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Clinicopathological and protein characterization of *BRAF*- and *K-RAS*-mutated colorectal cancer and implications for prognosis

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Recent evidence highlights the potential prognostic and predictive value of *BRAF* and *K-RAS* gene alterations in patients with colorectal cancer. However, a comprehensive evaluation of *BRAF* and *K-RAS* mutations and their specific clinicopathological features, histomorphological presentation and effect on protein expression have not been systematically analyzed. The aim of this study was to characterize the clinicopathological, histomorphological and protein expression profiles of *BRAF*- and *K-RAS*-mutated colorectal cancers and determine their impact on patient survival. Molecular analysis for microsatellite instability (MSI), *K-RAS* and *BRAF* was carried out on paraffin-embedded samples from 404 patients with primary colorectal cancer. Using tissue microarrays, 36 tumor-associated and 14 lymphocyte/inflammatory-associated markers were evaluated by immunohistochemistry. *BRAF* mutation was associated with right-sided tumor location ($p < 0.001$), higher tumor grade ($p = 0.029$), absence of peritumoral lymphocytic inflammation ($p = 0.026$) and MSI-H ($p < 0.001$). In right-sided tumors, loss of CDX2 expression was observed in 23 of 24 cases (95.8%). *BRAF* mutation was a poor prognostic indicator in patients with right-sided disease ($p = 0.01$). This result was maintained in multivariable analysis ($p < 0.001$; HR = 2.82; 95% CI: 1.5–5.5) with pT, pN and vascular invasion and independent of CDX2 expression. *K-RAS* mutation, in contrast, was not associated with any of the features analyzed. *BRAF* gene mutation is an adverse prognostic factor in right-sided colon cancer patients independent of MSI status and, moreover, in patients with lymph node-negative disease. These results indicate that molecular analysis for *BRAF* may be a useful biomarker for identifying patients with right-sided colon cancer with poor outcome who may benefit from a more individualized course of therapy.

Recent evidence suggests that the ERK/MAP kinase signaling pathway plays a critical role in colorectal cancer pathogenesis.[1] In fact, 30–35% of all patients with sporadic colorectal cancers have mutations in *K-RAS*, which are typically associated with tumors arising from the chromosomal instability (CIN) pathway following the classical adenoma-carcinoma sequence proposed by Vogelstein *et al.*[2] On the other hand, mutations in *BRAF*, a downstream molecule of *K-RAS*, occur in only 9–11% of patients with sporadic disease and are frequently observed in tumors with microsatellite instability (MSI) and arising from the serrated pathway.[3] Interestingly, mutations in *K-RAS* and *BRAF* genes seem to occur in a mutually exclusive manner, and both are suggested as integral components for an effective molecular classification of colorectal cancer.[4,5] Furthermore, both *K-RAS* and *BRAF* are currently being targeted as potential prognostic and predictive biomarkers in patients with metastatic disease treated with anti-EGFR therapies.[5,6] Although *K-RAS* and its prognostic value in colorectal cancer has been the subject of multi-institutional investigations dating from the 1990s, only recent evidence has shed light on the potential adverse prognostic impact of *BRAF* gene mutations.[7–10] Moreover, a comprehensive review of the literature would suggest that in fact little is known about colorectal cancers with *BRAF* mutation particularly with regard to their clinicopathological presentation, histomorphological characteristics and impact on protein expression.

On a clinicopathological level, *BRAF* mutations have been described as occurring more frequently in colon *versus* rectal cancers and often found in proximal compared to distal tumors.[11,12] Moreover, mutation in *BRAF* seems to be specific for sporadic disease, with mutation status suggested as an exclusion criterion for suspected Lynch syndrome-associated/hereditary nonpolyposis colorectal cancer.[13] Molecularly, *BRAF* mutations have been linked to high levels of MSI (MSI-H), MLH1 hypermethylation and CpG island methylator phenotype (CIMP)-high status.[4,14–19] On a protein level, downregulation of *BRAF* in cell lines has been shown to significantly decrease ERK1/2 phosphorylation and Cyclin D1 expression and increase expression of p27, results which have

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been confirmed using tissue from colorectal cancer patients.[20–22] Additional differences between *BRAF* mutated and wild-type colorectal cancers seem to include decreased expression of CDX2, loss of p16 and positivity for DNA methyltransferase-3B, a marker of *de novo* CpG island methylation or SIRT1 histone deacetylase expression.[23–26] Mutations in *BRAF* have been linked to unfavorable survival time in patients with microsatellite stable (MSS) colorectal cancers and seem to confer a poorer clinical outcome in metastatic patients independent of therapy branch chosen.[5,6,8–10]

The aim of this study was to perform a clinicopathological and protein characterization of *BRAF*-mutated and *K-RAS*-mutated colorectal cancer and determine the effect of mutation on patient survival. To this end, we evaluated an unselected cohort of 404 patients for *BRAF*, *K-RAS*, MSI, clinicopathological and histomorphological features as well as 36 tumor-associated and 14 lymphocyte and inflammatory-associated protein markers selected for their prominent roles in angiogenesis, cell adhesion, WNT, TGF-β, RAS/MAPK and pAKT signaling pathways and functions in immune and inflammatory responses.

Material and Methods

Patients and clinicopathological features

A total of 1,420 primary preoperatively untreated, unselected sporadic colorectal cancer patients treated at the University Hospital of Basel between the years 1987 and 1996 were initially included in this study. Hematoxylin and eosin (H&E)-stained slides were retrospectively collected from the Institute of Pathology, University Hospital of Basel, the Institute of Clinical Pathology, Basel, Switzerland and the Institute of Pathology, Stadtspital Triemli, Zürich, Switzerland. Histopathological criteria were reviewed by an experienced gastrointestinal pathologist (L.T.) and included tumor diameter, pT and pN classification, grade of differentiation, histologic subtype and the presence of tumor invasion into vessels. The tumor border configuration (pushing/expanding or infiltrating) and the presence of peritumoral lymphocytic inflammation at the invasive tumor front were scored according to Jass *et al.*[27] Briefly, tumor margins were identified as infiltrating when there was no recognizable margin of growth and a "streaming dissection" between normal structures of the bowel wall was present. Tumors with focal infiltrating growth patterns were considered as infiltrating. Margins were considered pushing when they were reasonably well circumscribed (or expanding) and often associated with a well-developed inflammatory lamina. Conspicuous peritumoral lymphocytic inflammation was regarded as present when there was a distinctive connective tissue mantle cap at the invasive margin seemingly "encapsulating" the tumor. Clinical data including patient age at diagnosis, tumor location and follow-up were retrieved from patient records. Clinical outcome of interest was cancer-specific survival time. Censored observations included patients who were alive at the last follow-up, died for reasons other than colorectal cancer or were lost to follow-up. Median follow-up time for all patients was 54 months with a 5-year cancer-specific survival rate of 50.1% (95% CI: 45–55). The use of patient material was approved by the local ethics committee at the University Hospital of Basel.

Specimen characteristics

A previously described single-punch tissue microarray was constructed including all 1,420 cases and 57 normal colorectal mucosa samples as control.[28,29] Of these 1,420 cases, paraffin-embedded surgical resection specimens from 600 cases were available and thus retrospectively collected from the archives of the Institute of Pathology, University Hospital Basel, Switzerland, for subsequent molecular analysis.

Assay methods

Molecular analysis of BRAF, K-RAS and mismatch repair genes. Genomic DNA was obtained from all 600 colorectal tissue blocks using NucleoMag 96 Tissue Kit (Macherey Nagel) protocol and processed in the Xiril X-100 robot (Xiril, Hombrechtikon, Switzerland). Briefly, punched tissue was lysed in proteinase K. B-beads and MB2 buffer were added to the cleared lysate, shaked for 5 min at RT. The supernatant was removed and MB3 was added followed by shaking and supernatant removal. The genomic DNA was eluted with MB6 buffer. Genomic DNA was amplified by PCR using AmpliTaq Gold polymerase (Applied Biosystem). *K-RAS* (exon 2 codon 12 and 13) and *BRAF* (exon 15 codon 600) were amplified by a first and a nested PCR. Residual primers were removed using the EXOSAPit (Amersham). Samples were then subjected to direct sequencing of single-stranded PCR products using the BigDye® Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and the ABI Prism® 3130 genetic analyzer (Applied Biosystems). All products were sequenced bidirectionally. Analysis of MSI status was based on the multiplex amplification of the 5 microsatellites (BAT25, BAT26, D2S123, D5S346 and D17S250). An initial denaturation step at 95°C for 10 min was followed by 42 cycles at 95°C for 40 sec, 54°C for 40 sec and 72°C for 60 sec. For the analysis, 1 μl of the DNA weight marker ROX 500 (Applied Biosystem) was added and 10 μl of deionized formamide in 3 μl of the PCR amplified solution. DNA was denaturated by incubation for 2 min at 95°C. The POP-7 polymer solution (Applied Biosystem) was used for the electrophoresis on the ABI Prism® 3130 genetic analyzer (Applied Biosystems). MSS and MSI-low (MSI-L) status were defined as instability at 0 and 1 markers, respectively. MSI-H was characterized by the presence of instability in $\geq$2 markers.

Immunohistochemistry

Immunohistochemistry was performed for 36 tumor-associated protein markers selected to represent the most important signaling pathways in colorectal tumorigenesis (WNT, RAS/MAP kinase, TGF-β and pAKT signaling) and in

Table 1. Tumor, lymphocyte and inflammatory cell protein markers

Signaling Pathway	Marker	Clone/Manufacturer/Dilution	Description
WNT	β-catenin	Dako, β-catenin-1; 1:100	Tumor promoter and mediator of WNT signaling
	APC	Santa Cruz, C20; 1:100	Tumor suppressor and promoter of β-catenin degradation
	CDX2	AbCam, clone AMT28; 1:50	Caudal-type homebox gene encoding a nuclear transcription factor involved in proliferation and differentiation of intestinal epithelial cells
	E-cadherin	Dako NCH-38; 1:100	Intracellular adhesion molecule
	CD133	Cell Signalling, clone 24139; 1:100	Transmembrane glycoprotein thought to function in maintaining stem cell properties by suppressing differentiation
RAS/MAPK	pERK	Cell Signalling, 20G11; 1:100	MAP kinase downstream of RAS
	EGFR	Ventana Medical Systems, c3C6; 3 mg/ml	Tyrosine kinase receptor involved in proliferation, differentiation and angiogenesis
	TOPK	Cell Signaling, polyclonal; 1:50	Oncogenic MEK involved in positive phosphorylation loop with ERK2
	Her2/neu	Dako, PN2A; 1:100	Tyrosine kinase receptor involved in cell proliferation and survival
	RHAMM	Novocastra, clone 2D6; 1:100	Receptor for hyaluronic acid
TGF-β	CD44v6	Bender MedSystems, clone Vff-18; 1:1,200	Splice variant of CD44, cell adhesion molecule
	CD44s	Dako, DF1485; 1:50	Cell adhesion molecule
	pSMAD2	Biocare Medical, polyclonal; 1:100	TGF-β signaling molecule
	TGF-β	AbCam, TB21; 1:1,000	Growth factor with tumor suppressing and promoting functions
	SMAD4	Biocare Medical, BC/B8; 1:100	Involved in TGF-β signaling
pAKT	pAKT	Cell Signalling, 244F9; 1:100	Involved in PI3-K signaling, prosurvival functions
Angiogenic/Metastasis	VEGF	Santa Cruz, VEGFA; 1:100	Marker of vascular permeability and angiogenesis
	uPA	American Diagnostica, 3689: 1:25	Urokinase plasminogen activator involved in extracellular matrix degradation and angiogenesis
	uPAR	American Diagnostica, 3936; 1:25	Urokinase plasminogen activator receptor involved in extracellular matrix degradation and angiogenesis
	RKIP	Upstate, polyclonal; 1:1,000	Metastasis suppressor and downregulator of mitogen activated protein kinase signaling
	EphB2	R&D Systems, AF467; 1:200	Tyrosine kinase receptor involved in deregulation of cell–cell interactions and metastasis
	Cox2	Dako, clone CX294; 1:100	Isoform of cyclooxygenase converting arachidonic acid to prostaglandin H2
Apoptosis/Cell cycle	Bcl2	Dako, clone 124; 1:400	Antiapoptotoc protein inhibiting release of cytochrome *c*

Table 1. Tumor, lymphocyte and inflammatory cell protein markers (Continued)

Signaling Pathway	Marker	Clone/Manufacturer/Dilution	Description
	p27	Dako, SX53G8; 1:100	Inhibitor of cyclin-dependent kinases
	Ki67	Dako, MIB-1; 1:100	Marker of proliferation
	MST1	Cell Signaling, polyclonal; 1:200	Proapoptotic protein
	APAF1	Novocastra, NCLAPAF-1; 1:40	Proapoptotic protein
	p53	Dako, DO-7; 1:200	Tumor suppressor involved in cell cycle arrest, apoptosis, angiogenesis and DNA repair
	p21	Novocastra, SX118; 1:20	Cell cycle arrest mediator
	ALDH1	AbCam, polyclonal isoform α1; 1:500	Isoform of alcohol dehydrogenase
Cell adhesion/Cytokeratins	CK20	Dako, clone Ks20.8; 1:50	Cytokeratin normally expressed in gastrointestinal epithelium
	CK7	Dako, clone OV-TL 12/30; 1:200	Cytokeratin detected in normal tissues and tumors of breast, ovary biliary tract and endometrium
	CD166	Novocastra, clone 110G/07; 1:200	Cell adhesion molecule
	EpCAM	Novocastra, clone VU-1D9; 1:200	Cell adhesion molecule
Mucins	MUC2	Cedarlane Laboratories, Ccp58; 1:100	Producer of gel-forming mucin specific goblet cells
	MUC1	Cedarlane Laboratories, 139H2; 1:100	Involved in cell adhesion, signal transduction and maintenance of cell polarity
Lymphocyte/Inflammatory	PD1	R&D Systems, AF1086; 1:40	Mainly germinal center-associated T cells
	CD68	Dako, M0876; 1:200	Macrophages and monocytes
	CD163	NeoMarkers, MS-1103; 1:40	Mature tissue macrophages and monocytes
	CD20	Dako, M0755; 1:50	Expression on B-cells
	MUM1	Dako, M7259; 1:50	Plasma cells, postgerminal B- and a few activated T cells
	CD56	Novocastra, NCL-CD56-1B6; 1:25	NK- and a small subset of activated T cells
	CD16	Novocastra, clone 2H7; 1:100	NK cells and in a small subset of monocytes
	Foxp3	Abcam, ab22510; 1:200	Regulatory T cells
	Mast cell tryptase	Dako, M7052; 1:2,000	Expressed on mast cells
	CD4	NeoMarkers, MS-1528; 1:40	Inducer, helper, regulator T cells and some monocytes
	CD8	Dako, C8\144B; 1:100	Cytotoxic/suppressor T cells and in a subset of NK cells
	iNOS	Abcam, ab15323, polyclonal; 1:100	Activated macrophages
	TIA-1	Immunotech, IM2550; 1:250	CD8+cytotoxic lymphocytes and in activated NK cells
	Granzyme	Novocastra, NCL-L-GRAN-B; 1:100	Activated CD8+ cytotoxic lymphocytes and NK cells

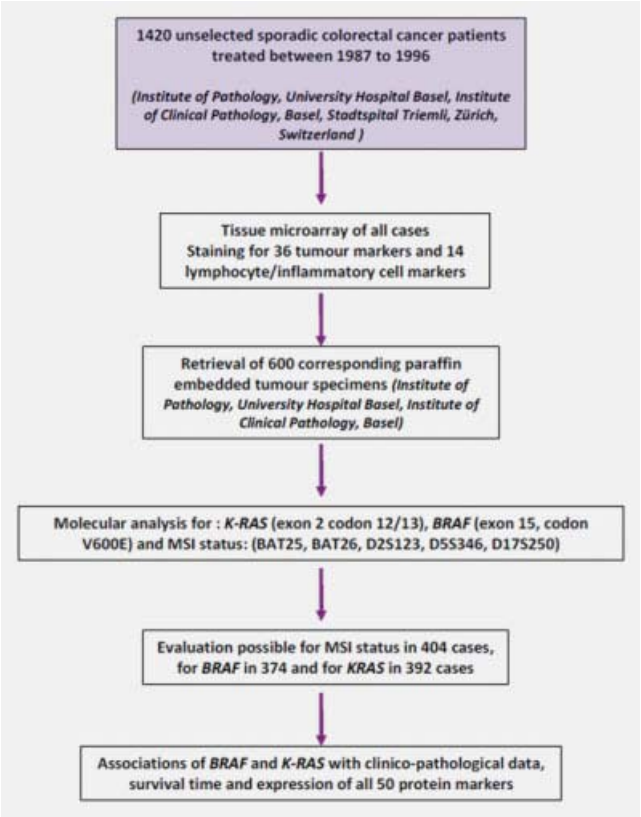


Figure 1. Study design. MSI: microsatellite instability status. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

processes of tumor progression (angiogenesis, metastasis, apoptosis, proliferation cell cycle, cell adhesion, cytokeratins and mucins). Immunohistochemistry was also carried out for 14 lymphocyte, and inflammatory-associated protein markers chosen to cover the widest possible range of different immune and inflammatory cell types including activated T-cells, regulatory, inducer and helper T-cells, macrophages, monocytes, B-cells and natural killer cells. A complete list of protein markers, brief descriptions of function or location, clone, manufacturer and antibody dilution are represented in Table 1.

The 600 colorectal cancers have previously been included on a larger tissue microarray of 1,420 cases.[29] Tissue microarray construction has been described in detail elsewhere.[28] Briefly, 1 tissue cylinder, 0.6 mm in diameter, was punched from representative tissue areas and brought into 1 recipient paraffin block (3 cm × 2.5 cm) using a homemade semiautomated tissue arrayer. Tissues were dewaxed and rehydrated in dH_2O. Following pressure cooker-mediated antigen retrieval in 0.001 M ethylenediaminetetraacetic acid pH 8.0, endogenous peroxidase activity was blocked using 0.5% H_2O_2. Sections were incubated with 10% normal goat serum for 20 min. After incubation with primary antibody, sections were incubated with HRP-conjugated secondary antibody (DakoCytomation) for 30 min at room temperature, immersed in 3-amino-9-ethylcarbazole+substrate-chromogen (DakoCytomation) for 30 min and counterstained with hematoxylin. Negative control tissues underwent the same protocol with the primary antibody omitted.

Evaluation of immunohistochemistry

Lymphocyte/inflammatory-associated protein markers were scored by analyzing the number of positive cells per tissue microarray punch. The total number of immunoreactive cells within the tumor microenvironment was evaluated, independent of localization (intratumoral or stromal). The total number of cells per tissue microarray punch were classified as negative when 0 positive cells were present, as low, moderate and high when 1–10 positive cells, 11–50 positive cells and >50 positive cells per punch could be observed, respectively. For PD1 and iNOS, cases were scored as the complete absence or presence of any positive cells. For tumor-associated protein markers, the percentage of immunoreactive tumor cells over the total number of tumor cells per punch was scored.

Study design

The study design is outlined in Figure 1.

Statistical analysis

Differences in categorical variables and lymphocyte/inflammatory-associated protein expression between *BRAF* and *K-RAS* wild-type and mutated colorectal cancers were analyzed using the Chi-square or Fisher's exact test, where appropriate, whereas age and tumor diameter were analyzed using Student's *t*-test because of the normal distribution of these variables. As tumor-associated protein expression follows a non-normal distribution, analysis of median protein expression values was performed using the Wilcoxon rank sum test. Odds ratios and 95% confidence intervals (CI) were obtained using logistic regression analysis. A Bonferroni correction for multiple comparisons was performed for the associations of immunohistochemical protein expression and mutation. Therefore, only *p*-values <0.001 were considered to represent statistically significant associations between *BRAF* or *K-RAS* and the features studied. Univariate survival analysis was carried out using the Kaplan–Meier and log-rank test, and in the multivariable setting with Cox regression analysis following verification of the assumption of proportional hazards. Analyses were performed using SAS (V9.1, Cary, NC).

Results

Representativity of patient cohort

Mutational investigations gave analyzable sequences in 374 cases for *BRAF* and 392 cases for *K-RAS*, whereas MSI status was determined in all 404 cases (Table 2). In a first step, we evaluated the representativity of our patient cohort stratified by MSI status. Indeed, 62 of 404 patients (15.4%) had MSI-H tumors compared to 84.6% with MSS/MSI-L cancers. MSI-H status compared to MSS/MSI-L was more frequently linked to right-sided tumors ($p < 0.001$), higher tumor grade ($p <$

Table 2. Characteristics of colorectal cancer patients ($n = 404$)

Clinicopathological features		Frequency *N* (%) All patients ($n = 404$; 100%)	MSS/MSI-L ($n = 342$; 84.6%)	MSI-H ($n = 62$; 15.4%)	*p*-value
Sex ($n = 401$)	Female	215 (53.6)	184 (54.3)	31 (50.0)	0.535
	Male	186 (46.4)	155 (45.7)	31 (50.0)	
Tumor location ($n = 399$)	Left-sided	262 (65.7)	234 (69.2)	28 (45.9)	<0.001
	Right-sided	137 (34.3)	104 (30.8)	33 (54.1)	
Histologic type ($n = 404$)	Mucinous	30 (7.4)	25 (7.3)	5 (8.1)	0.794
	Other	374 (92.6)	31 (92.7)	57 (91.9)	
pT stage ($n = 395$)	T1-2	83 (21.0)	71 (21.3)	12 (19.4)	0.727
	T3-4	312 (79.0)	262 (78.7)	50 (80.7)	
pN stage ($n = 388$)	N0	202 (52.1)	165 (50.2)	37 (62.7)	0.075
	N1-2	186 (47.9)	164 (49.9)	22 (37.3)	
Tumor grade ($n = 395$)	G1-2	372 (94.2)	320 (96.1)	52 (83.9)	<0.001
	G3	23 (5.8)	13 (3.9)	10 (16.1)	
Vascular invasion	Absent	276 (69.9)	229 (68.8)	47 (75.8)	0.267
	Present	119 (30.1)	104 (31.2)	15 (24.2)	
Tumor border configuration ($n = 393$)	Pushing	116 (29.5)	83 (25.1)	33 (53.2)	<0.001
	Infiltrating	277 (70.5)	248 (74.9)	29 (46.8)	
Peritumoral lymphocytes ($n = 395$)	Absent	305 (77.2)	264 (79.3)	41 (66.1)	0.023
	Present	90 (22.8)	69 (20.7)	21 (33.9)	
K-RAS codon 12/13 ($n = 392$)	Wild type	274 (69.9)	234 (69.4)	41 (73.2)	0.559
	Mutation	118 (30.1)	103 (30.7)	15 (26.8)	
BRAF codon V600E ($n = 374$)	Wild type	329 (88.0)	291 (91.8)	38 (66.7)	<0.001
	Mutation	45 (12.0)	26 (8.2)	19 (33.3)	
			Mean (range)		
Age ($n = 401$)	(years)	69.5 (40–95)	69.6 (40, 95)	68.6 (45, 89)	0.47
			5-year survival rate and 95%CI		
Survival time ($n = 401$)	(%)	50.1 (45–55)	48.9 (44–54)	56.4 (43–68)	0.239

0.001) pushing or expanding tumor border configuration ($p < 0.001$), presence of PTL inflammation at the tumor front ($p = 0.023$) and finally to a significantly greater number of *BRAF* mutations ($p < 0.001$). These findings are in agreement with the known distribution of these features in the population of colorectal cancer patients.

Association of BRAF and K-RAS mutation and clinicopathological features

Evaluating the entire patient cohort, *BRAF* mutation was associated with right-sided tumor location ($p < 0.001$), a frequent number of high-grade tumors ($p = 0.029$) and with the absence of PTL inflammation at the invasive tumor front ($p = 0.026$) (Table 3). As these features are all commonly associated with MSI-H, we stratified our analysis by MSI status. In MSS/MSI-L cancers, the absence of PTL inflammation was found to correlate with *BRAF* mutation in 24 of 25 cases (96%) and, moreover, was independent of tumor border configuration. *BRAF* mutation within the MSI-H cancers was frequently found on the right side ($p = 0.048$), in tumors with larger diameter ($p = 0.048$) and in older patients ($p = 0.048$). A trend toward absence of PTL inflammation with *BRAF* mutation was also observed ($p = 0.089$). No differences in survival time were noted for either the entire cohort of patients or for those with MSS/MSI-L or MSI-H cancers. *K-RAS* mutations were not linked to any other clinicopathological parameters or with survival time.

Association of BRAF and K-RAS mutation and lymphocyte/inflammatory-associated protein marker expression

We investigated differences in 14 lymphocyte/inflammatory-associated protein markers to determine the relationship of *BRAF* and *K-RAS* mutation with immunogenicity (Table 4). After considering multiple comparisons, only CD8-positive lymphocyte count was significantly associated with *BRAF* mutation ($p < 0.001$) but was restricted to tumors with MSI-

Table 3. Association of *BRAF*^{V600E} and *K-RAS* gene status with clinicopathological and molecular features in sporadic colorectal cancer

		BRAF^{V600E}			*K-RAS*		
		Wild-type	Mutation		Wild-type	Mutation	
Features		*N* (%)	*N* (%)	*p*-value[1]	*N* (%)	*N* (%)	*p*-value[1]
Gender	Female	176 (53.8)	24 (54.6)	0.928	139 (51.1)	68 (58.1)	0.203
	Male	151 (46.2)	20 (54.4)		133 (48.9)	49 (41.9)	
Tumor location	Left-sided	223 (68.6)	19 (43.2)	<0.001	184 (67.9)	73 (62.9)	0.343
	Right-sided	102 (31.4)	25 (56.8)		87 (32.1)	43 (37.1)	
pT stage	pT1-2	72 (22.4)	7 (15.9)	0.325	58 (21.7)	22 (19.0)	0.542
	pT3-4	249 (77.6)	37 (84.1)		209 (78.3)	94 (81.0)	
pN stage	pN0	163 (51.6)	22 (51.2)	0.958	138 (51.9)	56 (50.9)	0.864
	pN1-2	153 (48.4)	21 (48.8)		128 (48.1)	54 (49.1)	
Tumor grade	G1-2	306 (95.3)	38 (86.4)	0.029	250 (93.6)	110 (94.8)	0.651
	G3	15 (4.7)	6 (13.6)		17 (6.4)	6 (5.2)	
Histological subtype	Mucinous	22 (6.7)	5 (11.1)	0.35	14 (5.1)	10 (8.5)	0.173
	Other	307 (93.3)	40 (88.9)		260 (94.9)	104 (88.1)	
Vascular invasion	Absent	227 (70.7)	27 (61.4)	0.206	186 (69.7)	83 (71.6)	0.71
	Present	94 (29.3)	17 (38.6)		81 (30.3)	33 (28.5)	
Tumor border configuration	Infiltrating	227 (71.2)	30 (68.2)	0.683	187 (70.6)	82 (70.7)	0.981
	Pushing	92 (28.8)	14 (31.8)		78 (29.4)	34 (29.3)	
PTL inflammation	Absent	244 (76.0)	40 (90.9)	0.026	206 (77.2)	89 (76.7)	0.927
	Present	77 (24.0)	4 (9.1)		61 (22.9)	27 (23.3)	
MSI status	MSS/MSI-L	291 (88.5)	26 (57.8)	<0.001	233 (85.0)	103 (87.3)	0.559
	MSI-H	38 (11.6)	19 (42.2)		41 (15.0)	15 (12.7)	
		Mean (range)			Mean (range)		
Age at diagnosis	(years)	69.2 (40–95)	71.3 (44–89)	0.245	70.6 (40–95)	71.7 (44–93)	0.747
Tumor diameter	(mm)	48.9 (4–170)	55.7 (15–160)	0.064	52.2 (5–170)	54.7 (4–130)	0.554
		5-year survival rate (95% CI)			5-year survival rate (95% CI)		
Survival time	(%)	49.4 (44–55)	48.9 (33–63)	0.344	49.7 (44–56)	49.4 (40–58)	0.727

[1]Chi-square test or Fisher's exact test for categorical variables, where appropriate; Student's *t*-test for age and tumor diameter and log-rank test for differences in survival time. Abbreviations: MSS/MSI-L, microsatellite stable and instability-low; MSI-H, microsatellite instability-high; PTL, peritumoral lymphocyte.

H. No significant association of *K-RAS* mutation with any lymphocyte/inflammatory protein markers was observed.

Association of *BRAF* and *K-RAS* mutation and tumor-associated protein marker expression

To characterize *BRAF* and *K-RAS* mutation and their effect on the major pathways involved in colorectal cancer progression, we performed immunohistochemistry for 36 tumor-associated protein markers (Table 5). Despite strong associations of 3 protein markers (CDX2, TOPK and APAF-1; $p < 0.005$, each) with *BRAF* gene status, only loss of expression of CDX2 ($p < 0.001$) was significantly linked to *BRAF* mutation compared to wild-type tumors after adjustment for multiple comparisons. Using the previously described ROC curve-derived cutoff score for CDX2 of 95% tumor cell staining,[30] the odds of *BRAF* mutation with loss of CDX2 was 0.14 (95% CI: 0.07–0.3); $p < 0.001$. This association was independent of MSI-H status and therefore maintained in both MSS/MSI-L ($p < 0.001$) and MSI-H ($p = 0.004$) tumors. Strong associations of *K-RAS* mutation with increased protein expression were also observed for APC ($p = 0.005$), CD44s ($p = 0.005$), pSMAD2 ($p = 0.004$) and MUC2 ($p = 0.005$); however, none was considered significantly linked to *K-RAS* mutation after adjusting for multiple comparisons.

Association of *BRAF* mutation and tumor location

Because of the previously reported association of loss of CDX2 with right-sided cancers, mutation in *BRAF* was investigated by tumor location. In right-sided tumors, 23 of 24 cases (95.8%) with *BRAF* mutation showed a loss of CDX2 expression compared to 40 of 99 (40.4%) *BRAF* wild-type cases ($p < 0.001$). This strong correlation was maintained in both MSS/MSI-L and MSI-H cancers. Notably, of the right-sided cases with *BRAF* mutation, 10 of 11 (90.9%) MSS/MSI-

Table 4. Differences in lymphocyte and inflammatory-associated protein expression in patients with *BRAF*^{V600E} and *K-RAS* wild-type and mutated colorectal cancers

		BRAF^{V600E} wild-type	*BRAF*^{V600E} mutation		*K-RAS* wild-type	*K-RAS* mutation	
		N (%)	*N* (%)	*p*-value[1]	*N* (%)	*N* (%)	*p*-value[1]
PD1	Absent	319 (97.0)	43 (97.7)	1.0	268 (98.2)	110 (93.2)	0.026
	Present	10 (3.0)	1 (2.3)		5 (1.8)	8 (6.7)	
CD68	Negative	26 (8.0)	0 (0.0)	0.067	19 (7.0)	8 (6.8)	0.881
	Low	65 (19.9)	9 (20.5)		54 (20.0)	23 (19.5)	
	Moderate	138 (42.3)	15 (34.1)		110 (40.7)	53 (44.9)	
	High	97 (29.8)	20 (45.5)		87 (32.2)	34 (28.8)	
CD163	Negative	13 (4.0)	1 (2.3)	0.242	9 (3.4)	7 (6.0)	0.688
	Low	47 (14.6)	5 (11.4)		39 (14.6)	17 (14.7)	
	Moderate	131 (40.7)	13 (29.6)		106 (39.6)	44 (37.9)	
	High	131 (40.7)	25 (56.8)		114 (42.5)	48 (41.4)	
CD20	Negative	167 (52.5)	28 (62.2)	0.409	140 (52.2)	63 (55.3)	0.271
	Low	113 (35.5)	12 (26.7)		94 (35.1)	40 (35.1)	
	Moderate	33 (10.4)	3 (6.7)		30 (11.2)	7 (6.1)	
	High	5 (1.6)	2 (4.4)		4 (1.5)	4 (3.5)	
MUM1	Negative	95 (29.2)	9 (20.0)	0.607	76 (28.2)	29 (24.6)	0.376
	Low	99 (30.5)	16 (35.6)		81 (30.0)	45 (38.1)	
	Moderate	100 (30.8)	16 (35.6)		89 (33.0)	32 (27.1)	
	High	31 (9.5)	4 (8.9)		24 (8.9)	12 (10.2)	
CD56	Negative	263 (81.7)	41 (91.1)	0.247	216 (80.3)	104 (89.7)	0.07
	Low	52 (16.2)	2 (6.7)		45 (16.7)	11 (9.5)	
	Moderate	7 (2.2)	1 (2.2)		8 (3.0)	1 (0.9)	
CD16	Negative	4 (1.2)	1 (2.2)	0.003	5 (1.9)	1 (0.9)	0.544
	Low	77 (23.8)	2 (4.4)		59 (21.9)	28 (24.4)	
	Moderate	177 (54.8)	24 (53.3)		144 (53.3)	66 (57.4)	
	High	65 (20.1)	18 (40.0)		62 (23.0)	20 (17.4)	
Foxp3	Negative	73 (22.5)	13 (28.9)	0.788	65 (24.0)	27 (23.1)	0.642
	Low	107 (32.9)	13 (28.9)		81 (29.9)	24 (35.9)	
	Moderate	133 (40.9)	17 (37.8)		115 (42.4)	43 (36.8)	
	High	12 (3.7)	2 (4.4)		10 (3.7)	5 (4.3)	
Mast cells	Negative	40 (12.3)	6 (13.6)	0.608	28 (10.4)	17 (14.4)	0.585
	Low	149 (45.9)	16 (36.4)		120 (44.6)	55 (46.6)	
	Moderate	123 (37.9)	19 (43.2)		109 (40.5)	42 (35.6)	
	High	13 (4.0)	3 (6.8)		12 (4.5)	4 (3.4)	
CD4	Negative	189 (61.8)	27 (65.9)	0.92	160 (63.2)	67 (60.4)	0.195
	Low	92 (30.1)	11 (26.8)		74 (29.3)	33 (29.7)	
	Moderate	23 (7.5)	3 (7.3)		19 (7.5)	9 (8.1)	
	High	2 (0.7)	0 (0.0)		0 (0.0)	2 (1.8)	
CD8	Negative	104 (33.3)	13 (29.6)	<0.001	82 (31.5)	36 (31.9)	0.307
	Low	154 (49.4)	18 (40.9)		133 (51.2)	49 (43.4)	
	Moderate	52 (16.7)	7 (15.9)		38 (14.6)	25 (22.1)	
	High	2 (0.6)	6 (13.4)		7 (2.7)	3 (2.7)	
iNOS	Absent	210 (68.9)	28 (68.3)	0.942	176 (69.6)	77 (70.0)	0.934
	Present	95 (31.2)	13 (31.7)		77 (30.4)	33 (30.0)	
TIA-1	Negative	164 (55.6)	16 (40.0)	0.112	141 (57.6)	52 (48.2)	0.404
	Low	112 (38.0)	19 (47.5)		87 (35.5)	47 (43.5)	
	Moderate	18 (6.1)	4 (10.0)		16 (0.5)	8 (7.4)	
	High	1 (0.3)	1 (2.5)		1 (0.4)	1 (0.9)	

Table 4. Differences in lymphocyte and inflammatory-associated protein expression in patients with *BRAF*^{V600E} and *K-RAS* wild-type and mutated colorectal cancers (Continued)

		BRAF^{V600E} wild-type	*BRAF*^{V600E} mutation		*K-RAS* wild-type	*K-RAS* mutation	
		N (%)	*N* (%)	*p*-value[1]	*N* (%)	*N* (%)	*p*-value[1]
Granzyme	Negative	98 (32.0)	14 (33.3)	0.041	82 (32.2)	40 (36.4)	0.714
	Low	167 (54.6)	16 (38.1)		136 (53.3)	55 (50.0)	
	Moderate	37 (12.1)	10 (23.8)		33 (12.9)	12 (10.9)	
	High	4 (1.3)	2 (4.8)		4 (1.6)	3 (2.7)	

[1]Chi-square or Fischer's exact test, where appropriate.

Table 5. Protein expression differences between *BRAF*^{V600E} and *K-RAS* wild-type and mutated colorectal cancers

	BRAF^{V600E} wild-type		*BRAF*^{V600E} mutation			*K-RAS* wild-type		*K-RAS* mutation		
	Mean (%)	Median (%)	Mean (%)	Median (%)	*p*-value[1]	Mean (%)	Median (%)	Mean (%)	Median (%)	*p*-value[1]
ALDH1	10.0	0	10.9	0	0.268	10.1	0	12.4	0	0.731
APAF1	86.0	90	91.3	100	0.004	85.4	90	91.0	100	0.021
APC	70.6	90	66.6	80	0.328	67.5	80	78.6	90	0.005
β-catenin	13.9	5	7.0	0	0.044	12.1	5	16.4	5	0.216
Bcl2	36.8	10	22.7	0	0.014	33.4	10	36.8	10	0.736
CD133	23.4	5	20.8	5	0.64	25.0	5	20.0	5	0.609
CD166	56.8	70	42.9	35	0.06	52.9	60	58.4	80	0.249
CD44s	29.5	5	31.4	10	0.646	27.7	5	39.6	30	0.005
CD44v6	59.9	75	70.5	100	0.038	58.1	70	68.2	92·5	0.054
CDX2	85.3	100	48.4	60	<0.001	81.9	100	83.4	100	0.859
CK20	73.8	5	63.7	90	0.137	71.6	95	77.6	95	0.178
CK7	4.2	0	7.6	0	0.415	4.2	0	3.9	0	0.953
Cox2	59.6	100	94.5	100	0.106	90.3	100	59.4	100	0.477
E-cadherin	77.3	100	70.4	90	0.051	77.4	100	76.7	100	0.518
EGFR	62.1	80	73.4	90	0.043	61.1	80	66.7	90	0.251
EpCAM	92.2	100	97.0	100	0.378	91.2	100	95.3	100	0.192
EphB2	39.7	35	34.0	12.5	0.243	39.1	35	42.4	40	0.399
Her2/neu	7.6	0	4.5	0	0.363	8.1	0	7.4	0	0.502
Ki67	22.4	10	30.9	20	0.049	24.0	10	24.7	15	0.585
MST1	75.9	90	80.8	90	0.647	76.1	90	78.2	90	0.376
MUC1	29.1	10	39.9	30	0.113	29.6	5	29.7	10	0.791
MUC2	17.7	0	26.2	0	0.649	14.2	0	26.0	5	0.005
p21	9.2	5	10.2	5	0.293	8.5	5	10.5	5	0.038
p27	67.3	80	63.6	77·5	0.535	68.1	80	67.1	80	0.703
p53	41.9	20	39.3	10	0.832	44.2	30	38.1	5	0.3
pAKT	16.7	10	19.3	10	0.971	18.2	10	14.2	10	0.14
pERK	7.0	0	4.3	0	0.28	6.4	0	7.9	0	0.561
pSMAD2	55.0	70	48.0	50	0.362	56.1	70	44.1	40	0.004
RHAMM	74.7	90	83.5	90	0.265	75.2	90	79.6	90	0.375
RKIP	78.9	90	68.2	90	0.098	76.0	90	83.0	90	0.161
SMAD4	19.3	5	27.7	5	0.453	23.3	5	19.6	5	0.484
TGF-β	31.1	15	33.3	20	0.711	29.6	15	34.3	25	0.424
TOPK	57.0	75	77.3	95	<0.001	58.4	75	61.3	80	0.422
uPA	58.8	60	62.3	70	0.446	59.0	60	60.1	70	0.998
uPAR	69.8	75	66.9	70	0.824	69.9	75	68.5	75	0.72
VEGF	58.1	80	63.3	90	0·493	58·3	85	65.3	90	0.245

The mean and median number of immunoreactive tumor cells over all tumor cells per case (percentage staining) is shown.
[1]Wilcoxon Rank Sum Test for differences in median expression of tumor markers.

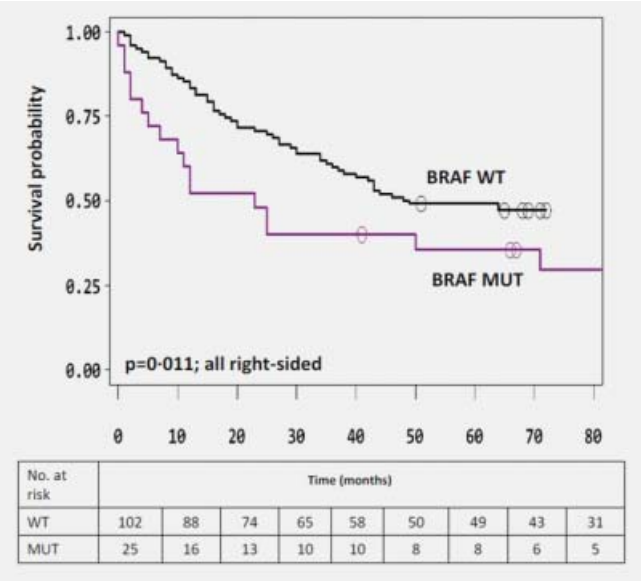


Figure 2. Kaplan–Meier survival curve illustrating differences in survival time for *BRAF* wild-type (WT) and mutated (MUT) right-sided colon cancers. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

L and all of 13 (100%) MSI-H cases had loss of CDX2 ($p < 0.001$; $p = 0.004$, respectively). On the left side, CDX2 was not discriminating for *BRAF* mutation with 10 of 19 cases showing a loss of expression.

In right-sided colon cancer, *BRAF* mutation had a significant and negative impact on prognosis (Fig. 2; $p = 0.01$). This adverse effect was maintained in multivariable analysis after adjusting for the effects of pT classification, pN classification, vascular invasion and MSI status (Fig. 3; Table 6). Specifically, the relative risk of death for patients with *BRAF* mutation was 2.82 (95% CI: 1.5–5.5) compared to patients with wild-type tumors ($p = 0.002$). Moreover, patients with lymph node-negative right-sided cancers showed a trend toward worse overall survival with *BRAF* mutation compared to those with wild-type disease ($p = 0.065$). Despite the strong correlation of loss of CDX2 expression with *BRAF* mutation, CDX2 expression in right-sided colon cancers was not associated with unfavorable outcome, neither in univariate ($p = 0.389$) nor in multivariable ($p = 0.869$) analysis. These results seem to indicate that *BRAF* mutation, and not CDX2 expression, is an independent prognostic factor in right-sided colon cancer. Patients with *BRAF* mutation and left-sided disease tended to experience a more favorable outcome ($p = 0.084$; Fig. 4). This result was however not confirmed in multivariable analysis with pT classification, pN classification, vascular invasion or MSI status, likely a result in part due to the small number of *BRAF*-mutated cases. No differences in survival time were observed for patients with *K-RAS* mutation compared to wild-type tumors with right-sided or left-sided cancers.

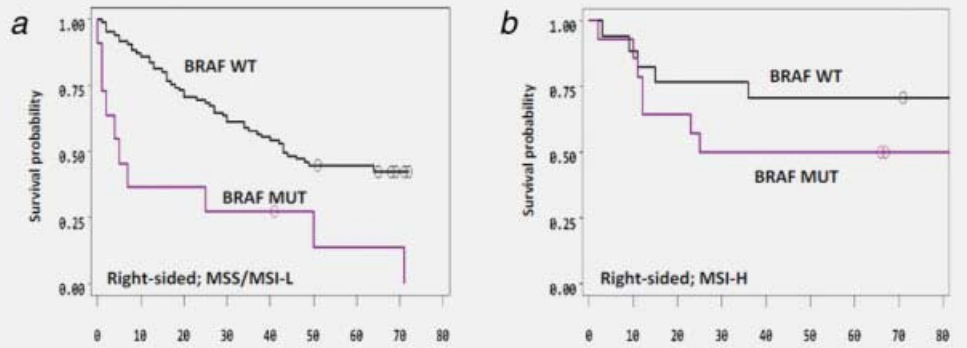
Discussion

In this study, using a representative cohort of 404 sporadic colorectal cancers, we identify *BRAF* mutation as a significant molecular biomarker of poor outcome in patients with right-sided disease, a result which appears to be independent not only of pT classification, pN classification and vascular invasion but also of MSI status. Using 50 protein markers, we additionally document 96% specificity for *BRAF* mutation in right-sided tumors with loss of CDX2 expression.

We found a significantly larger number of *BRAF* mutations in right-sided colon cancers and in tumors with a poorer degree of differentiation. These associations have previously been reported and appear to be due to the high frequency of *BRAF* mutation occurring in patients with MSI-H cancers who themselves are known to exhibit these features, as well as, paradoxically, lower rates of distant metastases and prolonged survival.[3] Interestingly, *BRAF* mutation in both MSS/MSI-L and MSI-H patients was only rarely observed in cases with PTL inflammation at the tumor border suggesting that the poor prognosis in patients with mutation could in part be due to the absence of this feature whose prognostic value was first described more than 20 years ago.[31] Moreover, a link between *BRAF* mutation and $CD8^+$ T cells was identified; however, this relationship was only maintained in MSI-H cancers known to exhibit abundant lymphocyte counts compared to their MSS/MSI-L counterparts.[32] With the exception of absence of PTL inflammation, no specific histopathological criteria appear to be useful for identifying either tumors with *BRAF* or *K-RAS* gene mutations, the latter showing no significant associations with the features analyzed.

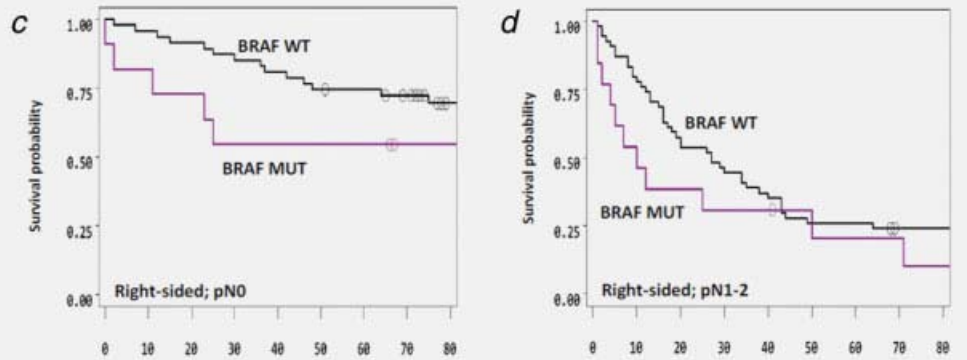
Although *K-RAS* mutations were not significantly linked to any of the 34 tumor markers after adjusting for multiple comparisons, CDX2 could be strongly related to *BRAF* gene status. Previous reports have suggested such a link as well as a loss of CDX2 expression in right-sided colon cancers.[23] In this study, we further show that 23 of 24 (96%) right-sided *BRAF* mutated colon cancers demonstrate loss of CDX2. CDX2 has been described as a tumor suppressor gene maintaining intestinal epithelium and regulating cell dynamics, adhesion, proliferation and apoptosis.[33,34] Therefore, the associations of loss of expression with *BRAF* mutation and poor tumor grade, although not necessarily supporting a functional relationship between these two genes, is in line with the tumor suppressive function of this molecule. Although current molecular laboratories support the analysis of *BRAF* gene status at a relatively low cost, a systematic prescreening of right-sided colon cancers for *BRAF* mutation using CDX2 as a surrogate marker may be a feasible alternative, which warrants further confirmation.

In this study, stratification of *BRAF* and evaluation of prognostic impact was made for right- *versus* left-sided colorectal cancers because of the association of mutation with proximal tumor location. The most important novel finding is the strong, negative impact of *BRAF* mutation in patients with right-sided colon cancer, and interestingly, independently of MSI status. Several lines of evidence seem to support this independence of *BRAF* mutation from MSI. First, several



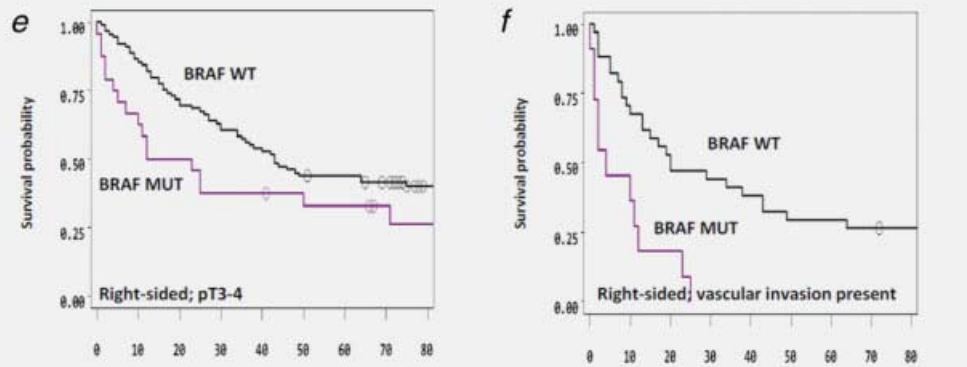
No. at risk	Time (months)								
WT	85	73	61	53	46	38	37	31	21
MUT	11	4	4	3	3	1	1	1	0

No. at risk	Time (months)								
WT	17	15	13	13	12	12	12	12	10
MUT	14	12	9	7	7	7	7	5	5



No. at risk	Time (months)								
WT	47	45	43	40	38	35	34	31	22
MUT	11	9	8	6	6	6	6	4	4

No. at risk	Time (months)								
WT	54	42	30	24	19	14	13	11	8
MUT	13	6	5	4	4	2	2	1	1



No. at risk	Time (months)								
WT	89	76	63	54	47	39	39	34	3
MUT	24	15	12	9	8	7	7	5	4

No. at risk	Time (months)								
WT	34	23	17	15	13	10	10	9	5
MUT	11	4	1	0					

Figure 3. Kaplan–Meier survival curves illustrating differences in survival time for right-sided (*a*) MSS/MSI-L cancers, (*b*) MSI-H cancers, (*c*) lymph node-negative cancers, (*d*) lymph node-positive cancers, (*e*) pT3-pT4 cancers and (*f*) cancer with vascular invasion stratified by *BRAF* gene status (WT: wild-type; MUT: mutation). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Table 6. Multiple Cox regression analysis showing the prognostic value of $BRAF^{V600E}$ mutation adjusting for the effects of pT stage, pN stage, vascular invasion and microsatellite instability (MSI) in right- and left-sided colorectal cancer

		Right-sided		Left-sided	
		HR (95%CI)	*p*-value	HR (95%CI)	*p*-value
$BRAF^{V600E}$	Wild-type	1.0	0.002	1.0	0.109
	Mutation	2.82 (1.5–5.5)		0.53 (0.3–1.2)	
pT stage	pT1-2	1.0	0.18	1.0	0.001
	pT3-4	2.69 (0.6–11.4)		2.39 (1.4–4.1)	
pN stage	pN0	1.0	0.024	1.0	0.005
	pN1-2	2.03 (1.1–3.7)		1.72 (1.1–2.5)	
	Present	2.23 (1.3–3.9)		2.04 (1.4–3.0)	
MSI status	MSS/MSI-L	1.0	0.015	1.0	0.622
	MSI-H	0.41 (0.2–0.8)		1.16 (0.6–2.1)	

authors have reported poor clinical and treatment outcomes in colorectal cancer patients in both MSS and MSI-H cases with *BRAF* mutation.[5,6,8–10] Second, recent work by Velho *et al.* investigated *BRAF*, *K-RAS* and PIK3CA mutations in colorectal serrated polyps and cancers.[35] They postulate that *BRAF* mutations are likely to precede MSI carcinomas because the frequency of mutation in serrated polyps is similar to that of MSI cancers but statistically different from the frequency in MSS tumors. Third, similar findings were observed by Kim *et al.* who describe *BRAF* mutations independently of CIMP and MSI in serrated polyps.[36] Together these observations suggest that *BRAF* mutation could occur before malignant transformation and may be a primary genetic event in colorectal carcinogenesis, which further suggests that the evaluation of *BRAF* by proximal or distal tumor location merits consideration.

Our results of adverse prognosis in *BRAF*-mutated patients with right-sided colon cancer may be to some extent affected by the lack of information regarding distant metastasis and adjuvant therapy. Moreover, the possible effect of anti-EGFR therapies in *BRAF*-mutated patients with right-sided disease cannot be discussed here, because all patients were treated before the anti-EGFR era. Despite this limitation, the unfavorable outcome associated with *BRAF* mutation in right-sided tumors was not only maintained in multivariable analysis with well-established prognostic features but also in patients with lymph node-negative colorectal cancers who by today's treatment guidelines are not generally considered for adjuvant chemotherapy.[37] These findings suggest that *BRAF* gene status in patients with pN0 disease could serve as an additional molecular biomarker to identify patients with poor outcome who may benefit from subsequent therapy. As information on family history was not available, it is possible that a small subset of patients with Lynch syndrome-associated colon cancers was included. As *BRAF* mutation among MSI-H patients is a feature of sporadic, rather than hereditary colorectal cancer and given that Lynch syndrome patients express a more abundant intratumoral lymphocytic reaction compared to sporadic

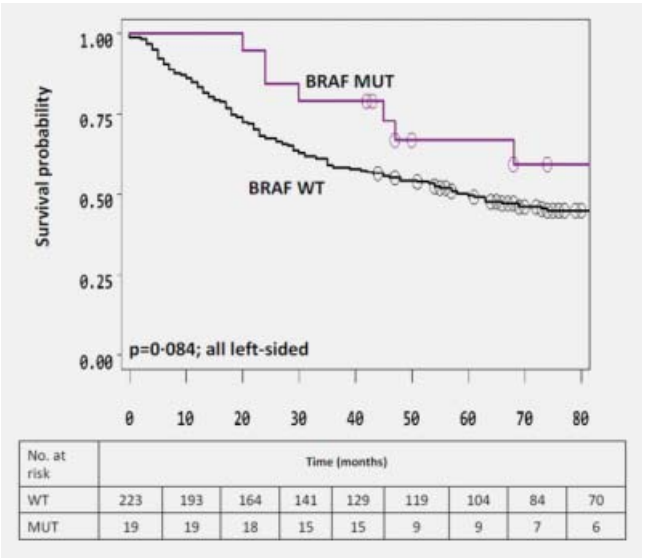


Figure 4. Kaplan–Meier survival curve illustrating differences in survival time for *BRAF* wild-type (WT) and mutated (MUT) left-sided colorectal cancers. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cases,[38] the lack of correlation of most lymphocyte markers with *BRAF* mutation in MSI-H cases may be to some extent explained by the inclusion of a small number of Lynch syndrome patients. In addition, protein profiling of 50 protein markers was performed using tissue microarray technology, a method which is often criticized for its lack of representation in cases with heterogeneous protein expression. Not only have several studies shown well-established correlations between protein expression and clinicopathological features using tissue microarrays with a single tumor punch but also our experience with this technique suggests that a high degree of interobserver reproducibility can be achieved.[29] Moreover, the ability to investigate a large panel of potential biomarkers, as in this study, is clearly not feasible using whole tissue sections. Our results pertaining to CDX2 expression and *BRAF*

mutations should therefore be interpreted with caution until validation can be accomplished. The major results of this study concerning the impact of *BRAF* mutation on poor patient outcome remain unaffected by protein marker expression.

In conclusion, *BRAF* gene mutation is an adverse prognostic factor in right-sided colon cancer patients with either MSS/MSI-L or MSI-H tumors and, moreover, in patients with lymph node-negative disease. These results indicate that molecular analysis for *BRAF* may be a useful biomarker for identifying patients with right-sided colon cancer with poor outcome who may benefit from a more individualized course of therapy.

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References

1. Fang JY, Richardson BC. The MAPK signalling pathways and colorectal cancer. *Lancet Oncol* 2005;6:322–7.
2. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM, Bos JL. Genetic alterations during colorectal-tumor development. *N Engl J Med* 1988;319: 525–32.
3. Jass JR. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. *Histopathology* 2007;50:113–30.
4. Ahlquist T, Bottillo I, Danielsen SA, Meling GI, Rognum TO, Lind GE, Dallapiccola B, Lothe RA. RAS signaling in colorectal carcinomas through alteration of RAS, RAF, NF1, and/or RASSF1A. *Neoplasia* 2008;10:680–6, 2 p following 6.
5. Tol J, Nagtegaal ID, Punt CJ. *BRAF* mutation in metastatic colorectal cancer. *N Engl J Med* 2009;361:98–9.
6. Di Nicolantonio F, Martini M, Molinari F, Sartore-Bianchi A, Arena S, Saletti P, De Dosso S, Mazzucchelli L, Frattini M, Siena S, Bardelli A. Wild-type *BRAF* is required for response to panitumumab or cetuximab in metastatic colorectal cancer. *J Clin Oncol* 2008;26:5705–12.
7. Andreyev HJ, Norman AR, Cunningham D, Oates JR, Clarke PA. Kirsten ras mutations in patients with colorectal cancer: the multicenter "RASCAL" study. *J Natl Cancer Inst* 1998;90:675–84.
8. French AJ, Sargent DJ, Burgart LJ, Foster NR, Kabat BF, Goldberg R, Shepherd L, Windschitl HE, Thibodeau SN. Prognostic significance of defective mismatch repair and *BRAF* V600E in patients with colon cancer. *Clin Cancer Res* 2008;14:3408–15.
9. Kakar S, Deng G, Sahai V, Matsuzaki K, Tanaka H, Miura S, Kim YS. Clinicopathologic characteristics. CpG island methylator phenotype, and *BRAF* mutations in microsatellite-stable colorectal cancers without chromosomal instability. *Arch Pathol Lab Med* 2008;132:958–64.
10. Ogino S, Nosho K, Kirkner GJ, Kawasaki T, Meyerhardt JA, Loda M, Giovannucci EL, Fuchs CS. CpG island methylator phenotype, microsatellite instability. *BRAF* mutation and clinical outcome in colon cancer. *Gut* 2009;58:90–6.
11. Deng G, Kakar S, Tanaka H, Matsuzaki K, Miura S, Sleisenger MH, Kim YS. Proximal and distal colorectal cancers show distinct gene-specific methylation profiles and clinical and molecular characteristics. *Eur J Cancer* 2008;44:1290–301.
12. Kalady MF, Sanchez JA, Manilich E, Hammel J, Casey G, Church JM. Divergent oncogenic changes influence survival differences between colon and rectal adenocarcinomas. *Dis Colon Rectum* 2009; 52:1039–45.
13. Domingo E, Niessen RC, Oliveira C, Alhopuro P, Moutinho C, Espin E, Armengol M, Sijmons RH, Kleibeuker JH, Seruca R, Aaltonen LA, Imai K, et al. *BRAF*-V600E is not involved in the colorectal tumorigenesis of HNPCC in patients with functional MLH1 and MSH2 genes. *Oncogene* 2005;24:3995–8.
14. Barault L, Charon-Barra C, Jooste V, de la Vega MF, Martin L, Roignot P, Rat P, Bouvier AM, Laurent-Puig P, Faivre J, Chapusot C, Piard F. Hypermethylator phenotype in sporadic colon cancer: study on a population-based series of 582 cases. *Cancer Res* 2008;68:8541–6.
15. Cheng YW, Pincas H, Bacolod MD, Schemmann G, Giardina SF, Huang J, Barral S, Idrees K, Khan SA, Zeng Z, Rosenberg S, Notterman DA, et al. CpG island methylator phenotype associates with low-degree chromosomal abnormalities in colorectal cancer. *Clin Cancer Res* 2008;14:6005–13.
16. de Vogel S, Weijenberg MP, Herman JG, Wouters KA, de Goeij AF, van den Brandt PA, de Bruine AP, van Engeland M. MGMT and MLH1 promoter methylation versus APC. KRAS and *BRAF* gene mutations in colorectal cancer: indications for distinct pathways and sequence of events. *Ann Oncol* 2009;20:1216–22.
17. Kumar K, Brim H, Giardiello F, Smoot DT, Nouraie M, Lee EL, Ashktorab H. Distinct *BRAF* (V600E) and KRAS mutations in high microsatellite instability sporadic colorectal cancer in African Americans. *Clin Cancer Res* 2009;15: 1155–61.
18. Nagasaka T, Koi M, Kloor M, Gebert J, Vilkin A, Nishida N, Shin SK, Sasamoto H, Tanaka N, Matsubara N, Boland CR, Goel A. Mutations in both *KRAS* and *BRAF* may contribute to the methylator phenotype in colon cancer. *Gastroenterology* 2008;134:1950–60, 60 e1.
19. Nosho K, Irahara N, Shima K, Kure S, Kirkner GJ, Schernhammer ES, Hazra A, Hunter DJ, Quackenbush J, Spiegelman D, Giovannucci EL, Fuchs CS, et al. Comprehensive biostatistical analysis of CpG island methylator phenotype in colorectal cancer using a large population-based sample. *PLoS One* 2008;3:e3698.
20. Ogino S, Nosho K, Irahara N, Kure S, Shima K, Baba Y, Toyoda S, Chen L, Giovannucci EL, Meyerhardt JA, Fuchs CS. A cohort study of cyclin d1 expression and prognosis in 602 colon cancer cases. *Clin Cancer Res* 2009;15:4431–8.
21. Ogino S, Shima K, Nosho K, Irahara N, Baba Y, Wolpin BM, Giovannucci EL, Meyerhardt JA, Fuchs CS. A cohort study of p27 localization in colon cancer, body mass index, and patient survival. *Cancer Epidemiol Biomarkers Prev* 2009;18: 1849–58.
22. Preto A, Figueiredo J, Velho S, Ribeiro AS, Soares P, Oliveira C, Seruca R. *BRAF* provides proliferation and survival signals in MSI colorectal carcinoma cells displaying *BRAF*(V600E) but not KRAS mutations. *J Pathol* 2008;214:320–7.
23. Baba Y, Nosho K, Shima K, Freed E, Irahara N, Philips J, Meyerhardt JA, Hornick JL, Shivdasani RA, Fuchs CS, Ogino S. Relationship of CDX2 loss with molecular features and prognosis in colorectal cancer. *Clin Cancer Res* 2009;15: 4665–73.
24. Nosho K, Shima K, Irahara N, Kure S, Baba Y, Kirkner GJ, Chen L, Gokhale S, Hazra A, Spiegelman D, Giovannucci EL, Jaenisch R, et al. DNMT3B expression might contribute to CpG island methylator phenotype in colorectal cancer. *Clin Cancer Res* 2009;15:3663–71.
25. Nosho K, Shima K, Irahara N, Kure S, Firestein R, Baba Y, Toyoda S, Chen L, Hazra A, Giovannucci EL, Fuchs CS, Ogino S. SIRT1 histone deacetylase expression is associated with microsatellite instability and CpG island methylator phenotype in colorectal cancer. *Mod Pathol* 2009;22:922–32.

26. Paya A, Alenda C, Perez-Carbonell L, Rojas E, Soto JL, Guillen C, Castillejo A, Barbera VM, Carrato A, Castells A, Llor X, Andreu M, et al. Utility of p16 immunohistochemistry for the identification of Lynch syndrome. *Clin Cancer Res* 2009;15:3156–62.
27. Jass JR, Atkin WS, Cuzick J, Bussey HJ, Morson BC, Northover JM, Todd IP. The grading of rectal cancer: historical perspectives and a multivariate analysis of 447 cases. *Histopathology* 1986;10: 437–59.
28. Sauter G, Simon R, Hillan K. Tissue microarrays in drug discovery. *Nat Rev Drug Discov* 2003;2:962–72.
29. Zlobec I, Terracciano L, Tornillo L, Gunthert U, Vuong T, Jass JR, Lugli A. Role of RHAMM within the hierarchy of well-established prognostic factors in colorectal cancer. *Gut* 2008;57:1413–19.
30. Lugli A, Tzankov A, Zlobec I, Terracciano LM. Differential diagnostic and functional role of the multi-marker phenotype CDX2/CK20/CK7 in colorectal cancer stratified by mismatch repair status. *Mod Pathol* 2008; 21:1403–12.
31. Jass JR, Love SB, Northover JM. A new prognostic classification of rectal cancer. *Lancet* 1987;1:1303–6.
32. Baker K, Zlobec I, Tornillo L, Terracciano L, Jass JR, Lugli A. Differential significance of tumour infiltrating lymphocytes in sporadic mismatch repair deficient versus proficient colorectal cancers: a potential role for dysregulation of the transforming growth factor-beta pathway. *Eur J Cancer* 2007;43:624–31.
33. Beck F. The role of Cdx genes in the mammalian gut. *Gut* 2004;53:1394–6.
34. Bonhomme C, Duluc I, Martin E, Chawengsaksophak K, Chenard MP, Kedinger M, Beck F, Freund JN, Domon-Dell C. The Cdx2 homeobox gene has a tumour suppressor function in the distal colon in addition to a homeotic role during gut development. *Gut* 2003;52:1465–71.
35. Velho S, Moutinho C, Cirnes L, Albuquerque C, Hamelin R, Schmitt F, Carneiro F, Oliveira C, Seruca R. *BRAF*, *KRAS* and *PIK3CA* mutations in colorectal serrated polyps and cancer: primary or secondary genetic events in colorectal carcinogenesis? *BMC Cancer* 2008;8:255.
36. Kim YH, Kakar S, Cun L, Deng G, Kim YS. Distinct CpG island methylation profiles and *BRAF* mutation status in serrated and adenomatous colorectal polyps. *Int J Cancer* 2008;123:2587–93.
37. Benson AB, III, Schrag D, Somerfield MR, Cohen AM, Figueredo AT, Flynn PJ, Krzyzanowska MK, Maroun J, McAllister P, Van Cutsem E, Brouwers M, Charette M, et al. American Society of Clinical Oncology recommendations on adjuvant chemotherapy for stage II colon cancer. *J Clin Oncol* 2004;22:3408–19.
38. Shia J, Ellis NA, Paty PB, Nash GM, Qin J, Offit K, Zhang XM, Markowitz AJ, Nafa K, Guillem JG, Wong WD, Gerald WL, et al. Value of histopathology in predicting microsatellite instability in hereditary nonpolyposis colorectal cancer and sporadic colorectal cancer. *Am J Surg Pathol* 2003;27:1407–17.