

# **BRAF Mutations in Colon Cancer Are Not Likely Attributable to Defective DNA Mismatch Repair<sup>1</sup>**

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## **Abstract**

Frequent *BRAF* mutations were reported recently in a variety of human malignancies, including colorectal cancer. In this study, we screened 293 colorectal cancers for mutations in exons 11 and 15, two regions containing hotspots for *BRAF* mutation. Of the 293 cancers, 170 had normal mismatch repair, and 123 had defective mismatch repair (originating from both somatic as well as germ-line mutations in several of the mismatch repair genes). A total of 63 exonic mutations (22%) were detected, 60 of which were V599E, and one each of D593G, G468E, and D586A. Of the tumors with defective mismatch repair, 34% (42 of 123) had a mutation in *BRAF*, whereas only 12% (21 of 170) of tumors with proficient mismatch repair demonstrated a mutation ( $P < 0.0001$ ). Interestingly, *BRAF* mutations were found most often in cases with an *hMLH1* abnormality (35 of 60) and rarely in cases with an *hMSH2* abnormality (1 of 39;  $P < 0.0001$ ). More interestingly, of the 31 *hMLH1* cases with a *BRAF* mutation, 30 occurred in tumors known to have hypermethylation of *hMLH1* promoter. Only 1 of the 15 cases with a germ-line mutation in *hMLH1* had a mutation in *BRAF*. In this series, *BRAF* mutations occurred rarely in tumors with defective mismatch repair attributable to the presence of germ-line mutation in either *hMLH1* or *hMSH2*. Furthermore, *BRAF* mutations were strongly associated with the epigenetic alteration of *hMLH1*. Overall, these data suggest that *BRAF* mutations are not a consequence of defective mismatch repair *per se*.

## **Introduction**

CRC<sup>3</sup> is the third most commonly diagnosed cancer and the third leading cancer cause of death in the United States (1). Approximately 10–15% of sporadic CRCs are caused by the presence of defective MMR (2). Such tumors are characterized by the presence of tumor MSI (MSI-H) and the absence of protein expression for any one of a number of genes involved in DNA mismatch repair, including *hMLH1*, *hMSH2*, *hMSH6*, or *PMS2* (2, 3). In sporadic CRC with defective MMR, ~90% of the cases are the result of inactivation of *hMLH1* attributable to promoter hypermethylation (4–6). The remaining cases are primarily the result of either somatic or germ-line mutations in the various MMR genes (2, 6).

The *Ras/Raf/MEK/MAP* kinase cascade is an essential component of intracellular signaling from activated cell surface receptors to transcription factors in the cell nucleus. Mutations of the *Raf* activator *Ras* are present in 30% of human cancers (7, 8), and their transforming potential is dependent on *Raf* (9). *BRAF* is one of three known *Raf* genes thought to have arisen from gene duplication (the other two are

*ARAF1* and *CRAF*). Recently, Davies *et al.* (10) reported the presence of *BRAF* somatic mutations in 66% of malignant melanomas and at a lower frequency in a wide range of other human cancers, including colon cancers. *BRAF* mutations in CRC were then reported to occur more frequently in those cases characterized by the presence of defective DNA MMR, with the authors suggesting that mutations in *BRAF* may be a consequence of defective MMR (11). In the current study, we confirm the observation that *BRAF* mutations are associated with the presence of defective MMR. In addition, however, we now demonstrate that *BRAF* mutations occur almost exclusively in tumors demonstrating the involvement of *hMLH1* attributable to promoter hypermethylation. *BRAF* mutations rarely occurred in the presence of germ-line mutations in MMR genes, suggesting that other genes and/or other factors have a more important role in the etiology of *BRAF* alterations rather than defective MMR *per se*.

## **Materials and Methods**

**Patient Population.** Paired normal/tumor tissue, and in some cases blood, was collected from 293 patients with colorectal cancer. Patients were selected from a number of ongoing studies specifically to enrich for cases with defective MMR. Tumor site and age at diagnosis were available for all but 7 cases. For tumor site, tumors of the proximal colon were defined as those CRCs occurring in the cecum, the ascending colon, and the transverse colon. Distal tumors were defined as those occurring in the descending or sigmoid colon and in the rectum.

**DNA Extraction.** DNA was extracted from microdissected frozen or paraffin-embedded tissue sections by a standard phenol/chloroform procedure or with a DNA extraction kit (Qiagen). For tumor DNA, only those areas containing >70% tumor cells were used. The corresponding normal control DNA for each patient was derived from adjacent normal mucosa or blood leukocytes. For blood specimens, DNA was extracted using the Puregene nucleic acid isolation kit (Gentra).

**MSI Testing.** For 240 of the cases, paired normal and tumor DNA were analyzed for microsatellite instability with six dinucleotide microsatellite markers (*D5S346*, *MYCL*, *D18S55*, *D17S250*, *D10S197*, and *ACTC*) and one mononucleotide repeat (*BAT 26*). For 53 of the cases, six dinucleotide (*D5S346*, *TP53*, *D18S34*, *D18S49*, *D18S61*, and *ACTC*) and four mononucleotide (*BAT 25*, *BAT 26*, *BAT 40*, and *BAT 34c4*) microsatellite markers were used. Tumors were classified as *MSI-H* if  $\geq 30\%$  markers demonstrated instability, *MSI-L* if  $< 30\%$  demonstrated MSI, and MSS if no marker exhibited MSI (12).

**Immunohistochemical Analysis.** The expression of *hMLH1* and *hMSH2* protein was assessed as described previously (6). Briefly, 5- $\mu$ m tissue sections from formalin-fixed, paraffin-embedded tissue were stained with antibody to *hMLH1* (clone G168 728; PharMingen; 1 mg/ml) and *hMSH2* (clone FE11; 0.5 mg/ml; Oncogene Science). Tumor cells that showed an absence of nuclear staining in the presence of normal positive staining in surrounding cells were interpreted as having an absence of expression of these proteins.

**Promoter Methylation of *hMLH1*.** The methylation status of the promoter regions of both *hMLH1* and *hMSH2* for 52 cases has been reported previously (6).

**Mutational Screening and Direct Sequencing of the *BRAF* gene.** The PCR primers for amplifying exons 11 and 15 were identical to those published

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<sup>3</sup> The abbreviations used are: CRC, colorectal cancer; MMR, mismatch repair; MSI, microsatellite instability; MSS, microsatellite stable; CSGE, conformation-sensitive gel electrophoresis; IHC, immunohistochemistry.

originally (10). A duplex PCR that simultaneously amplified the two exons was developed. PCR was performed for 30 cycles with initial denaturation at 94°C for 12 min, followed by 94°C for 20 s, 58°C for 30 s, and 72°C for 1 min. The reaction was processed in a total volume of 12.5 μl consisting of 200 μM each dATP, dGTP, and dTTP; 50 μM dCTP and 0.1 μl of [<sup>32</sup>P]dCTP; 2 mM MgCl<sub>2</sub>; 30 ng of template DNA; 1× AmpliTaq Gold buffer II; 0.5 unit of TaqAmpliGold DNA polymerase (Perkin-Elmer); and 6.25 pmol of each of the four primers. The PCR product was then denatured at 96°C for 5 min and cooled to 65°C over 30 min. The reannealed product (5 μl) was then mixed with 1 μl of loading dye (30% glycerol, 0.25% bromphenol blue, and 0.25% xylene cyanol FF). This mix (0.5 μl) was then loaded onto a CSGE gel consisting of 15% of acrylamide/1,4-bis(acryloyl)piperazine (19:1), 0.5× TTE buffer (44.4 mM Tris, 14.25 mM taurine, and 0.1 mM EDTA, pH 9.0), 15% formamide, and 10% ethylene glycol. The gel was run at 30 W for 5 h. When altered bands were detected, the patient samples were reamplified separately, and the purified PCR product, along with 3.8 pmol of sequencing primer, was mixed and sequenced using an ABI DNA sequencer. We also tested mixed samples to confirm that the CSGE was sensitive enough to detect mutation in tumors containing <50% tumor cells.

**Results**

Among the 293 CRC samples, a total of 63 exonic mutations (22%) were detected (Table 1). Normal/tumor pairs were examined in 48 of the 63 mutation-positive cases. When examined, all were somatic changes. The majority of mutations detected (60 of 63) had a T-to-A transversion at bp position 1796, resulting in a valine-to-glutamic acid substitution at codon 599 (V599E). We also observed one A-to-G transition at bp position 1778 leading to an aspartic acid-to-glycine substitution at codon 593 (D593G), one G-to-A transition at bp position 1403 producing a glycine-to-glutamic acid substitution at codon 468 (G468E), and one A-to-C transversion at bp position 1757 resulting in an aspartic acid to alanine substitution at codon 586 (D586A). The results of CSGE and DNA sequence analysis for representative alterations are shown in Fig. 1.

Given the high frequency of BRAF mutations in CRC, we examined their potential role in defective versus proficient MMR tumors, two distinct pathways in CRC development. Patients were selected from a number of ongoing studies specifically to enrich for cases with defective MMR (resulting from both somatic as well as germ-line mutations). All of the tumors (n = 293) were tested for the presence of MSI, and 188 were examined by IHC for hMLH1 and hMSH2 protein expression. Absence of protein expression by IHC was ob-

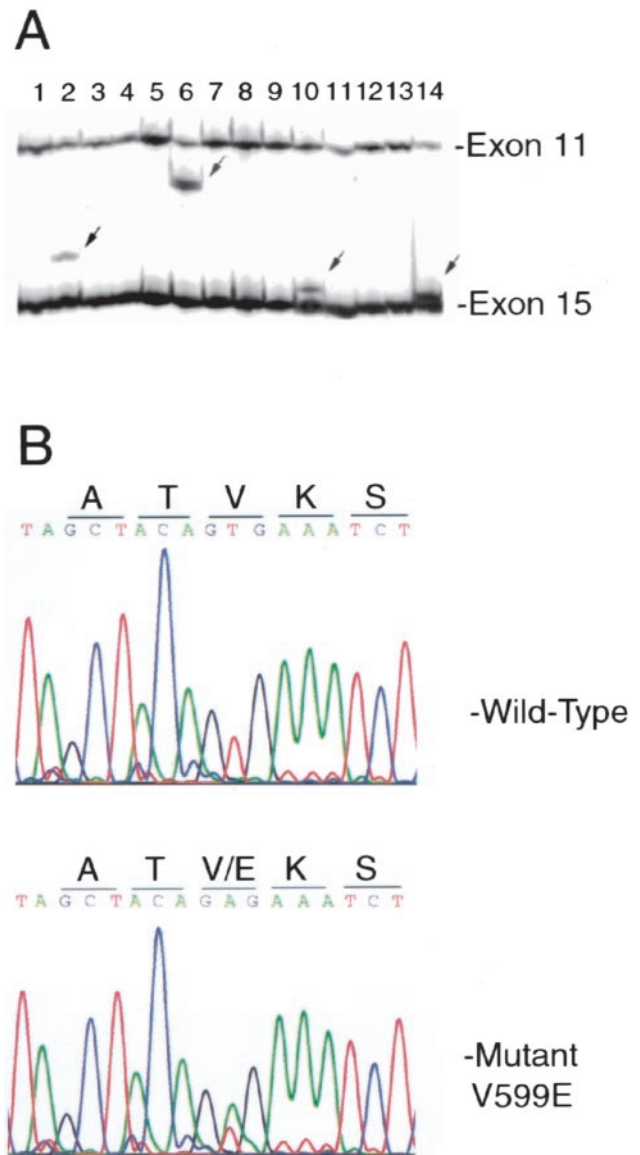


Fig. 1. CSGE and DNA sequence analysis for some representative specimens. For the CSGE, a duplex PCR that simultaneously amplifies exons 11 and 15 from tumor DNA was used in this study. When an altered band was detected, direct sequencing was performed on the product from a separate PCR reaction to confirm and characterize the sequence variant. A illustrates the CSGE banding pattern detected for the four mutations (arrows) identified in this series (Lane 2, D593G; Lane 6, G468E; Lane 10, V599E; and Lane 14, D586A). B illustrates the DNA sequence analysis for the most common mutation, V599E.

Table 1 BRAF mutations in CRCs with defective (MSI) and proficient (MSS) DNA MMR

CRCs	No. of cases	No. of mutants (%)	P <sup>a</sup>
All types	293	63 (22)	
MSS	170	21 (12)	
MSI-H	123	42 (34)	<0.0001
hMLH1	60	35 (58)	
Germ-line mutation	15	1 <sup>b</sup> (7)	
Hypermethylation	36	30 (83)	
Not determined	9	4 (44)	
hMSH2	39	1 (3)	<0.0001 <sup>c</sup>
Germ-line mutation	4	1 (25)	
Not determined	35	0 (0)	
IHC not performed	24	6 (25)	
Proximal <sup>d</sup>	169	54 (32)	
Distal <sup>d</sup>	117	9 (8)	<0.0001
Age <sup>d</sup>			
>81	27	9 (33)	
71-80	117	34 (29)	
61-70	58	15 (26)	
51-60	26	4 (15)	
<51	63	1 (2)	<0.0001

<sup>a</sup> Results from Fisher's exact test.

<sup>b</sup> Four of the 15 hMLH1 germ-line cases were also examined for hMLH1 hypermethylation; this case was the only one showing hMLH1 hypermethylation.

<sup>c</sup> Compares frequency of mutation in hMLH1 cases to that in hMSH2 cases.

<sup>d</sup> There were 7 cases without site information and 2 cases without age information.

served only in the MSI-H group of CRC. Overall, 34% (42 of 123) of the tumors with defective MMR had a mutation in BRAF, whereas only 12% (21/170) of tumors with proficient MMR demonstrated a mutation (Table 1). The difference in the mutation frequency between these two groups of tumors is statistically significant (P < 0.0001).

Because different genes and different mechanisms of gene inactivation underlie defective MMR in CRC, we examined the frequency of BRAF mutations as a function of the gene involved and the mode of gene inactivation (Table 1). Of the 123 cases with defective MMR, 60 were attributable to hMLH1, 39 were attributable to hMSH2, and 24 were not defined (IHC was not performed). Of the hMLH1 cases, 15 were carriers of a germ-line mutation (2 missense and 13 nonsense, frameshift, or splice), 36 were known to have hypermethylation of the promoter, and in 9 cases, the mechanism was not determined (Table 1). Of the hMSH2 cases, 4 were carriers of a germ-line mutation (1

missense, 1 splice, and 2 frameshifts), and in 35 cases, the mechanism was not determined (Table 1). When examined, *BRAF* mutations were found most often in cases with an *hMLH1* abnormality (35 of 60) and rarely in cases with an *hMSH2* abnormality (1 of 39;  $P < 0.0001$ ). Additionally, the presence of *BRAF* mutations was highly restricted to those cases with hypermethylation of the *hMLH1* promoter (30 of 36 hypermethylated cases versus 1 of 15 germ-line cases). Together, 30 of the 31 *hMLH1* cases (having a defined mechanism of gene inactivation) with a *BRAF* mutation occurred in those tumors with promoter hypermethylation of *hMLH1*. The germ-line mutation in the single *hMLH1* case with a *BRAF* alteration was a missense change. Furthermore, the *hMLH1* promoter was hypermethylated in this case, suggesting that the missense change was nonpathogenic. Because the frequency of *BRAF* mutations was considerably lower in the *hMSH2* cases, we mixed tumor DNA with normal control DNA before CSGE analysis to avoid missing detection of a mutation caused by the absence of a wild-type allele (because of loss of heterozygosity). DNA sequence analysis was also performed on several of these samples. No additional mutations were detected.

We also examined a variety of pathological and clinical features for associations with the presence of a mutation in *BRAF* (Table 1). When different sites of cancers were compared, tumors from the proximal colon were more likely to harbor somatic *BRAF* mutations than tumors from the distal colon ( $P < 0.0001$ ). For age, we divided those patients in five different groups with 10-year intervals. The frequency of mutations within the different age groups showed statistically significant differences ( $P < 0.0001$ ), with older patients having a higher frequency of the *BRAF* mutations compared with younger patients.

## Discussion

In this study, we examined 293 CRC cases for mutations in exons 11 and 15 of the *BRAF* oncogene, the two regions shown previously to contain hotspots for mutation (10). Our data confirmed the previous finding that *BRAF* mutations are frequent in CRC (10, 11), and that they are associated with the presence of defective MMR (11). However, after a more detailed analysis of those tumors with defective MMR, our data also suggest that mutations in *BRAF* are not the result of defective MMR, as suggested previously (11). Data supporting this argument include the following:

(a) *BRAF* mutations occurred rarely in tumors with defective MMR because of the presence of a germ-line mutation in either *hMLH1* or *hMSH2*. Only 2 mutations were identified among 19 cases with a known germ-line mutation in one of these two MMR genes. Although mutation information was not available for 35 of the *hMSH2* cases, the majority of these are likely to be germline.<sup>4</sup> Regardless of the mechanism of gene inactivation for *hMSH2*, however, only 1 of these 39 cases had a mutation in *BRAF*. Overall, only 2 of 54 cases confined to these subgroups of defective MMR demonstrated a mutation within the *BRAF* gene. When *BRAF* mutations were identified, they were more strongly associated with the presence of an epigenetic alteration of *hMLH1* (Table 1). Overall, 30 of the 31 *hMLH1* cases (having a defined mechanism of gene inactivation) with a *BRAF* mutation occurred in those tumors with promoter hypermethylation of *hMLH1*. To our knowledge, inactivation of *hMSH2* by promoter hypermethylation has not been reported (5).

(b) A fraction (21 of 170; 12%) of tumors with proficient MMR (MSS group) in our series also showed mutations in *BRAF*. In fact, based on the frequency of *BRAF* mutations determined in this study, it is possible to calculate the prevalence of such alterations in sporadic

CRC. That is, in a group of 100 sporadic CRCs, one would expect to find approximately 18–22 cases with *BRAF* mutations, 8–11 cases originating from the defective MMR group and 10–11 cases originating from the MSS group. Thus, although the relative frequency of *BRAF* mutations within the two groups is quite different, the absolute number is approximately the same. Overall, these data suggest that *BRAF* mutations are not a consequence of defective MMR *per se*. Rather, these data suggest the importance of other mechanisms.

Although our data suggest that *BRAF* mutations in CRC are not a consequence of defective MMR, the mechanism(s) responsible for their occurrence is, at this point, unknown. Because *BRAF* mutations were found more frequently in the sporadic cases with defective MMR compared with the germ-line cases with defective MMR, their occurrence may reflect fundamental differences in tumor initiation and/or progression between these two tumor types. Over time, such differences may favor the selection *BRAF* mutations in one group of tumors compared with the other. Unfortunately, few experiments have been performed that examine, in detail, the molecular and biochemical differences between the sporadic and the hereditary forms of colon cancers that have defective MMR. Another possible explanation is that *BRAF* mutations arise as a consequence of the inactivation of another gene or genes not involved in DNA MMR. If those tumors containing the epigenetic inactivation of *hMLH1* also exhibit more frequent and/or restricted promoter hypermethylation at other loci (compared with other tumors), then such a mechanism might help account for the *BRAF/hMLH1* association observed in this study. A number of other genes affecting the mutation rate, or the type of mutation, have been shown to be inactivated by promoter hypermethylation. For example, the inactivation of the *O<sup>6</sup>*-methylguanine-DNA methyltransferase gene by promoter hypermethylation has been reported to be associated with the presence of G:C to A:T transition mutations in p53 in human colorectal and brain tumors (13, 14). If this were the mechanism involved in these abnormal MMR cases, then a similar mechanism could be operating in the MSS cases. However, it is important to note that the role of an epigenetic mechanism for *BRAF* alterations is entirely speculative and is not supported by experimental data at this time. This is especially the case for the presence of *BRAF* mutations in the MSS group of cancers, which would account for approximately one-half of the cases expected in a group of sporadic CRCs. Finally, because we did not examine the entire *BRAF* gene, we cannot rule out the presence of other mutations in the other subgroups of CRC. This seems less likely, however, because the V599E mutation is the most common alteration identified to date (10, 11, 15–17).

As indicated above, the most common *BRAF* mutation identified to date (10, 11, 15–17), including those identified in our series, is V599E. This missense mutation has been demonstrated to maximally activate kinase activity of the *BRAF* protein by stimulating phosphorylation of endogenous extracellular signal-regulated kinases 1 and 2 (10). By transfection of the V599E mutant into NIH3T3 cells, the ability of the kinase-activated *BRAF* mutant to induce transformation has also been demonstrated (10). It is clear that this variant has a strong functional selection for growth advantage. However, what structural or sequence elements surrounding this variant make it prone to mutagenesis remain to be determined.

In summary, our data show that *BRAF* mutations are frequent in CRC and that they are associated with the presence of defective MMR. More specifically, however, in those cases with defective MMR, *BRAF* mutations occur primarily in the subgroup of cases defined by the epigenetic inactivation of *hMLH1*. Although the mechanism for this strong association is unknown, our data suggest that the etiology of mutations in *BRAF* is not likely attributable to defective MMR but more likely operates through an alternative mechanism.

<sup>4</sup> Cunningham, J. M. *et al.*, unpublished observations.

Clearly, additional experiments will have to be performed to better understand the etiology of mutations in the *BRAF* gene and the cause of their association in certain subgroups of CRC.

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