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ORIGINAL ARTICLE

EGFR Gene Amplification and *KRAS* Mutation Predict Response to Combination Targeted Therapy in Metastatic Colorectal Cancer

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Abstract Genetic variability in *KRAS* and *EGFR* predicts response to cetuximab in irinotecan refractory colorectal cancer. Whether these markers or others remain predictive in combination biologic therapies including bevacizumab is unknown. We identified predictive biomarkers from patients with irinotecan refractory metastatic colorectal cancer treated with cetuximab plus bevacizumab. Patients who received cetuximab plus bevacizumab for irinotecan refractory colorectal cancer in either of two Phase II trials conducted were identified. Tumor tissue was available for 33 patients. Genomic DNA was extracted and used for mutational analysis of *KRAS, BRAF,* and p53 genes. Fluorescence in situ hybridization was performed to assess EGFR copy number. The status of single genes and various combinations were tested for association with response. Seven of 33 patients responded to treatment. *KRAS* mutations were found in 14/33 cases, and 0 responded to treatment ($p = 0.01$). *EGFR* gene amplification was seen in 3/33 of tumors and in every case was associated with response to treatment ($p < 0.001$). *TP53* and *BRAF* mutations were found in 18/33 and 0/33 tumors, respectively, and there were no associations with response to either gene. *EGFR* gene amplification and *KRAS* mutations are predictive markers for patients receiving combination biologic therapy of cetuximab plus bevacizumab for metastatic colorectal cancer. One marker or the other is present in the tumor of half of all patients allowing treatment response to be predicted with a high degree of certainty. The role for molecular markers in combination biologic therapy seems promising.

Keywords Colorectal cancer · Cetuximab · Bevacizumab · Metastasis · EGFR · KRAS

Introduction

Colorectal cancer (CRC) is the third most common malignancy diagnosed in males and females and remains the third leading cause of cancer deaths in the United States, accounting for 49,700 deaths in 2015 [1]. Approximately 20% of patients have distant metastases at time of diagnosis, and an equal number will develop metastatic disease after treatment for clinically localized disease [2]. The management of patients with metastatic CRC has evolved considerably due to the introduction of new cytotoxic drug combinations and of novel targeted agents [3].

Cetuximab is a monoclonal antibody that blocks dimerization of epidermal growth factor receptor (*EGFR)*, thereby inhibiting downstream signal transduction. Bevacizumab is a monoclonal antibody that binds to and sequesters vascular endothelial growth factor (*VEGF*). The *VEGF* pathway, which indirectly regulates the *EGFR* pathway, plays a central role in promoting tumor angiogenesis [4]. Treatment with bevacizumab can enhance clinical response rate to cytotoxic drug combinations and deter tumorigenesis [5]. On the other hand, cetuximab has efficacy as a single agent in a minority of

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patients (10%) and, when combined with irinotecan, provides significant improvements in progression-free survival (PFS) and overall survival [6–9]. Previous reports by Saltz et al. showed that the combination of cetuximab, bevacizumab and irinotecan yield 20% response rates in patients with metastatic CRC who have failed prior chemotherapy [7]. Furthermore, since the success of this so-called Bond-2 study, there is limited data on molecular studies looking at the role of biomarkers in combination biologic therapy in metastatic CRC. The identification of molecular genetic variables that could accurately predict response could dramatically improve patient outcomes by assuring effective drug treatment for those who respond and by avoiding the toxicity and economic cost of ineffective therapy.

Several publications have examined the usefulness of tumor-derived molecular markers for prediction of response and resistance to cetuxamib [10–14]. *EGFR* protein is overexpressed in 60–80% of CRCs detected by immunohistochemistry (IHC) but does not predict response to cetuximab [6, 15–17]. More recent studies have shown that *EGFR* gene copy number and *KRAS* mutational status predict response to cetuximab in advanced CRC [10, 11]. Specifically, for patients with irinotecan refractory CRC, cancers harboring *KRAS* mutations do not respond to cetuximab. Furthermore, several reports show that tumors that have upregulated the EGFR signaling pathway by receptor amplification or ligand overexpression are drug sensitive. These data are important and exciting, but at present not all responses to cetuximab can be explained by molecular profiling. In addition, it is unclear how reliable these tumor markers will be in the setting of drug combinations that include bevacizumab.

In this study we provide data obtained from 33 patients with irinotecan refractory colorectal cancer who were treated with cetuximab plus bevacizumab to see if predictive markers for response to cetuximab remain valid. We test for the presence of four genetic alterations: *EGFR* gene amplification, *KRAS* mutations (codons 12, 13), *BRAF* mutations (V600E) and p53 mutations (exons 5–9) for their potential use as predictive markers for cetuximab and bevacizumab in metastatic CRC.

Methods

Patient Selection Patient selection and tumor block accrual were approved by the Institutional Review Board and Human Tissue Utilization Committee of Memorial Sloan-Kettering Cancer Center (MSKCC), respectively. Cases were obtained from multi-institutional studies (with MSKCC as lead site) for which tumor specimens were available [7]. Clinical trial eligibility included clinical documentation of failure after receiving at least one chemotherapy regimen for metastatic disease that contained either irinotecan or irinotecan and bevacizumab. Failure on therapy for metastatic disease was defined as progression on treatment or within 6 weeks after receiving the last dose of a given therapy. Progression was defined as any enlargement of a measurable or evaluable lesion, or any new lesion, which was felt by the treating physician to represent a clinical failure.

Gene Mutation Analysis Genomic DNA was extracted from paraffin tumor blocks using the DNA isolation kits (QIAGEN) and used for *KRAS, BRAF* and TP53 mutation analysis. KRAS and BRAF mutations were detected using PCR/LDR approaches as previously described [18–21].

Fluorescence in Situ Hybridization *EGFR* amplification was detected using fluorescence in situ hybridization (FISH) as previously described [22]. All 33 samples were detected *EGFR* gene and CEP 7 signals by two-color FISH. At least 30 cells of each sample were counted, and the ratio of 3.0 or more was taken as evidence of gene amplification.

Statistical Analysis Patients were scored as responders if they showed evidence of partial response (PR) or complete response (CR) to treatment on computed tomography (CT) scan as defined by the response evaluation criteria in solid tumors (RECIST) criteria. Non-responders had evidence of either stable disease (SD) or progression of disease (POD) as defined by this criterion. Skin toxicity was scored according to the common terminology criteria for adverse events (CTCAE) as defined by the National Cancer Institute.

Fisher's exact test (2-tailed) was used to examine associations of response to treatment. All statistical calculations were done using either SPSS version 12.0 for windows (SPSS, Inc., Chicago, IL) or GraphPad Prism version 3.03 for Windows (GraphPad Software, Inc. San Diego, CA). P values of <0.05 were considered significant.

Results

Patient Characteristics (Supplemental Table) The cohort consisted of 20 males and 13 females with a median age of 58 years (range 32 to 74 years). All patients had metastatic CRC that was irinotecan-resistant based on a poor response to prior chemotherapeutic regimens. Eleven individuals received a combination of cetuximab and bevacizumab (CB) while 22 received irinotecan in addition to cetuximab and bevacizumab (CBI). As defined by the RECIST criteria, 7 of 33 (21%) patients responded to treatment of CB or CBI. The non-responders included 20 with stable disease (SD) and 6 with progressive disease (POD). The median duration of response to cetuximab and bevacizumab was 223 days (range, 121–529 days). In the six patients with stable disease, the median duration of stabilization was 125 days (range, 41–387 days).

Ten patients had treatment associated with grade 1 skin toxicity and 11 had grade 2 or 3 skin toxicity.

Genetic Analysis and Association with Response to Therapy (Table 1) *KRAS* mutations were found in the tumors of 14 patients (42%). All mutations were found in the no response group while no mutations were found in the response group ($p = 0.01$). There were no BRAF mutations found in the entire cohort. The tumors of 18 patients (55%) were found to have p53 mutations within exons 5–9. Three of the 7 responders (43%) were found to have p53 mutations while 15 of 26 (58%) non-responders possessed this mutation. Three of 33 (9%) patient tumors possessed EGFR amplification and all amplifications were in the response group ($p < 0.001$).

FISH Analysis and Association with Response to Therapy (Fig. 1) *EGFR* amplification was seen in 3 of 33 (9%) cases. Figure 1a represents a positive control for EGFR amplification via FISH in human epidermoid carcinoma cell line A-431. Figure 1b is an example of 1 of 30 non-amplifications. Figure 1c and d portray 2 patients for which EGFR amplification was seen in representative cases. *EGFR* amplification was significantly associated with favorable response to target therapy ($p < 0.001$).

Progression-Free Survival Median time-to-tumor-progression was increased in responders compared to non-responders (291.7 vs. 124.4 days, $P = 0.0012$). Possession of *EGFR* amplification was not associated with a longer progression-free survival compared to patients without *EGFR* amplification (log-rank, $P = 0.91$) (Fig. 2a). Patients with tumors that had WT *KRAS* did not have a significantly longer progression-free survival than patients with mutant *KRAS* (log-rank, $P = 0.23$) (Fig. 2b).

Discussion

Multiple studies over the last decade using biomarker analysis support the feasibility of refining risk stratification in CRC by incorporating tumor pathology stage with molecular characteristics [23–26]. Though the benefit of targeted drugs has often been realized in metastasis, there remain conflicting reports hampering commonplace use of personalized therapy by genomic profiling [27]. Thus further studies are required to refine the role and candidacy of biomarkers to individualize patient therapy. Our primary aim was to determine if *EGFR* gene amplification and *KRAS* mutation are predictive of tumor response to cetuximab in combination with bevacizumab. Our secondary aim was to see if other genetic markers - mutations in the *BRAF* oncogene and mutations in the P53 tumor suppressor gene - might also be correlated with tumor response. Our study shows treatment response to cetuximab and bevacizumab in irinotecan refractory, metastatic CRC correlates with *KRAS* mutation status and *EGFR* gene amplification.

Patients carrying tumors with *KRAS* mutations have been reported to have a poorer prognosis and a diminished response to adjuvant chemotherapy [11–13, 23, 28, 29]. Initial studies on patients with metastatic CRC showed no relation between

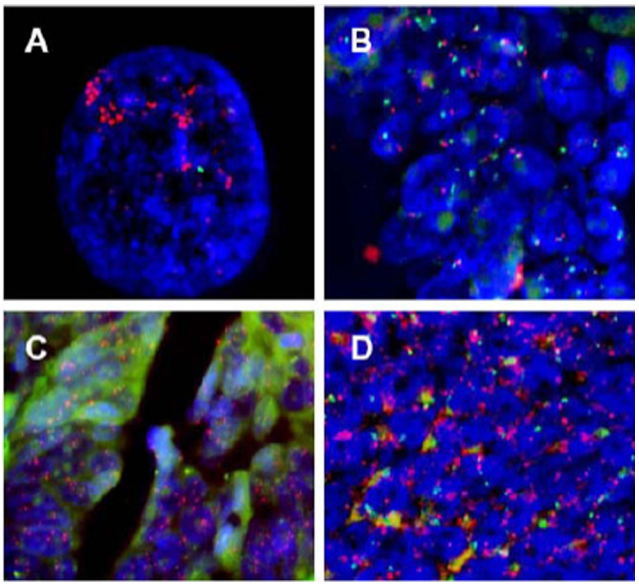

Fig. 1 Representative FISH analysis performed on formalin-fixed, paraffin-embedded human colon cancer samples. Dual-color FISH probes contain *EGFR* (red signals) and centromere of chromosome 7 (green signals). DAPI (blue) was used as counterstain. **a** shows *EGFR* gene amplification in human epidermoid carcinoma cell line A-431 with well-documented *EGFR* gene amplification. **b** shows colon cancer without *EGFR* amplification. **c** and **d** show colon cancer with EGFR amplification

Table 1 Genetic characteristics of KRAS, BRAF, p53, and EGFR in relationship to chemotherapeutic response

	KRAS		BRAF		TP53		EGFR	
	Wild type	Mutant	Wild type	Mutant	Wild type	Mutant	Amplification	No amplification
Response	7	0	7	0	4	3	3	4
No Response	12	14	26	0	11	15	0	26
	$p = 0.01$		NS		NS		$p < 0.001$	

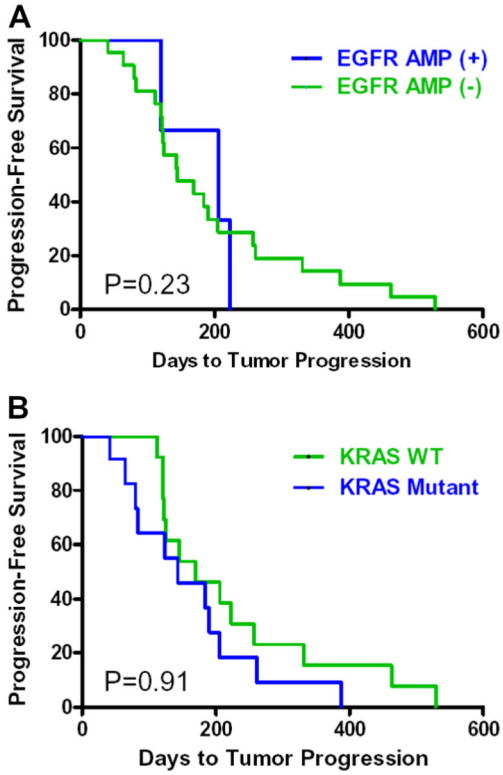

Fig. 2 Progression-free survival in patients with cetuximab and bevacizumab. The progression-free survival time was calculated by the Kaplan-Meier method. Panel A shows progression-free survival with *EGFR* amplification status ($P = 0.91$). Panel B shows progression-free survival with *KRAS* mutation status ($P = 0.23$)

KRAS mutation and response to cetuximab treatment, whereas subsequent studies showed that *KRAS* mutations were associated with lack of response [10–13, 23, 30]. Further, *EGFR* is overexpressed in 60–80% of CRC detected by immunohistochemistry (IHC). However, clinical studies demonstrated that many patients whose tumors express *EGFR* on IHC fail to respond to *EGFR* targeted therapy, and conversely, patients who respond may have tumors without *EGFR* expression [6, 15–17]. These discrepancy are probably explained by the different methods of *KRAS* and *EGFR* analysis in these studies, since it is more difficult to obtain high-quality DNA from paraffin-embedded tissue samples.

In our study, a *KRAS* mutation was identified in 41% of CRC tumors. Strikingly, none of the 14 patients with a *KRAS* mutation responded to cetuximab, while 7 (35%) of the 20 non-mutated patients had a response ($p = 0.04$). These results demonstrate that *KRAS* mutational status is highly predictive of tumor resistance to cetuximab. Partial responses meeting RECIST criteria were observed in 21% (7/33) of all patients. The three patients whose tumors had *EGFR* amplification were wild-type for *KRAS*, and notably all showed major drug responses. Four patients showed drug responses in the absence of *EGFR* amplification and also possessed a non-mutated *KRAS* gene. P53 mutations were detected in 55% of tumors, but these mutations had no predictive value for drug response. Similar findings in regards to *EGFR* amplification and *KRAS* status were reported by Lievre and colleagues showing that *EGFR* amplification and a wild-type *KRAS* genotype are predictors of response to cetuximab [11]. Similar trends were also seen by Moroni et al. and Sartore-Bianchii et al. [10, 14]. Our data indicate that *EGFR* gene amplification and *KRAS* mutation remain powerful and independent markers for predicting tumor response when bevacizumab is added to cetuximab.

Our data are concordant with results published for irinotecan refractory patients treated with cetuximab alone, panitumumab alone, or cetuximab plus irinotecan [6, 8, 9, 23, 31]. Though earlier studies supported a favorable response of bevacizumab to cetuximab, recent data has been mixed [24]. Investigators have recently shown clinical utility in the use of combination biologic therapy with these two agents [32, 33]. Thus, suggestions of a potential role for combination biologic therapy in the future treatment of metastatic CRC remain and warrant further testing. Though we are not the first group to identify predictive markers to cetuximab treatment, we are the first group to show that the predictive roles of *KRAS* and *EGFR* in cetuximab and bevacizumab treatment hold true – tumors with *KRAS* mutations remain drug resistant while those with wild-type *KRAS* and *EGFR* amplification remain drug sensitivity. If the sparing use of combination biologic therapy becomes standardized treatment in the future treatment of metastatic CRC, we believe biomarkers such as these should play a role in directing patient management.

The use of tumor markers to select patients for treatment with combination targeted therapy is promising, and clinical trials utilizing predictive markers to stratify drug treatment and optimize benefit are warranted. This study underlines the important role that molecular markers can play in predicting response to biologic agents in the treatment of CRC. In addition to guiding patient selection, these and other markers may prove useful in assessing the benefit of novel drug combinations designed to overcome resistance to biological agents.

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Compliance with Ethical Standards

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Conflict of Interest Sajid Khan, Zhaoshi Zeng, Jinru Shia, and Philip Paty declare no conflicts of interest.

Ethical Approval This retrospective study does not contain any studies with human participants by any of the authors. Tissue was obtained from

patients enrolled in clinical trials. These trials were in accordance with the ethical standards of the institutional review board and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Tumor block accrual were approved by the institutional review board.

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