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

Guoren Deng,1 Ian Bell,1 Suzanne Crawley,1 James Gum,1 Jonathan P. Terdiman,2 Brian A. Allen,2 Brindusa Truta,2 Marvin H. Sleisenger,1 and Young S. Kim1

1Department of Medicine, Gastrointestinal Research Laboratory, Veteran Affairs Medical Center and 2Cancer Center, University of California San Francisco, San Francisco, California

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).
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 with 5% CO₂ atmosphere. Primary tumors from sporadic colon cancer patients were obtained from San Francisco Veteran Affairs Medical Center and University of California San Francisco.

**Microdissection and Purity of Tumor DNA.** Archival pathological specimens were obtained, and 5-μm sections were cut and mounted on microscopic slides. H&E-stained slides were prepared. Tumor and normal mucosa was 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'-CTTTACTTACATACACCTCAG and 5'-TAACACCAAGCACTCTCAG (for activation domain of **BRAF** gene in exon 15), 5'-TTACAGTGGGACAAAGAATTGTG and 5'-TTATTGATGGCAAGACATGTAAT (for a glycine-rich loop region of **BRAF** gene in exon 11), 5'-GCCGTCTGAAAAGATGACTGAAT and 5'-TTATCCTGTACAAAAGAATGGTC (for codons 12 and 13 in exon 1 of K-ras), or 5'-CAGCTGAATAATCAGACTGTG and 5'-AATCAGTCCATAATGTGGC (for exon 2 of K-ras). The PCR products were separated by electrophoresis on a 2% agarose gel. 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; and codons 12 and 13 of the K-ras gene were 5'-CTTTACTTACATACACCTCAG (codon 580), 5'-CACCTCAGATATATTCTCTTACA (codon 585), 5'-GACCTCAGATATAAAATAGTGTTG (codon 593), 5'-CAAGCAGAAAAATAGTGATTGTTTG (codon 594), 5'-ACAGTAAAAATAGTTGATTTC (codon 595), 5'-GTTAAAATAAGATGTGTTTG (codon 596), 5'-TTAGTTGATTTGGCTACTGTA (codon 598), and 5'-GTAGTTGGTGGTCTAGCTACAG (codon 599). Their reverse primer was 5'-TAAACCTAGCAGCATCTCAGG. The forward primers for K-ras were 5'-AATCTGTGAGTGGAGCTGC (codon 12), 5'-GATGTTGAGCTGGTGT (codon 599). Their reverse primer was 5'-GAATTGCTGACTGGATTTT. The PCR product of K-ras was detected from 0.25 ng of Lovo DNA, whereas the mutant product was detected from 0.025 ng of Lovo 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 the cell lines Lovo (carrying wild-type **BRAF** as determined by sequencing) and KKO (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 KKO 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
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 to 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 (MEK)-extracellular signal-regulated kinase (ERK)], it is possible that when BRAF is

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

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumor</th>
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<td>42</td>
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*MSI, Microsatellite instability; Met, methylation; WT, wild type; Mut, mutant. P-values are based on χ² test.*

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For more information, please refer to the following references:

7. Ref. 7
8. Ref. 8
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 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.

**REFERENCES**


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