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Comparative Study of Mutations in Single Nucleotide Polymorphism Loci of *KRAS* and *BRAF* Genes in Patients Who Underwent Screening Colonoscopy, With and Without Premalignant Intestinal Polyps

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Abstract. *Aim: Our aim was to perform a comparison study of the mutation rate of V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS), and v-Raf murine sarcoma viral oncogene homolog-B (BRAF) genes between blood-based cell-free DNA (cfDNA), and tissue sample biopsies in individuals undergoing screening colonoscopy. Materials and Methods: All specimens were collected from January 2015 to January 2016. A total of 92 blood samples and colonic biopsy specimens were collected from healthy individuals with no polyps undergoing screening colonoscopy (group A, n=35), patients with colorectal cancer (group B, n=27), and patients with neoplastic intestinal polyps (group C, n=30). Peripheral blood was collected from each patient and a focal tissue biopsy was conducted. Results: We only found a limited statistically significant difference (p=0.046) in the mutation analysis for codon 12 of the KRAS gene when we compared tissue biopsies from patients in group B to those from group C. In the blood samples, only the rate of mutation in codon 12 of the KRAS gene in samples of group B was significantly higher than that in group A (p=0.013). Conclusion: Blood cfDNA may be a promising tool in CRC screening as it may discriminate patients with CRC compared to healthy individuals and those with colonic polyps, even though it does not appear useful in predicting the presence of colonic polyps.*

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Colorectal cancer (CRC) is the third most common malignancy in the Western world and therefore is considered a major public health issue (1). Approximately 1.4 million people worldwide were diagnosed with colorectal cancer in 2012, whilst it is estimated that 693,900 people died from the disease in the same year (2). According to data obtained from the World Health Organization mortality database and Eurostat, nearly 173,400 EU citizens will have died of colorectal cancer in 2016 (3). The high mortality rate is due to the fact that CRC usually remains undetected, until the disease progresses up to a point that it becomes difficult to cure (4). The vast majority of colon cancers evolve from precancerous colorectal lesions called adenomas (5). Therefore, early detection and diagnosis could have a significant impact on the reduction of mortality from CRC.

A new non-invasive method of detecting and monitoring CRC has attracted researchers' interest recently: the molecular characterization of circulating free DNA (cfDNA) in blood circulation. Identification of cf DNA was first reported more than half a century ago (6). These extracellular nucleic acids are active products of apoptosis and necrosis of both normal

and dead cells; they can also be released *via* complex interactions between tumor and adjacent healthy cells (7, 8). Outside the peripheral circulation (plasma, serum), cf DNA may also be found in other body fluids (9). What is essentially novel is that reports in the recent literature showed that cfDNA has a prognostic value in patients with CRC (10-12). According to these data, cfDNA can be used both as a diagnostic tool and as a prognostic marker, and it can also be used in the detection of tumor-specific mutations in the bloodstream of patients with CRC (13).

It is well known that the adenoma–carcinoma sequence constitutes a basic pathway of CRC carcinogenesis (14). The commonest mutations presenting in CRC tumor cells are of V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*). However, the clinical importance of *KRAS* mutation in CRC is debatable; a number of studies reported no association with survival (15, 16), whereas other studies suggested that patients with CRC with *KRAS* mutation have poorer outcome (17-19). *KRAS* and v-Raf murine sarcoma viral oncogene homolog-B (*BRAF*) mutations lead to the constitutive activation of endothelial growth factor receptor (EGFR) signaling through the oncogenic RAS/RAF/MEK/ERK pathway. Due to these mutations, CRC cells develop resistance to monoclonal antibodies to EGFR, as well as to widely used targeted EGFR therapies (20). In order to prevent this potential effect, it is advised that prior to their treatments, all patients eligible for anti-EGFR regimens should be tested for mutations in the *KRAS* gene. Concurrently, *BRAF* V600E point mutations should be identified, since they are found in 9% of cases and may potentially interfere with the targeted therapies (21). *BRAF* is found downstream of *KRAS* and contributes to the activation of the same intracellular pathways. According to several reports, the majority of *KRAS* and *BRAF* mutations found are mutually exclusive (22). A mutation in *BRAF* is considered a negative prognostic factor (23). Still, because of conflicting reports, *BRAF* mutational status has not been yet included in standard CRC treatment guidelines as a guide for anti-EGFR treatment (24-27).

Various methods are currently used in the analysis of the *KRAS* mutational status of a patients with CRC. In fact, the method of determining it from tumor sections of each individual patient is presented as one of the first examples of personalized medicine in the field of oncology (28). Detection of *BRAF* mutations is included in the screening of patients without *KRAS* mutations identified, in accordance with standard guidelines (29).

It is important to point out that although the molecular analysis of mutations is usually performed on tumor tissue, there are several concerns that this method does not reflect the biology of the disease at initiation of EGFR treatment (13). This is because it often takes several years from the time of the primary diagnosis of the tumor until the time of surgery/treatment. Moreover, repeated biopsies are not recommended, since they are deemed unethical and impractical. Therefore, the use of cfDNA for the detection of these tumor-specific genomic alterations may become a promising option for targeted-therapies selection.

The aim of our study was to evaluate the frequency of *KRAS* and *BRAF* mutations in cfDNA extracted from the blood of three groups of patients: Group A: healthy individuals undergoing screening colonoscopy and in whom no polyps were found; group B: patients with CRC; and group C: patients with neoplastic intestinal polyps, in comparison to their respective biopsy samples from intestinal tissue.

Materials and Methods

Participants and specimens. All specimens were collected at the Department of Gastroenterology, Evangelismos Hospital, Athens, Greece, from January 2015 to January 2016. A total of 92 blood samples and 92 colon biopsy specimens were collected from healthy individuals undergoing screening colonoscopy with no polyps (group A, n=35), patients with CRC (group B, n=27), and patients with neoplastic intestinal polyps (group C, n=30). Detailed data are listed in Table I. Peripheral blood was collected from each patient and focal tissue biopsies were conducted. All specimens were stored at −80˚C for further analysis. Patients with a family history of familial adenomas and polyps or hereditary nonpolyposis colorectal cancer (HNPCC) were excluded from this study. Our study was performed in accordance with the guidelines of the Hospital Ethics Committee. Informed consent was obtained from all participants.

Genotype analysis. DNA from biopsy samples was extracted using a commercial kit (Nucleospin Tissue; Macherey-Nagel, Düren Germany). cfDNA was isolated from plasma samples using High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The concentration of DNA was determined in a Nanodrop ND-100 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The presence of a mutation at codon 12 and codon 13 of *KRAS* was determined by an enriched polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) as previously described (30, 31). In summary, a mismatched upstream primer for codon 12 amplification, and a mismatched downstream primer for codon 13 amplification which introduced a *BstNI*, and a *HaeIII* restriction site in the wild-type allele, respectively, were used. The primers used were as follows: KRAS12F: 5'-ACTGAATATAAACTTGTGGTAGTTGGACCT-3', KRAS12R: 5'-CTGTATCAAAGAATGGTCCTGCACCAGTA-3', and KRAS13F: 5'-GTACTGGTGGAGTATTTGATAGTGTATTAA-3', KRAS13R: 5'-GTATCGTCAAGGCACTCTTGCCTAGG-3'. The underlined bases represent mismatches. The expected sizes of the PCR products are 162 bp and 159 bp for codon 12 and 13 respectively. *BstNI* digestion of wild-type codon 12 allele yields two bands of 133 and 29 bp, while the mutant type remains intact (162 bp). *HaeIII* digestion of wild-type codon 13 allele yields fragments of 85, 48 and 26 bp, while the mutant allele yields only two fragments of 85 and 74 bp because of an internal *HaeIII* site at nucleotide 85. As far as the V600E *BRAF* mutation is concerned,

Table I. *Clinical data of the participants*.

Group		Number (n)	Gender (n)		Age (years)	
			Male	Female	Range	Mean±SD
Group A (healthy individuals)		35	15	20	38-78	62.5±9.1
Group B (colorectal cancer)	Tubular adenocarcinoma	11	7	4	48-85	67.4±8.2
	Mucinous adenocarcinoma	3	2	1	41-68	58.2±9.6
	Papillary adenocarcinoma	1	1	0		44
	Mixed adenocarcinoma	11	6	5	61-74	68.2±4.5
	Squamous	1	0	1		58
Group C (neoplastic polyps)	Tubular adenoma	13	9	4	47-84	68.4±9.2
	Villous adenoma	4	2	2	43-69	57.2±10.7
	Tubulovillous adenoma	9	6	3	44-76	63.5±10.3
	Serrated adenoma	4	3	1	57-63	60.2±2.5

Table II. *Mutational analysis in biopsy samples. Data are the number of individuals*.

Group	*KRAS* codon 12		*KRAS* codon 13		*BRAF* (V600E)	
	+	−	+	−	+	−
A (healthy individuals)	1	34	0	35	6	29
B (colorectal cancer)	12	15	0	27	8	19
χ^2 (*vs*. group A)	13.49		-	-	0.74	
p-Value	0.0002		-	-	0.39	
C (neoplastic polyps)	5	25	0	30	8	22
χ^2 (*vs*. group A)	2.21		-	-	0.39	
p-Value	0.14		-	-	0.53	
χ^2 (*vs*. group B)	3.99		-	-	0.062	
p-Value	0.046		-	-	0.80	

+: Mutation(s) present; −: no mutation present.

Table III. *Mutational analysis in blood samples. Data are the number of individuals*.

Group	*KRAS* codon 12		*KRAS* codon 13		*BRAF* (V600E)	
	+	−	+	−	+	−
A (healthy individuals)	2	33	0	35	4	31
B (colorectal cancer)	9	18	0	27	4	23
χ^2 (*vs*. group A)	6.19		-	-	0.0001	
p-Value	0.013		-	-	0.99	
C (neoplastic polyps)	3	27	-	-	6	24
χ^2 (*vs*. group A)	0.03		-	-	0.37	
p-Value	0.86		-	-	0.54	
χ^2 (*vs*. group B)	3.36		-	-	0.03	
p-Value	0.06		-	-	0.87	

+: Mutation(s) present; −: no mutation present.

we used two forward primers with variations in their 3' nucleotides in a way that each was specific for the wild-type (V; AGGTG ATTTTGGTCTAGCTACAGT) or the mutated variant (E; AGG TGATTTTGGTCTAGCTACAGA), and one reverse primer (AS; TAGTAACTCAG-CAGCATCTCAGGGC), as previously described (31, 32). Sequence analysis of representative PCR products confirmed the results.

Statistical analysis. Frequency and susceptibilities of mutations were compared using the χ^2 test. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated with the corresponding χ^2 distribution test. The *p*-values obtained were two-tailed and were determined to be significant at $p<0.05$. The Hardy–Weinberg equilibrium was verified by calculating the expected frequencies and was tested separately in patients and in controls using the goodness-of-fit χ^2 test. All the comparisons were performed using GraphPad version 3.00 (GraphPad Software Inc., San Diego, CA, USA).

Results

A total of 92 participants had both biopsies and blood samples obtained for the reliable detection of *KRAS* and *BRAF* mutations. The mutation status in biopsies and blood samples is presented in Tables II and III.

The detected mutation rate in codon 12 of the *KRAS* gene in the biopsies of group B (CRC) was significantly higher than that for group C (neoplastic polyps) ($p=0.046$) and group A (healthy individuals) ($p<0.001$) but showed no significant difference between groups A and C. None of the patients or controls was found to carry mutations in codon 13 of the *KRAS* gene. Regarding the *BRAF* V600E mutation, the detection rate did not differ significantly among the groups tested.

In the blood samples, the detection rate of mutation in codon 12 of the *KRAS* gene in group C did not show any

significant differences when compared to the mutation rate in groups A and B. In contrast, the rate of mutation in codon 12 of the *KRAS* gene in the blood samples of group B was significantly higher than that of group A (p=0.013). None of the patients or controls were found to carry a mutation in codon 13 of the *KRAS* gene in their blood samples. Regarding the *BRAF* V600E mutation, the detection rate did not differ significantly among the groups tested.

Data derived after the comparison of the mutation status between matched biopsies and blood samples are presented in Table IV. Regarding the overall concordance between the mutation status in the biopsies and in the blood, there was a *KRAS* codon 12 mutation in group A and in one of the biopsy samples from a patient that also demonstrated *KRAS* mutation in the blood sample. There was a single case observed of a healthy individual who had a detectable mutation in the blood, but not in the colon biopsy specimen. For group B, seven patients had *KRAS* mutations in both biopsy and blood samples, five only in biopsies and two only in blood samples. In group C, three of the patients had mutations in both the biopsy and blood samples, while only two patients had mutations solely in blood samples.

Concerning the *BRAF* V600E mutation, six patients in group A were carriers of the mutation; in four others, the mutation was detected in both biopsy and blood samples, whereas in two patients it was present only in biopsy samples. In group B, three patients had the mutation in both biopsy and tissue samples, whereas five patients presented a mutation in the biopsy and one solely in the blood sample. In four patients of group C, the mutation was detected in both biopsy and blood samples, while in four patients, the mutation was found exclusively in biopsies and in one patient only in the blood sample.

Table IV. *Comparison of the presence of mutation between matched biopsy and blood samples. Data are number of cases.*

Group		*KRAS* codon 12 mutation	*BRAF* (V600E) mutation
A (healthy individuals)	Biopsy	0	2
	Blood	1	0
	Biopsy + blood	1	4
B (colorectal cancer)	Biopsy	5	5
	Blood	2	1
	Biopsy +blood	7	3
C (neoplastic polyps)	Biopsy	2	4
	Blood	0	2
	Biopsy + blood	3	4

Discussion

Nowadays it is well known that DNA mutations of *KRAS* and *BRAF* genes have a great impact on the progression of colorectal adenoma to carcinoma. In particular, over 50% of human CRCs and adenomas harbor *KRAS* mutations (14, 33), arising early in the polyp–adenoma–neoplasia sequence, most commonly in codons 12 and 13 (33, 34). In addition, *BRAF* mutations have been linked to high-grade, right-sided CRC, female gender, older age, and high level microsatellite instability in tumors (35, 36). Furthermore, a crucial role of *KRAS* and *BRAF* genotype was their valuable role in systemic chemotherapy, by predicting the therapeutic efficiency of anti-EGFR therapy. As already stated, the status of *KRAS* is the only established predictive biomarker in clinical practice for assessing the response to EGFR inhibitors used for CRC, such as centuximab or panitumumab, due to the fact that a mutant *KRAS* gene is associated with resistance to anti-EGFR immunotherapy. According to Amado *et al*., these monoclonal antibodies induce a response in 17-40% of patients with wild-type *KRAS* CRC tumors compared to 0% of patients with mutated *KRAS* tumors (37). A recent meta-analysis of Qiu *et al*., who investigated 22 studies, including 2,188 patients treated with anti-EGFR, showed that overall *KRAS* mutational status was associated with non-response and short overall survival (38). As far as the predictive role of *BRAF* mutation in EGFR-inhibiting therapy response is concerned, it is not as established as that for *KRAS* mutation and mutated BRAF is not yet an exclusion criterion for this type of therapy (39-41). Nevertheless, Chapman *et al*. demonstrated better survival with *BRAF* inhibiting therapy for melanoma in patients with *BRAF* V600E mutation (42).

In summary genotyping for *KRAS* and *BRAF* mutation status is a gold standard for categorizing CRC for clinical decisions. Unfortunately, tissue sampling of primary tumors is not always available, since it is sometimes difficult to obtain or takes years before metastasis occurs, while the mutation status of primary lesions may change over time (43, 44). To overcome these drawbacks, a non-invasive and cost-effective biomarker could be an ideal method to interpret the status of the entire tumor genome at various points during the course of the disease. Mandel and Metais in 1948 were the first researchers who discovered the existence of cell-free nucleic acids (cfNAs) in the bloodstream, separating the "affected patients from healthy controls" (6). Thus, it was a matter of time for cfNA usage as a noninvasive neoplasia detection method (45-47). The initial report referring to the discovery of "abnormal'' cfNAs in CRC individuals took place in the early 1990s when Sidransky *et al*. discovered *KRAS* gene mutation in stools of CRC-affected patients (48). Since DNA mutations were found in tumor cells of 'liquid biopsies', it is logical to suppose that these alterations are reflected in cfDNA released from neoplastic tissue into the blood. Furthermore, analysis of cfDNA is a non-invasive

technique that could provide invaluable information for early dissemination of tumor cells with significant clinical and biological implications. Thus it is feasible that the use of cfDNA as a liquid biopsy will contribute to this molecular follow-up investigation that is urgently needed.

We describe here a comparative study of mutations implied in single nucleotide polymorphism loci of *KRAS* and *BRAF* genes in individuals who performed screening colonoscopy. A total of 92 blood samples and 92 colon biopsy specimens were collected from healthy subjects undergoing screening colonoscopy with no polyps, patients with CRC, and patients with neoplastic intestinal polyps. *KRAS* and *BRAF* mutation status data (for codons 12 and 13, and V600E respectively) obtained from the analysis of tumor tissue by routine biopsy methods and of peripheral blood DNA using a PCR-RFLP method specifically designed to analyze cfDNA. According to the literature, similar studies comparing frequency of mutation status detection of these genes in bloodstream and biopsy of intestinal tissues are few in number and included very low patient numbers (49-53).

We only found a statistically significant difference (p=0.046) in the mutation analysis for codon 12 of the *KRAS* gene when we compared tissue biopsies from patients in group B (CRC) with group C (neoplastic polyps) and not in the blood-derived cfDNA of the groups compared, although the detection rate in group B was found to be higher than in group C. Moreover, statistical significance ($p\leq$0.013) was demonstrated when we compared the rate of mutation in codon 12 in the biopsies and in blood cfDNA of group B and group A (healthy individuals), a concordance which is supported by the literature (50, 54). To be more specific, 12 patients had a codon 12 *KRAS* mutation in their primary tumor, and identical mutations were found in the plasma DNA of nine of these patients. Similar findings were presented by Lecomte *et al*. who reported that the detection rate of mutations in the peripheral blood ranged between 9% (3/16) and 100% (5/5) (53). Recently, Yen *et al*. showed in their study, which consisted of 76 patients, a higher detection rate of mutation in blood samples, a rate of ~84.4% (52).

Regarding the mutation in codon 13 of the *KRAS* gene, none of the patients or controls were found to carry this specific mutation. Finally, as far as the V600E *BRAF* mutation is concerned, the detection rate did not differ significantly between the groups tested.

Concerning the comparison of the mutation status between matched biopsies and blood samples, a 26% concordance in the mutation status was demonstrated for the *KRAS* codon 12 mutation of group B and a 10% concordance for that of group C. The respective mutation status concordance for *BRAF* V600E was 13.3% for group C and 11.1% for group B patients.

In conclusion, this study evaluated the mutation rates of specific codons of *KRAS* and *BRAF* genes in cfDNA from blood and in biopsy samples of intestine, respectively. Even though the statistical difference was verified in all compared groups of patients, it was possible to detect genomic alterations, especially mutations in the *KRAS* gene, not only in tissue samples, but also in cfDNA extracted from blood samples of healthy individuals undergoing screening colonoscopy, patients with neoplastic intestinal polyps and patients with CRC. Most importantly, blood cfDNA may be a promising tool in CRC screening as it may discriminate patients with CRC from healthy individuals and those with colonic polyps, even though it does not appear useful in predicting the presence of colonic polyps.

Conflicts of Interest

No conflict of interest to declare.

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