

***BRAF* Mutation Is Frequently Present in Sporadic Colorectal Cancer with Methylated *hMLH1*, But Not in Hereditary Nonpolyposis Colorectal Cancer**

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ABSTRACT

Purpose: The *BRAF* gene encodes a serine/threonine kinase and plays an important role in the mitogen-activated protein kinase signaling pathway. *BRAF* mutations in sporadic colorectal cancer with microsatellite instability (MSI) are more frequently detected than those in microsatellite stable cancer. In this study, we sought to compare the frequencies of *BRAF* mutations in sporadic colorectal cancer with MSI with those in hereditary nonpolyposis colorectal cancer (HNPCC).

Experimental Design: We analyzed *BRAF* mutations in 26 colorectal cancer cell lines, 80 sporadic colorectal cancers, and 20 tumors from HNPCC patients by DNA sequencing and sequence-specific PCR. The methylation status of the *hMLH1* gene was measured by either sequencing or restriction enzyme digestion after NaHSO₃ treatment.

Results: We observed a strong correlation of *BRAF* mutation with *hMLH1* promoter methylation. *BRAF* mutations were present in 13 of 15 (87%) of the colorectal cell lines and cancers with methylated *hMLH1*, whereas only 4 of 91 (4%) of the cell lines and cancers with unmethylated *hMLH1* carried the mutations ($P < 0.00001$). Sixteen of 17 mutations were at residue 599 (V599E). A *BRAF* mutation was also identified at residue 463 (G463V) in one cell line. In addition, *BRAF* mutations were not found in any cancers or cell lines with *K-ras* mutations. In 20 MSI+ cancers from HNPCC patients, however, *BRAF* mutations were not detectable, including a subset of 9 tumors with negative *hMLH1* immunostaining and methylated *hMLH1*.

Conclusions: *BRAF* mutations are frequently present in sporadic colorectal cancer with methylated *hMLH1*, but not in HNPCC-related cancers. This discrepancy of *BRAF* mutations between sporadic MSI+ cancer and HNPCC might be used in a strategy for the detection of HNPCC families.

INTRODUCTION

BRAF, a member of RAF gene family, encodes a cytoplasmic serine/threonine kinase, which is an essential component of the mitogen-activated protein kinase signaling pathway. Mutations in the *BRAF* gene, predominantly at codon 599, have been detected in 66% of melanomas, 15% of sporadic colorectal cancers, and a smaller percentage of other cancers (1, 2). The frequency of *BRAF* mutations has been shown to be higher in sporadic colorectal cancer with microsatellite instability (MSI) than in microsatellite stable (MSS) cancer (31% versus 7%; Ref. 3). Most of the mutations in *BRAF* (28 of 32) were thymine to adenine transversion at nucleotide position 1796, leading to the substitution of valine for glutamate at amino acid residue 599 (V599E). In addition, there were no tumors that contained both *K-ras* mutations and V599E (3). In a more recent study, *BRAF* mutations were identified in 5.1% of the sporadic colorectal cancers, and 8 of 16 of these mutations were at amino acids other than residue 599. Of these eight tumors, five also contained *K-ras* mutation (4). MSI is observed in almost all colorectal cancers in hereditary nonpolyposis colorectal cancer (HNPCC) patients, whereas it occurs in approximately 15–20% of sporadic colorectal cancers (5, 6). Although a higher frequency of *BRAF* mutations has been reported to occur in sporadic colorectal cancers with MSI, its prevalence in HNPCC is not known. The purpose of this study was to clarify the relationship between *BRAF* and *K-ras* mutations and also to compare the frequency of *BRAF* mutation in microsatellite unstable cancers from sporadic and HNPCC patients.

MATERIALS AND METHODS

Cell Lines and Tumors. Colorectal cancer cell lines Caco2, Colo201, Colo320, H498, HCT8, HCT116, HRT18, HT29, Lovo, LS123, LS174T, SW48, SW620, SW1116, and SW1463 were obtained from American Type Culture Collection (Manassas, VA). Cell lines VACO5, VACO6, VACO10P, VACO411, VACO432, and VACO457 were kindly provided by Dr. Sanford D. Markowitz (Case Western Reserve University and University Hospitals of Cleveland, Cleveland, OH). Cell lines RW2982 and RW7213 were from Dr. Lance M. Tibbetts (Roger Williams General Hospital, Providence, RI). Cell line C1a was derived from 5583s, provided by Dr. Fred T. Bosman (Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland). Cell lines RKO and C were from Dr. Michael Brattain (Roswell Park Cancer Institute, Buffalo, NY). Cells were grown

Received 7/22/03; revised 9/29/03; accepted 9/30/03.

Grant support: Theodora Betz Foundation and Veterans Administration Medical Research Service.

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in DMEM supplemented with 10% fetal bovine serum at 37°C with 5% CO₂ atmosphere. Primary tumors from sporadic colorectal cancer patients were obtained from San Francisco Veteran Affairs Medical Center and University of California San Francisco. Tumors from HNPCC patients were from Cancer Center of University of California San Francisco.

Microdissection and Purity of Tumor DNA. Archival pathological specimens were obtained, and 5- μ m sections were cut and mounted on microscopic slides. H&E-stained slides were prepared. Tumor and normal mucosa were microdissected as described previously (7). To determine the frequency of *BRAF* and *K-ras* mutations in primary tumors, we first needed to consider the contamination of the tumor tissues with the adjacent normal tissues, which could lead to the underscoring of the mutations. The contamination was estimated by MSI analysis (7–9). The PCR products of the polymorphic alleles from the tumor DNA were compared with those from normal DNA of the same individual. The shifted product of the tumor compared with normal tissue indicated that the tumor is MSI+. In tumor samples, the ratio of the normal product over the sum of normal and shifted products represented the contamination rate of this microdissected tumor. The contamination rate in our microdissected tumors ranged from 5% to 30%, indicating that a >70% purity of tumor DNA can be obtained by microdissection. Thus, the mutation analysis in tumors would not be underscored.

Sequencing. Genomic DNA from colorectal cancer cell lines and microdissected primary tumors was amplified by PCR using primers 5'-CTTTACTTACTACACCTCAG and 5'-TA-CTCAGCAGCATCTCAGG (for activation domain of *BRAF* gene in exon 15), 5'-TTACAGTGGGACAAAGAATTG and 5'-TTATTGATGCGAACAGTGAATAT (for a glycine-rich loop region of *BRAF* gene in exon 11), 5'-GCCTGCTGAAA-ATGACTGAAT and 5'-TTATCTGTATCAAAGAATGGTC (for codons 12 and 13 in exon 1 of *K-ras*), or 5'-CACTGTA-ATAATCCAGACTGTG and 5'-AATTACTCCTTAATGT-CAGCTT (for codon 61 in exon 2 of *K-ras*). The PCR products were separated by electrophoresis on a 2% agarose gel and eluted with QIAquick gel extraction kit (Qiagen). The purified DNA was sequenced with an ABI PRISM 3100 automated sequencer.

Sequence-Specific PCR. Genomic DNA was amplified by PCR with a primer set covering the region to be analyzed. In each primer set, one primer was designed to contain the mutation sequence at the 3' end. The mutations to be determined were those described previously in the literature (2, 4, 10), including the first or second nucleotides at codons 463, 465, 467, and 468 (glycine-rich loop region); codons 580, 585, 593, 594, 595, 596, 598, and 599 (activation domain) of the *BRAF* gene; and codons 12 and 13 of the *K-ras* gene. These loci represented almost all of the mutations reported in the literature. The forward primers for glycine-rich loop region of *BRAF* gene were 5'-TTACAGTGGGACAAAGAATTGA, 5'-TTACAGT-GGGACAAAGAATTGT (codon 463), 5'-GGGACA-AAGAATTGGATCTGC, 5'-GGGACAAAGAATTGGATCT-GA, 5'-GGGACAAAGAATTGGATCTGT (codon 465), 5'-AAAGAATTGGATCTGGATCATG (codon 467), 5'-GAATT-GGATCTGGATCATTTGC, and 5'-GAATTGGATCTGGAT-CATTTGA (codon 468). Their reverse primer was 5'-TTATT-GATGCGAACAGTGAATAT. The forward primers for the

activation domain of the *BRAF* gene were 5'-CTTTACTTAC-TACACCTCAGG (codon 580), 5'-CACCTCAGATATATT-TCTTCATA (codon 585), 5'-GACCTCACAGTAAAAATAG-GTGT, 5'-GACCTCACAGTAAAAATAGGTGG (codon 593), 5'-CACAGTAAAAATAGGTGATTTG (codon 594), 5'-CA-CAGTAAAAATAGGTGATTTTC (codon 595), 5'-GTAAAA-ATAGGTGATTTTGGTG, 5'-GTAAAAATAGGTGATTTT-GGTTCG (codon 596), 5'-TAGGTGATTTTGGTCTAGCTA-T (codon 598), and 5'-GTGATTTTGGTCTAGCTACAGA (codon 599). Their reverse primer was 5'-TAACTCAGCAG-CATCTCAGG. The forward primers for *K-ras* were 5'-AACT-TGTGGTAGTTGGAGCTT, 5'-AACTTGTGGTAGTTGGA-GCTA, 5'-AACTTGTGGTAGTTGGAGCTC, 5'-ACTTGTG-GTAGTTGGAGCTGT, 5'-ACTTGTGGTAGTTGGAGCTGA, 5'-ACTTGTGGTAGTTGGAGCTGC (codon 12), 5'-GTGG-TAGTTGGAGCTGGTGT, 5'-GTGGTAGTTGGAGCTGGT-GA, and 5'-GTGGTAGTTGGAGCTGGTGC (codon 13). Their reverse primer was 5'-GAATGGTCTGCACCAGTAA. PCR was performed by 35 cycles of denaturing (94°C for 30 s), annealing (64°C for 30 s), and chain extension (72°C for 30 s). The PCR products were separated on a 2% agarose gel. The bands with the correct size indicated the mutations. In the sequence-specific PCR analysis, control PCR using primers with wild-type sequences were included.

Sensitivity and Specificity of Sequence-Specific PCR. To evaluate the specificity and sensitivity of the sequence-specific PCR, we used serially diluted DNA (250, 75, 25, 7.5, 2.5, 0.75, 0.25, 0.075, and 0.025 ng) of the cell lines Lovo (carrying wild-type *BRAF* as determined by sequencing) and RKO (carrying *BRAF* mutated at codon 599 as determined by sequencing) in the assay. No PCR product of *BRAF* mutant was observed from 250 ng of Lovo DNA, whereas the mutant *BRAF* product was detected from 0.25 ng of RKO DNA. We also performed *K-ras* mutation analysis by PCR with serially diluted DNA (250, 75, 25, 7.5, 2.5, 0.75, 0.25, 0.075, and 0.025 ng) of cell lines Caco2 (with wild-type of *K-ras* as determined by sequencing), SW1463 (with *K-ras* mutation of T for G at the first nucleotide of codon 12), LS123 (with *K-ras* mutation of A for G at the first nucleotide of codon 12), LS174T (with *K-ras* mutation of A for G at the second nucleotide of codon 12), SW1116 (with *K-ras* mutation of C for G at the second nucleotide of codon 12), SW620 (with *K-ras* mutation of T for G at the second nucleotide of codon 12), and Lovo (with *K-ras* mutation of A for G at the second nucleotide of codon 13). No PCR product of *K-ras* mutant was seen from 250 ng of Caco2 DNA in all assays, whereas the mutant products were detected with 0.25 ng of DNA from the above-mentioned cell lines with *K-ras* mutations. These assays suggest that sequence-specific PCR is sensitive and specific enough for analysis of the *BRAF* and *K-ras* mutations in the primary tumors.

MSI Analysis. The determination of MSI status in the colorectal cancer cell lines and primary tumors was performed as described previously (7–9).

Determination of hMLH1 Methylation. Methylation status of CpG sites in the hMLH1 promoter was analyzed based on the principle that cytidine in DNA is converted to thymidine after DNA is treated with NaHSO₃, whereas the methylated cytidine is resistant to the treatment. Thus, the unmethylated and methylated cytidine can be distinguished by sequencing or

Table 1 BRAF mutations in cell lines and primary tumors of colorectal cancer

	Cell line				Tumor				All			
	MSI ^a		MLH1 met		MSI		MLH1 met		MSI		MLH1 met	
	+	-	+	-	+	-	+	-	+	-	+	-
<i>BRAF</i>												
WT	4	13	0	17	15	57	2	70	19	70	2	87
Mut	7	2	7	2	7	1	6	2	14	3	13	4
% Mut	64	13	100	11	32	2	75	3	42	4	87	4
<i>P</i> ^b	<0.05		<0.001		<0.001		<0.001		<0.0001		<0.00001	

^a MSI, Microsatellite instability; Met, methylation; WT, wild type; Mut, mutant.

^b *P*s are based on χ^2 test.

digestion with a restriction enzyme that recognizes a sequence containing CpG. These two methods were described previously in Refs. 7 and 8.

Immunohistochemistry Analysis. To determine the expression of hMLH1 and hMSH2 proteins in tumors, paraffin sections were stained with anti-hMLH1 and anti-hMSH2 antibodies as described previously (7).

RESULTS

Frequencies of BRAF Mutations in Sporadic MSI+ and MSS Colorectal Cancers. *BRAF* mutations were determined in all 26 colorectal cancer cell lines and 15 tumors by direct sequencing and sequence-specific PCR. Mutations were observed in nine cell lines and two tumors by both methods. Because the mutation status obtained by sequencing and by sequence-specific PCR was identical in all 26 cell lines and 15 tumors, *BRAF* mutations in the other tumors were tested only by sequence-specific PCR. The mutations were observed in 6 of these 65 tumors. The mutations in these six tumors were confirmed by sequencing.

In 26 cell lines and 80 primary sporadic tumors, 17 *BRAF* mutations were identified [9 of 26 (35%) cell lines and 8 of 80 (10%) primary tumors]. Sixteen mutations were thymine to adenine transversion at nucleotide 1796, leading to V599E, whereas in one cell line, a guanine to thymine transversion at nucleotide 1388 was identified, resulting in the substitution of glycine for valine at residue 463 (G463V) in a glycine-rich loop region. The frequencies of mutations were higher in cell lines and sporadic tumors with MSI than in those with MSS [cell lines: 7 of 11 (64%) versus 2 of 15 (13%), $P < 0.05$; tumors: 7 of 22 (32%) versus 1 of 58 (2%), $P < 0.001$; Table 1]. Because *hMLH1* gene methylation and silencing have been reported in most sporadic colorectal cancers with MSI (8, 11, 12), we compared the frequencies of *BRAF* mutations in colorectal cancers with methylated versus unmethylated *hMLH1*. Seven of 7 cell lines with methylated *hMLH1* contained a *BRAF* mutation, whereas mutations were detected in only 2 of 19 cell lines with unmethylated *hMLH1* [11% ($P < 0.001$); Table 1]. Similarly, *BRAF* mutation frequencies were much higher in primary tumors with methylated *hMLH1* [6 of 8 tumors (75%)] than in those with unmethylated *hMLH1* [2 of 72 tumors (3%); $P < 0.001$; Table 1]. The significant increase in *BRAF* mutation frequencies (from 64% of MSI+ cell lines to 100% of cell lines with methylated *hMLH1* and from 32% of MSI+ tumors to 75%

of tumors with methylated *hMLH1*) is due to the fact that *BRAF* mutations do not exist in MSI+ cell lines and tumors with mutated *hMLH1* and *hMSH2* genes. In summary, *BRAF* mutations are more frequently detected in microsatellite unstable colorectal cancers [14 of 33 (42%)] than in MSS cancers [3 of 73 (4%); $P < 0.0001$]. However, *BRAF* mutations occur mainly in MSI+ cancers with methylated *hMLH1* [13 of 15 (87%)], indicating that *BRAF* mutation is closely correlated with *hMLH1* methylation in sporadic colorectal cancer.

In this study, most of the mutations in *BRAF* are valine to glutamate at residue 599 (V599E). The valine at residue 599 is adjacent to threonine at 598 and serine at 601 within the activation domain, which require phosphorylation to achieve maximal kinase activity. The mutation of V599E may mimic the transient phosphorylation of these two amino acid residues during normal signaling, resulting in the constitutive activation of BRAF. Another infrequent mutation, glycine to valine at residue 463 (G463V), is located in a different highly conserved region in exon 11 (glycine-rich loop). Both mutations, V599E and G463V, have been shown to induce elevated basal kinase activities compared with wild-type BRAF and dramatically increase the transformation activities in NIH 3T3 cells (2).

BRAF and K-ras Mutations in Sporadic Colorectal Cancers. The mutations of the *K-ras* gene were analyzed in 26 cell lines and 72 tumors by direct sequencing or sequence-specific PCR. *K-ras* mutations were identified in 11 of 26 (42%) cell lines and 30 of 72 (42%) tumors. *K-ras* mutation frequencies were higher in MSS cell lines [7 of 15 cell lines (47%)] and tumors [24 of 53 tumors (45%)] than in MSI+ cell lines [4 of 11 cell lines (36%)] and tumors [6 of 19 tumors (32%)]. In none of the 98 samples did we find any carrying both *K-ras* and *BRAF* mutations. The *K-ras* mutations were identified only in cell lines and tumors with wild-type *BRAF* (Table 2). We have also noticed that *K-ras* mutations were present only in cancers with unmethylated *hMLH1* (Table 2). This is explained by the fact that *BRAF* mutations are closely related with *hMLH1* methylation. The observations that *BRAF* mutations in colorectal cancer occur mainly in the hot spot at residue 599 (V599E) and that this mutation and *K-ras* mutations are mutually exclusive are consistent with previous reports (2–4). Because *BRAF* mutations, such as V599E, can activate the mitogen-activated protein kinase pathway [RAS-RAF-mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK)-extracellular signal-regulated kinase (ERK)], it is possible that when BRAF is

mutated, RAS mutation is not required for transformation. This hypothesis may explain why mutations of both *K-ras* and V599E were not observed in the same tumor.

BRAF Mutations and *hMLH1* Methylation in HNPCC-Related Cancers. To investigate the *BRAF* mutation status in HNPCC, we analyzed 20 tumors from patients of the University of California San Francisco High-Risk Colorectal Cancer Registry. These patients met at least one of the following requirements: (a) Amsterdam criteria for HNPCC (13); and (b) at least two first-degree relatives with colorectal cancer, and one of the cancers diagnosed at age < 50 years. All 20 of these tumors were microsatellite unstable. Seven of the unstable tumors showed negative hMSH2 immunostaining and came from patients with known *hMSH2* germ-line mutations, whereas 13 tumors (6 of which came from patients with known *hMLH1* germ-line mutations) showed negative hMLH1 immunostaining, (Table 3). Interestingly, no *BRAF* mutations were found in any of these 20 tumors, including 9 tumors with negative hMLH1 staining and demonstrable *hMLH1* methylation (Table 3). Of these nine tumors with methylated *hMLH1*, four came from HNPCC patients who harbored *hMLH1* germ-line mutations. This suggests that *hMLH1* methylation may act as the second hit for *hMLH1* inactivation in HNPCC in addition to somatic mutation and deletion, as has been described previously (14). However, in contrast to sporadic colorectal MSI+ cancers, *BRAF* mutations are not found in HNPCC-related cancers, regardless of the *hMLH1* methylation status.

DISCUSSION

Mutations in the *BRAF* gene have been reported in 66% of melanoma and 15% of sporadic colorectal cancer (1, 2). The frequency of *BRAF* mutations has been shown to be higher in sporadic MSI+ colorectal cancers than in MSS colorectal cancers (3). In this study, we found that *BRAF* mutations occurred frequently in MSI+ cell lines and tumors with methylated *hMLH1* (87%), whereas *BRAF* mutations were not present in four MSI+ cell lines with mutated *hMLH1*, nor were they found in any MSI+ tumors with mutated mismatch repair genes (*hMLH1*, *hMSH2*, and so forth). The underlying mechanisms involved in the close correlation between *hMLH1* methylation and *BRAF* mutation in sporadic colorectal cancer need to be investigated.

In addition, we did not find *BRAF* mutations in HNPCC-related cancers, even in those with methylated *hMLH1*. The high

Table 3 *BRAF* mutation and *hMLH1* methylation status in HNPCC^a

Patient no.	MSI	hMLH1 stain	hMSH2 stain	<i>hMLH1</i> met	<i>BRAF</i> mut
205 ^b	+	+	-	-	-
208 ^b	+	+	-	-	-
235 ^b	+	+	-	-	-
238 ^b	+	+	-	-	-
253 ^b	+	+	-	-	-
270 ^b	+	+	-	-	-
334 ^b	+	+	-	-	-
61	+	-	+	-	-
73	+	-	+	+	-
96	+	-	+	+	-
196 ^c	+	-	+	+	-
206	+	-	+	+	-
214	+	-	+	+	-
222 ^c	+	-	+	+	-
233 ^c	+	-	+	+	-
261 ^c	+	-	+	-	-
266 ^c	+	-	+	-	-
276	+	-	+	+	-
277	+	-	+	-	-
279 ^c	+	-	+	+	-

^a HNPCC, hereditary nonpolyposis colorectal cancer; MSI, microsatellite instability; met, methylation; mut, mutant.

^b Germ-line mutations of *hMSH2* have been identified.

^c Germ-line mutations of *hMLH1* have been identified.

frequency of *BRAF* mutation in sporadic colorectal cancer with MSI and methylated *hMLH1* gene and the lack of *BRAF* mutation in HNPCC might be useful for the identification of HNPCC families. Amsterdam criteria and Bethesda guidelines were developed to aid in clinical diagnosis of HNPCC (13, 15, 16). The detection of germ-line mutations in mismatch repair genes is an important supplement to clinical diagnosis, especially when the patient's family size is small, or the family history details are uncertain. However, germ-line tests are time-consuming and costly due to the heterogeneity of mutations. In addition to this, germ-line mutations of mismatch repair genes are not always detected in HNPCC patients or in families that meet Amsterdam criteria (9). Therefore, a strategy based on molecular analysis of tumor samples might be very beneficial. The difference in *BRAF* mutation status between the sporadic colorectal cancers with methylated *hMLH1* gene and HNPCC-related cancers may prove helpful in distinguishing HNPCC patients, in addition to other molecular assays, such as MSI analysis, hMLH1 and hMSH2 immunostaining, and *hMLH1* methylation analysis.

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Table 2 *BRAF*, *K-ras* mutations, and *hMLH1* methylation in colorectal cancer cell lines and tumors

	Cell line		Primary tumor	
	<i>K-ras</i>		<i>K-ras</i>	
	Mutant	Wild type	Mutant	Wild type
<i>BRAF</i>				
Mutant	0	9	0	6
Wild type	11	6	30	36
<i>MLH1</i> met ^a				
+	0	7	0	6
-	11	8	30	36

^a Met, methylation.

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