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Increased expression of EHMT2 associated with H3K9me2 level contributes to the poor prognosis of gastric cancer

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Abstract. Di-methylated lysine 9 of histone H3 (H3K9me2), regulated by histone methyltransferases, is involved in the epigenetic regulation of tumor-associated genes. The present study aimed to evaluate whether the H3K9me2 methylation level is associated with the expression level of euchromatic histone lysine methyltransferase 2 (EHMT2) in the prognosis of gastric cancer (GC). H3K9me2 methylation level and EHMT2 expression level were detected by immunohistochemistry in 118 GC samples. The clinicopathological significance of H3K9me2 and EHMT2 in patients with GC was assessed using a paired Student's t-test, χ^2 test, Kaplan-Meier analysis with a log-rank test and Cox's proportional hazard analysis. Strong positive immunostaining of H3K9me2 and EHMT2 was observed in cancerous tissues compared with adjacent non-cancerous tissues. Positive immunostaining of EHMT2 and H3K9me2 was associated with lymph node metastasis, pathological grade and tumor-node-metastasis stage. H3K9me2 expression level was increased in tumor tissue and associated with worse specific-disease and disease-free survival time. In addition, EHMT2 protein expression levels were associated with the expression levels of H3K9me2. Low expression levels of H3K9me2 and EHMT2 predicted a better prognosis of patients with GC. The survival time of patients with a high expression of H3K9me2 and/or EHMT2 was significantly shorter compared with that of the patients with a low expression of H3K9me2 and/or EHMT2. In conclusion, an overexpression pattern of H3K9me2 and/or EHMT2 may be associated with clinicopathological features of GC and may be predictor markers of progression and prognosis in patients with GC, in addition to putative therapeutic targets.

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Key words: di-methylated lysine 9 of histone H3, euchromatic histone lysine methyltransferase 2, histone methylation, gastric cancer, chromatin remodeling

Introduction

Gastric cancer (GC) is one of the most severe tumor types with a high mortality rate (1,2) and poor prognosis (3,4). The global pattern of histone modifications may serve as a predictor of the risk of recurrence of human cancer (5,6). Histone modification, as a notable component of epigenetics, occurs in a diverse range of biological processes. Aberrant post-translational modification of histone tails by methylation is closely associated with tumor development, progression, prognosis and recurrence (7). For example, di-methylation of lysine 9 of histone H3 (H3K9me2) is correlated with gene repression and serves a well-established function in heterochromatin formation and gene transcription regulation in human cancer (8). Among well-studied histone methylations, the methylation pattern of H3K9 is associated with gene regulation including repression (9). Euchromatic histone lysine methyltransferase 2 (EHMT2; also known as G9a), which is a lysine methyltransferase that contributes to the epigenetic silencing of tumor suppressor genes, is required for H3K9me2 (10). EHMT2 may catalyze a modification at histone 3 lysine 9 including H3K9me1 and H3K9me2; H3K9me1 is associated with gene activation, whereas H3K9me2 is predominant in silenced genes (11). EHMT2-dependent H3K9me2 is associated with gene silencing and functions primarily through the recruitment of H3K9me2-binding proteins that prevent transcriptional activation (12). EHMT2 has been reported to be overexpressed in pancreatic (13), breast (14,15), lung (16,17), hepatocellular (18), colorectal carcinoma (19) and GC (20).

The abnormal expression level of EHMT2 and H3K9me2 has been identified in multiple types of cancer, including hematologic malignancies (21). However, the clinical signifi-

cance of EHMT2, H3K9me2 and their interactions in solid tumor types, including in GC, remains unclear. A previous study has revealed that H3K9me2 may contribute to DNA methylation via DNA (cytosine-5-) methyltransferase 3 αb to repress E-cadherin in the epithelial-mesenchymal transition-associated metastasis of GC (22). Additionally, the hypoxic silencing of tumor suppressor Runt-related transcription factor 3 may also be mediated by upregulated EHMT2 and histone deacetylase 1 in GC cells (20). Increased EHMT2 levels in GC tissues may also promote tumor invasion and metastasis, and are associated with an with advanced stage and shorter overall survival time in a SET domain-independent manner (23). Previously, accumulating evidence has indicated that investigation into the clinical importance of EHMT2 levels and H3K9me2 methylation patterns may be of help for the diagnosis and treatment of GC (24-26).

The aim of the present study was to evaluate the methylation pattern of H3K9me2 and EHMT2 expression levels in GC and adjacent healthy tissues, and to reveal the association between the increased EHMT2 expression and H3K9me2 methylation levels.

Materials and methods

Clinical cases with GC. A total of 118 archived paraffin-embedded GC specimen blocks were selected retrospectively from the Department of Pathology of Yancheng Hospital (Jiangsu, China). The specimens were collected from patients (82 men and 36 women) with GC who underwent surgery between March 2010 and December 2011. Medical records, including clinicopathological parameters and follow-up data, were also obtained. The inclusion criteria were as follows: i) No other serious or fatal diseases and ii) if the patients died during the follow-up period, the cause of mortality should be the secondary change, including tumor progression, cachexia, recurrence and metastasis, and follow-up data were complete. The American Joint Committee on Cancer staging system (8th edition) (27) was used for pathological tumor-node-metastasis (pTNM) staging. The mean and median follow-up period was 38.1 months and 41.0 months (range, 1-60 months), respectively. The present study was ethically approved and supervised by the Committee for Ethical Review of Research of Yancheng Hospital.

Table I. Clinicopathological characteristics of patients with gastric carcinoma (n=118).

Characteristics	Patient, n	Percentage, %
Sex		
Male	82	69.5
Female	36	30.5
Age, years		
≤60	59	50.0
>60	59	50.0
Differentiation degree		
Well-differentiated	16	13.6
Moderately differentiated	40	33.9
Poorly differentiated	62	52.5
Lymph node metastasis		
Negative	51	43.2
Positive	67	56.8
Distal metastasis		
Negative	110	93.2
Positive	8	6.8
pTNM		
I	30	25.4
II	33	28.0
III	47	39.8
IV	8	6.8
Survival time, months following operation		
≤5	75	63.6
>5	43	36.4

pTNM, pathological tumor-node-metastasis.

Immunohistochemical staining. The selected paraffin blocks were sliced into 4-μm tissue sections, heated for 60 min at 65˚C and cooled down. The sections were subsequently deparaffinized, rehydrated and placed with ethylene diamine tetraacetic acid antigen retrieval solution (pH 8.0) to retrieve the antigens, followed by incubation with 3% H_2O_2 for 25 min at room temperature (RT). Following blocking with 3% BSA for 30 min at RT, the sections were treated with primary antibodies against H3K9me2 (1:200; cat. no. ab1220; Abcam) and EHMT2 (1:800; cat. no. ab185050; Abcam) in a wet box at 4˚C overnight. The sections were further incubated with horseradish peroxidase-conjugated secondary antibodies (cat. no. K5007; Dako; Agilent Technologies GmbH) for 50 min at RT. The staining was visualized using freshly prepared 3,3'-diaminobenzidine reagent (cat. no. G1211; Wuhan Servicebio Technology Co., Ltd.). The chromogenic reaction was stopped when the nuclei appeared brown/yellow under the microscope; subsequently, the nuclei were counterstained with hematoxylin staining solution at RT. Following dehydration and washing, the slides were mounted with coverslips, and the staining was evaluated under an Axiocam 105 light microscope (magnification, x100 and x400; Carl Zeiss AG). Cells treated with PBS instead of the primary antibody were used as a negative control.

Evaluation of immunostained epithelial tissues. The staining was blindly examined by two associate professors from the Department of Pathology, Medical School, Southeast University (Nanjing, China). The analyzed areas were tumor cells in the cancerous tissues and epithelial cells in the adjacent healthy epithelial tissues. The scoring system was described in previous studies (28,29). In brief, the final score of immunostaining was calculated as the sum product of the scale of staining intensity and the scale of staining area. The scale of intensity was as follows: 0=negative; 1=weak staining; 2=moderate staining and 3=intensive staining; and the scale of staining area was as follows: 0=0-5% posi-

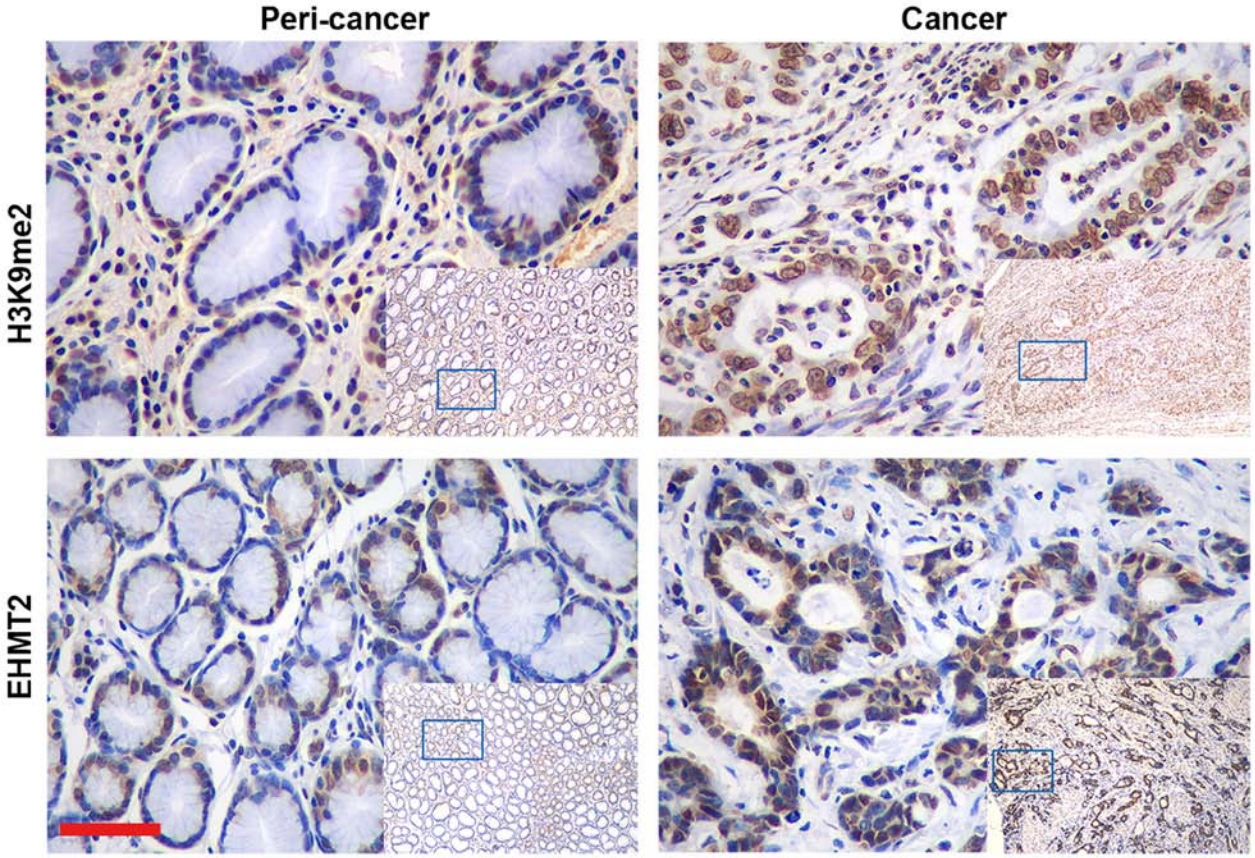

Figure 1. Correct identification of H3K9me2 and EHMT2-stained nuclei in gastric cancer and adjacent healthy tissues. The Ariol system trainer overlay demonstrates the correct identification of negative H3K9me2 and EHMT2 nuclei expression in adjacent healthy tissues (indicated by blue dots) and positive nuclei expression in cancer tissues (yellow/brown dots). Tissue microarray slides were scanned using a magnification, x20. Scale bar, 100 mm. H3K9me2, di-methylated lysine 9 of histone H3; EHMT2, euchromatic histone lysine N-methyltransferase 2.

tive cells; 1=6-25% positive cells; 2=26-50% positive cells; 3=51-75% positive cells and 4=76-100% positive cells. The expression patterns were classified into two groups: Low (score >8) and high (score >8) scoring group. The patients were divided into three groups according to H3K9me2 and EHMT2 expression: i) Low expression group for patients with low expression levels of H3K9me2 and EHMT2; ii) high expression group for patients with high expression levels of H3K9me2 and EHMT2 and iii) other group for patients with high H3K9me2 expression and low EHMT2 expression, and vice versa.

Statistical analysis. Statistical analysis was performed using SPSS software (v.19; IBM Corp.). Data are presented as the mean ± standard error of the mean. The histone modification levels of cancerous and adjacent healthy tissues were assessed using a paired Student's t-test. The associations between clinicopathological variables and the immunostaining scores of histone methylation were analyzed using a χ^2 test or Fischer's exact test. Kaplan-Meier (K-M) analysis with a log-rank test was used to determine the contribution of the clinicopathological features and the immunostaining expression patterns to the patients' survival time. Multivariate analysis using Cox's proportional hazard regression was used to examine the clinical value of the levels of the studied protein and the clinicopathological parameters of the patients. Statistical analyses were performed using SPSS Statistics v.17 (SPSS Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinicopathological profiles of the patients. In the present study, the tissue samples from 82 male and 36 female patients with GC were studied; the median age was 59.7 years (age range, 33-83 years), and the clinicopathological variables are summarized in Table I. The samples comprised 16 well-differentiated cases, 62 poorly differentiated cases and 40 moderately differentiated cases. The pTNM staging was as follows: 30 cases were stage I (25.4%), 33 were stage II (28%), 47 were stage III (39.8%) and 8 were IV (6.8%). Among the 118 patients, the 5-year survival rate following gastrectomy was 36.4%.

H3K9me2 and EHMT2 expression patterns are associated with clinicopathological characteristics in patients with GC. The expression of histone methylation marker H3K9me2 and histone methyltransferase EHMT2 were evaluated in the surgical samples from 118 patients with GC. H3K9me2 was localized in the nuclei of epithelial cells, whereas EHMT2 mainly labelled the nucleus with partial staining in the cytoplasm (Fig. 1). Example tissues exhibiting low and high expression areas are presented in Fig. 2. The assessment of the positive immunoreaction in the epithelial

Table II. Differential expression of histone methylation between gastric cancer and peri-cancer tissues.

Histone methylation	Cancer	Peri-cancer	P-value
H3K9me2	7.557±0.206	7.311±0.236	<0.001[a]
EHMT2	6.765±0.216	4.319±0.228	<0.001[a]

[a]P<0.001. H3K9me2, di-methylated lysine 9 of histone H3; EHMT2, euchromatic histone-lysine N-methyltransferase 2.

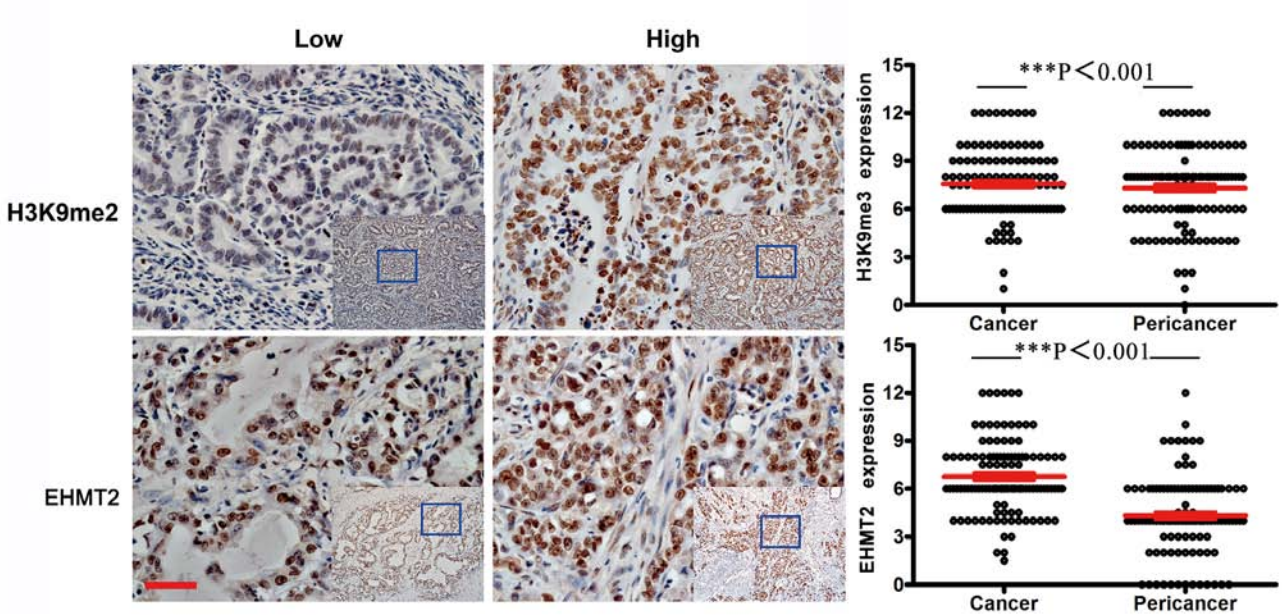

Figure 2. Expression patterns of H3K9me2 and EHMT2 in cancer and peri-cancer tissues. Left, representative images of the low and high expression of H3K9me2 and EHMT2 in cancerous tissues. Scale bar, 100 mm. The graphs on the right-hand side demonstrate the differential expressions of H3K9me2 and EHMT2 between gastric cancer and peri-cancer tissues. ***P<0.001. H3K9me2, di-methylated lysine 9 of histone H3; EHMT2, euchromatic histone-lysine N-methyltransferase 2.

cells of cancerous and non-cancerous tissues demonstrated that cancerous tissues exhibited significantly stronger immunostaining compared with adjacent healthy tissues (P<0.001; Table II).

To clarify the association between patient clinicopathological characteristics, EHMT2 expression and H3K9me2 methylation, the data were analyzed using a χ^2 test; the results demonstrated that EHMT2 overexpression and H3K9me2 methylation levels were significantly associated with the degree of differentiation (P=0.025 and P=0.031, respectively), lymph node involvement (P=0.021 and P=0.021, respectively) and pTNM stage (P=0.036 and P=0.022, respectively), but not sex, age or distal metastasis (Table III; Fig. 3). The results of distal metastasis may have been affected by the low ratio of positive cases (6.8%) in the studied samples.

EHMT2 overexpression was significantly associated with H3K9me2 methylation level (P=0.040; data not shown). The results indicated that EHMT2 and H3K9me2 expression patterns exhibited notable consistency. As presented in Fig. 3, H3K9me2 exhibited similar ratios of cases of each stage of pTNM to EHMT2 when analyzed based on the expression level, which further confirmed their positive association.

Effect of H3K9me2 and EHMT2 on the survival of patients with GC. To understand the impact of H3K9me2 and EHMT2 expression patterns on the survival of patients with GC, overall survival time based on H3K9me2 and EHMT expression levels was further analyzed by K-M analysis with a log-rank test. Patients with low expression levels of H3K9me2 exhibited a significantly longer survival time compared with those in the high expression group (P<0.05). Overexpression of EHMT2 presented the same trend (P<0.05; Fig. 4; Table S1). Mean progression-free survival time of patients in the low and high H3K9me2 expression groups were 45.521±2.195 and 34.061±3.861 months, respectively, and in the low and high EHMT2 expression level groups were 41.761±2.399 and 32.243±3.364 months, respectively (Table S1). The median survival period exhibited similar tendencies. Univariate K-M survival curves demonstrated that tumor differentiation degree, lymph node involvement, distal metastasis and pTNM

Table III. Association between H3K9me2 or EHMT2 expression patterns and clinicopathological characteristics of patients with gastric carcinoma (n=118).

	H3K9me2 expression				EHMT2 expression			
Characteristics	Low	High	χ^2	P-value	Low	High	χ^2	P-value
Sex			0.798	0.372			0.031	0.861
Male	57	25			51	31		
Female	22	14			23	13		
Age, years			0.957	0.328			0.145	0.703
≤60	37	22			38	21		
>60	42	17			36	23		
Differentiation degree			4.661	0.031[a]			5.027	0.025[a]
Well + moderately differentiated	43	13			41	15		
Poorly differentiated	36	26			33	29		
Lymph node metastasis			5.352	0.021[a]			5.346	0.021[a]
Negative	40	11			38	13		
Positive	39	28			36	31		
Distal metastasis			1.114	0.291			0.554	0.457
Negative	75	35			68	42		
Positive	4	4			6	2		
pTNM			5.217	0.022[a]			4.392	0.036[a]
I+II	48	15			45	18		
III+IV	31	24			29	26		

[a]P<0.05. H3K9me2, di-methylated lysine 9 of histone H3; EHMT2, euchromatic histone-lysine N-methyltransferase 2; pTNM, pathological tumor-node-metastasis.

stage were significantly associated with a shorter survival time of patients with GC (P<0.001; Table S2). However, the sex and age of patients exhibited no association with patient survival time in the present study.

Combination of EHMT2 overexpression and H3K9me2 level is an effective prognostic marker for patients with GC. To identify the independent risk factors for the prognosis of patients with GC, multivariate Cox's regression analysis was conducted to evaluate the clinicopathological features of patients with GC and the expression levels of H3K9me2 and EHMT2. The results demonstrated that the expression patterns of H3K9me2 and EHMT2, differentiation degree, lymph node involvement, distal metastasis and pTNM stage exhibited independent significant prognostic effects on patient survival time (P<0.05; Table IV). To present the significant factors more accurately, the patients were regrouped by combining the expression patterns of H3K9me2 and EHMT2. The expression patterns were classified into two groups: Low (score ≤8) and high (score >8) scoring groups. The patients were allocated into three groups: i) Low expression group for patients with low expression levels of H3K9me2 and EHMT2; ii) high expression group for patients with high expression levels of H3K9me2 and EHMT2 and iii) other group for patients with high H3K9me2 expression and low EHMT2 expression, and vice versa. The survival analysis revealed

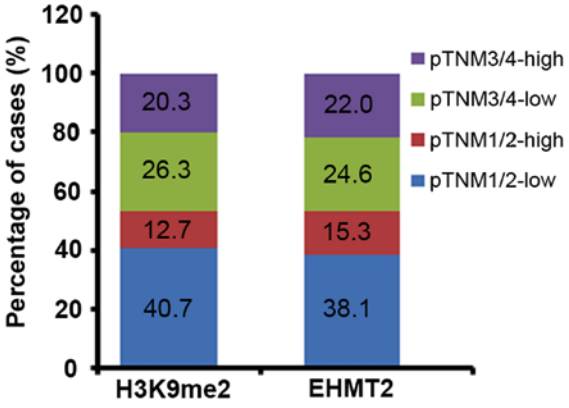

Figure 3. Percentage of cases at each pTNM stage in different H3K9me2 and EHMT2 expression groups. H3K9me2 expression pattern of pTNM exhibited similar ratios to EHMT2 when examined according to the expression level, which further confirmed the association between H3K9me2 and EHMT2. H3K9me2, di-methylated lysine 9 of histone H3; EHMT2, euchromatic histone-lysine N-methyltransferase 2; pTNM, pathological tumor-node-metastasis.

that the high expression group exhibited the shortest survival time (31.467±5.671 months), the low expression group exhibited the longest survival period (46.000±2.711 months) and the other group exhibited an intermediate survival duration

Table IV. Multivariate Cox analysis of overall survival based on individual and combined groups of the histone signatures.

A, Individual groups of histone signatures

Characteristics	HR	95% CI	P-value
Sex	1.088	0.633-1.868	0.939
Age	0.773	0.462-1.293	0.539
Differentiation degree	0.514	0.299-0.882	0.016[a]
Lymph node metastasis	0.230	0.113-0.467	<0.001[c]
Distal metastasis	0.402	0.185-0.872	0.021[a]
pTNM	0.110	0.044-0.271	<0.001[c]
H3K9me2	1.050	0.637-1.729	0.042[a]
EHMT2	1.004	0.599-1.683	0.045[a]

B, Combined groups of histone signatures

Characteristics	HR	95% CI	P-value
Differentiation degree	0.531	0.313-0.901	0.019[a]
Lymph node metastasis	0.234	0.116-0.474	<0.001[c]
Distal metastasis	0.388	0.183-0.822	0.013[a]
pTNM	0.106	0.043-0.258	<0.001[c]
Low expression group	0.009[b]		
High expression group	1.178	0.709-1.957	0.018[a]
Other groups	1.434	0.715-2.876	0.020[a]

[a]P<0.05, [b]P<0.01, [c]P<0.001. HR, hazard ratio; CI, confidence interval; pTNM, pathological tumor-node-metastasis; H3K9me2, di-methylated lysine 9 of histone H3; EHMT2, euchromatic histone-lysine N-methyltransferase 2.

(37.943±2.830 months; Table S1). Therefore, the combination of H3K9me2 and EHMT2 expression patterns may be used as a more accurate indicator for the overall survival time of patients with GC compared with either H3K9me2 or EHMT2 alone (P=0.036; Table S1). In addition, multivariate Cox analysis revealed that the combined expression patterns of H3K9me2 and EHMT2, differentiation degree, lymph node involvement, distal metastasis and pTNM stage significantly predicted the survival time of patients with GC (P<0.05; Table IV).

Discussion

Alterations of epigenetic regulation genes, including DNA methylation, histone modifications, chromatin remodeling and non-coding RNA regulation, have been detected in early carcinogenesis and cancer progression (30-34). A number of them have been proposed as biomarkers for cancer detection and tumor prognosis (35-37). A number of diverse factors, including DNA damage, DNA and histone methylation, in addition to environmental influence, are associated with GC-associated mortality in China (17). Previously, increasing evidence has revealed that aberrant epigenetic regulation serves an important function during tumorigenesis. Histone methylations and their corresponding catalytic enzymes were the focus of a previous study; among various best-studied histone methylations, H3K9 methylation is associated with gene repression (9). Epigenetic alterations of tumor genes are present during carcinogenesis and may provide novel biomarkers for diagnosis (38-40).

EHMT2 was first identified as a gene located in the major histocompatibility complex locus in mice and human leukocyte antigen locus in humans (41). Previous studies have indicated that EHMT2 serves an important function during the carcinogenesis and development of a tumor (13,14,17,42,43). EHMT2 is also a crucial factor in a variety of biological progresses, including behavior plasticity, lymphocyte development, stem cell differentiation and tumor cell growth (44). The clinical importance of EHMT2 expression in numerous cancer tissues has been studied (16); however, the expression pattern of EHMT2 and its significance in GC is largely unclear. Although the crucial functions of H3K9me2 and EHMT2 have been elucidated in several tumor types (45,46), the associations between H3K9me2 methylation pattern and EHMT2 expression level in GC are unknown. EHMT2 is significantly upregulated in numerous different tumor types compared with matched normal controls, and knocking down EHMT2 or the pharmacological inhibition of its activity suppresses tumor cell growth and invasion, indicating that EHMT2 may be an oncogenic and metastatic factor (47,48). In addition, high expression levels of EHMT2 are correlated with a poor overall survival in patients with lung adenocarcinoma (16).

EHMT2 is a main histone lysine methylation enzyme which catalyzes the modification at histone 3 lysine 9,

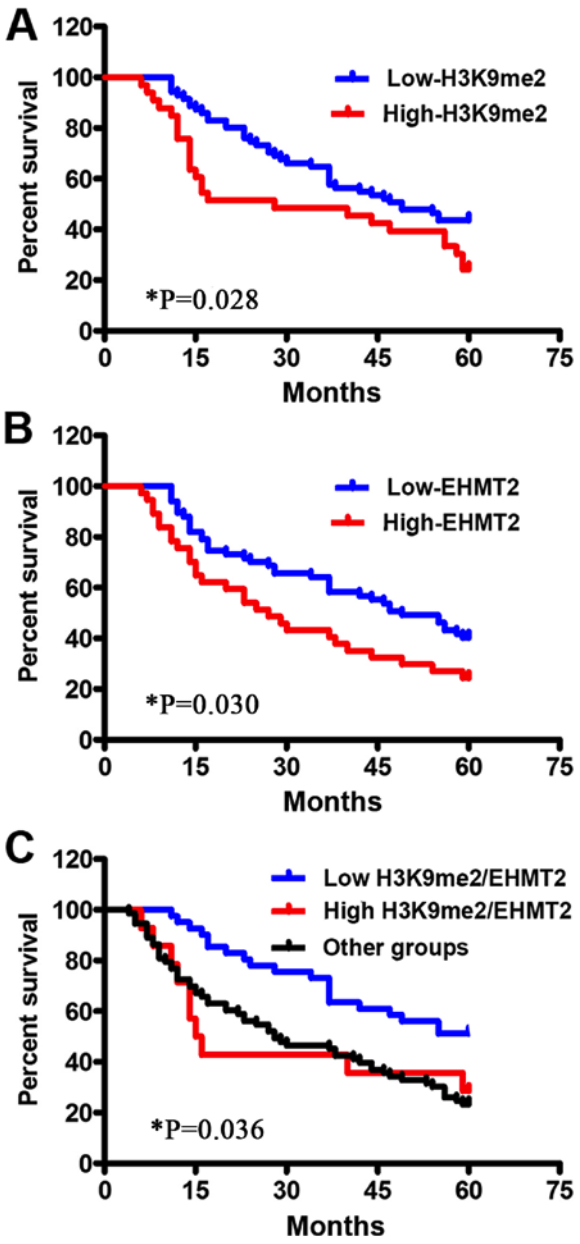

Figure 4. Kaplan-Meier plots of overall survival time, according to H3K9me2 and EHMT2 expression levels in patients with gastric cancer. Kaplan-Meier curves based on the (A) histone methylation levels of H3K9me2, (B) EHMT2 expression levels and (C) combined histone methylation levels of H3K9me2 and EHMT2 expression levels. High H3K9me2 and EHMT2 expression was significantly associated with a poorer overall survival time of patients with gastric cancer. *P<0.05 vs. low expression group. H3K9me2, di-methylated lysine 9 of histone H3; EHMT2, euchromatic histone-lysine N-methyltransferase 2.

including H3K9me1 and H3K9me2. The patterns of H3K9me1 or H3K9me2 are different during development, as there are either more mono- or dimethylations at H3K9 during the maturation of the auditory system (49). During the development of the zebrafish retina, EHMT2 expression and H3K9me2 markers have been noted to be closely associated (50).

H3K9 methylation is a crucial event in reprogramming to pluripotency (51). Numerous studies have demonstrated that the global level of H3K9me2 is associated with the prognosis of prostate and kidney cancer (52,53). These results indicate that there is an association between EHMT2 expression and H3K9me2 markers.

The focus of the present study was the function of EHMT2 and H3K9me2 in the processes associated with a poor prognosis of GC. H3K9me2 and EHMT2 expression exhibited strong immunostaining in GC tumor tissues compared with adjacent healthy tissues. There was an association between the levels of H3K9me2 and EHMT2. The results also demonstrated that EHMT2 expression was associated with the differentiation degree and lymph node metastasis. These results were consistent with a previous study (23), which demonstrated that increased EHMT2 expression in GC tissues correlated with an advanced stage and promoted tumor invasion and metastasis. Depletion of EHMT2 may be of therapeutic value by inhibiting cell proliferation and inducing apoptosis in GC (54). High expression levels of H3K9me2 or EHMT2 were significantly associated with a worse overall survival time in patients with GC. Patients with combined lower levels of H3K9me2 and EHMT2 exhibited a better survival rate and prognosis compared with those with combined higher levels of H3K9me2 and EHMT2, in addition to the other group. Furthermore, the results of the present study suggested that the development of GC is associated with pathological grade, lymph node metastasis and TNM stage.

In conclusion, EHMT2 expression level and H3K9me2 methylation level may be associated with the development risk and prognosis of GC. Patients with increased EHMT2 and H3K9me2 levels exhibited worse overall survival and a poorer prognosis compared with other patients. Overexpression of EHMT2 and H3K9me2 levels may be predictor markers of progression and prognosis in patients with GC.

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Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

PC conceived the study and was a major contributor in writing the manuscript. QQ and ZZ contributed to the analysis and interpretation of the data. XS and SY were involved in drafting the manuscript and the re-analysis of the immunohistochemistry scores. ZY acquired the data and performed statistical analysis. RS also acquired the data. YL performed the immunostaining. DG made contributions to the experimental studies. HF designed the research protocols and gave final approval of the version to be published. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was reviewed and ethically approved by the Committee for Ethical Review of Research of Yancheng Hospital (Yancheng, China), and written informed consent was obtained from the patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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