

Mutation pattern of *KRAS* and *BRAF* oncogenes in colorectal cancer patients

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Received August 8, 2011 / Accepted January 17, 2012

The aim of this study was to identify *KRAS* and *BRAF* gene mutations in colorectal cancer patients and to assess whether they are linked with clinicopathological features. The results of *KRAS* and *BRAF* mutation analysis could be used in the selection of patients for anti-EGFR therapy. All specimens were obtained during routine surgery of patients with colorectal carcinoma. The diagnoses were established by standard procedures and confirmed histopathologically. After DNA extraction, *KRAS* mutations were analyzed using quantitative real-time PCR and *BRAF* mutations were analyzed using real-time PCR by fluorescence melting curve analysis. Our results show that *KRAS* gene mutations were detected in 35.6% samples and the most frequent mutation was Gly12Val. *BRAF* gene mutation Val600Glu was detected in 8.5% samples. Statistical analysis revealed a significant association between the *KRAS* mutation and Dukes' stage ($p=0.034$), with the lowest frequency in Dukes'A, and between the *KRAS* mutation and histological grade ($p=0.044$), with no *KRAS* mutation found in poor differentiated tumors. The first data about *KRAS* and *BRAF* mutational status in the sample of Croatian population with colorectal cancer shows that the incidence of *KRAS* and *BRAF* mutations is within generally valid limits. Prospective studies are to be continued in order to determine whether these mutations contribute to progression of colorectal cancer.

Key words: colorectal cancer, *KRAS*, *BRAF*

Colorectal cancer (CRC) is one of the most common forms of malignant neoplasia and frequently takes a fatal course following metastasis (1). The development of CRC is a multistep process which involves successive genetic alterations (2). Mutations of the genes encoding the Kirsten rat sarcoma viral oncogene homologue (*KRAS*) and the v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) have been implicated in colorectal carcinogenesis (3). Their products mediate cellular responses through RAS/RAF/MAP kinase signal pathway. Activating mutations of these oncogenes are point mutations (missense mutations) and they arise early during the development of colorectal cancer (3). *KRAS* gene mutations have been detected in 30-40% of all patients with CRC and have been linked to poor outcomes. *BRAF* gene mutations have been detected in 5-15% of CRCs, but *KRAS* and *BRAF* mutations appear to be mutually exclusive (4,5,6).

The epidermal growth factor receptor (EGFR), a tyrosine kinase receptor belonging to the ErbB family, has been

found to play a significant role in the pathogenesis of CRC by inducing downstream signaling pathways, such as the phosphatidylinositol-3-kinase/Akt and RAS/RAF/MAP-activated protein kinase (MAPK) pathways, which are crucial in the regulation of cell growth, proliferation, migration, angiogenesis, invasion and apoptosis (7). The EGFR signaling pathway is overexpressed in about 80% of CRCs and has been the focus of a new drug development (8). Consequently, antibodies targeting EGFR, such as cetuximab and panitumumab, have been examined for therapeutic efficacy in CRC patients (9). Initial evaluation of these new drugs as monotherapy in patients with EGFR-expressing chemotherapy-refractory tumors yielded response rates of approximately 10% (10-13). The realization that detection of positive EGFR expression by immunostaining does not reliably predict clinical outcome of EGFR-targeted treatment has led to an intense search for alternative predictive biomarkers. Oncogenic activation of signaling pathways downstream of the EGFR, such as mutation of *KRAS* or

BRAF, is important for the progression of colorectal cancer (14). Tumor *KRAS* mutations have emerged as an important predictive marker of resistance to panitumumab or cetuximab treatment (5,15,16). Seven different somatic missense mutations in codons 12 and 13 are relevant for therapeutic efficacy of anti-EGFR therapy (5). In addition, among colorectal tumors carrying wild-type *KRAS*, mutation of *BRAF* or *PIK3CA* or loss of *PTEN* expression may be associated with resistance to EGFR-targeted monoclonal antibody treatment, although these additional biomarkers require further validation before incorporation into clinical practice (10). The activating mutations in *KRAS* oncogene are found mostly in codons 12 and 13 (>90%) but also affect codons 61 and 146 (17,18). The most frequently observed types of mutations are G>A transitions, G>T and G>C transversions (19). Identification of somatic-activating mutations of *BRAF* has been reported in various cancers, with by far the most common mutation being a 1799T>A transversion leading to a Val600Glu (V600E) substitution (4). Until *KRAS* mutation is accepted, *BRAF* is still evolving as a powerful predictive biomarker for anti-EGFR therapy in CRC (20).

Although the role of *KRAS* in the pathogenesis and progression of CRC is widely accepted, there is no agreement on independent prognostic role of *KRAS* and *BRAF* oncogenes in CRC. With the aim to investigate their role in progression of CRC, the incidence of *KRAS* and *BRAF* mutations was established in a population of patients with CRC in Croatia and compared with clinicopathological features of tumors and patients.

Materials and methods

Tumor samples. Tissue specimens from 75 colorectal cancers were obtained from patients who underwent curative surgical resection. All specimens were paraffin embedded and stored in the archive. The patient group consisted of 29 women and 46 men aged 18-80 years (mean=60.2). Informed consent was obtained from patients and the study was performed in conformance with the Declaration of Helsinki ethical guidelines. Data on tumor size were not available for two patients, histological grade and Dukes' stage for one patient each. The presence of *KRAS* gene mutations was not known in two patients, and of *BRAF* gene mutations in another four patients. For 74 tumor samples, clinicopathological features were as follows: 13.5% (10/74) tumors were classified as Dukes' A, 23% (17/74) as Dukes' B and 63.5% (47/74) tumors as Dukes' C. The majority of tumors (67/74) were classified as well/moderately differentiated (low histological grade) while seven were poorly differentiated tumors (high histological grade). Overall, tumor size ranged from 10 to 110 mm and 28.8% (21/73) tumors were larger than 5 cm. (Table 1)

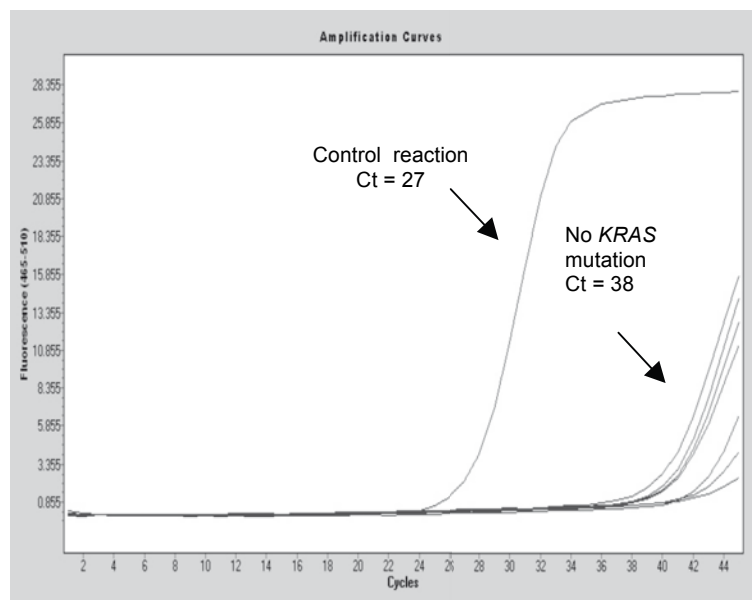
Mutation analysis. Mutational analysis for seven common somatic *KRAS* mutations and one *BRAF* point mutation was performed on 75 samples. DNA was extracted using the Qiaamp QIAamp FFPE Tissue Kit (Qiagen GmbH, Germany)

Table 1. Clinical features of the sample

Feature		Total sample
Age (years) (means+/-d.f.)		60.24+/-11.74
Gender	Male	46 (61.3)
N(percent)	Female	29 (38.7)
Tumor size	≤5	52 (71.2)
N(percent)	>5	21 (28.8)
Histological grade (differentiation)	Well/moderate	67 (90.5)
N(percent)	Poor	7 (9.5)
Dukes' stage	A	10 (13.5)
N(percent)	B	17 (23.0)
	C	47 (63.5)

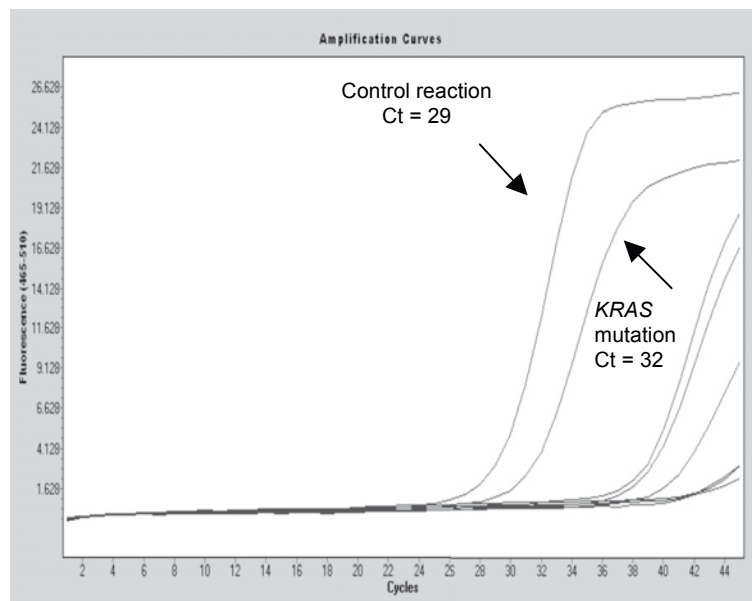
following the manufacturer's instructions. The specimen amounts used for extraction were four to six 10- μ m sections of formalin-fixed, paraffin-embedded tumor samples. After DNA extraction, we used 200-500 ng tumor DNA per reaction, depending on the available amount of the tumor tissue. Mutant *KRAS* was detected on instrument Roche LightCycler[®]480 Real-Time PCR System using *KRAS* Mutation Kit (TheraScreen[®]KRAS Mutation Kit CE-IVD, DxS, UK) that identifies seven common somatic point mutations located in exon 2 (codons 12 and 13): Gly12Asp, Gly12Ala, Gly12Val, Gly12Ser, Gly12Arg, Gly12Cys and Gly13Asp, using quantitative real-time polymerase chain reaction (Q-RT-PCR) with allele-specific primers (ARMS and Scorpions technology, ARMS/S)). Eight assays are supplied: the control assay used to assess the total DNA in a sample, and seven mutation assays used to detect the mutant DNA. Each assay is able to detect 1% mutation in the background of wild-type DNA. A central laboratory (HistoGeneX, Antwerp, Belgium) validated the assay for analytic and diagnostic performance, established acceptance criteria, and included appropriate quality controls for each assay. This method uses absolute quantification as a technique which enables us to quantify a single target sequence and express the final result as an absolute value (1% Δ Ct value for the assay) (Figures 1 and 2). It is established having very high sensitivity (1% mutant/wild-type ratio) and specificity of ARMS/S method.

Mutation in exon 15 of the *BRAF* gene were detected on instrument Roche LightCycler[®]2.0 Real-Time PCR using real-time PCR and fluorescence melting curve analysis (FMCA). All primers, probes and positive control for the most frequent *BRAF* somatic mutation Val600Glu (V600E) were purchased from TIBMolbiol (LightMix[®]KitBRAF V600E, Germany). Amplification was performed in glass 20- μ l capillaries containing Roche LightCycler Fast Start DNA Master Hybridization Probes Kit (Roche Diagnostics). Fluorescence melting peaks were obtained by plotting the negative derivative of fluorescent signal corresponding to the temperature (-dF/dT). A melting peak at 61°C indicates the presence of the *BRAF* V600E mu-



Ct = cycle threshold; $\Delta Ct = 11 > 1\% > \Delta Ct$

Figure 1. Q-RT-PCR: Negative result for *KRAS* mutation

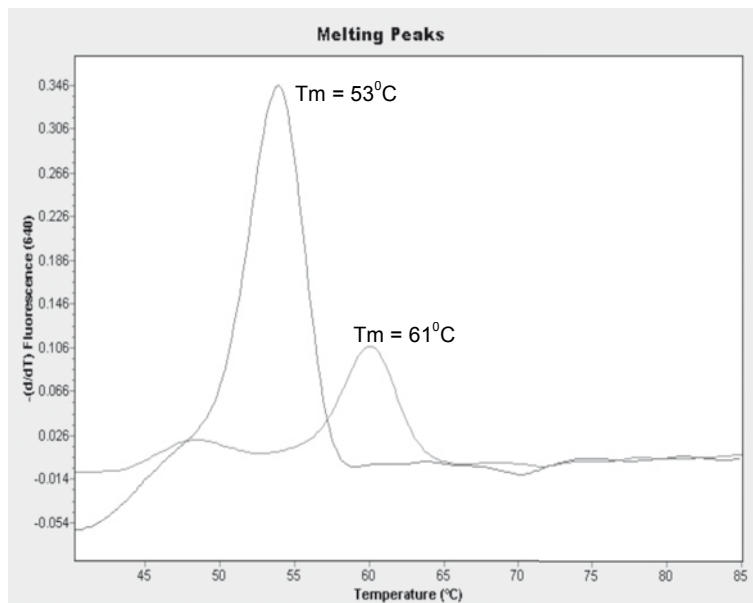


Ct = cycle threshold; $\Delta Ct = 3 < 1\% \Delta Ct$

Figure 2. Q-RT-PCR: Positive result for *KRAS* mutation Gly12Val

tated genotype while wild-type samples generate a baseline result or display a peak at 53°C due to incomplete suppression of the wild-type DNA (Figure 3). As a negative control, we used healthy colorectal mucosa. The observed sensitivity of LightMix*BRAF* V600E Kit is 0.2% mutants of *BRAF* V600E DNA since amplification of the wild-type DNA is suppressed by a competitor.

Statistical analysis. We used descriptive statistics for clinical data presentation. Differences in clinical and pathological variables between the groups of patients with and without *BRAF* and *KRAS* mutations were analyzed using t-tests for independent samples for continuous variables and Fisher exact tests for categorical variables, as appropriate. Statistical analysis was performed using SPSS version 13.0.



Tm = melting temperature; negative result Tm = 53°C (negative control, healthy colorectal mucosa), positive result Tm = 61°C (tumor tissue)

Figure 3. FMCA: Negative and positive result for BRAF Val600Glu mutation

Results

Our results of mutational analysis show that KRAS gene mutations are detected in 26/73 samples (35.6%) while BRAF gene mutations were comparatively rare events detected in 6/71 samples (8.5%).

The most frequent KRAS mutation is Gly12Val (GGT>GTT) detected in 12 samples (46.1%). Seven patients had Gly12Asp (GGT>GAT) mutation (26.9%), three patients Gly13Asp (GGC>GAC) mutation (11.6%), two patients had Gly12Cys (GGT>TGT) mutation (7.7%) and two patients had Gly12Ser (GGT>AGT) mutation (7.7%). Consistent with literature reports, the majority of KRAS mutations were found in codon 12, with a smaller number of nucleotide substitutions in codon 13. KRAS gene alterations consisted of 14 transversions (G>T) and 12 transitions (G>A), out of which twenty three mutations affected codon 12 and three mutations affected codon 13 (Table 2). Statistical analysis revealed a significant association between the KRAS mutation and Dukes' stage (p=0.034), with the lowest

frequency in Dukes' A, and between the KRAS mutation and histological grade (p=0.044), with no KRAS mutation found in poor differentiated tumors. We found no association between mutations and other clinicopathological features (Table 3).

BRAF gene mutation Val600Glu was detected in 6 samples (8.5%). All mutations were detected in tumors classified as Dukes' C. Five of six BRAF positive samples (83%) were well to moderately differentiated tumors in men, one half of positive samples were smaller and half larger than 5 cm. All BRAF mutations were found in tumors with wild-type status of KRAS gene. We found no association between BRAF mutations and clinicopathological features (Table 4).

Discussion

Mutations in the KRAS and BRAF oncogenes are thought to occur at an early stage in the adenoma-carcinoma sequence, with the frequency of mutations increasing with the tumor size (3). Specific KRAS mutations induce different

Table 2. The number and type of KRAS mutations, affected codons and corresponding altered amino acids

Codon	Type of point mutation	Number of point mutations	Wild-type codon (amino acid)	Mutated codon (amino-acid)	Putative altered amino-acid
12	G>A	9 (35%)	GGT (Gly)	GAT (Asp)	7 (27%)
			GGT (Gly)	AGT (Ser)	2 (8%)
12	G>T	14 (54%)	GGT (Gly)	GTT (Val)	12 (46%)
			GGT (Gly)	TGT (Cys)	2 (8%)
13	G>A	3 (11%)	GGC (Gly)	GAC (Asp)	3 (11%)

Table 3. Associations between *KRAS* mutation and clinicopathological features of colorectal cancer

Feature		<i>KRAS</i> (wild type)	<i>KRAS</i> (mutation)	Test
*Age (years) (means+/-d.f.)		60.67+/-10.31	59.29+/-14.33	t=0.477, df=71, p=0.635
+Gender N(percent)	Male	29 (63.0)	16 (59.3)	p=0.806
	Female	17 (37.0)	11 (40.7)	
+Tumor size N(percent)	≤5	29 (63.0)	21 (84.0)	p=0.101
	>5	17 (37.0)	4 (16.0)	
+Histological grade N(percent) (differentiation)	Well/moderate	39 (84.8)	26 (100)	p=0.044
	Poor	7 (15.2)	0 (0)	
+Dukes' stage N(percent)	A	4 (8.7)	4 (15.4)	p=0.034
	B	7 (15.2)	10 (38.5)	
	C	35 (76.1)	12 (46.1)	

*Data were calculated using two-tailed t test for independent samples;

+Data were calculated using fisher exact test for categorical variables

Table 4. Associations between *BRAF* mutation and clinicopathological features of colorectal cancer

Feature		<i>BRAF</i> (wild type)	<i>BRAF</i> (mutation)	Test
*Age (years) (means+/-d.f.)		60.25+/-12.06	61.5+/- 7.53	t=0.248, df=69, p=0.804
+Gender N(percent)	Male	40 (61.5)	5 (83.3)	p=0.404
	Female	25 (38.5)	1 (16.7)	
+Tumor size N(percent)	≤5	47 (87.0)	3 (50.0)	p=0.343
	>5	17 (13.0)	3 (50.0)	
+Histological grade N(percent) (differentiation)	Well/moderate	60 (92.3)	5 (83.3)	p=0.423
	Poor	5 (7.7)	1 (16.7)	
+Dukes' stage N(percent)	A	10 (15.4)	0 (0)	p=0.134
	B	17 (26.2)	0 (0)	
	C	38 (58.4)	6 (100)	

* Data were calculated using two-tailed t test for independent samples;

+ Data were calculated using fisher exact test for categorical variables

biological consequences by affecting differently the structural conformation and the function of the mutated protein (21). In particular, disease-free survival of CRC patients and overall survival were shown to depend on the type of *KRAS* mutation (15). In CRC, about 3000 point mutations (missense mutations) of the *KRAS* gene have been identified, with predominance in codons 12 and 13 (17,22). Approximately 90% of the activating mutations were found in codons 12 (wild-type GGT) and 13 (wild-type GGC), identifying these codons as hot spot mutation points. These two codons, coding for the two adjacent glycine residues, play an important role in the catalytic activity of *KRAS* proteins. The substitutions of 12 and 13 amino acid residues (Gly) in the *KRAS* protein with any other amino acid (except proline) altered its GTP-ase activity to a different extent and/or its ability to interact with its regulators, depending on the substituted amino acid residue (23). The *KRAS* mutations can be detected in approximately 30-40% of all patients with colorectal cancer. The most frequently observed types of mutations among the point mutations in human cancers are

C>T and G>A transitions (24). These G>A transition mutations occurred either by misreplication of the unrepaired, endogenously produced O⁶-methylguanidine from faulty S-adenosylmethionine methylation or due to exposure to nitrosamines (25-27). It was shown that *KRAS* G>C and G>T transversions in CRC were related to worse prognosis than G>A transitions (28).

In our study, we found a *KRAS* mutation in 35.6% (26/73) samples of colorectal cancer: 88% in codon 12 and 12% in codon 13. These results are consistent with reported results about frequency of *KRAS* mutations in CRC where the majority of *KRAS* mutations were found in codon 12. Among mutations in codon 13, the glycine to aspartate mutation was the most common and accounted for over 80% of the changes that occur in this codon. We found three glycine to aspartate mutations in codon 13. Most of the mutations that were identified in this study are substitutions of amino acid glycine with valine 46.1% (12/26). This substitution was reported as the second most frequent codon 12 mutation (22.5%) and also, connected with increased risk of recur-

rence and death (29). We did not find Gly12Val mutation in tumors classified as Dukes' A. Among mutations in codon 12, the substitution of glycine with aspartate has been reported as the most frequent change (32.5%), but in our study we ranged Gly12Asp only as the second most frequent mutation 26.9% (7/26).

Furthermore, the rate of transversions (G>T) in our study was found to be 54% (14/26) and of transitions (G>A) 46% (12/26), which is consistent with an observation that these are the most frequently observed types of mutations in CRC (30).

The gold standard for prognostication is clinicopathological staging or Dukes' stage but tumor grade and size have also been established as prognostic factors in CRC (31,32). We found a significant association between the *KRAS* mutation and Dukes' stage ($p=0.034$) and between the *KRAS* mutation and histological grade ($p=0.044$). The lowest frequency of *KRAS* mutations was found in Dukes' A and no *KRAS* mutation found in poor differentiated tumors. We found no association between *KRAS* mutations and other clinicopathological features. A number of studies published in the past 20 years have produced different results regarding the association between *KRAS* mutations and prognosis in CRC. Some studies have demonstrated significant differences between *KRAS* mutations in general or specific types of *KRAS* mutations and tumor stage, grade, survival and/or other clinicopathological variables, whereas others have not (15,33). The small sample size of several studies and the different methods used to detect mutations might be the basis of the discordant conclusions reached by these investigations.

In the present study, mutations in *BRAF* were identified in 8.5% (6/71) of colorectal cancers and they never occurred in association with *KRAS* mutation. Tumors with detected *BRAF* V600E mutation were classified as Dukes' C. All these data are consistent with reported results which suggest that *KRAS* and *BRAF* mutations are two different epigenetic alterations among CRCs, mutually exclusive, with possible negative prognostic role in this disease (34).

High sensitivity (1% mutant/wild-type ratio) and specificity of ARMS/S method enable us to detect the presence of *KRAS* mutation in heterogeneous specimens at a low allelic concentration without the need for confirmation by direct sequencing (35,36). For detection of *BRAF* V600E mutation the melting curve analysis was previously described indicating the melting temperature for wild type *BRAF* at 53°C, whereas the V600E mutant melted at 61°C (37). The LightCycler PCR and post-PCR melting curve analysis method for detection of *BRAF* V600E mutation with TIBMolbiol primers and probes was validated and considered a sensitive method which can be used for diagnostic purposes (38). The observed sensitivity of LightMix *BRAF* V600E Kit is 0.2% mutants of *BRAF* V600E DNA since amplification of the wild-type DNA is suppressed by competitor and was used in the recent updated analysis of overall survival according to tumor *KRAS* and *BRAF* mutation status in colorectal cancer (39).

According to recent statistics of World Health Organization, Croatia is taking the first place in the world against mortality of colorectal cancer and, because of the low rates of screening, only small number of cases are diagnosed in localized stage of this disease. As our set of colorectal cancer tissue samples was obtained from patients who underwent curative surgical resection in University Hospital Center Zagreb (year 2009/10.), we observed only small percent of tumor samples classified as Dukes' A.

This is the first study about incidence of *KRAS* and *BRAF* gene mutations in colorectal cancer of Croatian patients. Our data on *KRAS* and *BRAF* mutational status in the studied sample of colorectal cancer shows that the incidence of *KRAS* (35.6%) and *BRAF* (8.5%) mutations is within commonly accepted limits of 30-40% and 5-15%, respectively. Significant association between the *KRAS* mutations and Dukes' stage was found and suggests that the presence of these mutations may play a role in the progression of this disease in our group of patients. However, significantly higher incidence of mutation in well/moderate differentiated tumours opposes to this findings (considering the small number of poor differentiated tumours in the overall study sample). These data should be confirmed on a larger study group in prospective studies in order to determine whether these mutations contribute to progression of CRC. All obtained results could serve in selection of patients for anti-EGFR monoclonal antibody therapy and as predictive biomarkers of responsiveness to the same therapy.

Acknowledgements: This study has been carried out as part of Project No. 108-1080058-0047, Croatian Ministry of Science, Education and Sports.

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