Original Article

DNA hypermethylation of *MAL* gene may act as an independent predictor of favorable prognosis in patients with colorectal cancer

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Background: Aberrant DNA methylation could be used as biomarkers for colorectal cancer (CRC) detection and assessment of prognosis. The aim of our study was to investigate the potential possibility of *MAL* methylation as a prognostic biomarker for postoperative CRC patients.

Methods: We followed up 298 sporadic postoperative CRC patients and detected *MAL* methylation in tumor tissues and adjacent non-tumor tissues by methylation-sensitive high resolution melting (MS-HRM) analysis. Univariate, multivariate Cox regressions were performed to evaluate the potential possibility of *MAL* methylation as a predictor of prognosis. Propensity score (PS) analysis was used to control confounders.

Results: The *MAL* methylation level in adjacent non-tumor tissues was significantly lower than that in tumor tissues (P<0.001). The *MAL* methylation had no significant correlation with clinicopathologic characteristics. *MAL* hypermethylation was detected in 63.4% (189/298) tumor tissues. The overall 5-year survival rates in hypermethylation and hypomethylation group were 70.78% and 55.69% (P=0.007). *MAL* hypermethylation was significantly associated with a favorable clinical outcome, the hazard ratio (HR) were 0.650 [95% confidence interval (CI): 0.454–0.929, P=0.018], 0.613 (95% CI: 0.422–0.889, P=0.010) and 0.692 (95% CI: 0.481–0.996, P=0.047) in univariate, multivariate Cox and PS method, respectively. The subgroup analysis showed that *MAL* hypermethylation in CRC patients with lower diagnosis age (<60) and colon cancer had a lower risk of death than *MAL* hypomethylation patients.

Conclusions: *MAL* was frequently hypermethylated in CRC tumor tissues. *MAL* hypermethylation might act as an independent prognostic predictor of survival advantage in postoperative patients with CRC.

Keywords: Colorectal cancer (CRC); methylation; prognosis; *MAL*

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Introduction

Colorectal cancer (CRC) had a high estimated death of 881,000 in 2018. And the mortality ranked second in the world (1). The global burden of CRC is expected to reach 1,100,000 cancer deaths by 2030 (2). The 5-year relative survival rate for CRC patients has remained less than 50% in low-income countries (3). Surgery to remove the primary tumor is the most common treatment for CRC patients; however, approximately one-half of postoperative patients will experience a recurrence in the first three years (4). Survival of postoperative CRC patients is not only tightly related to pathological staging and specific histological features, but also related to multiple genetic and epigenetic

alterations.

CRC is biologically a multi-step carcinogenic disease that is characterized by genetic and epigenetic disorders of signal transduction cascades. Various epigenetic mechanisms are involved in the tissue-specific expression and maintenance of chromosome stability, which provide opportunities for early detection, diagnosis, prognosis and management of CRC patients (5,6). DNA methylation, as the most popular epigenetic alteration, can regulate gene expression by the mechanisms which include chromatin complex alterations and recruitment of methyl-CpG domain-binding proteins around CpG islands (6). Large and sufficiently powered clinical studies have demonstrated the feasibility of using specific methylated DNA signatures as prognostic biomarkers in CRC tissues. The DNA hypermethylation of *CDKN2A*, *EVL* and *IGFBP3* was associated with poor prognosis (7-9). Moreover, DNA methylation can reflect the expression of specific gene, which can be utilized as a surrogate for RNA- or protein-based expression analysis of tumor (10).

MAL gene encodes the integral membrane protein, which is a component of the protein machinery for raft remodeling by playing an essential role in T-cell development (11,12). *MAL* gene also encodes part of the protein machinery for glycolipid-enriched membrane-mediated apical transport in epithelial polarized cells, which was also important in membrane signal transduction (13-15). Although the expression of *MAL* was frequently decreased in gastric, esophageal and cervical carcinoma cells, it was increased by treatment with the demethylation reagent 5-aza-2'-deoxycytidine (16-18). *MAL* over-expression could decrease cellular motility and increase levels of apoptosis, which exhibited diminished tumorigenicity via the Fas signaling pathway (17). In addition, Zanotti *et al.* revealed that higher *MAL* mRNA levels were significantly associated with shorter overall survival (OS) and poor progression free survival (PFS) in high-grade serous ovarian carcinoma patients (19). Buffart *et al.* indicated that *MAL* hypermethylation in gastric cancer patients had a better survival compared with unmethylation patients (20). *MAL* hypermethylation was unequally distributed in a region close to the transcription start point and significantly associated with reduced gene expression *in vitro* in CRC (21,22). Therefore, we speculated that *MAL* methylation might act as a biomarker for prognosis of CRC patients. In the present study, we aimed to evaluate the CpG island methylation of *MAL* in CRC, and investigate the association between *MAL* methylation and prognosis of postoperative patients with CRC.

Methods

Patient cohort and materials collection

Two hundred and ninety-eight sporadic non-metastatic CRC patients (295 are Han, 2 are Manchu and 1 is Korean), confirmed by pathological diagnosis, were included in this study. The subjects were from a follow-up study of 453 patients who underwent surgical resection in the Third Affiliated Hospital of Harbin Medical University (Harbin, Heilongjiang, China) from November 2004 to July 2005 and from May 2007 to January 2008. Patients with any other cancer history were excluded. No patient had received pre-operative radiotherapy or chemotherapy before. The tumor tissues and adjacent non-tumor tissues (>10 cm distance to primary tumor) were immediately frozen in liquid nitrogen after resection and stored at –80 ℃ for further experiments. Clinical data were also collected, which included age at diagnosis, gender, tumor markers [preoperative carcinoembryonic antigen (CEA), preoperative CA19-9], clinicopathologic characteristics [tumor location, tumor-node-metastasis (TNM) stages, tumor invasion, lymph nodes involved, metastasis status, pathological classification, histological classification and differentiation degree] and clinical information of disease diagnosis and treatment (cancer detection methods, operation methods, the use of intestinal stapler, intraoperative chemotherapy, postoperative chemotherapy and postoperative radiotherapy). All patients provided informed consent at the time of sample collection. The study was approved by the Research Ethics Committee of Harbin Medical University.

The OS time was used as a measure of prognosis, which was defined as the time from surgery to the patient's death of all causes or the last follow-up visit. The date of the last follow-up was March 15, 2014 (109 months).

DNA extraction and sodium bisulfate modification

Genomic DNA was extracted from tumor tissue and adjacent non-tumor tissue specimens using the classical phenol-chloroform procedure and stored at –80 ℃.

Genomic DNA was bisulfate treated using a commercially available DNA modification kit (EpiTect Bisulfite Kit®, Qiagen, Hilden, Germany) and stored at –20 ℃. All procedures were followed according to manufacturer's instructions.

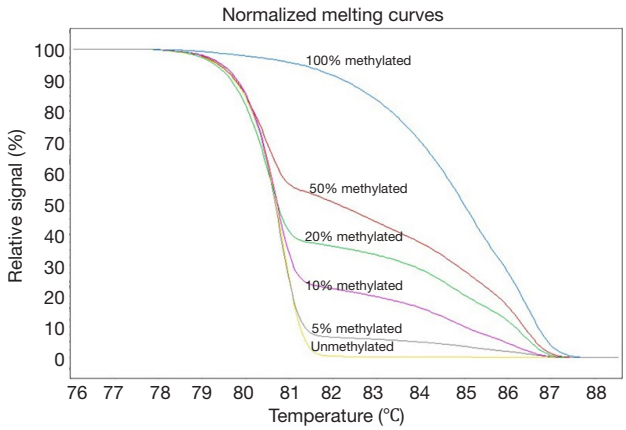

Figure 1 Profile of fluorescence obtained at the melting temperature for serial dilutions of methylated DNA (100%, 50%, 20%, 10% and 5%) in *MAL* gene.

Methylation analysis of MAL

Methylation-sensitive high resolution melting (MS-HRM) was performed on a LightCycler 480 (Roche Applied Science, Mannheim, Germany) equipped with Gene Scanning software (version 2.0) to detect and analyze *MAL* methylation, as previously published (23). Universal unmethylated (0% methylated) and methylated (100% methylated) human whole genomic DNA samples (Zymo Research Corp., Irvine, CA, USA) were used as the unmethylated control and methylated DNA for MS-HRM analysis, respectively. A series of standards, including 100%, 50%, 20%, 10%, 5% and 0% methylated DNA (*Figure 1*), in a background of universal unmethylated DNA, were constructed by serially diluting the methylated control DNA into the unmethylated control according to mass concentration.

The *MAL* primers for MS-HRM analysis were as follows: forward primer, 5'-TGG TGA AGA TAG AGA AGT TAT TGG GTA GG-3'; and reverse primer, 5'-AAA ACC CCC AAA CCA CTA AAC TC-3', as reported previously (24). The amplification length was 160 bp, which was located at the CpG island close to the transcription start point of *MAL* gene. The whole reaction volume was 5.0 μL, which contained 2.5 μL of 1× LightCycler 480 High Resolution Melting Master Mix (Roche, Mannheim, Germany), 0.7 μL $MgCl_2$ (25 mM), 0.1 μL of each forward and reverse primer(10 μM), 1.1 μL of polymerase chain reaction (PCR)-grade water and 0.5 μL of bisulfate-treated DNA. The cycling protocol started with one cycle at 95 ℃ for 10 min, followed by 50 cycles at 95 ℃ for 10 s, a touchdown of 64–58 ℃ for 30 s (0.4 ℃/step) followed by 5 delays, 72 ℃ for 20 s, and a HRM step at 95 ℃ for 1 min, 40 ℃ for 1 min, and 70 ℃ for 5 s. The melting step was performed using a continuous acquisition between 70 and 93 ℃ at 40 acquisitions per 1 ℃. Each plate included a water-blank as a negative control. A series of standard substances with known methylation ratios were included in each assay. To ensure the veracity and repeatability of the experiment, all samples were conducted independently in duplicate for each experiment. MS-HRM data were analyzed using Gene Scanning Software (Roche, Mannheim, Germany). Data processing included normalization and temperature shifting using LightCycler 480. The *MAL* methylation of tissue from CRC patients was determined by comparing curves of each sample to be tested with the series of standard substances in gene scanning module.

Statistical analysis

The cut-off value of *MAL* methylation was determined by using receiver operator characteristic (ROC) curve for permitting the clearest discrimination between tumor tissues and adjacent non-tumor tissues and for distinguishing hypermethylation from hypomethylation of tumor tissues. Missing values of the investigation data were generated using multiple imputation method provided by SPSS. All results were derived from pooled data of dataset obtained by multiple imputation method. The association between *MAL* methylation and clinicopathologic characteristics was evaluated with χ^2 test. The survival rates were estimated using life table method. The comparison of survival rates between hypomethylation and hypermethylation groups

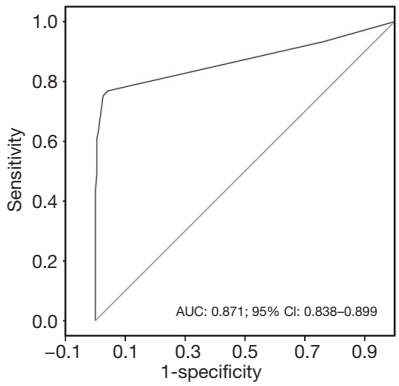

Figure 2 ROC curve of *MAL* methylation from tumor tissues and adjacent non-tumor tissues. ROC, receiver operator characteristic; AUC, area under the curve; CI, confidence interval.

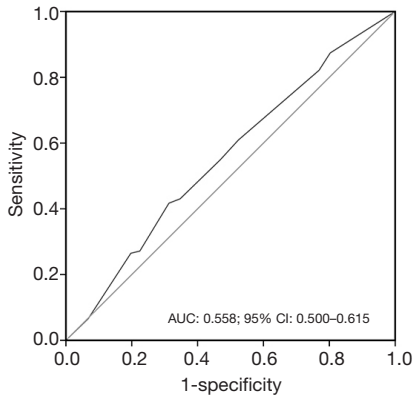

Figure 3 ROC curve of *MAL* methylation from tumor tissues. ROC, receiver operator characteristic; AUC, area under the curve; CI, confidence interval.

was performed using log-rank tests. Associations of *MAL* methylation, clinicopathologic characteristics and clinical information of disease diagnosis and treatment with OS were analyzed by univariate Cox regression. Multivariate Cox regression was then employed to estimate the independent prognostic effect of *MAL* methylation. Hazard ratio (HR) and corresponding 95% confidence interval (CI) were calculated by Cox regression. Survival curve was constructed using the GraphPad Prism 7.0. All statistical analyses were performed using IBM SPSS Statistics 23.0 and two-sided P values <0.05 were considered to be statistically significant.

Propensity score (PS) method was used for accounting for baseline differences in characteristics between methylation groups. Multivariate logistic regression model was developed to estimate the PS, including the variables that are related to both outcomes and group decision or outcomes only.

Results

MAL methylation in tumor and adjacent non-tumor tissues

We examined *MAL* methylation of 298 primary tumor tissue specimens and 193 adjacent non-tumor tissue specimens. In tumor and adjacent non-tumor tissues, the median of *MAL* methylation were 20.0% (ranged from 0.0% to 60.0%) and 2.5% (ranged from 0.0% to 20.0%), respectively. The *MAL* methylation level of adjacent non-tumor tissues was significantly lower than tumor tissues (Mann-Whitney U test, P<0.001).

The cut-off value of *MAL* methylation was 2.5%, which had a high predictive ability to distinguish tumor tissues from adjacent non-tumor tissues. The sensitivity and specificity were 0.768 and 0.960, respectively, with the area under the curve (AUC) 0.871 (95% CI: 0.838–0.899) (*Figure 2*). There were 229 (76.8%) samples with *MAL* methylation >2.5% in tumor tissues, whereas there were only 8 (4.0%) samples in adjacent non-tumor tissues.

For further survival analysis, the cut-off value of *MAL* methylation for distinguishing the survival status was ≤7.5%, which meant that the tumor tissue with methylation ≤7.5% was defined as hypomethylation, others were defined as hypermethylation. The sensitivity and specificity were 0.422 and 0.689, respectively, with AUC 0.558 (95% CI: 0.500–0.615) (*Figure 3*). Thirty-six point six percent (109/298) CRC patients were in hypomethylation group, whereas 63.4% (189/298) CRC patients were in hypermethylation group.

The association between MAL methylation of tumor tissues and clinicopathologic characteristics of CRC patients

The median age of diagnosis for 298 CRC patients was 58 years old (ranging from 25–80 years old), and the male-to-female ratio was 1.42. The result showed that *MAL* methylation had no significant correlation with clinicopathologic characteristics (P>0.05) (*Table 1*).

MAL methylation in tumor tissues and prognosis of CRC patients

At the end of the 109 months' follow-up, 41.3% (123/298)

Table 1 Demographic and clinicopathologic characteristics of patients and *MAL* methylation in tumor tissues (N=298)

Characteristics	Total (%)	*MAL* methylation, n (%)		P
		Hypomethylation (N=109)	Hypermethylation (N=189)	
Age at diagnosis				0.103
<60 years	162 (54.4)	66 (60.6)	96 (50.8)	
≥60 years	136 (45.6)	43 (39.4)	93 (49.2)	
Gender				0.623
Male	175 (58.7)	62 (56.9)	113 (59.8)	
Female	123 (41.3)	47 (43.1)	76 (40.2)	
CEA				0.627
<5 ng/mL	123 (41.3)	43 (39.4)	80 (42.3)	
≥5 ng/mL	175 (58.7)	66 (60.6)	109 (57.7)	
CA19-9				0.711
<37 U/mL	217 (72.8)	78 (71.6)	139 (73.5)	
≥37 U/mL	81 (27.2)	31 (28.4)	50 (26.5)	
Tumor location				0.953
Colon	110 (36.9)	40 (36.7)	70 (37.0)	
Rectum	188 (63.1)	69 (63.3)	119 (63.0)	
TNM stages				0.198
I–II	173 (58.1)	58 (53.2)	115 (60.8)	
III–IV	125 (41.9)	51 (46.8)	74 (39.2)	
Tumor invasion				0.279
T1–T3	149 (50.0)	50 (45.9)	99 (52.4)	
T4	149 (50.0)	59 (54.1)	90 (47.6)	
Lymph nodes involved				0.232
N0	172 (57.7)	58 (53.2)	114 (60.3)	
N1–N2	126 (42.3)	51 (46.8)	75 (39.7)	
Metastasis status				0.617
M0	284 (95.3)	103 (94.5)	181 (95.8)	
M1	14 (4.7)	6 (5.5)	8 (4.2)	
Pathological classification				0.157
Prominence	201 (67.4)	68 (62.4)	133 (70.4)	
Others	97 (32.6)	41 (37.6)	56 (29.6)	
Histologic classification				0.841
Adenocarcinoma	227 (76.2)	85 (78.0)	142 (75.1)	
Mucinous adenocarcinoma	60 (20.1)	20 (18.3)	40 (21.2)	
Others	11 (3.7)	4 (3.7)	7 (3.7)	
Differentiation degree				0.424
Poor	48 (16.1)	20 (18.3)	28 (14.8)	
Moderate or well	250 (83.9)	89 (81.7)	161 (85.2)	

CEA, carcinoembryonic antigen; TNM, tumor-node-metastasis.

Table 2 The overall survival rates at 1-, 3-, 5- and 8-year in groups stratified by *MAL* methylation in tumor tissues (N=298)

Groups	1-year		3-year		5-year		8-year	
	OSR (SE)	P	OSR (SE)	P	OSR (SE)	P	OSR (SE)	P
All patients (N=298), n (%)	0.9292 (0.0149)		0.7775 (0.0244)		0.6530 (0.0283)		0.5121 (0.0381)	
Methylation levels, n (%)		0.274		0.026		0.007		0.013
Hypomethylation (N=109)	0.9079 (0.0278)		0.7080 (0.0442)		0.5569 (0.0491)		0.4287 (0.0641)	
Hypermethylation (N=189)	0.9416 (0.0171)		0.8171 (0.0284)		0.7078 (0.0339)		0.5593 (0.0475)	

OSR, overall survival rate; SE, standard error.

of the patients died and 45.0% (134/298) were still alive. The median OS time was 58 months for hypomethylation group, 74 months for hypermethylation group and 73 months for all patients (the follow-up time ranged from 1–109 months). In hypermethylation group, the survival rates at 1-, 3-, 5-, and 8-year were 94.16%, 81.71%, 70.78% and 55.93%, respectively. In addition, 3-, 5- and 8-year survival rate in hypermethylation group were significantly higher than that rate in hypomethylation group (*Table 2*).

The univariate Cox analysis was performed regarding all variables and *MAL* methylation (*Table 3*). Subsequently, the multivariate Cox regression was performed, which included age at diagnosis (P=0.522), gender (P=0.503) and other significant variables revealed in univariate Cox analysis (*Table 3*). PS was estimated by covariates model including age at diagnosis, gender, preoperative CA19-9, TNM stages, differentiation degree and operation methods. The multivariate Cox regression analysis showed that the preoperative CA19-9, TNM stages, differentiation degree, operation methods and *MAL* methylation were independent predictors of prognosis for postoperative CRC patients (*Table 3*, *Figure 4*). Also, *MAL* hypermethylation was significantly associated with a favorable clinical outcome with the HR of 0.613 (95% CI: 0.422–0.889, P=0.010) and 0.692 (95% CI: 0.481–0.996, P=0.047) in multivariate Cox regression and PS analysis respectively in all patients (*Table 4*).

The differences in OS rates stratified by age at diagnosis, gender, tumor location and TNM stages were also analyzed. The CRC *MAL* hypermethylation patients in subgroup of age at diagnosis <60 and colon cancer had a significantly favorable outcome in the three models (*Table 4*). In multivariate Cox regression, *MAL* hypermethylation in male CRC and stages III–IV patients had a favorable outcome significantly with the HR of 0.590 (95% CI: 0.353–0.986, P=0.044) and 0.548 (95% CI: 0.336–0.892, P=0.016), respectively (*Table 4*).

Discussion

It is well known that biomarkers can be used for disease diagnosis, for predicting disease prognosis and for optimizing treatment strategies. Evidence had shown that methylated DNA signatures can develop into prognostic biomarkers in CRC (5). The *MAL* gene encodes a non-glycosylated membrane protein that is expressed in a restricted pattern of cell types including T lymphocytes (11), myelin-forming cells (25) and polarized epithelial cells (26). Mal protein is a component of the integral protein machinery for apical transport in polarized epithelial cells (26) and participates in membrane signal transduction (27). Mori *et al.* reported that *MAL* promoter hypermethylation was found in primary colon cancer, while the methylation level was significantly lower or absent in normal colonic mucosae (28). However, the implications of *MAL* methylation on the prognosis of postoperative CRC patients are not clear. To the best our knowledge, this is the first report investigating the association between *MAL* methylation and prognosis of postoperative CRC patients.

The investigated CpG island of *MAL* was located in the first exon, which is close to the transcription start position. As a simple, reliable and high sensitive technique, MS-HRM can even assess individual CpG site and detect low-abundance (as low as 0.1–1.0%) methylation (29,30). This technique is more convenient and cost-effective than pyrosequencing, which counts every CpG site (29,31-33). Liu *et al.* (34), Candiloro *et al.* (35) and Liu *et al.* (36) had demonstrated significant consistency of gene methylation between the detection of MS-HRM and pyrosequencing methods.

As a result, we found that the *MAL* methylation level was significantly higher in tumor tissues than that in adjacent non-tumor tissues. The tumor tissues hypermethylation were found in 76.8% patients, whereas only 4.0% patients in adjacent non-tumor tissues. Our results are in keeping

Table 3 Univariate and multivariate Cox analysis for association between methylation of *MAL*, different factors and OS

Variables	Number		Univariate Cox		Multivariate Cox	
	Patients (N=298)	Deaths (N=123)	Crude HR (95% CI)	P	Adjusted HR (95% CI)	P
Age at diagnosis				0.522		0.121
<60 years	162	64	1.000		1.000	
≥60 years	136	59	1.122 (0.788–1.599)		1.349 (0.924–1.970)	
Gender				0.503		0.978
Male	175	70	1.000		1.000	
Female	123	53	1.130 (0.791–1.615)		0.995 (0.673–1.470)	
CEA				0.001		0.711
<5 ng/mL	123	36	1.000		1.000	
≥5 ng/mL	175	87	1.935 (1.312–2.855)		0.915 (0.571–1.466)	
CA19-9				0.000		0.000
<37 U/mL	217	55	1.000		1.000	
≥37 U/mL	81	68	5.628 (3.917–8.084)		4.756 (3.012–7.511)	
Tumor location				0.471		–
Colon	110	42	1.000		–	
Rectum	188	81	1.147 (0.790–1.665)		–	
TNM Stages				0.000		0.000
I–II	173	46	1.000		1.000	
III–IV	125	77	3.209 (2.223–4.632)		2.442 (1.663–3.585)	
Pathological classification				0.001		0.086
Prominence	201	69	1.000		1.000	
Others	97	54	1.913 (1.312–2.788)		1.392 (0.954–2.031)	
Histologic classification				–[†]		–
Adenocarcinoma	227	95	1.000		–	
Mucinous adenocarcinoma	60	23	0.969 (0.614–1.529)		–	
Others	11	5	1.094 (0.445–2.689)		–	
Differentiation degree				0.012		0.028
Poor	48	25	1.000		1.000	
Moderate or well	250	98	0.554 (0.350–0.876)		0.564 (0.338–0.940)	
Cancer detection methods				0.281		–
Symptoms appear	291	119	1.000		–	
Others	7	4	1.731 (0.638–4.694)		–	
Operation methods				0.000		0.000
Radical surgery	285	110	1.000		1.000	
Others	13	13	17.358 (9.196–32.766)		6.056 (3.080–11.905)	

Table 3 (*continued*)

Table 3 (*continued*)

Variables	Number		Univariate Cox		Multivariate Cox	
	Patients (N=298)	Deaths (N=123)	Crude HR (95% CI)	P	Adjusted HR (95% CI)	P
Intestinal stapler				–†		–†
Yes	223	82	1.000		1.000	
No	63	34	1.594 (1.062–2.392)*		1.674 (1.102–2.542)*	
Unknown	12	7	1.575 (0.733–3.384)		1.179 (0.525–2.649)	
Intraoperative chemotherapy				–†		–
Yes	83	30	1.000		–	
No	200	82	1.145 (0.751–1.745)		–	
Unknown	15	11	2.220 (1.106–4.458)*		–	
Postoperative chemotherapy				0.865		–
Yes	135	57	1.000		–	
No	163	66	1.031 (0.723–1.471)		–	
Postoperative radiotherapy				0.013		0.131
Yes	14	10	1.000		1.000	
No	284	113	0.424 (0.215–0.833)		0.586 (0.293–1.172)	
MAL methylation				0.018		0.010
Hypomethylation	109	53	1.000		1.000	
Hypermethylation	189	70	0.650 (0.454–0.929)		0.613 (0.422–0.889)	

†, pooled data was not provided by SPSS 23.0; *, statistically significant association. OS, overall survival; HR, hazard ratio; CI, confidence interval; CEA, carcinoembryonic antigen; TNM, tumor-node-metastasis.

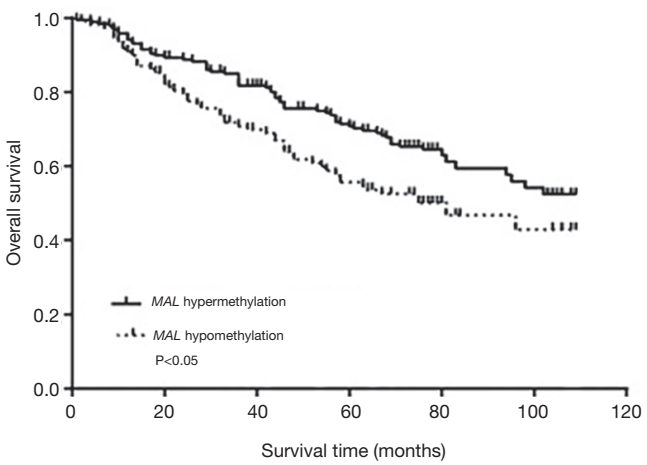

Figure 4 Survival curve of CRC patients with *MAL* hypermethylation and *MAL* hypomethylation. CRC, colorectal cancer.

with those from previously published studies (21,37,38). The sensitivity and specificity were 0.768 and 0.960, respectively, with AUC of 0.871 (95% CI: 0.838–0.899) for tumor tissues versus adjacent non-tumor tissues. Lind *et al.* reported that multi-gene in combination with *MAL* resulted in a biomarker panel with a sensitivity of 94% and a specificity of 98% (37), which was consistent with the stability and reliability of the results in our study. The high specificity would increase the positive predictive value in judging CRC tumor tissue and adjacent non-tumor tissue.

We collected comprehensive clinicopathologic characteristics data including preoperative CEA, preoperative CA19-9, pathological classification, histologic classification and differentiation degree in our study. The results showed that *MAL* methylation had no significant correlation with these clinicopathologic characteristics. Lind *et al.* (37) and

Table 4 Subgroup analysis on the association between *MAL* methylation and OS

Subgroup	Methylation	Number		Univariate Cox		Multivariate Cox		Propensity score	
		Patients	Deaths	Crude HR (95% CI)	P value	Adjusted HR† (95% CI)	P value	Adjusted HR‡ (95% CI)	P value
All					0.018		0.010		0.047
	Hypomethylation	109	53	1.000		1.000		1.000	
	Hypermethylation	189	70	0.650 (0.454–0.929)		0.613 (0.422–0.889)		0.692 (0.481–0.996)	
Age									
<60 years	Hypomethylation	66	33	1.000	0.006	1.000	0.001	1.000	0.013
	Hypermethylation	96	31	0.502 (0.307–0.821)		0.406 (0.233–0.704)		0.527 (0.317–0.876)	
≥60 years	Hypomethylation	43	20	1.000	0.522	1.000	0.574	1.000	0.917
	Hypermethylation	93	39	0.838 (0.488–1.439)		0.851 (0.485–1.493)		0.968 (0.522–1.796)	
Gender									
Male	Hypomethylation	62	27	1.000	0.378	1.000	0.044	1.000	0.421
	Hypermethylation	113	43	0.805 (0.497–1.304)		0.590 (0.353–0.986)		0.820 (0.506–1.330)	
Female	Hypomethylation	47	26	1.000	0.010	1.000	0.134	1.000	0.055
	Hypermethylation	76	27	0.489 (0.285–0.841)		0.654 (0.375–1.141)		0.560 (0.309–1.014)	
Tumor location									
Colon	Hypomethylation	40	20	1.000	0.031	1.000	0.008	1.000	0.024
	Hypermethylation	70	22	0.512 (0.279–0.942)		0.402 (0.206–0.787)		0.491 (0.265–0.910)	
Rectum	Hypomethylation	69	33	1.000	0.222	1.000	0.156	1.000	0.492
	Hypermethylation	119	48	0.758 (0.487–1.182)		0.711 (0.443–1.139)		0.853 (0.541–1.344)	
TNM stage									
I–II	Hypomethylation	58	20	1.000	0.083	1.000	0.409	1.000	0.086
	Hypermethylation	115	26	0.597 (0.333–1.070)		0.751 (0.380–1.483)		0.586 (0.318–1.078)	
III–IV	Hypomethylation	51	33	1.000	0.193	1.000	0.016	1.000	0.166
	Hypermethylation	74	44	0.740 (0.470–1.164)		0.548 (0.336–0.892)		0.718 (0.449–1.148)	

†, controlling for the variables which were revealed statistically significant differences in univariate Cox analysis; ‡, covariates model including age at diagnosis, gender, preoperative CA19-9, TNM stages, differentiation degree and operation methods. OS, overall survival; HR, hazard ratio; CI, confidence interval; CEA, carcinoembryonic antigen; TNM, tumor-node-metastasis.

Kang *et al.* (39) also reported that there were no statistically significant differences between *MAL* methylation and gender, age, tumor location and TNM stages in patients with CRC. In addition, there was also no statistically significant difference between *MAL* methylation and the clinicopathologic characteristics of patients with gastric cancer (16,20). Unfortunately, the proportion of non-Han population was only 1.0% (3/298), so that the analysis was impossible. Consequently, combined with the above results, *MAL* methylation might independently affect the survival outcome of CRC patients, rather than affecting the outcome via clinicopathologic characteristics.

Our study is also the first to evaluate the survival rates of CRC patients with different *MAL* methylation. *MAL* hypermethylation group had a survival benefit with a longer survival time over 16 months. *MAL* hypermethylation group had higher OS rates at 3-, 5- and 8-year compared to the hypomethylation group (*Table 2*) except for 1 year, which

indicated that *MAL* hypermethylation had a significant impact on the long-term survival of postoperative patients with CRC.

Multivariate Cox proportional hazard regression showed that *MAL* methylation, preoperative CA19-9, TNM stages, differentiation degree and operation methods might be independent prognostic predictors for postoperative patients with CRC. *MAL* hypermethylated patients had a survival advantage. Usually, as a tumor-suppressor gene, *MAL* silencing has been associated with promoter hypermethylation, which could re-induce gene expression after demethylation treatment in colon cancer cell lines. In addition, *MAL* protein was not expressed in the malignant cells (21). Our results were contradicted with the hypothesis that silencing of *MAL* by hypermethylation is associated with a better prognosis for the CRC patients. Furthermore, other researchers had the same conclusion with ours. For example, Hsi *et al.* found that Hodgkin lymphoma patients with *MAL* expression had a poor outcome compared with patients without *MAL* (40). In addition, Buffart *et al.* also revealed that the hypermethylation of *MAL* promoter correlated with a significantly better survival rate of patients with gastric cancer (20). Therefore, we hypothesized that the *MAL* hypermethylation might lead to additional genetic changes (41) or *MAL* methylation interact with other gene methylation (42), rather than dependent on *MAL* methylation alone.

The Cancer Genome Atlas (TCGA) dataset was utilized to validate our results. The CRC datasets of Illumina Human Methylation 450 including colon cancer and rectal cancer were downloaded from UCSC Xena (https://xena.ucsc.edu/) respectively and merged. The dataset included a total of 396 specimens, of which 88 died. The follow-up period ranged from 0.19 to 145.23 months. Only cg22403344 site was located in the same region of our amplification. There was no statistically significant association between methylation of cg22403344 and prognosis of CRC patients, with HR of 1.236 (95% CI: 0.811–1.884, P=0.324). However, with the 20 CG sites in our amplification, there were no similar results as our research.

Our subgroup analysis calculated the differences in OS rates, which was stratified by age at diagnosis, gender, tumor location and TNM stages. First, we found that age at diagnosis <60 years old patients with *MAL* hypermethylation had a better survival outcome. According to Toyota *et al.*, methylation type A was defined as slight amount methylated in normal mucosa and frequently methylated in tumor tissues, which was associated with aging-specific methylation (41). Based on this, we assumed that *MAL* methylation might act as type A methylation since they shared same age associated methylation pattern. In addition, we speculated that the association between *MAL* hypermethylation and a favorable prognosis might be masked in higher age group. Secondly, our result showed that *MAL* hypermethylation patients had a lower risk of death in colon cancer, whereas difference of OS rates between hypermethylation and hypomethylation was not obvious in rectal cancer group. Thirdly, since patients with early CRC (TNM stages I–II) generally had a better prognosis than those with TNM stages III–IV, we extended the findings that *MAL* hypermethylation patients with TNM stages III–IV had a higher OS rate. PS has been proved to be a useful, innovative and creative statistical method for evaluating intervention effects in non-experimental or observational studies. The PS analysis included age at diagnosis, gender, preoperative CA19-9, TNM stages, differentiation degree and operation methods into a single covariate, which was used to adjust for baseline differences. The PS analysis confirmed the stability and reliability of the results of multivariate survival analysis.

It is worthy of note that this is a novel study about *MAL* methylation on the prognosis of postoperative patients with CRC in a patient cohort. We tested tumor tissues and adjacent non-tumor tissues from surgical patients using MS-HRM. Compared with other forms of clinical samples, tissue samples were more stable and reliable. However, there are limitations to be considered. Tumor-specific death was not assessed in our study. The data collected on cancer treatment were limited, and we were restricted from analyzing the association between the *MAL* methylation and treatment decision, which can be utilized to establish more personalized treatment strategies.

Conclusions

MAL hypermethylation was frequently observed in tumor tissues of patients with CRC and it might be an independent predictor of survival advantage in postoperative patients with CRC. Compared with *MAL* hypomethylation patients, the hypermethylation patients had a favorable prognosis in younger than 60-year-old patients group and colon cancer patients group.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.09.04). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). All patients provided informed consent at the time of sample collection. The study was approved by the Research Ethics Committee of Harbin Medical University.

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