RESEARCH ARTICLE

Prognostic Significance of Overexpression of EZH2 and H3k27me3 Proteins in Gastric Cancer

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Abstract

The enhancer of zeste homolog 2 (EZH2) methyl transferase and histone 3 lysine 27 (H3K27me3) protein can repress gene transcription, and their aberrant expression has been observed in various human cancers. This study determined their expression levels in gastric cancer tissues with reference to clinicopathological features and patient survival. We collected 117 gastric cancer and corresponding normal tissues for immunohistochemistry analysis. In gastric cancers, 82/117 (70.1%) were positive for EZH2 and 66/117 (56.4%) for H3K27me3 proteins in contrast to only 5.41% and 7.25% of normal gastric mucosa specimens, respectively. Kaplan-Meier survival data showed the average overall and disease-free survival of EZH2 high expression patients was 25.2 and 20.2 months, respectively, shorter than that with EZH2 low expression (40.5 and 35.9 months). The average overall survival and disease-free survival of high H3K27me3 expression patients was 23.4 and 17.4 months, shorter than without H3K27me3 expression (37.6 and 34.5 months). The average overall survival and disease-free survival of patients with both EZH2 and H3K27me3 expression was 18.8 and 12.9 months, respectively, shorter than that with either alone (34.7 and 31.2 months) or with low levels of both (43.9 and 39.9 months). Multivariate Cox regression analysis showed that H3K27me3 and EZH2 expression, tumor size differentiation and clinical stage were all independent prognostic factors for predicting patient survival. This study demonstrated that detection of both EZH2 and H3K27me3 proteins can predict poor survival of gastric cancer patients, superior to single protein detection. In addition, H3K27me3 and EZH2 protein expression could predict lymph node metastasis.

Keywords: Gastric cancer - prognosis - enhancer of zeste homolog 2 - trimethylation of lysine 27 on histone H3

Asian Pacific J Cancer Prev, **13**, 3173-3178

Introduction

Gastric cancer is the fourth most common malignancy in the world, the second leading cause of cancer death in men, and the fourth among women according to 2007 statistical data (www.cancer.org; Global Cancer Facts & Figures 2007), although the worldwide incidence and mortality of gastric cancer have significantly decreased recently. Approximately 70% of new gastric cancer cases occur in developing countries with 800,000 cases of cancer-related death annually worldwide, and the 5-year survival rate of gastric cancer patients after surgery is approximately 10–30% (Harrison et al., 1998; Msika et al., 2000; Green et al., 2002). Like most other cancers, the first option for treatment of gastric cancer is surgery followed by chemotherapy and other types of therapy. The molecular mechanisms responsible for gastric cancer development and progression are still under investigation, and cancer prevention has been insufficient to dramatically reduce tumor incidence. Thus, the development of biomarkers to predict patient survival and treatment response is necessary to improve the quality of life for patients. To date, the most informative prognostic factors are tumor stage (which measures both the depth of invasion and the extent of metastasis in the clinic), size, and classification, although there are some other biomarkers that predict gastric cancer patient prognosis. For example, several proteins (such as TGFα, EGFR, and CDC25B) have been identified as markers of gastric cancer (Yasui et al., 2001). These genes are associated with gastric cancer development and progression, which are a multi-step process and a result of the accumulation of various oncogene activations and tumor suppressor gene inactivation (Yasui et al., 2000).

It has been suggested that epigenetic changes are involved in the silencing of various tumor suppressor genes, facilitating tumorigenesis and/or progression of human cancers (Strahl and Allis, 2000; Esteller, 2008). To this end, histone methylation has been found to play an important role in the regulation of gene expression and chromatin functions (Strahl and Allis, 2000). Trimethylation of lysine 27 on histone H3 (H3K27me3), a marker of transcriptionally silent chromatin, is methylated by enhancer of zeste homolog 2 (EZH2) (Cao et al., 2002).

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EZH2 is the catalytic subunit of the polycomb repressive complex 2 (PRC2) protein complex. These proteins are required for the long-term epigenetic silencing of chromatin and play an important role in cell differentiation and embryonic development; for example, they have a role in X chromosome inactivation during embryonic development. Alterations of these proteins are associated with aggressiveness and poor prognosis of hepatocellular carcinoma (Cai et al., 2011), esophageal squamous cell carcinoma (He et al., 2010), nasopharyngeal carcinoma (Cai et al., 2011), and ovarian cancer (Rao et al., 2010). Previous studies have documented that H3K27me3 alteration was associated with tumorigenesis, progression, and survival of various types of human cancers (e.g., prostate, breast, ovarian, pancreatic, and esophageal cancers) (Yu et al., 2007; Wei et al., 2008; He et al., 2009; Tzao et al., 2009). These studies have encouraged us to demonstrate the role of H3K27me3 and EZH2 proteins in gastric cancer. Therefore, we recruited 117 gastric cancer patients to provide both cancerous and the corresponding noncancerous gastric tissues for immunohistochemical analysis of these two proteins. We then associated the clinicopathological with survival data from the gastric cancer patients.

Materials and Methods

Patients and tissue samples

In this study, we retrospectively recruited formalin-fixed, paraffin-embedded tissues from 117 patients with gastric cancer, who underwent initial surgical resection between March 2001 and August 2003 from the archives of the Department of Pathology, the First Hospital Affiliated with Sun Yat-sen University (Guangzhou, China). This cohort consisted of 72 (61.5%) men and 45 (38.5%) women, with a median age of 61 years old. The average follow-up time was 28.5 months, and the median follow-up time was 23.0 months (ranged from 5.0 to 80.0 months). Clinicopathological characteristics, including patient age and gender, tumor differentiation, stage, and relapse, were collected and are shown in Table 1. Tumor differentiation was based on the criteria proposed by the World Health Organization (WHO) Classification of Tumors (2003). Tumor stages were defined according to the American Joint Committee on Cancer/International Union Against Cancer tumor-node-metastasis (TNM) classification system. This study was approved by the Institutional Research Medical Ethics Committee at Sun Yat-sen University, and the patients or their guardians agreed to participate in this study. The tissue microarray (TMA) was constructed according to the standard method described previously (Beecher Instruments, Silver Spring, MD, USA). Briefly, the individual donor tissue block and the corresponding histological hematoxylin-eosin stained slides were overlaid for tissue TMA sampling. The tissues (gastric cancer tissues and normal stomach tissues taken from the same patients) were sampled using a tissue arraying instrument (Beecher Instruments). A 0.6 mm-diameter cylinder of tissue was removed. Subsequently, the tissue cylinder was re-embedded into a predetermined position in a recipient paraffin block. In our constructed stomach TMA, three cores of sample were selected from each primary gastric cancer and normal stomach tissue. Multiple sections (5 μm thick) were cut from the TMA block and mounted on microscope slides.

Immunohistochemistry

TMA sections were first deparaffinized in xylene, rehydrated through graded alcohol, immersed in 3% hydrogen peroxide in phosphate-buffered saline (PBS) for 10 min to block endogenous peroxidase activity, and antigen-retrieved in a pressure cooker for 3 min in citrate buffer (pH 6.0). TMA sections were then blocked for nonspecific binding by incubation in 10% normal goat serum at room temperature for 30 min. Subsequently, the sections were incubated with a mouse monoclonal antibody anti-EZH2 (BD Transduction Laboratories, Franklin Lakes, NJ, USA) at a dilution of 1:100 or a rabbit monoclonal antibody anti-H3K27me3 (Cell Signaling Technology, Beverly, MA, USA) at a dilution of 1:100 at 4 °C overnight in a moist chamber. The next day, the sections were sequentially incubated with a secondary antibody (Envision, Dako, Denmark) for 1 h at room temperature, and color was developed with DAB (3, 3-diaminobenzidine) as a chromogen. The sections were then counterstained with Mayer's hematoxylin, dehydrated, and mounted. A negative control was obtained by replacing the primary antibody with a normal murine or rabbit IgG, and known positive immunostained sections were used as positive controls. The sections were finally reviewed and scored under a microscope.

Evaluation of the stained TMA sections

Expression of EZH2 and H3K27me3 proteins on tissue sections was assessed by two independent pathologists who were blindly to any clinicopathological and survival data. The stained TMA sections were evaluated according to nuclear localization of EZH2 and H3K27me3 proteins and the percentage of nuclei staining for high and low expression of EZH2 and H3K27me3 proteins, but the staining intensity was not counted. The high expression of these two proteins in each case was counted if more than 50% of the tumor cells in the triplicate tissue spots on the TMA sections were stained; otherwise, it was recorded as low expression (Figure 1). The quantification criteria were defined according to previous studies (He et al., 2010; Cai et al., 2011a; Cai et al., 2011b). In the current study, a minimum of 500 epithelial cells was counted for each normal or tumor sample.

Statistical analysis. Association of EZH2 and H3K27me3 expression with patient survival was estimated by using the Mantel-Cox log-rank test. Association of these two protein expressions with different clinicopathological patient data was analyzed using the chi-square test, stage-match univariate survival analysis, and multiple Cox proportional hazards regression analysis. SPSS statistical software package (SPSS Standard version 13.0, SPSS Inc., Chicago, IL, USA) was used to generate the P-value for each test. The difference was considered to be significant if the P-value from a two-tailed test was less than 0.05.

Results

Differential protein expressions of EZH2 and H3K27me3 proteins in gastric cancer and the corresponding adjacent normal tissues

In this study, we immunochemically analyzed EZH2 and H3K27me3 protein expression in gastric cancer and the corresponding adjacent normal tissues. The data showed that EZH2 protein was mainly expressed in the cell nuclei of gastric mucosa cells (Figure 1) and that the scope of its expression was between 0–100%. Particularly, EZH2 protein was expressed in 82 cases (70.0%; SE, 4.3%) of gastric cancer and 7 cases (8.3%; SE, 3.0%) of adjacent normal gastric tissues (P < 0.001). Similarly, expression of H3K27me3 protein was also observed mainly in the cell nuclei of gastric mucosa cells, although H3K27me3 protein occasionally was localized in both the cell cytoplasm and nuclei. H3K27me3 protein was expressed in 66 cases (56.4%; SE, 3.9%) of gastric cancer tissues, whereas it was only expressed in 11 cases (13.1%; SE, 3.7%) of normal gastric mucosae (P < 0.001). These data indicate that both EZH2 and H3K27me3 proteins were overexpressed in gastric cancer tissues compared to the adjacent normal mucosae.

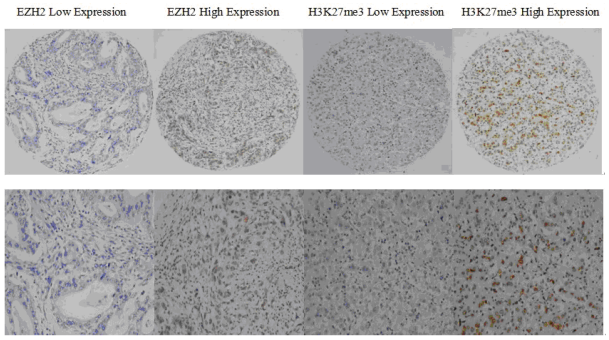

Figure 1. Differential Expression of EZH2 and H3K27me3 Proteins in Gastric Cancer Tissues Compared to the Adjacent Normal Mucosae. Note: magnification for the top panel is 40×, while it is 100× in the bottom panel

Association of EZH2 and H3K27me3 expression with clinicopathological parameters and survival of patients with gastric cancer

Expression of EZH2 and H3K27me3 proteins was in 82/117 (70.09%) and 66/117 (56.41%) of gastric cancer tissues, respectively. The chi-square test data showed that high expression of EZH2 and H3K27me3 protein was associated with advanced clinical stages and lymph node metastasis of gastric cancer (P < 0.05; Table 1) but not with age and gender of the patients or tumor grade (P > 0.05, Table 1).

Further analysis demonstrated a significant association between H3K27me3 and EZH2 expression in gastric cancer tissues, i.e., the number of EZH2-expressed tumor cases was significantly associated with the number of high H3K27me3-expressed tumor cases (52/82 cases, 63.41% vs. 14/35 cases, 40.00% in both negative cases; P = 0.019). Furthermore, we also determined the association of these two protein expressions with overall survival and disease-free survival of the gastric cancer patients. Kaplan-Meier survival data showed that the average overall patient survival with high EZH2 expression was 25.2 months, which was significantly shorter than that of the patients with low expression (40.5 months; P = 0.001 using a log-rank test; Figure 2A). Similarly, the average overall patient survival with high expression of H3K27me3 was 23.4 months, which was significantly shorter than that

Table 1. Association of EZH2 and H3K27me3 expression with clinicopathological features of gastric cancer

Variable		EZH2 expression				H3K27me3 expression		
	N	Low	High	P-value[a]	N	Low	High	P-value[a]
Age (years)				0.889				0.315
≤ 61[b]	58	17 (29.3%)	41 (70.7%)		59	23 (38.9%)	36 (62.1%)	
> 61	59	18 (30.5%)	41 (69.5%)		58	28 (48.3%)	30 (51.7%)	
Sex				0.307				0.814
Male	72	24 (33.3%)	48 (66.7%)		72	32 (44.4%)	40 (55.6%)	
Female	45	11 (24.4%)	34 (75.6%)		45	19 (42.2%)	26(57.8%)	
Tumor differentiation				0.271				0.1
Well	5	3 (60.0%)	2 (40.0%)		5	2 (40.0%)	3 (60.0%)	
Moderate	36	12 (33.3%)	24 (66.7%)		36	21 (58.3%)	15 (41.7%)	
Poor	76	20 (26.3%)	56(73.7%)		76	28 (26.9%)	48 (63.1%)	
Tumor stage				0				0.003
I and II	28	14 (50.0%)	14(50.0%)		28	20 (71.4%)	8 (29.6%)	
III	49	17 (34.7%)	32 (65.3%)		49	17 (34.7%)	32 (65.3%)	
IV	40	4 (10.0%)	36 (90.0%)		40	14 (35.0%)	26 (65.0%)	
Clinical stage				0.091				0.019
T1	5	3 (60.0%)	2 (40.0%)		5	2 (50.0%)	3 (50.0%)	
T2	10	4 (40.0%)	6 (60.0%)		10	6 (58.0%)	4 (42.0%)	
T3	69	23 (33.3%)	46 (66.7%)		69	36 (52.2%)	33 (47.8%)	
T4	33	5 (15.2%)	28 (84.8%)		33	7 (21.2%)	26 (78.8%)	
Lymph node metastasis				0.015				0.403
NO (N0)	24	12 (50.0%)	12 (50.0%)		24	4 (16.7%)	20 (83.3%)	
YES (N1)	93	23 (24.7%)	70 (75.3%)		93	23 (24.7%)	70 (75.3%)	
Distant metastasis				0.357				0.887
M0	98	31 (31.6%)	67 (68.4%)		98	43 (43.9%)	55 (56.1%)	
M1	19	4 (21.1%)	15 (78.9%)		19	8 (42.1%)	11 (57.9%)	

[a]Chi-square test; [b]Median age

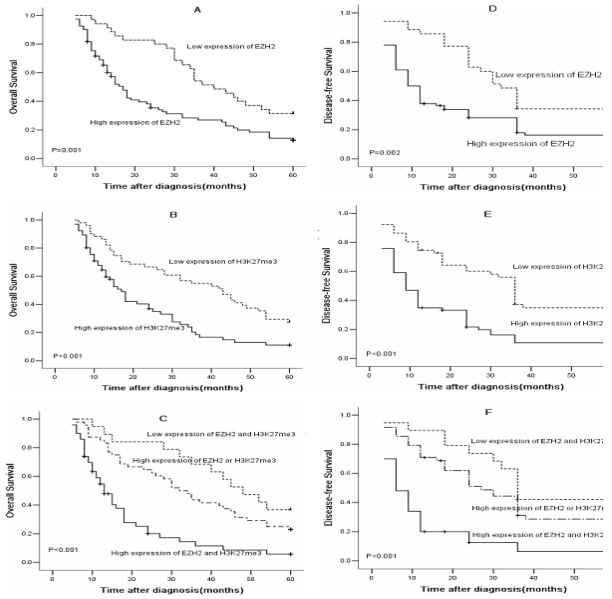

Figure 2. Kaplan-Meier Survival Analysis of EZH2 and H3K27me3 Expression in Gastric Cancer Patients Using A Log-rank Test. The probability of overall survival (A) and disease-free survival (D) of patients with gastric cancer: low expression of EZH2, n = 35; high expression of EZH2, n = 82. The probability of overall survival (B) and disease-free survival (E) of patients with gastric cancer: low expression of H3K27me3, n = 51; high expression of H3K27me3, n = 66. The probability of overall survival (C) and disease-free survival (F) of patients with gastric cancer: low expression of EZH2 and H3K27me3, n = 19; high expression of EZH2 or H3K27me3, n = 48, high expression of EZH2 and H3K27me3, n = 50

of patients with low expression (37.6 months; P = 0.001 using a log-rank test; Figure 2B). However, the average overall patient survival with both EZH2 and H3K27me3 expression was 18.8 months, which was obviously shorter than that of patients with either expression (34.7 months) or with low expression of both (43.9 months; P < 0.001 using a log-rank test; Figure 2C). Similarly, Kaplan-Meier survival data showed that the average disease-free patient survival with high EZH2 expression was 20.2 months, which was significantly shorter than that of patients with low expression (35.9 months; P = 0.002 using a log-rank test; Figure 2D). The average disease-free patient survival with high expression of H3K27me3 was 17.4 months, which was significantly shorter than that of patients with low expression (34.5 months; P < 0.001 using a log-rank test; Figure 2E). However, the average disease-free patient survival with both EZH2 and H3K27me3 expression was 12.9 months, which was obviously shorter than that of patients with either expression (31.2 months) or with low expression of both (39.9 months; P < 0.001 using a log-rank test; Figure 2F).

Multivariate Cox regression analysis

After that, we performed multivariate Cox regression analysis to associate all clinicopathological data and these two protein expressions with patient survival because these variables observed to have a prognostic association for survival by using the univariate analysis may be covariate (Table 2). We found that H3K27me3 expression was an independent prognostic factor for poor patient

Table 2. Cox Multivariate Analyses of Prognostic Factors on Overall Survival

Characteristic	Hazards ratio	95% CI	P-value
Age (≤ 61 vs. > 61)	1.215	0.748-1.682	0.592
Sex (Male vs. Female)	1.103	0.694-1.755	0.678
Tumor size, cm (≤ 3 vs. > 3)	1.606	0.964-2.677	0.069
Stage (I-II vs. III-V)	8.831	3.574-21.823	<0.001
Differentiation (Well-moderate vs. poor)	1.258	0.777-2.039	0.351
EZH2 (low vs. high)	1.885	1.172-3.032	0.009
H3K27me3 (low vs. high)	3.441	1.894-6.252	<0.001

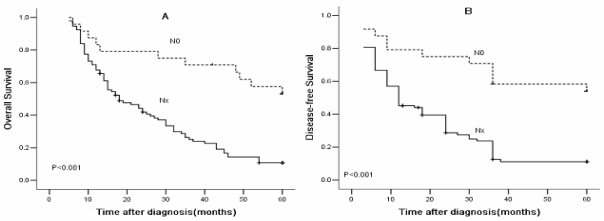

Figure 3. Kaplan-Meier Survival Analysis of the Probability of Overall Survival (A) and disease-free survival (B) with or without lymphatic metastasis using the log-rank test. Patients without lymphatic metastasis (N0) = 24; Patients with lymphatic metastasis (N1) = 93

survival (hazard ratio, 3.441; 95% CI, 1.894–6.252; P < 0.001; Table 2). Similar data were also observed for EZH2 protein (hazard ratio, 1.885; 95% CI, 1.172–3.032; P = 0.009; Table 2). In addition, tumor size, differentiation, and clinical stages as independent prognostic factors were useful to predict patient survival.

Furthermore, we found that 93/117 patients (79.49%) with gastric cancer had lymph node metastasis. Kaplan-Meier survival analysis showed that overall patient survival with lymph node metastasis (N1) was 25.4 months, which was clearly shorter than that of those without lymph node metastasis (N0) (45.8 months; P < 0.001 using a log-rank test; Figure 3A). In addition, Kaplan-Meier survival analysis showed that disease-free patient survival with lymph node metastasis (N1) was 19.39 months, which was clearly shorter than that of those without lymph node metastasis (N0) (42.8 months; P < 0.001 using a log-rank test; Figure 3B). Expression of either EZH2 or H3K27me3 protein and lymphatic metastasis were present in 70/93 (75.27%) of gastric cancer cases, and Kaplan-Meier survival analysis showed that overall patient survival with high EZH2 expression and lymph node metastasis (70/93) was 22.5 months, which was significantly shorter than that of those without EZH2 expression and lymph node metastasis (23/93) (34.3 months; P = 0.001 using a log-rank test; Figure 4A). Similarly, the overall patient survival with H3K27me3 expression and lymph node metastasis (70/93) was 20.9 months, which was significantly shorter than that of those without H3K27me3 expression and lymph node metastasis (23/93) (38.6 months; P < 0.001 using a log-rank test; Figure 4B). Moreover, the average overall patient survival with both EZH2 and H3K27me3 expression (55/93) was 18.4 months, which was obviously shorter than that of patients with only one protein expression (30/93) (33.3 months) or with low expression of both (8/93) (42.3 months; P < 0.001 using a log-rank test; Figure 4C).

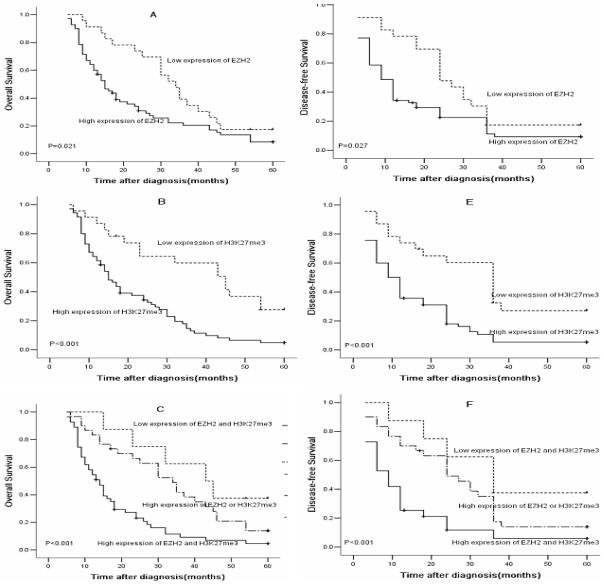

Figure 4. Kaplan-Meier Survival Analysis of EZH2 and H3K27me3 Expression in Gastric Cancer Patients with Lymphatic Metastasis Using the Log-rank Test. The probability of overall survival (A) and disease-free survival (D) of patients with lymphatic metastasis: low expression of EZH2, n = 23; high expression of EZH2, n = 70. The probability of overall survival (B) and disease-free survival (E) of patients with lymphatic metastasis: low expression of H3K27me3, n = 23; high expression of H3K27me3, n = 70. The probability of overall survival (C) and disease-free survival (F) of patients with lymphatic metastasis: low expression of EZH2 and H3K27me3, n = 8; high expression of EZH2 or H3K27me3, n = 30, high expression of EZH2 and H3K27me3, n = 55

Similarly, Kaplan-Meier survival data showed that the average disease-free patient survival with high EZH2 expression and lymph node metastasis was 17.3 months, which was significantly shorter than that of the patients with low expression (28.7 months; P = 0.027 using a log-rank test; Figure 4D). The average disease-free patient survival with high expression and lymph node metastasis of H3K27me3 was 15.6 months, which was significantly shorter than that of patients with low expression (32.9 months; P < 0.001 using a log-rank test; Figure 4E). However, the average disease-free patient survival with both EZH2 and H3K27me3 expression and lymph node metastasis was 13.6 months, which was obviously shorter than that of patients with expression of only one protein (26.9 months) or with low expression of both proteins (37.9 months; P < 0.001 using a log-rank test; Figure 4F).

Discussion

In the current study, we detected EZH2 and H3K27me3 protein expression in gastric cancer tissue specimens and found differential expression of these two proteins in gastric cancer and normal gastric mucosae. We also found that overexpression of EZH2 and H3K27me3 proteins in gastric cancer tissues was significantly associated with poor patient survival compared to that of the patients with low expression of either protein or both. Multivariate Cox regression analysis showed that H3K27me3 and EZH2 expression, tumor size and differentiation, and clinical stages were all independent prognostic factors for predicting patient survival. Thus, our study revealed that detection of these two proteins could serve as a biomarker in the prediction of gastric cancer patient survival and gastric cancer metastasis. Further study will verify the current data before it can be translated into clinical practice.

Similarly, a recent study (Bachmann et al., 2006) has shown that a strong EZH2 expression is associated with increased tumor cell proliferation and clinicopathological data of patients with melanoma and endometrial, prostate, and breast cancers. Matsukawa et al. (2006) found that high EZH2 expression levels predicted a poor gastric cancer prognosis. Recent studies have shown that different gene promoter methylation mediated by EZH2 methyl transferase was able to promote the malignant phenotypes of cancer cells (Schlesinger et al., 2007; Tonini et al., 2008), which was due to the loss of tumor suppressor gene functions and led to stem cells or progenitor cells transforming cancer cells (Widschwendter et al., 2007), such as hepatocarcinoma and prostate, breast, ovarian, and esophageal cancers. Altered expression of EZH2 protein affected cancer patient prognoses to various degrees (Bryant et al., 2005; Weikert et al., 2005; Collett et al., 2006; Yonemitsu et al., 2009; Cai et al., 2011c). EZH2 overexpression has been found to be associated with tumor size, depth of invasion, vessel invasion, lymph node metastasis, and clinical stages of gastric cancer (Matsukawa et al., 2006). Taken together, EZH2 protein detection could be used as a novel biomarker for predicting poor survival or tumor aggressiveness.

Nevertheless, compared with EZH2 protein, there have been only a few studies on H3K27me3 expression in cancer tissues. Moreover, to date, there have been no reports on the evaluation of these two proteins together to predict cancer patient prognosis. In our current study, we observed a statistically significant association between H3K27me3 and EZH2 protein expression. Our data showed that EZH2 expression levels in gastric cancer tissues are significantly higher than those in normal gastric tissues. In addition, patients with high EZH2 expression levels have lower survival rates than those with low expression levels; this data is consistent with the results obtained by Matsukawa et al. (2006). Similarly, the overall patient survival with high H3K27me3 expression was also shorter than that of patients with low H3K27me3 expression. Moreover, a combination of both proteins is able to predict poor gastric cancer patient survival, which is better than using only one protein. Indeed, it is clear that as a histidine methyltransferase, EZH2, regulates H3K27me3 (Tonini et al., 2008), and then these proteins induce transcriptional repression and thereby participate in controlling gene expression patterns in the cells. Thus, the combined effects of EZH2 and H3K27me3 proteins could be greater than that of each one for predicting overall survival of gastric cancer patients.

In addition, lymphatic metastasis is a major event for tumor progression. A previous study conducted by the Japanese National Cancer Center Hospital showed that lymphatic metastasis was able to predict the prognosis of gastric cancer patients and that the relative risk was 4.39 (Maruyama et al., 1992). Our current data also

drew a similar conclusion that the overall survival of the 93 patients with lymphatic metastasis was significantly shorter than that of those without lymphatic metastasis. Moreover, we combined lymphatic metastasis and expression of these two proteins to predict the overall survival of gastric cancer patients and found that overall patient survival with high EZH2 expression and lymph node metastasis was significantly shorter than that of those without EZH2 expression and lymph node metastasis. Similarly, the overall patient survival with H3K27me3 expression and lymph node metastasis was significantly shorter than that of those without H3K27me3 expression and lymph node metastasis. Moreover, the average overall patient survival with both EZH2 and H3K27me3 expression and lymph node metastasis was obviously shorter than that of patients with expression of only one of the proteins or low expressions of both proteins and without lymph node metastasis. Therefore, this study provides a novel strategy to treat gastric cancer by blocking EZH2 and H3K27me3 protein expression or activity in gastric cancer. In addition, this finding could be potentially used in the clinic as a biomarker to predict gastric cancer prognosis.

Acknowledgements

The study is supported in part by grants from the National Natural Science Foundation of China (#81072044) and the Guangdong Natural Science Foundation (#S2011010004653).

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