Mutation profile of *KRAS* and *BRAF* genes in patients with colorectal cancer: association with morphological and prognostic criteria

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Genet. Mol. Res. 14 (4): 16793-16802 (2015)
Received August 1, 2015
Accepted October 30, 2015
Published December 14, 2015
DOI http://dx.doi.org/10.4238/2015.December.14.6

ABSTRACT. *KRAS* and *BRAF* mutations are well-recognized molecular alterations during colorectal carcinogenesis, but there is little agreement on their effect on tumor characteristics. Therefore, we aimed to evaluate the distribution of the most common *KRAS* and *BRAF* mutations in Greek patients with colorectal cancer and their possible associations with clinical histopathological parameters. In this study, 322 and 188 colorectal carcinomas were used for the mutation analysis of *KRAS* (exon 2) and *BRAF* (exon 15) genes, respectively. The mutational status of both genes was evaluated by polymerase chain reaction and sequencing analysis. Although the overall frequency of *KRAS* mutations (36.6%) seemed to be similar to those reported for other populations, the rate of point mutations at codon 13 was significantly lower (12%) in Greek patients with colorectal cancer and associated with male gender ($P < 0.05$). Tumors with G>T codon 12 transversions and G>C transitions showed more frequent lymph

node metastasis (P < 0.05, P < 0.005, respectively). The rate of *KRAS* mutations gradually decreased with increasing histological grade (P < 0.05), as opposed to *BRAF* mutations, which were strongly associated with poorly differentiated tumors (P < 0.005). Additionally, we found that the histological features of preexisting adenoma were associated with the absence of *BRAF* mutations, in contrast to *KRAS* (P < 0.05). Our data suggested that there seems to be a correlation between morphological criteria and discrete genetic pathways in colorectal carcinogenesis. Moreover, ethnic or geographic factors may have an impact on genetic background of colorectal carcinomas, and specific types of *KRAS* mutations may influence the metastatic potential of colorectal tumors.

Key words: Colorectal cancer; KRAS mutations; $BRAF^{V600E}$ mutation; Histopathological criteria

INTRODUCTION

Colorectal cancer (CRC) remains one of the most frequent and life-threatening types of cancer in the Western world, including Greece. According to recent epidemiological data, CRC constitutes the third most common cause of cancer-related deaths worldwide (Siegel et al., 2012).

Accumulating knowledge in epidemiology and molecular genetics of CRC demonstrate that this cancer encompasses a heterogeneous group of tumors that harbor complex morphologic, genetic and epigenetic abnormalities (Ogino et al., 2011). Until now, many crucial molecular alterations underlying colorectal carcinogenesis have been elucidated, such as mutations of the *KRAS* and *BRAF* genes. p21RAS protein controls intracellular signaling networks participating in cell proliferation, apoptosis and differentiation (Graziano et al., 2011). Activating point mutation is the most common mechanism for *KRAS* gene transformation (Ma et al., 2009), and approximately 35% of colorectal carcinomas carry a point mutation at codon 12 or 13 of *KRAS*, which leads to the constitutive activation of *KRAS* pathways (Benvenuti et al., 2007; Souglakos et al., 2009). Although more than 75 research groups worldwide have published data on the significance of *KRAS* in CRC, there is little agreement about the relation of *KRAS* mutations to other histological and clinical factors (Andreyev et al., 1998).

Recently, the *BRAF* gene is also of interest as being a potential prognostic and predictive biomarker in patients with CRC. *V-raf* murine sarcoma viral oncogene homolog B1 (*BRAF*), a member of the *RAF* gene family, encodes a serine-threonine protein kinase that is a downstream effector of activated *KRAS* (Sridhar et al., 2005). Activating *BRAF* mutations are found in approximately 7% of human cancers and specifically in 4-15% of sporadic colorectal carcinomas (Di Nicolantonio et al., 2008). The best studied and most prevalent *BRAF* mutation (>95%) in tumors concerns the kinase activation domain of the BRAF protein and results from the substitution of valine with glutamic acid at codon 600 ($BRAF^{V600E}$) (Ikenoue et al., 2003; Fransén et al., 2004). Besides the constitutive activation of the RAF-MAPK-ERK pathway, the signaling changes resulting from a V600E mutation are still unclear (Custodio et al., 2013).

An assiduous review of the literature would suggest that there is little knowledge and consensus in colorectal carcinomas about *BRAF* mutation status, particularly with regard to their clinicopathological status and histomorphological features.

The aim of this study was to characterize the *KRAS* and *BRAF* mutation profile in Greek CRC patients to determine the potential association of these mutations with specific clinical and histological parameters. Therefore, we assessed the distribution of the most common *KRAS* and *BRAF* mutations in a large group of Greek patients with CRC.

MATERIAL AND METHODS

Patients

In the present study, formalin-fixed and paraffin-embedded tissue samples of 322 colorectal carcinomas were retrieved from the pathologic archives of the University Hospital of Larissa. All patients were surgically treated and histologically diagnosed with CRC during 2008-2012. Clinical data including patient's age at diagnosis and primary tumor location were retrieved from patient records. Histopathological criteria, such as pT and pN classification (Edge et al., 2010), grade of differentiation (histological grade), histological subtype, presence of vascular invasion, and pre-existing adenoma were also studied.

The study was based on informed consent and approval by the Ethics Committee of the University of Thessalia, Larissa, Greece and conducted according to the Declaration of Helsinki.

DNA extraction

After evaluating the standard hematoxylin/eosin-stained slides from each specimen, appropriate samples were specifically chosen by a pathologist to include predominantly (>70%) tumor cells without significant necrosis or inflammation (Allegra et al., 2009). From each selected block, seven to ten sections of 10-µm thickness were cut and placed in 2-mL sterile Eppendorf tubes. After xylene/ethanol deparaffinization of each sample, DNA was extracted using the QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer instructions. DNA quality was evaluated by both agarose gel electrophoresis and spectrophotometry at 260/280 nm.

Amplification of *KRAS* and *BRAF* genes

Genomic DNA was amplified for *KRAS* (exon 2, codons 12, 13) and *BRAF* (exon 15, codon 600) genes by using specific primers (Table 1) and AmpliTaq DNA polymerase (Applied Biosystems, USA). The amplification mixture consisted of 5 µL 10X reaction buffer, 2.5 mM $MgCl_2$, 0.5 µM each oligonucleotide primer, 2.5 U AmpliTaq DNA polymerase and 2 µL template DNA in a final volume of 50 µL. Samples were amplified as follows: an initial denaturation step at 94°C for 10 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 52 or 58°C for 1 min for *KRAS* and *BRAF*, respectively, and extension at 72°C for 1 or 2 min for *KRAS* and *BRAF*, respectively. In all reactions, negative control was included. PCR-amplified products were analyzed in a 3% agarose gel, using a 100-bp DNA ladder (Invitrogen, Life Technologies, USA) and ethidium bromide staining.

Sequencing

All PCR products were purified using the QIAquick PCR Purification kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer protocol. Subsequently, the purified products were subjected to bidirectional sequence analysis on an ABI 3500 Genetic Analyzer (Applied Biosystems, USA).

Table 1. Primers for the amplification of the *KRAS* and *BRAF* genes.

Gene	Primer sequence	PCR product (bp)
KRAS exon 2	F: ACTGAATATAAACTTGTGGTAGTTGGACCT R: TCAAAGAATGGTCCTGGACC	156
BRAF exon 15	F: TCATAATGCTTGCTCTGATAGGA R: GGCCAAAATTTAATCAGTGGA	224
	F: forward primer, R: reverse primer	

Statistical analysis

All quantitative variables are presented as the mean and standard deviation (SD), whereas absolute and relative (%) frequencies are used for the categorical variables. The Pearson chi-square test or Fisher exact test was used to evaluate the associations between different biological variables. As for quantitative variables, the r correlation coefficient and relative *t*-test were used. Differences were considered to be statistically significant with $P < 0.05$ and all P values were two-tailed.

RESULTS

Patient characteristics and histopathological parameters

In the present study, we analyzed 322 patients with histologically confirmed CRC. A hundred and eighty male (55.9%) and a hundred and forty-two female patients (44.1%) were included in our study. The median age at the time of diagnosis was 67 years (SD 10.7 years) with no significant age differences between male and female patients. Patients' and tumor characteristics are shown in Table 2. The characteristics of these patients are in accordance with larger populations with CRC reported elsewhere (Beahrs et al., 1992; Andreyev et al., 1998).

Male patients featured more the common poorly differentiated carcinomas (15.5%) compared to women (7.7%) ($P < 0.05$). No other difference was observed in the frequencies of histopathologic tumor parameters between the sexes. As for tumor location, carcinomas located in the right colon (ascending and transverse colons) were mostly poorly differentiated (22.5%) compared to tumors located in the left colon and rectum (6.6%) ($P < 0.05$). Tumors with proximal location also featured more common advanced pT3/T4 stages (92%) compared to those of distal location (80%) ($P < 0.05$). Besides male gender and tumor location, poor histological differentiation was also associated with the presence of vascular invasion ($0.01 < P < 0.025$), as well as with the presence of lymph node metastasis ($P < 0.005$).

KRAS mutations and clinicopathological features

Evaluating the entire patient cohort, a total of 118 patients (36.6%) had a *KRAS* mutation in the tumor specimen; 44 and 34 patients had the Gly12Asp and Gly12Val mutations, respectively. Moreover, at codon 12, the mutations were Gly12Cys (10 patients), Gly12Ala (7 patients), Gly12Ser (4 patients) and Gly12Arg (3 patients). The other observed mutations were at codon 13: Gly13Asp (14 patients) and Gly13Arg and Gly13Cys (1 patient each) (Table 3). G to A occurred in 52.4% of mutations, and G to T or C in 38.3 and 9.3% of mutations, respectively. Mutations at the first base of codon 12 or 13 occurred in 15% and at the second base in the remaining 85% of cases.

Table 2. Demographics and clinical characteristics of our patients-correlation with *KRAS* mutation status.

Parameters	Overall N = 322	*KRAS* wild type N = 204 (63.4%)	*KRAS* mutation N = 118 (36.6%)	P value
Gender				
Male	180 (55,9%)	119 (66,1%)	61 (33,9%)	ns
Female	142 (44,1%)	85 (59,9%)	57 (40,1%)	
Location				
Right colon	111 (34.5%)	62 (55,9%)	49 (44,1%)	P < 0.05
Left colon	152 (47.2%)	103 (67,8%)	49 (32,2%)	
Rectum	59 (18.3%)	39 (66,1%)	20 (333,9)	
Histologic subtype				
Adenocarcinoma	290 (90.1%)	184 (63,4%)	106 (36,6%)	ns
Mucinous adenocarcinoma	26 (8.1%)	14 (53,9%)	12 (46,1%)	
Neuroend. Differentiation	5 (1.5%)	5	0	
Adenosquamous carcinoma	1 (0.3%)	1	0	
Differentiation degree (Grade)				
Well (G1)	38 (11,8%)	13 (34,2%)	25 (65,8%)	P < 0.005
Moderate (G2)	202 (62,7%)	125 (61,9%)	77 (38,1%)	P < 0.005
Moderate to poor (G2-G3)	43 (13,4%)	31 (72,1%)	12 (27,9%)	
Poor (G3)	39 (12,1%)	35 (89,7%)	4 (10,3%)	
Preexisting adenoma				
Present	61 (19%)	31 (51%)	30 (49%)	P < 0.05
Absent	261 (81%)	173 (66%)	88 (34%)	
Vascular invasion				
Present	136 (42%)	88 (65%)	48 (35%)	ns
Absent	186 (58%)	116 (62%)	70 (38%)	
pT stage				
T1	7 (3%)	4 (57%)	3 (43%)	ns
T2	43 (13%)	31 (72%)	12 (28%)	
T3	234 (72%)	145 (62%)	89 (38%)	
T4	38 (12%)	24 (63%)	14 (37%)	
pN stage				
N0	143 (44.4%)	86 (60%)	57 (40%)	ns
N1	102 (31.6%)	62 (61%)	40 (39%)	
N2	77 (24%)	56 (73%)	21 (27%)	

ns = not significant.

Table 3. Distribution of *KRAS* mutation types.

Codon	Point mutation types	Patients N (%)
12 [N = 102 (86%)]		
G>A	GGT (Gly) > GAT(Asp)	44 (37%)
G>T	GGT (Gly) > AGT(Ser)	4 (3.4%)
G>C	GGT (Gly) > GTT(Val)	34 (29%)
	GGT (Gly) > TGT(Cys)	10 (8.5%)
	GGT (Gly) > GCT(Ala)	7 (6%)
	GGT (Gly) > CGT(Arg)	3 (2.5%)
13 [N = 16 (14%)]		
G>A	GGC (Gly) > GAC (Asp)	14 (12%)
G>C	GGC (Gly) > TGC (Arg)	1 (0.8%)
G>T	GGC (Gly) > CGC (Cys)	1 (0.8%)

The mutation rate at both codons 12 and 13 was not significantly different between the two sexes. Nevertheless, the detailed study of the mutation types in relation to the patient's gender revealed that men harbored the more common mutations at codon 13 (20%) compared to female patients (7%) (P < 0.05). In relation to tumor location, *KRAS* mutations occurred more frequently at proximal carcinomas (44%) compared to those located in the left colon (32%) or rectum (34%) (P < 0.05). Additionally, the incidence of mutations on both codons was significantly higher in the cases of preexisting adenomas (49%), compared to those without corresponding histological findings.

Concerning histological differentiation, 25 (66%) of 38 well-differentiated carcinomas, 77 (38%) of 202 moderately differentiated carcinomas and 4 (10%) of 39 poorly differentiated carcinomas harbored a *KRAS* mutation. Forty-three tumors expressed major histological heterogeneity and were classified as moderately to poorly differentiated carcinomas, of which 12 (28%) harbored a *KRAS* mutation. Therefore, there was a gradual decrease in mutation rate with higher histological grade. When the chi-square test was applied to compare consecutively all differentiation grades in relation to *KRAS* mutation rate, a statistically significant difference was obtained ($P < 0.005$).

There was no apparent correlation between the presence of *KRAS* mutation and other histopathological criteria, such as histological subtype, vascular invasion and pathologic tumor (pT) or lymph node (pN) classification. Nevertheless, specific mutations did correlate with the presence of lymph node metastasis (Table 4). In particular, colorectal tumors that harbored a G>T transversion in codon 12 had more commonly (51%) lymph node metastatic spread compared to those that harbored a G>A transition (31%) ($P < 0.05$). Additionally, carcinomas with G>C transversion in codon 12 exhibited exclusively lymph node metastatic disease ($P < 0.005$).

Table 4. Correlation of *KRAS* mutations at codon 12 with pN classification.

Mutation type in codon 12	pN0 (Duke's B stage) N (%)	pN1/pN2 (Duke's C stage) N (%)	P value
G>A	33 (69%)	15 (31%)	P < 0.05
G>T	21 (49%)	23 (51%)	P < 0.005
G>C	0	10 (100%)	

BRAF mutation and clinicopathological features

For *BRAF* analysis, we examined 188 out of 204 *KRAS* wild-type patients because of the DNA quantity. Seventeen cases (9%) had a *BRAF* mutation, while the remaining 171 (91%) were classified as *BRAF* wild-type carcinomas. All samples carried the substitution of valine with glutamic acid at amino acid 600 ($BRAF^{V600E}$) of the *BRAF* gene. In all cases of *KRAS* and *BRAF* mutation, the two genes were mutually exclusive. The basic clinicopathological characteristics of the primary tumor in relation to *BRAF* mutations are shown in Table 5.

As expected, *BRAF* mutations were significantly related to the right-sided tumor than those located in the left colon ($P < 0.005$), although this statistically significant difference did not hold in the comparison with rectal carcinomas. The presence of *BRAF* mutation (13%) was higher in women compared to male patients (6.5%), although this difference did not prove to be statistically significant ($0.10 < P < 0.25$).

We also investigated the variation in the rate of *BRAF* mutations between tumors with different histological grades; none of the 15 well-differentiated carcinomas, 5 (4.1%) of 122 moderately differentiated carcinomas, 5 (25%) of 20 moderately to poorly differentiated carcinomas and 7 (23%) of 31 poorly differentiated carcinomas harbored a $BRAF^{V600E}$ mutation. Unlike *KRAS,* the presence of *BRAF* mutations was strongly associated with high grade tumors (moderately to poorly or poorly differentiated tumors) ($P < 0.005$).

There was no significant association of *BRAF* mutation with the presence of vascular invasion. On the other hand, histological features of preexisting adenoma were associated with the absence of this specific mutation ($P < 0.05$).

There was no apparent correlation between the presence of *BRAF* mutations and pathological tumor (pT) or lymph node (pN) classification (Table 5).

Table 5. Distribution of tumor characteristics according to tumor *BRAF* status.

Tumor parameters	*BRAF* wild type N = 17 (9%)	*BRAF* mutation N = 171 (91%)	P value
Gender			
Male	103 (66%)	7 (34%)	0.10 < P < 0.25
Female	68 (60%)	10 (40%)	
Location			
Right colon	56 (82.4%)	12 (17.6%)	P < 0.005
Left colon	80 (97.5%)	2 (2.5%)	ns
Rectum	35 (92%)	3 (8%)	
Histologic subtype			
Adenocarcinoma	161 (91%)	15 (9%)	-
Mucinous adenocarcinoma	8 (80%)	2 (20%)	
Neur. Differentiation	2	0	-
Differentiation degree (Grade)			
Well (G1)	15 (100%)	0	P < 0.005
Moderate (G2)	117 (96%)	5 (4%)	P < 0.005
Moderate to poor (G2-3)	15 (75%)	5 (25%)	
Poor (G3)	24 (77%)	7 (23%)	
Preexisting adenoma			
Present 30	30 (100%)	0	P < 0.05
Absent 158	141 (89%)	17 (11%)	
Vascular invasion			
Present	72 (91%)	7 (9%)	-
Absent	99 (91%)	10 (9%)	
pT stage			
T1/T2	27 (93%)	2 (7%)	ns
T3/T4	144 (90%)	15 (10%)	
pN stage			
N0	85 (90%)	9 (10%)	ns
N1	49 (98%)	1 (2%)	
N2	37 (84%)	7 (16%)	

ns = not significant.

DISCUSSION

To our knowledge, this is the first study concerning *KRAS* and *BRAF* mutational status in a large number of Greek patients with CRC. Our aim was to investigate the distribution of these mutations, as well as their association with clinical histopathological features.

In the present study, we found that male patients featured more commonly poorly differentiated carcinomas compared to women (P < 0.05). Besides the risk of CRC, it is possible that lifestyle differences between the two sexes may also have an impact on histological differentiation of colorectal carcinomas (Newcomb et al., 2007; Wallace et al., 2009).

We also concluded that tumor histopathological parameters may also vary by initial site. Carcinomas located in the right colon were more frequently poorly differentiated and featured more commonly advanced pT3/T4 stages. These findings are in agreement with previous studies (Derwinger et al., 2011; Yamauchi et al., 2012; Weiss et al., 2012). Although the frequency of *KRAS* mutations is consistent with larger CRC studies reported in the past (Andreyev et al., 1998; Samowitz et al., 2000), the relative proportions of specific types of *KRAS* mutations, mainly the mutation frequency at codon 13, appear to differ significantly in the Greek population, compared to that reported from the USA (Samowitz et al., 2000) or in other larger multicenter studies (Andreyev et al., 1998; Andreyev et al., 2001).

In the multicenter “RASCAL” study (Andreyev et al., 1998), when the geographic variation was examined with the mutation rate, there was no consistent difference in the predominance of a single type of mutation in individual regions. However, its dataset was too small to rule out with

certainty such a difference. Moreover, in the same study, Andreyev et al. (1998) found that the spectrum of point mutations in codon 12 of *KRAS* gene was significantly different in a small group (37 patients) from Yugoslavia and in a larger group (192 patients) from Switzerland compared to the rest of the collaborating groups.

In our study, the relative proportions of specific mutations at codon 12 were similar to the findings of the studies mentioned above, but the G to A mutation rate at codon 13 appeared to be significantly lower (12%). Besides potential variations in methods of mutation detection, ethnic or geographic parameters may have an effect on the molecular characteristics of CRC as well. In our study, the overall mutation rate in male and female patients was not significantly different. However, when specific mutations were considered, we found an association between male gender and mutation rates at codon 13 of the *KRAS* gene. These findings are in contrast to a recent multicenter Japanese study, where these mutations were more common in female patients (Uetake et al., 2011). Moreover, in the study of Samowitz et al. (2000), male patients were significantly more likely to have a G to A transition at codon 12. It is possible that various lifestyle or environmental factors to which men and women are differentially exposed, decrease or increase the likelihood of specific mutations.

We also observed that *KRAS* mutations were significantly detected in proximal tumors than those of distal location, a finding that is consistent with previous studies (Elnatan et al., 1996; Samowitz et al., 2000; Rosty et al., 2013). In the latter study, the authors also observed that *KRAS*-mutated carcinomas frequently develop in contiguity with a residual polyp, which we also observed. There was no apparent correlation between the presence of *KRAS* mutations and histological subtype, vascular invasion and pathological tumor (pT) or lymph node (pN) classification (Wadler et al., 1997; Hardingham et al., 1998; Kressner et al., 1998; Beránek et al., 1999; Andreyev et al., 2001).

However, when specific mutations were evaluated, patients that harbored a G>T transversion in codon 12 had more commonly lymph node metastasis compared to those that harbored a G>A transition at the same codon ($P < 0.05$). Additionally, colorectal carcinomas with G>C transversion at codon 12 presented exclusively with lymph node metastatic disease ($P < 0.005$). Similar findings were presented in a previous study included 31 patients with Dukes' stage B and 42 patients with Dukes' stage C (Moerkerk et al., 1994). These investigators found that codon 12 G>A transitions occurred exclusively in the first group of patients (Dukes' B), whereas G>C transversions of the same codon were found exclusively in the second group. Based on these results, it could be assumed that patients with CRC with or without lymph node metastatic spread may have significant differences in their molecular profiles and type of specific mutations. Interestingly, in a retrospective Spanish report of 230 CRC patients, a higher percentage of *KRAS* mutations was detected in primary tumors of patients with lung metastases than in those with liver metastases, suggesting a role for these mutations in the propensity of primary CRC to metastasize to the lung (Cejas et al., 2009).

So far, little is known about the relation of *KRAS* mutations to histopathological grade in colorectal carcinomas. In our study we found that *KRAS* mutation rate was gradually reduced according to the aggravation of the histological grade. Besides a few other studies (Andreyev et al., 1998; Beránek et al., 1999), most of the relative studies have not shown such a relationship. On the contrary, we found that the presence of *BRAF* mutation was strongly associated with poorly differentiated tumors, a finding that is consistently reported in many other studies (Zlobec et al., 2010; Van Cutsem et al., 2011; Benedix et al., 2012). Based on these results we can assume that poorly differentiated tumors have distinct molecular abnormalities, and since *KRAS* and *BRAF* mutations are thought to be an early event in colorectal tumorigenesis, they may have a major impact on tumor morphology. Additionally, we found that the histological features of preexisting

adenoma were associated with the absence of *BRAF* mutations, as opposed to *KRAS*. These morphological associations, both in histological grading and residual adenoma, support the idea of the existence of distinct parallel pathways that rarely cross over (Rajagopalan et al., 2002). Lately, there has been increasing evidence for an alternative molecular pathway in colonic carcinogenesis different from chromosomal and microsatellite instability (MSI) pathways. This CpG island methylator phenotype (CIMP) has been strongly associated with somatic *BRAF* mutations (Weisenberger et al., 2006; Ogino et al., 2009; Curtin et al., 2011).

Moreover, the development of serrated neoplasia has been associated with activation of the MAPK-ERK signaling pathway and abnormal silencing of certain genes by DNA methylation (Park et al., 2003; McGivern et al., 2004; Wynter et al., 2005; Jass et al., 2005).

In conclusion, our data suggest that there is a correlation between morphological criteria and discrete genetic pathways in colorectal carcinogenesis. Moreover, ethnic or geographic factors may have an impact on genetic makeup of colorectal carcinomas, and specific types of mutations may influence the metastatic potential of a colorectal tumor.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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