Effect of classification based on combination of mutation and methylation in colorectal cancer prognosis

HARUHIKO AOYAGI[1], SATORU IIDA[1], HIROYUKI UETAKE[2], TOSHIAKI ISHIKAWA[2], YOKO TAKAGI[2], HIROTOSHI KOBAYASHI[1], TETSURO HIGUCHI[1], MASAMICHI YASUNO[1], MASAYUKI ENOMOTO[1] and KENICHI SUGIHARA[1]

Departments of [1]Surgical Oncology and [2]Translational Oncology, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan

Received August 23, 2010; Accepted November 3, 2010

DOI: 10.3892/or.2010.1118

Abstract. Colorectal cancer (CRC) is caused by an accumulation of genetic alterations and epigenetic alterations. The molecular classification of CRCs based on genetic alterations and epigenetic alterations is evolving. Here, we examined mutations and methylation status in CRCs to determine if the combination of genetic and epigenetic alterations predicts prognosis. We examined 134 sporadic CRCs. We used the direct sequencing method to identify mutations in BRAF and AKT1, which are downstream of KRAS and PIK3CA, respectively, in the EGFR pathway. We used the Methylight method to determine the methylation status of hMLH1, p16, MINT1, MINT2 and MINT31. Both BRAF and AKT1 mutations were found in only one case (0.75%). Aberrant methylation of hMLH1, p16, MINT1, MINT2 and MINT31 was detected in 22.4, 35.1, 32.8, 59.7 and 41.0% of cases, respectively. The clinicopathological factors were not significantly correlated to mutation or methylation. Among the patients who had no mutation in the EGFR pathway, the overall survival was significantly shorter in the patients with methylation compared to the patients with no methylation in hMLH1 and p16 (p=0.0318). Methylation could play a key role in the prognosis of patients without mutations in the EGFR pathway. The combination of genetic and epigenetic alterations may be a good marker for the prognosis of CRC patients.

Introduction

Colorectal cancer (CRC) is the third most common cancer in the world and the second most common cause of cancer-related death (1). Of patients who undergo potentially curative surgery, 17% develop local recurrence or distant metastasis leading to a shorter survival time (2). Therefore, it is important to identify molecular markers of biological and prognostic significance and predictive value in patients with advanced CRC (3,4).

CRC develops as a result of progressive accumulation of genetic alterations and epigenetic alterations (3-5). The elucidation of the human genome sequence (6) has showed that about 50-70 gene mutations are detected in CRC. Many studies have reported the importance of the mutations in the EGFR pathway, including the RAS/RAF pathway and the PI3K/AKT pathway (7-10). Gene mutations in the EGFR pathway are related to the efficiency of cetuximab or panitumumab therapy in metastatic CRC (11-14). The RAS/RAF pathway mediates the cellular response to extracellular signals that regulate cell growth, differentiation, and apoptosis (15). The PI3K/AKT pathway plays a central role in carcinogenesis since it is frequently activated and deregulated in the carcinogenic process of various human cancers (16). We previously examined the mutation of KRAS and PIK3CA in CRC patients and found that PIK3CA mutation is predictive of poor survival (17).

Gene methylation has been recognized as a third mechanism of Knudson's two-hit theory, and it is clear that methylation is associated with not only carcinogenesis but also the evolution and metastatic processes of cancer (18,19). Epigenetic changes usually begin early in carcinogenesis, are potentially reversible, and can advance to gene alterations. Therefore, the detection of aberrant methylation is important for the early diagnosis, prognosis, and treatment of patients with CRC (20-22).

In the present study, we examined the mutation of BRAF and AKT1, which are downstream of KRAS and PIK3CA, respectively, and the methylation status of hMLH1, p16, MINT1, MINT2 and MINT31 to clarify whether the combination of genetic and epigenetic alterations might be used as parameters to predict prognosis in CRC.

Materials and methods

Patients and tissue samples. A total of 158 patients who had undergone surgical resection for primary sporadic colorectal

Correspondence to: Dr Satoru Iida, Department of Surgical Oncology, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan
E-mail: s-iida.srg2@tmd.ac.jp

Key words: mutation, methylation, colorectal cancer, prognosis

cancer at the Department of Surgical Oncology, Tokyo Medical and Dental University (Tokyo, Japan), between March 2000 and April 2003 were targeted in our previous study. Of them, 134 patients, for whom genomic DNA was available were included in this study. This research was approved by the institutional review board of Tokyo Medical and Dental University, and written informed consent was obtained from all participants. The patients comprised 86 men and 48 women, ranging in age from 37 to 88 (mean, 64.5 years). Tumors were classified as proximal (proximal to the splenic flexure) or distal. There were 48 cancers in the proximal colon and 86 cancers in the distal colon, including the rectum. Histological classification and tumor staging were performed according to the International Union Against Cancer Tumor-Node-Metastasis (TNM) classification. No patient received preoperative chemotherapy or radiotherapy. After surgery, patients with stage III CRC received oral or intravenous 5-fluorouracil (5-FU)-based adjuvant chemotherapy, and patients with stage IV tumors received 5-FU-based systemic chemotherapy without any radiotherapy. Patients were prospectively followed-up after surgery for a median of 49 months. All resected specimens were fixed in 10% pH-neutral formalin and embedded in paraffin. In all cases, archival H&E slides of the primary tumors were retrieved and reviewed to confirm pathological features.

DNA extraction and mutation analysis. Tissue blocks were cut into 10-μm thick sections with a microtome. The blade was changed and the microtome was cleaned after each specimen. After the specimens were deparaffinized and washed, tumor tissue was manually dissected with a razor blade in comparison to H&E slide. Tumor tissues were incubated overnight with proteinase K in digestion buffer, and then genomic DNA was extracted by a standard phenol-chloroform method. Exon 1 of the KRAS gene, exons 9 and 20 of the PIK3CA gene, exon 15 of the BRAF gene, and exon 4 of the AKT1 gene were selected for mutation analysis, because mutations cluster in these regions. The exons were sequenced after PCR amplification. Primer sequences and PCR conditions are available upon request. PCR products were purified with Microcon YM-100 Centrifugal Filters (Millipore, MA) and Centri-Sep Columns (Princeton Separations, Adelphia, NJ) and then directly sequenced with a Big Dye Terminator Cycle Sequencing kit (3130 Genetic Analyser, Applied Biosystems, Foster City, CA).

Methylight analysis. Sodium bisulfite conversion and DNA recovery was performed using EpiTect Bisulfite (Qiagen). Following sodium bisulfite conversion, genomic DNA was analyzed by the Methylight technique, a fluorescence-based, real-time PCR (Q-PCR) assay (23) and the ABI Prism 7300 Real-Time PCR System (Taqman; Applied Biosystems). Six sets of primers and probes designed specifically for bisulfite-converted DNA were used. One set was used to detect methylation in the gene of interest and the other five sets served as reference sets for ß-actin (ACTB) to normalize for input DNA. The reference primers and probes were designed in a region of the ACTB gene that lacks CpG dinucleotides, thus allowing for equal amplification regardless of the methylation levels. Primer and probe sequences are available upon request. *Sss*I-treated HCT-15 DNA was used as a fully methylated positive control (100% methylation ratio). Parallel TaqMan PCR was performed with specific primers for the bisulfite-converted methylated sequence for a particular locus and with the ACTB reference primers. In each case, triplicate threshold cycle (Ct) values were obtained and averaged, and expression levels were then evaluated by the 2-ΔΔCt method (24). As an internal standard, each individual sample was normalized to its ß-actin (ACTB) content and compared to the gene expression level of *Sss*I-treated HCT-15 DNA (calibration sample) as follows: $2^{-\Delta\Delta Ct}$, where ΔΔCt = (Ct-target-Ct-reference) treated-sample - (Ct-target-Ct-reference) calibrator sample. We defined the percentage of fully methylated reference (PMR) to be $2^{-\Delta\Delta Ct}$ x 100%.

Statistical analysis. All statistical analyses were performed with StatView Software (version 5.0). To estimate differences between groups, the χ^2 test, Fisher's exact test, Student's t-test and log-rank test were used as appropriate. The Kaplan-Meier method was used to estimate survival. Survival was calculated from the date of surgery. P-values <0.05 were considered to be significant.

Results

Mutation and methylation status in relation to clinicopathological parameters of 134 CRCs. KRAS mutations in exon 1 were found in 30.6% (41/134) of the cases, and PIK3CA mutations in exons 9 and 20 were found in 13.4% (18/134) of the cases. Both BRAF and AKT1 mutations were found in only one case (0.75%). One case with an AKT1 mutation also had a PIK3CA mutation, while one case with a BRAF mutation had no other mutations. There were no correlations between mutations in KRAS, BRAF, PIK3CA, and AKT1. There were no correlations between the RAS/RAF and PIK/AKT pathways. There were also no statistically significant differences between patients with mutations and patients without mutations in these pathways. The frequency of mutations is summarized in Table I.

Aberrant methylation of hMLH1, p16, MINT1, MINT2 and MINT31 was detected in 22.4% (30/134), 35.1% (47/134), 32.8% (44/134), 59.7% (80/134), and 41.0% (55/134), respectively. Aberrant methylation of p16 was significantly associated with tumor depth. The frequency of methylation is summarized in Table I.

Mutation or methylation status was not significantly correlated to the clinicopathological data (Tables I and II).

Relationship between the RAS/RAF and PIK/AKT pathways and methylation of hMLH1 or p16 in CRCs. The relationship between the RAS/RAF and PIK/AKT pathways and methylation of hMLH1 or p16 is summarized in Table III. Although not statistically significant, hMLH1 methylation tended to be associated with p16 methylation (p=0.06).

Prognostic value of mutations in the RAS/RAF and PIK/AKT pathways and methylation of hMLH1 and p16 in CRCs. There was no statistically significant difference in overall survival between patients with and without a mutation in the RAF/RAF and PIK/AKT pathways (p=0.2436; Fig. 1). Of

Table I. Mutation status in relation to clinicopathological parameters of 134 CRC.

		KRAS			PIK3CA			BRAF			AKT1		
		Wt	Mut	P-value	Wt	Mut	P-value	Wt	Mut	P-value	Wt	Mut	P-value
No. of cases	134	93	41		116	18		133	1		133	1	
Gender													
Male	86 (64.2)	63	23	0.1952	75	11	0.7705	85	1	0.4533	86	0	0.1791
Female	48 (35.8)	30	18		41	7		48	0		47	1	
Tumor site													
Proximal	48 (35.8)	29	19	0.0917	39	9	0.1775	47	1	0.1791	48	0	0.4533
Distal	86 (64.2)	64	22		77	9		86	0		85	1	
Histology													
Well	46 (34.3)	32	14	0.9765	38	8	0.3313	46	0	0.468	45	1	0.165
Others	88 (65.7)	61	27		78	10		87	1		88	0	
pT													
T1, T2	22 (16.4)	17	5	0.3809	18	4	0.4749	22	0	0.6564	22	0	0.6564
T3, T4	112 (83.6)	76	36		98	14		111	1		111	1	
pN													
Positive	59 (44.0)	39	20	0.3809	52	7	0.8144	59	0	0.3661	59	0	0.3661
Negative	75 (56.0)	54	21		64	11		74	1		74	1	
TNM stage													
I, II	65 (48.5)	45	20	0.7391	57	8	0.7109	64	1	0.3011	64	1	0.3011
III, IV	69 (51.5)	48	21		59	10		69	0		69	0	
Lymphatic invasion													
Positive	101 (75.4)	69	32	0.6331	91	10	0.036	100	1	0.5661	100	1	0.5661
Negative	33 (24.6)	24	9		25	8		33	0		33	0	
Venous invasion													
Positive	124 (92.5)	86	38	0.9666	111	13	0.0004	123	1	0.7756	123	1	0.7756
Negative	10 (7.5)	7	3		5	5		10	0		10	0	

Wt, wild-type; Mut, mutated type.

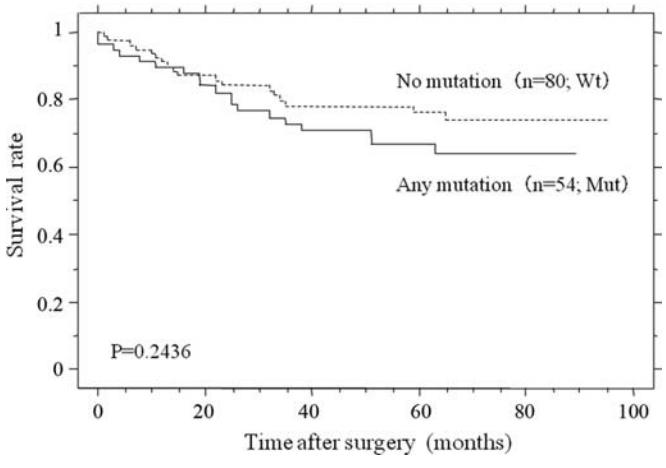

Figure 1. Overall survival in relation to mutation in the RAS/RAF and PIK/AKT pathways.

134 patients with CRC, 54 had a mutation in the RAS/RAF or PIK/AKT pathway. Among these 54 patients, there was no significant difference in overall survival based on methylation of hMLH1 or p16 (p=0.5463; Fig. 2). Of the 134 patients with CRC, 80 had no mutations in the RAS/RAF and PIK/AKT pathways. Among these wild-type patients, patients with methylated hMLH1 or p16 had a significantly shorter overall survival than those without methylation (p=0.0318; Fig. 3).

Discussion

In the present study, we examined mutations in the RAS/RAF and PIK/AKT pathways and the methylation status of hMLH1, p16, MINT1, MINT2 and MINT31. We analyzed their correlations with clinicopathological factors and prognosis to determine whether these factors are novel prognostic markers

Table II. Methylation status in relation to clinicopathological parameters of 134 CRC.

		hMLH1			p16			MINT1			MINT2			MINT31		
		Unm	Met	P-value	Unm	Met	P-value	Unm	Met	P-value	Unm	Met	P-value	Unm	Met	P-value
No. of cases	134	104	30		87	47		90	44		54	80		79	55	
Gender																
Male	86 (64.2)	69	17	0.33	55	31	0.9887	59	27	0.6436	34	52	0.8094	50	36	0.7972
Female	48 (35.8)	35	13		32	16		31	17		20	28		29	19	
Tumor site																
Proximal	48 (35.8)	35	13	0.33	29	19	0.4415	30	18	0.3904	16	32	0.2194	25	23	0.227
Distal	86 (64.2)	69	17		58	28		60	26		38	48		54	32	
Histology																
Well	46 (34.3)	35	11	0.7595	27	19	0.2935	31	15	0.9677	21	25	0.361	29	17	0.4867
Others	88 (65.7)	69	19		60	28		59	29		33	55		50	38	
pT																
T1, T2	22 (16.4)	18	4	0.6047	10	12	0.0391	11	11	0.0608	9	13	0.9491	10	12	0.1591
T3, T4	112 (83.6)	86	26		77	35		79	33		45	67		69	43	
pN																
Positive	59 (44.0)	45	14	0.8792	41	18	0.2563	44	15	0.1709	27	32	0.3177	36	23	0.8248
Negative	75 (56.0)	59	16		46	29		46	29		27	48		43	32	
TNM stage																
I, II	65 (48.5)	55	10	0.0591	40	25	0.4613	42	23	0.542	24	41	0.6739	39	26	0.8114
III, IV	69 (51.5)	49	20		47	22		48	21		30	39		40	29	
Lymphatic invasion																
Positive	101 (75.4)	82	19	0.0823	70	31	0.0685	72	29	0.0754	42	59	0.5955	60	41	0.8528
Negative	33 (24.6)	22	11		17	16		18	15		12	21		19	14	
Venous invasion																
Positive	124 (92.5)	95	29	0.3286	81	43	0.7485	85	39	0.2296	51	73	0.4901	73	51	0.9493
Negative	10 (7.5)	9	1		6	4		5	5		3	7		6	4	

Unm, unmethylated; Met, methylated.

Table III. Relationship between mutation in EGFR pathway and methylation of hMLH1 and p16 in CRC.

	PIK3CA			p16			hMLH1		
	Mut	Wt	P-value	High	Low	P-value	High	Low	P-value
KRAS									
Mut	6	35		20	21		8	33	
Wt	12	81	0.787	27	65	0.03	22	71	0.596
PIK3CA									
Mut	-	-	-	6	12		5	13	
Wt	-	-	-	41	74	0.848	25	91	0.556
p16									
High	-	-	-	-	-	-	15	32	
Low	-	-	-	-	-	-	15	71	0.06

Wt, wild-type; Mut, mutated type.

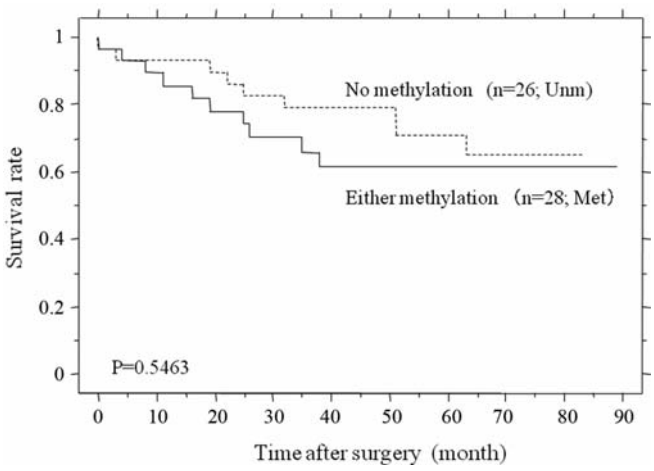

Figure 2. Overall survival in relation to hMLH1 and p16 methylation in the mutation group. Unm, unmethylated group; Met, methylated group.

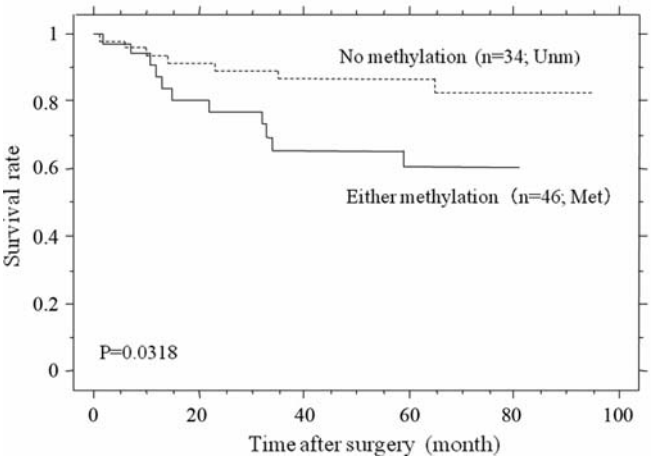

Figure 3. Overall survival in relation to hMLH1 and p16 methylation in the wild-type group. Unm, unmethylated group; Met, methylated group.

of CRC. We found that the combination of mutation and methylation may be a good prognostic marker for CRC.

Mutations in the RAS/RAF and PIK/AKT pathways are present in CRC (7-10,25). We previously examined the mutation of KRAS and PIK3CA in CRC patients, and found that PIK3CA mutation is predictive of poor survival in these patients (17). In the present study, we examined the mutation status of BRAF and AKT1, which are downstream of KRAS and PIK3CA, respectively. BRAF and AKT1 mutations were detected in one case each. BRAF mutation has been reported to occur in about 15% of CRC cases, while V600E accounts for approximately 90% of the mutations (26,27). The frequency of BRAF mutation in CRC patients differs among ethnic groups. Brim *et al* (28) analyzed BRAF mutation in CRC patients of different ethnic groups, African American, Omani and Iranian. Among these CRC patients, BRAF mutation was detected in 10% of the African Americans, 19% of the Omanis, and 2% of the Iranians. The frequency of BRAF mutation in Asia tends to be low, reported at approximately 5% (29-32). These differences among ethnic groups may be due to different lifestyle factors such as diet, alcohol and smoking (33,34). Our study indicates that the frequency of BRAF mutations in Asian patients with CRC is lower than in other ethnic groups. BRAF mutation in CRC is associated with microsatellite instability-high colorectal cancer (MSI-H CRC) (9,10,15,27). MSI-H CRC is often detected in the early stage of cancers. Most samples in the present study were from CRC patients in an advanced stage, so it is possible that the frequency of BRAF mutation was low. Carpen *et al* (35) found AKT1 mutation in 6% of CRC patients, but other studies have reported smaller frequencies of AKT1 mutation. Kim *et al* (36) found no AKT1 mutations in 104 CRC patients. In a study of 88 CRC patients, Bleeker *et al* (37) found only one case with an AKT1 mutation. Therefore, it is possible that the frequency of AKT1 mutation in CRC cases is lower than the 6% reported by Carpen *et al*. In the present study of CRC, the frequency of BRAF and AKT1 mutations was less than the frequency of KRAS and PIK3CA mutations; therefore, it is possible that the mutation of KRAS and PIK3CA is more important than the mutation of BRAF and AKT1 in carcinogenesis of CRC.

We examined the methylation status of five genes, but found no significant correlation between methylation status and clinicopathological factors. Many reports have found a relationship between methylation of these genes and CRC (38-40). The hMLH1 gene is methylated in MSI-H CRC, and the relationship between methylated hMLH1 and CRC prognosis has been discussed in many studies (41-43). Wettergren *et al* (44) reported that p16 hypermethylation may be a prognostic marker in CRC patients. Therefore, we focused on methylation of these two genes, hMLH1 and p16.

The combination of genetic alterations and epigenetic alterations may provide a good marker for the prognosis of CRC patients. Shen *et al* (45) analyzed both mutation and methylation in primary CRC and found that CRC consists of three distinct subclasses, each of which is fairly homogeneous. Lee *et al* (29) divided CRC patients into four groups based on classification of the RAS/RAF mutation and CIMP, and showed that this classification may be a very effective prognostic marker. Similarly, Ogino *et al* (46) showed that patients with CIMP-low and mutated BRAF have a shorter survival than those with other CIMP/BRAF types.

In the present study, overall survival was not associated with mutations in the RAS/RAF and PIK/AKT pathways. Thus, genetic classification was not useful as a prognostic marker among these patients. Overall survival of patients with mutations was not associated with the methylation status of hMLH1 and p16. However, among the patients without mutations, overall survival was significantly shorter in patients with any methylation than in those without methylation (p=0.0318). Thus, the combination of genetic and epigenetic classification has potential as a good prognostic marker among CRC patients. One possible reason for the lack of prognostic significance of epigenetic and genetic parameters among patients with mutations is that the genetic alterations may predominate in carcinogenesis of CRC; therefore, it is reasonable that the overall survival of wild-type patients is significantly shorter in when methylation occurs compared to no methylation; that is, methylation may play a central role in carcinogenesis of wild-type CRC. Thus, the combination

of genetic and epigenetic alterations may be used as a good marker for prognosis in CRC patients.

In conclusion, we found that genetic alteration by itself was not significantly associated with prognosis; however, the combination of genetic alteration and epigenetic alteration may be a good marker for the prognosis of CRC.

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