SPANDIDOS PUBLICATIONS

Mutational analysis of the *BRAF* gene in colorectal mucinous carcinoma in association with histological configuration

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Abstract. Genetic alterations and their association with clinicopathological features in colorectal mucinous carcinoma (MC) remain unknown. In particular, little is known about the mutational status of the *BRAF* gene, which is activated by oncogenic *Ras*. This study aimed to evaluate the status of *BRAF* together with K-*ras*, p53 and mismatch-repair deficiency to clarify their association with tumorigenesis of colorectal MC. *BRAF* and K-*ras* mutations were determined in 43 colorectal MCs by direct sequencing. p53 alteration was investigated immunohistochemically. The status of mismatch-repair deficiency was assessed by microsatellite analyses and immunohistochemistry for hMLH1. We also examined the association between these molecular alterations and clinicopathological features including histological configuration. *BRAF* mutation was detected in 4 (9.3%) tumors and was located at codon 599 of exon 15 in all cases. K-*ras* mutation was detected in 13 tumors (30.2%). No *BRAF* and K-*ras* mutations were identified simultaneously in the same tumor. The incidence of mismatch-repair deficiency tended to be higher in MC with *BRAF* mutation than without. In terms of histological configuration, we classified the cases according to growth type by tumor edge morphology. All MCs with *BRAF* mutation and 9 of 13 MCs (69.2%) with K-*ras* mutation were classified as polypoid type. *BRAF* and K-*ras* mutation did not affect patient prognosis, but non polypoid type was significantly more aggressive than polypoid type. Our findings indicate that *BRAF* mutation plays an important role in the tumorigenesis of colorectal MC and in tumor edge morphology, similar to K-*ras* mutation.

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Introduction

Colorectal mucinous carcinoma (MC), one of the subsets of colorectal adenocarcinomas, is diagnosed when extracellular mucin constituted more than 50% of the carcinoma volume, according to the World Health Organization (WHO) definition (1). MC represents 5-15% of colorectal carcinomas (2,3). Although the clinicopathologic significance of colorectal MC has been recognized for a long time, its biological features are still under investigation and discussion.

Colorectal carcinoma has a multi-step process characterized by a sequence of genetic alterations in cell growth regulatory genes (4). Mutational activation of the *RAS* gene, in particular the K-*ras* oncogene, is an early event and is considered to play a role in the progression of size and grade of atypia (5,6). *RAS* is part of the Ras/Raf/MEK/MAP kinase cascade, which is an essential component of intracellular signaling from activated cell surface receptors to transcription factors in the cell nucleus (7). Recently, activating mutation of *BRAF*, a member of the *RAF* gene family, has been found in malignant melanomas and in a wide range of human carcinomas. In colorectal carcinoma, although the incidence of *BRAF* mutation is reportedly approximately 10%, no study has focused on this mutation in MC (8). More recent data indicated that *BRAF* mutation is associated with a high frequency of microsatellite instability (MSI) and inactivation of the mismatch-repair (MMR) gene in colorectal carcinoma.

In the present study, we analyzed the status of *BRAF* together with K-*ras*, p53, hMLH1 and MSI to clarify their association with tumorigenesis in colorectal MC. Additionally, we examined the association between these molecular alterations and clinicopathological features.

Materials and methods

Patients and tissue samples. We obtained 43 samples of colorectal MC by surgical resection from 43 patients at Dokkyo University School of Medicine Hospital between 1988 and 2005. Patients with familial adenomatous polyposis, hereditary nonpolyposis colorectal cancer or inflammatory bowel disease were excluded from this study. The study was performed with the approval of Dokkyo University Surgical

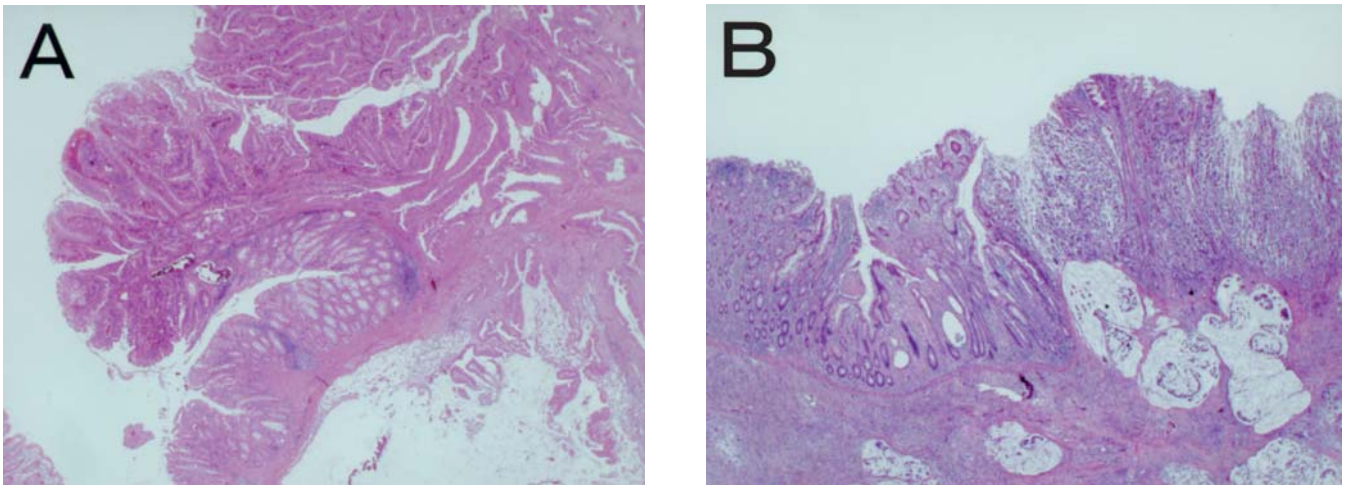

Figure 1. Tumor edge morphology of colorectal MC. (A) Polypoid-type tumor growing above the level of the normal mucosa at the tumor margin. (B) Nonpolypoid-type tumor does not grow above the level of the normal mucosa at the tumor margin (staining by hematoxylin and eosin, magnification x2).

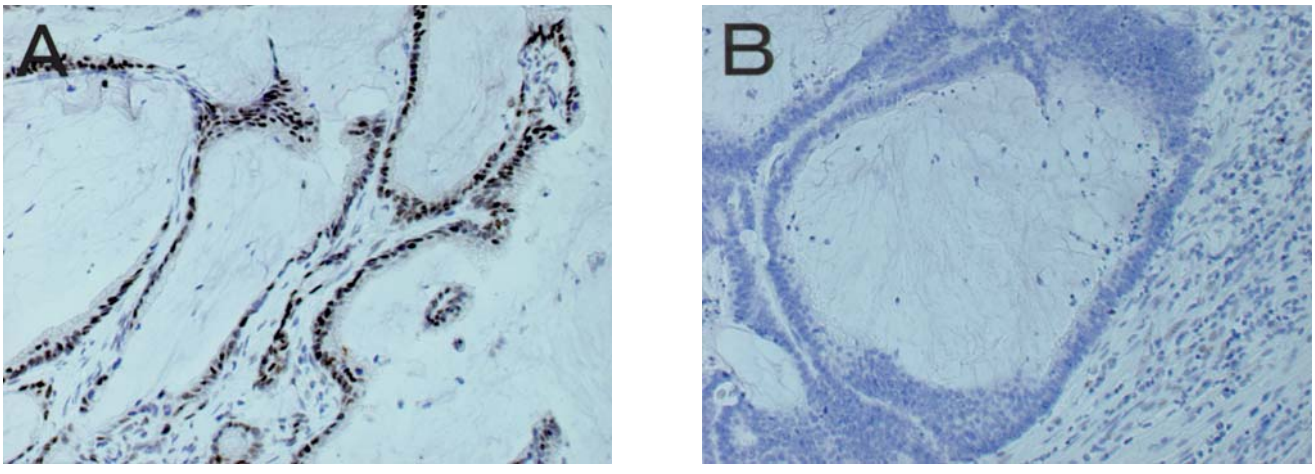

Figure 2. Immunohistochemical staining of hMLH1. (A) hMLH1-positive colorectal MC. Immunoreactivity of hMLH1 in nuclei of colorectal MC cells. (B) Loss of hMLH1. No immunoactivity of hMLH1 in nuclei of colorectal MC cells (magnification x12.5).

Pathology Committee, and informed consent was obtained from all patients.

Patient age and gender, and tumor location and stage, were ascertained from the pathology report. All cases were staged using Turnbull's modification of Dukes' classification (9). Classifications of growth type were assessed by tumor edge morphology and invasive margin morphology. The former was classified as polypoid type when the tumor edge was obviously elevated above the level of the normal mucosa at the tumor margin (Fig. 1A) and as nonpolypoid type when the tumor edge was at the level or below the normal mucosa (Fig. 1B), using the criteria of George *et al* (10). The latter was classified as expanding type when the advancing front of the tumor was clearly demarcated and pushed the surrounding tissue, and as infiltrating type when the cancer cells spread into the surrounding tissue without a distinct border, using the criteria of Jass *et al* (11). We also graded MC for the extent of differentiation according to the WHO classification; well-differentiated type was defined as glands lined by a columnar mucus-secreting epithelium together with intestinal mucin, and poorly differentiated type was defined as chains or irregular clusters of cells surrounded by mucus (1). The clinicopathologic features of the samples are summarized in Table I.

DNA extraction. Formalin-fixed, paraffin-embedded samples were cut serially at a thickness of 10 μm. Based on histopathological findings, the tumor and corresponding normal tissues were microdissected from each of five serial sections and the tissues were deparaffinized in xylene and rehydrated in a graded ethanol series. DNA was then extracted from the whole dissected tissue using a DNA Isolator PS Kit (Wako Pure Chemical, Osaka, Japan) according to the supplied protocol.

Screening of BRAF and K-ras mutation. The complete coding sequences of exon 11 (G loop region) and 15 (activation segment) of *BRAF* and exon 1 of K-*ras* were amplified using the polymerase chain reaction (PCR). The primer sequences were: for exon 11 of *BRAF*, forward 5'-GCT TGT CAC TTA TAA AGG AAA CTA-3', reverse 5'-TCC CTC TCA GGC ATA AGG TAA-3'; for exon 15 of *BRAF*, forward 5'-CCG GTT TTT AAA TTA GTC ACC T-3', reverse 5'-TCA TAA TGC TTG CTC TGA TAG GA-3'; and for exon 1 of K-*ras,* forward 5'-ACT GAA TAT AAA CTT GTG GTA G-3', reverse 5'-AGT TTC TTA CCA GGA CGT-3'. Direct sequencing of the amplified DNA was performed as follows. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Tokyo, Japan), then sequenced on an ABI PRISM

Clinicopathological features of the patients with carcinoma in the colorectum.

Gender	
Man	30 (69.8%)
Woman	13 (30.2%)
Age (year, mean ± SD)	64.2±13.6 (29-90)
Tumor location	
Proximal	24 (55.8%)
Distal	19 (44.2%)
Dukes' stage	
A/B	10 (23.3%)
C/D	33 (76.7%)
Differentiation	
Well/moderate	31 (72.1%)
Poor/signet	12 (27.9%)
Tumor edge morphology	
Polypoid	20 (46.5%)
Nonpolypoid	18 (41.9%)
Unclassifiable	5 (11.6%)
Invasive margin morphology	
Expanding	21 (48.8%)
Infiltrating	19 (44.2%)
Mixed	3 (7.0%)

Proximal, from cecum to transverse colon; distal, from splenic flexure to rectum.

3700 DNA Analyzer (Applied Biosystems, Foster City, CA) using an ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). The same primers were used for both amplification and sequencing. The resulting sequencing data were analyzed using Gene Scan Analysis Software (Applied Biosystems) in accordance with the manufacturer's protocol. All sequences were verified in both the forward and reverse directions.

Analysis of MSI. MSI was evaluated at five microsatellite markers (D5S346, BAT25, BAT26, D2S123 and D17S250) recommended by the National Cancer Institute panel (12). PCR was performed using fluorescent labeled multiplex primers. The amplified PCR products were analyzed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) with Gene Scan Analysis Software provided by the manufacturer. If more than one microsatellite marker of tumor tissue exhibited differences from the patient's matched normal tissue, the tumor was classified as an MSI tumor.

Immunohistochemical analysis of hMLH1 and p53. Immunohistochemical staining for p53 or hMLH1 protein was carried out with an anti-human p53 antibody (NCL-p53-CM1; Novocastra Laboratories, Newcastle, UK; diluted 1:2000) or with an anti-human hMLH1 antibody (BD PharMingen, San Diego, CA; diluted 1:30) on formalin-fixed, paraffin-embedded tissue sections using a Labeled Streptavidin-Biotin Kit (Dako Japan, Kyoto, Japan). For p53, positive controls and negative controls were used for each set of experiments. Sections of colorectal adenocarcinomas that had been confirmed to overexpress this protein were used as positive controls, and the antibody was not applied to negative controls. Immunoreactivity was considered positive if at least focal nuclear accumulation of p53 protein was detected. For hMLH1, negative tumor was determined when tumor cells showed no nuclear staining but the surrounding normal epithelial cells showed positive-nuclear staining (Fig. 2).

Table II. Relationship between *BRAF* and K-*ras* mutation.

	K-*ras* mutation	
	Positive	Negative
BRAF mutation		
Positive	0	4
Negative	13	26

Statistics. The Chi-square test (Fisher's exact test when the expected number of any cell was ≤5 cases) was performed to determine correlations among the various parameters. Cumulative survival rate was assessed by the Kaplan-Meier method and compared by log-rank test. Multivariate analysis was performed using the Cox proportional hazards model with hazard ratios (HR) and 95% confidence intervals (CI) to evaluate independent prognostic factors. Survival was defined as the time from the date of surgery for colorectal MC to the date of death. Others deaths unrelated to colorectal MC were excluded for the purpose of survival analysis. Differences at $P<0.05$ were considered to be statistically significant.

Results

BRAF and K-ras mutation. *BRAF* mutation was detected in 4 (9.3%) tumors, and in all cases was located at the hot spot codon 599 of exon 15. All *BRAF* mutations were CTG→GAG (V599E). K-*ras* mutation was detected in 13 (30.2%) tumors, including 10 (76.9%) at codon 12 and 3 (23.1%) at codon 13. The most frequent mutation at codon 12, GGT→GAT (G12D), was detected in 8 cases (80%). All mutations at codon 13 were GGC→GAC (G13D). Overall, 17 of 43 MCs (39.5%) harbored mutations in either *BRAF* or K-*ras*. No *BRAF* and K-*ras* mutations were identified simultaneously in the same tumor (Table II).

Relationship of BRAF and K-ras mutation with clinicopathological features. The relationship of *BRAF* and K-*ras* mutation with clinicopathological features is shown in Table III. We failed to detect any significant relationship of *BRAF* and K-*ras* mutation with age, gender, tumor location, Dukes' stage or differentiation. With regard to classification by tumor edge morphology, 20 of 43 MCs (46.5%) were classified as polypoid type and 18 (41.9%) were classified as nonpolypoid type. The remaining five were unclassifiable because one edge showed polypoid type and the other nonpolypoid type. All of 4 MCs with *BRAF* mutation and 9 of 13 MCs (69.2%) with K-*ras* mutation were classified as polypoid type. MCs with *BRAF*

Table III. Relationship of *BRAF* and K-*ras* mutation with clinicopathological features.

	BRAF (+) (n=4) (%)	K-*ras* (+) (n=13) (%)	*BRAF* (+) or K-*ras* (+) (n=17) (%)	*BRAF* (-) and K-*ras* (-) (n=26) (%)
Age (year, mean ± SD)	66.0±13.5	67.2±14.7	66.9±14.0	61.8±13.3
Gender				
Man	4 (100)	7 (53.8)	11 (64.7)	19 (73.1)
Woman	0	6 (46.2)	6 (35.3)	7 (26.9)
Tumor location				
Proximal	3 (75.0)	7 (53.8)	10 (58.8)	14 (53.9)
Distal	1 (25.0)	6 (46.2)	7 (41.2)	12 (46.1)
Dukes' stage				
A/B	2 (50.0)	4 (30.8)	6 (35.3)	5 (19.2)
C/D	2 (50.0)	9 (69.2)	11 (64.7)	21 (80.8)
Differentiation				
Well/moderate	4 (100)	10 (76.9)	14 (82.4)	16 (61.5)
Poor/signet	0	3 (23.1)	3 (17.6)	10 (38.5)
Tumor edge morphology				
Polypoid	4 (100)	9 (69.2)	13 (76.5)	8 (30.8)
Nonpolypoid	0	3 (23.1)	3 (17.6)	14 (53.8)
Unclassifiable	0	1 (7.7)	1 (5.9)	4 (15.4)
Invasive margin morphology				
Expanding	3 (75.0)	6 (46.2)	9 (52.9)	12 (46.1)
Infiltrating	1 (25.0)	6 (46.2)	7 (41.2)	12 (46.1)
Mixed	0	1 (7.6)	1 (5.9)	2 (7.7)

BRAF (+), *BRAF* mutation positive; K-*ras* (+), K-*ras* mutation positive.

Table IV. Relationship of *BRAF* and K-*ras* mutation with MSI and accumulation of p53 and hMLHI.

	BRAF (+) (n=4) (%)	K-*ras* (+) (n=13) (%)	*BRAF* (+) or K-*ras* (+) (n=17) (%)	*BRAF* (-) and K-*ras* (-) (n=26) (%)
MSI status				
Instability	2 (50.0)	1 (7.7)	3 (17.6)	2 (7.7)
Stability	2 (50.0)	12 (92.3)	14 (82.4)	24 (92.3)
p53 status				
Positive	1 (25.0)	4 (30.8)	5 (29.4)	10 (38.5)
Negative	3 (75.0)	9 (69.2)	12 (70.6)	16 (61.5)
hMLHI status				
Positive	2 (50.0)	12 (92.3)	14 (82.4)	24 (92.3)
Negative	2 (50.0)	1 (7.7)	3 (17.6)	2 (7.7)

BRAF (+), *BRAF* mutation positive; K-*ras* (+), K-*ras* mutation positive.

mutation showed polypoid type more frequently than those without *BRAF* or K-*ras* mutation (100% vs. 30.8%, P=0.033). Similarly, although the difference was not statistically significant, MCs with K-*ras* mutation showed polypoid type more frequently than those without *BRAF* or K-*ras* mutation (69.2% vs. 30.8%, P=0.071). On the other hand, we failed to detect any significant relationship of these mutations with invasive margin morphology.

Relationship of BRAF and K-ras mutation with MSI status, expression of p53 and hMLH1. The relationship of *BRAF* and K-*ras* mutation with MSI, p53 and hMLH1 status is shown in

 MSI and loss of hMLH1 were detected in 5 of 43 (11.6%). The incidences of MSI and loss of hMLH1 occurred in 2 of 4 MCs (50.0%) with *BRAF* mutation, compared with 1 of 13 (7.7%) with K-*ras* mutation, and 2 of 26 (7.7%) without *BRAF* or K-*ras* mutation. The incidences of MSI and loss of hMLH1 tended to be higher in *BRAF* mutated MC than in non-*BRAF* mutated MC (50% vs. 8.3%, P=0.060). Expression of p53 was detected in 15 of 43 MCs (34.9%). There was no significant relationship of *BRAF* and K-*ras* mutation with p53 expression.

Survival analysis. Log-rank statistics showed that *BRAF* and K-*ras* mutation was not associated with patient prognosis, whereas tumor edge morphology and invasive margin morphology were found to affect patient prognosis. In terms of tumor edge morphology, nonpolypoid type had a closer association with poor patient prognosis than polyoid type (P=0.016). In terms of invasive margin morphology, infiltrating type had a closer association with poor patient prognosis than expanding type (P=0.006).

Multivariate analysis using Cox regression and correcting for gender and age at surgery, Dukes' stage (A/B or C/D) and differentiation (well/moderate or poor/signet) showed that tumor edge morphology and invasive margin morphology were independent prognostic factors [HR 3.3 (95% CI 1.2-9.3); P=0.028 and HR 6.4 (95% CI 1.8-22.7); P=0.026, respectively]. Dukes' stage and differentiation were also shown to significantly affect patient prognosis [HR 4.1 (95% CI 1.1-16.1); P=0.014 and HR 4.4 (95% CI 1.6-12.3); P=0.005, respectively].

Discussion

In this study, we examined the incidence of *BRAF* mutation in colorectal MC, and clarified the relationship of *BRAF* mutation with other genetic alterations and clinicopathological features. *BRAF* mutation was observed in 9.3% of colorectal MCs and in all cases was located at the hot spot codon 599 of exon 15. *BRAF* mutation in all these cases was CTG→GAG, resulting in the substitution of valine (V) by glutamic acid (E). The incidence of *BRAF* mutation was similar to that reported previously in colorectal carcinoma. K-*ras* mutation was observed in 30.2% of colorectal MCs, being at codon 12 in 76.9% and codon 13 in 23.1%. These results suggest that *BRAF* mutation would play an important role in the tumorigenesis of colorectal MC, similar to K-*ras* mutation.

Both *BRAF* and K-*ras* are members of the Ras/Raf/MEK/MAP kinase cascade, which transduces various growth signals from the cell surface to the nucleus. As in previous studies, we found no colorectal MC that concurrently contained both *BRAF* mutation at codon 599 of exon 15 and K-*ras* mutation (13,14). The combined incidence of *BRAF* and K-*ras* mutation was approximately 40% in colorectal MC, suggesting that activation at various points of the Ras/Raf/MEK/MAP kinase cascade would constitute a key event in the tumorigenesis of colorectal MC.

Approximately 15% of sporadic colorectal carcinomas show MSI, and the majority of those with MSI demonstrate inactivation of the MMR gene due to hypermethylation of the *hMLH1* gene (15-17). Recent data have shown that *BRAF* mutation is associated with a high frequency of MSI and inactivation of the MMR gene in colorectal carcinoma (14). Lubomierski *et al* reported that the incidence of *BRAF* mutation was approximately five times (27% vs. 5%) higher in MSI tumors than in non-MSI tumors (18). In this study, although the number of MCs examined was not large enough to allow definite statistical correlations, the incidences of MSI and loss of hMLH1 tended to be higher in *BRAF* mutated MC than in non-*BRAF* mutated MC (50% vs. 8.3%, P=0.060). These results were nearly consistent with those of previous studies of colorectal carcinoma (14,19).

In histological configuration of colorectal carcinomas, there are two distinct subtypes: protrusive and flat carcinomas. These may represent different pathways of colorectal tumorigenesis. Shimoda *et al* classified the growth of early colorectal carcinoma into two types, polypoid and nonpolypoid, on the basis of tumor edge morphology, and suggested that polypoid carcinomas would develop from adenomatous polyps whereas nonpolypoid carcinomas would develop from flat polyps or *de novo* in nonadenomatous mucosa (20). In addition, a number of mutational data have supported the contention that polypoid and nonpolypoid tumors are separate entities, because the incidences of K-*ras* and *APC* gene mutation in nonpolypoid tumors is lower than that in polypoid tumors (21-25). In advanced colorectal carcinoma, George *et al* likewise classified growth type and reported that K-*ras* mutation was more frequent in polypoid carcinomas than in nonpolypoid carcinomas, and that the latter were significantly more aggressive than the former (10). In this study, we focused on the relationship between histological configuration and several genetic alterations including *BRAF* mutation in colorectal MC, using this morphological classification.

We found that 46.5% of the classifiable carcinomas were polypoid and that 41.9% were nonpolypoid roughly, thus showing roughly the same incidence. All of 4 MCs with *BRAF* mutation and 9 of 13 MCs with K-*ras* mutation were classified as polypoid type. The incidence of polypoid MC with *BRAF* mutation was higher than that without *BRAF* or K-*ras* mutation (100% vs. 30.8%, P=0.033), and the incidence of polypoid MC with K-*ras* mutation also tended to be higher than that without *BRAF* or K-*ras* mutation (69.2% vs. 30.8%, P=0.071). Similar to K-*ras* mutation, *BRAF* mutation was also associated with polypoid carcinomas. This result suggests that activation of the MAP kinase cascade by *BRAF* or K-*ras* mutation would be associated with polypoid carcinomas, and that polypoid and nonpolypoid MC may develop through distinct pathways. On the other hand, differentiation according to the WHO definition and invasive margin morphology, which was one of the prognostic factors classified by Jass *et al* (11), were not associated with *BRAF* or K-*ras* mutation. Thus, it is conceivable that, in colorectal MC, *BRAF* and K-*ras* mutation may play an important role in tumor edge morphology, but not in differentiation and tumor invasion morphology.

Many studies have reported that genetic alterations affect the prognosis of patients with carcinoma. It has been reported that K-*ras* mutation seems to be associated with poor prognosis in colorectal carcinoma (26-28). However, conflicting results have also been reported (29-31). In addition no studies have investigated the association between *BRAF* mutation and the prognosis of patients with colorectal carcinoma. In this study,

we investigated whether activation of the MAP kinase cascade by *BRAF* and K-*ras* mutation influences the prognosis of patients with colorectal MC. We also investigated the association between growth pattern and prognosis. Our data showed that activation of the MAP kinase cascade did not affect the prognosis of patients with colorectal MC, whereas tumor edge morphology and invasive margin morphology have an effect. As reported previously, nonpolypoid and infiltrating types were significantly more aggressive than polypoid and expanding types in colorectal carcinoma. These data suggest that tumor morphology rather than genetic alterations would predict poor prognosis in patients with colorectal MC. In this study, however, due to the small sample size, it was not possible to determine the actual prognostic value of *BRAF* or K-*ras* in colorectal MC. Further studies using a large number of cases will be necessary to clarify the effect of these mutations on the prognosis of patients with colorectal MC.

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