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Advances in Brief

Similarity of the Phenotypic Patterns Associated with *BRAF* and *KRAS* Mutations in Colorectal Neoplasia[1]

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Abstract

Activation of the RAS/RAF/extracellular signal-regulated kinase-mitogen-activated protein kinase/extracellular signal-regulated kinase/mitogen-activated protein kinase pathway by *RAS* mutations is commonly found in human cancers. Recently, we reported that mutation of *BRAF* provides an alternative route for activation of this signaling pathway and can be found in melanomas, colorectal cancers, and ovarian tumors. Here we perform an extensive characterization of *BRAF* mutations in a large series of colorectal tumors in various stages of neoplastic transformation. *BRAF* mutations were found in 11 of 215 (5.1%) colorectal adenocarcinomas, 3 of 108 (2.8%) sporadic adenomas, 1 of 63 (1.6%) adenomas from familial adenomatous polyposis (FAP) patients, and 1 of 3 (33%) hyperplastic polyps. *KRAS* mutations were detected in 34% of carcinomas, 31% of sporadic adenomas, 9% of FAP adenomas, and no hyperplastic polyps. Eight of 16 *BRAF* mutations were *V599E*, the previously described hotspot, and none of these was associated with a *KRAS* mutation in the same lesion. The remaining eight mutations involve other conserved amino acids in the kinase domain, and 62.5% have a *KRAS* mutation in the same tumor. Our data suggest that *BRAF* mutations are, to some extent, biologically similar to *RAS* mutations in colorectal cancer because both occur at approximately the same stage of the adenoma-carcinoma sequence, both are associated with villous morphology, and both are less common in adenomas from FAP cases. By contrast, colorectal adenocarcinomas with *BRAF* mutations are associated with early Dukes' tumor stages ($P = 0.006$) and no such relationship was observed for *KRAS* mutations. The presence in some colorectal neoplasms of mutations in both *BRAF* and *KRAS* suggests that modulation of the RAS-RAF-extracellular signal-regulated kinase-mitogen-activated protein kinase/extracellular signal-regulated kinase/mitogen-activated protein kinase signaling pathway may occur by mutation of multiple components.

Introduction

Colorectal cancer develops through a multistep process of mutation and clonal expansion. It has been shown that somatic mutation of the *RAS* genes, in particular *KRAS*, is an early event in colorectal carcinogenesis, predominantly occurring during the transformation of a small to intermediate sized adenoma (1).

The RAS proteins participate in the RAS-RAF-MEK-ERK-MAP kinase[3] pathway, which mediates cellular responses to growth signals (2). There are three *RAF* genes, each encoding cytoplasmic serine/threonine kinases that are regulated by binding to RAS (2, 3). We have previously reported that *BRAF* is somatically mutated in a number of human cancers, including malignant melanoma, colorectal carcinoma, and ovarian borderline (low malignant potential) tumors (4). Mutations in *BRAF* occur in two regions of the *BRAF* kinase domain, the G loop (which mediates binding of ATP) and the activation segment (which protects the substrate binding site). Mutated forms of *BRAF* that have been studied thus far have elevated kinase activity and can transform NIH3T3 cells (4).

We now investigate in greater detail the occurrence and spectrum of *BRAF* mutations in colorectal cancer and in particular their biological relationship to *KRAS* mutations.

Materials and Methods

Colorectal Tumor Samples. Samples from colorectal cancer surgical resections and polyps were collected at Queen Mary Hospital between 1990 and 2001. All specimens were received fresh from the operating theater or endoscopy room. Representative tumor and normal blocks (if available) were snap frozen in liquid nitrogen and stored at −70°C. Frozen sections were cut for histology evaluation. Blocks with tumor occupying >70% of the section area were used. The remainder of each specimen was fixed in 10% buffered formalin and processed through paraffin for histology. DNA was extracted by standard protocols using proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. The study was approved by the Ethics Committee of the University of Hong Kong.

Mutation Screening. Screening for mutations was performed as described previously (4). In brief, we used a capillary-based modified heteroduplex method optimized to run on an ABI PRISM 3100 Genetic Analyzer. PCR primers were designed to amplify the exons plus at least 50 bp of flanking intronic sequence (details of the primers used are available in the supplementary information of our previous paper (4). Genomic DNA, 12 ng from the test sample, was mixed with 3 ng of control genomic DNA and amplified using standard PCR conditions in which one of the primers was labeled with FAM, NED, or VIC dye. The resulting samples were then analyzed on an ABI PRISM 3100 Genetic Analyser under semidenaturing conditions using optimized separation medium and run conditions. The resulting traces were analyzed using proprietary software to identify samples that produce a shift in peak migration relative to a standard normal control, indicating the presence of a putative sequence variation. Samples that produced a heteroduplex shift were directly sequenced on both strands using the BigDye terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according to the manufacturer's protocol and analyzed on an ABI PRISM 3100 Genetic Analyser. Analysis of microsatellites for the presence of instability in colorectal cancers was performed as previously described (5). The following loci were used: *BAT26*, *BAT25*, *BAT40*, *TGFβRII* (transforming growth factor-β receptor II),

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[3] The abbreviations used are: RAS-RAF-ERK-MEK-MAP kinase, RAS-RAF-extracellular signal-regulated kinase-mitogen-activated protein kinase/extracellular signal-regulated kinase/mitogen-activated protein kinase; FAP, familial adenomatous polyposis; MSI, microsatellite instability.

Table 1 *Spectrum of BRAF mutation in 11 colorectal cancers, their associated clinicopathological features, and KRAS mutation status*

BRAF mutations									
Nucleotide	aa[a]	Sex	Age (yr)	Tumor side	MSI	Dukes' stage	Mucinous	Differentiation	*KRAS*
T1796A	*V(599)E*[b]	M	75	R	MSI-H	B	Yes	Moderate	Nil
T1796A	*V(599)E*[b]	M	62	L	MSS	C	No	Moderate	Nil
T1796A	*V(599)E*	F	67	R	MSI-H	B	No	Poor	Nil
T1796A	*V(599)E*	F	38	L	MSS	B	Yes	Moderate	Nil
C1793T	*T(598)I*	M	64	L	MSI-H	B	No	Moderate	*G13D*
G1783C	*G595R*	F	62	L	MSS	B	No	Well	Nil
A1778T	*D593V*	M	43	L	MSI-L	B	No	Moderate	*G12D*
A1778G	*D593G*	M	43	L	MSS	B	No	Moderate	*G13C*
A1739G	*N580S*	M	87	L	MSS	B	No	Well	*Q22K*
G1403A	*G468E*[b]	F	76	L	MSS	B	No	Moderate	Nil
T1400G	*F467C*	F	40	L	MSS	A	No	Moderate	*G12D*

[a] aa, amino acid.
[b] These mutations have been reported in our previous study (4).

D2S123, *D5S346*, *TP53*, *D18S58*, *D3S1067*, *D5S82*, and *DCC* (deleted in colon cancer). At least five loci were analyzed in each case, including both dinucleotide and mononucleotide loci. Tumors were designated high-level microsatellite instability (MSI-H) when at least 40% loci showed altered electrophoretic mobility relative to the corresponding normal tissue. Tumors with some (but <40%) altered loci were designated low-level microsatellite instability (MSI-L), and those without altered loci were designated microsatellite stable (MSS). For colonic polyps, the MSI status was defined by shifts in *BAT26* and *BAT25* as corresponding normal tissue was not available.

Results

We studied 215 colorectal adenocarcinomas from colectomies (212 cases) or local resections of liver metastases (3 cases); 122 were from male and 93 from female patients. The patients' age ranged from 24 to 89 years (mean age, 58 years). There were 45 tumors in the right colon and 170 in the left colon. There were 28 Dukes' A, 71 Dukes' B, 83 Dukes' C, and 33 Dukes' D cancers. Nineteen were well-differentiated, 168 were moderately differentiated, and 28 were poorly differentiated. Twenty of these cases have been included in our previous study reporting the incidence of *BRAF* mutation in various types of human cancer (4).

We also studied 113 sporadic colorectal polyps; 72 were from male patients, and 41 were from female patients. The patients' age ranged from 20 to 87 years (mean age, 62 years). There were 66 tubular adenomas, 25 tubulovillous adenomas, 17 villous adenomas, 1 retention polyp, 3 hyperplastic polyps, and 1 Peutz-Jegher polyp. Among these, 4 showed no dysplasia, 32 showed mild dysplasia, 34 showed moderate dysplasia, and 33 showed severe dysplasia. 10 showed early malignant transformation into invasive cancers. 88 were located in the left colon and 24 in the right colon (1 case had unknown tumor location). The size of the polyps ranged from 3 to 45 mm in maximum dimension (mean, 10.88 mm; see Table 6 for size distribution). In addition, 63 polyps from 3 patients with FAP[4] were studied. The diagnosis of FAP in these individuals was made on the basis of clinical criteria and confirmed by identification of germline adenomatous polyposis coli mutations (patient 1, deletion exons 4–7; patient 2, c.1349–1355delTCTGTGT; patient 3, c.3284–3285delAG). The FAP polyps ranged from 3 to 20 mm in maximum dimension (mean, 6.3 mm). These tumors were generally small tubular adenomas with low-grade dysplasia (see Table 6 for size distribution); 21 were located in the left colon and 42 were found in the right colon. There were 58 tubular, 1 villous, and 4 tubulovillous adenomas. Forty-two had mild dysplasia, 19 had moderate dysplasia, and 2 had severe dysplasia.

The spectrum of *BRAF* mutations found in both colorectal adenocarcinomas and colonic polyps, their association with clinicopathological features, and *KRAS* mutations are shown in Tables 1 and 2. Of 215 colorectal adenocarcinomas studied, there were 11 cases (5.1%) with *BRAF* mutations (3 of these were previously reported (4). The mutations were absent in the corresponding normal mucosae and hence were somatically acquired. Of these colorectal cancers, 208 were successfully analyzed for *KRAS* mutations; in 70 (33.7%), a *KRAS* mutation was identified. The spectrum of *KRAS* mutations is shown in Table 3.

Of the 11 colorectal cancers with *BRAF* mutations, 5 were from female and 6 were from male patients. Two arose from the right colon and nine from the left colon. Two were well differentiated, eight were moderately differentiated, and one was poorly differentiated. Two were mucinous adenocarcinomas (from a total of 15 mucinous carcinomas examined). One tumor was Dukes' stage A (of 27 examined), 9 were Dukes' stage B (of 71 examined), 1 was Dukes' stage C (of 83 examined), and none was Dukes' stage D (of 33 examined) (Table 4). Overall, there was no significant association of *BRAF* mutations with patient gender, age, tumor differentiation, mucinous morphology, or location. There was a statistically significant association of *BRAF* mutations with early Dukes' stages (Dukes' stage A and B *versus* C and D, $P = 0.006$, χ^2 test). MSI status was available in 107 cancers. 3 of 11 cancers (27%) with *BRAF* mutation were MSI-H, compared with 19 of 96 cancers (20%) without *BRAF* mutations ($P = 0.851$). We did not observe any association of *KRAS* mutations with patient gender, age, tumor location, differentiation, mucinous morphology, or tumor stage.

Of the 113 sporadic colonic polyps studied, 4 had *BRAF* mutations (3.5%). *BRAF* mutations were found in 3 of 108 adenomas (2.8%), 1 of 3 hyperplastic polyps (33%), and none in the single retention and Peutz-Jegher polyps. We successfully analyzed 108 polyps for *KRAS* mutations, and in 32 (29.6%) a *KRAS* mutation was identified [all

Table 2 *Spectrum of BRAF mutation in 5 colonic polyps, their associated clinicopathological features, and KRAS mutation status*

BRAF mutations										
Nucleotide	aa	Sex	Age (yr)	Sporadic/FAP	Tumor side	MSI	Tumor size (mm)	Morphology	Dysplasia	*KRAS*
T1796A	*V(599)E*	M	62	Sporadic	L	MSS	5.5	Hyperplastic	Nil	Nil
T1796A	*V(599)E*	M	49	Sporadic	R	MSS	13	Villous	Mild	Nil
T1796A	*V(599)E*	M	60	Sporadic	R	MSS	10	Villous	Moderate	Nil
T1796A	*V(599)E*	M	65	Sporadic	R	MSS	9	Villous	Severe	Nil
T1782G	*F(594)L*	M	68	FAP	R	MSS	5	Tubular	Mild	Nil

Table 3 *Spectrum of KRAS mutation in 70 colorectal cancers, 32 sporadic adenomas, and 5 FAP adenomas*

Nucleotide change	Amino acid change	No. of CRCs	No. of adenomas	No. of FAP adenomas
G35A	*G12D*	21	14	2
G35T	*G12V*	15	6	1
G34A	*G12S*	4		
G34T	*G12C*		3	1
G35C	*G12A*	2	1	
G34C	*G12R*	1		
G38A	*G13D*	19	5	
G37T	*G13C*	2	1	
C64A	*Q22K*	1		
C176A	*A59E*	1		
G175A	*A59T*	1		
A182G	*Q61R*	1		
A183T, A183C	*Q61H*	2	2	
G274T	*D92Y*			1

Table 4 *Relationship of BRAF mutation with tumor stage in 215 colorectal cancers*

Dukes' stage[a]	*BRAF* mutation	
	Negative	Positive
A	27	1
B	62	9
C	82	1
D	33	0

[a] Stage A and B *versus* stage C and D, $P = 0.006$.

occurring in the adenomas (31%)]. Of the 63 adenomas from FAP patients, only 1 (1.6%) showed *BRAF* mutations. We successfully analyzed 54 FAP adenomas for *KRAS* mutations, and 5 (9%) were positive. The incidence of *KRAS* mutation was significantly lower in FAP polyps than in sporadic ones ($P = 0.004$, χ^2 test). The incidence of *BRAF* mutations also appeared lower in FAP adenomas. The spectrum of *KRAS* mutations in polyps is shown in Table 3.

The relationship of *BRAF* and *KRAS* mutations with morphology and size of sporadic and FAP adenomas are summarized in Tables 5 and 6. All three sporadic adenomatous polyps with *BRAF* mutations showed villous morphology. Although the number of adenomatous polyps with *BRAF* mutations was small, the association with villous morphology reach borderline statistical significance ($P = 0.056$, tubular *versus* villous/tubulovillous, Fisher's exact test). The trend persisted ($P = 0.064$) even taking the FAP adenomas into statistical calculation (Table 5). Overall, a modest association for right-sided location was observed; all 4 adenomas (sporadic and FAP) with *BRAF* mutation arose in the right colon (from a total of 65) and 0 from the left colon (from a total of 105; $P = 0.035$, Fisher's exact test). *BRAF* mutation was absent in sporadic and FAP adenomas <5 mm in diameter (Table 6). There was no significant association of *BRAF* mutation with patient's age, gender, polyp size, and degree of dysplasia.

Adenomas with *KRAS* mutations were more often associated with villous or tubulovillous morphology (Tables 5 and 6; $P < 0.001$, χ^2 test). *KRAS* mutation was also absent in sporadic and FAP adenomas less than 5 mm in size (Table 6). In addition, adenomas with *KRAS* mutation were significantly larger (mean size with mutation, 13.51 ± 6.55 mm; mean size without mutation, 8.09 ± 3.85 mm; $P < 0.001$ by Student *t* test) and were more common in adenomas with higher grade dysplasia and those with transformation to invasive cancer (8 of 67 mild dysplasia, 11 of 46 moderate dysplasia, 14 of 34 severe dysplasia, and 4 of 10 adenomas with malignant transformation; $P = 0.006$, χ^2 test). Otherwise, there was no association of *KRAS* with patient age, gender, and location of tumor.

Eight of the 16 *BRAF* mutations were *T1796A* (resulting in the substitution of valine 599 by glutamate), a previously documented hotspot (4). None of the colorectal tumors with *V599E* carried a *KRAS* mutation. The remaining *BRAF* mutations were *G468E* (4), *F594L*, and six novel mutations, *N580S*, *D593V*, *D593G*, *G595R*, *T598I*, and *F467C*. The novel mutations all changed highly conserved amino acids (Fig. 1). Five of eight tumors containing non-*V599E BRAF* mutations had *KRAS* mutations. Although one of these, *Q22K*, is unusual, it has been previously reported and is associated with transforming activity (6). The incidence of *KRAS* mutations was significantly higher in colorectal tumors (cancer and polyps) with non-*V599E BRAF* mutations (five of eight) than in those with *V599E* mutations (none of 8; $P = 0.012$, Fisher's exact test), and also those with no *BRAF* mutation (102 of 354; $P = 0.039$, χ^2 test).

Discussion

Several conclusions are suggested by the data presented: (*a*) *BRAF* mutations are present predominantly in intermediate sized adenomas. Although the incidence of *BRAF* mutation in colorectal adenomas (2.7%) was lower than in carcinomas (5.1%), these results were not significantly different and overall suggest that *BRAF* mutations occur in adenoma stage. The stage in the adenoma-carcinoma sequence at which *BRAF* mutations arise is therefore similar to that at which *KRAS* mutations occur; (*b*) colorectal adenomas with *BRAF* mutations, similar to those with *KRAS* mutations, are strongly associated with villous morphology; (*c*) the frequency of *BRAF* mutations is lower in adenomas from FAP cases, similar to the pattern for *KRAS* mutations; (*d*) colorectal carcinomas with *BRAF* mutations tend to have a lower Dukes' stage; and (*e*) supporting the previously reported observations (4), tumors with *BRAF V599E* do not have *KRAS* mutations, whereas other *BRAF* mutations are often accompanied by *KRAS* mutations in the same tumors.

We observed a lower incidence of *BRAF* mutations in colorectal cancer than in our previous study of a few cases (4). The lower incidence is unlikely to be a result of lower sensitivity of the detection methods, given that the same methodology has been used in both studies. Indeed, using this detection method, we found *KRAS* mutations in 33.7% of cases, an incidence that compared well with our previous series and a compilation of world literature series of *KRAS* mutations in colorectal cancer (7, 8). Moreover, there were no obvious differences in the stage, grade, or histology of the new cases in this report compared with those previously described (4). Reconciliation

Table 5 *Relationship of BRAF and KRAS mutation with morphology of sporadic and FAP colorectal adenomas*

	BRAF mutation		*KRAS* mutation	
	Negative	Positive	Negative	Positive
Tubular	123	1	100	12
Villous	15	3	8	10
Tubulovillous	29	0	12	15
P	$P = 0.064$, tubular *vs.* villous/tubulovillous, Fisher's exact test		$P < 0.001$, χ^2 test	

Table 6 *Relationship of BRAF and KRAS mutations with size of sporadic and FAP colorectal adenomas*

Size of adenoma (mm)	Sporadic adenomas		Adenomas from FAP patients	
	BRAF mutation (%)	*KRAS* mutation (%)	*BRAF* mutation (%)	*KRAS* mutation (%)
<5	0 (8)[a]	0 (8)	0 (17)	0 (15)
5–10	3 (33)	18.2 (33)	2.4 (42)	5.7 (35)
10–15	4.3 (47)	40.1 (42)	0 (1)	100 (1)
>15	0 (20)	45 (20)	0 (3)	66.7 (3)
Total	2.8 (108)	31 (103)	1.6 (63)	9.3 (54)

[a] Numbers in parentheses, number analyzed.

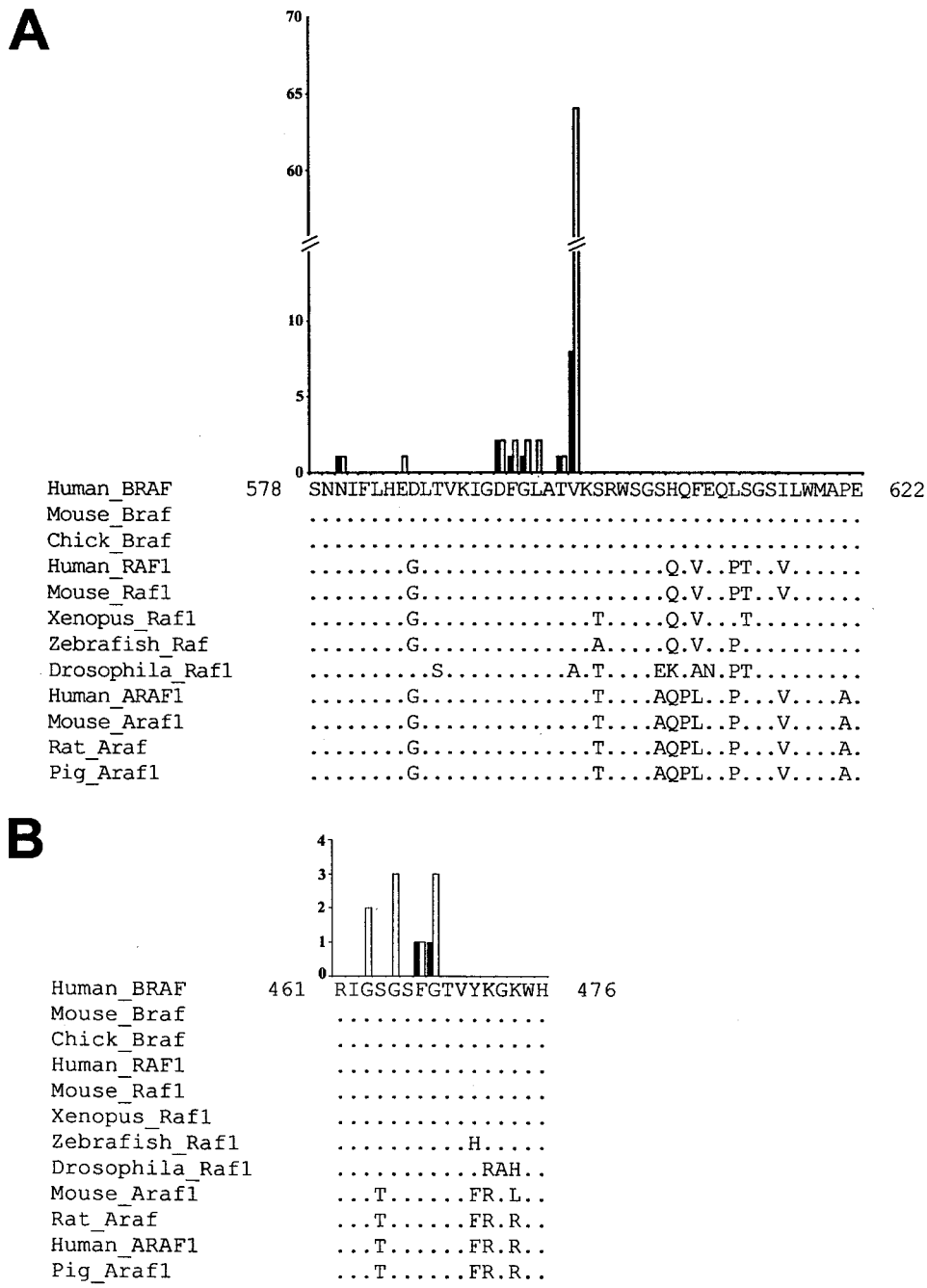

Fig. 1. Sequence conservation and mutations in the *BRAF* activation domain (*A*) and G loop (*B*) in colorectal tumors analyzed in this report (■) and total of all published cancers with *BRAF* mutations (□) (4).

of these frequency estimates of *BRAF* mutations in colorectal cancer will require additional studies.

Our data indicate that *BRAF* mutations are more common in Dukes' stage A/B colorectal carcinomas than in Dukes' stage C/D. This may suggest a less invasive biological behavior for those cancers that harbor *BRAF* mutations. Follow-up data are available in seven of nine cases with Dukes' stage B tumors carrying *BRAF* mutations. They all have survived without recurrence (mean follow-up 60 months, range 5–87) compared with 75% 5-year survival in *BRAF*-negative Dukes' B cancers from this series (this comparison does not reach statistical significance). Study of a larger series will be necessary to evaluate further if colorectal cancers with a *BRAF* mutation are associated with better survival.

We observed many similarities in tumors with *BRAF* or *KRAS* mutation that may reflect a common role of the RAS-RAF-extracellular signal-regulated kinase-mitogen-activated protein kinase/extracellular signal-regulated kinase/mitogen-activated protein kinase pathway in colorectal tumorigenesis. *BRAF* and *KRAS* mutations occur at the adenoma stage of the adenoma-carcinoma sequence (although there is a statistically nonsignificant tendency in our series for *BRAF* mutations to be more common in carcinomas than in adenomas). Like *KRAS* (9), *BRAF* mutations are preferentially more common in villous and rare in tubular adenomas. Also adenomas arising from FAP patients rarely have *KRAS* (1) or *BRAF* mutation. Because the FAP adenomas in our series are generally smaller than the sporadic adenomas and most are tubular adenomas, this may partly explain the rarity of *KRAS* and *BRAF* mutations. We also found a *BRAF* mutation in a hyperplastic polyp and a small percentage of these have previously been shown to harbor *KRAS* mutations (10, 11). In contrast, the association of

BRAF mutations with Dukes' stage A/B colorectal cancers was not observed for *KRAS* mutations.

We previously showed that a mutation in the activation segment, conversion of valine 599 to glutamic acid, is a hotspot for *BRAF* mutation in human cancer (4). This mutation results in the insertion of a negatively charged residue adjacent to a site of regulatory phosphorylation at T598, which may mimic regulatory phosphorylation, thus leading to constitutive activation of *BRAF* independent of *RAS*. Here we confirmed that *V599E* is the most common mutation in colorectal tumors, accounting for 50% of mutation observed. Consistent with the proposed autonomous nature of this mutation in the RAS-RAF-MEK-ERK-MAP kinase signaling pathway (4), mutation of *RAS* is not required and hence not observed in any of the tumors carrying *V599E*. We identified six novel *BRAF* mutations involving other conserved amino acids in the vicinity of the activation segment or glycine-rich loop. Most of these were associated with *KRAS* mutations in the same cancers. This is consistent with our previous observations on other non-*V599 BRAF* mutations and suggests modulation of the RAS-RAF-MEK-ERK-MAP kinase signaling pathway by mutation of multiple components in colorectal cancer.

It is interesting to speculate on the functional consequences of three of the novel *BRAF* mutations, *D593V*, *D593G*, and *T598I*, all located in the kinase activation segment. In *BRAF*, both *T598* and *S601* require phosphorylation to achieve maximal kinase activity. Phosphorylation of these two residues occurs after recruitment of *BRAF* to the membrane by activated RAS. In experimental systems, replacement of these two residues by acidic amino acids can mimic regulatory phosphorylation and results in RAS-independent *BRAF* activation. However, replacement by nonpolar amino acids results in complete abrogation of BRAF kinase activity (12). Similarly, previously described experimental mutants of *BRAF D593* abolish kinase activity. Although all of the cancer-associated *BRAF* mutants previously studied were associated with increased kinase activity and transforming activity in NIH3T3 cells (4), the current results suggest that some *BRAF* mutations found in human cancer may reduce or abolish kinase and transforming activities. Clearly, more studies are required to functionally characterize these mutants.

Note Added in Proof

While this paper was being considered for publication, a similar study was published by Rajagopalan *et al.*, Nature, *418*: 934, 2002.

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Similarity of the Phenotypic Patterns Associated with *BRAF* and *KRAS* Mutations in Colorectal Neoplasia

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