Upregulated expression of G9a is correlated with poor prognosis of gastric cancer patients

Chi Zhang, PhD, Shaozhong Wei, PhD, Junjie Hu, PhD, Zhiguo Xiong, PhD

Abstract
As one of the most serious cancers, gastric cancer (GC) represents the third leading cause of malignancy-related deaths. G9a is a histone lysine methyltransferase and has been reported to be involved in the progression of some human cancers. In the present study, we aimed to explore the expression patterns and clinical value of G9a in GC patients.

The expression of G9a in 142 paired GC tissues and adjacent non-cancerous tissues (no less than 5 cm from tumor edge) was examined with quantitative real-time polymerase chain reaction (qRT-PCR). To estimate the association of G9a expression with clinical characteristics of GC patients, Chi-square test and t test were conducted. Kaplan–Meier survival and multivariate Cox regression analyses were performed to explore the prognostic value of G9a in GC.

Upregulated expression of G9a was found in GC tissues compared with noncancerous tissues (P < .001). Elevated G9a expression was significantly correlated with patients' lymph node metastasis (P = .007) and TNM stage (P < .001). Kaplan–Meier survival curves demonstrated that patients with high G9a expression had shorter survival time than those with low expression (log-rank test, P < .05), reaching a median OS of 24 months. According to the results of Cox regression, G9a could be considered as an independent prognostic biomarker in patients with GC (HR = 3.912, 95% CI = 2.213–6.915, P < .001). Additionally, the diagnosis cut-off value of G9a in GC patients was 1.515.

Taken together, G9a expression was upregulated in GC tissues and could be an effective prognostic biomarker for GC.

Abbreviations: AUC = area under the curve, CDK5 = cyclin-dependent kinase 5, EMT = Epithelial-Mesenchymal Transition, GC = gastric cancer, qRT-PCR = quantitative real-time polymerase chain reaction, RBM4 = RNA-binding motif 4, ROC = receiver operating characteristic curve, SD = standard deviation.

Keywords: G9a, gastric cancer, prognosis

1. Introduction
Gastric cancer (GC) is one of the most frequent malignant tumors and ranks the third position among main reasons for cancer-related deaths all over the world. Up to now, some therapeutic methods were used for cancer treatment, including surgery, chemotherapy, and radiotherapy. However, the prognosis of GC patients is still unsatisfactory. Lacking clinical symptoms in early stage, most of GC patients are diagnosed at advanced stage, leading to poor prognosis. Besides, current treatment options are limited and long-term survival is poor. Therefore, improving diagnostic detection and prognostic prediction would be urgent for GC. Great efforts have been made to identify novel prognostic markers which could improve the treatment and survival rate of GC patients.

G9a is a histone lysine methyltransferase belonging to Su (Var) 3 family, and can catalyze dimethylation and histone H3 lysine 9, causing gene silencing at transcriptional level. Evidences indicate that histone methylation is a major reason for the silencing of tumor suppressor genes. Thus, G9a has been reported to be involved in the progression of different cancers, such as ovarian cancer, lung cancer and so on. Increased expression of G9a in human cancers suggests that it may be a cancer promoter. In addition, previous studies on GC reported that the knockdown of G9a could induce cell apoptosis in GC, suggesting its crucial role in this malignancy. However, there are few studies on the prognostic significance of G9a in GC.

In the present study, we attempted to evaluate the expression patterns of G9a and its prognostic value in GC through Kaplan–Meier survival analysis and Cox regression assay.

2. Materials and methods

2.1. Patients and tissue specimens
In this study we collected 142 GC patients who received surgery at Hubei Cancer Hospital. No cases had received any therapy...
(chemotherapy or radiotherapy) before operation. One hundred forty-two pairs of GC tissues and adjacent noncancerous tissues (no less than 5 cm from tumor edge) were collected and immediately frozen in liquid nitrogen before stored at −80°C for standby use. All patients were diagnosed by 2 pathologists based on the diagnosis criteria from the World Health Organization. Clinicopathological features of these patients were summarized in Table 1, including age, gender, disease type, pathological type, differentiation (low grade: well/moderate differentiated and low-grade/moderate malignancy; and high grade: poorly differentiated and high-grade malignancy, based on bipolar grading), lymph node metastasis and TNM stage. Moreover, all of the patients were followed up for 5 years, and relevant information was collected through telephone calls or letters. This research was approved by the Ethics Committee of Hubei Cancer Hospital (approval number: 20150844). Written informed consents were signed by the participators or their families.

2.2. RNA extraction and qRT-PCR

TRIzol reagent (Invitrogen, Carlsbad, CA) was adopted to extract total RNA from both cancer tissues and normal samples following the producer’s instructions. The concentration of RNA was estimated with NanoDrop ND-1000 (NanoDrop, Wilmington, DE). The first chain of cDNA was synthesized using PrimeScript reverse transcriptase (RT) reagent kit (TaKaRa, Shiga, Japan). 7300 Real-Time PCR System (Applied Biosystems, USA) and YBR Green PCR master mix (Applied Biosystems, USA) were applied for qRT-PCR reaction. Primers used in this reaction were designed with Primer Express software. GAPDH gene was adopted as the internal control to normalize G9a expression. Total primers were as follows: G9a forward: 5'-TTCAGTCTCCTACTATGATTIT-3', reverse: 5'-ATCATAGTAGACTGAAATT-3'; GAPDH forward: 5'-ATGGGCACGCGTAGAAA-3'; reverse: 5'-TGAAGGGTCATTGATGGCA-3'. Final relative G9a expression was calculated with 2−ΔΔCt method.

3. Results

3.1. Increased G9a mRNA expression in GC

In this study, the expression of G9a in tissue samples from 142 GC patients was assessed with qRT-PCR. The results in Figure 1 indicated that G9a expression was higher in GC specimens than in the matched noncancerous ones (P < .001).

3.2. Association of G9a expression with clinicopathological features of GC patients

Using Chi-square test, we investigated the relationship between G9a expression and clinicopathological characteristics of GC patients. Clinical information was listed in Table 1. The results showed that the overexpression of G9a was correlated with

| Table 1 Association of G9a expression with the clinicopathological features of GC patients. |

| Features                  | Total No. | Low (n = 71) | High (n = 71) | P values |
|---------------------------|-----------|--------------|---------------|----------|
| Age (years)               | n = 142   | 58           | 30            | .733     |
| ≤ 60                      | 84        | 41           | 43            | .603     |
| Gender                    |           |              |               |          |
| Female                    | 53        | 25           | 28            | .863     |
| Male                      | 89        | 46           | 43            |          |
| Disease type              |           |              |               |          |
| SRC                       | 55        | 28           | 27            | .855     |
| MGC                       | 87        | 43           | 44            |          |
| Pathological type         |           |              |               |          |
| Papillary                 | 43        | 21           | 22            |          |
| Tubular                   | 99        | 50           | 49            |          |
| Differentiation           |           |              |               |          |
| Well/moderate             | 69        | 40           | 29            | .065     |
| Poor                      | 73        | 31           | 42            |          |
| Lymph node metastasis     |           |              |               | .007     |
| Negative                  | 76        | 46           | 30            |          |
| Positive                  | 66        | 25           | 41            | <.001    |
| TNM stage                 |           |              |               |          |
| I-II                      | 73        | 49           | 24            |          |
| III-IV                    | 69        | 22           | 47            |          |

MGC=mixed gastric cancer, SRC=signet ring cell.

![Figure 1](image-url)
lymph node metastasis ($P = .007$) and TNM stage ($P < .001$). However, G9a expression was not associated with other clinical features, either age, gender, disease type pathological type or differentiation (all $P > .05$). Meanwhile, the comparison of G9a expression was conducted among GC patient with different ages, genders, disease types, pathological types, differentiation situations, lymph node metastasis statuses and TNM stages through $t$ test. The results showed significantly difference in the expression of G9a between GC patients with different differentiation situations, lymph node metastasis statuses and TNM stages ($P < .05$, Fig. 2).

3.3. Elevated G9a expression predicted poor outcomes of GC patients

In order to elucidate the prognostic significance of G9a expression in GC patients, all participants were followed up for 5 years Kaplan–Meier survival curves were constituted for them. The results of survival analysis showed that patients with high G9a expression had poorer overall survival than those with low expression (log-rank test, $P < .05$) (Fig. 3) and that the median OS was 24 months. Moreover, multivariate Cox regression analysis revealed that increased expression of G9a was an independent prognostic factor for GC (OS: HR = 3.912, 95% CI = 2.213–6.915; $P < .001$; PFS: HR = 4.070, 95%CI = 2.310–7.199, Table 2). In other words, increased G9a expression predicted poor prognosis of GC. Moreover, ROC curve showed that the sensitivity and specificity of G9a in GC diagnosis were 82.4% and 97.2%, respectively, while the area under the curve (AUC) was 0.954 with 95% CI (0.931–0.977), and the diagnosis cut-off value was 1.515 (Fig. 4).

4. Discussion

GC is considered as a serious disease threatening human health and reducing the patients’ quality of life. Its mortality rate is fairly high, especially in East Asia where more than half of total deaths caused by this malignancy appear. Evidences demonstrate that early diagnosis of GC is rare due to the lack of clinical manifestations at early stage, which leads to miserable outcomes among the cases. Despite advances in therapeutic strategies, such as clinical surgery, and chemo and radiation therapies, the prognosis of GC remains dismal. Therefore, novel molecular markers should be exploited to improve the diagnosis and treatment of GC. Currently, various prognostic markers have been proposed for GC. For example, Sun et al showed that the expressions of CDK5 and p27 were down-regulated in GC cases and related to the poor prognosis of GC.

In the present study, we investigated G9a expression and its prognostic potential in GC patients.

G9a is a euchromatic methyltransferase, and involved in gene silencing via the methylation of histone 3 lysine 9. Reportedly, it can combine with other transcription factors to manage the expression of certain genes. G9a dysregulation has been investigated in many human cancers. For instance, G9a expression was found to be upregulated in head and neck squamous cell carcinoma tissues and was proved to serve as a novel therapeutic target in this cancer. Francesco and his colleagues detected the role of G9a in human cancers, and implied that G9a was involved in the initiation and progression of different cancers. In addition, Chen et al revealed that G9a represented an effective antineoplastic target. Consequently, we considered that there might be a potential association between G9a and GC prognosis.

In the current study, we detected the expression level of G9a mRNA using qRT-PCR for paired GC and adjacent noncancer-
ous tissue samples. The results showed G9a expression was significantly up-regulated in GC tissues compared with normal ones, which was in accordance with findings in the previous study of Lin et al.\cite{19} Moreover, we examined the influences of clinicopathological features on G9a expression in patients with GC. The results revealed that G9a expression was influenced by tumor differentiation, lymph node metastasis and TNM stage. However, age, gender, disease type, and pathological type were not correlated with G9a expression. These results implied G9a might be involved in the progression of GC.

In the current study, we focused on the prognostic value of G9a expression in GC. Kaplan–Meier survival curves demonstrated that overall survival was shorter among patients with high levels of G9a than those with low G9a expression. In order to further verify the prognostic value of G9a, multivariate Cox regression was conducted, which revealed G9a might be an effective prognostic marker for GC. Showing a polyfactorial and multistep process, GC is influenced by multiple genetic and environmental factors. So, it is difficult for only 1 factor to independently predict GC prognosis. Moreover, until now, multiple factors have been reported to be associated with GC prognosis, and G9a was also an effective prognostic factor for this malignancy in our study. G9a combining with other factors may exactly predict the prognosis of GC, but this matter needs to be explored in further study.

In this research, we conducted a preliminary analysis on the prognostic significance of G9a in GC. However, some limitations should be noted. The sample size was relatively small and might cause certain bias in final results. In this study, GC tissues and adjacent normal ones were detected, but those from healthy people were not considered due to difficulty in sampling. The influence of G9a on some reported major genes for GC was not

Table 2

|                | Univariate      | Multivariate     |
|----------------|-----------------|------------------|
|                | P value | HR (95%CI) | P value | HR (95%CI) |
| G9a OS         | <.001   | 3.912 (2.213–6.915) | <.001   | 3.912 (2.213–6.915) |
| G9a PFS        | <.001   | 4.070 (2.310–7.199) | <.001   | 4.070 (2.310–7.199) |

OS = overall survival, PFS = progression-free survival.
explored. Therefore, in further studies, we will verify the results in this study, based on better design, more consideration of the above limitations and larger sample size.

In conclusion, G9a expression was upregulated in GC and correlated with the disease progression. The overexpression of G9a was a prognostic biomarker for GC. Although G9a expression is proved to be a crucial indicator in GC, its molecular mechanisms need to be exploited in further studies.

Author contributions
Conceptualization: Chi Zhang.
Data curation: Chi Zhang, Shaozhong Wei.
Formal analysis: Chi Zhang.
Funding acquisition: Chi Zhang, Shaozhong Wei, Junjie Hu, Zhiguo Xiong.
Investigation: Chi Zhang.
Methodology: Chi Zhang, Shaozhong Wei, Zhiguo Xiong.
Project administration: Chi Zhang, Zhiguo Xiong.
Resources: Chi Zhang, Shaozhong Wei.
Software: Chi Zhang, Shaozhong Wei, Junjie Hu.
Supervision: Chi Zhang, Shaozhong Wei, Junjie Hu.
Validation: Chi Zhang, Junjie Hu.
Visualization: Chi Zhang.
Writing – original draft: Chi Zhang, Shaozhong Wei, Junjie Hu.
Writing – review & editing: Chi Zhang, Zhiguo Xiong.

Figure 4. ROC curve analysis, sensitivity was as ordinate and 1-specificity was as abscissa. The area under the curve (AUC) was calculated to express the diagnosis value of G9a. The sensitivity and specificity were 82.4% and 97.2%, respectively and AUC was 0.954 (0.931–0.977). The diagnosis cut-off value of G9a in GC was 1.515.

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