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K-Ras gene mutation status as a prognostic and predictive factor in patients with colorectal cancer undergoing irinotecan- or oxaliplatin-based chemotherapy

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Keywords: colorectal cancer, *B-Raf* mutation, *K-Ras* mutation, irinotecan, oxaliplatin, anti-EGFR therapy, RAS/RAF/mitogen-activated protein kinase

Background: CRC caused more than 600,000 estimated deaths in 2008. Dysregulated signaling through the RAS/RAF/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathway due to mutations in *K-Ras* and *B-Raf* are common events in CRC.

Methods: Incidence of mutations in codons 12 and 13 of *K-Ras* and exons 11 and 15 of *B-Raf* were analyzed in amplified PCR products from primary tumors of 273 patients with CRC, and their prognostic and predictive significance was assessed. The prognostic role of clinical and pathological factors was also examined.

Results: *K-Ras* mutations were present in 89 patients (32.6%), of whom 76 (85.4%) had mutations in codon 12 and 10 (11.2%) had mutations in codon 13. *B-Raf* gene mutations were present in 17 patients (6.9%), of whom 6 (35.3%) had mutations in exon 15. Multivariate analysis revealed a predictive significance for *K-Ras* mutations with respect to time to progression in patients treated with irinotecan and oxaliplatin as first-line chemotherapy. There was no predictive significance for *B-Raf* gene mutation status in these patients. The following risk factors were found to affect overall survival (OS) rates: primary tumor location, lymph node involvement grade, carcinoembryonic antigen (CEA) level before treatment, and performance status according to WHO criteria.

Conclusions: Based on the results of this study, *K-Ras* mutation status may be a suitable indicator of patient eligibility and a prognostic indicator for responsiveness to anti-EGFR therapy alone, or in combination with chemotherapy. Also, *K-Ras* mutation status may predict time to progression in patients treated with irinotecan and oxaliplatin.

Introduction

Colorectal cancer (CRC) is the third most common cancer in men (663,000 cases) and second in women (571,000) in the world, with more than one million newly diagnosed cases reported annually. Approximately 608,000 CRC deaths are estimated worldwide each year, accounting for 8% of all cancer deaths and making it the fourth most common cause of death from cancer.[1]

Ras proteins are proto-oncogenes that function as molecular switches. In response to various hormones, cytokines, mitogens, and differentiation and growth factors such as epidermal growth factor (EGF) acting via the EGF receptor (EGFR), GTP-bound RAS regulates a number of critical cellular processes, including gene expression, mitosis, embryogenesis, cell differentiation, movement, metabolism and programmed death.[2] RAS maintains these cellular phenotypes by regulating the activation of multiple downstream effector pathways, including the RAF/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathway.[3-6]

Dysregulated signaling through this pathway due to mutations and genetic alterations in pathway components and/or upstream activators can lead to constitutive activation independent of EGFR signaling and uncontrolled cell proliferation. Indeed, constitutive activation of this pathway is found in many human cancers. Approximately 15–30% of all cancers have mutations in RAS family genes,[7] with mutations in the *K-Ras* gene accounting for nearly 80% of these[8] and 40% of all CRC.[9,10] K-RAS codons 12 and 13 are the most common sites of oncogenic activation, with over 90% of mutations.[11] Amino acid alterations at these codons, which are adjacent to the GDP/GTP binding pocket, reduce or abolish GTPase activity of K-RAS and lock the protein in an active, GTP-bound state. As a result, this "dominant

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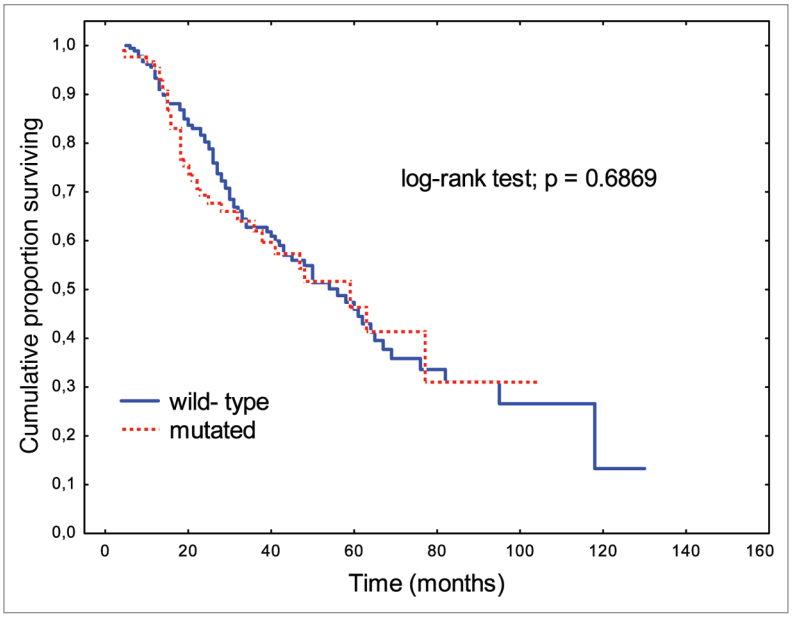

Figure 1. Overall survival (OS) rates in patients with *K-Ras* gene mutations relative to those with wild-type gene.

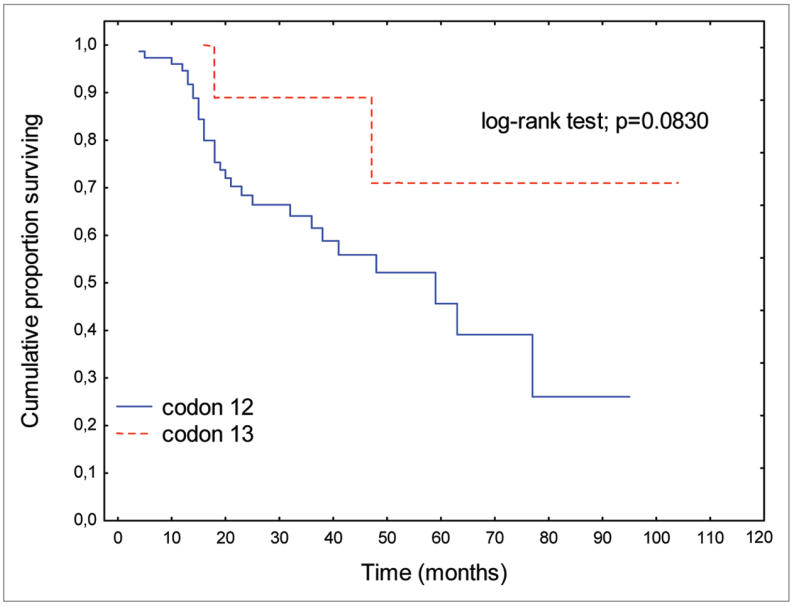

Figure 2. OS rates in patients with *K-Ras* gene mutations in codon 12 relative to codon 13.

active" mutant KRAS and its downstream effectors become independent of epidermal growth factor (EGFR), among others.

Somatic mutations in BRAF are associated with malignant melanomas,[12] CRC,[13] ovarian cancer[14] and papillary thyroid carcinomas.[15] Over 30 single-site missense mutations in the *B-Raf* gene have been identified in human cancers, mostly within the kinase domain.[16] These mutations likely insert a negatively charged residue adjacent to sites of regulatory phosphorylation, mimicking it in the activation segments of BRAF. A *Glu* for *Val* substitution at residue 599 in the activation segment accounts for over 90% of BRAF mutations in human cancers. This V599E BRAF mutant shows highly elevated kinase activity and stimulates ERK activity constitutively independent of RAS activation.[16,17]

The introduction of molecular biological techniques has facilitated the identification of hitherto unknown factors that influence both prognosis (prognostic markers) and response to previously administered anticancer therapy (predictive markers).

The aim of this study was to analyze the incidence of mutations in the *K-Ras* and *B-Raf* genes in patients with CRC, and to assess their significance as prognostic and predictive factors. Additionally, we also examined the potential role of selected clinical and pathological variables as prognostic factors.

Results

Patient characteristics. Patient characteristics are summarized in **Table 1**. The median age of the patients included in this study was 65 y (181 women, 92 men). Most underwent primary tumor resection (260/273 patients, 95.2%), while secondary metastatic disease was diagnosed in 194 patients (71.1%), of whom 70 (36.1%) underwent resection and 22 (11.3%) underwent thermoablation. The primary tumor was located in the colon in 112 patients (41.1%), sigmoid colon in 100 patients (36.6%) and rectum in 61 patients (22.3%). The metastases were located in the liver in 129 (66.5% of patients with metastases), in the lungs in 39 (20.1%), and in other organs in 126 patients (64.9%). Pretreatment carcinoembryonic antigen (CEA) levels were elevated above the normal range in 89 patients (32.6%).

***K-Ras* and *B-Raf* gene mutation status.** *K-Ras* gene mutations were present in 89 patients (32.6%), of whom 76 (85.4%) had mutations in codon 12 and 10 (11.2%) had mutations in codon 13. Women showed a higher incidence of *K-Ras* gene mutations relative to men ($p = 0.0290$). No significant differences were observed with respect to tumor size, lymph node involvement grade, histological grade, histopathological type, primary tumor localization, performance status, age, or pretreatment CEA level.

B-Raf gene mutations were present in 17 patients (6.9%), of whom 6 (35.3%) had mutations in exon 15. One patient had a mutation in exon 11, while mutation status was not determined in 10 patients (58.8%). A higher incidence of *B-Raf* gene mutations was detected in patients with low-grade neoplasm ($p < 0.0001$), primary tumor localization outside the sigmoid colon ($p = 0.0467$) and with non-tubular neoplasms ($p = 0.0468$). Other parameters assessed were not statistically different.

Prognostic significance of *K-Ras* and *B-Raf* gene mutation status. There were no significant differences in OS rates between

Table 1. Patient and tumor characteristics

Patients (n = 273)	
Age in years (median age, age range)	65 (25–85)
Gender	
Female	181 (66.3%)
Male	92 (33.7%)
***K-Ras* gene mutation status**	
Mutation	89 (32.6%)
Codon 12	76 (27.8%)
Codon 13	10 (3.7%)
Undetermined localization	3 (1.1%)
Wild-type	184 (67.4%)
***B-Raf* gene mutation status**	
Mutation	17 (6.2%)
Exon 11	1 (0.3%)
Exon 15	16 (5.9%)
Undetermined status of mutation	0 (3.7%)
Wild-type	46 (90.1%)
Primary tumor localization	
Colon	112 (41.1%)
Sigmoid colon	100 (36.6%)
Rectum	61 (22.3%)
Localization of metastases	
Liver	129 (66.5%)
Lungs	39 (20.1%)
Other localizations	126 (64.9%)

Table 2. Univariate and multivariate analysis of OS rate (log-rank test)

Univariate analysis			
Clinical parameter	**n**	**Median OS (months)**	**p value**
Age			
< 75 years	231	58.9	0.0472
≥ 75 years	38	36.7	
Gender			
Male	92	42.6	0.0328
Female	181	62.7	
Primary tumor localization			
Sigmoid colon	100	68.0	0.0039
Colon/Rectum	173	43.5	
WHO performance status			
0-1	258	58.4	0.0027
2-3	15	19.0	
Karnofsky performance status			
≤ 80	16	19.4	0.0036
> 80	257	58.1	
Lymph node involvement grade			
Involved lymph nodes	60	65.3	0.0031
Uninvolved lymph nodes	231	46.3	
Pretreatment CEA level (ng/ml)			
≤ 5	170	25.6	< 0.0001
> 5	89	76.3	

Multivariate analysis		
Clinical parameter	**Multivariate analysis**	
	HR (95% CI)	**p value**
Primary tumor localization		
Sigmoid colon vs. Rectum/Colon	0.53 (0.35–0.81)	0.0032
Lymph node involvement grade		
Involved vs. Uninvolved	1.94 (1.17–3.24)	0.0107
WHO performance status		
0–1 vs. 2	0.34 (0.18–0.64)	0.0008
Karnofsky performance status		
≤ 80 vs. > 80	NS	> 0.05
Pretreatment CEA level (ng/ml)		
≤ 5 vs. > 5	2.68 (2.09–3.44)	< 0.0001
Age		
≤ 75 vs. > 75 years	NS	> 0.05
Gender		
Male vs. female	NS	> 0.05

NS, not significant.

patients with *K-Ras* mutations and wild-type *K-Ras* genes (p = 0.6869; **Fig. 1**). A perceptible trend to prolongation of OS was apparent when *K-Ras* mutations were present in codon 13 relative to codon 12 (p = 0.0830; **Fig. 2**).

Similarly, mutations in the *B-Raf* gene showed no prognostic significance (**Fig. 3**). Patients with disseminated CRC (M+) and *B-Raf* gene mutations tended toward shorter OS relative to those with wild-type *B-Raf* genes (p = 0.06723).

Clinical and pathological variables identified by univariate analysis as potential prognostic factors for OS rate. These results are summarized in **Table 2**. Univariate analysis identified the following prognostic factors as influencing OS rate in this patient cohort: Age, patients 75 years and older lived for 36.7 mo relative to those younger than 75 (58.9 mo) (p = 0.0472); Gender, female patients lived for 62.7 mo relative to 42.6 mo for male patients (p = 0.0328); Primary tumor localization, patients with primary tumors in the sigmoid colon lived for 68.0 mo compared with 43.5 mo in patients with primary tumors located in the colon or rectum (p = 0.0039); Performance status, patients with a good performance score e.g., WHO 0–1 (58.4 mo) and Karnofsky status 81–100% (58.1 mo) lived longer relative to those with poor performance status (19.0 and 19.4 mo for patients with WHO 2–3 and Karnofsky status ≤ 80%, respectively) (p = 0.0027 and p = 0.0036, respectively); Lymph node involvement grade, survival in patients without lymph node metastases was 65.3 mo relative to 46.3 mo in patients presenting with metastases (p = 0.0031); Pretreatment CEA level, median time to progression in patients with normal pretreatment CEA level ≤ 5 ng/ml was 76.3 mo relative to 25.6 mo in patients with increased pretreatment CEA level (p < 0.0001; **Table 2**).

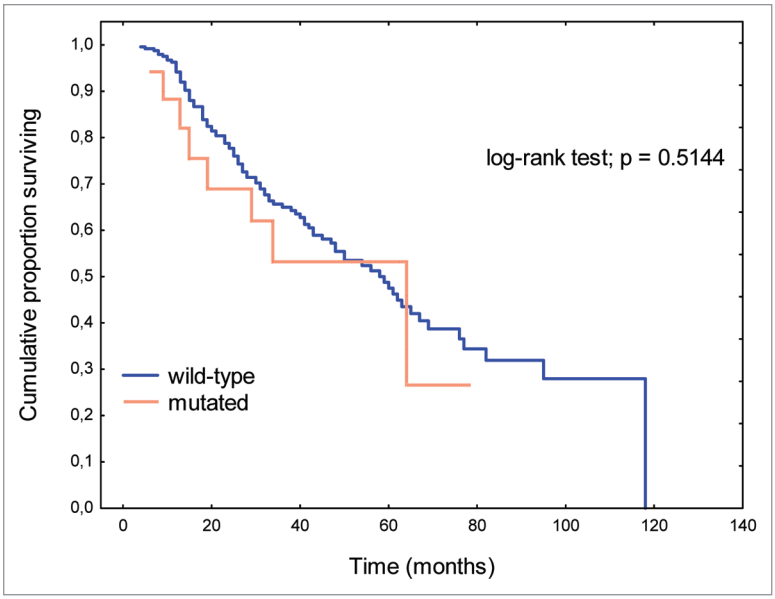

Figure 3. OS rates in patients with *B-Raf* gene mutations relative to those with wild-type gene.

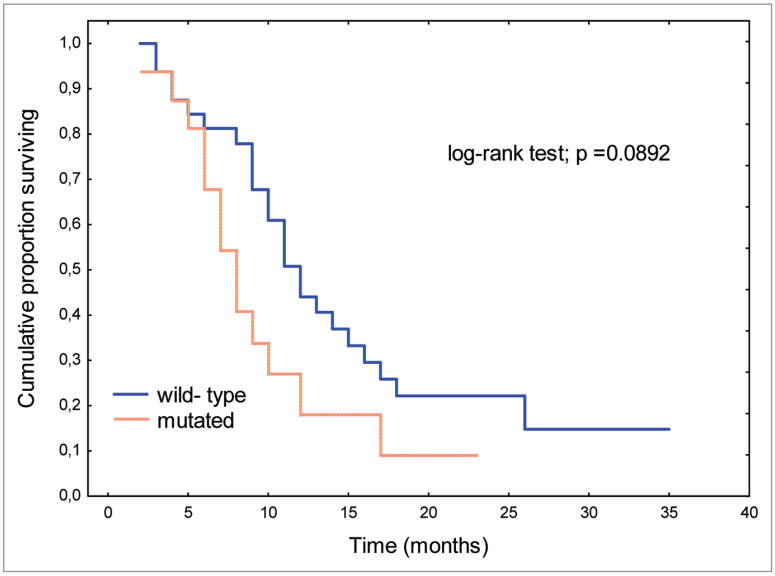

Figure 4. Time to progression according to *K-Ras* gene mutation status.

Other clinical parameters such as histological differentiation grade and primary tumor size showed no significant differences between groups.

Clinical and pathological variables identified by multivariate analysis as potential prognostic factors for OS rate. These results are summarized in **Table 2**. Multivariate analysis identified the following independent prognostic factors affecting OS rates: primary tumor localization (HR 0.53; p = 0.0032); pretreatment CEA level (HR 2.68; p < 0.0001); WHO performance status (HR 0.34; p = 0.0008); lymph node involvement grade (HR 1.94; p = 0.0107).

Other clinical parameters such as age, gender, and Karnofsky performance status showed no significant differences in this analysis.

Predictive roles of *K-Ras* and *B-Raf* mutations on time to progression in CRC patients treated with irinotecan-based first-line palliative chemotherapy on the basis of univariate analysis. These results are summarized in **Table 3**. Patients with higher pretreatment levels of CEA (> 5 ng/ml) showed a median time to progression of 9.0 mo relative to 13.0 mo in patients with normal levels (≤ 5 ng/ml, p = 0.0085). Patients without resection of metastases showed a median time to progression of 9.0 mo relative to 14.0 mo in patients who underwent resection (p = 0.0131). Patients with *K-Ras* gene mutations showed a median time to progression of 9.0 mo relative to 11.0 mo in those with the wild-type *K-Ras* gene (p = 0.05883). Other clinical parameters including histological differentiation grade, primary tumor location and size, lymph node involvement grade, and *B-Raf* gene mutation status showed no predictive significance in this analysis.

Predictive roles of *K-Ras* and *B-Raf* mutations on time to progression in CRC patients treated with irinotecan-based first-line palliative chemotherapy on the basis of multivariate analysis. These results are summarized in **Table 3**. Multivariate analysis identified the following independent favorable predictive factors in patients with disseminated CRC treated with irinotecan-based first-line palliative chemotherapy: Wild-type *K-Ras* gene (HR 0.59; p = 0.0459) and normal pretreatment CEA levels (HR 0.52; p = 0.0065).

However, this analysis did not reveal any significant differences between patients with and without resection of metastases, with different histological types of neoplasms and *B-Raf* gene mutation status.

Predictive roles of *K-Ras* and *B-Raf* mutations on time to progression in CRC patients treated with oxaliplatin-based first-line palliative chemotherapy on the basis of univariate analysis. These results are summarized in **Table 4**. Univariate analysis of time to progression in patients treated with oxaliplatin-based first-line chemotherapy regimens reveals that increased CEA levels and resection of metastases exerted significant influences on median time to progression. Patients with increased pretreatment CEA levels had a time to progression of 8.0 mo compared with 13.0 mo in patients with normal CEA levels (p = 0.0084). Patients without resection of metastases had a time to progression of 9.0 mo relative to 16.0 mo in patients who underwent resection (p = 0.0226). Patients with tubular tumors showed a time to progression of 9.0 mo compared with 13.0 mo in those with other histological types (p = 0.0462). Patients with *K-Ras* gene mutations did not show a significant difference in time to progression when treated with oxaliplatin chemotherapy, when compared with those with the wild-type *K-Ras* gene (**Fig. 4**). The significance of *B-Raf* gene status, WHO performance status, and Karnofsky performance status could not be assessed.

Predictive roles of *K-Ras* and *B-Raf* mutations on time to progression in CRC patients treated with oxaliplatin-based first-line palliative chemotherapy on the basis of multivariate analysis. These results are summarized in **Table 4**. Multivariate analysis identified resection of metastases (HR 0.43; p = 0.0249) and wild-type *K-Ras* gene (HR 0.49; p = 0.0451) as independent favorable predictive factors in patients with disseminated CRC who were treated with oxaliplatin-based first-line palliative chemotherapy regimens.

However, no statistically significant effects of CEA levels and types of neoplasm could be seen. The significance of *B-Raf* gene status, WHO performance status, and Karnofsky performance status could not be assessed due to the small number of patients.

Discussion

Cancer treatment is increasingly based on targeted therapy, i.e., morphological identification of tumor histology, tumor staging and identification of target pathways and molecules. New insights into signaling processes gone astray in carcinogenesis broaden the scope of molecular diagnosis in cancer. Identification and validation of new prognostic and prognostic markers allow physicians to offer patient-targeted therapy from a broader range of options.

Presently known biomarkers for CRC include the genetic instability status of the tumor, KRAS mutation status as a negative predictive marker for the overall rate of response to anti-EGFR treatment in patients with metastatic cancer, and BRAF mutation as an unfavorable prognostic marker.[18]

The introduction of molecularly targeted drugs for the treatment of advanced CRC is based on emerging data on the molecular mechanisms responsible for its origin and development. Disturbances in the RAS/RAF/MEK/ERK signaling pathway are the most frequent and perhaps the most important observed defects, with activating mutations in the *K-Ras* and *B-Raf* genes playing key roles.

The aims of this study were to evaluate the incidence of *B-Raf* and *K-Ras* gene mutation in patients with CRC regardless of disease stage, and to determine the prognostic significance of these mutations on time to progression in response to treatment with palliative chemotherapy. The role of select clinical and pathological variables as potential prognostic factors was also examined.

Our analysis revealed *K-Ras* gene mutations in our patient population with an incidence of 32.6% with most *K-Ras* mutations located in codon 12 (27.8%) compared with codon 13 (3.7%), similar to previously reported data.[8,19] We estimate the incidence of *B-Raf* gene mutations at 6.2%, occurring predominantly in exon 15. Further, our analysis shows that *B-Raf* mutations in exon 15 (V599E) account for nearly 90% of all mutations. These results are similar to previously published data.[16,17,20]

Interestingly, women present with a higher rate of *K-Ras* gene mutations relative to men. A higher incidence of *B-Raf* mutations was seen in patients with low-grade neoplasms, primary tumor location outside the sigmoid colon, and neoplasms other than tubular.

In our analysis, no significant influence on survival was seen in patients with mutations either in the *K-Ras* or *B-Raf* genes relative to the general population. However, patients with *K-Ras* mutations in codon 12 showed significantly decreased survival rates compared with those with mutations in codon 13.

Table 3. Univariate and multivariate analysis of time to progression (log-rank test) for irinotecan-based chemotherapy

Univariate analysis			
Clinical parameter	**n**	**Median time to progression (months)**	**p value**
Age			
< 75 years	79	11.0	0.9099
≥ 75 years	1	-	
Gender			
Male	48	12.0	0.1598
Female	32	9.0	
Primary tumor localization			
Sigmoid colon	27	11.0	0.6440
Colon/Rectum	53	10.1	
WHO performance status			
0–1	79	11.0	0.3185
2–3	1	-	
Karnofsky performance status			
≤ 80	79	-	0.3185
> 80	1	11.0	
***B-Raf* gene mutation status**			
Mutation	4	10.5	0.2909
Wild-type	69	11.0	
***K-Ras* gene mutation status**			
Mutation	4	9.0	0.05883
Wild-type	76	11.0	
Pretreatment CEA level (ng/ml)			
≤ 5	38	13.0	0.0085
> 5	40	9.0	
Resection of metastases			
Yes	27	14.0	0.0131
No	53	9.0	

Multivariate analysis		
Clinical parameter	**Multivariate analysis**	
	HR (95% CI)	**p value**
Histological type		
Tubular vs. others	NS	> 0.05
***K-Ras* gene mutation status**		
Mutation vs. wild-type	0.59 (0.35–0.99)	0.0459
***B-Raf* gene mutation status**		
Mutation vs. wild-type	NS	> 0.05
Pretreatment CEA level (ng/ml)		
≤ 5 vs. > 5	0.52 (0.33–0.83)	0.0065

NS, not significant.

Table 4. Univariate and multivariate analysis of time to progression (log-rank test) for oxaliplatin-based chemotherapy

Univariate analysis			
Clinical parameter	**n**	**Median time to progression (months)**	**p value**
Age			
< 75 years	47	10.0	0.9252
≥ 75 years	2	-	
Gender			
Male	25	11.0	0.6149
Female	24	9.7	
Primary tumor localization			
Sigmoid colon	24	11.6	0.2375
Colon/Rectum	25	9.0	
WHO performance status			
0–1	49	10.0	0.3185
2–3	0	-	
Histological type			
Tubular	22	13.0	0.0462
Others	27	9.0	
Pretreatment CEA level (ng/ml)			
≤ 5	25	13.0	0.0084
> 5	21	8.0	
Resection of metastases			
Yes	18	16.0	0.0226
No	31	9.0	

Multivariate analysis		
Clinical parameter	**Multivariate analysis**	
	HR (95% CI)	**p value**
Histological type		
Tubular vs. others	NS	> 0.05
Resection of metastases vs. no resection of metastases	0.43 (0.21–0.90)	0.0249
***K-Ras* gene mutation status**		
Mutation vs. wild-type	0.49 (0.24–0.99)	0.0451
Pretreatment CEA level (ng/ml)		
≤ 5 vs. > 5	NS	> 0.05

NS, not significant.

Previous studies have shown that mutations of the K-Ras gene in patients with metastatic CRC are a predictive marker of poor response to anti-EGFR therapy alone or in combination with chemotherapy, relative to patients with WT tumors.[21-28] However, Richman et al.[23] could not establish any prognostic significance of *K-Ras* and *B-Raf* mutations in patients with disseminated CRC and treated only with chemotherapy.

Our study did not establish a prognostic role for *B-Raf* mutation status in CRC patients in contrast to the results obtained by Tol et al.,[29] who observed significantly shorter OS in patients with these mutations. Their retrospective analysis was conducted in a relatively small group of patients with stage IV disease being treated with anti-EGFR therapy, which may have significantly affected survival rates in patients with WT tumors. Similarly, a very high incidence of mutations may account for the differences between our data and results from a previous study in which *B-Raf* mutations had a prognostic significance in patients with stage II or III CRC.[30] In the present analysis, a subgroup of patients with disseminated CRC (M+) and wild-type B-Raf genes tended toward longer survival rates relative to those with B-Raf mutations, although this difference was not statistically significant.

In this study, univariate analysis of the role of clinical and pathological variables revealed a positive, statistically significant influence of the following factors on overall patient survival: female gender, primary tumor localization in sigmoid colon, CEA level within normal limits, good performance status (WHO: 0–1 or Karnofsky Performance Status Scale 81–100%) and lack of metastases in regional lymph nodes. Multivariate analysis identified primary tumor localization in sigmoid colon, lack of metastases in regional lymph nodes, CEA level within normal limits and good performance status according to WHO criteria (0–1) as favorable independent prognostic factors.

Lagautriere et al.[31] conducted a retrospective analysis of CRC patients being treated surgically to determine prognostic factors, and identified age, preoperative CEA level, performance status, ileus, and clinical and pathological staging as influencing OS. On the other hand, clinical parameters such as gender, primary tumor localization, and pathological staging had no influence. The retrospective nature of their analysis and differences in inclusion criteria between studies may account for observed differences. Other studies did not show an effect of age on patient survival,[32,33] although the prognostic significance of primary tumor localization relative to other localizations has been observed.[34]

The predictive significance of molecular factors in response to treatment is a fundamental problem in oncology. Available data concerning possible influence of molecular parameters on chemotherapy treatment is strictly limited. Therefore, we performed an analysis of the influence of *K-Ras* and *B*-Raf mutations on time to progression in CRC patients being treated with palliative first-line chemotherapy based on irinotecan and oxaliplatin.

Multivariate analysis revealed a predictive significance for *K-Ras* mutations with respect to time to progression in patients treated with chemotherapy based on irinotecan and oxaliplatin as first-line chemotherapy. However, there was no predictive significance for *B-Raf* gene mutation status in patients treated with irinotecan or oxaliplatin (evaluation not performed due to a small n). Both univariate and multivariate analyses of time to progression in patients treated with irinotecan showed that pretreatment CEA level was a predictive factor. Resection of metastases was found to be a statistically significant predictive factor by univariate, but not by multivariate analysis. Additionally, univariate analysis revealed that pretreatment CEA level and histopathological type of neoplasm also influence time to progression. However, these factors were not identified by multivariate analysis.

To sum up, *K-Ras* mutation status, pretreatment CEA level and resection of metastases appear to be predictive of time to progression in CRC patients treated with chemotherapy regimens based on irinotecan and oxaliplatin in first-line therapy. Our results regarding CEA level and resection of metastases are similar to those published by Fong et al.[35] and confirms the predictive significance of *K-Ras* and *B-Raf* gene status in patients treated with targeted anti-EGFR therapy.[23-26,35-38]

There is not much evidence for the predictive significance of K-Ras and B-Raf gene mutation status in patients treated solely with chemotherapy, including that based on irinotecan and oxaliplatin.[23] Further, most studies examined the effects of treatment with single-agent anti-EGFR therapy or its combination with chemotherapy. Similarly, predictive significance could not be established for *K-Ras* gene mutations in patients with colorectal cancer, non-small cell lung carcinoma and other solid tumors treated with conventional chemotherapy.[38]

Our results suggest that determination of K-Ras and B-Raf mutation status in patients qualified for anti-EGFR therapy alone or in combination with chemotherapy can greatly assist in predicting the success or failure of these treatments. Moreover, K-Ras mutation status should be determined in patients qualified for chemotherapy based on irinotecan or oxaliplatin. The role of B-Raf mutation status remains unclear.

Patients and Methods

Ethical approval for research. The research approved by the appropriate local ethical committees (reference numbers WIM-50/2008 and WIM-45/2009).

Patients. Two hundred and seventy-three consecutive patients (median age 65 y, range 25–85 y) with CRC who were treated between 2006 and 2010 at the Oncology Department of the Military Institute of the Heath Services, Warsaw, were included in this study (see **Table 1** for an overview of patient characteristics).

Inclusion criteria consisted of a confirmed histopathological diagnosis of colorectal cancer, availability of adequate primary tumor material and a lack of effect of chemotherapy or radiotherapy on the tumor.

Surgically removed primary tumor tissue specimens were fixed in formalin and converted into paraffin blocks for further analysis.

Tumor specimens and histological examination. Primary tumor tissue collected from colorectal cancer patients was fixed in 10% neutral buffered formalin for 24 h and converted into paraffin blocks. Serial 5 μm-thick sections of each paraffin block corresponding to representative areas of the tumors were stained with hematoxylin/eosin (H&E) and the presence of tumor tissue verified by an experienced pathologist.

DNA isolation. DNA from paraffin-embedded tissue was prepared from 10–30 μm sections after macrodissection, to ensure they contained at least 80% tumor cells. Tissue samples were extracted with xylene and ethanol to remove paraffin and placed in 1% SDS/proteinase K (10 mg/ml) at 56°C overnight. DNA was isolated using the NucliSens easyMAG platform (bioMérieux) for automated nucleic acid extraction.

***K-Ras* and *B-Raf* mutation analysis.** Mutation analysis at codons 12 and 13 of the *K-Ras* gene, and exons 11 and 15 of the *B-Raf* gene was performed by direct sequencing of amplified PCR products. Genomic DNA was amplified by PCR using the following primers: FS 5'-TCA TTA TTT TTA TTA TAA GGC CTG CTG-3', RS 5'-CAA GAT TTA CCT CTA TTG TTG GAT CA-3' (for codons 12 and 13 in exon 2 of *K-Ras*), BF11 5'-TCC CTC TCA GGC ATA AGG TAA-3', BR11 5'-TTA TTG ATG CGA ACA GTG AAT AT-3' (for a glycine-rich loop region in exon 11 of the *B-Raf* gene), B2F 5'-TCA TAA TGC TTG CTC TGA TAG GA-3', B1R 5'-TAACTCAGCAGCATCTCAGG-3' (for activation domain in exon 15 of the *B-Raf* gene). PCRs were performed in a total volume of 10 μl containing 2 μl of extracted genomic DNA, 1X PCR buffer, 1.5 mmol/L $MgCl_2$, 0.2 μmol/L of each primer, 0.1 mmol/L dNTPs and 1U of Taq DNA polymerase (EURx Ltd.).

PCR conditions were as follows: 95°C for 10 min and 40 cycles of 95°C for 20 sec, 56°C for 30 sec (*K-Ras* and *B-Raf* in exon 11), 57°C for 30 sec (*B-Raf* in exon 15), 72°C for 30 sec, and finally 5 min at 72°C. Amplification products were purified using the DNA Gel-Out Kit (DNA GDANSK). Automated sequencing was performed using the Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems).

Sequencing reactions were purified using the ExTerminator Kit (DNA GDANSK), and analyzed on an ABI PRISM 377 DNA sequencer (Applied Biosystems). A wild-type control DNA sample (without *K-Ras* and *B-Raf* mutations) and a known mutation sample were also included in the experiment. The presence of a mutation was confirmed by sequencing at least two independent PCR products.

Enriched PCR-RFLP analysis for *K-Ras* codon 12 mutations detection. Detection of *K-Ras* mutations in codon 12 was performed by enriched non-radioactive single-step PCR-restriction fragment length polymorphism (RFLP) as described previously (Banerjee et al., 1997), with some modifications.

First-round PCR primers K1 *5'-ACT GAA TAT AAA CTT GTG GTA GTT GGA CCT-3'* and DD5P *5'-TCA TGA AAA TGG TCA GAG AA-3'* were designed to create a restriction site for the restriction endonuclease BstOI (Promega) within the amplified product. The upstream primer K1 is immediately upstream of *K-Ras* codon 12 and introduces a G to C substitution at the first position of codon 11, creating a BstOI restriction site (5'-CCTGG-3') in the amplified fragment. This site overlaps with the first 2 nucleotides of codon 12 and is lost when a codon 12 mutation is present. As a result, the restriction endonuclease BstOI recognizes the sequence 5'-CCTGG-3' in *K-Ras* codon 12 wild-type PCR products and digests them, without affecting mutant PCR products.

Second-round PCR primers K1 and K2 5'-TCA AAG AAT GGT CCT GGA CC-3' created another restriction site in the final segment of the PCR product, which served as an internal control for the restriction digestion. PCR products containing codon 12 mutations were mainly amplified in the second round, because wild-type products were digested in the previous step. These products will contain only one restriction site for BstOI near their 3'-end. Any non-digested PCR products containing

wild-type codon 12 sequence that are amplified during the second PCR round will contain two BstOI restriction sites—one identical to that in the mutant molecules and the second overlapping with the codon 12 sequence (introduced by the K1 primer).

The products of the second PCR amplification were also digested with BstOI. The digestion products were electrophoresed on a 3% agarose gel and stained with ethidium bromide. Non-restricted PCR products were 157 bp, wild-type products were 113 bp and mutant codon 12 products were 142 bp in size. A normal control DNA sample (without the *K-Ras* codon 12 mutation) and a known mutation sample were included in all experiments. The results of PCR detection were verified by direct DNA sequencing.

Statistical analysis. The chi-square test was used to investigate the differences between the two treatment groups with respect to baseline characteristics and response rates. Time to disease progression and overall survival (OS) were summarized as Kaplan-Meier estimates. The log-rank test was used in the Kaplan-Meier survival analyses to assess the effect of variables on time to disease progression and OS.

Multivariate analyses of time to disease progression and OS were performed by Cox proportional-hazard regression using the forward stepwise method; all variables found to be significant in the univariate analysis were included in the multivariate analysis. Statistical calculations were performed using STATISTICA for Windows Version 7.0 software.

Disclosure of Potential Conflicts of Interest

The authors declare that they have no competing interests.

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