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ORIGINAL ARTICLE

The role of K-RAS and B-RAF mutations as biomarkers in metastatic colorectal cancer

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Summary

Purpose: Unlike cetuximab, there is a paucity of biomarkers for bevacizumab as predictors of outcome in metastatic colorectal cancer (mCRC) patients. Obviously exploring the worth of some potential markers in this setting is warranted. The purpose of this study was to investigate the predictive value of the presence of K-RAS and B-RAF mutations on the outcome of patients with mCRC treated with FOLFIRI and bevacizumab combination therapy.

Methods: A total of 172 patients with mCRC were evaluated. K-RAS and B-RAF mutations were analyzed by quantitative PCR. Median progression-free survival (PFS) and overall survival (OS) were compared utilizing chi-square and Mann-Whitney U tests, respectively.

Results: Forty-four percent (N=77) of the patients were found to harbor K-RAS mutations and 6 (7.5%) were positive for B-RAF mutations. In baseline no difference in PFS and OS was observed between the groups with or without K-RAS mutation. No relationship was established between K-RAS and B-RAF mutation status and baseline CEA and CA19-9 tumor markers levels.

Conclusion: K-RAS and B-RAF mutations do not seem to be predictive of treatment outcome as potential biomarkers for bevacizumab therapy in mCRC. However, not only the presence of K-RAS and B-RAF mutations but also the different biological behavior of the various subtypes of mutations should be considered as potential determinants in the final outcome of this disease.

Key words: bevacizumab, biomarker, B-RAF, chemotherapy, K-RAS, metastatic colon cancer

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Introduction

CRC is the third most common cancer diagnosed in both men and women and the third leading cause of cancer related deaths. Vascular endothelial growth factor-A (VEGF) is a protein released from hypoxic tumor cells and and mainly promotes endothelial cell migration, proliferation and survival in addition to increasing vascular permeability, by binding to its specific receptor. VEGF is important in the regulation of physiological and pathological angiogenesis. It is overexpressed in many types of cancer, particularly in CRC. Targeted biological agents have recently come into use in addition to standard chemotherapy in advanced CRC. With the advent of new targeted biological agents in the treatment protocols of advanced CRC, treatment outcomes have significantly changed [1]. Bevacizumab is a monoclonal antibody directed not against the tumor cells themselves but to VEGF, while cetuximab and panitumumab are monoclonal antibodies directed against the epidermal growth factor receptor (EGFR) on the tumor cells. All agents are usually used in combination with chemotherapy.

Addition of bevacizumab to fluoropyrimidine-based chemotherapies is a standard first-line therapy in mCRC treatment. Addition of bevacizumab to standard chemotherapy, such as 5-fluorouracil/leucovorin (5-FU/LV), irinotecan plus 5-FU/LV (FOLFIRI) and 5-FU/LV+oxaliplatin (FOLFOX), is associated with increased response rate (RR), OS and PFS [2]. Toxicity is generally tolerable. However, there have been fatal adverse effects observed including gastrointestinal perforation and arterial thromboembolism.

Kirsten ras (K-RAS) is the oncogene that encodes ras signal protein. B-RAF is a cytoplasmic serine/threonine kinase, directly interacting with RAS and regulates its activity by triggering a cytoplasmic phosphorylation cascade which leads to the activation of transcription factors that control cell growth, differentiation and apoptosis. The B-RAF mutation, a thymine to adenine transversion mutation that results in the substitution of the amino acid valine with glutamate in the final protein, appears in 4-15% of CRC [3,4].

K-RAS and B-RAF mutations are potential biomarkers for CRC. The presence of K-RAS mutations has been shown to be associated with poor prognosis and reduced response to treatment. Activation of Ras/Raf/Mek/Erk pathway has been reported to be associated with increased VEGF expression and the suppression of negative regulators for angiogenesis. This finding suggests that K-RAS and B-RAF aberrations might potentially affect the response to antiangiogenic therapy. While certain studies reported the presence of K-RAS gene mutations as a predictor of non-response to targeted therapy related with EGFR, some of them demonstrated that bevacizumab was actually associated with longer OS independent of Ras/Raf/Mek/Erk pathway [4].

We carried out additional analyses to better describe the clinical benefit of bevacizumab treatment in mCRC as related to the K-RAS and B-RAF mutation status. The purpose of this study was to investigate the potential association of K-RAS and B-RAF status with the outcome of bevacizumab therapy and their potential roles as prognostic biomarkers.

Table 1. Details of K-RAS and B-RAF mutation status

K-RAS mutations	*N*	*%*	*B-RAF mutations*	*N*	*%*
12ALA (GGT>GCT)	5	2.9	Negative	74	92.5
12ARG (GGT>CGT)	3	1.7	Positive	2	2.5
12ASP (GGT>GAT)	24	14	Positive (v600a)	1	1.2
12CYS (GGT>TGT)	7	4.1	Positive (v600e)	1	1.2
12SER (GGT>AGT)	3	1.7	Positive (v600krm)	2	2.5
12VAL (GGT>GTT)	18	10.5			
13ASP (GGC>GAC)	16	9.3			
Negative	90	53.5			
Negative sequence	5	3			
Total	172	100	Total	80	100

Table 2. Patient and primary tumor characteristics

Characteristics	*N (%)*
Age (years)	
Median	60.5
Range	27-83
Gender	
Male	64 (37.2)
Female	108 (62.8)
Stage at diagnosis	
I	1 (0.6)
II	20 (11.6)
III	50 (29.1)
IV	87 (50.6)
Missing data	14 (8.1)
Primary tumor localization	
Rectum	63 (36.6)
Sigmoid	43 (25)
Left colon	28 (16.3)
Right colon	17 (9.9)
Caecum	13 (7.6)
Transverse colon	2 (1.2)
Tumor histology	
Adenocarcinoma	131 (76.3)
Mucinous carcinoma	27 (15.7)
Other	14 (8)

Methods

The medical records of 172 patients with CRC being on follow up for metastatic disease between 2000 and 2010 in our clinic were retrospectively analyzed. The patients, regardless of what kind of adjuvant chemotherapy they had received previously,were treated with FOLFIRI and bevacizumab combination as their first-line treatment for metastatic disease. The patients were stratified according to their primary tumor localization and site of metastasis, and K-RAS and B-RAF mutations were analyzed by pathologists experienced in gastrointestinal (GI) tumors utilizing real-time PCR and the Qia Gen Kit (Manchester, UK) for the 7 mutations specific for codons 12 and 13 of K-RAS gene. In addition, a portion of samples was amplified from the isolated DNA that was performed using DNA extraction, and B-RAF mutation was detected by melting curve analysis. RFS and OS were determined. PFS1 showed the time till progression for patients who presented initially with metastasis. PFS1 was also used to define the time to recurrence in patients who had undergone curative resection followed by adjuvant therapy. PFS2 defined the time till the second progression.

The mutations screened for K-RAS and B-RAF were as follows (Table 1): Gly12Ala(GGT>GCT), Gly12Asp (GGT>GAT), Gly12Arg (GGT>CGT), Gly12Cys (GGT>TGT), Gly12Ser (GGT>AGT), Gly12Val (GGT>GTT), Gly13Asp (GGC>GAC).

Statistics

Categorical and continuous variables were compared with Chi-square and Mann-Whitney U tests, respectively. PFS and OS were estimated by using the Kaplan-Meier method. Log-rank test was used to evaluate differences between groups. Univariate analysis was performed to assess the significance of clinicopathological features as prognostic factors. A p-value < 0.05 was considered significant.

Results

The median patient age was 60.5 years (range 27-83). Sixty-two percent of the patients were male and 38% female. Tumor localizations were as follows: 36.6% rectum, 25% sigmoid, 16.3% left colon, 1.2% transverse colon, 9.9% right colon, and 7.6% caecum. Demographic characteristics of the patients are summarized in Table 2. K-RAS mutation was positive in 44% (75 out of 172) of the patients. B-RAF analysis was performed in a total of 80 patients and mutation was detected in only 6 (7.5%) of them. No relationship was established between K-RAS and B-RAF mutation status and baseline CEA and CA19-9 levels. The primary tumors of the 6 patients with B-RAF mutation were in the rectosigmoid, and localizations of tumors with K-RAS mutations were as follows: rectosigmoid 43/103 (41.7%), left colon 16/28 (57%), and tumors in the transverse and ascending colo-caecum 18/32 (56%). While 67 (39%) patients had only liver metastases, the total number of patients with liver plus other metastases was 108 (62.7%) and while 17 (9.9%) patients had only lung metastasis, the total number of patients with lung plus other metastases was 43 (25%). In 46% of patients with liver metastasis,K-RAS

Table 3. Tumor localization according to K-RAS status

K-RAS status	*Rectosigmoid* *N (%)*	*Left colon* *N (%)*	*Right, transverse, caecum* *N (%)*
K-RAS mutant type	43 (41.7)	16 (57)	18 (56)
K-RAS wild type	60 (58.3)	12(43)	14 (44)
Total	106	31	35

Table 4. K-RAS and B-RAF mutation status in frequently seen pathologies

	K-RAS wild *N (%)*	*K-RAS mutant* *N (%)*	*B-RAF wild* *N (%)*	*B-RAF mutant* *N (%)*
Adenocarcinoma	63 (52.9)	56 (47.1)	51 (92.7)	4 (7.3)
Mucinous carcinoma	11 (42.3)	15 (57.7)	12 (85.7)	2 (14.3)

mutation was positive, and K-RAS mutation rate was 50% for patients with lung metastasis. K-RAS mutation rate was 61% for patients with both liver and lung metastasis (Table 3). While 11 (6.4%) patients had only peritoneal metastasis, the total number of patients with peritoneal plus other metastases was 44 (25%). No patient had only bone metastasis or only brain metastasis whereas the total number patients with bone metastasis was 3 (1.8%) and with brain metastasis was 1 (0.6%). No significant difference was found between the histological subtypes of the tumors and K-RAS and B-RAF mutations (Table 4).

When the relationship between K-RAS mutation and PFS1 was investigated, there was no difference in PFS between the groups with or without K-RAS mutation with regard to the selection of first-line chemotherapy (oxaliplatin-based/FOLFOX or XELOX vs FOLFIRI + bevacizumab) in the group of patients who presented with metastatic disease. Figure 1 shows PFS1 in patients receiving oxaliplatin-based therapy or FOLFIRI + bevacizumab as per K-RAS mutation status.

When the relationship between K-RAS mutation and PFS2 was investigated, no difference in PFS was found between the groups with or without K-RAS mutation with regard to the selection of second-line chemotherapy (oxaliplatin based/ FOLFOX or XELOX vs FOLFIRI + bevacizumab) in the group of patients who presented with metastatic disease. Figure 2 shows PFS2 in patients receiving oxaliplatin-based therapy and FOLFIRI + bevacizumab as per

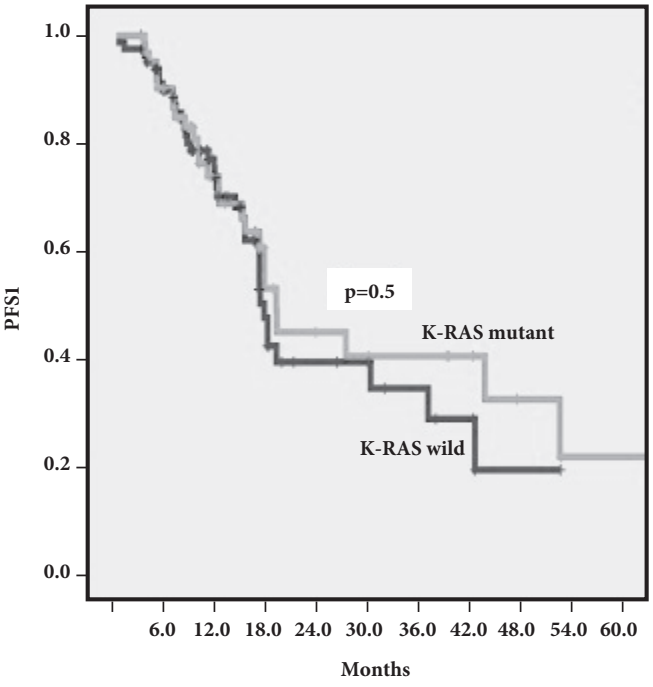

Figure 1. Relation of K-RAS mutation status and progression-free survival of patients who received first-line oxaliplatin/irinotecan chemotherapy or bevacizumab.

K-RAS mutation status. OS for the whole group of patients at 36 months was 81% (Figure 3), and no significant difference was detected in OS as per K-RAS status (whole group, Figure 4). This analysis could not be done regarding B-RAF status due to the small number of patients with mutation (Figure 5). K-RAS and B-RAF mutations were present in 34.5 and 6.5% of the patients, respectively. B-RAF-mutated tumors were more likely to develop in the right colon, were

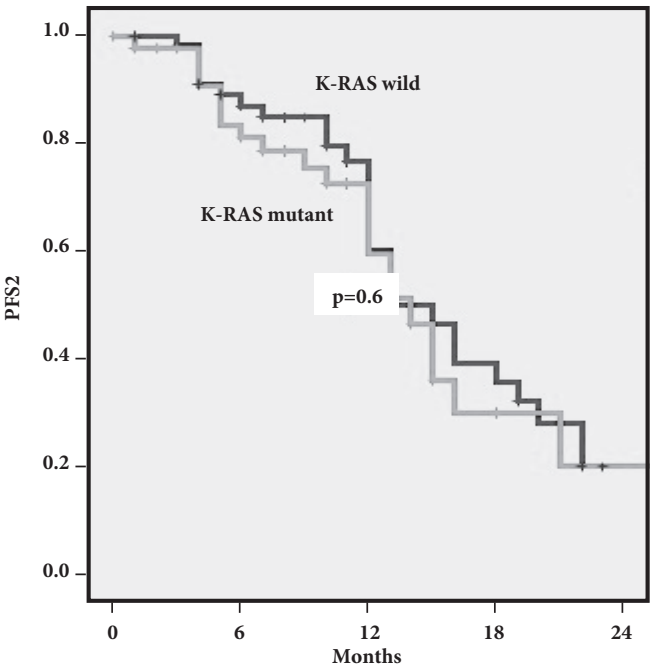

Figure 2. Relation of K-RAS mutation status and progression-free survival of patients who have received second-line oxaliplatin/irinotecan chemotherapy or bevacizumab.

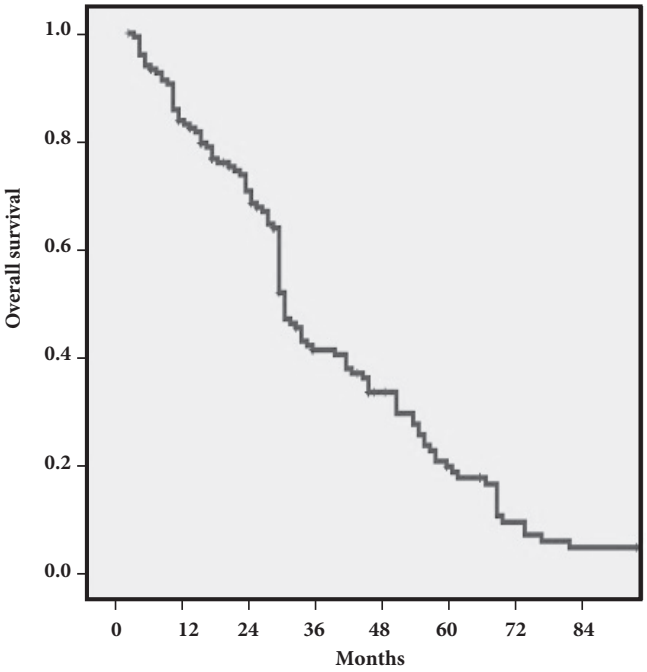

Figure 3. Overall survival for all patients who had gone through mutation testing.

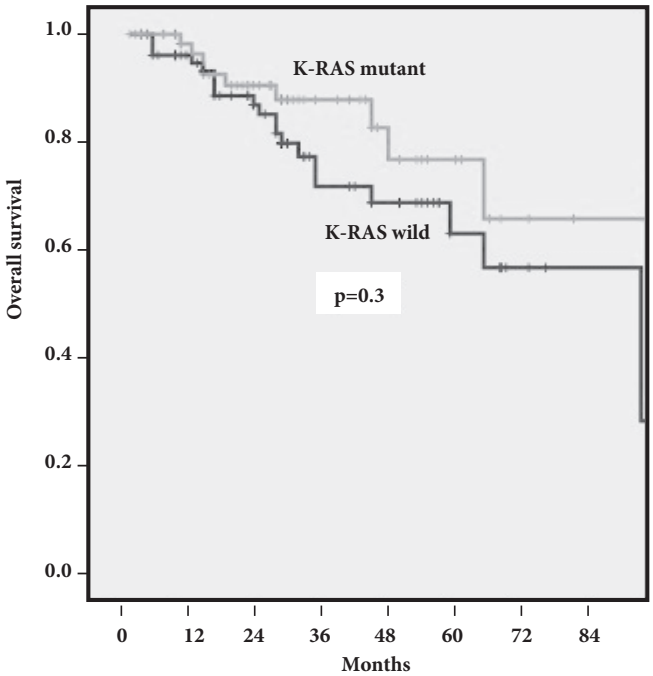

Figure 4. Overall survival according to K-RAS status.

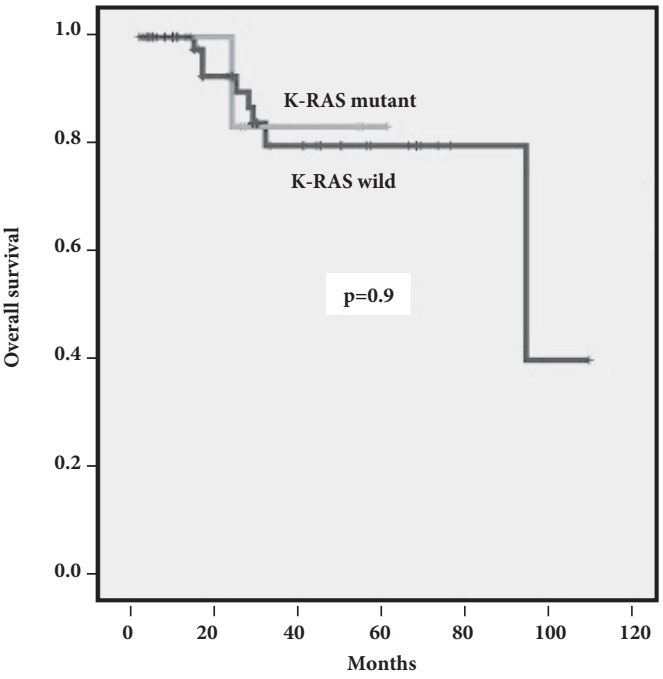

Figure 5. Overall survival according to B-RAF status.

more likely to be poorly differentiated adenocarcinomas or mucinous carcinomas with a higher rate of peritoneal metastasis. The median OS for B-RAF mutation-positive and K-RAS mutation-positive patients was 11.0 and 27.7 months, respectively, which was significantly worse than that for patients with wild-type K-RAS and B-RAF (40.6 months ;$p < 0.05$).

Discussion

Bevacizumab in combination with chemotherapy is now considered standard treatment in advanced CRC in combination with chemotherapy as it has been unequivocally shown to improve survival. Thus, median OS of mCRC has been increased to 23.5 months with the use of targeted biological agents [5].

Several trials [1,6] have shown that K-RAS mutations upregulate VEGF and many other angiogenic factors in tumor cells. When the potential mechanisms of resistance are taken into account, patients with mutated (m)K-RAS and wild type (wt) K-RAS tumors benefit from bevacizumab similarly and VEGF is thought to be the main angiogenic factor in CRC regardless of the K-RAS mutation status.

mK-RAS gene,on the other hand,is a predictive marker for the decreased efficacy of EGFR-targeting monoclonal antibodies when used alone or in combination with chemotherapy. Thus, the presence of mK-RAS is important in terms of selecting a patient subgroup who are resistant to the inhibition of EGFR with monoclonal antibodies. Despite the fact that only one third of the wt K-RAS subgroup responds to EGFR inhibition, K-RAS is the only biomarker used in clinical practice for mCRC, due to the role of the biomarkers of EGF, VEGF and IGF receptor pathways in mCRC [7].

Metastatic CRC patients with mK-RAS tumors do not benefit from cetuximab-or panitumumab-based therapies targeting EGFR [5,7]. At present, K-RAS mutation status is a widely accepted biomarker to select mCRC patients for therapies targeting EGFR. When standard 5-fluorouracil, oxaliplatin, irinotecan and bevacizumab-based treatments fail, EGFR targeted rescue treatment may be considered for the individuals with wt K-RAS tumors. However, the biology of K-RAS mutant disease and predictions about the treatment outcome still require urgent elucidation [8].

Over the last years, OS of advanced CRC has been significantly prolonged by the introduction of biological agents into the standard chemotherapy [2]. Two major targets for the biological agents are EGFR and VEGF-A. The agents targeting EGFR and VEGF, although effective, add substantially to the cost of standard chemotherapy. Therefore, predictive markers have to be clarified and potentially responsive populations well-defined in order to use these expensive therapies in the most cost-efficient way.

Although mK-RAS indicates resistance to anti-EGFR treatment, there is merely clinical evidence regarding the efficiency of antiangiogenic treatment in the presence of K-RAS and B-RAF mutations. In a trial [9] investigating the effects of adding anti-VEGF agent into the first-line chemotherapy on PFS, no conclusive evidence was demonstrated, suggesting that K-RAS or B-RAF status is a predictor regarding the efficiency of bevacizumab. In addition, it was found that improvements in PFS were independent from K-RAS or B-RAF status and there was no difference between K-RAS and B-RAF status and OS improvement, and no evident relationship was established regarding PFS improvement and K-RAS mutation status when bevacizumab was added into IFL combination chemotherapy. This lends significant support to the concept that K-RAS and B-RAF status does not have any effect on the response to antiangiogenic efficacy. K-RAS mutation, as an unfavorable prognostic factor, still remains a controversial issue.

In the RASCAL trial (Kirsten ras in Colorectal Cancer Collaborative Group) [10], which is one of the most important studies investigating K-RAS mutation and its effect on prognosis, K-RAS status was evaluated in CRC patients who had previously received chemotherapy and did not receive active treatment at present; no suggestion was made regarding K-RAS mutation as a prognostic marker for PFS or OS. RASCAL-II metaanalysis of 3439 patients suggested specific importance of codon 12 glycine-to-valine mutations, but these occurred in less than 10% of the patients [9]. In another trial [11], K-RAS gene status was not found to be of any prognostic value in patients receiving bevacizumab and capecitabine/

oxaliplatin or capecitabine/irinotecan chemotherapy and was not associated with any impact on the efficacy of chemotherapy combination with bevacizumab in terms of RR, PFS and OS.

A trial evaluating the effect of K-RAS and B-RAF gene mutation status on PFS, OS and RR in patients receiving combination therapy with bevacizumab determined that there is currently no predictive marker available to assist in the selection of patients eligible for anti-VEGF therapy [12]. VEGF and EGFR pathways have been shown to interact with increased angiogenesis in solid tumors. Therefore, K-RAS and VEGF activating mutations are likely to affect the response to antiangiogenic treatment via the MAPK pathway through upregulation of VEGF and other key angiogenesis mediators.

When targeted treatment options for mCRC therapy are considered, there is still ambiguity in selecting the optimal treatment for patients with wtK-RAS tumors. Although these tumors are potentially susceptible to EGFR targeting monoclonal antibodies, a retrospective analysis has determined that the results in patients receiving bevacizumab were better for patients with wtK-RAS tumors and that mK-RAS influences the prognosis [13].

K-RAS gene mutation status was not prognostic for PFS and OS in patients with advanced mCRC in the MAX trial [11]. On the other hand, mB-RAF seems likely to have a prognostic value. IFL/bevacizumab data has suggested that B-RAF gene status has a prognostic value on survival as mentioned above. The risk of mortality was significantly reduced in patients with wtB-RAF. This study has shown that B-RAF gene mutation status is more prognostic for OS rather than PFS in comparison with K-RAS gene mutation status. In the K-RAS and B-RAF status analysis of the MAX study, addition of bevacizumab to chemotherapy did not have any therapeutic effect regardless of K-RAS and B-RAF mutation status. K-RAS status was prognostic neither for PFS nor for OS in mCRC. However, while B-RAF was prognostic for unfavorable OS it was not prognostic for disease progression [14].

This trial has shown significant relations between K-RAS status and treatment efficacy in each of the efficacy endpoints [14]. K-RAS mutation status has been confirmed as a strong predictive biomarker for the efficacy of cetuximab plus FOLFIRI treatment. B-RAF mutation was a strong marker for poor prognosis [6]. The results have verified the efficacy of cetuximab plus FOLFOX-4 therapy in the first-line treatment of patients with wtK-RAS mCRC, and that K-RAS mutation status is an efficient predictive biomarker. However, as in our study, in this trial the small number of mB-RAF tumors impeded to make a definite deduction regarding the prognostic or predictive use of this biomarker [15]. This trial indicates that K-RAS mutation is more likely to be found in patients with liver metastasis, whereas liver metastasis is predicted in our study [4].

It is recommended to definitely specify K-RAS status of CRC patients who are to receive EGFR targeted treatment. Also, evidence suggests that patients with K-RAS mutation should not receive these treatments. Additionally, despite conflicting results, B-RAF kinase mutations (V600E) also seem to determine sensitivity to EGFR inhibitors.

In addition, although the precise prognostic value of K-RAS mutation is still debated, B-RAF and K-RAS mutations are considered as prognostic for poor survival. On the other hand, evidence for the association of B-RAF with poor prognosis is overwhelming [9].

Another study showed better treatment outcomes with the addition of oxaliplatin in patients with B-RAF-mutated tumors, but this did not reach statistical significance [12].

Data from current reports has shown that K-RAS status does not predict the clinical benefit of the addition of bevacizumab into the first-line IFL chemotherapy, independently from K-RAS status which is prognostic in mCRC. In relation to adding bevacizumab into IFL chemotherapy, the indirect benefit in PFS and OS was that a higher RR was observed when bevacizumab was added into IFL in patients with wtK-RAS. There was not a similar benefit in patients with K-RAS mutation [16].

A study investigating the relevance of K-RAS and B-RAF mutations with tumor markers has shown that CA19.9 carries a significant predictive value for OS, whereas CEA and CA 19.9 declines are not pre-

dictive for OS in mCRC patients, independently from K-RAS status. In addition, only patients with a high level of CA19.9 benefited significantly from bevacizumab administration [17].

Over the last years, the knowledge about the relationship between the optimal use of monoclonal antibodies and mutations in mCRC has increased significantly. Alternative opinions have also emerged regarding this issue. While some authors have shown that K-RAS mutations do not correlate with worse outcome in mCRC [1], some others support the prognostic relevance of K-RAS mutations in mCRC [14]. These findings confirm the concept that K-RAS may not be a predictive factor for efficacy of cytotoxic chemotherapies.

Our study proposes that the efficacy of anti-VEGF therapy with bevacizumab seems independent from the K-RAS status, in contrast to the EGFR targeted monoclonal antibodies. Considering daily practice, K-RAS and perhaps B-RAF testing should not be a prerequisite in patients who are to receive bevacizumab.

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