Clinical stage and risk of recurrence and mortality: interaction of DNA methylation factors in patients with colorectal cancer

Hsien-Feng Chang,[1] Chang-Chieh Wu,[2] Chien-An Sun,[3] Chi-Ming Chu,[1] Fu-Gong Lin,[1] Jih-Fu Hsieh,[1] Chih-Hsiung Hsu,[4] Chi-Hua Huang,[1] Tsan Yang,[5] Yang-Ming Tsai,[1] Jen-Chun Kuan,[6] Yu-Ching Chou[1]

[1]School of Public Health, National Defense Medical Center, Taipei, Taiwan
[2]Department of Surgery, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan
[3]Department of Public Health, College of Medicine, Fu-Jen Catholic University, New Taipei City, Taiwan
[4]Graduate Institute of Medical Sciences, National Defense Medical Center, Taipei, Taiwan
[5]Department of Health Business Administration, Meiho University, Pingtung, Taiwan
[6]Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan

Correspondence to
Dr Yu-Ching Chou, School of Public Health, National Defense Medical Center, No.161 Sec. 6, Minquan E. Rd., Neihu Dist, Taipei 11490, Taiwan; trishow@mail.ndmctsgh.edu.tw

J-CK and Y-CC contributed equally to this work.

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ABSTRACT

Aberrant DNA methylation plays a crucial role in cancer development; however, prospective evidence of an interaction between molecular biomarkers and cancer staging for predicting the prognosis of colorectal cancer (CRC) is still limited. We examined DNA methylation in tumors and adjacent normal tissues from patients who underwent CRC surgical resection, and evaluated the interaction between cancer staging (advanced vs local) and DNA methylation to predict the prognosis of CRC. We recruited 132 patients with CRC from Tri-Service General Hospital in Taiwan and used the candidate gene approach to select 3 tumor suppressor genes involved in carcinogenesis pathways. ORs and 95% CIs were computed using logistic regression analyses while adjusting for potential covariates. Advanced cancer stage was correlated with cancer recurrence (OR 7.22, 95% CI 2.82 to 18.45; p<0.001). In addition, after stratification by promoter methylation in 3 combined genes in the matched normal tissues, we observed a joint effect after adjusting for sex, age at surgery, and adjuvant chemotherapy, yielding a significant OR of 20.35 (95% CI 4.16 to 99.57; p<0.001). DNA methylation status would significantly increase the recurrence risk of CRC with a significant impact on joint effect between DNA methylation and clinical stage, particularly in matched normal tissues. This was attributed to molecular changes that could not be examined on the basis of clinical pathology. Our interaction results may serve as a reference marker for evaluating the risk of recurrence in future studies.

Significance of this study

What is already known about this subject?

- DNA methylation is one of the most well-defined epigenetic changes, providing a new generation of cancer biomarkers.
- Although tumor-node-metastasis classification remains the most powerful factor for predicting cancer prognosis, the interaction between molecular factors and staging for predicting recurrence and mortality in patients with colorectal cancer (CRC) remains unclear.
- The 5-year survival rates of CRC are from 91.0% to 80.0% in histological stages I and II to ~61.7–23.2% in histological stages III and IV.

What are the new findings?

- We examined the interaction between the DNA methylation status of tumor suppressor genes and clinical stage in patients who underwent surgical resection of CRC.
- DNA methylation status significantly increased the recurrence risk of CRC with a significant effect on the interaction between DNA methylation and clinical stage, particularly in matched normal tissues.
- The possible reason is molecular changes that could not be examined on the basis of clinical pathology.

How might these results change the focus of research or clinical practice?

- The findings provide new insights into the interaction between DNA methylation in CRC and different clinical cancer staging and demonstrate the interaction occurring in adjacent normal tissues.

INTRODUCTION

Colorectal cancer (CRC), a common cause of death, is increasingly recognized as a critical public health concern. An annual incidence of 132 700 new cases and mortality of 49 700 deaths were reported in the USA for 2015, with 134 490 new cases and 49 190 deaths projected for 2016.[1]

The prognosis of CRC is poor in advanced stages.[2] The 5-year survival rate of CRC varies across studies, ranging from 91.0% to 80.0% in histological stages I and II to ~61.7–23.2% in histological stages III and IV.[3 4] Moreover, 42% of patients develop local recurrence or distant metastasis in stages II and III.[5]

Studies have indicated that chronic inflammation contributes to cancer[6] because oxidative stress increases cancer risk, and different tumor

development stages are based on the presence of leukocytes in the neoplastic tissue.[7] The mediators of inflammatory responses release cytokines, oxyradicals, chemokines, and growth factors that might cause DNA damage, leading to point mutations or methylation in tumor suppressor genes. Cellular protein expression is involved in mismatch repair (MMR), apoptosis, and cell cycle.[8 9]

DNA methylation is one of the most well-defined epigenetic changes, providing a new generation of cancer biomarkers.[10–14] The DNA methylation status has been associated with CRC in numerous studies.[15–17] Loss of MMR and cell cycle control function are the major causes of tumor progression in CRC, which is related to DNA methylation at gene promoter regions located at the transcription start site.[18] Previous studies have identified that DNA methylation-related genes are correlated with carcinogenesis pathways by gene silencing, including cell cycle control encode cyclin-dependent kinase inhibitor 2A (*CDKN2A*), MMR system O-6-methylguanine DNA methyltransferase (*MGMT*), and human mutL homolog 1 (*hMLH1*).[19 20] However, the interaction effect between different stages and DNA methylation remains unclear in patients with CRC.

The prognosis of CRC, which differs in each histological stage, may be responsible for cancer-specific gene promoter region methylation. We selected three genes on the basis of two carcinogenesis pathways and loss of function in MMR and cell cycle control, both of which are related to DNA hypermethylation in transcription factor binding sites.[21] We examined DNA methylation status in tumors and adjacent normal tissues (matched normal) from patients who underwent surgical resection of CRC. We hypothesized that, in specific tumor suppressor genes, DNA methylation enhances the association between histological stage and CRC prognosis. Our results provide new insights into how DNA methylation in CRC interacts with different histological stages and demonstrate the interaction occurring in matched normal tissues.

MATERIALS AND METHODS

Participants' characteristics

We recruited all patients who received a diagnosis of invasive CRC between 2006 and 2010 and were eligible for surgical resection. We obtained informed consent from all patients and followed them to evaluate recurrence and survival. This study was approved by the Tri-Service General Hospital (TSGH) Institutional Review Board at National Defense Medical Center (TSGHIRB approval number: 098-05-292). The method of follow-up was based on medical records linked to data in a cancer registration database containing patients' information on the cause of death. According to the clinical practice guidelines of the Division of Colon and Rectum of TSGH, patients undergoing surgical resection should return for a checkup once every 3 months after surgery (in the first year) and once every 3–6 months thereafter. Therefore, we identified patients with cancer recurrence by reviewing their medical records from subsequent checkups, and recurrence was defined as local recurrence or metastasis. In contrast, the patients without recurrence were followed until their most recent checkup as the study end point. Patient characteristics (sex, age at surgery, stage, recurrence, all-cause mortality, lymphovascular invasion, histological grade, and tumor location) were obtained from their medical records. On the basis of the inclusion criteria, specimen pairs were obtained from 132 patients with CRC (264 samples). The mean follow-up period was 25.2 months, and the last patients were followed up for 8.8 months since 2012. A colon tissue sample was collected in the operating room while simultaneously resecting tumor and normal tissues; the normal tissues were taken from an incision at least 10 cm from the tumor sites. The obtained surgical specimens were immediately frozen in liquid nitrogen and stored at −80°C for further experiments. The surgical procedure was reviewed by the Colon and Rectal Surgery Division, TSGH.

DNA purification and bisulfite conversion

Genomic DNA was isolated from the colon and rectal tissue samples by cellulose-coated magnetic beads, using the MagCore Compact Automated Nucleic Acid Extractor (Cat. No.: MCA0801; RBC Bioscience, Taipei, Taiwan) and the Genomic DNA Tissue Kit according to the manufacturer's protocol. The isolated DNA was treated using sodium bisulfite conversion using the EZ DNA Methylation Kit (Zymo Research Corporation, Orange, California, USA). A positive control of methylated DNA was generated by using SssI methylase (Zymo Research Corporation, Orange, California, USA).

Methylation-specific PCR

We used the candidate gene approach to select tumor suppressor genes (*CDKN2A*, *hMLH1*, and *MGMT*) involved in the pathways correlated to cancer stages and prognosis such as the cell cycle and MMR, which are correlated with cancer stages and prognosis. Bisulfite-converted DNA was subjected to a methylation-specific PCR (MS-PCR) by using primer pairs designed to specifically amplify the promoter regions. The reaction solution (25 μL) contained HotStart Taq Premix (12.5 μL, RBC Bioscience, Taipei, Taiwan), 1.2 μL aliquots of forward and reverse primers, and bisulfite-converted DNA.

For the MS-PCR, we used the following oligonucleotide primers for *CDKN2A*: 5′-TTATTAGAGGGTGGGGCGGATCGC-3′ (forward primer) and 5′-GACCCCGAACCGCGACCGTAA-3′ (reverse primer) to amplify the methylated sequence (PCR product size: 150 bp), and 5′-TTATTAGAGGGTGGGGTGGATTGT-3′ (forward primer) and 5′-CAACCCCAAACCACAACCATAA-3′ (reverse primer) to amplify the unmethylated sequence (PCR product size: 151 bp); *hMLH1*: 5′-ACGTAGACGTTTTATTAGGGTCGC-3′ (forward primer) and 5′-CCTCATCGTAACTACCCGCG-3′ (reverse primer) to amplify the methylated sequence (PCR product size: 118 bp), and 5′-TTTTGATGTAGATGTTTTATTAGGGTTGT-3′ (forward primer) and 5′-ACCACCTCATCATAACTACCCACA-3′ (reverse primer) to amplify the unmethylated sequence (PCR product size: 124 bp); and *MGMT*: 5′-TTTCGACGTTCGTAGGTTTTCGC-3′ (forward primer) and 5′-GCACTCTTCCGAAAACGAAACG-3′ (reverse primer) to amplify the methylated sequence (PCR product size: 81 bp), and 5′-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3′ (forward primer) and 5′-AACTCCACACTCTTCCAAAAACAAAACA-3′ (reverse primer) to amplify the unmethylated sequence (PCR

product size: 93 bp). PCR cycling conditions were as follows: 10 min at 95°C; 35 cycles of 30 s denaturation at 95°C; 30 s annealing at 62°C, 60°C, and 53°C; 30 s extension at 72°C; and a final extension of 4 min at 72°C. After the amplification, PCR products were mixed with a loading buffer, electrophoresed on 2% agarose gel by using 0.2 μL gel-stained dye for 25 min, and visualized using a ultraviolet transilluminator.

Statistical analysis

Baseline characteristics were compared for sex, age at surgery (continuous), cancer stage (I, II, III, and IV), recurrence, mortality status, adjuvant chemotherapy, lymphovascular invasion, histological grade, tumor location, and the methylation status of gene promoter regions in the candidate genes (*CDKN2A*, *hMLH1*, and *MGMT*). To examine the possible interaction among DNA methylation, clinical stage, CRC recurrence risk, and all-cause mortality, we separately evaluated the various stages and divided them into two subgroups (local and advanced stages) on the basis of the different pathological types of tissue: tumor and adjacent normal tissues (matched normal). Previous studies have reported that age, sex, and adjuvant chemotherapy are the most critical confounding variables in the statistical analysis of several cancers.[22–26] Therefore, we adjusted these confounding factors in multiple analyses. ORs and 95% CIs for the association between cancer stage (local and advanced) and prognosis, including cancer recurrence and all-cause mortality after surgical resection in patients with CRC, were computed using logistic regression models. For the interaction assessment, we assessed the modification effect of two factors by using two different stratification analyses. We initially assessed the interaction on the basis of the joint effect of target genes methylation status and clinical stages on the risk of CRC recurrence and all-cause mortality by comparing the binomial stage and expected joint effects of a risk factor and DNA methylation within the same reference group in terms of recurrence and mortality. In addition, we assessed the stratified effect by considering that two causes may operate independently to produce the effect. All statistical analyses were performed using IBM Statistical Package for the Social Sciences (SPSS) V.22 (IBM SPSS Statistics 22). All statistical tests were two-tailed, and values of $p<0.05$ were considered statistically significant.

RESULTS

During the study period, we obtained 132 tumor samples from patients with CRC as well as matched normal samples from the TSGH tumor bank. Among the study patients, 48.5% were men; their mean age was 66.1 years (SD 13.7 years). The patients were classified into four clinical subgroups: stage I (15.9%), stage II (37.1%), stage III (28.8%), and stage IV (18.2%). The prognoses in this study indicated that among all the patients, 31.1% had cancer recurrence or metastasis and 23.5% died during the study period. In addition, we classified the patient characteristics into three individual gene groups (*CDKN2A*, *hMLH1*, and *MGMT*) and combined the groups (≥1 genes) of gene promoter methylation status stratified by different variables (sex, age at surgery, stage, recurrence, survival, and other pathological information) on the basis of the tumor and matched normal tissues of the patients (table 1).

In the multivariable analysis, compared with local cancer stages (stages I and II, reference group), advanced cancer stages (stages III and IV) were more highly associated with cancer recurrence (OR 8.82; 95% CI 3.62 to 21.46; $p<0.001$). Moreover, compared with local cancer stages, the survival status of advanced cancer stages was borderline significantly associated with mortality (OR 2.14; 95% CI 0.94 to 4.87; $p=0.071$). After multivariable adjustment for sex, age, and adjuvant chemotherapy, the OR of recurrence in the advanced cancer stage group was identical to that in the crude analysis (OR 7.22; 95% CI 2.82 to 18.45; $p<0.001$), and recurrence was significantly associated with mortality (OR 2.71; 95% CI 1.09 to 6.75; $p=0.032$; table 2).

We further examined the interaction between CRC prognosis and the different cancer stages (local and advanced) in the methylation status of the three genes in the tumor and normal tissues (table 3). We observed that a significant joint effect increased the association of recurrence with the combined methylation status of ≥1 genes in advanced cancer stages (Me/advanced) with an OR of 30.00 (95% CI 6.53 to 137.88; $p<0.001$); the OR after adjusting for confounders was 20.35 (95% CI 4.16 to 99.57; $p<0.001$). The ORs of *CDKN2A*, *hMLH1*, and *MGMT* methylation in advanced stages were 34.40 (95% CI 3.51 to 336.99; $p=0.002$), 10.81 (95% CI, 0.83 to 140.40; $p=0.069$), and 13.90 (95% CI 2.03 to 95.21; $p=0.007$), respectively. In addition, we observed an interaction pattern between cancer stages and gene promoter methylation status in tumor tissues with an OR of 10.81 (95% CI 2.15 to 54.26; $p=0.001$; table 3). Compared with the interaction in the normal tissues, combined DNA methylation status of ≥1 genes and advanced stages showed a strong joint effect (figure 1A); however, a slight effect was observed in tumor tissues (figure 1B).

The stratified effect showed a higher OR of recurrence (OR 17.65; 95% CI 1.91 to 163.52; $p<0.011$) in the normal tissues with the combined methylation status of ≥1 genes in the Me/advanced group than in the Me/local reference group after adjusting for confounders (table 4). Thus, an association was observed between cancer stages and prognosis after stratification by methylation status in combined genes, particularly in the normal tissues.

We did not observe an association between gene promoter region methylation and different cancer stages for survival in patients with CRC because of limited all-cause mortality events with gene methylation in the normal tissues. In addition, we did not observe a significant association and interaction in the tumor tissues (table not shown).

DISCUSSION

DNA methylation is generally associated with the epigenetic silencing of gene functions located at the gene promoter;[27] however, methylation might be crucial in a significant proportion of genes.[28] Several studies have determined the cause and role of aberrant DNA methylation in colorectal carcinogenesis. In our study, we observed an increase in the frequency of gene promoter hypermethylation and the extent of carcinogenic colorectal tissue growth for the three genes under investigation. This finding is in accordance with a review article by Kim *et al*.[29]

Table 1 Characteristics and distribution of methylation status in patients with CRC (n=132)

		Methylation status							
		CDKN2A		MLH1		MGMT		≥1 of genes	
Variables	*Total*	Normal	Tumors	Normal	Tumors	Normal	Tumors	Normal	Tumors
Sex, n (%)									
Male	64 (48.5)	9 (14.1)	32 (50.0)	1 (1.6)	10 (15.6)	4 (6.3)	27 (42.2)	13 (20.3)	44 (68.8)
Female	68 (51.5)	13 (19.1)	23 (33.8)	3 (4.4)	11 (16.2)	5 (7.4)	32 (47.1)	20 (29.4)	43 (63.2)
Age at surgery									
Mean (SD)	66.1 (13.7)	63.1 (16.0)	63.2 (16.0)	66.3 (14.8)	59.5 (2.6)	69.1 (13.5)	60.1 (8.4)	66.2 (12.9)	62.9 (14.3)
<65, n (%)	59 (44.7)	10 (16.9)	24 (40.7)	4 (6.8)	8 (13.6)	7 (11.9)	28 (47.5)	18 (30.5)	40 (67.8)
≥65, n (%)	73 (55.3)	12 (16.4)	30 (41.1)	0 (0.0)	13 (17.8)	2 (2.7)	30 (41.1)	15 (20.5)	47 (64.4)
Stage, n (%)									
I	21 (15.9)	5 (23.8)	5 (23.8)	1 (4.8)	2 (9.5)	1 (4.8)	6 (28.6)	6 (28.6)	11 (52.4)
II	49 (37.1)	10 (20.4)	21 (42.9)	1 (2.0)	11 (22.4)	2 (4.1)	24 (49.0)	13 (26.5)	35 (71.4)
III	38 (28.8)	6 (15.8)	17 (44.7)	2 (5.3)	5 (13.2)	3 (7.9)	20 (52.6)	10 (26.3)	27 (71.1)
IV	24 (18.2)	3 (12.5)	13 (54.2)	1 (4.2)	3 (12.5)	3 (12.5)	9 (37.5)	6 (25.0)	15 (62.5)
Recurrence, n (%)									
No	91 (68.9)	15 (16.5)	34 (37.4)	3 (3.3)	16 (17.6)	4 (4.4)	42 (46.2)	21 (23.1)	59 (64.8)
Yes	41 (31.1)	9 (22.0)	22 (53.7)	2 (4.9)	5 (12.2)	5 (12.2)	17 (41.5)	14 (34.1)	29 (70.7)
All-cause mortality, n (%)									
No	101 (76.5)	44 (43.6)	12 (38.7)	4 (4.0)	17 (16.8)	8 (7.9)	48 (47.5)	29 (28.7)	69 (68.3)
Yes	31 (23.5)	9 (20.8)	22 (21.4)	1 (3.2)	4 (12.9)	1 (3.2)	11 (35.5)	6 (19.4)	19 (61.3)
Adjuvant chemotherapy, n (%)									
No	56 (42.4)	12 (21.4)	20 (35.7)	1 (1.8)	8 (14.3)	2 (3.6)	26 (46.4)	15 (26.8)	37 (66.1)
Yes	76 (57.6)	12 (15.8)	36 (47.4)	4 (5.3)	13 (17.1)	7 (9.8)	33 (43.4)	20 (26.3)	51 (67.1)
Lymphovascular invasion, n (%)*									
No	45 (34.1)	7 (15.6)	19 (42.2)	3 (6.7)	9 (20.0)	3 (7.1)	23 (51.1)	12 (26.7)	32 (71.1)
Yes	3 (2.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (33.3)	0 (0.0)	1 (33.3)
Histological grade, n (%)*									
Well	2 (1.5)	0 (0.0)	1 (50.0)	1 (50.0)	1 (50.0)	0 (0.0)	1 (50.0)	1 (50.0)	2 (100.0)
Moderately	92 (69.7)	18 (19.6)	40 (43.5)	4 (4.3)	14 (15.2)	8 (8.7)	37 (40.2)	26 (28.3)	59 (64.1)
Poor or undifferentiated	8 (6.1)	1 (12.5)	4 (50.0)	0 (0.0)	1 (12.5)	0 (0.0)	5 (62.5)	1 (12.5)	7 (87.5)
Tumor location, n (%)*									
Colon	91 (68.9)	15 (16.5)	38 (41.8)	5 (5.5)	15 (16.5)	7 (7.7)	38 (41.8)	24 (26.4)	59 (64.8)
Rectum	16 (12.1)	6 (37.5)	10 (44.9)	0 (0.0)	2 (12.5)	1 (6.3)	8 (50.0)	6 (37.5)	13 (81.3)

*The total number of patients with CRC does not correspond because of missing data.
CDKN2A, cyclin-dependent kinase inhibitor 2A; CRC, colorectal cancer; MGMT, O-6-methylguanine DNA methyltransferase; MLH1, mutL homolog 1.

Table 2 Gender and age at surgery adjusted ORs for prognosis in different cancer stages in patients with colorectal cancer

Prognosis	Number of participants	Number of cases (%)	Crude OR (95% CI)	Multivariable adjusted OR (95% CI)
Recurrence*				
Local (1 and 2)	70	8 (11.4)	1.00 (referent)	1.00 (referent)
Advanced (3 and 4)	62	33 (53.2)	8.82 (3.62 to 21.46)	7.22 (2.82 to 18.45)
All-cause mortality†				
Local (1 and 2)	70	12 (17.1)	1.00 (referent)	1.00 (referent)
Advanced (3 and 4)	62	19 (30.6)	2.14 (0.94 to 4.87)	2.71 (1.09 to 6.75)

Adjusted for gender, age at surgery (continuous), and adjuvant chemotherapy.
*Number of recurrence cases in different cancer stages.
†Number of all-cause mortality cases in different cancer stages.

The effect of these outcomes, potentially enhanced by molecular mechanisms, remains unclear. Although numerous factors, including genetic and environmental factors, have been proposed as independent predictors of CRC prognosis,[30 31] the clinical cancer stage and tumor-node-metastasis (TNM) classification remains the most powerful factor for predicting cancer prognosis.[32] However, a study indicated the need to stratify the clinical

Table 3 Joint effect between gene promoter region methylation and different cancer stages for cancer recurrence in patients with colorectal cancer

	Normal tissues				Tumor tissues			
Recurrence in different stages by methylation	**Number of participants**	**Number of cases (%)**	**Crude OR (95% CI)**	**Adjusted OR (95% CI)**	**Number of participants**	**Number of cases (%)**	**Crude OR (95% CI)**	**Adjusted OR (95% CI)**
CDKN2A								
UnMe/local (1 and 2)*	55	7 (12.7)	1.00 (referent)	1.00 (referent)	44	5 (11.4)	1.00 (referent)	1.00 (referent)
UnMe/advanced (3 and 4)†	53	25 (47.2)	6.12 (2.35 to 15.97)	5.40 (1.99 to 14.62)	32	14 (43.8)	6.07 (1.89 to 19.43)	4.95 (1.46 to 16.83)
Me/local (1 and 2)‡	15	1 (6.7)	0.49 (0.06 to 4.33)	0.50 (0.06 to 4.47)	26	3 (11.5)	1.02 (0.22 to 4.66)	1.09 (0.23 to 5.23)
Me/advanced (3 and 4)§	9	8 (88.9)	54.86 (5.93 to 507.60)	34.40 (3.51 to 336.99)	30	19 (63.3)	13.47 (4.10 to 44.30)	12.21 (3.38 to 44.11)
p Value¶			<0.001	0.002			<0.001	<0.001
MLH1								
UnMe/local (1 and 2)*	68	8 (11.8)	1.00 (referent)	1.00 (referent)	57	7 (12.3)	1.00 (referent)	1.00 (referent)
UnMe/advanced (3 and 4)†	59	31 (52.5)	8.30 (3.38 to 20.37)	6.74 (2.61 to 17.38)	54	29 (53.7)	8.29 (3.19 to 21.53)	6.81 (2.49 to 18.65)
Me/local (1 and 2)‡	2	0 (0.0)	NA**	NA**	13	1 (7.7)	0.60 (0.07 to 5.31)	0.62 (0.07 to 5.72)
Me/advanced (3 and 4)§	3	2 (66.7)	15.00 (1.22 to 184.81)	10.81 (0.83 to 140.40)	8	4 (50.0)	7.14 (1.45 to 35.23)	5.85 (1.12 to 30.47)
p Value¶			0.035	0.069			0.016	0.036
MGMT								
UnMe/local (1 and 2)*	67	7 (10.4)	1.00 (referent)	1.00 (referent)	40	4 (10.0)	1.00 (referent)	1.00 (referent)
UnMe/advanced (3 and 4)†	56	29 (51.8)	9.21 (3.59 to 23.62)	7.41 (2.75 to 19.97)	33	20 (60.6)	13.85 (3.98 to 48.18)	9.64 (2.61 to 35.62)
Me/local (1 and 2)‡	3	1 (33.3)	4.29 (0.34 to 53.53)	2.62 (0.20 to 34.19)	30	4 (13.3)	1.38 (0.32 to 6.05)	1.15 (0.26 to 5.17)
Me/advanced (3 and 4)§	6	4 (66.7)	17.14 (2.64 to 111.14)	13.90 (2.03 to 95.21)	29	13 (44.8)	7.31 (2.06 to 25.93)	6.12 (1.66 to 22.58)
p Value¶			0.003	0.007			0.002	0.007
≥1 of genes								
UnMe/local (1 and 2)*	51	6 (11.8)	1.00 (referent)	1.00 (referent)	24	2 (8.3)	1.00 (referent)	1.00 (referent)
UnMe/advanced (3 and 4)†	47	21 (44.7)	6.06 (2.17 to 16.93)	5.15 (1.77 to 14.99)	20	10 (50.0)	11.00 (2.03 to 59.75)	7.51 (1.31 to 43.24)
Me/local (1 and 2)‡	19	2 (10.5)	0.88 (0.16 to 4.80)	0.77 (0.14 to 4.32)	46	6 (13.0)	1.65 (0.31 to 8.88)	1.50 (0.27 to 8.23)
Me/advanced (3 and 4)§	15	12 (80.0)	30.00 (6.53 to 137.88)	20.35 (4.16 to 99.57)	42	23 (54.8)	13.32 (2.77 to 64.00)	10.81 (2.15 to 54.26)
p Value¶			<0.001	<0.001			0.001	0.004

*UnMe/local (1 and 2): gene promoter region unmethylated with cancer stage 1 or 2.
†UnMe/advanced (3 and 4): gene promoter region unmethylated with cancer stage 3 or 4.
‡Me/local (1 and 2): gene promoter region methylated with cancer stage 1 or 2.
§Me/advanced (3 and 4): gene promoter region methylated with cancer stage 3 or 4.
¶p for the joint effect interaction.
**NA due to limited numbers of cases.
CDKN2A, cyclin-dependent kinase inhibitor 2A; MGMT, O-6-methylguanine DNA methyltransferase; MLH1, mutL homolog 1; NA, not available.

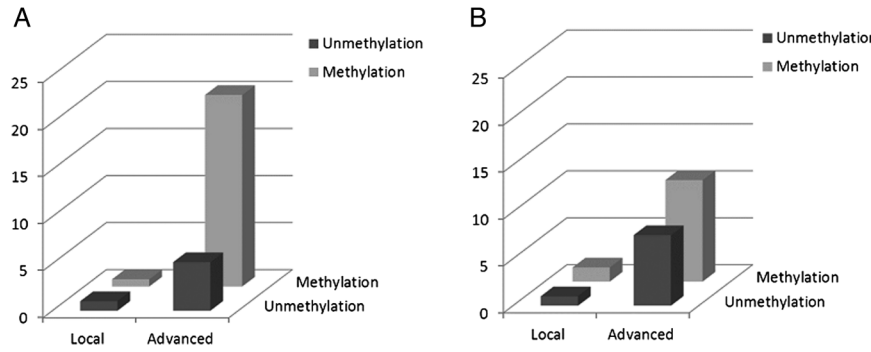

Figure 1 Bar chart of association between a dichotomous cancer stage ('local' for stages I and II vs 'advanced' for stages III and IV) and at least one gene promoter region methylation status ('methylation' vs 'unmethylation') of three genes (*CDKN2A*, *hMLH1*, and *MGMT*) resulted in cancer recurrence. (A) In matched normal tissues, in three categories of effect compared with a referent (local stage with unmethylation, UnMe/local), the OR was much greater in joint effect for individuals who were diagnosed advanced cancer stage and with gene promoter region methylation (advanced stage with methylation, Me/advanced), than independent effect for individuals who were either classified in advanced cancer stage or gene promoter region methylation. (B) The joint effect of cancer stage and gene promoter region methylation was slightly greater than the independent effects compared with a referent. CDKN2A, cyclin-dependent kinase inhibitor 2A; MGMT, O-6-methylguanine DNA methyltransferase; hMLH1, human mutL homolog 1.

classification system for patients in the same tumor stage and having the same molecular factors,[33] which is consistent with our study of using DNA methylation patterns in tumor suppressor genes to perform further stratification. According to the TNM Classification of Malignant Tumors published in affiliation with the American Joint Committee on Cancer, cancer development is based on the anatomical information and is categorized into tumor size, regional lymph node status, and distant metastases.[34] Therefore, we defined stages I and II as local stages and stages III and IV as advanced stages for further analysis.

Aberrant DNA methylation accounts for the histological heterogeneity and pathological diversity of cancers. The mechanism of DNA methylation in the gene promoter region has been widely discussed in previous studies. The biological interaction may be based on interleukin-6, which is an inflammatory cytokine that increases the DNA hypermethylation of the tumor suppressor gene *p53* families but decreases the DNA methylation of the growth factor receptors in epithelial cells.[35 36] In the complex concept of chronic inflammation caused by inflammation-mediated cytokine damage, their products may provide a mechanistic link between chronic inflammation and cancer development[9] in which damaged products may promote aberrant DNA methylation in human cancers. These chronic inflammation-related mediators, such as tumor-associate macrophages, cytokines, chemokines, and growth factors, induce epigenetic changes in tumor suppressor genes during somatic cells proliferation, and these altered changes further strengthen the association between chronic inflammation and cancer development.[8]

An observational study investigating the role of hypermethylation of the *hMLH1* promoter region in the normal colonic mucosa reported that DNA methylation in the normal colonic mucosa is closely associated with age and microsatellite instable (MSI) CRC development.[37] Moreover, the hypermethylation of the *hMLH1* promoter region is one of the molecular pathways associated with CRC development, constituting a crucial oncogenic molecular pathway in CRC that is present in ~12–15% of all colorectal malignant tumors.[38] In summary, the association between DNA methylation and clinical stage may explain the occurrence of chronic inflammation and MSI in patients with CRC after surgical resection.

Sato *et al*[39] evaluated DNA methylation in noncancerous tissues obtained from patients with lung adenocarcinomas and observed aberrant DNA methylation in several genes in precancerous stages. Aberrant DNA methylation of adjacent normal tissues in early cancer stages determines tumor aggressiveness during the disease progression to developed lung adenocarcinoma. This result is consistent with our finding that molecular changes might not be observed through clinicopathological examination, such as immunochemistry staining with certain cancer targeting antibodies; however, DNA methylation alterations might occur in normal tissues adjacent to the tumor sites. Therefore, compared with cancer tissues, aberrant DNA methylation in adjacent normal tissues lead to a poor prognosis after surgical resection.

The strengths of this study include its retrospective design in obtaining all tissue samples and clinical information after CRC diagnosis as well as collecting information on the recurrence and survival status after surgical resection. We used two factors, DNA methylation and clinical stage, to assess the CRC prognosis. Moreover, the evaluation of the joint effect between these two factors enabled us to determine the independent effects and association of CRC recurrence.

However, our study has several limitations that should be addressed. First, the study involved only patients with CRC and not normal healthy individuals. Establishing an acceptable protocol could assist in studying the methylation of tumor suppressor genes, their distribution in promoter regions, their distribution in the colon and rectum, and their time sequence dependence in healthy individuals, particularly in those who develop cancer. Although an animal model was used to simulate the methylation status of the adenoma–carcinoma sequence, which is a precursor of animal cancer progression, it was not performed in humans. Second, we did not have sufficient information on chronic inflammation to examine this finding in our study.

Table 4 Stratified effect between gene promoter region methylation and different cancer stages for cancer recurrence in patients with colorectal cancer

	Normal tissues				Tumor tissues			
Recurrence in different stages by methylation	**Number of participants**	**Number of cases (%)**	**Crude OR 95% CI**	**Adjusted OR 95% CI**	**Number of participants**	**Number of cases (%)**	**Crude OR 95% CI**	**Adjusted OR 95% CI**
CDKN2A								
Unmethylation								
Local (1 and 2)	55	7 (12.7)	1.00 (referent)	1.00 (referent)	44	5 (11.4)	1.00 (referent)	1.00 (referent)
Advanced (3 and 4)	53	25 (47.2)	6.12 (2.35 to 15.97)	5.56 (2.04 to 15.16)	32	14 (43.8)	6.07 (1.89 to 19.43)	6.09 (1.71 to 21.70)
Methylation								
Local (1 and 2)	15	1 (6.7)	1.00 (referent)	1.00 (referent)	26	3 (11.5)	1.00 (referent)	1.00 (referent)
Advanced (3 and 4)	9	8 (88.9)	112.00 (6.13 to 2045.17)	38.97 (1.26 to 1204.16)	30	19 (63.3)	13.24 (3.22 to 54.45)	11.49 (2.47 to 53.53)
p Value*			0.001	0.036			<0.001	0.002
MLH1								
Unmethylation								
Local (1 and 2)	68	8 (11.8)	1.00 (referent)	1.00 (referent)	57	7 (12.3)	1.00 (referent)	1.00 (referent)
Advanced (3 and 4)	59	31 (52.5)	8.30 (3.39 to 20.37)	6.80 (2.64 to 17.54)	54	29 (53.7)	8.29 (3.19 to 21.53)	6.88 (2.48 to 19.08)
Methylation								
Local (1 and 2)	2	0 (0.0)	1.00 (referent)	1.00 (referent)	13	1 (7.7)	1.00 (referent)	1.00 (referent)
Advanced (3 and 4)	3	2 (66.7)	NA†	NA†	8	4 (50.0)	12.00 (1.02 to 141.34)	21.31 (0.78 to 579.63)
p Value*			NA†	NA†			0.048	0.070
MGMT								
Unmethylation								
Local (1 and 2)	67	7 (10.4)	1.00 (referent)	1.00 (referent)	40	4 (10.0)	1.00 (referent)	1.00 (referent)
Advanced (3 and 4)	56	29 (51.8)	9.21 (3.59 to 23.62)	7.86 (2.89 to 21.34)	33	20 (60.6)	13.85 (3.98 to 48.18)	11.75 (2.98 to 46.37)
Methylation								
Local (1 and 2)	3	1 (33.3)	1.00 (referent)	1.00 (referent)	30	4 (13.3)	1.00 (referent)	1.00 (referent)
Advanced (3 and 4)	6	4 (66.7)	4.00 (0.21 to 75.66)	NA†	29	13 (44.8)	5.28 (1.47 to 19.03)	7.42 (1.62 to 33.91)
p Value*			0.355	NA†			0.011	0.010
≥1 of genes								
Unmethylation								
Local (1 and 2)	51	6 (11.8)	1.00 (referent)	1.00 (referent)	24	2 (8.3)	1.00 (referent)	1.00 (referent)
Advanced (3 and 4)	47	21 (44.7)	6.30 (2.25 to 17.66)	6.13 (2.04 to 18.36)	20	10 (50.0)	11.00 (2.03 to 59.75)	13.36 (1.73 to 103.11)
Methylation								
Local (1 and 2)	19	2 (10.5)	1.00 (referent)	1.00 (referent)	46	6 (13.0)	1.00 (referent)	1.00 (referent)
Advanced (3 and 4)	15	12 (80.0)	25.50 (4.00 to 162.38)	17.65 (1.91 to 163.52)	42	23 (54.8)	8.07 (2.82 to 23.09)	6.56 (2.17 to 19.83)
p Value*			<0.001	0.011			<0.001	0.001

Adjusted for gender and age at surgery (continuous).
*p for the stratified effect interaction.
†NA due to limited numbers of cases.
CDKN2A, cyclin-dependent kinase inhibitor 2A; MGMT, O-6-methylguanine DNA methyltransferase; MLH1, mutL homolog 1; NA, not available.

In conclusion, the results of this study indicate that DNA methylation status significantly increases the recurrence risk of CRC with a significant effect on the interaction between DNA methylation and clinical stage, particularly in the matched normal tissues. The possible reason is molecular changes that could not be examined on the basis of clinical pathology. We suggest using these interaction results in the matched normal tissues of patients with CRC as a reference marker for evaluating the risk of recurrence in future studies.

Contributors H-FC helped draft the manuscript, conduct the literature review and prepare the materials, methods and the discussion sections of the text. C-AS helped supervise the field activities, designed the study's analytic strategy and critical revision. C-CW helped for study participants diagnosed and biospecimen collection. C-MC helped for consulting of data analysis and literature discussion. F-GL helped conduct the results of data analysis. J-FH helped for experimental condition testing and biospecimen storage. C-HHsu helped for experimental consulting. C-HHuang helped for experimental assistant. TY helped for the multivariable analysis and the interaction effect in the study topic. Y-MT helped for collection and assembly of data. J-CK helped draft the manuscript, conduct the literature review and prepare the materials, methods and the discussion sections of the text. Y-CC designed the study and directed its implementation, including quality assurance and control and final approval of manuscript.

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