Original Article

Incidence and Prognostic Impact of KRAS and BRAF Mutation in Patients Undergoing Liver Surgery for Colorectal Metastases

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BACKGROUND: Molecular biomarkers offer the potential for refining prognostic determinants in patients undergoing cancer surgery. Among patients with colorectal cancer, KRAS and BRAF are important biomarkers, but their role in patients undergoing surgical therapy for liver metastases is unknown. In this study, the incidence and prognostic significance of KRAS and BRAF mutations were determined in patients undergoing surgical therapy of colorectal liver metastases (CRLM). **METHODS:** KRAS and BRAF analysis was performed on 202 patients undergoing surgery for CRLM between 2003 and 2008. Tumor samples were analyzed for somatic mutations using sequencing analysis (KRAS, codon12/13, BRAF, V600E). The frequency of mutations was ascertained, and their impact on outcome was determined relative to other clinicopathologic factors. **RESULTS:** KRAS gene mutations were detected in 58/202 patients (29%). In contrast, mutation in the BRAF gene was identified in very low frequency in this surgical cohort, found in only 4/202 (2%) patients. On multivariate analysis, KRAS mutation was associated with worse survival (hazard ratio [HR], 1.99; 95% confidence interval [CI], 1.21-3.26), as well as recurrence risk (HR, 1.68; 95% CI, 1.04-2.70). Although other clinicopathologic features, including tumor number, carcinoembryonic antigen, and primary stage were also associated with survival, KRAS status remained independently predictive of outcome. The low incidence of BRAF mutation limited assessment of its prognostic impact. **CONCLUSION:** Whereas KRAS mutations were found in approximately one third of patients, BFAF mutations were found in only 2% of patients undergoing surgery for CRLM. KRAS status was an independent predictor of overall and recurrence-free survival. Molecular biomarkers such as KRAS may help to refine our prognostic assessment of patients undergoing surgical therapy for CRLM. ***Cancer* 2013;119:4137-44**. © *2013 American Cancer Society*.

KEYWORDS: hepatectomy; predictors; factors; molecular; personalized therapy.

INTRODUCTION

Understanding of molecular tumor characteristics is rapidly changing the management of cancer patients. By identifying distinct mutational patterns, it is possible to offer more accurate prognosis, enhance our capacity to predict tumor response to therapy, and ultimately reach an improved understanding of the mechanisms of the disease that will hopefully lead to more personalized treatment approaches. Many such alterations have been recognized in malignancies ranging from breast cancer[1] to leukemia,[2] rendering our management more efficient and our knowledge of these processes more complete.

Although surgical therapy for patients with resectable colorectal liver metastases (CRLM) offers a potential for cure, with reported 5-year survival rates of up to 50% in most series, many patients will develop recurrence and may not derive long-term benefit from resection.[3-6] As the indications for liver-directed therapy broaden, the ability to predict outcome is increasingly important to guide surgical treatment according to individual recurrence risk. Several clinicopathologic factors—including number and size of metastases, preoperative carcinoembryonic antigen (CEA) level, and interval between primary tumor and hepatic metastases—have been shown to be useful in stratifying the risk of recurrence following liver resection.[3-6] Although helpful in defining outcomes following surgical therapy, the application of molecular biomarkers may help refine our prognostic assessment in these patients.

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See editorial on pages 4083-5, this issue.

DOI: 10.1002/cncr.28347, **Received:** March 25, 2013; **Revised:** May 30, 2013; **Accepted:** June 28, 2013, **Published online** September 19, 2013 in Wiley Online Library (wileyonlinelibrary.com)

In recent years, studies in colorectal cancer have focused on the mutation of 2 proto-oncogenes, KRAS and BRAF, which have been associated consistently with primary and secondary resistance to therapy[7-11] as well as poor prognosis (BRAF).

The importance of KRAS in the biology of colorectal cancer (CRC) was recognized early, along with APC and p53, as part of the process giving rise to adenocarcinoma of the large intestine.[12] In contrast to APC and p53 mutations, however, some have suggested the ability of KRAS mutation to independently predict prognosis in both primary and metastatic CRC.[13] In addition, it is well established that the presence of mutant KRAS (mKRAS) confers primary and secondary resistance to epidermal growth factor receptor (EGFR) inhibitor therapies.[14-16] Presence of the V600E mutation of the BRAF gene (mBRAF) in colorectal cancer patients, on the other hand, has been clearly shown to be a predictor of increased cancer mortality, regardless of stage and other clinicopathologic characteristics,[17-20] as well as possibly predicting resistance to anti-EGFR therapies.[21-23] In the majority of these studies on CRC, patient populations were evaluated based on stage of disease, including unselected patients with advanced disease. The purpose of this study was to assess these biomarkers in patients with resectable CRLM. Specifically, we aimed to determine the incidence of mKRAS and mBRAF in patients undergoing surgical therapy for CRLM and evaluate their impact on postoperative prognosis.

MATERIALS AND METHODS

Study Population

Two hundred thirty consecutive patients undergoing curative intent surgical treatment for CRLM at the Johns Hopkins Hospital between January 2003 and December 2008 were identified from our Institutional Review Board-approved institutional prospective database. Patients were excluded if they had undergone incomplete (R2) palliative surgery or if they had been treated with anti-EGFR agents during the perioperative period (n = 8). In addition, 20 patients were excluded due to an inadequate specimen for molecular analysis. Patients were considered eligible if they underwent complete (R0 or R1) liver resection, with or without complete tumor ablation. In some cases, synchronous resections were performed along with the primary tumor. Patients undergoing ablation alone, either surgical or percutaneous, were excluded. In patients who underwent staged resections, the second resection was considered the operative date. In total, 202 patients met the inclusion and exclusion criteria and were considered for further analysis.

Tumor DNA

The paraffin-embedded tumor blocks for each case were identified from the hospital pathology archives. Hematoxylin and eosin staining was used to verify whether satisfactory cellularity was present for mutational analysis as well as to accurately determine presence of tumor and confirm diagnosis. Blocks were cut at 10 μm and the slides were subsequently deparaffinized by immersing them in xylene (30 minutes) and, subsequently, in alcohol. After removing the paraffin, the delineated tumor was microdissected from the slide and digested overnight with proteinase K at 55°C. DNA was extracted with glycogen (100 mg) using a DNAeasy Blood and Tissue Kit (Qiagen Sciences, Germantown, MD).

KRAS/BRAF Mutation Analysis

The extracted DNA was evaluated for the presence of the most common mutations of the KRAS (codon 12/13) and BRAF (V600E) genes. These regions of interest were amplified using polymerase chain reaction (PCR) and the reaction product underwent agarose gel electrophoresis against known positive and negative controls to assess the presence and size of the amplified product. The PCR protocol settings used were: initial denaturation of 95°C for 5 minutes, followed by 40 cycles of amplification at 95°C for 40 seconds, 57°C for 40 seconds, and 72°C for 40 seconds and a final elongation at 72°C for 10 minutes. For amplification of the codon 12/13 region of the KRAS gene, the oligonucleotide primers used were 5′-TCATTATTTTTATTATAAGGCCTGCTG-3′ (sense) and 5′-TTGGATCATATTCGTCCACAA-3′ (anti-sense). To evaluate for the V600E mutation of the BRAF gene, the primers were 5′-TGAAGACCTCACAGTAAA AATAGGTG-3′ (sense) and 5′-CCACAAAATG GATCCAGACA-3′ (anti-sense). The amplified products were subsequently column-purified using a GeneJET PCR Purification Kit (Fermentas ThermoScientific, Glen Burnie, MD) and then sequenced.

Clinical Data Collection

The following data were collected for each patient: disease status, demographics, laboratory data, operative details, date of last follow-up, type of chemotherapy, administration and timing of chemotherapy, and date of death, as well as tumor number, size, and location. Tumor size and number were defined by the resection specimen. Data on recurrence were defined using time of diagnosis of

recurrent disease based on follow-up CT imaging as the outcome parameter.

Statistical Analysis

Correlation of the incidence of mKRAS and mBRAF with the presence of clinical or pathologic characteristics was assessed using a chi-square test. The characteristics tested, which were chosen on the basis of previous clinical knowledge, were: age and sex of the patients, location of primary tumor (colon or rectum), disease-free interval (defined as time between the operation on the primary and the diagnosis of metastatic disease), N stage of the primary tumor, size of largest liver metastasis, number of liver metastases, preoperative CEA level, use of preoperative chemotherapy, use of adjuvant chemotherapy, intraoperative use of tumor ablation, presence of extrahepatic metastases synchronous with liver metastases, and presence of microscopically positive final resection margins. The decision was made to dichotomize the variables disease-free interval ($\leq$1 vs $>$1 year), N stage (N-positive vs N-negative), CEA level ($\leq$200 vs $>$200 ng/mL), age ($\leq$60 vs $>$60 years), number of liver metastases ($<$3 vs $\geq$3 foci), and size of largest liver metastasis ($\leq$5 vs $>$5 cm).

Overall survival (OS) and recurrence-free survival (RFS) was described using the Kaplan-Meier method, and a proportional hazards regression analysis (Cox) model was used to describe the relation between patients' OS and RFS and the previously enumerated variables. In the multivariate model, initially all the above variables were included, and a backward stepwise regression was then performed to ensure that only those variables that correlated with survival to a statistically significant extent were included in the final model. Statistical significance was defined as $P<.05$ (2-tailed).All statistical analyses where performed using STATA SE version 11.0 (StataCorp LP, College Station, TX).

RESULTS

Patient Characteristics

Among the 202 patients treated with surgical therapy, 152 underwent liver resection only, whereas 50 were treated with a combination of resection and ablation. Twenty (10%) patients had synchronous extrahepatic metastatic disease that was also resected with curative intent. The median age was 61 years (range, 33-84 years). The overall median follow-up was 1.5 years, 2.7 years among survivors.

A total of 162 (81%) patients received preoperative chemotherapy, either as part of neoadjuvant treatment or as conversion chemotherapy, while 130 patients (65%) received adjuvant chemotherapy in the postoperative period (within 6 months following liver surgery). No patients received anti-EGF receptor therapy at any time prior to liver surgical therapy or in the adjuvant setting, as imposed by the exclusion criteria.

TABLE 1. Patient Clinicopathologic Characteristics and KRAS Mutation Status

Characteristics	wtKRAS	mKRAS	Total	*P*
Total no. of patients	144 (71)	58 (29)	202 (100)	
Age, y				.72
≤60	71 (49)	27 (47)	98 (49)	
>60	73 (51)	31 (53)	104 (51)	
Sex				.42
Male	98 (68)	36 (62)	134 (66)	
Female	46 (32)	22 (38)	68 (34)	
Primary tumor N stage				.23
Negative	53 (39)	17 (30)	70 (36)	
Positive	83 (61)	40 (70)	123 (64)	
Tumor location				
Right side	30 (21)	24 (41)	54 (27)	**.011**
Left side	69 (48)	22 (38)	91 (45)	
Rectum	45 (31)	12 (21)	57 (28)	
Preoperative CEA, ng/mL				.42
>200	8 (7)	2 (4)	10 (6)	
≤200	105 (93)	50 (96)	155 (94)	
Disease-free interval				.59
>1 y	53 (37)	19 (33)	72 (36)	
≤1 y	91 (63)	39 (67)	130 (64)	
Preoperative chemotherapy				.44
Yes	118 (82)	44 (78)	162 (81)	
No	26 (18)	13 (22)	39 (19)	
Adjuvant chemotherapy				.78
Yes	94 (65)	36 (63)	130 (65)	
No	50 (35)	21 (37)	71 (35)	
Size of largest liver metastasis				.30
>5 cm	20 (15)	5 (9)	25 (12)	
≤5 cm	116 (85)	50 (91)	166 (88)	
No. of liver metastases				.46
1-2	94 (65)	41 (71)	135 (67)	
≥3	50 (35)	17 (29)	67 (33)	
Bilateral disease				.67
Yes	39 (27)	14 (24)	53 (26)	
No	105 (73)	44 (76)	149 (74)	
Final resection margin				.29
R0	130 (90)	55 (95)	185 (92)	
R1	14 (10)	3 (5)	17 (8)	
Concomitant extrahepatic metastases				.7
Yes	15 (12)	5 (9)	20 (10)	
No	129 (88)	53 (91)	182 (90)	
Surgical therapy				.90
Resection + ablation	36 (25)	14 (24)	50 (25)	
Resection only	108 (75)	44 (76)	152 (75)	
BRAF mutant				.20
Yes	4 (3)	0 (0)	4 (2)	
No	140 (97)	58 (100)	198 (98)	

Abbreviations: CEA, carcinoembryonic antigen; mKRAS, mutant KRAS; wtKRAS, wild-type KRAS.
Data are presented as no. (%). Boldface values are $P<.05$.

KRAS and BRAF Mutations

Among the 202 tumor samples evaluated, KRAS mutations were detected in 58 (29%) samples via direct

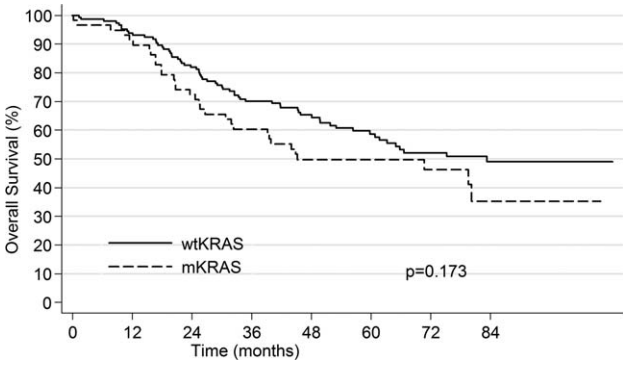

Figure 1. Overall survival after hepatic surgery for colorectal liver metastasis depicted by KRAS mutation status (Kaplan-Meier method).

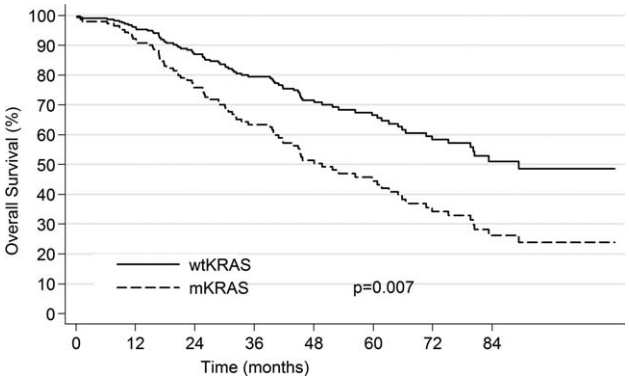

Figure 2. Overall survival after hepatic surgery for colorectal liver metastasis depicted by KRAS mutation status (multivariate Cox model).

sequencing. The mutations observed were at codon 12 GGT→GAT (G12D) (n = 17), GGT→GTT (G12V) (n = 13), GGT→AGT (G12S) (n = 5), GGT→TGT (G12C) (n = 4), and GGT→GCT (G12A) (n = 2), as well as GGT→GAC (G13D) (n = 14) and GGT→TGC (G13C) (n = 1) at codon 13. No correlation was found between the specific types of mutation and other clinicopathologic characteristics.

Various clinical and pathologic features were assessed and compared according to KRAS status (Table 1). The presence of mKRAS was more frequent among patients with right-side primary tumors ($P = 0.011$) but was not significantly associated with the other variables examined.

The V600E mutation in BRAF was detected in 4 out of the 202 (2%) patients. Among tumors harboring mBRAF, 75% originated from the right side of the colon, compared with 26% for those without the mutation ($P = 0.030$). The average age for patients with mBRAF was 71 years, but was only 59 years for patients with wild-type BRAF ($P = 0.024$). Based on the low incidence of mBRAF in this study, any further definitive association analysis was not possible.

Overall Survival

The median overall survival of the entire cohort was 70.7 months and the 5-year survival was 55.1%. The effect of a number of clinicopathologic factors, including presence of mKRAS and mBRAF, on patient OS following hepatic surgery for CRLM was assessed. In univariate analysis, mKRAS was associated with increased mortality (hazard ratio [HR], 1.34; 95% confidence interval [CI], 0.88-2.04) although not statistically significant (Fig. 1). The median survival among patients with mKRAS was 45.2 months compared with 71.9 months for patients with wild-type KRAS, with a 5-year survival of 49.8% and 57.4%, respectively. Once adjusted for known predictors of patient survival through a multivariate Cox model, mKRAS was associated with significantly worse OS after liver surgery for CRLM (HR, 1.99; 95% CI, 1.21-3.26) (Fig. 2). This difference in the influence of KRAS mutation was the effect of adjusting for preoperative CEA levels (≥200 ng/mL or <200 ng/mL), use of ablation during surgery, node-positive primary tumor, presence synchronous extrahepatic metastases, and presence residual

TABLE 2. Overall Survival and Recurrence-Free Survival After Liver Surgery (Cox Proportional Hazards Model)

	Univariate Analysis		Multivariate Analysis	
Feature	HR (95% CI)	*P*	HR (95% CI)	*P*
Overall survival				
KRAS mutant	1.34 (0.88-2.04)	.173	1.99 (1.21-3.26)	**.007**
Ablation therapy	2.05 (1.36-3.08)	**.001**	2.41 (1.50-3.89)	**<.001**
CEA >200 ng/mL	2.59 (1.29-5.18)	**.007**	2.78 (1.27-6.07)	**.011**
Node-positive primary	2.14 (1.34-3.43)	**.002**	2.06 (1.25-3.40)	**.005**
R1 resection	1.93 (1.03-3.62)	**.041**	2.10 (0.94-4.66)	.069
Other synchronous metastases	3.10 (1.85-5.19)	**<.001**	2.92 (1.57-5.41)	**.001**
Recurrence-free survival				
KRAS mutant	1.53 (0.99-2.36)	.057	1.68 (1.04-2.70)	**.034**
Ablation therapy	2.55 (1.69-3.84)	**<.001**	3.17 (1.99-5.06)	**<.001**
CEA >200 ng/mL	3.01 (1.48-6.12)	**.002**	2.68 (1.27-5.66)	**.010**
Node-positive primary	2.25 (1.40-3.62)	**.001**	2.35 (1.41-3.91)	**.001**
Other synchronous metastases	1.96 (1.10-3.47)	**.022**	2.08 (1.07-4.03)	**.030**
Preoperative chemotherapy	3.04 (1.47-6.27)	**.003**	2.73 (1.29-5.75)	**.008**

Abbreviations: CEA, carcinoembryonic antigen; CI, confidence interval; HR, hazard ratio.
Boldface values are $P < .05$.

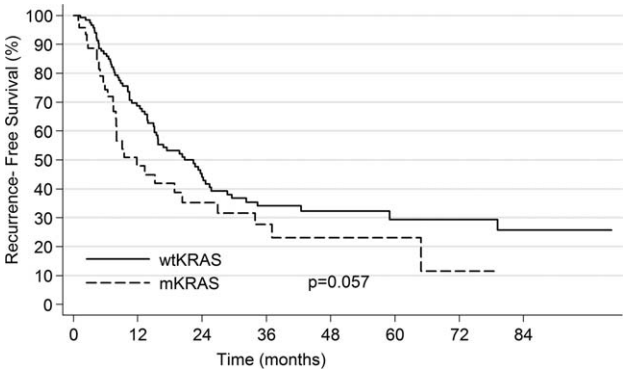

Figure 3. Recurrence-free survival after hepatic surgery for colorectal liver metastasis depicted by KRAS mutation status (Kaplan-Meier method).

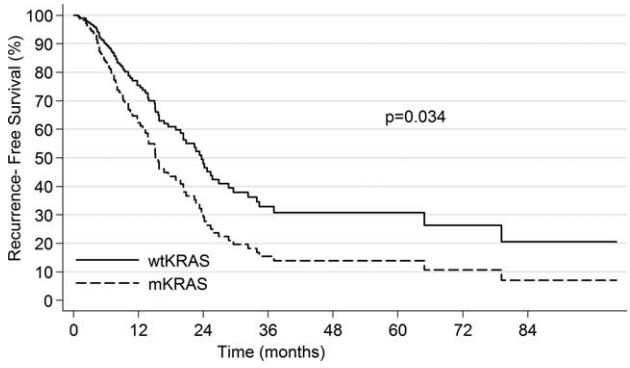

Figure 4. Recurrence-free survival after hepatic surgery for colorectal liver metastasis depicted by KRAS mutation status (multivariate Cox model).

microscopic disease (R1 resection), all of which were found to be independent predictors of OS (Table 2). In the subgroup of patients who underwent liver resection with ablation as part of CRLM treatment, the effect of KRAS mutation appeared to be even more pronounced (univariate HR, 2.55; 95% CI, 1.25-5.20; multivariate HR, 7.13; 95% CI, 2.85-17.85) (data not shown).The ability to determine the effect of mBRAF on survival was limited due to the low observed mutation frequency. However, while not significant (HR, 1.9; $P = 0.274$), overall survival in those few patients with BRAF mutation was poorer compared with those with wild-type BRAF tumors (median, 25.4 months vs. 70.7 months; 3-year survival, 25% vs 68%).

Recurrence-Free Survival

The median time to recurrence for the entire cohort was 18.9 months, and the 3-year RFS was 32.2%. Patients with mKRAS tumors presented with decreased RFS in both univariate (HR, 1.53; 95% CI, 0.99-2.36) (Fig. 3) and multivariate (HR, 1.68; 95% CI, 1.04-2.70) (Fig. 4) analyses. The median RFS among patients with mKRAS was 11.8 months compared with 20.8 months for patients with wild-type KRAS, with a 3-year RFS of 27.7% and 34%, respectively. Preoperative CEA levels >200 ng/mL, use of ablation during surgery, N-positive primary tumor, presence of synchronous extrahepatic metastases, and administration of preoperative chemotherapy were also significant predictors of RFS (Table 2).

DISCUSSION

Hepatic surgery for CRC has a sound rationale, supported by clinical data demonstrating potential for long-term benefit.[24] Yet, the majority of patients will experience recurrence within 5 years.[4,25] Although various clinicopathologic factors can impact prognosis, our ability to predict outcome in a clinically significant manner remains poor. Currently, there are few biomarkers for predicting which patients have more aggressive disease or which may derive the greatest benefit from surgical therapy. The frequency and prognostic implications of KRAS and BRAF mutation status have been described in both localized and metastatic CRC, but their characteristics in patients with potentially resectable liver metastases have not been elucidated. The current study demonstrates that only 29% of patients undergoing surgical therapy for metastases harbor tumors with mKRAS, and only 2% are found to have mBRAF. In addition, we found a prognostic effect of KRAS mutation status independent of EGFR inhibitor therapy. This finding was observed both in OS and in RFS.

In contrast to most published studies reporting the incidence of KRAS mutation in localized and metastatic CRC, ranging from 35% to 45%,[7,9,14,18] we found KRAS mutations in only 29% of tumors from patients undergoing liver surgery. In addition, BRAF mutations were exceedingly rare in this cohort, being present in only 4 tumors (2%). Most studies report an incidence in stage IV CRC of 8%-10%.[18,20] The reason for the difference observed in KRAS and BRAF status in particular is intriguing. While the knowledge of the mutational status was uncommonly known by the treating clinicians, it is likely that patient selection plays a role. There is a growing body of literature validating BRAF mutation as a significantly poor prognostic factor in metastatic CRC. Although mBRAF was too low to correlate with other

known prognostic factors in our study, it is possible that mBRAF patients had apparently worse biology (eg, rapid progression, resistance to preoperative chemotherapy) independent of technical resectability, possibly limiting any incentive to recommend liver resection. Alternatively, perhaps nonmutated KRAS and BRAF tumors more likely present with oligometastases confined to the liver. Indeed, one study reported that mKRAS tumors are more likely to develop pulmonary metastases.[26] In another study, Tran et al[27] found that mBRAF tumors were more likely to develop peritoneal and distant nodal metastases compared with wild-type BRAF tumors. Although the presence of mBRAF is likely a highly significant poor prognostic factor in patients undergoing hepatic metastatectomy and may be a contraindication to surgical therapy, the low frequency of mBRAF in our series raises doubts regarding the usefulness of preoperative BRAF testing in patients who are being considered for curative-intent hepatic surgery for CRC. Specifically, based on these data, testing for BRAF is not recommended routinely for selection of patients for surgical therapy of CRLM.

Recent studies on surgical CRLM patients have also investigated the correlation between the presence of KRAS mutations and patient overall survival, as well as patterns of recurrence. Stremitzer et al[28] studied 60 patients undergoing hepatic surgery for CRLM after administration of neoadjuvant chemotherapy including bevacizumab, revealing a potential prognostic role of mKRAS among those patients. Andreou et al[29] analyzed 341 CRLM patients who received adjuvant chemotherapy for their primary tumor and showed that treatment with FOLFOX was associated with worse prognosis after hepatectomy and higher incidence of somatic mutations, including KRAS. However, in contrast to both studies that included only patients that had undergone preoperative chemotherapy, the current study included the entire cohort of surgical CRLM patients, including both those receiving and not receiving preoperative chemotherapy.

KRAS mutations are highly predictive of resistance to EGFR-directed therapy. In contrast, the prognostic implications of KRAS mutation status are less well defined, and various studies in both localized and metastatic disease have produced inconsistent results. Most studies demonstrate that patients who have metastatic colorectal cancer with mKRAS tumors have an OS similar to that of patients who have nonmutated KRAS tumors independent of EGFR therapy.[20,30-34] Specifically, despite predictive value for this targeted therapy, several large randomized trials including the PACCE study,[30] the CAIRO2 study,[34] and the AGITG MAX trial[20] reported lack of prognostic significance of a KRAS mutation. In contrast, retrospective mutational analyses of other large randomized trials (eg, the FOCUS,[18] COIN,[35] and CRYSTAL[36] trials) suggested a prognostic role of KRAS.

In our study, KRAS mutation appeared prognostic for both OS and RFS. Although univariate analysis failed to reach statistical significance, both OS and RFS were independently significant after adjusting for known predictors of outcome. Specifically, the 5-year overall survival was 57% in nonmutated KRAS tumors compared with 50% 5-year survival in patients whose tumors had KRAS codon 12/13 mutations. The 3-year RFS observed was 34% and 28% in the nonmutated vesrus mutated KRAS tumors, respectively.

Our study has several limitations. The sample size, although fairly representative, was relatively small. In addition, we could not obtain a complete chemotherapy history for all patients. Specifically, details of the remote use of EGFR-directed therapy were not available in many patients. Although patients undergoing this therapy were excluded when used prior to or within 6 months following resection, it is possible that some of these patients received cetuximab or panitumumab following a recurrence, possibly impacting OS in favor of the wild-type KRAS group. However, because EGFR therapy was excluded in the postoperative adjuvant setting, the observed difference in recurrence would not have been impacted by this therapy. In another similar study, Nash et al[37] evaluated 126 patients undergoing resection of CRLM in an era prior to these targeted therapies and similarly reported improvement in disease-specific survival in patients with wild-type KRAS tumors. Finally, the KRAS testing performed in this study using direct nucleotide sequencing and PCR accounts for only mutations in codons 12 and 13, and not for less common mutations.[38] Specifically, mutations in codon 61 and 146 have been shown to be associated with shorter progression-free survival compared with wild-type KRAS patients.[39]

In conclusion, the current study identifies KRAS mutation as a predictor for increased risk of recurrence and poorer survival for patients with surgically treated hepatic colorectal metastases. Currently, these findings should not encourage KRAS status to be used in current practice as a primary determinant for the optimal surgical management of advanced colorectal cancer. Yet, this biomarker may be helpful toward improving prognostic models and clinical decision-making based on molecular as well as clinicopathologic features. While BRAF mutations were found to be highly uncommon in these surgical patients, future studies evaluating the role of other

biomarkers in patients with hepatic colorectal metastases, including NRAS, PIK3CA, MET, MMR, and PTEN, could add additional value to our prognostic information. Hopefully, with future refinements in the understanding of molecular genetics of colorectal cancer, these tools can help in the designing personalized cancer therapy in patients with surgically treatable metastases.

FUNDING SOURCES

No specific funding was disclosed.

CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

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