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ORIGINAL ARTICLE

G12V and G12A *KRAS* mutations are associated with poor outcome in patients with metastatic colorectal cancer treated with bevacizumab

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Abstract The v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutations are found in 35–45 % of colorectal cancer (CRC) cases. Although the association between the RAS signaling and angiogenesis is well known, the negative predictive value of *KRAS* mutation has not been established in patients treated with bevacizumab. The aim of this study was to evaluate the association between specific *KRAS* mutation types and outcome of patients with metastatic CRC treated with bevacizumab. The study included 404 patients with metastatic CRC (mCRC) treated with bevacizumab. Clinical data obtained from the clinical registry CORECT were retrospectively analyzed. The shortest survival was observed in patients with tumors harboring G12V or G12A *KRAS* mutation (G12V/A). The median progression-free survival (PFS) and overall survival (OS) for patients with tumors harboring G12V/A *KRAS* mutation was 6.6 and 16.8 compared to 11.6 and 26.3 months for patients with tumors harboring other *KRAS* mutation type ($p<0.001$ and $p<0.001$), while the survival of patients harboring other *KRAS* mutation types was comparable to those with tumors harboring wild-type *KRAS* gene. In the Cox multivariable analysis, *KRAS* G12V/A mutation type remains a significant factor predicting both PFS (HR=2.18, $p<0.001$) and OS (HR=2.58, $p<0.001$). In conclusion, the results of the present study indicate that there is a significant difference in biological behavior between tumors harboring G12V/A and other *KRAS* mutations. Moreover, comparison of the survival of patients with tumors harboring G12V/A *KRAS* mutations with those harboring wild-type *KRAS* gene revealed that G12V/A *KRAS* mutations are prognostic biomarker for inferior PFS and OS in patients with mCRC treated with bevacizumab in univariate as well as multivariable analyses.

Keywords Colorectal cancer · Bevacizumab · Chemotherapy · KRAS · Mutation

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Background

Colorectal cancer (CRC) is one of the most common causes of morbidity and mortality in developed countries [1]. Considerable progress in the treatment of metastatic CRC (mCRC) has been reached in recent years, and several novel active agents have been approved for the systemic therapy of mCRC patients. Bevacizumab is a recombinant humanized monoclonal antibody that blocks angiogenesis targeting vascular endothelial growth factor A (VEGF-A). The efficacy and safety of bevacizumab in the treatment of patients with mCRC have been demonstrated in phase III clinical trials as well as in observational studies [2–6], but, so far, no reliable biomarker predicting response to bevacizumab has been established. The negative prognostic significance of gene mutations in v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) in patients with locally advanced or metastatic CRC has been widely reported [7–12]. Although the association between the activation of RAS signaling and angiogenesis is well known, the negative predictive value of *KRAS* mutation has not been, to the best of our knowledge, established in patients treated with bevacizumab. The aim of this study was to evaluate the association between specific *KRAS* mutations types with outcome of patients with mCRC treated with bevacizumab.

Patients and methods

Patients and treatment

Clinical data of 404 adult patients with histologically confirmed mCRC treated between 2005 and 2014 with bevacizumab-based therapy at one of three oncology centers in the Czech Republic including Department of Oncology and Radiotherapy, Charles University Medical School and Teaching Hospital Pilsen; Department of Oncology, Charles University First Faculty of Medicine and Thomayer Hospital; and Department of Oncology, Palacký University Medical School and Teaching Hospital Olomouc were retrospectively analyzed. Bevacizumab (Avastin, F. Hoffman-La Roche Ltd., Basel, Switzerland) was administered in combination with chemotherapy or as a single agent in standard approved doses (5.0 mg/kg every 14 days or 7.5 mg/kg every 21 days). The chemotherapy regimens included 5-fluorouracil and leucovorin in combination with oxaliplatin (FOLFOX) or irinotecan (FOLFIRI) or 5-fluorouracil and leucovorin (FUFA); capecitabine in combination with oxaliplatin (XELOX) or irinotecan (XELIRI) or capecitabine alone; oxaliplatin alone; and irinotecan alone. None of the patients has received prior anti-angiogenic therapy. The assessment of *KRAS* gene status was performed at the time of diagnosis of metastatic disease. As it is a standard practice in the Czech Republic, sample analysis was performed either at the treating center using standardized methods including direct sequencing, real-time PCR (2008–2010), and reverse hybridization method (StripAssay) (since 2010). The detailed methodology is available as Supplementary Online Data. Participating centers sent *KRAS* gene findings (wild-type or mutated) to the CORECT registry.

Data source

The data were obtained from the clinical registry CORECT. This clinical registry (http://corect.registry.cz/) is a non-interventional post-registration database of epidemiological and clinical data of patients with mCRC treated with targeted therapies in the Czech Republic. The registry contains anonymized individual baseline patient data collected at the start of targeted therapy including demographics, initial stage, and disease characteristics, as well as data on survival and adverse events. The data are updated at least twice a year. Clinical data from the registry were validated against hospital medical records. Data on type of *KRAS* mutation in codons 12 and 13 were extracted from the hospital information systems and merged with the registry data. The protocol was approved by the independent ethics committee of the University Hospital Pilsen and complied with the International Ethical Guidelines for Biomedical Research Involving Human Subjects, Good Clinical Practice guidelines, the Declaration of Helsinki, and local laws. Outcome of part of the same cohort have been recently published in a report that covered all centers in the country but did not include details of *KRAS* mutational status [13].

Outcome assessment

The clinical status of the patients was assessed continuously during visits at pre-specified time points. Physical examination and routine laboratory tests were performed every 2 weeks, and computed tomography (CT) was performed every 3 to 4 months during the treatment. The objective tumor response was assessed locally by the attending physician using Response Evaluation Criteria in Solid Tumors (RECIST) [14].

Statistical analysis

Standard frequency tables and descriptive statistics were used to characterize sample data set. The significance of differences in baseline characteristics between patients with tumors harboring wild-type *KRAS* gene and those with tumors harboring *KRAS* mutation as well as between subgroups of patients with tumors harboring different *KRAS* mutation types were assessed using Fisher's exact test or Mann–Whitney test. Progression-free survival (PFS) and overall survival (OS) were estimated using Kaplan Meier method, and all point estimates were accompanied by 95 % confidence intervals. PFS was defined from the date of initiation of bevacizumab administration until the date of first documented progression or death due to any cause. OS was defined from the date of bevacizumab initiation until the date of death due to any cause. Statistical significance of the differences in survival was assessed using the log-rank test. Patients, who were to the date of analysis still alive or without progression, were censored at the date of last visit. Moreover, multivariable Cox proportional hazards model was used to assess the effect of type of *KRAS* mutation on survival in the presence of other potential predictive and prognostic factors. Due to non-proportionality of hazards, sex was incorporated to the model as a stratification factor. Standard level of significance $\alpha=0.05$ was used. Exception was post hoc pairwise comparison of PFS and OS according to *KRAS* mutation in which Bonferroni correction was applied, therefore alpha was set to 0.05/3= 0.017.

Results

Patient characteristics

Records of 404 patients were analyzed. The results of *KRAS* mutation analysis and the distribution of mutation types are summarized in Table 1. Only patients with tumors harboring activating *KRAS* mutations in codons 12 and 13 ($N=135$) were included in the present analysis along all patients with wild-type *KRAS* tumors ($N=223$). Patients with insufficient data were excluded ($n=45$). Only one patient has mutation in codon 61 and was also excluded from the analysis. Baseline patient characteristics according to the present of *KRAS* mutation are summarized in Table 2.

Table 1 Results of *KRAS* mutation testing in codons 12 and 13

KRAS test results	*n* (%)
Wild-type *KRAS*	223 (55.2)
KRAS mutation	181 (44.8)
Codon 12	107 (26.5)
G12A	11 (2.7)
G12C	9 (2.2)
G12D	35 (8.7)
G12F	1 (0.2)
G12L	1 (0.2)
G12R	3 (0.7)
G12S	14 (3.5)
G12V	33 (8.2)
Codon 13	28 (6.9)
G13D	28 (6.9)
Total	404 (100)

Survival of patients according to *KRAS* mutation status and specific *KRAS* mutation type

The median PFS and OS for patients with tumors harboring *KRAS* mutation was 9.2 and 22.8 months compared to 10.8 and 29.2 months for patients with wild-type *KRAS* genes ($p=0.309$ and $p=0.003$, respectively) (Fig. 1). The PFS and OS data for patients with tumors harboring wild-type *KRAS* gene, *KRAS* mutation, and the more common (occurring in more than 9 cases) specific types of *KRAS* mutation (G12A, G12C, G12D, G12S, G12V, and G13D) are summarized in Table 3. The shortest survival was observed in patients with tumors harboring G12V or G12A *KRAS* mutation (G12V/A). The median PFS and OS for patients with tumors harboring G12V/A *KRAS* mutation was 6.6 and 16.8 months compared to 11.6 and 26.3 months for patients with tumors harboring other *KRAS* mutation type ($p<0.001$ and $p<0.001$, respectively), while the survival of patients with tumors harboring other *KRAS* mutation types was comparable to those harboring wild-type *KRAS* gene (Fig. 2). The survival data of patients with tumors harboring G12V/A *KRAS* mutation, other *KRAS* mutations, and wild-type *KRAS* gene are summarized in Table 4. In the Cox multivariable analysis, *KRAS* G12V/A mutation type remains a significant factor predicting both PFS (HR=2.18, $p<0.001$) and OS (HR=2.58, $p<0.001$) (Table 5).

Discussion

Our data suggest that the presence of G12V/A *KRAS* mutations could be an independent prognostic biomarker for inferior PFS and OS in patients with mCRC treated with bevacizumab while the prognosis of patients with tumors harboring other *KRAS* mutations seems to be comparable to the patients with wild-type *KRAS* tumors.

The *KRAS* oncogene is a member of a human *RAS* oncogene family producing a self-inactivating guanosine triphosphate (GTP) binding signal transducer, located on an inner surface of the cell membrane. *KRAS* gene mutations can compromise the intrinsic GTPase activity, resulting in constitutively active KRAS protein that triggers various downstream effector signaling pathways [15, 16]. The *KRAS* gene mutations have been detected in many human tumor types. The incidence of *KRAS* mutations in patients with CRC is relatively high, estimated between 35 and 45 % of cases [17, 18]. It has been demonstrated that *KRAS* mutation is the major predictive biomarker of resistance to monoclonal antibodies targeting epidermal growth factor receptor (EGFR), cetuximab, and panitumumab [11, 19–21].

Table 2 Baseline patient's characteristics according to *KRAS* mutation

	Wild-type *KRAS*	*KRAS* mutation			p*
	(n=223)	All *KRAS* mutations (n=135)	G12V/A (n=44)	Other type (n=91)	
Males/Females, n (%)	137/86 (61.4/38.6)	85/50 (63.0/37.0)	26/18 (59.1/40.9)	59/32 (64.8/35.2)	0.823; 0.571
Age (years)					
Median (range)	61.3 (31–82)	63.0 (31–83)	62.4 (33–77)	63.2 (1–83)	0.105; 0.598
Location, n (%)					
Colon	132 (59.2)	78 (57.8)	26 (59.1)	52 (57.1)	0.825; 0.855
Rectum	91 (40.8)	57 (42.2)	18 (40.9)	39 (42.9)	
History of thromboembolism, n (%)	10 (4.5)	7 (5.2)	1 (2.3)	6 (6.6)	0.801; 0.427
History of hypertension, n (%)	78 (35.0)	59 (43.7)	18 (40.9)	41 (45.1)	0.116; 0.713
Synchronous metastases, n (%)					
No	86 (38.6)	47 (34.8)	17 (38.6)	30 (33.0)	0.500; 0.566
Yes	137 (61.4)	88 (65.2)	27 (61.4)	61 (67.0)	
Prior surgery, n (%)	189 (84.8)	120 (88.9)	39 (88.6)	81 (89.0)	0.341; 0.999
Prior radiotherapy, n (%)	36 (16.1)	23 (17.0)	9 (20.5)	14 (15.4)	0.883; 0.472
Adjuvant chemotherapy, n (%)	65 (29.1)	36 (26.7)	15 (34.1)	21 (23.1)	0.630; 0.214
Number of metastatic sites (%)					
Not available	41 (18.4)	24 (17.8)	6 (13.6)	18 (19.8)	
1	97 (43.5)	57 (42.2)	16 (42.1)	41 (56.2)	0.695; 0.262
2	62 (27.8)	36 (26.7)	16 (42.1)	20 (27.4)	
≥3	23 (10.3)	18 (13.3)	6 (15.8)	12 (16.4)	
Chemotherapy regimens, n (%)					
FOLFOX or XELOX	152 (68.2)	94 (69.6)	30 (68.2)	64 (70.3)	0.577; 0.959
FOLFIRI or XELIRI	34 (15.2)	22 (16.3)	8 (18.2)	14 (15.4)	
Other types	33 (14.8)	19 (14.1)	6 (13.6)	13 (14.3)	
No chemotherapy	4 (1.8)	0 (0.0)	0 (0.0)	0 (0.0)	
Line of therapy, n (%)					
First line	173 (77.6)	115 (85.2)	37 (84.1)	78 (85.7)	0.099; 0.801
Second and higher line	50 (22.4)	20 (14.8)	7 (15.9)	13 (14.3)	
Subsequent treatment with anti-EGFR monoclonal antibodies, n (%)	117 (52.5)	0 (0.0)	0 (0.0)	0 (0.0)	

*Fisher exact test or Mann–Whitney test. First p value correspond to comparison of wild-type *KRAS* (n=223) and all mutated (n=135) and second one to comparison of two subgroups of mutated *KRAS* patients (n=44 vs. n=91)

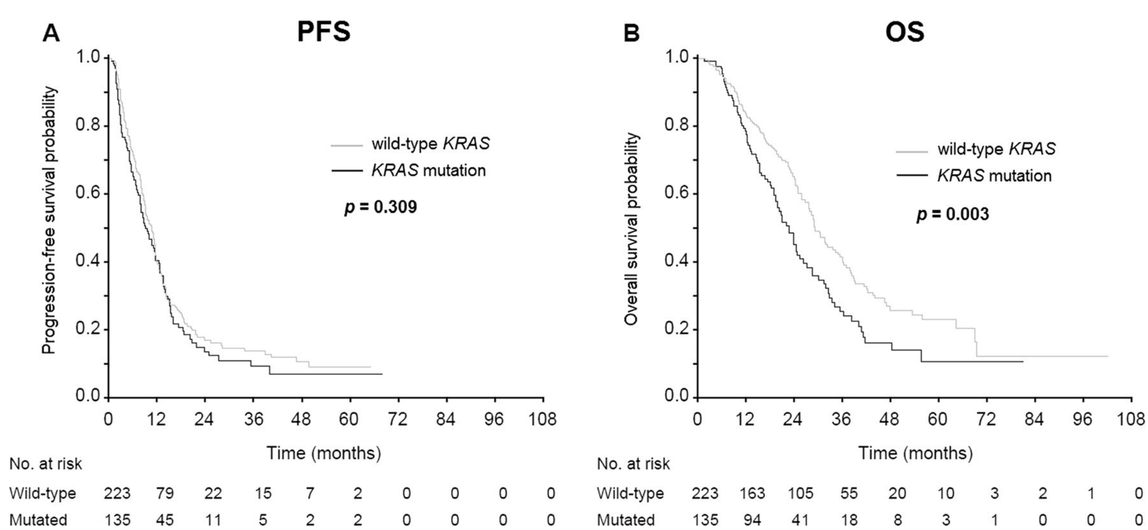

Fig. 1 Kaplan-Meier estimates of progression-free survival (PFS) and overall survival (OS) according to presence of *KRAS* mutation (**a**, **b**)

Table 3 Progression-free and overall survival of patients with the most common *KRAS* mutation types in the present cohort

KRAS mutation type, *n* (%)	*N*	Median PFS (95 % CI)	Median OS (95 % CI)
G12A	11	3.5 months (1.7–5.2)	16.1 months (6.0–26.1)
G12C	9	10.6 months (6.8–14.3)	27.4 months (10.0–44.8)
G12D	35	15.1 months (7.3–22.9)	28.7 months (17.1–40.2)
G12S	14	11.5 months (0.5–22.5)	32.7 months (14.5–51.0)
G12V	33	6.6 months (3.5–9.8)	19.2 months (12.5–25.9)
G13D	28	11.3 months (8.6–14.1)	22.8 months (11.6–34.0)

Although it has been reported that various pro-angiogenic growth factors such as VEGF, TGF-alpha, or TGF-beta can be induced or strongly upregulated by mutant *RAS* oncogenes, the relevance of *KRAS* mutation status in the efficacy of anti-angiogenic agents remains unclear [22, 23]. The results of studies focusing on predictive or prognostic role of *KRAS* mutation in patients with mCRC treated with bevacizumab are controversial. The retrospective study by Diaz-Rubio et al. and meta-analysis by Petrelli et al. reported longer PFS and OS for patients with tumors exhibiting wild-type *KRAS* gene compared to patients with tumors harboring *KRAS* mutation [24, 25]. On the other hand, other studies failed to demonstrate predictive or prognostic role of *KRAS* mutation status [26–29]. Similarly, a recent study based on the same CORECT registry reported similar outcome of patients treated with the combination of bevacizumab and chemotherapy regardless of *KRAS* mutational status [13]. However, outcomes were not analyzed according specific *KRAS* mutation types. Recently, it has been hypothesized that different specific *KRAS* mutation types result in different biological behavior, but there is only limited clinical data to support this hypothesis. *KRAS* mutations associated with poor outcome (G12V/A) represent only about a third of cases with tumors harboring *KRAS* mutation, so it is not surprising that the negative prognostic impact of these mutations might be diluted in an unselected population. Different proportions of patients with G12V/A mutations could possibly explain conflicting results in different cohorts of patients.

The aim of the present study was to evaluate the potential role of *KRAS* mutation status and the role of specific *KRAS* mutation types in patients with mCRC treated with chemotherapy and bevacizumab in general clinical practice. Significantly shorter OS was observed for patients with tumors harboring any *KRAS* mutation, while the difference in PFS was not significant. Subsequently, the role of specific *KRAS* mutation types was analyzed. The lowest survival rates were observed for patients harboring G12V/A *KRAS* mutation. Significantly shorter PFS and OS were observed for patients with tumors harboring G12V/A *KRAS* mutation compared to patients with tumors harboring other *KRAS* mutation type. Notably, both compared groups were well-balanced according to baseline clinical characteristics. The survival rates for patients with tumors harboring other *KRAS* mutation types were comparable to patients with tumors harboring wild-type *KRAS* gene. The contradictory results have been reported in a retrospective study by Bruera et al. showing the association of G12D *KRAS* mutation type with worse prognosis of mCRC patients treated with intensive triplet chemotherapy (FIr-B/

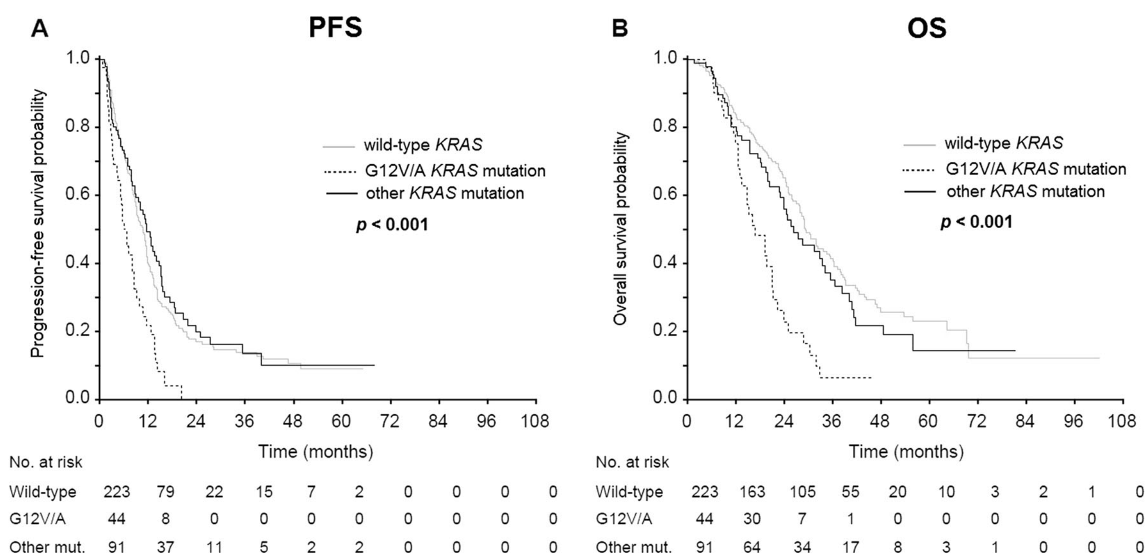

Fig. 2 Kaplan-Meier estimates of progression-free survival (PFS) and overall survival (OS) according to specific *KRAS* mutation type (**a**, **b**)

Table 4 Progression-free and overall survival according to *KRAS* status and type of mutation

KRAS test results, *n* (%)	*n*	Median PFS (95 % CI)	Pairwise comparison		
Wild-type *KRAS*	223	10.8 months (9.2–12.3)	X	X	-
G12V/A *KRAS* mutation *KRAS*	44	6.6 months (4.8–8.4)	X	-	X
Other *KRAS* mutation type	91	11.6 months (9.0–14.3)	-	X	X
Log-rank test *p* value		<0.001	<0.001	0.485	<0.001
KRAS test results, *n* (%)	*n*	Median OS (95 % CI)	Pairwise comparison		
Wild-type *KRAS*	223	29.2 months (26.3–32.1)	X	X	-
G12V/A *KRAS* mutation	44	16.8 months (12.1–21.4)	X	-	X
Other *KRAS* mutation type	91	26.3 months (19.1–33.5)	-	X	X
Log-rank test *p* value		<0.001	<0.001	0.270	0.001

FOx) plus bevacizumab, although the cohort was small, including only 59 patients (27 patients with *KRAS* mutation) [30].

Patients in the present cohort were treated mostly before the introduction of anti-EGFR agents into the first line of mCRC therapy. Actually, improved OS (and the discrepancy between no difference in PFS and improved OS) of patients with wild-type *KRAS* could be partly explained by the fact, that many patients with wild-type *KRAS* tumors received anti-EGFR agents (cetuximab or panitumumab) after failure of bevacizumab whereas patients with tumors harboring *KRAS* mutation did not. Currently, both anti-EGFR antibodies and bevacizumab are used in the first-line therapy of mCRC, but only bevacizumab remains a therapeutic option in patients with tumors harboring *KRAS* mutations.

The G12V *KRAS* mutation is encountered frequently in primary mCRC and is associated with decreased OS, suggesting that this mutation type may confer a more aggressive CRC phenotype [31–34]. Several experimental studies have shown that G12V *KRAS* mutation possesses increased oncogenic potential and is associated with more aggressive cancer behavior compared to G12D *KRAS* mutation [10, 35, 36]. The GTPase activity of G12V-mutated *KRAS* protein was found to be only one quarter of the activity of G12D-mutated *KRAS* protein and one tenth of wild-type KRAS protein activity [37]. The functional differences associated with specific amino acid substitutions cause differential activation of signaling pathways, and proteins resulting from different *KRAS* mutation have different downstream signaling properties. The G12V-mutated *KRAS* protein interacts primarily with RAF signaling through the ERK pathway, whereas G12D-mutated *KRAS* protein signals primarily through the PI3K/AKT, JNK, p38, and FAK pathways [35]. However, little is known about biological behavior of G12A *KRAS* mutation from experimental studies. Mizutani et al. has reported that G12A-mutated *KRAS* protein also interacts primarily with RAF through the ERK signaling pathway [38]. The crucial link between angiogenesis an the effect of specific *KRAS* mutation types is based on the observation that the expression of VEGF and other important regulators of angiogenesis is regulated mainly by RAS/

Table 5 Multivariable Cox-proportional hazards model for progression-free and overall survival

Parameter	Category	Progression-free survival		Overall survival	
		HR (95 % IS)	*p* value	HR (95 % IS)	*p* value
KRAS status	Wild-type *KRAS*	1.00	-	1.00	-
	G12V/A *KRAS* mutation	2.18 (1.48–3.22)	<0.001	2.58 (1.66–4.02)	<0.001
	Other *KRAS* mutation type	0.99 (0.72–1.36)	0.934	1.21 (0.83–1.77)	0.329
Age	<65 years	1.00	-	1.00	-
	≥65 years	0.99 (0.75–1.31)	0.930	0.95 (0.68–1.32)	0.751
Location	Colon	1.00	-	1.00	-
	Rectum	1.05 (0.80–1.39)	0.719	1.36 (0.98–1.89)	0.066
Synchronous metastases	No	1.00	-	1.00	-
	Yes	1.35 (1.02–1.79)	0.036	1.68 (1.19–2.36)	0.003
Number of metastatic sites	1	1.00	-	1.00	-
	2 and more	1.88 (1.42–2.48)	<0.001	2.21 (1.60–3.06)	<0.001
Line of therapy	First line	1.00	-	1.00	-
	Second and higher	2.09 (1.49–2.92)	<0.001	3.06 (2.07–4.52)	<0.001

RAF/MEK/ERK signaling pathway, suggesting that different *KRAS* mutation types may differentially stimulate tumor angiogenesis [39–42]. Based on the present results together with the experimental studies mentioned above, we hypothesize that G12V/A-mutated *KRAS* proteins stimulate tumor angiogenesis and influence the response to anti-angiogenic therapy by aberrant activation of RAS/RAF/MEK/ERK signaling pathway, whereas other types of mutated *KRAS* proteins do not possess these properties because of signaling through another pathways. Thus, G12V/A *KRAS* mutation types predict poor outcome in patients with metastatic colorectal cancer treated with bevacizumab.

The principal limitations of the present study are the retrospective nature and relatively limited number of patients with resulting heterogeneity, especially regarding chemotherapy backbone regimens. The *KRAS* mutation analyses were performed either at the treatment center, with the result that different technologies were used, potentially introducing a bias. The present study also did not include control group not treated with bevacizumab, and, therefore, it cannot be concluded with certainty that patients harboring G12V/A *KRAS* mutation will not benefit from adding bevacizumab to chemotherapy. This question should be answered in prospective randomized trials in the future. Nevertheless, this is the largest study published so far evaluating the prognostic role of specific *KRAS* mutation types in patients with mCRC treated with bevacizumab and also the first study showing G12V/A *KRAS* mutations as an independent prognostic biomarker.

In conclusion, the results of the present retrospective study indicate that there is a significant difference in biological behavior between tumors harboring G12V/A and other *KRAS* mutations. Moreover, comparison of survival of patients with tumors harboring G12V/A *KRAS* mutations with those harboring wild-type *KRAS* gene revealed that G12V/A *KRAS* mutations are prognostic biomarkers for inferior PFS and OS in patients with mCRC treated with bevacizumab in univariate as well as multivariable analyses. Prospective studies on the predictive role of specific *KRAS* mutations should be performed to confirm these results and to evaluate whether G12V/A *KRAS* mutations are feasible predictive biomarker for the selection of patients for the treatment with bevacizumab in clinical practice.

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Compliance with ethical standards

Conflicts of interest JF has received honoraria from Astra Zeneca, Roche, and Novartis for consultations and lectures unrelated to this project. BM has received honoraria from Astra Zeneca, Roche, Merck, Amgen, and Novartis for consultations and lectures unrelated to this project. TB has received honoraria from Roche for consultations and lectures unrelated to this project. OF, VMM, LH, JK, ZB, MB, VL, OT, and MS declare that they have no actual or potential conflict of interest including any financial, personal, or other relationships with other people or organizations that could inappropriately influence this work.

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