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Global changes of 5-hydroxymethylcytosine and 5-methylcytosine from normal to tumor tissues are associated with carcinogenesis and prognosis in colorectal cancer*

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Abstract: Aberrant DNA methylation has raised widespread attention in tumorigenesis. In this study, we aimed to investigate the changes of global DNA methylation and hydroxymethylation from normal to tumor tissues in colorectal cancer (CRC) and their association with the prognosis. The levels of genomic 5-hydroxymethylcytosine (5hmC) and 5-methylcytosine (5mC) in cancerous tissues were significantly lower than those in corresponding adjacent normal tissues. The genomic levels of 5mC were significantly positively correlated with 5hmC in normal and cancerous tissues (all $P<0.05$). The ratio of 5mC in cancerous tissues to matched normal tissues (C/N-5mC) was also significantly positively correlated with the ratio of 5hmC in cancerous tissues to matched normal tissues (C/N-5hmC) ($P=0.01$). The 5mC levels and C/N-5mC ratios decreased with age (all $P<0.05$). Higher 5mC and 5hmC levels were found in rectal than in colon tissues (all $P<0.05$). High levels of 5mC in cancerous tissues and high C/N-5hmC ratios were each associated with lymph node metastasis (all $P<0.05$). Survival analysis indicated that the C/N-5mC ratio ($P=0.04$) is an independent protective factor for overall survival. The data showed that patients with a combination of high C/N-5hmC and low C/N-5mC ratios tended to have a worse prognosis ($P<0.01$). Our findings showed that the C/N-5mC ratio may be an independent prognostic factor for CRC outcome. Patients with both a high C/N-5hmC ratio and a low C/N-5mC ratio exhibited the worst survival, suggesting that 5mC and 5hmC can be used as critical markers in tumorigenesis and prognosis estimation.

Key words: 5-Hydroxymethylcytosine (5hmC); 5-Methylcytosine (5mC); Prognosis; Colorectal cancer

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1 Introduction

Over recent years, colorectal cancer (CRC) has been well-studied. It has also become one of the most common cancers. Its pathogenesis is characteristic of sequential genetic and epigenetic changes (van

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Engeland *et al*., 2011). Epigenetic modification denotes heritable traits of gene expression that do not include alterations of the DNA sequences. Epigenetic changes mainly affect chromatin structure stability, gene expression, tissue-specific modulation, genome integrity, genomic imprinting, embryonic development, and X-chromosome inactivation in females. Epigenetic changes often refer to reversible DNA methylation as well as histone acetylation, chromatin looping, and small noncoding RNAs (Turek-Plewa and Jagodzinski, 2005).

Aberrant DNA methylation occurs in many different kinds of cancer and plays a key role in tumorigenesis. In many human cancers, global genomic hypomethylation as well as hypermethylation in promoter regions has been extensively studied. These alterations lead to genomic instability and gene regression or even silencing (Bird, 2002; Robertson, 2005; Baylin and Jones, 2011; Dawson and Kouzarides, 2012). In CRC, low genomic 5-methylcytosine (5mC) was first found in tumor tissues in 1988 (Feinberg *et al*., 1988). Global DNA demethylation is age-dependent, even preceding the process of carcinogenesis (Suzuki *et al*., 2006). Previous findings have shown that lower levels of 5mC correlate well with advanced malignancy grade in breast cancer and colon cancer (Barciszewska *et al*., 2007). Some studies have shown that the *Line-1* (long interspersed nuclear element 1) methylation level, which may represent global methylation status, is negatively associated with T-stage and is significantly reduced in lymph node metastases in CRC (Sunami *et al*., 2011; Morikawa *et al*., 2012). Furthermore, early studies found that 5mC is an independent marker of poor survival outcome in CRC patients (Frigola *et al*., 2005; Ogino *et al*., 2008; Li *et al*., 2014). However, there are few studies analyzing the relationship between clinicopathological features and the degree of change in 5mC from normal to tumor tissues in CRC patients.

Kriaucionis and Heintz (2009) used the technologies of high-pressure liquid chromatography, thin-layer chromatography, and mass spectrometry to identify 5-hydroxymethylcytosine (5hmC) in the brain. Another group reported that ten eleven translocation (TET) can convert 5mC to 5hmC in cultured cells and in vitro (Sun *et al*., 2013). Just like 5mC, 5hmC exists ubiquitously and stably in human tissues, and its reduction in cancerous tissues has been reported in a variety of malignancies including hematological malignancies and solid tumors, as well as in CRC (Robertson, 2005; Jin *et al*., 2011; Dawson and Kouzarides, 2012; Zhang *et al*., 2013; Wang *et al*., 2014). A recent study of acute myeloid leukemia showed that high levels of 5hmC are correlated with inferior overall survival (Kroeze *et al*., 2014). However, in melanoma, a reduced level of 5hmC is a marker of worse prognosis and is associated with dysplastic cytomorphological features and tumor progression (Larson *et al*., 2014). In solid tumors, low 5hmC levels represent poor overall survival and high cumulative recurrence (Liu *et al*., 2014). In addition, 5hmC levels have also been highly correlated with tumor stage (Chen *et al*., 2013). So far, studies have reported that 5hmC has its own additional biological function as an intermediate base in DNA demethylation (Pfeifer *et al*., 2013). Ficz *et al*. (2011) found that 5hmC in the promoter regions is associated with an increased transcriptional level. Also, Robertson *et al*. (2011) reported that the presence of 5hmC at the promoter negatively regulates gene expression. So the exact function of 5hmC is still unclear. A recent study of CRC indicated that 5hmC in promoters resists DNA hypermethylation and highlighted that 5hmC plays an important role in cancerous cell proliferation (Uribe-Lewis *et al*., 2015). Therefore, given the potential roles of 5mC and 5hmC in carcinogenesis, we explored their association with clinicopathological parameters and outcomes in CRC patients.

2 Materials and methods

2.1 Participants

From 2006 to 2012, 71 CRC patients (40 men and 31 women) were recruited from Taizhou Hospital in Zhejiang Province, China. Primary cancerous colorectal tissues and corresponding adjacent normal tissues from the surgical margin were collected after surgery. No patients received radiotherapy or chemotherapy before surgery. The diagnosis of CRC was made by senior pathologists. The pathological features and clinical stages of primary tumors were defined on the basis of the criteria of the American Joint Commission on Cancer/International Union Against Cancer (AJCC/UICC). Patients were followed up until death or censored at five years from the date of

surgery. All the tissues in this study, including tumor tissues and corresponding normal tissues, were the primary tissues. No metastasis tissue was analyzed in this study. However, we compared the difference between patients with and without lymph node metastasis or distant metastasis. The protocol was approved by the Research Ethics Committee of the School of Medicine, Zhejiang University, Hangzhou, China.

2.2 Genomic DNA extraction

Genomic DNA was extracted from all 142 samples (cancerous and adjacent normal tissues) using an E.Z.N.A.® DNA/RNA isolation kit (Omega Bio-Tek, Norcross, GA, USA) and quantified using a spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA).

2.3 Determination of genomic 5hmC and 5mC levels

Genomic 5hmC and 5mC levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (MethylFlash Hydroxymethylated/Methylated DNA Quantification Kit (Fluorometric); Epigentek, Farmingdale, NY, USA). Genomic DNA (100 ng) was used to assess the levels of 5hmC and 5mC, following the manufacturer's protocols (Li and Liu, 2011). The percentages of 5mC (5mC%) and 5hmC (5hmC%) in total DNA were calculated using the following formulae:

$$5mC\%=(OD_{sample}-OD_{negative})/(slope\times 2\times S)\times 100\%,$$
$$5hmC\%=(OD_{sample}-OD_{negative})/(slope\times 5\times S)\times 100\%,$$

where OD_{sample} and $OD_{negative}$ are the optical densities of sample and negative control, respectively. S is the amount of input sample DNA in nanograms.

2.4 Statistical analysis

SPSS 19.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. For variables with non-normal distributions, medians and interquartile ranges are presented. The Wilcoxon signed-rank test for paired non-parametric data was used to determine the differences in 5mC and 5hmC levels between cancerous and adjacent normal tissues in overall and subgroup analysis. Correlations between 5hmC and 5mC levels were evaluated by the Spearman's rank correlation coefficient. The associations between 5hmC and 5mC levels and clinicopathological parameters were calculated using the Mann-Whitney test. The continuous variables, 5mC and 5hmC levels, were classified into categorical variables according to the receiver operating characteristic curves combined with the median value. The cut-off values were the points at the base of the maximal Youden index (sensitivity+specificity–1) (Liang *et al*., 2015). Kaplan-Meier survival analysis was used to calculate the overall survival time, and prognostic differences were compared with the log-rank test. The adjusted hazard ratio (HR) was measured using the multivariate Cox proportional hazard model. Statistical significance was set at $P<0.05$ (two-tailed). Graphs were created using GraphPad Prism 5 (San Diego, CA, USA).

3 Results

3.1 Basic characteristics of participants

The demographic characteristics of the 71 patients are summarized in Table 1. The age at diagnosis ranged from 28 to 84 years (median, 64 years); 37 patients (52.1%) had colon cancer and the others had rectal cancer; 38 had lymph node metastasis and 8 had distant metastasis; in 35, the tumor had penetrated to the visceral peritoneum or other organs; and the 5-year overall survival rate of the cohort was 60.6%.

3.2 5hmC and 5mC levels in CRC and adjacent normal tissues

The overall and subgroup percentage levels of 5mC and 5hmC are presented in Table 1 and Fig. 1. The median level (P25–P75) of 5hmC in cancerous tissue was 0.05% (range, 0.01%–0.10%), lower than in normal tissue (0.07% (0.03%–0.13%); $P<0.01$). Among the CRC patients, 87.3% (62/71) had lower 5hmC levels in the cancerous tissues than in the normal tissues. These differences were consistent even in stratified analysis by age, gender, CRC location, tumor-node-metastasis (TNM) stage, and depth of tumor invasion, as well as lymph node metastasis and distant metastasis (Table 1).

As for 5mC, its percentage level in cancerous tissues (4.46% (3.47%–5.75%)) was lower than in normal tissues (6.15% (4.32%–8.16%); $P<0.01$). Among the CRC patients, 74.6% (53/71) had lower

Table 1 Associations of 5mC and 5hmC levels with clinical pathological factors in CRC patients

Variable	Number	Level of 5mC (%)				Level of 5hmC (%)			
		Normal tissues	Cancerous tissues	P^b	C/N	Normal tissues	Cancerous tissues	P^b	C/N
Age (year)									
≤60	30	6.58 (4.59–8.80)	5.32 (4.18–7.52)	<0.01	0.86 (0.63–1.07)	0.07 (0.03–0.11)	0.05 (0.01–0.07)	<0.01	0.61 (0.39–0.85)
>60	41	5.81 (4.21–8.02)	4.15 (3.09–5.28)	<0.01	0.69 (0.55–0.93)	0.08 (0.03–0.16)	0.04 (0.01–0.12)	<0.01	0.53 (0.36–0.83)
P^a		0.36	<0.01		0.04	0.58	0.87		0.44
Gender									
Male	40	5.54 (4.25–8.04)	4.53 (3.46–5.72)	<0.01	0.79 (0.63–1.00)	0.07 (0.02–0.15)	0.04 (0.01–0.11)	<0.01	0.61 (0.40–0.81)
Female	31	6.71 (4.41–9.16)	4.42 (3.45–5.99)	<0.01	0.70 (0.55–1.02)	0.08 (0.05–0.13)	0.05 (0.02–0.08)	<0.01	0.53 (0.37–1.02)
P^a		0.18	0.98		0.31	0.73	0.54		0.91
Location									
Colon	37	4.73 (4.11–6.05)	4.03 (3.23–5.20)	<0.01	0.83 (0.64–1.05)	0.04 (0.02–0.08)	0.01 (0.01–0.05)	<0.01	0.52 (0.29–0.68)
Rectum	34	7.86 (6.45–8.53)	5.05 (4.01–6.42)	<0.01	0.69 (0.52–0.95)	0.12 (0.07–0.23)	0.10 (0.04–0.14)	<0.01	0.65 (0.48–0.89)
P^a		<0.01	0.04		0.17	<0.01	<0.01		0.01
Tumor-node-metastasis (TNM) stage									
I–II	29	5.67 (4.23–7.50)	4.32 (3.40–4.93)	<0.01	0.70 (0.53–1.06)	0.10 (0.03–0.19)	0.05 (0.01–0.11)	<0.01	0.48 (0.34–0.75)
III–IV	42	6.37 (4.36–8.23)	4.97 (3.49–6.42)	<0.01	0.83 (0.63–0.95)	0.07 (0.03–0.11)	0.04 (0.01–0.08)	<0.01	0.63 (0.45–0.85)
P^a		0.33	0.08		0.69	0.29	0.88		0.11
Depth of tumor invasion (T)									
T1–T3	36	5.37 (4.18–7.82)	4.61 (3.68–5.95)	<0.01	0.88 (0.61–1.07)	0.10 (0.04–0.16)	0.05 (0.01–0.12)	<0.01	0.61 (0.45–0.84)
T4	35	6.52 (4.72–8.36)	4.32 (3.11–5.67)	<0.01	.69 (0.54–0.91)	0.07 (0.03–0.11)	0.03 (0.01–0.07)	<0.01	0.52 (0.33–0.75)
P^a		0.26	0.33		0.05	0.21	0.16		0.30
Lymph node metastasis (N)									
N0	33	6.12 (4.41–7.94)	4.07 (3.17–4.93)	<0.01	0.69 (0.49–1.06)	0.09 (0.04–0.19)	0.05 (0.01–0.12)	<0.01	0.48 (0.34–0.75)
N1–N2	38	6.05 (4.21–8.26)	5.05 (3.85–6.73)	<0.01	0.83 (0.65–0.98)	0.07 (0.03–0.11)	0.04 (0.01–0.08)	<0.01	0.63 (0.48–0.85)
P^a		0.62	0.02		0.20	0.21	0.77		0.07
Distant metastasis (M)									
M0	63	6.12 (4.22–8.09)	4.50 (3.62–5.78)	<0.01	0.78 (0.59–1.05)	0.08 (0.03–0.15)	0.05 (0.01–0.10)	<0.01	0.56 (0.37–0.83)
M1	8	6.29 (4.84–8.24)	3.72 (2.58–5.86)	0.01	0.75 (0.41–0.89)	0.04 (0.02–0.08)	0.02 (0.01–0.04)	0.09	0.56 (0.41–0.95)
P^a		0.72	0.25		0.24	0.16	0.25		0.79

All cases were classified according to the 7th edition of the pathologic tumor-node-metastasis (pTNM) classification of the AJCC/UICC. Data are expressed as median (P25–P75); P^a values were determined with the Mann-Whitney test, and P^b values with the Wilcoxon signed-rank test

5mC levels in cancerous tissues than in normal tissues. Similar decreases in the 5mC levels in cancerous tissues were found in the subgroups (Table 1).

The genomic level of 5mC was significantly positively correlated with 5hmC in both cancerous (Spearman's ρ=0.25, P=0.03) and normal tissues (Spearman's ρ=0.36, P<0.01). The cancerous tissue: normal tissue (C/N) ratio of 5mC was also positively correlated with the C/N ratio of 5hmC (Spearman's ρ=0.31, P=0.01).

3.3 Associations of 5mC and 5hmC levels with clinicopathological characteristics in CRC

The associations between the 5mC and 5hmC levels and clinicopathological characteristics are shown in Table 1. Patients >60 years old had lower 5mC levels and C/N-5mC ratios than those ≤60 years old (P<0.05). No significant difference was found between male and female patients. In tumor and normal tissues, 5mC and 5hmC levels were higher in

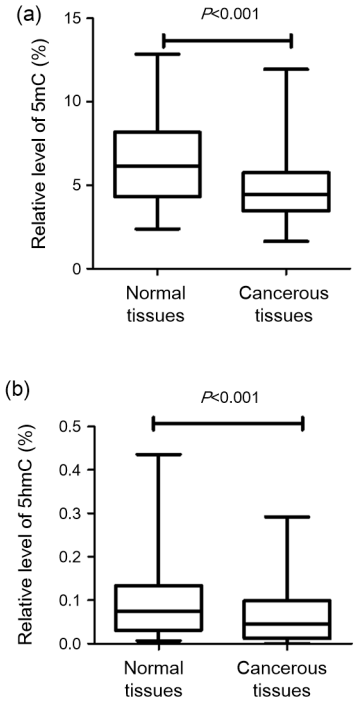

Fig. 1 Levels of 5mC and 5hmC in colorectal cancer and adjacent normal tissues

Percentage levels of 5mC (a) and 5hmC (b) in normal and cancerous tissues. Data are expressed as median (P25–P75) using box-plot and the whiskers on vertical bars show minimum and maximum values. *P* values were determined by the Wilcoxon signed-rank test

rectal cancer than in colon cancer (all $P<0.05$). Similar results were found for C/N-5hmC ratios ($P<0.05$). Lower C/N-5mC ratios were found in patients with infiltrative growth (Stage T4) ($P=0.05$). Those with lymph node metastasis tended to have higher 5mC levels ($P=0.02$), as well as C/N-5hmC ratios ($P=0.07$) in tumor tissues. Higher 5mC levels and C/N-5hmC ratios in tumor tissues were found in advanced TNM stages ($0.05<P<0.1$).

3.4 Associations of 5mC and 5hmC levels with overall survival

The overall survival of patients stratified by 5mC and 5hmC levels is presented in Table 2 and Fig. 2. Among the 71 patients, the median survival time was 50.7 months (interquartile range, 27.6–58.7 months), and the overall survival rate was 59.7%. According to the receiver operating characteristic curves, the cut-off value was 5.81% for 5mC and 0.07% for 5hmC in normal tissues and 4.51% for 5mC and 0.05% for 5hmC in cancerous tissues. The cut-off value for the C/N ratio was 0.92% for 5mC and 0.48% for 5hmC. Kaplan-Meier analysis revealed that CRC patients with a higher C/N-5mC ratio had a longer survival of 54.0 months (standard error of the mean (SEM)=3.0 months) than those with a lower ratio (mean=42.9 months; SEM=2.9 months; $P=0.01$). Shorter survival was found in patients with high levels of 5mC in normal tissues ($P=0.07$), high levels of 5hmC in cancerous tissues ($P=0.04$), and high C/N-5hmC ratios ($P=0.08$). After adjustment for age, sex, and TNM stage with the multiple Cox regression model, significant associations were found in the C/N-5mC ratios (HR=0.36; 95% confidence interval (CI): 0.13–0.96; $P=0.04$) and in the 5mC levels in normal tissues ($P<0.04$). However, neither the 5hmC levels in cancerous tissues nor the C/N-5hmC ratios had a significant correlation with survival rate based on the multiple Cox regression model (all $P<0.05$).

3.5 Combined effect of 5mC and 5hmC on survival

The combined effect of the C/N-5mC and C/N-5hmC ratios on survival is presented in Table 3 and Fig. 2. Based on the different values of the C/N-5mC and C/N-5hmC ratios, the 71 patients were divided into four groups. Comparison of the combined signature analysis for all 71 patients showed a significant correlation with overall survival based on Kaplan-Meier curves ($P<0.01$). Patients with both high C/N-5hmC and low C/N-5mC ratios had a shorter survival of 36.2 months than the others (mean=53.1 months; SEM=2.4 months; $P<0.01$; Table 3). In the multiple Cox regression model with adjustment for age, sex, and TNM stage, patients with high C/N-5hmC and low C/N-5mC ratios also showed a worse survival outcome (HR=3.48, 95% CI: 1.54–7.88; $P<0.01$).

4 Discussion

We found that the 5mC and 5hmC levels were significantly reduced in CRC, and were correlated with some of the clinicopathological features. Furthermore, high C/N-5hmC and low C/N-5mC ratios were associated with an unfavorable outcome in CRC patients.

Table 2 Survival analyses of 5mC and 5hmC levels in patients with CRC

Variable	Number	Survival rate (%)	Survival time (month)*	P^a	HR (95% CI)	P^b
5mC levels in normal tissues						
Low	34	70.6	51.5±2.9		1	
High	37	51.4	42.5±3.4	0.07	2.29 (1.02–5.16)	0.04
5mC levels in cancerous tissues						
Low	38	65.8	48.1±3.2		1	
High	33	54.5	45.2±3.3	0.39	1.63 (0.73–3.64)	0.23
C/N-5mC						
Low	46	50.0	42.8±3.0		1	
High	25	80.0	54.0±3.0	0.01	0.36 (0.13–0.96)	0.04
5hmC levels in normal tissues						
Low	36	63.9	48.9±2.9		1	
High	35	57.1	44.6±3.6	0.57	1.39 (0.65–2.98)	0.40
5hmC levels in cancerous tissues						
Low	36	72.2	51.8±2.6		1	
High	35	48.6	41.7±3.6	0.04	1.84 (0.84–4.05)	0.13
C/N-5hmC						
Low	24	75.0	51.5±3.8		1	
High	47	53.2	44.3±2.8	0.08	2.01 (0.78–5.41)	0.14

P^a values for survival were determined with the log-rank test, and P^b values were measured by multivariate analyses of overall survival (Cox proportional hazards regression model) after adjustment for age, sex, and TNM stage. * Data were expressed as mean±SEM

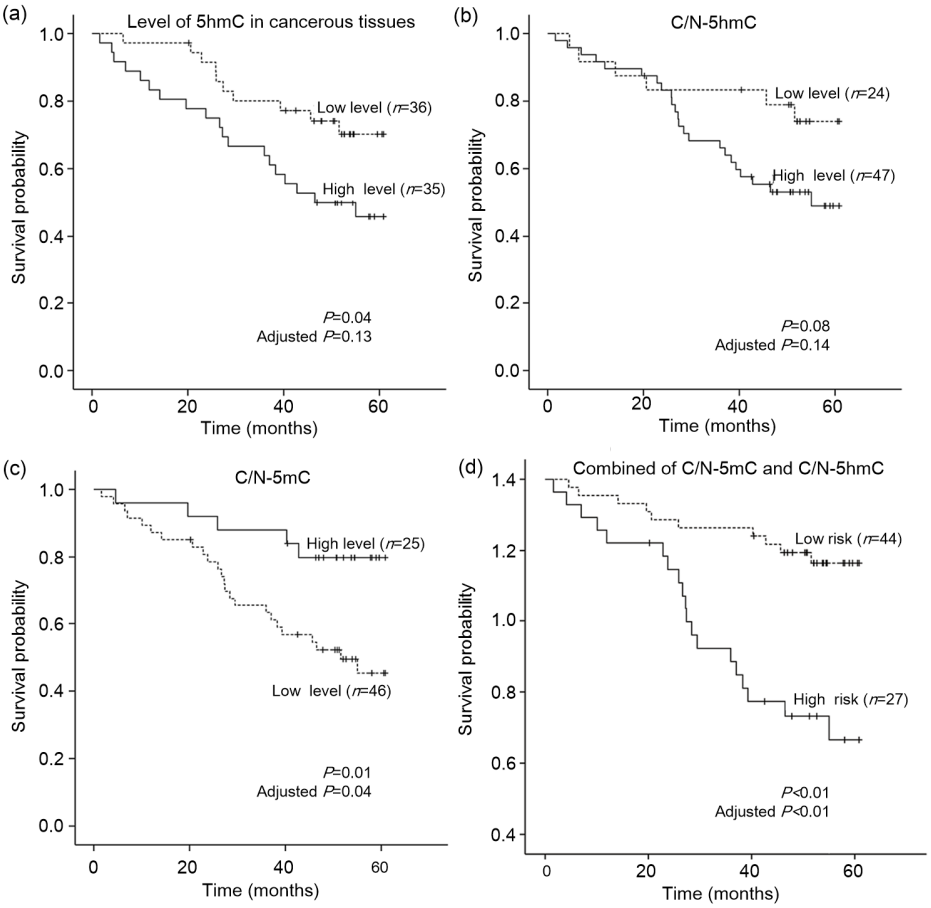

Fig. 2 Associations of 5mC and 5hmC levels with survival in colorectal cancer patients

Overall survival of 71 CRC patients in relation to the 5hmC levels in cancerous tissues (a), C/N-5hmC (b), C/N-5mC (c), and risk level of the combined model (d). Survival rates were analyzed using the Kaplan-Meier survival test. *P* values were calculated using the log-rank test and adjusted *P* values were assessed by multivariate analyses after adjustment for age, sex, and TNM stage

Table 3 Combined effect of C/N-5mC and C/N-5hmC on survival

Group	C/N-5hmC	C/N-5mC	Number	Survival rate (%)	Survival time (month)*	P^a	HR (95% CI)	P^b
A	High	Low	27	33.3	36.2±3.8	<0.01	1	
B	Low	High	5	80.0	49.6±10.1		0.30 (0.04–2.40)	0.25
C	High	High	20	80.0	55.1±2.7		0.24 (0.08–0.73)	0.01
D	Low	Low	19	73.7	52.0±3.9		0.33 (0.12–0.92)	0.03
B+C+D (low risk)			44	68.2	53.1±2.4		1	
A (high risk)			27	33.3	36.2±3.8	<0.01	3.48 (1.54–7.88)	<0.01

P^a values for survival were determined with the log-rank test, and P^b values were measured by multivariate analyses of overall survival (Cox proportional hazards regression model) after adjustment for age, sex, and TNM stage. * Data were expressed as mean±SEM

It has been suggested that epigenetic alteration is an early and major event during carcinogenesis, especially DNA-methylated modification. So far, many different methods have been developed to assess global DNA methylation and hydroxymethylation levels, such as bisulfite sequencing, liquid chromatography-mass spectrometry, and methylated/hydroxymethylated DNA immunoprecipitation. In this study, we used ELISA to quantify the genome-wide 5hmC and 5mC levels because of its convenience, cost-effectiveness, and relatively high sensitivity and specificity (Jin *et al*., 2010; 2011; Li and Liu, 2011; Zhu *et al*., 2014).

First, the levels of 5mC and 5hmC differed between cancerous and corresponding adjacent tissues, in line with previous studies (Li and Liu, 2011; van Engeland *et al*., 2011; Kudo *et al*., 2012). A significant reduction of 22.39% for 5mC and 44.07% for 5hmC was found in cancerous tissues relative to normal tissues (Feinberg *et al*., 1988). A further reduction of 5hmC was noted in cancerous tissues compared to 5mC (P<0.01). Higher levels of 5hmC and 5mC were found in rectal than in colon tissues, supporting the evidence that the distribution of 5hmC and 5mC is tissue-dependent (Li and Liu, 2011). These results may be affected by individual heterogeneity and variation, which existed in our study as well as in previous research (Zheng *et al*., 2007). To solve this problem, we used the C/N-5hmC and C/N-5mC ratios, in which the 5hmC and 5mC levels in cancerous tissues were corrected by those in normal tissues.

Global DNA hypomethylation is one of the first epigenetic changes in CRC to be recognized, and we also found lower 5mC levels in different CRC subgroups than in adjacent normal tissues. We further found that decreased C/N-5mC ratios were significantly associated with older cancer patients and those with deeper tumor invasion, which was consistent with the reduction of 5mC levels in cancerous tissues. Moreover, lower 5mC levels were found at early TNM stages than in corresponding normal tissues, suggesting that 5mC plays an important role in the initiation of neoplastic transformation (Suzuki *et al*., 2006). Further reduction of C/N-5mC ratios was found in tumors with infiltrative growth. However, there was no significant correlation between C/N-5mC ratios and TNM stages. One explanation is that DNA hypomethylation occurs in the early stages of tumor development and does not progress with advancing stages, supporting the transient nature of the DNA demethylation process (Haffner *et al*., 2011). Global hypomethylation increases chromosomal instability, while hypermethylation at CpG dinucleotide-dense regions suppresses gene expression, especially the inactivation of tumor suppressor genes. Some studies have also shown that "cancer-germline" genes are activated as a consequence of genome hypomethylation (de Smet and Loriot, 2010; 2013). Cancer germline genes aberrantly activated in cancer are tissue-specific, and encode tumor-specific antigens. These results revealed a dual effect of DNA hypomethylation in suppression at a later stage of colorectal tumorigenesis, but promotion of early lesions in CRC (Yamada *et al*., 2005). In addition, Kaplan-Meier survival analysis revealed that high C/N-5mC ratios are associated with a better survival outcome and may be an independent prognostic marker.

5hmC, an oxidation product of 5mC, is not only an intermediate in DNA demethylation, but also a stable DNA base involved in gene regulation and

tumor genesis (Kudo *et al*., 2012; Pfeifer *et al*., 2013; Uribe-Lewis *et al*., 2015). In this study, we found that in cancerous tissues, 5hmC levels were also lower in different CRC subsets than in adjacent normal tissues. Furthermore, significantly positive correlations between 5mC levels and 5hmC levels were found both in CRC tissues and adjacent normal tissues. Studies of embryonic stem cells have reported that 5hmC may be involved in the regulation of cell differentiation, and its loss in CRC may provide support for the hypothesis that 5hmC plays an important role in cellular differentiation. Since the generation of 5hmC requires 5mC as a substrate, the decreased 5hmC levels may be partly due to reduced 5mC levels (Pfeifer *et al*., 2013). However, in some CRC cases, high levels of 5mC corresponded with low 5hmC levels, suggesting the existence of other mechanisms underlying the reduction in 5hmC. The mRNA levels of the *Dnmt* (DNA methyltransferase) and *Tet* families were also assessed, and the correlations with 5hmC levels were analyzed (data not shown). The expression of *Dnmt3b* was negatively associated with 5hmC levels in tumor tissues, while no significant relationship was found between *Tets* and 5hmC levels. Other than the *Tet* family, many enzymes are involved in the process of demethylation, like isocitrate dehydrogenase and thymine DNA glycosylase, which also contributed to the complexity of the results in our study (Kohli and Zhang, 2013). Some researchers have reported that Dnmt3a and Dnmt3b not only serve as DNA methyltransferases, but also serve as DNA dehydroxymethylases (Chen *et al*., 2012; Kudo *et al*., 2012). Other studies have also reported that 5hmC can resist DNA hypermethylation (Uribe-Lewis *et al*., 2015). Furthermore, we cannot ignore the possibility that enhanced proliferation leads to a "passive" dilution of 5hmC (Lian *et al*., 2012).

Finally, we analyzed the relationships between 5hmC levels and the clinical features of CRC. With regard to the 5hmC ratios, a higher C/N-5hmC value was associated with an advanced TNM stage and the presence of lymph node metastasis. Interestingly, whether in cancerous tissues or in C/N-5hmC ratio, our study showed that a low 5hmC level was associated with a better survival rate using Kaplan-Meier with the P values of 0.04 and 0.08, respectively. However, there was no significant difference with the Cox proportional hazards regression model. The difference might be due to the different sensitivities between the Cox proportional hazards regression model and Kaplan-Meier. High 5hmC levels correlated with poor survival has also been reported in acute myeloid leukemia (Kroeze *et al*., 2014); but studies of other malignancies have shown that 5hmC is a protective factor in the prognosis. Thus, the role of 5hmC in prognosis needs further exploration.

We demonstrated that epigenetics plays an important role during tumorigenesis by showing the clinical relevance of 5mC and 5hmC levels in a cohort of CRC patients. Our findings support the hypothesis that loss of 5mC and 5hmC is involved in CRC tumorigenesis. In addition, our results showed that C/N-5mC and C/N-5hmC ratios may be independent prognostic factors for CRC survival outcomes. As far as we know, this is the first study to evaluate the combined effect of C/N-5mC and C/N-5hmC ratios on survival. Patients with either high C/N-5hmC or low C/N-5mC ratios tended to have a worse prognosis (HR=3.48, 95% CI: 1.54–7.88; P<0.01), further confirming that both 5hmC and 5mC play important roles in CRC and their combined effect could be used as a prognostic biomarker. Further studies with large clinical samples are needed.

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Compliance with ethics guidelines

Yi-ping TIAN, Ai-fen LIN, Mei-fu GAN, Hao WANG, Dan YU, Chong LAI, Dan-dan ZHANG, Yi-min ZHU, and Mao-de LAI declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study. Additional informed consent was obtained from all patients for which identifying information is included in this article.

References

Barciszewska, A.M., Murawa, D., Gawronska, I., *et al*., 2007. Analysis of 5-methylcytosine in DNA of breast and colon cancer tissues. *IUBMB Life*, **59**(12):765-770. http://dx.doi.org/10.1080/15216540701697412

Baylin, S.B., Jones, P.A., 2011. A decade of exploring the cancer epigenome-biological and translational implications. *Nat. Rev. Cancer*, **11**(10):726-734. http://dx.doi.org/10.1038/nrc3130

Bird, A., 2002. DNA methylation patterns and epigenetic memory. *Gene Dev*., **16**(1):6-21. http://dx.doi.org/10.1101/gad.947102

Chen, C.C., Wang, K.Y., Shen, C.K., 2012. The mammalian *de novo* DNA methyltransferases DNMT3A and DNMT3B are also DNA 5-hydroxymethylcytosine dehydroxymethylases. *J. Biol. Chem*., **287**(40):33116-33121. http://dx.doi.org/10.1074/jbc.C112.406975

Chen, M.L., Shen, F., Huang, W., *et al*., 2013. Quantification of 5-methylcytosine and 5-hydroxymethylcytosine in genomic DNA from hepatocellular carcinoma tissues by capillary hydrophilic-interaction liquid chromatography/quadrupole TOF mass spectrometry. *Clin. Chem*., **59**(5):824-832. http://dx.doi.org/10.1373/clinchem.2012.193938

Dawson, M.A., Kouzarides, T., 2012. Cancer epigenetics: from mechanism to therapy. *Cell*, **150**(1):12-27. http://dx.doi.org/10.1016/j.cell.2012.06.013

de Smet, C., Loriot, A., 2010. DNA hypomethylation in cancer: epigenetic scars of a neoplastic journey. *Epigenetics*, **5**(3):206-213. http://dx.doi.org/10.4161/epi.5.3.11447

de Smet, C., Loriot, A., 2013. DNA hypomethylation and activation of germline-specific genes in cancer. *Adv. Exp. Med. Biol*., **754**:149-166. http://dx.doi.org/10.1007/978-1-4419-9967-2_7

Feinberg, A.P., Gehrke, C.W., Kuo, K.C., *et al*., 1988. Reduced genomic 5-methylcytosine content in human colonic neoplasia. *Cancer Res*., **48**(5):1159-1161.

Ficz, G., Branco, M.R., Seisenberger, S., *et al*., 2011. Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. *Nature*, **473**(7347):398-402. http://dx.doi.org/10.1038/nature10008

Frigola, J., Sole, X., Paz, M.F., *et al*., 2005. Differential DNA hypermethylation and hypomethylation signatures in colorectal cancer. *Hum. Mol. Genet*., **14**(2):319-326. http://dx.doi.org/10.1093/hmg/ddi028

Haffner, M.C., Chaux, A., Meeker, A.K., *et al*., 2011. Global 5-hydroxymethylcytosine content is significantly reduced in tissue stem/progenitor cell compartments and in human cancers. *Oncotarget*, **2**(8):627-637. http://dx.doi.org/10.18632/oncotarget.316

Jin, S.G., Kadam, S., Pfeifer, G.P., 2010. Examination of the specificity of DNA methylation profiling techniques towards 5-methylcytosine and 5-hydroxymethylcytosine. *Nucleic Acids Res*., **38**(11):e125. http://dx.doi.org/10.1093/nar/gkq223

Jin, S.G., Jiang, Y., Qiu, R., *et al*., 2011. 5-hydroxymethylcytosine is strongly depleted in human cancers but its levels do not correlate with IDH1 mutations. *Cancer Res*., **71**(24):7360-7365. http://dx.doi.org/10.1158/0008-5472.CAN-11-2023

Kohli, R.M., Zhang, Y., 2013. TET enzymes, TDG and the dynamics of DNA demethylation. *Nature*, **502**(7472):472-479. http://dx.doi.org/10.1038/nature12750

Kriaucionis, S., Heintz, N., 2009. The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. *Science*, **324**(5929):929-930. http://dx.doi.org/10.1126/science.1169786

Kroeze, L.I., Aslanyan, M.G., van Rooij, A., *et al*., 2014. Characterization of acute myeloid leukemia based on levels of global hydroxymethylation. *Blood*, **124**(7):1110-1118. http://dx.doi.org/10.1182/blood-2013-08-518514

Kudo, Y., Tateishi, K., Yamamoto, K., *et al*., 2012. Loss of 5-hydroxymethylcytosine is accompanied with malignant cellular transformation. *Cancer Sci*., **103**(4):670-676. http://dx.doi.org/10.1111/j.1349-7006.2012.02213.x

Larson, A.R., Dresser, K.A., Zhan, Q., *et al*., 2014. Loss of 5-hydroxymethylcytosine correlates with increasing morphologic dysplasia in melanocytic tumors. *Modern Pathol*., **27**(7):936-944. http://dx.doi.org/10.1038/modpathol.2013.224

Li, J., Huang, Q., Zeng, F., *et al*., 2014. The prognostic value of global DNA hypomethylation in cancer: a meta-analysis. *PLoS ONE*, **9**(9):e106290. http://dx.doi.org/10.1371/journal.pone.0106290

Li, W., Liu, M., 2011. Distribution of 5-hydroxymethylcytosine in different human tissues. *J. Nucleic Acids*, **2011**:870726. http://dx.doi.org/10.4061/2011/870726

Lian, C.G., Xu, Y., Ceol, C., *et al*., 2012. Loss of 5-hydroxymethylcytosine is an epigenetic hallmark of melanoma. *Cell*, **150**(6):1135-1146. http://dx.doi.org/10.1016/j.cell.2012.07.033

Liang, J.Z., Li, Y.H., Zhang, Y., *et al*., 2015. Expression of ETV6/TEL is associated with prognosis in non-small cell lung cancer. *Int. J. Clin. Exp. Pathol*., **8**(3):2937-2945.

Liu, W.R., Tian, M.X., Jin, L., *et al*., 2014. High expression of 5-hydroxymethylcytosine and isocitrate dehydrogenase 2 is associated with favorable prognosis after curative resection of hepatocellular carcinoma. *J. Exp. Clin. Cancer Res*., **33**:32. http://dx.doi.org/10.1186/1756-9966-33-32

Morikawa, T., Kuchiba, A., Qian, Z.R., *et al*., 2012. Prognostic significance and molecular associations of tumor growth pattern in colorectal cancer. *Ann. Surg. Oncol*., **19**(6):1944-1953. http://dx.doi.org/10.1245/s10434-011-2174-5

Ogino, S., Nosho, K., Kirkner, G.J., *et al*., 2008. A cohort study of tumoral LINE-1 hypomethylation and prognosis in colon cancer. *J. Natl. Cancer Inst*., **100**(23):1734-1738. http://dx.doi.org/10.1093/jnci/djn359

Pfeifer, G.P., Kadam, S., Jin, S.G., 2013. 5-Hydroxymethylcytosine and its potential roles in development and cancer. *Epigenet. Chromatin*, **6**(1):10. http://dx.doi.org/10.1186/1756-8935-6-10

Robertson, K.D., 2005. DNA methylation and human disease. *Nat. Rev. Genet*., **6**(8):597-610. http://dx.doi.org/10.1038/nrg1655

Robertson, J., Robertson, A.B., Klungland, A., 2011. The presence of 5-hydroxymethylcytosine at the gene promoter

and not in the gene body negatively regulates gene expression. *Biochem. Bioph. Res. Commun.*, **411**(1):40-43. http://dx.doi.org/10.1016/j.bbrc.2011.06.077

Sun, M., Song, C.X., Huang, H., *et al*., 2013. HMGA2/TET1/HOXA9 signaling pathway regulates breast cancer growth and metastasis. *Proc. Natl. Acad. Sci. USA*, **110**(24):9920-9925. http://dx.doi.org/10.1073/pnas.1305172110

Sunami, E., de Maat, M., Vu, A., *et al*., 2011. LINE-1 hypomethylation during primary colon cancer progression. *PLoS ONE*, **6**(4):e18884. http://dx.doi.org/10.1371/journal.pone.0018884

Suzuki, K., Suzuki, I., Leodolter, A., *et al*., 2006. Global DNA demethylation in gastrointestinal cancer is age dependent and precedes genomic damage. *Cancer Cell*, **9**(3):199-207. http://dx.doi.org/10.1016/j.ccr.2006.02.016

Turek-Plewa, J., Jagodzinski, P.P., 2005. The role of mammalian DNA methyltransferases in the regulation of gene expression. *Cell. Mol. Biol. Lett.*, **10**(4):631-647.

Uribe-Lewis, S., Stark, R., Carroll, T., *et al*., 2015. 5-Hydroxymethylcytosine marks promoters in colon that resist DNA hypermethylation in cancer. *Genome Biol.*, **16**(1):69. http://dx.doi.org/10.1186/s13059-015-0605-5

van Engeland, M., Derks, S., Smits, K.M., *et al*., 2011. Colorectal cancer epigenetics: complex simplicity. *J. Clin. Oncol.*, **29**(10):1382-1391. http://dx.doi.org/10.1200/JCO.2010.28.2319

Wang, J., Tang, J., Lai, M., *et al*., 2014. 5-Hydroxymethylcytosine and disease. *Mutat. Res.-Rev. Mutat.*, **762**:167-175. http://dx.doi.org/10.1016/j.mrrev.2014.09.003

Yamada, Y., Jackson-Grusby, L., Linhart, H., *et al*., 2005. Opposing effects of DNA hypomethylation on intestinal and liver carcinogenesis. *Proc. Natl. Acad. Sci. USA*, **102**(38):13580-13585. http://dx.doi.org/10.1073/pnas.0506612102

Zhang, L.T., Zhang, L.J., Zhang, J.J., *et al*., 2013. Quantification of the sixth DNA base 5-hydroxymethylcytosine in colorectal cancer tissue and C-26 cell line. *Bioanalysis*, **5**(7):839-845. http://dx.doi.org/10.4155/bio.13.28

Zheng, Z., Chen, T., Li, X., *et al*., 2007. DNA synthesis and repair genes *RRM1* and *ERCC1* in lung cancer. *N. Engl. J. Med.*, **356**(8):800-808. http://dx.doi.org/10.1056/NEJMoa065411

Zhu, X., You, Y., Li, Q., *et al*., 2014. BCR-ABL1-positive microvesicles transform normal hematopoietic transplants through genomic instability: implications for donor cell leukemia. *Leukemia*, **28**(8):1666-1675. http://dx.doi.org/10.1038/leu.2014.51

中文概要

题　目：正常组织与肿瘤组织中总体 5hmC 和 5mC 水平的变化程度与结直肠癌肿瘤发生及预后有关

目　的：研究 DNA 总体甲基化、羟甲基化水平与结直肠癌发生、发展及预后的关系。

创新点：采用肿瘤与正常组织总体 5-甲基胞嘧啶（5mC）、5-羟甲基胞嘧啶（5hmC）的比值并探讨其与结直肠癌临床病理变化和预后的关系。

方　法：采用酶联免疫吸附试验（ELISA）方法检测肿瘤与相应正常组织基因组中总体 5hmC 和 5mC 水平。

结　论：C/N-5mC 比值的水平对结直肠癌的预后是一个独立的保护性因子（P=0.04）。当病人同时具有高水平的 C/N-5hmC 和低水平的 C/N-5mC，其预后更差（P<0.01）。因此，可以使用 5mC 和 5hmC 对结直肠癌的发生和预后进行评估。

关键词：5-羟甲基胞嘧啶；5-甲基胞嘧啶；预后；结直肠癌