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ORIGINAL ARTICLE

KRAS and *BRAF* mutations in Serbian patients with colorectal cancer

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Summary

Purpose: *Mutations of KRAS and BRAF genes represent molecular biomarkers of response to targeted therapy in patients with metastatic colorectal cancer (mCRC). Since these mutations have been shown to exert different biological effects and impacts on patients' outcome, there is a need to determine reliably the frequency and types of KRAS mutations for diagnostic and individual therapeutic purposes. Despite having a wild type (wt) KRAS, some patients fail to respond to treatment. BRAF V600E mutation is an additional molecular determinant of response to the same therapy. In this study we described the KRAS and the BRAF V600E mutation spectra and frequencies in a group of Serbian mCRC specimens.*

Methods: *KRAS mutations were determined with DxS TheraScreen® K-RAS Mutation Kit and KRAS StripAssay™, and for the BRAF V600E mutation we applied High Resolution Melting (HRM) analysis.*

Results: *KRAS mutations were present in 34.7% of 190 analyzed samples. The 7 most frequent mutation types observed were: G12D 43.9%, G12V 21.2%, G12A 10.6%, G12C 7.6%, G12S 4.5%, G12R 1.5%, G13D 10.6%. Among the wt KRAS patients, 17.8% carried the BRAF V600E mutation.*

Conclusions: *We have shown that the spectrum and frequency distribution of the identified KRAS and BRAF mutations in Serbian study population are in good accordance with literature data. We believe that our results are significant concerning aspects related to tumor molecular biology as well as to patient selection in the diagnostic settings.*

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

Introduction

CRC is the third most common cause of cancer-related death worldwide [1]. Novel therapeutic agents such as monoclonal antibodies (mAbs) targeting EGFR, (cetuximab and panitumumab), have been introduced for the treatment of its metastatic form (mCRC). These molecules bind to the extracellular domain of EGFR, leading to inhibition of its downstream signaling. Recent studies have demonstrated that mutations in the *KRAS* and *BRAF* genes, members of the cascade activated by EGFR, are predictors of a negative response to EGFR targeted therapies in patients with mCRC [2-4].

KRAS gene encodes a GDP/GTP-binding protein involved in the regulation of cellular proliferation, differentiation and senescence. Activating mutations cause the KRAS protein to accumulate in its active GTP-bound form, leading to constitutive activation of downstream signal transduction. About 30-40% of CRCs acquire mutations in this gene early in carcinogenesis [5]. The majority (98%) of these mutations in CRC are somatic point mutations located in codon 12 (approximately 82% of all reported *KRAS* mutations) and codon 13 (approximately 17%) of exon 2 [6,7]. According to literature data, the pattern of *KRAS* mutations is tumor-type specific [8]. It has been also shown that *KRAS* mutations present in primary colorectal tumors are also typically present in metastases [9,10]. Furthermore, some data indicate that not all *KRAS* mutations are equal in their biological characteristics, and that a portion of patients (<10%) with *KRAS*-mutated tumors is able to respond to anti-EGFR therapy [8,11,12]. Constitutively activated *KRAS* not only promotes tumor initiation but also tumor growth, survival, progression, local invasion, metastasis formation, angiogenesis, and even immune response [13]. Thus, reliable and sensitive

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determination of the *KRAS* mutation status has become especially important in individual treatment decisions. Despite having a wild-type *KRAS* status (wt *KRAS*), only 40-60% of the patients will respond to treatment [14]. The identification of additional genetic markers of response is therefore of a great importance.

BRAF gene encodes a serine/threonine protein kinase which is involved in intracellular signaling and cell growth. The gene product is also a principal downstream effector of *KRAS* within the *RAS/RAF/MAPK* pathway [15]. *BRAF* activating mutations have been reported in various types of cancers [16]. In colon cancer these mutations are found in approximately 10-15% [17]. All mutations in the *BRAF* gene occur within the kinase domain, resulting in an elevated kinase activity of the BRAF protein. More than 95% of *BRAF* mutations in CRC occur in exon 15 as a point mutation, p.Val600Glu (V600E) [18]. *BRAF* mutations are significantly associated with right-sided tumors, older age, high grade, and microsatellite instability (MSI)-high tumors [19, 20]. The detection of *BRAF* mutations would significantly improve better selection of patients with mCRC for anti-EGFR therapy. In particular, determination of *BRAF* status is recommended in wt *KRAS* patients who did not respond to the mentioned therapy, since these mutations have been reported to be mutually exclusive [21].

The aim of the present study was to describe the *KRAS* mutation spectrum and frequencies in a group of Serbian mCRC specimens (n = 190) referred to our laboratory for *KRAS* mutation analysis, as well as to determine the frequency of *BRAF* V600E mutation in patients with wt *KRAS* status.

Methods

Patient samples

This study included formalin-fixed and paraffin-embedded (FFPE) tumor samples from 190 patients with mCRC. Tissue samples were referred to our laboratory from various cancer centers in Serbia for routine *KRAS* testing. The group of patients comprised 121 males (63.7%) and 69 females (36.3%) with the median age of 60 years (range 27-80). All individuals included in the present study were of Caucasians.

DNA isolation

Depending on the size of the tissue sample, 1-6 sections of the FFPE blocks were used for genomic DNA extraction with the QIAamp® DNA FFPE Tissue kit (QIAGEN, UK), according to the manufacturer's instructions. Concentration (μg/μL) and absorbance (A260/280 ratio) were measured with a UV spectrophotometer (BioPhotometer, Eppendorf, Germany).

KRAS mutation analysis using the DxS TheraScreen® KRAS Mutation Kit

For determining the 7 most common mutations (G12A, G12D, G12R, G12C, G12S, G12V and G13D) in codons 12 and 13 of the *KRAS* gene we used the TheraScreen® KRAS Mutation Kit (QIAGEN Manchester Ltd., UK). Mutation analysis was performed according to the manufacturer's instructions on an ABI PRISM® 7500 PCR instrument (Applied Biosystems Inc., Foster City, CA). For each sample one control reaction and 7 primer specific reactions were prepared. In particular, the total volume for each reaction was 25 μL: 20 μL of reaction mix plus 5 μL of sample DNA (DNA concentration: 100-200 ng/μL). PCR profile included an initial denaturation step at 95° C for 4 min and two-step amplification for 40 cycles with a denaturation at 95° C for 30 s and annealing at 60° C for 1 min. The fluorescence was acquired at the 60° C step. Non-template controls (water) and mixed standards (provided by the manufacturer) were used in order to monitor the results.

KRAS Mutation analysis using the KRAS StripAssay™

KRAS StripAssay™ (ViennaLab Diagnostics, Vienna, Austria) simultaneously covers ten mutations in codons 12 and 13 of the *KRAS* gene namely G12A, G12D, G12R, G12C, G12S, G12V, G12L, G12I, G13D and G13C. Mutation detection was carried out following manufacturer's instructions. Total volume of each sample reaction was 25 μL: 15 μL of amplification mix, 5 μL of diluted Taq DNA polymerase and 5 μL of sample DNA template (DNA concentration: 8-10 ng/μL). PCR profile consisted of pre-PCR step (94° C/2 min) followed by thermocycling (94° C/1 min - 70° C/50 s - 56° C/50 s - 60° C/1 min (35 cycles)) and final extension (60° C/3 min). PCR products were analyzed for presence and quality by gel electrophoresis on a 3% agarose gel. Following PCR, biotinylated amplification products were hybridized to teststrips strictly controlling temperature at 45° C (± 0.5° C), and bound sequences were visualized using streptavidin-alkaline phosphatase conjugate and color substrates. The genotype of a sample was determined using the enclosed Collector™ sheet. Placing the processed teststrip into one of the designated fields, aligning it to the schematic drawing and checking the positive and negative control lines enabled the detection of respective *KRAS* mutations.

Detection of BRAF V600E mutation using High Resolution Melting (HRM) analysis

PCR and HRM were consecutively done on a LightCycler® 480 II (Roche Diagnostics GmbH, Mannheim, Germany) in one single run, and all reactions were performed in duplicate. Non-template control (water) along with two other controls, previously confirmed by sequencing to be wild-type (DNA from colorectal cell line LS174 is homozygous for the wild-type allele) and mutated (DNA from a patient carrying *BRAF* V600E heterozygous mutation), were added for each tested amplicon. Primers, selected to flank the *BRAF* V600E mutation (GTG>GAG), gave an amplicon of 250 bp and their sequences were as follows: forward-5´-CTCTTCATAATGCTTGCTCTGATAGG-3´and reverse-5´-TAGTAACTCAGCAGCATCTCAGG-3´. Each reaction mixture contained about 50-100 ng of DNA, 200 nM primers (HPLC purified), 5 μL of LightCycler HRM Master Reaction Mix (Roche), 3 mM $MgCl_2$, and water to a final volume of 10 μL. PCR conditions were: 95° C for 10 min, followed by 45 cycles of 10 s at 95° C, a touchdown of 64° C to 54° C for 10 s (1° C/cycle), and 20 s at 72° C. After amplification, the PCR product was denatured at 95° C for

1 min and cooled down to 40° C for 1 min to allow heteroduplex formation. The final HRM step was performed from 65° C to 95° C with an increase of 1° C/s with 25 acquisitions/ºC. Final cooling was at 40° C for 10 s. The HRM curve analysis was performed using the accompanying Gene Scanning Software.

Results

The *KRAS* genotyping was successfully performed in all 190 mCRC patients using two commercially available diagnostic kits (DxS TheraScreen® K-RAS Mutation Kit and KRAS StripAssay™). *KRAS* point mutations in codons 12 and 13 were present in 66 cases (34.7%), while 124 (65.3%) analyzed samples had wt *KRAS* status. The seven most frequent of the ten tested mutation types were observed with both assays; among them the frequencies of mutations in codon 12 were G12D 43.9%, G12V 21.2%, G12A 10.6%, G12C 7.6%, G12S 4.5%, G12R 1.5% and the only one in codon 13 was G13D 10.6% (Figure 1). Our study showed that the most common mutations G12D, G12V, G12A and G13D accounted for 86.4% (57/66) of all mutations. Examples of the results obtained with DxS TheraScreen® K-RAS Mutation Kit and with KRAS StripAssay™ are shown on Figure 2 and Figure 3, respectively.

Furthermore, the rate of base pair transitions (59.1%) was found to be higher than the transversions' rate (40.9%). The only detected transition in both codons was (G>A), with 12 GGT>GAT (G12D) being the most frequent one. Two types of transversions (G>T, G>C) occurred only in codon 12.

The *BRAF* V600E testing was successful in 101 out of 124 wt *KRAS* patients due to the poor DNA quality. Among the tested patients, 83 (82.2%) samples had the wt *BRAF* genotype and 18 (17.8%) samples carried the tested *BRAF* mutation. The examples of obtained results are shown on Figure 4.

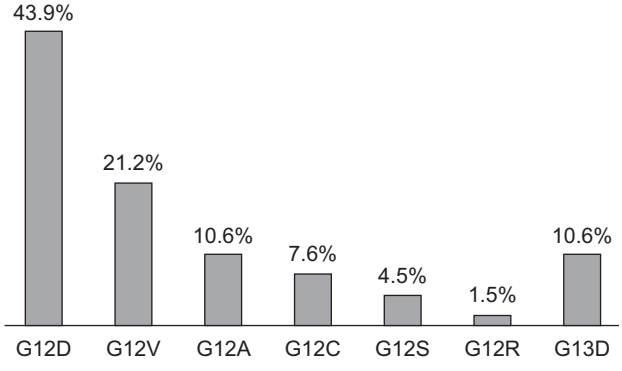

Figure 1. Distribution of detected *KRAS* mutation types.

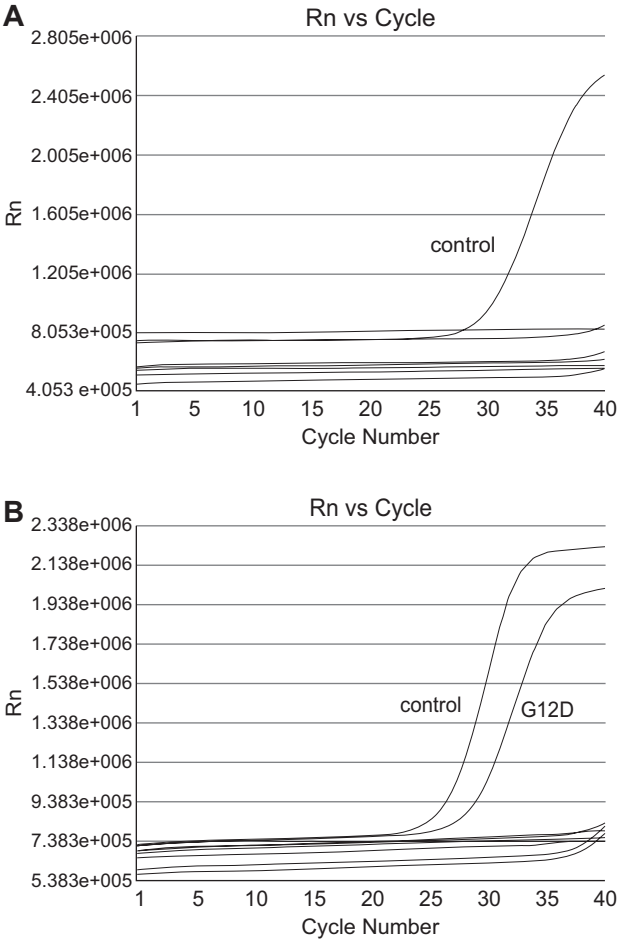

Figure 2. The detection of *KRAS* mutations with DxS TheraScreen® K-RAS Mutation Kit. **A:** Amplification plot of a wild-type sample. **B:** Amplification plot of a sample carrying G12D mutation. Rn - normalized reporter.

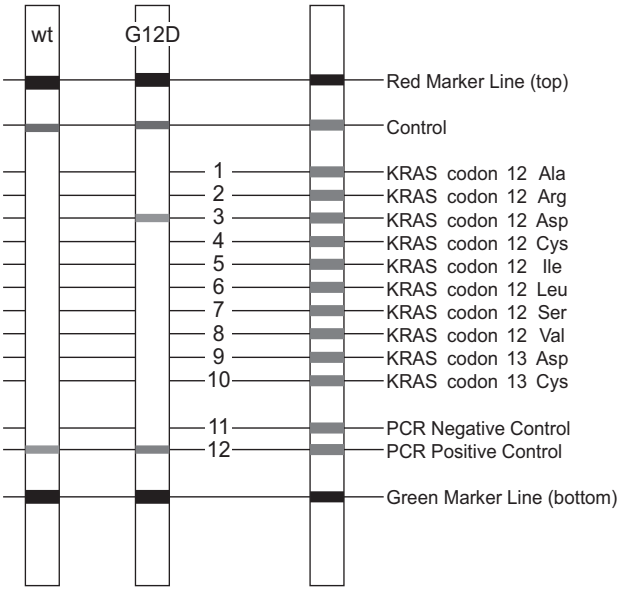

Figure 3. *KRAS* StripAssay results for wild-type (wt) and G12D mutated samples.

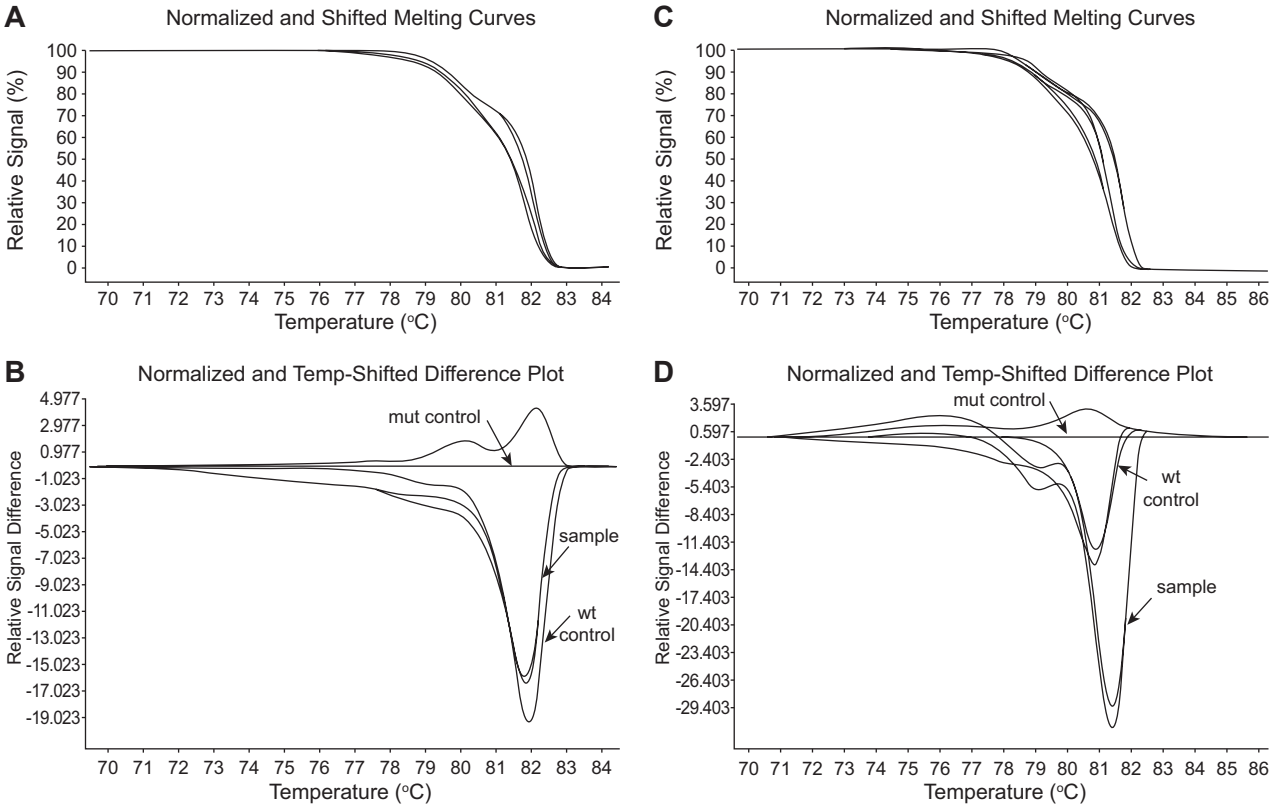

Figure 4. The detection of V600E mutation in *BRAF* gene. Melting curves **(A, C)** and difference plots **(B, D)** for wt *BRAF* and altered *BRAF* samples, respectively; mut control - *BRAF* V600E heterozygous patient, wt control - cell line LS174.

Discussion

In the time of targeted cancer therapy, molecular determination of particular genetic markers, such as *KRAS* and *BRAF* mutations, in colorectal tumors provides more individualized patient treatment. Previous studies have shown that only CRCs with wt *KRAS* respond to EGFR-targeted antibody treatment [2, 4, 22]. Seven different DNA base pair substitutions within codons 12 and 13, each leading to an amino acid substitution in the protein, are the most frequently observed genetic events within the *KRAS* gene in CRC. Therefore, analysis of these hotspot clustered mutations is compulsory before treatment and for large routine diagnostic tests reliable frequency and types of *KRAS* mutations have to be established. These data have not been reported until now In Serbian population.

In the present study, in order to identify the suitable patients for therapy with cetuximab we performed two CE-marked diagnostic assays (detection limit approximately 1% of mutant in a background of wild-type genomic DNA). The use of DxS TheraScreen® K-RAS Mutation Kit for detecting *KRAS* mutations in our laboratory has been standardized and certified in the 2010 Ring Trial for the molecular-pathological detection of *KRAS* mutations in colorectal cancers by QuIP (Quality Initiative in Pathology) by the German Society for Pathology (DGP) and the Federation of the German Pathologist (BDP).

According to the literature data *KRAS* mutations are not only important for the development of CRC, but also for its progression [5]. It has been reported that their presence increased the risk of death by 26% [23]. Secondly, the pattern of *KRAS* mutations is tumor-type specific. Although CRCs have both codon 12 and codon 13 mutations (79% and 17.6% respectively; in our study 89.4% and 10.6%), *KRAS* mutated pancreatic tumors (75% - 95%) almost invariably carry codon 12 mutations, and in non-small cell lung cancer, more than 90% of *KRAS* mutations are placed in codon 12 [8]. The results obtained on 190 Serbian patients with mCRC are consistent with other literature reports. We detected 124 wild-type patients (65.3%) and 66 (34.7%) patients with *KRAS* point mutations in codons 12 and 13.

Regarding the transforming phenotype, it was shown that different mutations may have different effects. For example, any mutation of guanine (G) to thymine (T), but not to adenine (A) or to cytosine (C), increased the risk of death by 44%. Specifically, glycine to valine (G>T) mutations on codon 12 predispose to more aggressive biological behavior in patients with Dukes' C tumors, in whom it is associated with a 50% increased

risk of relapse or death [23]. In our study this mutation was the second most prevalent one (21.2%) (Figure 1). On the contrary, G>A transitions, which result into glycine to aspartate or to serine mutations, have less aggressive transforming phenotypes [12]. In addition, G>A nucleotide substitutions score the second place among the point mutations in human cancers in the Human Gene Mutational Database for all human somatic missense mutations and in our study they represent the most common alterations (G12D 43.9 being the most frequent). Similar to other literature data, we also report a cluster of four mutation types (G12D, G12V, G12A and G13D), which accounts for 86.4% (57/66) of all mutations.

Some reports indicate that a small part of patients (<10%) with *KRAS* mutated tumors can respond to anti-EGFR therapy [11] and that about 15% have long-term disease stabilization [24]. In these patients' tumors, codon 13 mutations were in excess compared with the overall *KRAS* mutated tumor population. Finally, it was shown for the first time that there is a positive association between *KRAS* G13D mutations and cetuximab treatment in regard to better overall and progression-free survival [8]. Mutations in codon 13 are generally among the most frequent mutation types in CRCs and in the current study they account for 10.6%. All these data emphasize that it is no longer sufficient to look for the presence or absence of mutations in tumors only, but in addition research should focus on their individual effects.

The selection of patients for anti-EGFR therapy on the basis of *KRAS* mutational status seems to be not sensitive enough. Specifically, around 40-60% of the patients with wt *KRAS* fail to respond to this treatment [14]. Thus, it is important to verify another molecular determinant of response to EGFR-targeted mAb therapy, such as *BRAF* genotype.

BRAF activating mutations are seen most commonly in melanoma (>60%), but have also been detected in lung, thyroid, ovarian carcinomas, while in rectal cancer they are extremely rare [16, 25, 26]. The frequency of these mutations in CRC is found to be approximately 10-15%% [17]. More than 95% of *BRAF* mutations in CRC occur as a point mutation V600E [18]. Moreover, some authors have shown a significant association of *BRAF* mutations with right-sided colorectal tumors, older age, high grade, and MSI-high tumors [19, 20]. The high frequency of *BRAF* mutations in human cancer and their appearance in early carcinogenesis suggest that it may function as an oncogene [26, 27]. The utility of HRM analysis as a reliable and sensitive technique for the detection of the *BRAF* V600E mutation in CRC has been previously demonstrated [28]. The sensitivity of HRM was higher than the sequencing one, and was comparable to real-time allele-specific PCR (both methods being able to detect 1% of mutated DNA).

Given that mutations in *BRAF* and *KRAS* genes have been reported to be mutually exclusive [21], in this study we have only tested the wt *KRAS* patients for the presence of *BRAF* V600E mutation. In that group, 18/101 (17.8%) patients were found with the *BRAF* V600E mutation, which is slightly higher than the published data. In terms of responsiveness to anti-EGFR treatment, the determination of the mutational status of *KRAS* and *BRAF* markers can identify generally up to 55% of nonresponders, which is very important for clinical practice [3,29].

In this study we have shown that the spectrum and frequency distribution of the identified mutations in *KRAS* and *BRAF* genes in Serbian study population are in good accordance with literature data. To our knowledge, these data have not yet been determined in Serbia. We believe that our results are significant under the aspects related to tumor molecular biology as well as to patient selection in the diagnostic settings. Assessment of *KRAS* and *BRAF* mutational spectra in patients with colorectal cancer is clearly needed for developing personalized therapeutic strategies.

Study approval

The authors state that any necessary ethics committee approval was secured for the reported study by the Institute for Oncology and Radiology of Serbia in Belgrade.

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