

# Highly conserved sequence of exon 15 *BRAF* gene and *KRAS* codon 12 mutation among Greek patients with colorectal cancer

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**ABSTRACT: Background:** The RAS/RAF/MEK/MAP kinase pathway is essential to intracellular signaling transduction regulating cell proliferation, differentiation and death. We investigated the occurrence of exon 15 *BRAF* and *KRAS* codon 12 mutations among Greek patients with colorectal cancer.

**Methods:** Sixty-one samples from patients with sporadic colorectal adenocarcinomas were studied for exon 15 *BRAF* mutations. DNA from surgically resected specimens was analyzed by a combination of polymerase chain reaction and direct sequencing. *KRAS* codon 12 mutational analysis was technically possible in 58 samples (58/61) by a combination of polymerase chain reaction and restriction fragment length polymorphism.

**Results:** No exon 15 *BRAF* mutations were detected in any of the colon cancer specimens. The frequency of *KRAS* codon 12 mutations was 29.3% (17/58). Patients aged  $\leq 70$  years more frequently presented carcinomas harboring *KRAS* codon 12 mutations than patients aged  $>70$  years ( $p=0.028$ ). Patients between 61 and 70 years of age were more likely to be carriers of this mutation ( $p=0.040$ ).

**Conclusions:** Despite the limited study sample, our data suggest that *BRAF* mutations might be present less frequently than *KRAS* mutations in Greek patients with colorectal carcinomas. Further research involving larger patient series will be necessary to confirm these findings and to assess possible ethnic, environmental and lifestyle influences on *BRAF* and *KRAS* mutagenesis. (Int J Biol Markers 2007; 22: 12-8)

**Key words:** Colorectal cancer, Mutational analysis, Exon 15 *BRAF*, V600E, *KRAS* codon 12 mutations, Restriction fragment length polymorphism

## INTRODUCTION

There are approximately 1 million new cases of colorectal cancer diagnosed worldwide and half a million deaths caused by the disease each year (1). Carcinogenesis of the colon and rectum develops through a multistep process of genetic and epigenetic events resulting in activation of oncogenes and inactivation of tumor suppressor genes. Events such as mutations, loss of heterozygosity (LOH), epigenetic silencing of gene transcription by promoter hypermethylation and gene amplification allow escape from the tight constraints that control normal cells (2, 3).

The *RAS* family genes (*KRAS*, *HRAS*, and *NRAS*) encode GTP binding proteins. Early observations identified these molecules as having an impact on cell transformation and tumorigenesis. Cumulative evidence strongly supports the implication of activated *RAS* genes in vari-

ous human malignancies (4-6). *RAS* genes contribute to tumorigenesis through accumulation of mutations resulting in altered protein forms with increased GTPase activity (7). Point mutation of the *KRAS* gene in codon 12 is an early event in colorectal carcinogenesis, mostly occurring during the transformation of a small to intermediate size adenoma (7, 8).

The RAF family proteins are RAS-regulated kinases involved in cellular growth responses. The RAS/RAF/MEK/MAP kinase (RAS-RAF-extracellular signal-regulated kinase-mitogen-activated protein kinase/extracellular signal-regulated kinase/mitogen-activated protein kinase) cascade transduces signals from cell surface to nucleus (9, 10). Three known RAF genes, resulting from gene duplication (*BRAF*, *ARAF1* and *CRAF*), encode for cytoplasmic serine/threonine kinases, their regulation being dependent on RAS binding (10, 11). Activating mutations within *BRAF* have been report-

ed in a high percentage of skin melanomas and at a lower frequency in various other cancer types including colorectal carcinomas (12, 13). Current evidence suggests that the identified *BRAF* mutations occur within the kinase domain (12). The most common *BRAF* mutation results in a single substitution of T to A on exon 15 at nucleotide position 1799 (1799T/A), previously named T1796A (12, 14, 15). This mutation converts a valine residue to a glutamic acid (V600E) at amino acid position 600, previously reported as V599E (12, 14, 15). Mutated *BRAF* proteins possess elevated kinase activity and are capable of transforming NIH3T3 cells independent of the RAS function (12). Additionally, *BRAF* mutations, such as V600E, were described only in *KRAS*-negative colon carcinomas, suggesting that *BRAF/KRAS* activating mutations might be alternative genetic events in colon cancer (12, 16).

In the present study, we performed mutational analysis of exon 15 *BRAF* and *KRAS* codon 12 mutation screening in colorectal carcinomas among Greek patients in order to assess the role of possible *BRAF* and *KRAS* mutations within this population group.

## METHODS

### *Colorectal tumor samples and patient population*

Surgical resection samples from colorectal cancers were collected at Laiko General Hospital of Athens during a period of 3 years. Representative tumor specimens were snap frozen and stored at  $-80^{\circ}\text{C}$ . The colon cancer samples used in this study were histologically examined by an experienced pathologist prior to DNA extraction. An extreme effort was made to avoid any adjacent normal tissue and to isolate areas of tissue containing  $>70\%$  of tumor cells.

Sixty-one sporadic colorectal adenocarcinomas were examined. All patients were Greek, white subjects and had a negative family history for colorectal tumors. The mean age of the 61 patients was 67.1 years (standard deviation  $\pm 10.2$  years). Data available for this series included tumor site, Dukes' stage (17), histological grade and mucinous status.

The institutional ethics committee of Laiko General Hospital, Athens, approved the present study. All participating specimen donors gave their written informed consent. The investigation conforms to the principles outlined in the Declaration of Helsinki.

### *DNA extraction, oligonucleotide primers and PCR amplification*

DNA was extracted by standard protocols using proteinase K digestion, phenol-chloroform purification and ethanol precipitation, as previously described (18). Spe-

cific primers (forward: 5'-TCATAATGCTTGCTCTGATAGGA-3', and reverse: 5'-GGCCAAAAATTTAATCAGTGGA-3', MWG Biotech AG, Ebersberg, Germany) amplified the polymerase chain reaction (PCR) product of the exon 15 *BRAF* region as previously reported (12). PCR procedures were carried out at an annealing temperature of  $55^{\circ}\text{C}$  with an  $\text{MgCl}_2$  concentration of 3.0 mM using Taq Qiagen Polymerase. Primers (forward: 5'-ACTGAATATAAAGTGTGGTAGTTGGACCT-3', and reverse: 5'-TCAAAGAATGGTCTGGACC-3') covering codon 12 of *KRAS* amplified a 157-bp product. The PCR procedure was carried out at an annealing temperature of  $58^{\circ}\text{C}$  with an  $\text{MgCl}_2$  concentration of 3.0 mM using Taq Qiagen Polymerase. PCR products were run on a 2% agarose gel and stained with ethidium bromide to visualize the DNA.

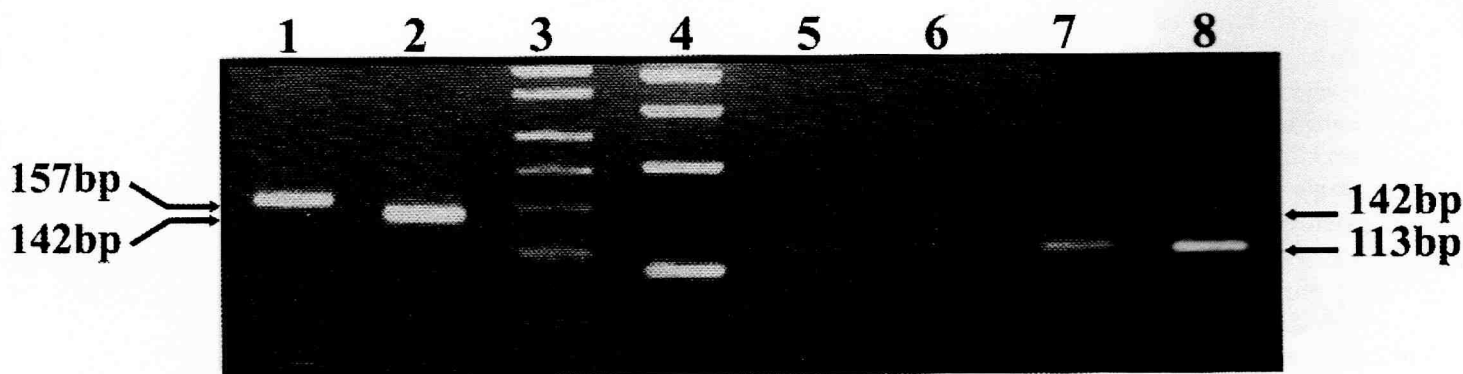
### *Sequencing analysis for BRAF and RFLP analysis of KRAS*

PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA), subjected to direct sequencing on both strands (BigDye™ Terminator Cycle Sequencing Ready Reaction Kit [Applied Biosystems, Foster City, CA, USA]) according to the manufacturer's manual and analyzed on an ABI PRISM 3100 Avant Genetic Analyzer. The sequences were initially analyzed by visual inspection (secondary peaks or nontypical background signals). Subsequently, all the samples were analyzed twice by aligning and comparing all sequences with the corresponding wild-type *BRAF* sequence obtained from an international database ([www.ensembl.org](http://www.ensembl.org)).

PCR amplification products (10  $\mu\text{L}$ ) of codon 12 of *KRAS* were digested by means of restriction endonuclease *MvaI* (Roche Diagnostics GmbH, Mannheim, Germany). The digested PCR products were electrophoresed on a 3% agarose gel and visualized under a UV transilluminator. *MvaI* digestion of the PCR fragment yielded 2 major bands at 142 bp and 113 bp in the mutant samples and a single band at 113 bp in the wild-type samples. DNA from the SW480 cell line carrying a homozygous mutation in *KRAS* codon 12 was used as a positive control. In *KRAS* PCR products, restriction fragment length polymorphism (RFLP) procedures were carried out twice. Samples were considered positive only when the mutational pattern was reproducible. Seven randomly selected samples, positive for *KRAS* mutations after RFLP analysis, were sequenced and the initial results were fully confirmed.

### *Statistical analysis*

Statistical analysis of the results was performed with the package SPSS 14.0 for Windows. Because of the data type (nominal data grouped into categories), statistical analysis was carried out through contingency tables. Variables such as age groups, sex, tumor site, Dukes'



**Fig. 1** - Detection of *KRAS* codon 12 point mutation after RFLP analysis in colorectal cancer. Lane 1: SW480 undigested PCR product (157 bp). Lane 2: Digested SW480 PCR product (142 bp) with *Mva*I restriction enzyme, homozygously mutated in *KRAS* codon 12. Lane 3: molecular weight marker pUC19/*Msp*I. Lane 4: 100 bp DNA ladder. Lanes 5 and 6: Positive samples for *KRAS* mutation (142 bp and 113 bp) in 3% agarose gel electrophoresis. Lanes 7 and 8: wild-type *KRAS* colorectal samples (113bp).

stage, histological grade and mucinous status were analyzed. We conducted all tests at an  $\alpha=0.05$  level of significance.

**RESULTS**

In this study we performed direct sequencing analysis of exon 15 of the *BRAF* gene in 61 sporadic colorectal adenocarcinomas. We found that the coding sequence of exon 15 was perfectly conserved in all samples. No nucleotide exchange that could interfere by modifying the amino acid sequence of the *BRAF* protein was detected.

Molecular analysis for the detection of *KRAS* mutations in codon 12 was possible for 58 adenocarcinomas. Seventeen cases (17/58) were positive for *KRAS* mutations (Fig. 1). All *KRAS* mutations were detected in heterozygous status. Among the 17 colorectal tumors with *KRAS* codon 12 mutations, 12 patients were male (among 35 male cases examined) and 5 were female (among 23 female cases examined). Fifteen of the 39 patients who were aged 70 years or less and 2 of the 19 patients who were over 70 were carriers of *KRAS* codon 12 mutations ( $p=0.028$ , Pearson's chi-square test) (Tab. I). Eleven of the 24 patients in the age group from 61 to 70 years developed tumors with *KRAS* mutations while 4 of the 15 patients aged  $\leq 60$  years and 2 of the 19 who were over 70 years presented the mutant pattern ( $p=0.040$ , Pearson's chi-square test) (Tab. II). Males between 61 and 70 years were more commonly carriers of *KRAS* mutations compared to younger or older patients, with a marginal level of statistical insignificance ( $p=0.047$ , Pearson chi-square test and  $p=0.059$ , Fisher exact test) (Tab. III). Eight tumors with *KRAS* mutations were located in the proximal colon (cecum, ascending colon, and transverse colon) among a total of 21 tumors in this region, and 9 tumors with *KRAS* mutations were located in the distal colon (descending or sigmoid colon and rectum) among

**TABLE I** - *KRAS* CODON 12 MUTATIONS IN PATIENTS  $\leq$  OR  $>70$  YEARS

Age	<i>KRAS</i> codon 12 mutation	
	Positive n (%)	Negative n (%)
$\leq 70$ years	15 (38.5)	24 (61.5)
$>70$ years	2 (10.5)	17 (89.5)
P value	0.028	

a total of 37 tumors in this region. One *KRAS* mutation-positive tumor was Dukes' stage A (of the 5 Dukes' stage A tumors examined), 4 were Dukes' B (of the 14 Dukes' B tumors examined), 9 were Dukes' C (of the 26 Dukes' C tumors examined) and 3 were Dukes' D (of the 11 Dukes' D tumors examined). Tumor stage was uncertain in 2 cases. Data on histological grade were available for 51 cases and on mucinous status for 44 cases. Overall, we did not observe any significant association between *KRAS* mutations and tumor site or stage, grade of differentiation, and mucinous status (Tab. II).

**DISCUSSION**

Our results revealed the absence of *BRAF* mutations in the colorectal carcinomas analyzed (0/61). Davies et al reported a frequency of 18% and 12% for the occurrence of *BRAF* mutations in colorectal cancer cell lines (40 samples) and in primary colorectal cancers (33 samples), respectively (12), while Yuen et al reported a frequency of 5.1% in colorectal adenocarcinomas (16). In a recent study, *BRAF* mutations were detected in 9.1% of 44 human primary colorectal tumors (19). In 2 large studies, the mutation was found in 8% of 275 colorectal

**TABLE II - KRAS CODON 12 MUTATIONS AND CLINICOPATHOLOGICAL FEATURES OF THE COLORECTAL ADENOCARCINOMAS**

Clinicopathological features of the examined colorectal carcinomas	Total (n)	KRAS codon 12 mutation positive n (%)	KRAS codon 12 mutation negative n (%)	P value
<b>Age</b>				
≤60 years	15	4 (26.7)	11 (73.7)	0.040
61-70 years	24	11 (45.8)	13 (54.2)	
>70 years	19	2 (10.5)	17 (89.5)	
<b>Sex</b>				
Male	35	12 (34.3)	23 (65.7)	NS
Female	23	5 (21.7)	17 (78.3)	
<b>Tumor location</b>				
Proximal	21	8 (38.1)	13 (61.9)	NS
Distal	37	9 (24.3)	28 (75.7)	
<b>Tumor stage<sup>a</sup></b>				
Dukes' A	5	1 (20.0)	4 (80.0)	NS
Dukes' B	14	4 (28.6)	10 (71.4)	
Dukes' C	26	9 (34.6)	17 (65.4)	
Dukes' D	11	3 (27.3)	8 (72.7)	
<b>Histological grade<sup>b</sup></b>				
Well/moderate	43	12 (27.9)	31 (72.1)	NS
Poor	8	3 (37.5)	5 (62.5)	
<b>Mucinous status<sup>c</sup></b>				
Negative	39	10 (25.6)	29 (74.4)	NS
Positive	5	2 (40.0)	3 (60.0)	

NS, non-significant

<sup>a</sup>Data available for 56 cases<sup>b</sup>Data available for 51 cases<sup>c</sup>Data available for 44 cases**TABLE III - KRAS CODON 12 MUTATIONS AMONG FEMALE AND MALE PATIENTS IN DIFFERENT AGE GROUPS**

Age groups and KRAS codon 12 mutation	Total (n)	Sex	
		Female (n)	Male (n)
<b>≤60 years</b>			
Positive	4	2	2
Negative	11	5	6
<b>61-70 years</b>			
Positive	11	1	10*
Negative	13	6	7
<b>&gt;70 years</b>			
Positive	2	2	0
Negative	17	7	10

\*p=0.047 (Pearson's chi-square test) and p=0.059 (Fisher exact test)

cancers (20) and over 20% of 293 cancers analyzed (21). Ikehara et al found *BRAF* mutations in 7.2% by screening 83 sporadic adenocarcinomas (22). In a previous study, it was reported that the discrepancies in *BRAF* mutation frequencies found were unlikely to be related to methodological differences and the authors suggested that further research should reconcile the differences in frequency estimates of *BRAF* mutations in colorectal tumors (16). Supporting this observation, we suggest that patterns or

attitudes related to different population subgroups may favor, at least in part, selection differences in *BRAF* mutation incidence in colorectal cancer.

Malignant melanomas of soft tissues share clinical, histological and immunohistochemical features with malignant melanomas of the skin but exon 15 *BRAF* mutations are significantly less frequent among the former (23). Similarly, absence of exon 15 *BRAF* germline mutations was observed in familial melanomas (24). Finally, Edwards et al reported that none of the UV-protected mucosal melanomas in their study had an exon 15 *BRAF* mutation. The authors suggested that the possible presence of a not yet defined oxidative damage serves as a precursor for the *BRAF* T/A transversion and perhaps inflammation-associated oxidative changes influence *BRAF* mutagenesis (25). Panagopoulos et al, commenting on their results by testing malignant soft-tissue melanomas, supported the hypothesis that *BRAF* mutations are unlikely to result from an intrinsic genomic mechanism (23). We suggest that *BRAF* mutagenesis might be strongly influenced by extrinsic, environmental or micro-environmental factors even among tumor types that present a "traditional" tendency to harbor this mutational pattern. This may explain, at least in part, the variation observed in *BRAF* mutation frequency in various studies.

Glarakis et al reported an incidence of *KRAS* mutations in approximately 36% of Greek patients with col-



rectal cancer (18). In our study, *KRAS* mutations at codon 12 were detected in approximately 30% of cases, an incidence in agreement with previous studies on *KRAS* mutations in colorectal cancer (26, 27). *RAS* mutations in colorectal cancer occur mainly at codon 12 of *KRAS* (77-82%) (28, 29). In a population-based study, *KRAS* mutations were detected in 31.8% of 1413 examined cases with colorectal cancer. Of these, 77.9% were found within codon 12 (29). Without ignoring the reported association between specific *KRAS* mutations within codon 13 and clinical outcome (risk of relapse or death) (30), we focused on screening codon 12, which traditionally shows the highest incidence of *KRAS* mutations. We found that the frequency of *KRAS* mutations was higher in younger ( $\leq 70$  years) than in older patients ( $>70$  years). This finding could be significant if we consider that age  $>70$  years was found to have negative independent prognostic value for overall survival in patients with colorectal cancer in a recent study (31). Patients aged between 61 and 70 years are more likely to be carriers of the mutation. Male patients in the age group of 61-70 years presented a higher frequency of a *KRAS* mutant pattern than patients of different age or sex, although this was of borderline significance. Perhaps the onset of *KRAS* mutagenesis in colorectal cancer is an earlier event, as it is probably in alignment with sex-related attitudes or patterns. Samowitz et al stressed the possible influence of lifestyle patterns to which men and women are differentially exposed; for example, use of alcohol or tobacco, dietary factors and hormone replacement therapy may positively or negatively influence the likelihood of these mutations (29). Perhaps the onset of the oncogenic process and the time or duration of exposure represent additional parameters to be considered.

As previously reported, *KRAS* mutations, specifically at codon 12, were found to be significantly more common in advanced-stage tumors (29). Although we did not find a statistically significant association between *KRAS* mutations and Dukes' tumor stage, our results confirm that tumors at Dukes' stages C and D harbored over 60% of the observed mutations. Further research is needed to investigate why exon 15 of *BRAF* possesses high sequence stability and *KRAS* codon 12 mutations among Greek patients show similar incidence rates as studies conducted elsewhere.

Events such as the epigenetic inactivation of the mismatch repair gene *MLH1* seem to be responsible for the progressive accumulation of mutations that define the mutational spectrum in the majority of sporadic tumors with microsatellite instability (MSI) (32). Summarizing the evidence, *BRAF* and *KRAS* mutations lead to different pathways of senescence-related neoplasia in the colon-rectum, the former with CpG island methylator phenotype (CIMP)-high and MSI-high status and the latter with CIMP-low and MSI-low status (33). The role of methylation involving both RAF and RAS pathways moves into

the direction of silencing proapoptotic and cell cycle inhibition genes, as was emphasized in a recently published report focusing on senescence (33). Furthermore, the methylation status of multiple promoters can be predicted through knowledge of *BRAF* and, to a lesser extent, *KRAS* activating mutations, indicating their tight association with different patterns of DNA hypermethylation in colorectal cancer (34). The low frequency of *BRAF* mutations observed in mismatch-repair-proficient tumors might be explained by the absence of *MLH1* hypermethylation (15, 35, 36). In 2 recent studies, the frequencies of *BRAF* mutations in MSI-negative tumors were 4% and 5%, respectively, and in MSI-positive tumors 39% and 52%, respectively (20, 37). Although *BRAF* mutations are much more common in MSI-positive tumors, the comparatively lower frequency of this phenotype means that a considerable proportion occur in MSI-negative tumors (20). We did not analyze our tumor samples for MSI and this is a limitation of our study. However, we would have expected at least a minimum *BRAF* mutation incidence rate, comparable to the lowest rates reported in the literature.

The *BRAF*V600E has been identified as a convenient marker to discriminate between MSI-positive tumors that are sporadic or hereditary nonpolyposis colorectal cancer (38-40). The detection of a *BRAF*V600E mutation in colorectal cancer suggests a sporadic origin of the disease. These findings have a potential impact on the genetic testing for hereditary nonpolyposis colorectal cancer and suggest the potential use of *BRAF* to exclude this condition (41). In our study, the total absence of V600E represents an interesting point. Perhaps a "time-dimension" model needs to be considered. The onset of *BRAF* mutagenesis in colorectal cancer is observed as a later event, probably related to advanced age (21). Enrolling older patients might increase the likelihood of finding these mutations. The mechanisms interacting to render the V600E variant prone to mutagenic alteration (21) and how those mechanisms influence the frequency rate of V600E remain to be established.

Cigarette smoking was recently found to be associated with an increased risk of colon cancer with CpG island methylator phenotype and/or *BRAF*V600E mutations, irrespective of the MSI status. This illustrates how stratification of tumors on the basis of molecular characteristics could reveal associations with risk factors that were masked up till now because of the genetic heterogeneity of cancer (42). In keeping with this, findings from another study suggest that *BRAF* mutations could identify a subgroup of colorectal cancer with distinctive clinical, pathological and molecular features independent of the MSI status (20). Furthermore, results from a recent study evaluating diet and lifestyle associations with CpG island methylator phenotype and incidence of *BRAF* mutations in colorectal cancer suggest the involvement of multiple pathways to colon cancer through direct, inverse or no

correlations between CpG island methylator phenotype status and the various diet or lifestyle factors analyzed (43). The link between the biological behavior and sporadicity of colorectal cancer deserves to be further researched in order to gain insight into its potential clinical application (44).

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