four most relevant MMR proteins: hMLH-1, hMSH-2, hMSI-6, and hPMS-2; loss of protein expression demonstrates a good correlation with high MSI [11, 15].

as a predictive marker

5-fluoruracil. Almost all first-line CRC chemotherapy regimens include 5-fluoruracil (5-FU) or its prodrug capecitabine. In tumor cells, 5-FU is converted into several active metabolites, cytotoxicity results from misincorporation of fluoronucleotides into both DNA and RNA and from inhibition of the enzyme thymidylate synthase (TS) which is essential in nucleotide synthetic functions [16]. MSI has also been reported as a predictive marker for the lack of response to 5-FU-based chemotherapy in UICC stage II and III CRC [17, 18], but this finding remains controversial. The background of MSI (hereditary versus sporadic) and the UICC stage of the tumor may influence the predictive impact of MSI [10, 18, 19]. Hutchins et al. suggest that patients with MSI, in spite of their favorable prognosis, should not be precluded from chemotherapy if it is indicated due to other unfavorable tumor characteristics [such as pT4 or lymphangioinvasion (L1)].

irinotecan. Irinotecan (CPT-11) is administered as a prodrug which is converted by carboxylesterases to its active metabolite, SN38. Defective MMR was studied in an adjuvant trial (CALGB 89803), disease-free survival was significantly improved in MSI compared with microsatellite stable (MSS) tumors upon addition of irinotecan to 5-FU/leucovorin treatment [20]; however, these results were not supported by the PETACC-3 trial in which 5-FU/leucovorin with or without irinotecan was compared, and the addition of irinotecan did not improve survival in patients with MSI versus MSS tumors [21]. Therefore, the utility of MSI/defective MMR as a predictive marker requires further study [19].

KRAS

as a prognostic marker

KRAS is part of the EGFR-signaling pathway downstream to EGFR, a receptor tyrosine kinase which is activated through extracellular ligand binding. Activation of the pathway ultimately leads to the modulation of angiogenesis, cell migration, proliferation, cell adhesion, metastasis formation, and survival [22, 23].

The prognostic effect of KRAS has extensively been studied and rendered conflicting results. While the initial RASCAL collaborative study [24] found KRAS mutations to be a poor prognostic marker, the subsequent RASCAL II analysis found only one specific KRAS mutation to be prognostic in UICC stage III only [25]. KRAS was found to be prognostic for poor OS in the MRC FOCUS trial [26], but not in the PETACC 3 trial of UICC stage II and III tumors [27].

as a predictive marker for anti-EGFR therapy

Cetuximab and panitumumab are the two currently licensed monoclonal antibodies targeting the EGFR pathway. The attempt to predict response to EGFR treatment by assessing EGFR expression by immunohistochemistry [28] in analogy to HER2/new in breast cancer turned out to be unsuccessful, which led to extensive research of the prognostic capacities of other signaling pathway constituents, especially KRAS and BRAF. Lievre et al. [5] reported activating KRAS mutations as predictors of resistance to anti-EGFR treatment in 2006. Such mutations are thought to occur as an early event in the adenoma–carcinoma sequence [1] and even as an initial event in CRCs arising from traditional serrated adenomas (so-called mixed type or serrated CRC) [3, 29]. Mutation of the KRAS gene results in a constitutively active KRAS protein and mitogen activated protein kinase (MAPK) pathway signaling independent from EGFR. Inhibition of EGFR is likely to be ineffective in these tumors since it acts upstream of the activated protein. This hypothesis has been proven in several prospective, randomized trials: CRYSTAL, OPUS, PACE, CAIRO2 [30–32], PRIME [33], and CONSORT [34].

In vitro and mouse model analysis showed that p.G13D-mutated cells were sensitive to cetuximab, as were KRAS wild-type cells, while p.G12V-mutated cells were insensitive
to cetuximab treatment [35]. Recent post-hoc analyses of clinical trials suggest that mutations in codon 13 (p.G13D) may in fact not interfere with response to anti-EGFR treatment [35]. De Roock et al. found in a pooled analysis of 579 patients from several clinical trials (BOND, SALVAGE, EVEREST, and others) that patients with p.G13D-mutated tumors (n = 32) treated with cetuximab had longer OS [adjusted HR, 0.50; 95% confidence interval (95% CI) 0.31–0.81; P = 0.005] and longer progression-free survival (adjusted HR, 0.51; 95% CI 0.32–0.81; P = 0.004). There was a significant interaction between KRAS mutation status (p.G13D versus other KRAS mutations) and OS benefit with cetuximab treatment (adjusted HR, 0.30; 95% CI 0.14–0.67; P = 0.003). Modest et al. [36] also published pooled retrospective data suggesting comparable efficacy of cetuximab-based and bevacizumab-based first-line therapy in patients with p.G13D mutant mCRC. However, Gajate et al. could not show any difference in response to cetuximab between different KRAS mutations in their cohort of 110 patients [37]. Again, all these data come from retrospective analyses and therefore have to be evaluated with caution; to date, there is not enough evidence to change clinical practice. Therefore, evaluation of cetuximab therapy in tumors with p.G13D mutations in prospective randomized trials may be warranted.

In general, KRAS mutational analyses concentrate on mutations in codon 12 and 13, commercially available kits such as the ‘Therascreen KRAS Mutation Test kit’ tests only six mutations in codon 12 and one mutation in codon 13, which make up for ~96% of all observed mutations. However, other activating mutations have been identified in these codons and, additionally, in codon 61 and 146 of the KRAS gene. Approximately 1% of tumors with wild type at codons 12 and 13 will have mutations in codon 146 and an additional 7% of these will be mutated in codon 61 [38, 39]. These mutations may very well predict resistance to anti-EGFR treatment as may mutations of the Neuroblastoma RAS viral oncogene homolog (NRAS) gene. It remains to be seen, whether expanded mutational analyses of KRAS and NRAS adds substantial additional predictive value. To answer this question, data from many trials will have to be analyzed to warrant sufficient patients with each genotype (Figure 1).

B-Raf murine sarcoma viral oncogene homolog B1 (BRAF)
as a prognostic marker

BRAF is downstream of KRAS in the MAPK-signaling pathway; it is known to be subject to activating mutations with a hotspot in exon 15, codon 600. The most frequent mutation

Figure 1. Prevalence of molecular alterations in the EGFR pathway and response to anti-EGFR treatment with monoclonal antibodies in chemotherapy-refractory mCRC (adapted from [87]).
is p.V600E. The frequency of BRAF mutations in CRC decreases with advancing UICC stage, ~8% of all CRC carry a BRAF mutation which is mutually exclusive to KRAS mutations [26, 40]. After being primarily discussed as a potent predictive marker for resistance to anti-EGFR treatment [41], BRAF mutation has meanwhile been reported as a marker for poor prognosis in mCRC in a number of retrospective analyses of large clinical trials [11, 26, 27, 42-44]. The prognostic value of BRAF mutation, however, is obviously influenced by the MSI status. Samowitz et al. [45] reported a negative prognostic effect of BRAF mutations only for MSS patients, but not for patients with MSI. In fact, patients with BRAF mutation and MSI had a favorable prognosis when compared with MSS/ BRAF wild-type patients.

Testing of the BRAF status in MSI cases will most likely be of additional use for the detection of unknown cases of HNPCC. According to current knowledge, the BRAF mutation status allows the discrimination of sporadic MSI tumors (MSI/HNPCC. According to current knowledge, the BRAF mutation status allows the discrimination of sporadic MSI tumors (MSI/BRAF mutant) from HNPCC (MSI/BRAF wild type) [46].

as a predictive marker for anti-EGFR therapy

Data from a recent pooled retrospective analysis of the expanded CRystal and Opus trials suggest that BRAF mutations are not predictive for resistance to cetuximab in combination with standard first-line therapy in patients with KRAS wild-type mCRC [44], but indicate worse prognosis independent of the given treatment. BRAF mutant tumors may respond to anti-EGFR treatment but to a much lesser degree than BRAF wild-type tumors [44, 47]. The initial report from Di Nicolantonio et al. [41] indicating a strong predictive effect for BRAF mutations in anti-EGFR treatment of a small cohort of mCRC patients could not be corroborated by other authors. However, the relatively low frequency of BRAF mutations in CRC makes it rather difficult to draw conclusions based on post-hoc analyses of phase II and III trials [47].

gene expression profiles

Since the development of array-based technologies, their utility has been tested in multiple studies to provide additional prognostic and predictive information. The majority of these array-based technologies have failed to extend into commercially viable and robust platforms. Up to date, the commercial viability and robustness of these array-based technologies has not been achieved. However, the relatively low frequency of BRAF mutations in CRC makes it rather difficult to draw conclusions based on post-hoc analyses of phase II and III trials [47].

loss of heterozygosity of 17p and 18q

Loss of heterozygosity (LOH) of 17p and 18q have so far only been studied as ‘prognostic’ markers. LOH of the short arm of chromosome 17 (17p) and the long arm of chromosome 18 (18q) have been implicated in colorectal carcinogenesis early on and occur in as many as 70% of all CRCs [1, 52, 53]. Several important tumor suppressor genes are located in these regions: DCC, SMAD2, and SMAD4 on 18q and p53 on 17p. Despite extensive research, the findings regarding LOH of 17p and 18q are still conflicting [54, 55].

Immunohistochemical studies of p53 [56, 57] and SMAD4 [55, 58, 59] produce equally conflicting data.

PIK3CA

Phosphatidylinositol-3-kinases (PI3K) are lipid kinases promoting various biologic processes, including cellular proliferation and survival. Mutations of the PIK3CA gene, encoding for the p110α catalytic subunit of PI3K, have been identified in many human solid tumors, including colon, breast, brain, ovarian, liver, and lung cancers [60, 61]. PIK3CA mutations are found in 10%–20% of CRCs and have been reported to be associated with specific clinicopathologic features and molecular events, such as proximal tumor location, MSI, and KRAS mutation [62-67]. Mutations occur mainly in exons 9 and 20 of the PIK3CA gene.
as a prognostic marker

The prognostic significance of PIK3CA mutation in CRC remains uncertain. Liao et al. recently published a study done on 1170 CRCs (UICC stage I–IV) in which he could show that the coexistence of PIK3CA exon 9 and 20 mutations, but not PIK3CA mutations in either exon 9 or 20 alone, is associated with poor prognosis of patients with CRC [61]. Patients with mutations in both exons showed significantly worse cancer-specific survival (P = 0.031; multivariate HR = 3.51; 95% CI 1.28–9.62) and OS (P = 0.0008; multivariate HR = 2.68; 95% CI 1.24–5.77). Ogino et al. [62] could show an adverse effect of PIK3CA mutations on cancer-specific survival after curative resection of UICC stage I–III colon cancers, this effect was more prominent in KRAS wild-type tumors. Tol et al. [68] could not show any prognostic relevance in UICC stage IV patients, while Farina Sarasqueta et al. [69] found a prognostic effect of exon 20 mutations in a cohort of UICC stage I–III patients.

as a predictive marker for anti-EGFR therapy

There is also conflicting data on the predictive value of PIK3CA mutations. While Sartore-Bianchi et al. [63] could show no objective response to either cetuximab or panitumumab in 110 patients with mCRC, Tol et al. [68] did not observe any predictive value of PIK3CA mutations in 599 patients enrolled in the CAIRO2 study. De Roock et al., however, showed in a cohort of 1022 patients treated with cetuximab that only mutations in exon 20 of the PIK3CA gene were significantly associated with nonresponse to anti-EGFR treatment. The response rate for mutated tumors was 0%, while 36.8% of the wild-type tumors responded (P = 0.029), the median progression-free survival was 11.5 compared with 24 weeks [70] (Figure 1).

additional predictive markers for anti-EGFR therapy

EGFR ligands amphiregulin/epiregulin

All the predictive markers discussed so far are negative predictive markers for anti-EGFR therapy. There is some evidence that the upregulation of the EGFR ligands amphiregulin and epiregulin will help to identify patients who will benefit from anti-EGFR treatment, thus maybe constituting the first positive predictive marker for panitumumab and cetuximab [71]. These two markers are currently tested on bigger cohorts, it remains to be seen whether the results can be reproduced in a larger number of patients and whether the test for epiregulin and amphiregulin mRNA is robust enough to become a standard diagnostic test.

PTEN

The phosphatase and tensin homolog (PTEN) is a tumor suppressor protein that regulates the activity of PI3K/AKT (v-akt murine thymoma viral oncogene homolog). The loss of PTEN results in AKT-mediated hyperphosphorylation, which protects cells from apoptosis. In sporadic breast, lung, and colorectal (30%–40%) cancers, the PTEN loss can occur by mutation, promoter methylation, and microRNA suppression [72]. Similar to PIK3CA, PTEN, and KRAS mutations are not mutually exclusive. Loupakis et al. [73] analyzed the role of PTEN loss in the activity of cetuximab plus irinotecan in mCRC. Their data from metastatic tissue demonstrated that in a PTEN-positive group, 12 of 33 patients (36%) were responders, whereas only 1 of 22 (5%) PTEN-negative patients responded (P = 0.007). The median progression-free survival (PFS) was 4.7 months versus 3.3 months for patients with PTEN-positive and PTEN-negative tumors, respectively (HR, 0.49; P = 0.005), suggesting that the loss of PTEN in CRC metastases may predict resistance to cetuximab plus irinotecan. The analysis of 162 samples by Laurent-Puig et al. [74] reported the PTEN null expression rate of 19.9% with an association of poorer OS in the KRAS-WT population (P = 0.013), suggesting that mCRC-PTEN-positive patients have a better survival outcome than those with PTEN loss. A recent small study corroborated these data; Sood et al. [75] found a significant negative effect of PTEN loss on OS, but not on progression-free survival in patients treated with cetuximab. In KRAS wild-type patients, the negative effect of PTEN loss was significant for both, progression-free and OS. However, interpreting PTEN immunohistochemical data can be challenging, because the results can be variable. A standard, universally accepted PTEN testing and scoring system allowing for comparisons of data across the globe, has yet to be established [76].

EGFR polysomy

Increases in the EGFR gene copy number are more frequent in CRGs than EGFR mutations (≈20%) [74]. Laurent-Puig et al. and others could show that an increased copy number of EGFR is associated with response to anti-EGFR treatment [63, 74, 77, 78], thus indicating that this marker could be used as a positive predictive marker for anti-EGFR treatment. EGFR copy number changes are detected by either fluorescence in situ hybridization or chromogenic in situ hybridization. The distribution of EGFR polysomy within CRCs can be very heterogeneous and reproducibility is therefore hard to obtain. Time will tell, if a standard scoring protocol such as for Her2/new in breast cancer can be established and proven to be clinically useful in CRC.

anti-VEGF predictive markers

Vascular endothelial growth factor acts as a potent angiogenic factor and is a key player in tumor angiogenesis. It induces the proliferation of endothelial cells, increases vascular permeability and promotes the extravasation of proteins from the tumor vessels, thus contributing to the formation of the tumor stroma [76]. Bevacizumab, an anti-VEGF monoclonal antibody, was approved for the treatment of mCRC after Hurwitz et al. [79] could show in a phase III trial that bevacizumab significantly improved the patient survival. No conclusive evidence yet exists that VEGF is a predictive biomarker of efficacy for treatment with antiangiogenic therapy [76], no other predictive markers have been determined thus far.

anti-IGF1R predictive markers

The insulin-like growth factor receptor (IGF1R) is a major mediator of growth hormones and its roles in oncogenic...
processes include cell growth, transformation, differentiation, motility, cell-cycle progression, and inhibition of apoptosis [76]. In 2009, Zha et al. [80] reported that at least some CRC cell lines were sensitive to the inhibition of IGFR1 by a monoclonal antibody MoAb h10H5. Their data showed that the expression levels of IGFR1 correlated with response to MoAb h10H5, suggesting that it could have predictive value for this treatment.

Numerous new anti-IGFR1 therapies are being developed and have entered early phase trials in multiple solid tumors. This pathway has shown potential to add another class of ‘targeted’ agents to the management of mCRC in the near future. Ongoing research continues to unravel the complexities of understanding the IGF1R pathway, including its interaction with the EGFR and VEGF pathways [76].

predictive markers for 5-FU, irinotecan, and oxaliplatin
Response prediction is not confined to ‘targeted’ therapies but plays an important role in ‘conventional’ chemotherapy as well. Even though this paper is focused on ‘targeted’ therapies, it should be mentioned that predictive markers for 5-FU, irinotecan, and oxaliplatin are discussed in the literature, although, to our knowledge, none of these markers have been validated in prospective clinical trials.

For 5-FU, several enzymes involved in the 5-FU metabolism are thought to have an influence on response to treatment: thymidylate synthase (TS), dihydropyrimidine dehydrogenase, and thymidine phosphorylase. Many studies have established TS as a predictor of response and resistance to 5-FU in mCRC [81].

In addition to a possible influence of MSI on response to ‘irinotecan’, there is a genetic variant of the enzyme UGT1A1, catalyzing its hepatic glucuronidation step, that is thought to be associated with higher irinotecan associated toxicity [82]. However, this finding was not corroborated in the MRC FOCUS trial, therefore genotyping of UGT1A1 is probably not necessary in routine clinical practice [83]. A systematic search for biomarkers of irinotecan and oxaliplatin efficacy identified high expression of topoisomerase 1 in the CRCs as a favorable marker [14], the validation of these data is currently underway [84].

As summarized in a recent comprehensive review, a large number of studies have evaluated if high ERCC1 by IHC, RT-PCR, or genotyping predicted a poor outcome in patients with CRC treated with ‘oxaliplatin’-based chemotherapy. All of these published studies were generated in the metastatic setting. Most of them evaluated ERCC1 by genotyping, some by either immunohistochemistry or RT-PCR [85]. The data suggest so far that high ERCC1 mRNA expression predicts resistance to oxaliplatin, while immunohistochemical analyses of ERCC1 expression were inconsistent with regard to their predictive value. The ERCC1 genotyping (118C_T SNP) data remain inconclusive to date [85].

collection
Our knowledge about colorectal carcinogenesis and potential prognostic/predictive markers is continuously growing. The value of each molecular marker needs to be scrutinized against the gold standard of UICC/TNM (tumour–node–metastasis) classification and thorough histopathologic workup [84, 86]. Each new marker has to be tested for its validity in prospective clinical trials; robust test platforms, standards of quality assurance, and quality control have to be defined, and at last, the cost and benefit of each new marker has to be carefully evaluated in the multidisciplinary management of CRC.

To date, there is a clear indication for MSI testing for recurrence prediction and HNPPC screening with possible modifications of follow-up and chemotherapy. KRAS testing is needed whenever anti-EGFR therapy is considered. The role of individual KRAS mutations has to be investigated in large prospective trials. Although multiple studies were undertaken to find new predictive markers, KRAS remains the only validated predictive marker in CRC thus far.

The subdivision of CRC in different subentities generates the necessity of multicenter trials, since no single center will be able to ascertain significant numbers of all the tumor subtypes we are aware of now (let alone those that we will be aware of in the future). In all these trials, FFPE tissue should be collected and investigated for potential prognostic/predictive markers in order to make personalized treatment a reality in CRC.

disclosure
US has declared no conflict of interest. GBB and DEA both serve as members for an advisory board from AMGEN.

references


