PIK3CA mutation and methylation influences the outcome of colorectal cancer

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Abstract. Colorectal cancer (CRC) occurs through the accumulation of genetic and epigenetic alterations. The epigenetic abnormalities, in cooperation with genetic alterations, are capable of causing aberrant gene function that results in cancer. In the present study, we examined mutations and methylation status in 164 CRCs to determine whether the combination of genetic and epigenetic alterations may be used to classify CRC patients in relation to their clinicopathological characteristics and outcomes. Mutation analyses for the *KRAS* and *PIK3CA* genes were performed using direct sequencing, and the MethyLight method was used to determine the methylation status of *BNIP3*, *p16* and *hMLH1*. The combination of the *KRAS* mutation with methylation status did not have any association with clinicopathological characteristics and outcomes. However, patients with the *PIK3CA* mutation and/or high methylation (2 or 3 methylated genes) had significantly poorer outcomes in disease-specific survival (DSS) compared with those with wild-type *PIK3CA* and 0 or 1 methylated genes (P=0.0059). Additionally, multivariate analysis revealed that the *PIK3CA* mutation and/or a high level of methylation predicts a poor DSS independently of clinicopathological characteristics. Our results suggest that a combination of genetic and epigenetic alterations is a potent biomarker for predicting the prognosis of CRC.

Introduction

Colorectal cancer (CRC) is among the most common types of cancer in both males and females and is associated with high mortality, particularly at advanced stages (1). Markers for defining individual risk signatures in CRC patients are of great clinical value, as they may allow for targeted therapies to improve the outcomes of CRC patients.

CRC arises through the accumulation of multiple genetic and epigenetic changes. Somatic mutations in *APC*, *BRAF*, *KRAS*, *PIK3CA* and *TP53*, as well as in other genes, have been frequently observed in CRC and are thought to drive colorectal tumorigenesis (2). We previously examined the mutation of genes in the EGFR pathway and found that the *PIK3CA* mutation is associated with poor prognosis in CRC patients (8,11).

In addition to genetic alterations, epigenetic silencing is a prevalent mechanism by which abnormal gene inactivation may occur in cancer and is involved in initiation and promotion (3). A predominant mode of epigenetic alteration in cancer is gene silencing via the hypermethylation of the CpG island promoter. Hypermethylation results in abnormal silencing of a number of tumor suppressors in numerous human malignancies including CRC (3).

It has been suggested that epigenetic abnormalities are able to cooperate with genetic alterations to cause aberrant gene function and result in cancer (4). Thus, in the present study, we examined *KRAS* and *PIK3CA* mutations and the methylation status of *BNIP3*, *p16* and *hMLH1* and analyzed the correlation between these molecular alterations and the clinicopathological characteristics of CRC. We aimed to clarify whether a combination of genetic and epigenetic alterations could be used to classify CRC patients in relation to their clinicopathological characteristics and outcomes.

Materials and methods

Patients and tissue samples. After providing informed consent, a total of 165 CRC patients who underwent surgical resection at Tokyo Medical and Dental University Hospital between March 2000 and April 2003 were recruited in this study. This study was approved by the institutional review board of the Tokyo Medical and Dental University. The study population (Table I) consisted of 62 females and 103 males, with a mean age of 64.5±10.3 years (range, 37-89). Sixty-two

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Table I. Clinicopathological characteristics according to the mutation and methylation status of each gene.

	KRAS			*PIK3CA*			*BNIP3*			*p16*			*hMLH1*		
Characteristics	Mut 52 (32%)	Wt 113 (69%)	P	Mut 20 (12%)	Wt 145 (88%)	P	Met 84 (58%)	Unm 61 (42%)	P	Met 48 (33%)	Unm 97 (67%)	P	Met 30 (21%)	Unm 115 (79%)	P
Age (years)	64.6 (37-89)	64.6 (41-88)	0.898	67.8 (54-84)	64.1 (37-89)	0.145	65.0 (37-89)	63.8 (41-88)	0.614	66.2 (48-87)	64.0 (37-89)	0.292	65.7 (37-88)	64.4 (41-89)	0.496
Gender			0.216			0.811			0.456			0.661			0.288
Female	23 (37)	39 (63)		8 (13)	54 (87)		28 (54)	24 (46)		17 (31)	38 (69)		13 (24)	42 (76)	
Male	29 (28)	74 (72)		12 (12)	91 (88)		56 (60)	37 (40)		31 (34)	59 (64)		17 (19)	73 (81)	
Location			0.372			0.221			0.918			0.305			0.338
Proximal	22 (35)	40 (65)		10 (16)	52 (84)		31 (58)	22 (42)		20 (38)	32 (68)		13 (25)	39 (75)	
Distal	30 (29)	73 (71)		10 (10)	93 (90)		53 (58)	39 (42)		28 (30)	65 (70)		17 (18)	76 (82)	
Differentiation			0.773			0.886			0.466			0.331			0.529
Wel	23 (34)	44 (66)		9 (13)	59 (87)		32 (53)	28 (47)		22 (38)	36 (62)		11 (19)	47 (81)	
Mod	24 (28)	62 (72)		10 (12)	74 (88)		43 (59)	30 (41)		23 (31)	51 (69)		15 (20)	59 (80)	
Por	4 (36)	7 (64)		1 (8)	11 (92)		8 (73)	3 (27)		2 (17)	10 (83)		4 (33)	8 (67)	
T			0.223			0.346			0.641			0.371			0.558
Tis	5 (56)	4 (44)		0 (0)	9 (100)		4 (67)	2 (33)		1 (17)	5 (83)		0 (0)	6 (100)	
T1	2 (14)	12 (86)		3 (23)	10 (77)		8 (67)	4 (33)		6 (55)	5 (45)		1 (9)	10 (91)	
T2	6 (33)	12 (67)		1 (6)	17 (94)		9 (64)	5 (36)		6 (43)	8 (57)		3 (21)	11 (79)	
T3	22 (36)	39 (64)		6 (10)	55 (90)		35 (61)	22 (39)		19 (33)	38 (67)		14 (25)	43 (75)	
T4	17 (27)	47 (73)		10 (16)	54 (84)		28 (50)	28 (50)		16 (28)	41 (72)		12 (21)	45 (79)	
N			0.325			0.570			0.295			0.404			0.592
N0	28 (29)	69 (71)		13 (13)	84 (87)		45 (54)	38 (46)		30 (36)	53 (64)		16 (19)	67 (81)	
N1-3	24 (36)	43 (64)		7 (10)	60 (90)		39 (63)	23 (37)		18 (30)	43 (70)		14 (23)	47 (77)	
M			0.242			0.098			0.290			0.503			0.0005
M0	45 (33)	92 (67)		14 (10)	123 (90)		69 (56)	54 (44)		39 (32)	83 (68)		19 (16)	103 (84)	
M1	6 (21)	22 (79)		6 (21)	22 (79)		15 (68)	7 (32)		9 (39)	14 (61)		11 (48)	12 (52)	

Mut, mutated; Wt, wild-type; Met, methylated; Unm, unmethylated; Wel, well-differentiated; Mod, moderately differentiated; Por, poorly differentiated; TNM classification according to the International Union Against Cancer TNM staging.

cancers were located in the proximal site and 103 were located in the distal site, including the rectum. Histological classification and tumor staging were performed according to the International Union Against Cancer Tumor-Node-Metastasis classification guidelines. No patient received preoperative chemotherapy or radiotherapy. Following surgery, patients with stage III CRC received oral or intravenous 5-fluorouracil (FU)-based adjuvant chemotherapy and patients with stage IV tumors received 5-FU-based systemic chemotherapy without radiotherapy. Patients were prospectively followed up following surgery for a median period of 64 months (range, 0.07-107.6). Resected specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. For all cases, archival hematoxylin and eosin (H&E) slides of primary tumors were retrieved and reviewed to confirm pathological features.

DNA extraction. Tissues were cut into 10-μm sections from paraffin-embedded blocks. After specimens were deparaffinized and washed, tumor tissue was manually dissected for comparison with H&E slides. The same amount of dissected tissue was used for each case. Genomic DNA was extracted using standard proteinase K (Invitrogen, Carlsbad, CA, USA) digestion, phenol/chloroform extraction, and ethanol precipitation, as previously described (5).

Mutation analysis. Exon 1 of the *KRAS* gene and exons 9 and 20 of the *PIK3CA* gene were selected for mutation analysis, since mutations are known to cluster in these regions (6,7). Primer sequences and PCR conditions have been reported previously (8). Following the purification of PCR products using a Microcon YM-100 Centrifugal Filter (Millipore Corporation, Billerica, MA, USA) and Centri-Sep Columns (Princeton Separations, Adelphia, NJ, USA), direct sequencing was carried out using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and analyzed using an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems). Somatic mutations were further validated by independent PCR amplification and sequencing and matched with normal mucosa.

MethyLight analysis. Sodium bisulfite conversion and DNA recovery was performed using EpiTect Bisulfite (Qiagen, Hilden, Germany). Following sodium bisulfite conversion, genomic DNA was analyzed using the MethyLight technique, a fluorescence-based real time PCR (Q-PCR) assay (9) and the ABI Prism 7300 Real Time PCR system (Taqman; Applied Biosystems). Four sets of primers and probes designed specifically for bisulfite-converted DNA were used. Three sets were used to detect methylation in the gene of interest and the remaining set served as a reference for β-actin (ACTB) to normalize for input DNA. Reference primers and probes were designed in a region of the *ACTB* gene lacking CpG dinucleotides, thus allowing for equivalent amplification regardless of methylation levels. Primer and probe sequences were previously reported (10,11). *Sss*I-treated HCT-15 DNA was used as a fully methylated positive control (100% methylation ratio). Parallel TaqMan PCR reactions were performed using primers specific for the bisulfite-converted methylated sequence of a particular locus and with *ACTB* reference primers. In each case, triplicate threshold cycle (Ct) values were obtained and averaged and expression levels were evaluated using the $2^{-\Delta\Delta Ct}$ method. As an internal standard, each sample was normalized to its ACTB content and compared with the gene expression level of *Sss*I-treated HCT-15 DNA as positive controls (calibration sample) as follows: $2^{\Delta\Delta Ct}$ where $\Delta\Delta$Ct=(Ct-target-Ct-reference) treated sample - (Ct-target-Ct-reference) calibrator sample. We defined the percentage of the fully methylated reference (PMR) to be $2^{\Delta\Delta Ct}$ x100%. To define *p16* or *hMLH1* methylation status, a PMR cut-off value of 4 was used based on previously validated data (9). Based on the distribution of PMR values in normal colon epithelial tissue (10), a PMR cut-off value of >0 was used to define positive methylation status for *BNIP3*.

Statistical analysis. Statistical analysis was carried out using StatView Software (version 5.0). To estimate the differences among the groups, the Chi-square test, Fisher's exact test, Student's t-test and log-rank test were used where appropriate. The Kaplan-Meier method was used to estimate survival. Survival was calculated beginning from the date of surgery. Prognostic factors were estimated using multivariate analysis and the Cox proportional hazards model. P<0.05 was considered to indicate a statistically significant result.

Results

Mutation of KRAS and PIK3CA and methylation of BNIP3, p16 and hMLH1. *KRAS* mutations in exon 1 were observed in 32% of cases and *PIK3CA* mutations in exons 9 and 20 in 12% of cases. Methylation frequencies of the examined genes were 58% for *BNIP3*, 33% for *p16* and 21% for *hMLH1*. Although *hMLH1* methylation was significantly associated with distant metastasis (P=0.0005), other associations were not observed between clinicopathological characteristics and the mutation or methylation status of individual genes (Table I).

Correlation between mutation and methylation. Associations among gene mutations and gene methylations are shown in Table II. The *KRAS* mutation was significantly associated with *BNIP3* (P<0.0001) and *p16* methylation (P=0.04) as well as with the number of methylated genes (P=0.0082). *p16* methylation was associated with *BNIP3* methylation (P<0.0001) and *hMLH1* methylation (P=0.03).

Correlation between molecular markers and outcomes. The correlation between the molecular parameters and disease-specific survival (DSS) were analyzed (Table III). Patients with *hMLH1*-methylated tumors had significantly shorter DSS compared with those without methylation [hazards ratio (HR), 2.231; P=0.05], but mutation or methylation of other single genes did not affect DSS. Having mutations in both the *KRAS* and *PIK3CA* genes did not correlate with DSS. By contrast, when cases were divided into 2 groups, namely a low methylation group (0 or 1 methylated genes) and a high methylation group (2 or 3 methylated genes), the high methylation group had significantly shorter DSS than the low methylation group (HR, 2.681; P=0.01). Integration of the *KRAS* mutation and methylation status of multiple genes did not reveal an association with DSS. However, CRCs were divided into 4 groups:

Table II. Concordance between the mutation and methylation status of each gene.

	PIK3CA		*BNIP3*		*p16*		*hMLH1*		Methylation[a]	
	Mut	Wt	Met	Unm	Met	Unm	Met	Unm	High	Low
KRAS	P=0.918		P<0.0001		P=0.0402		P=0.467		P=0.0082	
Mut	7	45	39	9	21	26	8	39	24	23
Wt	13	88	45	52	27	71	22	77	28	70
PIK3CA			P=0.827		P=0.982		P=0.428		P=0.775	
Mut			10	8	6	12	5	13	7	11
Wt			74	53	42	85	25	102	45	82
BNIP3					P<0.0001		P=0.541			
Met					42	39	19	64		
Unm					4	54	11	48		
p16							P=0.0295			
Met							15	33		
Unm							15	81		

[a]Methylation high, 2 or 3 methylated genes; low, 0 or 1 methylated genes. Mut, mutated; Wt, wild-type; Met, methylated; Unm, unmethylated.

Table III. Correlation of molecular parameters with DSS.

		HR	95% CI	P
KRAS	(Mutant vs. wild)	0.930	0.418-2.071	0.8590
PIK3CA	(Mutant vs. wild)	1.429	0.540-3.779	0.4721
BNIP3	(Methylated vs. unmethylated)	2.233	0.944-5.284	0.0675
p16	(Methylated vs. unmethylated)	1.950	0.912-4.169	0.0849
hMLH1	(Methylated vs. unmethylated)	2.231	1.000-4.977	0.0500
Mutation[a]	(Mutant vs. wild)	1.001	0.468-2.139	0.9983
Methylation[b]	(High vs. low)	2.681	1.253-5.747	0.0110
Mutation/methylation	(Mutant and/or high vs. wild and low)	1.499	0.634-3.546	0.3569
KRAS/methylation	(Mutant and/or high vs. others)	1.675	0.766-3.663	0.1963
PIK3CA/methylation	(Mutant and/or high vs. others)	2.924	1.312-6.494	0.0087

[a]Mutant, at least one mutation gene; wild, no mutation. [b]Methylation high, 2 or 3 methylated genes; low, 0 or 1 methylated genes. CI, confidence interval; HR, hazards ratio.

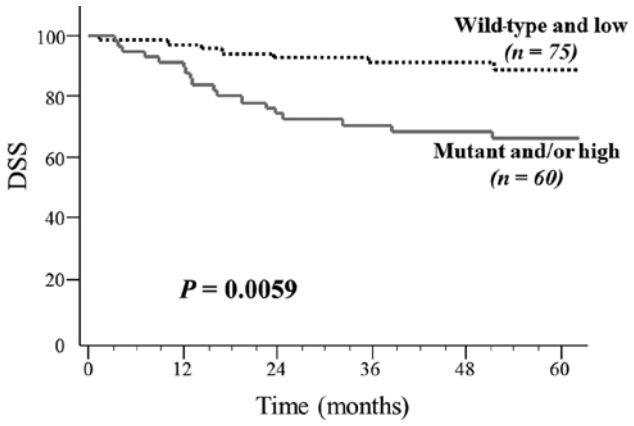

Figure 1. Kaplan-Meier estimate of DSS according to *PIK3CA* mutation and methylation classification. DSS, disease-specific survival.

CRC containing i) *PIK3CA* mutation and high methylation (5.2%); ii) *PIK3CA* mutation and low methylation (8.1%); iii) no mutation and high methylation (31.1%); and iv) no mutation and low methylation (55.6%). Patients with the *PIK3CA* mutation and/or high methylation (groups i, ii and iii) had a significantly poorer outcome in DSS compared with those with wild-type *PIK3CA* and low methylation (group iv; HR, 2.924; P=0.0087) (P=0.0059, log-rank test; Fig. 1).

Results of the univariate and multivariate analyses using the Cox proportional hazards model are shown in Table IV. Univariate analysis revealed that the *PIK3CA* mutation and/or high methylation was a significant prognostic factor for DSS, when used with the depth of the tumor, lymph node metastasis, distant metastasis, histological differentiation, lymphatic invasion and vessel invasion. Variables with P<0.05 in the

Table IV. Cox proportional hazards model of prognostic factors of DSS.

Characteristics		Univariate analysis			Multivariate analysis		
		HR	95% CI	P	HR	95% CI	P
Gender	(Female vs. male)	0.424	0.171-1.050	0.0636			
Age	(≥65 vs. <65)	0.699	0.328-1.487	0.3526			
Location	(Distal vs. proximal)	0.839	0.389-1.808	0.6539			
T	(T1,2 vs. T3,4)	8.547	1.157-62.50	0.0354	2.451	0.270-22.20	0.4253
N	(N- vs. N+)	3.559	1.558-8.130	0.0026	1.368	0.504-3.717	0.5377
M	(M0 vs. M1)	7.874	3.448-17.86	<0.0001	7.092	2.732-18.18	<0.0001
Differentiation	(Por vs. wel/mod)	3.031	1.046-8.781	0.0411	0.611	0.172-2.172	0.4466
Ly	(Ly0,1 vs. ly2,3)	4.831	2.247-10.31	<0.0001	3.891	1.379-10.99	0.0102
v	(v0,1 vs. v2,3)	4.049	1.399-11.76	0.0099	1.761	0.540-5.747	0.3482
PIK3CA/methylation	(Mutant and/or high vs. others)	2.924	1.312-6.494	0.0087	2.793	1.220-6.410	0.0151

HR, hazards ratio; CI, confidence interval; TNM classification according to the International Union Against Cancer TNM staging; por, poorly differentiated; wel, well-differentiated; mod, moderately differentiated; ly, lymphatic invasion; v, vessel invasion; high methylation, 2 or 3 methylated genes.

univariate analysis were used for the multivariate analysis. *PIK3CA* mutation and/or high methylation, as well as distant metastasis and lymphatic invasion, were found to be independent and significant prognostic factors for DSS.

Discussion

In this study, we examined mutations in *KRAS* and *PIK3CA*, which comprise components of the EGFR pathway, and the methylation status of *BNIP3*, *p16* and *hMLH1* in CRCs to evaluate patients with the *PIK3CA* mutation and/or 2 or more methylations with a poorer prognosis compared with other patients. Our results suggest that a combination of epigenetic and genetic alterations is a potent biomarker for predicting CRC prognosis.

Integrated analysis of epigenetic and genetic alterations is increasingly significant in cancer research. Since CRC develops as a result of accumulating genetic and epigenetic alterations, the combination of genetic and epigenetic profiles could confer differential clinical phenotypes and potential variability in survival. Ward *et al* reported that although DNA methylation is associated with a worse outcome in CRC patients, this adverse prognostic effect is lost in methylated tumors with microsatellite instability (12). By integrating genetic and epigenetic analyses, Shen *et al* revealed that colon cancers correspond to 3 molecularly distinct subclasses of disease, each of which is relatively homogeneous (13). In the present study, CRCs were divided into 4 groups according to *PIK3CA* mutations and the number of methylated genes: CRCs with i) *PIK3CA* mutation and 2 or 3 methylations (5.2%); ii) *PIK3CA* mutation and less than 2 methylations (8.1%); iii) no mutation and 2 or 3 methylations (31.1%); and iv) no mutations and less than 2 methylations (55.6%). The last group was associated with better prognosis than the former 3 groups. CRC patients with the *PIK3CA* mutation and/or more than 1 methylation had worse outcomes compared with the other groups. This finding suggests that mutation analysis and determining methylation status may provide significant predictive information regarding tumor behavior and clinical outcome in CRC patients.

BNIP3 is a pro-apoptotic member of the Bcl-2 family (14). The expression of BNIP3 is induced by hypoxia, such as that which occurs during cardiac ischemia and in the hypoxic regions of tumors, and it acts against pro-survival proteins, including Bcl-2 and Bcl-xl (15-18). Transcriptional silencing of the *BNIP3* gene has recently been observed in multiple human cancer cell lines, including colorectal, gastric, pancreatic, and hematopoietic tumors (19). Murai *et al* reported that aberrant hypermethylation of *BNIP3* was detected in 66% of primary colorectal and 49% of primary gastric cancers, but not in normal tissue; these authors also revealed that *BNIP3* is silenced by DNA methylation (20). Akada *et al* and Erkan *et al* revealed that loss of BNIP3 expression correlates with poor prognosis and increased chemoresistance in patients with pancreatic cancer (21,22). Manka *et al* demonstrated that *BNIP3* silencing induces or increases the metastatic growth of breast cancer in distant organs (23). Moreover, our previous study also indicated that *BNIP3* methylation is associated with poor clinical outcome and chemoresistance in primary CRC (10). These previous studies suggest that reduced BNIP3 expression contributes to carcinogenesis of the colon and rectum and may be a predictive factor for the prognosis of CRC. Therefore, the *BNIP3* gene, as well as the *hMLH1* and *p16* genes, were tested in our methylated gene sets for predicting the outcome of CRC.

Activation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway is thought to be critical in CRC development and clinical behavior (6,8,24,25). *PIK3CA* encodes the p110α catalytic subunit of PI3K and is mutated in 10-32% of CRCs (6,8,24,26-29). *PIK3CA* mutations elevate kinase activity, thereby activating the PI3K/AKT pathway and contributing to tumorigenesis through decreased apoptosis, loss of contact inhibition and increased tumor invasion (6,30); these factors have been associated with poor outcomes in CRC

patients (8,24,31). Although previous studies have investigated the role of the *PIK3CA* mutation and gene methylation in CRC, the data are inconclusive. Certain studies reported a significantly higher frequency of the *PIK3CA* mutation in the CpG island methylator phenotype (CIMP)-high tumor compared with CIMP-negative tumors and a significant correlation between the *PIK3CA* mutation and *RASSF2* gene methylation (29,32,33), whereas other studies failed to observe this correlation (11,34). In the present study, although CIMP status was not examined, the *PIK3CA* mutation did not exhibit an association with the methylation status of the genes examined. Moreover, few studies have described the effect of the combination of the *PIK3CA* mutation with gene methylation on patient outcome. Ogino *et al* demonstrated that the effect of the *PIK3CA* mutation on CRC patient survival is not significantly modified by CIMP (31). Their result was inconsistent with our finding that integration of the *PIK3CA* mutation and methylation status may be used to predict outcomes for CRC patients. It is possible that the methylated gene set used in this study was different from that used in the Ogino study. The *BNIP* gene included in our methylated set was not included in CIMP markers used to date. Moreover, there have been no studies regarding the influence of integrating the *PIK3CA* mutation and methylation status of the *BNIP3* gene on outcomes of CRC. It would be useful to examine whether mutating other genes from the EGFR pathway also affects CRC prognosis in cooperation with *BNIP3* methylation.

In conclusion, by integrating genetic and epigenetic alterations, we revealed that CRC patients with poor prognosis could be identified. Further studies are necessary to confirm our finding that a combination of the *PIK3CA* mutation with gene methylation may be useful for predicting outcomes of CRC patients and to elucidate the underlying mechanism behind these findings.

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