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Prognostic and Predictive Biomarkers in Resected Colon Cancer: Current Status and Future Perspectives for Integrating Genomics into Biomarker Discovery

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ABSTRACT

The number of agents that are potentially effective in the adjuvant treatment of locally advanced resectable colon cancer is increasing. Consequently, it is important to ascertain which subgroups of patients will benefit

from a specific treatment. Despite more than two decades of research into the molecular genetics of colon cancer, there is a lack of prognostic and predictive molecular biomarkers with proven utility in this setting. A secondary objective of the Pan European Trials in Adjuvant Colon Cancer-3 trial, which compared irinotecan in combination with 5-fluorouracil and leucovorin in the postoperative treatment of stage III and stage II colon cancer patients, was to undertake a translational research study to assess a panel of putative prognostic and predictive markers in a large colon cancer patient cohort. The Cancer and Leukemia Group B 89803 trial, in a similar design, also investigated the use of prognostic and predictive biomarkers in this setting. In this article, the authors, who are co-investigators from these trials and performed similar investigations of biomarker discovery in the adjuvant treatment of colon cancer, review the current status of biomarker research in this field, drawing on their experiences and considering future strategies for biomarker discovery in the postgenomic era. *The Oncologist* 2010;15:390–404

INTRODUCTION

Colorectal cancer (CRC) is a major cause of cancer mortality worldwide, with approximately 500,000 recorded deaths from the disease in 2002 [1]. A significant proportion of patients presenting with stage I, II, or III disease (75% of patients) can be cured by surgical intervention, with U.S. 5-year survival rate figures of 93.2%, 82.5%, and 59.5%, respectively, compared with only 8.1% for stage IV disease [2]. Following resection, there is a considerable risk for tumor recurrence in patients with stage III and high-risk stage II disease, which can be significantly reduced by treating with 5-fluorouracil (5-FU)-based adjuvant chemotherapy [3–5]. The addition of oxaliplatin to 5-FU–based chemotherapy (5-FU, leucovorin, and oxaliplatin—the FOLFOX-4 regimen) is now a standard adjuvant treatment for colon cancer, with a higher 5-year disease-free survival (DFS) rate (73.3% versus 67.4%) and significantly higher overall survival (OS) rate at 6 years in stage III patients (78.5% versus 76%), compared with 5-FU–based treatment alone [6]. The combination of irinotecan with 5-FU failed to result in a higher DFS rate than with 5-FU–based therapy alone in the Pan European Trials in Adjuvant Colon Cancer (PETACC)-3 and Cancer and Leukemia Group B (CALGB) 89803 trials [7, 8].

In the absence of adjuvant therapy, approximately 50% of colon cancer patients with resectable disease are cured by surgery alone, whereas 50% relapse. Using adjuvant chemotherapy following surgery rescues approximately 15% of patients from the relapsing group. In current practice, the majority of colon cancer patients receive treatment unnecessarily, either because they were cured or because they will relapse despite treatment. It is therefore essential to identify patients who will benefit from adjuvant therapy, sparing others needless toxicity and the financial burden of chemotherapy that will not work. The availability and application of various treatment modalities in colon cancer has resulted in intense interest in the elucidation of prognostic and predictive biomarkers that will improve outcome through patient classification and selection for specific therapies. A prognostic biomarker provides information about the patient's overall outcome, regardless of therapy, whereas a predictive biomarker gives information about the effect of a particular therapeutic intervention. Currently, the tumor–node–metastasis stage is the only proven prognostic marker to aid in the identification of patients with aggressive disease [3, 9]. Thus, there is an urgent need for prognostic and predictive biomarkers to guide adjuvant therapy for colon cancer and a need for large cohorts of randomized patients in which to test and validate biomarkers in this setting.

A secondary objective of the PETACC-3 trial was to undertake a translational research study to assess a panel of putative prognostic and predictive markers in a large colon cancer patient cohort [10]. The preliminary findings of that study and the status of other large randomized adjuvant trials, including the CALGB 89803 trial, were reviewed and placed in the context of biomarker discovery for adjuvant treatment of colon cancer at a meeting of a panel of experts held in Boston (U.S.) in May 2008 sponsored by Pfizer. In this article, we review the current status of biomarkers for the adjuvant treatment of colon cancer and consider future strategies for biomarker discovery and development in the postgenomic era.

BIOMARKERS IN COLON CANCER: PRESENT STATUS

Molecular Genetics of Colon Cancer

Our knowledge of the molecular etiology of colon cancer has facilitated the identification of a number of promising prognostic and/or predictive biomarkers. A simplified model of tumor progression from adenoma to carcinoma has been proposed, which includes the stepwise accumulation of genetic events to several key genes and genetic loci: disruption to WNT signaling, activation of the *KRAS* proto-oncogene, allelic imbalance (AI) on chromosome 18q, reduced expression of *SMAD4*, and mutation of the *TP53*

Table 1. Genes commonly involved in sporadic CRC

Gene	Protein function	Defect in CRC	Frequency (%)
APC	Negative regulator of WNT signaling involved in controlling cell proliferation in the colon and small intestine	Inactivation by mutations leading to loss of function of APC protein (protein truncation) and constitutive activation of WNT signaling	85
MLH1	DNA single nucleotide mismatch repair	Epigenetic silencing leading to loss of protein expression and the accumulation of cellular mutations	15–25
TP53	Transcription factor regulating downstream target genes involved in cell cycle regulation	Inactivating (nonsense and misense) mutations leading to loss of function of wild-type protein	35–55
SMAD4	Component of the TGF-β signaling pathway	Target of AI on chromosome 18q, gene inactivation by homozygous deletion/mutation	10–35
KRAS	GDP/GTP binding protein facilitating ligand dependent TK growth factor signaling	Activation (most commonly through codon 12/13 misense mutations) leading to activation of the RAF–MEK–ERK pathway	35–45
BRAF	Serine–threonine protein kinase that acts as a downstream effector of KRAS-mediated signaling	Activation most commonly through a valine-to-glutamic acid amino acid (V600E) substitution	8–12

Abbreviations: AI, allelic imbalance; CRC, colorectal cancer; ERK, extracellular signal–related kinase; MEK, mitogen-activated protein kinase/extracellular signal–related kinase kinase; TGF-β, transforming growth factor β; TK, tyrosine kinase.

tumor suppressor gene [11–15]. A summary of the genes involved in sporadic colon cancer development is shown in Table 1. A more detailed molecular analysis of colon cancers revealed colon tumors to be heterogeneous with regard to molecular alterations and potentially categorizable into specific tumor phenotypes based on their molecular profiles. Two of these represent genetic instability classes. The majority of sporadic cases (up to 85%) display chromosomal instability (CIN), which manifests as aneuploid and polyploid karyotypes and multiple structural chromosomal changes [12, 13, 16, 17]. This phenotype is thought to arise through defects in a number of processes, including aberrant expression or mutation of mitotic checkpoint genes, microtubule spindle defects, and telomere dysfunction [18].

In contrast, the remaining 15% of sporadic colon cancers demonstrate a microsatellite instability (MSI) phenotype, in which tumors display insertion–deletion mutations, most commonly in short tandemly repeated nucleotides (microsatellites) [19, 20]. Chromosome losses are rarer in these tumors, which tend to have a diploid karyotype [17, 21]. The underlying genetic mechanism responsible for this phenotype is loss of function, predominantly through gene silencing of DNA mismatch repair (MMR) genes, in particular, *MLH1* in sporadic CRC [22, 23]. Consequently, this phenotype is also often referred to as the MMR deficient (dMMR) phenotype, and in 2%–3% of CRC is caused by germline mutations to one of a number of MMR genes

(*MLH1*, *MSH2*, *MSH6*, and *PMS2*) that form part of the presentation of Lynch syndrome, or hereditary nonpolyposis CRC [24]. Whereas patients with an MSI/dMMR tumor phenotype have a relatively stable karyotype, a deficient repair process in tumors leads to loss of function mutations in tumor suppressor genes, including *TGFBR2*, *IGF2R*, and *PTEN*, and is associated with gain-of-function mutations in oncogenes such as *BRAF* [25–27]; this phenomenon, in turn, is often referred to as a "mutator" phenotype.

Finally, the analysis of CpG island methylation in the silencing of genes in colon tumors has led to the identification of the CpG island methylator phenotype, which appears to partially overlap the MSI phenotype [28, 29].

A summary of the clinical utility of a number of promising candidate markers in the adjuvant setting is presented in Table 2 and reviewed below.

Genomic Instability Phenotypes as Biomarkers

MSI

MSI can be detected in tumors by a number of complementary approaches. Using the polymerase chain reaction (PCR) to amplify specific microsatellite repeats, the presence of instability can be monitored through a comparison of the length of repeats obtained from normal DNA (typically extracted from adjacent normal mucosa cells) with those from the DNA extracted from the tumor cells. A ref-

Table 2. Summary of studies investigating candidate genes and phenotypes as independent prognostic and predictive biomarkers in adjuvantly treated colon cancer patients

Candidate biomarker	Prognostic utility[a]		Predictive utility[b]		General comments
	In favor	**Against**	**In favor**	**Against**	**General comments**
Tumor MSI-H phenotype (or dMMR)	MSI-H is associated with longer survival [32–34, 36, 40, 42, 107]	No evidence that MSI-H is associated with longer survival [152, 153]	MSI-H is associated with longer survival in patients receiving adjuvant 5-FU–based therapy than those receiving surgery alone [38, 61] MSI-H is predictive of response to irinotecan plus 5-FU and LV in stage III colon cancer patients [41]	No evidence that MSI-H is associated with longer survival in patients receiving adjuvant therapy [33, 36, 39, 40, 42, 152]	MSI-H phenotype is largely associated with good prognosis There is a call for patients with stage II colon cancers with MSI-H tumors to not receive adjuvant chemotherapy [39, 43] The predicative value of the MSI-H phenotype is being tested in the ongoing E5202 adjuvant colon cancer trial
Tumor 18q AI	18q AI is associated with shorter survival [45–48, 50, 51, 154]	No evidence that 18q AI is associated with shorter survival [34, 49]	NR	NR	The prognostic and predictive value of 18q AI is being examined in the ongoing E5202 adjuvant colon cancer trial
Tumor p53 expression/ mutation	*p53* mutation/overexpression is associated with poor patient prognosis—lower DFS [62], RFS [66], and OS [67] rates	No evidence that p53 status provides prognostic value [63, 68]	*p53* mutation and overexpression is associated with shorter survival in patients receiving 5-FU–based adjuvant therapy [61, 67, 69]	No evidence that p53 status is predictive in treated or untreated patients [66, 70]	Different methodologies have been used to assess p53 status, making comparison between studies difficult
Tumor *KRAS* mutation	*KRAS* mutation is associated with shorter survival [69, 74, 75]	No evidence that *KRAS* mutation has prognostic value [62, 77, 78, 80–84]	Wild-type *KRAS* patients benefit from adjuvant therapy [69]	CALGB 89803 study suggests that *KRAS* gene mutations are not predictive of outcome for patients receiving adjuvant irinotecan plus 5-FU and LV.	Tumor *KRAS* mutation status is not a prognostic factor in large adjuvant trials and in mCRC patients receiving BSC [79] *KRAS* mutation status is predictive of outcome with EGFR-targeted agents in mCRC patients Adjuvant trials (PETACC-8) are under way to investigate tumor *KRAS* status in patient response to EGFR-targeted agents
Tumor TYMS expression	High TYMS expression is associated with shorter survival (mainly in patients receiving 5-FU–based adjuvant therapy) [65, 66, 101–104] High TYMS expression is associated with longer survival in patients receiving adjuvant therapy [101, 102, 106] Low TYMS expression is associated with shorter survival in patients receiving surgery alone [105]	No evidence that TYMS expression has prognostic value [68, 107, 108]	High TYMS expression is associated with longer survival in patients receiving 5-FU–based adjuvant therapy [101, 102]	No evidence that High TYMS expression is predictive of response to adjuvant therapy [66, 108]	TYMS expression has been determined by a number of different technologies— RT-PCR, IHC (different scoring systems used)—the clinical value of TYMS expression remains to be determined
TYMS genotypes	High *TYMS* expression genotypes and haplotypes have been associated with tumor recurrence in patients with stage II and stage III colon cancer [113] High *TYMS* expression genotypes have been associated with longer DFS and OS in patients receiving adjuvant therapy [115]	NR	Low *TYMS* expression genotypes in patients treated with 5-FU–based adjuvant therapy have been associated with longer survival [114]	NR	The relationship between germline variation and *TYMS* gene function remains to be elucidated and the clinical value needs to be further determined

Only studies from published (peer-reviewed) reports in which ≥100 patients were studied and in which biomarkers were shown to be independently associated with clinical outcome are shown.

[a]Prognostic utility was assessed in relation to reported data from meta-analyses or analyses (retrospective and prospective) of patient clinical samples from single-arm studies, large population- based studies, or large collaborative group studies.

[b]Predictive utility was assessed in relation to reported data from studies in which patients receiving adjuvant 5-FU– based chemotherapy and nontreated patients were described and compared including: single randomized trials, large intergroup studies, and meta-analyses.

Abbreviations: 5-FU, 5-fluorouracil; AI, allelic imbalance; BSC, best supportive care; DFS, disease-free survival; dMMR, deficient mismatch repair; EGFR, epidermal growth factor receptor; IHC, immunohistochemistry; LV, leucovorin; mCRC, metastatic colorectal cancer; MSI-H, microsatellite instability high; NR, no published reports; OS, overall survival; PETACC, Pan European Trials in Adjuvant Colon Cancer; RFS, relapse-free survival; RT-PCR, reverse transcription-polymerase chain reaction.

erence panel of 5–10 microsatellite loci is used to diagnose MSI cases [30], for which three categories have been established: MSI-High (MSI-H), unstable for 30% of markers used; MSI-Low (MSI-L), unstable for 10%–30% of markers used; and microsatellite stable (MSS), for cases that display no MSI. Lack of expression of MMR proteins as assessed by immunohistochemistry (IHC) (primarily using antibodies to the MLH1 protein) is diagnostic for dMMR and is often used in MSI tumor analysis as an alternative to PCR, and additionally in the clinical setting to complement genetic testing for Lynch syndrome patients [24].

In clinical studies, MSI rates have been shown to vary with tumor stage—22% reported in stage II, 12% reported in stage III, and 2% reported in stage IV disease [31]. In the adjuvant setting, MSI tumor status has been shown to be a significant prognostic marker. The majority of retrospective studies (Table 2) demonstrate that patients with MSI-H (or dMMR) colon cancers have higher survival rates than those with MSS tumors [32–34]. These findings were confirmed in a meta-analysis of 32 trials, which confirmed the prognostic advantage in patients with MSI-H tumors and those treated with 5-FU–based adjuvant therapy [35]. In the PETACC-3 study, the prognostic value of MSI status was found to be more significant in patients with stage II disease than in stage III cases [36]. In addition, in a multivariate analysis of stage II colon cancer patients from the QUick and Simple And Reliable (QUASAR) study, Kerr and colleagues demonstrated that MMR deficiency (hazard ratio [HR], 0.31; 95% confidence interval [CI], 0.15–0.63; $p <$ .001) and T4 stage (HR, 1.94; 95% CI, 1.35–2.79; $p =$ .005) (together accounting for 25% of patients) were independent prognostic factors for tumor recurrence [37]. Similar findings were reported in a multivariate analysis of the PETACC-3 data [36].

The value of MSI tumor status as a predictive marker of adjuvant therapy is less clear. An early study suggested that MSI-H was predictive of response to 5-FU–based adjuvant therapy in patients with stage III colon cancer [38]. However, an accumulating body of evidence suggests that patients with MSI-H tumors do not benefit from 5-FU–based adjuvant therapy, compared with patients with MSS tumors [33, 35, 39, 40]. This is particularly relevant for patients with stage II disease, for whom adjuvant chemotherapy (5-FU alone) is reported to increase survival by approximately 3%, and has led some investigators to recommend that stage II colon tumors should be analyzed for dMMR status to guide decisions on the use of adjuvant therapy [39].

Recently the CALGB 89803 study reported a higher 5-year DFS rate in stage III colon cancer patients with MMR-deficient/MSI-H tumors treated with irinotecan plus

5-FU than in patients treated with the same regimen with intact MMR proteins: this was not observed in patients treated with 5-FU and leucovorin (LV) alone, suggesting that tumor MSI status might be predictive of response to irinotecan in stage III colon cancer [41]. In contrast, the PETACC-3 study, in 1,327 patients, failed to demonstrate a predictive effect of tumor MSI status for patients treated with irinotecan, 5-FU, and LV, compared with those receiving 5-FU alone [36, 42].

To date, MSI is considered to be a strong and well-validated prognostic marker in adjuvant CRC, and it is currently the only such biomarker in this setting. In the appropriate clinical setting, we would advocate that MSI data may be used in clinical decision making, particularly in stage II colon cancers, for which a favorable outcome of patients with MSI-H tumors suggests that these patients should not receive adjuvant chemotherapy [43]. The assessment of MSI tumor status as a predictive marker for adjuvant therapy requires more data. It should also be considered, however, that the value of MSI tumor status as a prognostic or predictive marker in the adjuvant setting may be effected by mutations to other genes involved in colon cancer etiology, such the *BRAF* gene (discussed below) [44].

Chromosome 18q AI/CIN

Chromosome 18q AI has been associated with poor prognosis in stage II and stage III CRC patients in some studies [45–48], but not others [34, 49] (Table 2). Watanabe and colleagues reported that patients with stage III MSS colon tumors with no 18q AI had a higher survival rate following 5-FU–based treatment (70% versus 50%) than those whose tumors displayed 18q AI [50]. In the CALGB 89803 study, stage III colon cancer patients with 18q AI had lower 5-year DFS (0.78 versus 0.93) and OS (0.85 versus 0.98) rates than patients whose tumors displayed no 18q AI [51]. However, drawing conclusions from comparing chromosome 18q AI studies in colon cancer is difficult, and differences in the methodologies used, including the scoring of AI, possibly explains the contradictory findings reported. Thus, the inconsistency of the genetic markers used among studies leads to analysis of AI in different regions on chromosome 18q.

An additional complication comes from the stage-specific effects of biomarkers. The PETACC group presented, at the 2009 American Society of Clinical Oncology Annual Meeting [36], that tumor 18q AI status was not found to be prognostic in stage II tumors, whereas an effect was found in stage III tumors on univariate analysis. This is important because the patient population most in need of prognostic markers is stage II patients, for whom treatment versus no treatment is based on the inherent prognostic features. Cur-

rently, in the E5202 clinical trial (discussed below) [52], 18q AI status is being used to differentiate between low- and high-risk stage II tumors in an extrapolation of the stage III data, which in reality may not be biologically correct. In addition, when the PETACC group evaluated the effect of 18q loss of heterozygosity (LOH) in univariate, compared with multivariate, models (containing MSI and tumor node status), it was found that 18q LOH status lost significance if MSI was included in the model [36], suggesting that these markers do not act independently and correct prognostication will have to take into account several markers.

A further problem in assessing tumor 18q AI status is determining what is actually being measured by 18q AI, which is currently generally unclear. Unless carefully analyzed, AI can be scored as the consequence of a number of different genetic events arising from different molecular causes, with possibly different functional and biological consequences. Thus, AI may be generated by loss or gain of chromosomal material. Where loss is the proven mechanism of the AI, the assumption commonly made is that the clinical significance is a result of the loss of function of specific genes within the chromosomal region (*SMAD7*, *SMAD4*, *DCC*, and *SMAD2*). If this is indeed the case, then 18q AI association studies should incorporate data derived from quantitative assays measuring target gene or protein expression, as has been reported in metastatic CRC (mCRC) for *SMAD4* [53]. In stage III colon cancer, lower expression of *SMAD4* was reported to be associated with poor prognosis in patients treated with 5-FU–based chemotherapy [54–56].

However, it is also possible that chromosome 18q AI may simply be a surrogate marker for the complex CIN phenotype found in the majority of colon tumors [57, 58]. Thus, AI assays restricted to chromosome 18q are not able to discriminate between 18q-related gene-inactivation events and more general aneuploidy (a characteristic of CIN, which may also nevertheless lead to the inactivation or diminution of 18q gene function). This has implications in relation to our understanding of the contribution of chromosome 18q imbalance to colon tumor biology and response to therapy, and its role as a biomarker. Thus, tumor phenotypes might be masked or conflated using one technology to assess imbalance at 18q, which may in turn explain the contradictory findings on 18q AI and prognosis in colon cancer in the literature [34, 45, 46, 49].

Examining CIN as a prognostic marker in colon cancer has proven difficult, first because the phenotype is poorly defined and second because a number of different technologies, including AI, flow cytometry, and array-based comparative genomic hybridization (a-CGH), have been used to measure CIN. A recent meta analysis of 63 studies (10,126

CRC patients of all stages) found CIN to be associated with a worse prognosis in CRC, including patients with locally advanced disease [59]. In that analysis, CIN was assessed in studies using techniques to measure chromosome ploidy (flow cytometry and image analysis), and hence the data include chromosome 18 numerical alterations. Further, the predictive value for patients receiving 5-FU–based chemotherapy could not be determined. The authors called for CIN to be evaluated as a prognostic marker together with MSI status in clinical trials of colon cancer patients involving adjuvant therapy.

Candidate Genes as Biomarkers
A number of important colon cancer genes have been identified and extensively studied as candidate biomarkers in colon cancer in the adjuvant setting and are reviewed below.

TP53
The *TP53* gene encodes a transcription factor, and in response to a variety of cellular stresses, including DNA damage, activated TP53 protein binds to the regulatory sequences of a number of target genes to initiate a program of cell cycle arrest, DNA repair, apoptosis, and angiogenesis [60]. Loss of function of TP53 is critical in tumorigenesis, and alterations to the *TP53* gene (mutations, often resulting in protein overexpression) are frequent events in colon cancer, often associated with the CIN phenotype and inversely correlated with the MSI tumor phenotype [61, 62]. Associations of *TP53* tumor alterations with patient prognosis and response to adjuvant chemotherapy have been widely studied, and findings are contradictory (Table 2) [63, 64]. For example, TP53 protein expression and gene mutation have been associated with poor prognosis in colon cancer patients, although other studies report no prognostic value [63, 68]. In clinical studies in which adjuvant chemotherapy–treated and nontreated groups could be analyzed, stage III CRC patients whose tumors demonstrated no *TP53* alterations experienced significantly longer survival following 5-FU–based chemotherapy than patients whose tumors overexpressed p53 [61, 67, 69]. However, other studies in colon cancer patients failed to demonstrate correlations between *TP53* alterations and benefit from adjuvant therapy [66, 70]. The contradictory nature of these studies may reflect differences in the methodologies used to assess TP53 status, including different antibodies used to detect the protein (with varying sensitivities for wild-type or mutant protein), different immunostaining techniques, and different scoring systems used for assessing expression. Indeed, the reported value for TP53 overexpression in the literature covers a wide range (27%–76%), which may

reflect these issues. It is generally accepted that the detection of p53 protein by IHC is a poor indicator of *TP53* gene mutation status, because alternative molecular mechanisms can lead to protein stabilization in tumors, and some mutations lead to loss of protein stability [71]. Studies in which *TP53* mutations were detected by gene sequencing report associations with poor prognosis in colon cancer patients [62, 67, 72]. It has been suggested that, to analyze the gene properly in clinical studies, *TP53* mutation status should be assessed by DNA sequencing and data must be combined with TP53 protein expression information as determined by IHC.

KRAS

The *KRAS* proto-oncogene encodes a 21-kDa guanosine triphosphate/guanosine diphosphate binding protein involved in facilitating cellular response to extracellular stimuli. Mutations within the *KRAS* gene (primarily at codons 12 and 13) abrogating GTPase activity and leading to downstream activation of RAS/RAF signaling are common (35%–42%) and early events in colon tumorigenesis [73].

However, the role of *KRAS* mutation status as a prognostic and predictive biomarker in the adjuvant setting is controversial (Table 1). In a large meta-analysis, codon 12 glycine-to-valine mutations were found to be prognostic in patients with stage III disease [74, 75]. Smaller studies have shown *KRAS* mutation status to be associated with poor prognosis in patients with stage II [76] and stage III disease [69, 74, 75]. However, recent analyses from the CALGB 89803 (stage III colon cancer) and PETACC-3 (stage II and III) trials demonstrated *KRAS* mutation to not be a prognostic marker for patients treated with adjuvant 5-FU–based chemotherapy [77, 78]. In addition, the National Cancer Institute of Canada CO.17 trial recently demonstrated that tumor *KRAS* mutation status had no prognostic effect for OS in pretreated stage IV patients receiving best supportive care [79].

As a predictive marker in the adjuvant setting, most studies report no association between *KRAS* mutations and response to standard chemotherapy [62, 80–83]. In a Southwest Oncology Group trial, patients with stage III tumors with *KRAS* mutations gained no additional benefit from receiving 5-FU/LV compared with observation or LV alone. In contrast, patients with *KRAS* wild-type tumors significantly benefited from 5-FU/LV therapy [69]. Data from the CALGB 89803 study suggest that *KRAS* tumor mutation status is not prognostic or predictive for treatment with irinotecan plus 5-FU and LV in stage III colon tumors [84].

In contrast, because of the central role of KRAS downstream in the EGFR signaling pathway, there is currently intense interest in *KRAS* mutation status as a predictive bio-marker in patients with advanced CRC treated with therapies targeted to EGFR. *KRAS* gene mutations activate the EGFR signaling pathway independently of ligand stimulation of the receptor, and thus bypass the efficacy of EGFR-targeting drugs. Single-arm studies [85–87] and large randomized studies in first-line [88, 89] and in previously treated [79, 90] mCRC patients have demonstrated *KRAS* tumor mutations to be predictive of a lack of response to the EGFR-targeted antibodies cetuximab and panitumumab. It is now common practice to reserve treatment with EGFR-targeting agents to wild-type *KRAS* CRC patients.

BRAF

The *BRAF* gene encodes a serine–threonine protein kinase that acts as a downstream effector of KRAS signaling and belongs to the RAS–RAF–mitogen-activated protein kinase/extracellular signal–related kinase kinase (MEK)–extracellular signal–related kinase (ERK) kinase pathway [91]. *BRAF* gene mutations are important in colorectal tumorigenesis [91, 92]. The most frequently reported *BRAF* tumor mutation is a valine-to-glutamic acid amino acid (V600E) substitution that leads to the aberrant activation of the MEK–ERK pathway [93]. *BRAF* and *KRAS* mutations tend to be mutually exclusive events in tumors [94], with *BRAF* mutations occurring more frequently in MSI than in MSS tumors [44, 95].

In patients with stage IV CRC, *BRAF* mutations have been reported to be associated with poor prognosis [96], and in chemotherapy-refractory mCRC patients *BRAF* mutations have been reported to be predictive of a lack of response to EGFR-targeted agents [96]. In stage II and stage III colon cancer patients in the PETACC-3 study, *BRAF* mutations occurred in 7.9% of tumors and were found to not be prognostic of relapse-free survival, but they were prognostic for OS, particularly in patients with MSI-L and MSS tumors (HR, 2.2; $p = .0003$) [77]. Other retrospective studies have also demonstrated an association between *BRAF* mutation and poor prognosis in stage II–III [44] and stage I–IV [97] CRC patients. Interestingly, in those studies the good prognosis associated with patients with MSI-H tumors was abrogated in the presence of coincident *BRAF* mutations [44, 97].

In the adjuvant setting, *BRAF* mutation status appears to be a valid prognostic marker; however, associations of *BRAF* tumor mutations with different molecular subgroups may have to be considered in order to assess the impact of *BRAF* mutation status as a predictive marker for treatment in future studies in this setting.

TYMS

The thymidylate synthase gene *TYMS* encodes a key enzyme for pyrimidine biosynthesis and is an essential component of the DNA synthesis pathway. TYMS protein activity is inhibited by 5-FU (a pyrimidine analog), leading to cell cycle arrest and apoptosis [98]. In vitro data indicate that TYMS expression is a determinant of 5-FU sensitivity, suggesting that the expression of the gene may also determine tumor sensitivity in vivo [99, 100]. However, conflicting data make the role of this gene as a prognostic or predictive marker in the adjuvant setting controversial (Table 2). High levels of tumor TYMS protein are reported to be associated with poor prognosis in CRC patients, particularly in those receiving surgery alone, although the reasons for this remain unclear [65, 66, 101–104]. Study of the expression of other enzymes in the pyrimidine biosynthesis pathway—dihydropyrimidine dehydrogenase (DPD) and thymidine phosphorylase—has shown low tumor expression of TYMS and DPD to be associated with worse prognosis in stage II and stage III CRC patients treated with surgery alone [105]. Patients receiving adjuvant 5-FU–based chemotherapy with high levels of tumor TYMS expression were reported to experience significantly longer survival times [101, 102, 106], with TYMS expression reported to be predictive of response to adjuvant chemotherapy [101, 102]. However, other studies found no prognostic [68, 107, 108] or predictive [66, 108] value of response to adjuvant chemotherapy for TYMS expression in colon cancer.

Some studies have investigated TYMS mRNA levels in tumors, and high levels of tumor TYMS mRNA and failure to respond following 5-FU–based chemotherapy have been reported [109, 110]. Germline variants in the *TYMS* gene have been shown to alter TYMS protein and gene expression [111, 112], and have been associated with response, time to tumor progression, OS, and time to tumor recurrence after 5-FU–based chemotherapy, although the data are conflicting [113–115]. The clinical significance and relationships between mRNA and protein levels in tumors and between germline variation and *TYMS* gene function remain to be elucidated in colon cancer.

The Use of Randomized Clinical Trials for Biomarker Validation in Adjuvant Colon Cancer: The PETACC-3 Study

The PETACC-3 trial encompassed a translational study to validate current candidate biomarkers in a large colon cancer cohort of 3,278 patients. The main aims of the translational study were: (a) to assess the feasibility of biomarker analysis on archival formalin-fixed, paraffin-embedded (FFPE) material collected prospectively from 368 collaborating centers in 31 European countries, (b) to evaluate or confirm the prognostic relevance of selected biological markers using 3-year DFS and OS endpoints, and (c) to assess the predictive utility of specific markers in patients receiving irinotecan in combination with 5-FU and LV, compared with those receiving 5-FU and LV alone [10, 36, 42].

FFPE tissue blocks were available from 1,564 patients and were processed in a central laboratory, where 20–25 sections were cut per patient tissue block for subsequent analysis. Biomarkers were assessed using validated and robust methodologies [10]. All data were collected and analyzed at the Swiss Group for Clinical Cancer Research. Biomarker data were available from 1,452 cases, with 1,401 evaluable for matched normal and tumor tissue. The success rate for the number of samples evaluable for specific markers was high: >80% for IHC analysis and >95% for DNA mutation analysis using techniques optimized for use on degraded DNA extracted from FFPE tissues. The frequency of specific biomarker alterations in the PETACC-3 study was consistent with that found in the literature [10], with sufficient statistical power to detect an HR of 0.7 for DFS if the proportion of single-marker detection is 80% [10, 116]. Thus, a reassessment of the significance of *TP53* mutation and IHC, *KRAS* mutation, *TYMS* genotype and IHC, and MSI in this cohort is ongoing [36, 42, 77], on which many of the same biomarkers are being tested [8, 41, 51, 78]. Clearly, these two studies provide useful independent test and validation cohorts of patients in which to investigate candidate biomarker utility.

The Current Status of Biomarkers in Adjuvant Colon Cancer: A Summary

Extensive colon cancer research over the last decade has provided some promising biomarkers. In some cases, we are close to using these in meaningful prospective clinical studies. For example, the E5202 study is currently determining the role of MSI and 18q AI as predictive factors to guide decision making for stage II colon cancer patients. In that trial, the risk for relapse after adjuvant treatment for stage II CRC is being assessed based on initial stratification by MSI status and 18q AI; low-risk patients are subject to observation whereas high-risk cases receive FOLFOX and bevacizumab [52]. Other candidate biomarkers are a long way from having their utility in the clinical setting confirmed, with many of the studies providing evidence of their suitability being limited by the following common features:

1. Many studies are retrospective analyses of single-arm investigations performed in small and often heterogeneous cohorts of patients in which rectal tumors have

been examined together with colon tumors, and patients have not been stratified by stage, gender, or age. Thus, many have been statistically underpowered to provide meaningful results. Using large cohorts of patients, such as those in the PETACC-3 and CALGB 89803 trials, may address many of these issues and provide an accurate assessment of the prognostic and predictive (of response to irinotecan-based treatment) capabilities of the promising biomarkers described.

2. In many studies, a lack of standardization of methodologies for marker measurement has resulted in data that are not comparable. It is hoped in the approaches used in the PETACC-3 [116] and CALGB 89803 studies to centralize and standardize sample handling and methodologies that these important issues will be addressed.

3. Often, the methodology chosen in studies does not represent a comprehensive analysis of multiple components of a specific biological pathway, each of which may incur defects in multihit tumorigenesis. Significant associations between molecular lesions in a pathway and clinical parameters may therefore be missed. A prime example of this is the analysis of chromosome 18q in colon cancer patients undergoing adjuvant treatment. It is recommended that future studies employ methods that can discriminate among different molecular pathologies, such as combinations of a-CGH, single nucleotide polymorphism (SNP) arrays, and other methods that detect DNA ploidy. The concurrent consideration of gene or protein expression data for candidate genes on 18q might also be necessary to demonstrate meaningful associations.

4. Often, not all mutations within a given gene are screened. An example is the *KRAS* gene, for which the frequently occurring mutations at codons 12 or 13 are measured but other, less common, mutations at codons 61 and 146 are not assessed. This can also be a source of potential bias in an investigation. Furthermore, other candidate genes in related signaling pathways will need to be examined to provide a pathway-centric approach to make sense of some of the observations made with single candidate genes. For example, *KRAS* mutation status should be judged in conjunction with the PI3K–AKT axis because there is extensive crosstalk between these pathways [117].

Studies to date have demonstrated the urgent need for biomarker development and have highlighted the methodological challenges of this research. Attempts have been made to provide guidelines for the validation and optimization of biomarkers for use in the clinical setting, prime examples being: the tumor marker utility grading system (TMUGS) [118]; reporting recommendations for tumor marker prognostic studies (REMARK) [119]; guidelines for gene expression localization experiments; and the minimum information specification for in situ hybridization and immunohistochemistry experiments (MISFISHIE) [120].

FUTURE CONSIDERATIONS FOR BIOMARKER INVESTIGATIONS IN THE ADJUVANT SETTING IN THE POSTGENOMIC ERA

In this postgenomic era, technological developments have occurred in which the whole genome can be rapidly and cost-effectively investigated with high-throughput approaches. We now review how a combination of functional genomics and molecular profiling in conjunction with carefully designed clinical trials can be applied to identifying biomarkers and provide a vision for the future for adjuvant colon cancer.

Postgenomic Technologies

A number of technology platforms have been developed to detect genomewide alterations in tumors. These tools also have the potential to provide predictive profiles for patient prognosis and response to chemotherapy and are being applied to CRC in general. Gene expression microarrays allow the analysis of global gene-expression patterns in mRNA extracted from tissue samples. Changes in tumor DNA copy number have been traditionally characterized using a-CGH, based on bacterial artificial chromosome construct probes [58, 121]. Although copy number–dependent AI and copy number–neutral AI have been assessed by SNP microarrays [57], often the two technologies are combined to allow a more precise definition of the molecular basis of AI events occurring in tumors [57]. It has also been reported that SNP-bead arrays can discriminate between both copy number–dependent and copy number–neutral AI events [122, 123]. Furthermore, with the development of high-throughput gene sequencing and mutation detection capabilities, a detailed picture of the mutation spectrum of many genes in individual tumors can be realistically achieved [124]. Recently, many of these technology platforms and methods were adapted for use with DNA or RNA extracted from FFPE tissues [123, 125–127]. Following the identification of candidate genes of interest through expression profiling, the feasibility of developing quantitative reverse transcription PCR assays for use on FFPE material from colon cancer patients in a clinical setting was demonstrated [128]. These developments are important for biomarker validation in large retrospective analyses, in which FFPE material is often the only tissue available for study.

Integrating Genomics Into Biomarker Identification in Adjuvant Colon Cancer

Hypothesis-Driven Candidate Gene Approaches

Whereas postgenomic technologies offer powerful tools for biomarker discovery and validation, significant challenges must be met in order for these methodologies to produce changes in clinical practice. Hypothesis-driven approaches seek to correlate molecular alterations of functionally relevant genes (cell cycle, apoptosis, drug metabolism) with patient groups classified by clinical parameters (e.g., responders to chemotherapy versus nonresponders). In the PETACC-3 study, such an approach might be to identify genes or molecular profiles that are thought to be associated with response to irinotecan. Thus, in mCRC, the UK MRC FOCUS trial of chemotherapy for bowel cancer [Fluorouracil, Oxaliplatin, and Irinotecan (CPT11) Use and Sequencing] demonstrated that patients whose tumors express high levels of topoisomerase 1 protein (a target for irinotecan) gain significantly more clinical benefit from receiving irinotecan with 5-FU and LV first line, compared with patients whose tumors are low or negative for topoisomerase 1 [129]. Yu and colleagues examined the expression of 24 genes involved in the irinotecan pathway in matched normal and tumor tissues from 52 patients with Dukes' C CRC [130]. They found that patients could be classified into three groups based on statistically significant differences in the levels of gene expression, and concluded that expression profiling of the irinotecan pathway genes may be valuable for predicting response to irinotecan-based therapy in colon cancer patients [130].

In similar approaches, cell lines have been used as in vitro models for drug sensitivity, with candidate genes identified by microarray analysis (differentially expressed transcripts) and gene knockdown technologies. This approach has led to the identification of potential predictors of taxane response in breast cancer [131, 132], and to the establishment of the CINATRA trial (Chromosomal Instability and Anti-Tubulin Response Assessment, EudraCT no 2006–006073-240) to identify predictors of response to the microtubule-stabilizing agent epothilone 906 in mCRC patients [133]. Similar strategies have described integrated genomic-based approaches to identify oncogenic pathways to predict sensitivity of cancer patients to specific chemotherapy regimens [134–137].

Genomic Profiling

There is clear heterogeneity in the biology of CRC tumors classified by clinical parameters; thus, in stage II disease some patients appear to have a higher risk for tumor recurrence following surgery. This suggests heterogeneity among individual patients in the molecular pathways disrupted in tumors, and data from early rectal cancer trials support this idea [122]. This would explain why, after 20 years of research, there remains a paucity of molecular biomarkers in the clinical setting. A strategy to identify more useful biomarkers might therefore be to reclassify patients into subgroups based on the similarities of their tumor molecular profiles using microarray or other genomic-profiling technologies.

Using this approach, investigators have interrogated microarray data using unsupervised hierarchical clustering analysis, in which patients are grouped according to the similarity of their gene-expression profiles. This was pioneered in early breast cancer, for which the intrinsic subtype gene-expression model classifies breast tumors into subgroups with different clinical outcomes [138, 139]. Subsequently, several validated tumor genomic profiles associated with relapse in early breast cancer patients were reported [139–145]. In addition, five gene expression–based models were compared in a single data set of 295 patients, in which the 70-gene set and recurrence score (RS) models demonstrated 77%–81% agreement in their outcome predictions for individual samples, suggesting that they may be tracking a common set of biological phenotypes [146]. However, that study was limited because the data set used for comparison was also used to develop one of the expression signatures, and secondly, it was underpowered for comparing similar signatures. More recently, a large meta-analysis of publically available breast cancer gene expression and clinical data, comprising 2,833 breast tumors, demonstrated how prognostic signatures can be successfully computed on different microarray platforms using a simple approach [147], and confirmed the prognostic values of these signatures, revealing that many of them are broadly equivalent because of the inclusion of genes associated with cellular proliferation [148]. Finally, for the best prognostic value, it was shown that gene-expression values should not be used in isolation but combined with clinical variables that measure the extent of tumor progression, such as tumor size and nodal status [147]. We anticipate that this experience will be of great value when crosstrial comparisons of genomic and expression data are performed in CRC patients.

In colon cancer studies in the adjuvant setting, genomic profiling identified a 23-gene signature reported to predict recurrence in colon cancer patients with Dukes' B disease, yielding a 78% prognosis prediction accuracy [149]. This was validated in an independent study that yielded a 67.7% mean prognosis profile [150] and identified a 30-gene expression profile that produced highly variable prediction accuracy across training and validation sets. The authors

concluded that microarray expression profiling is able to predict, to some extent, prognosis in stage B colon cancer patients and that resampling techniques should be used to objectively assess the performance of microarray-based prognosis predictors [150].

More recently, a multivariate analysis (including stage, grade, nodes, and MSI status) of four developmental studies in colon cancer patients identified 18 genes (seven prognostic genes and six genes predictive for 5-FU and LV benefit, and five reference genes) and separate prognostic RS and treatment predictive score (TS) algorithms [37]. In a validation analysis on material from the QUASAR study, the RS was validated as an independent predictor of individualized recurrence risk for stage II colon cancer patients, although the TS was not validated as a predictor of benefit from 5-FU and LV therapy [37].

Although studies in colon cancer are promising, caution is required because investigators have yet to meet the high standards for study reproducibility and generalizability required for the use of genomic profiling in the classification of cancer patients for clinical purposes (reviewed by Michiels et al. [151]). Robust prediction rules must be developed in order to correlate gene-expression profiles with clinical outcome. A major question to be addressed with this approach is whether or not gene-expression profiles improve on existing prognostic systems. In breast cancer, clinical trials have been established to determine the prognostic and predictive values of gene-expression profiles. In the TAILORx study [Trial Assigning IndividuaLized Options for Treatment (Rx)], the National Cancer Institute is investigating the breast cancer RS model in 10,000 women recruited across the U.S. and Canada with estrogen receptor– and/or progesterone receptor–positive, human epidermal growth factor receptor 2/neu-negative breast cancer that has not yet spread to the lymph nodes. The European Organization for Research and Treatment of Cancer has designed a trial (Microarray In Node negative Disease May Avoid ChemoTherapy, MINDACT) to study the 70-gene signature in 6,000 node-negative breast cancer patients recruited across Europe. The design of similar trials will be the gold standard for the investigation of the prognostic and predictive value of genomic profiles in adjuvant colon cancer.

CONCLUSIONS

Despite significant methodological progress, CRC research has not yet provided biomarkers for clinical use in guiding adjuvant colon cancer treatment, although MSI is promising. Preliminary biomarker data from the PETACC-3 and CALGB 89803 trials suggest that it is possible to perform translational studies on FFPE material derived from large multicenter clinical trials carried out in the adjuvant setting. Furthermore, the approaches used provide a model for use in the laboratory of material collected during the course of other randomized adjuvant studies. Although the development of platforms for genomewide analysis of molecular alterations in tumors will facilitate biomarker discovery, it is important that our studies are not driven primarily by the availability of superior technology. The selection of hypotheses for testing must be guided in the first instance by putative clinical relevance, irrespective of whether the questions are focused on increasing our understanding of tumor biology or are part of patient reclassification/treatment selection procedures. Ultimately, the end product of a translational study must be a clinically relevant biomarker that can be easily assayed in the clinical setting, producing a direct benefit for the individual patient.

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