Clinicopathological significance of G9A expression in colorectal carcinoma

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Abstract. G9A, the primary histone methyltransferase (HMTase) for histone H3 lysine 9, is upregulated in numerous types of cancer and is critical for tumor cell proliferation. The present study aimed to investigate the G9A expression level in colorectal carcinoma (CRC) to evaluate the clinical significance of G9A in CRC. First, the present study detected the expression of G9A protein in 100 pairs of CRC specimens by immunohistochemistry staining and analyzed the correlations between G9A expression and pathological tumor features. It was found that G9A expression was increased markedly in CRC tumor specimens and the high expression was associated with tumor distant metastasis. Oncomine database analysis demonstrated an elevated expression level of G9A in various types of CRC. In total, 6 public available data sets from the Gene Expression Omnibus (GEO) were used and Gene set enrichment analysis (GSEA) was conducted. The results of the bioinformatics analysis demonstrated that high G9A expression was associated with American Joint Committee on Cancer staging, tumor differentiation, tumor relapse of CRC, and may serve a role in CRC cell proliferation. These findings suggested that G9A was overexpressed in CRC and involved in the tumorigenesis and distant metastasis of CRC. The expression level may also serve as a potential indicator for tumor recurrence in CRC. The present findings aided in the understanding of the crucial role of G9A in tumorigenesis and also offered novel ideas for CRC therapy.

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Introduction

Aberrant epigenetic regulations have been found in various types of cancer and numerous types of human disease. The cancer epigenome is characterized by global changes in DNA methylation and altered histone modification patterns (1). The global pattern of histone modifications may serve as a predictor for the risk of recurrence of human cancers (2,3). It seems that epigenetics have reached the mainstream in pathogenesis research of numerous types of human disease, particularly in cancer. Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the world (4). Cumulative evidence has proved that the disruption of epigenetic regulation may drive the initiation and progression of CRC, such as histone modifications (5-7). Emerging evidences reveal that altered expression of histone methyltransferases (HMTs) and histone demethylases (HDMs) may be involved in cancer progression of CRC, such as Enhancer of zeste homolog 2 (EZH2) and lysine demethylase 4C (5,8,9). The functions of HMTs and HDMs in the pathogenesis of CRC require further investigation.

G9A is the primary HMT for mono- and dimethylation of H3K9 *in vivo* (10). Among various best-studied histone methylations, H3K9 methylation is thought to be associated with gene repression (11). Recently, G9A has been reported to perform critical roles in a number of biological progresses, such as behavior plasticity, lymphocyte development, stem cells differentiation and tumor cell growth (12-17). It has been found that the expression level of G9A is increased in numerous types of cancer as compared with their corresponding normal tissues, such as melanoma, lung cancer, neuroblastoma, leukemia and hepatocellular carcinoma (HCC) (17-19). It has been demonstrated that decreasing G9A expression level or inhibiting its activity reduces cellular proliferation and induces autophagy related cell death in colon cancer cells, breast cancer cells and neuroblastoma cells (17,20,21). A recent study also reported that G9A suppression induces DNA damage in colorectal cancer cells (21).

Although G9A has been reported to be crucial in numerous types of cancer, the function in colorectal cancer progression remains unknown. In the present study, the expression profile of G9A in CRC was examined to explore the function in CRC progression. The immunohistochemistry analysis of 100 pairs of tumor specimens revealed that G9A protein expression was markedly increased in CRC and the high expression may be related to distant metastasis. A significant elevated mRNA expression level of G9A in various colorectal carcinomas compared with normal colon and rectum tissues by Oncomine database analysis was also identified. Furthermore, the present study investigated the association between G9A expression level and the clinicopathological features of CRC using 6 publicly available datasets from Gene Expression Omnibus (GEO), and found that G9A expression was associated with American Joint Committee on Cancer (AJCC) staging, tumor differentiation, and tumor relapse. The present findings provide novel evidence to further understand the crucial role of G9A in tumorigenesis, and also offers significant ideas for CRC therapy.

Materials and methods

Patients. In total, 100 patients were diagnosed with CRC (53 with colon cancer and 47 with rectal cancer; Table I), which was classified with the 7th edition of the International Union against Cancer TNM staging system (classified into T1, T2, T3 and T4 based on the size and the extension of the primary tumor; classified into N0, N1 and N2 based on the degree of spread to regional lymph nodes; classified into M0 and M1 based on the presence of distant metastasis), the Dukes' staging system (classified into A, B, C and D) and histological grading (classified into well, moderately, or poorly differentiated) (22-24). All patients underwent surgical resection of tumors at the Renmin Hospital of Wuhan University in Wuhan, China. Ethical approval for the study was granted by the Renmin Hospital's ethics committee. Informed written consent was obtained from all participants involved in the study. The key clinical characteristics of the patients are summarized in Table I. Normal specimens were obtained from adjacent, grossly normal-appearing tissue taken at least 10 cm away from the cancer. None of the patients included in this study had chemotherapy or radiotherapy prior to surgery. There was no follow-up information available for these patients.

Immunohistochemistry. Immunostaining was performed as described previously (25). Briefly, deparaffinized sections were treated with 3% H_2O_2 and subjected to antigen retrieval by citric acid (pH 6.0). Subsequent to overnight incubation with primary antibody of G9A (1:100; cat. no. ab133482; Abcam, Cambridge, UK) at 4˚C, the sections were incubated for 15 min at room temperature with horseradish peroxidase-labeled polymer conjugated with secondary antibody (MaxVision™ HRP-Polymer anti-Rabbit IHC Kit; Maixin-Bio, Fuzhou, China) and incubated for 1 min with diaminobenzidine. The sections were then lightly counterstained with hematoxylin. The sections without primary antibody served as negative controls. The positive brown staining was visualized and then photographed using a light microscope at x200 or x400 magnification (BX51, Olympus Corporation, Tokyo, Japan).

Evaluation of immunohistochemical staining. The immunohistochemical staining results of all sections were evaluated by two independent observers (Dr Zhi Zeng and Dr Jian Qin, the co-authors), who were unaware of the disease outcome. Expression levels were ascertained according to the two observers' evaluations. As G9A is mainly expressed in the cell nucleus, the percentage of nucleus staining-positive cells were graded as 0 (<10%), 1 (≥10%, and <25%), 2 (≥25%, and <50%), 3 (≥50%, and <75%), 4 (≥75%) (Fig. 1A-F). For analysis the G9A protein expression levels were divided into two groups: Low expression level group (score value ≤2) and high expression level group (score value ≥3).

Oncomine database analysis. The Oncomine database (https://www.oncomine.org) (26), which is a cancer microarray database and web-based data-mining platform, was interrogated to validate the expression status of G9A mRNA in various types of CRC. Filter indexes were set in Oncomine based on research interests. Primary filters were set as differential analysis (cancer vs. normal) and cancer type (colorectal cancer). Dataset filters were set as data type (mRNA). Datasets were ordered by overexpression with P-value. Datasets were set by P-value (1E-6) and fold change (1.5+). The datasets were then sorted to analyze the expression of G9A mRNA associated with different cancer types vs. normal tissue.

Publicly available data analysis. The data sets of the patients with CRC and the corresponding clinical data were downloaded from the publicly available Gene Expression Omnibus (GEO) datasets (http://www.ncbi.nlm.nih.gov/gds/; National Center for Biotechnology Information, Bethesda, USA). A total of 6 independent data sets from GSE38832 (27) (n=122), GSE37892 (28) (n=130), GSE28722 (29) (n=125), GSE17536 (30,31) (n=177), GSE18088 (32) (n=53), and GSE33113 (33,34) (n=96) were utilized to analyze the expression level of G9A in CRC. The patients were divided into two groups according to their G9A expression level (top 50%, high vs. bottom 50%, low). For GSE38832, GSE37892, GSE17536, GSE18088 and GSE33113, Log2 intensity of probe 202326 were used to represent the expression level of G9A. For GSE28722 and GSE6988, Log2 intensity of probe 10023819278 and AA434117 were used to represent the expression level of G9A, respectively. The significance was defined as $P<0.05$.

Gene set enrichment analysis (GSEA). JAVA program for GSEA (http://www.broadinstitute.org/gsea) (35,36) was utilized to analyze the potential genes influenced by G9A high expression. CRC patient gene profiling data (GSE37892 and GSE18088) was obtained from the GEO site. The patients were divided into two groups according to their G9A expression level (top 50%, high vs. bottom 50%, low) and GSEA was carried out to assess the effects of G9A expression level on various biological gene sets. MsigDB c5 (GO gene sets, 1,454 gene sets) was used. Gene sets with a false discovery rate value (FDR) of <0.25 and normal values of $P<0.05$ subsequent to performing 1,000 permutations were regarded as having significant enrichment.

Table I. Association between G9A protein expression and clinicopathological features of CRC.

Clinicopathological features	Case size, n	G9A expression, n (%)		P-value[a]
		Low	High	
Diagnosis age (years)				0.028
≤60	49	22 (44.90)	27 (55.10)	
>60	51	34 (66.67)	17 (33.33)	
Sex				1.000
Male	50	28 (56.00)	22 (44.00)	
Female	50	28 (56.00)	22 (44.00)	
Size (diameter)				0.349
<5 cm	47	24 (51.06)	23 (48.94)	
≥5 cm	53	32 (60.38)	21 (39.62)	
Depth of invasion				0.106
T1	13	8 (61.54)	5 (38.46)	
T2	42	24 (57.14)	18 (42.86)	
T3	35	22 (62.86)	13 (37.14)	
T4	10	2 (20.00)	8 (80.00)	
Nodal metastasis				0.799
N0	57	33 (57.89)	24 (42.11)	
N1	23	12 (52.17)	11 (47.83)	
N2	18	9 (50.00)	9 (50.00)	
Distant metastasis				0.043
M0	89	53 (59.55)	36 (40.45)	
M1	11	3 (27.27)	8 (72.73)	
TNM stage				0.106
I	13	8 (61.54)	5 (38.46)	
II	42	24 (57.14)	18 (42.86)	0.035[b]
III	35	22 (62.86)	13 (37.14)	0.020[b]
IV	10	2 (20.00)	8 (80.00)	
Dukes stage				0.209
A	14	9 (64.29)	5 (35.71)	
B	41	23 (56.10)	18 (43.90)	
C	34	21 (61.76)	13 (38.24)	
D	11	3 (27.27)	8 (72.73)	0.049[c]
Differentiation				0.631
Well	20	13 (65.00)	7 (35.00)	
Moderately	57	30 (52.63)	27 (47.37)	
Poorly	23	13 (56.52)	10 (43.48)	

[a]Chi-square test was used to statistically analyze the association between title category and G9A protein expression (all unmarked P-values); [b]Compared with TNM stage IV; [c]Compared with Dukes stage C.

Statistical analysis. Experimental data were analyzed with SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL, USA). The χ^2 and Fisher's exact tests were used to analyze the statistical significance of the relationship between G9A expression and the clinicopathological features. For univariate recurrence analysis, recurrence curves were obtained with the Kaplan-Meier method and compared using the log-rank test. P<0.05 was considered to represent a statistically significant difference.

Results

G9A expression was increased in CRC tumor tissues. To investigate the role of G9A in colorectal cancer, we examined G9A protein expression level in 100 pairs of colorectal cancer tissues and the corresponding non-cancerous tissues by immunohistochemistry. G9A positively staining cells exhibited brown particles that localized in nuclei (Fig. 1A-G). Results showed that G9A protein expression was significantly increased

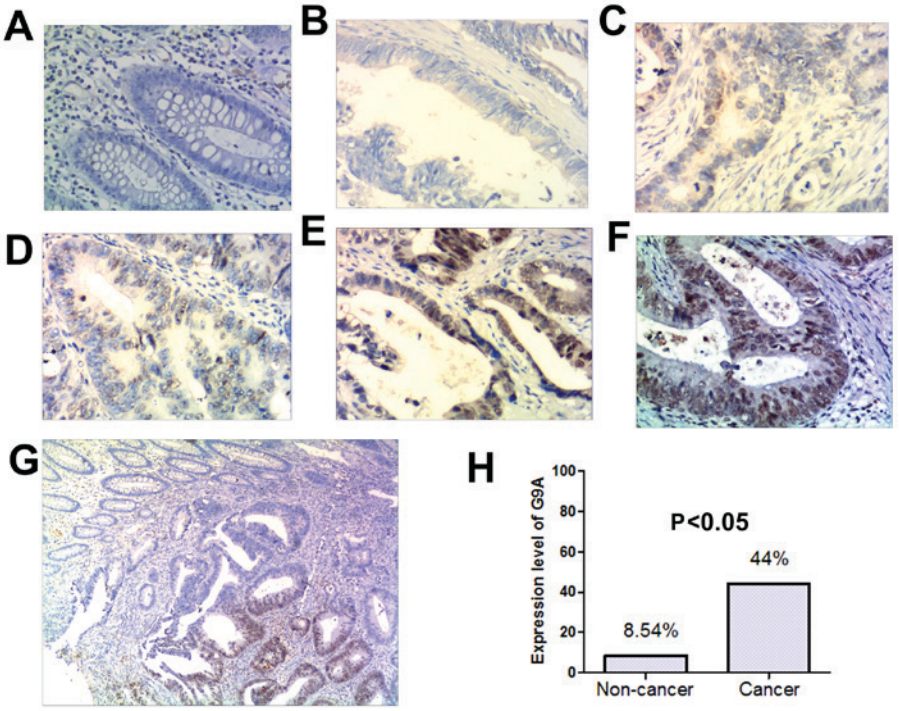


Figure 1. Immunohistochemical staining of G9A proteins in representative tissue specimens. (A) The expression level of G9A in normal colorectal tissues (absent from staining, score=0). (B-F) The expression of G9A in CRC tissues. The percentage of nucleus staining-positive cells were graded as: B, <10% (score=0); C, 10-25% (score=1); D, 25-50% (score=2); E, 50-75% (score=3); F, 75-100% (score=4). Original magnification, x100. (G) The expression status of G9A in the tissue from the junction between non-cancer and cancer. (H) The difference of G9A expression between non-cancer and cancer groups. G9A protein expression was significantly higher in the CRC tumor samples compared with that in the adjacent non-cancer tissues.

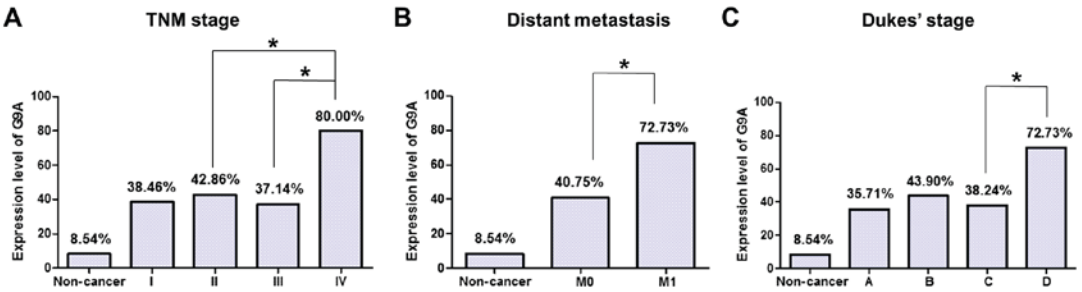
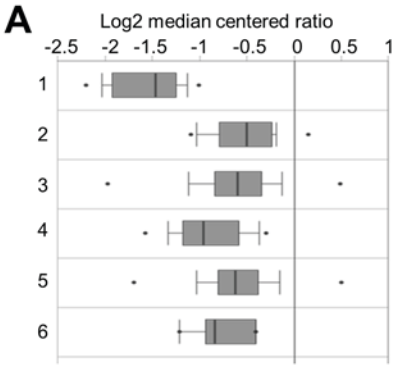


Figure 2. The expression of G9A correlates with TNM stages, distant metastasis, and Dukes stage in CRC. G9A expression was strongly associated with (A) TNM staging, (B) distant metastasis, and (C) Dukes staging. Expression levels of G9A were quantified via the ratio of the amount of the high expression level samples. *P<0.05.

in tumor samples (44%, n=100) in comparison to that in the adjacent non-cancer tissue samples (8.54%, n=82) (Fig. 1H). The difference was significant (P<0.05). In Fig. 1G, the represented specimen was from the junction between tumoral and non-tumoral tissue. As illustrated in Fig. 1G, G9A protein is strongly stained in the tumoral region, whereas the non-tumoral region is weakly stained. The present results indicate that G9A expression in CRC tumor samples is significantly higher than that in adjacent noncancerous tissue samples (Fig. 1H; P<0.05).

Association between G9A expression and clinicopathological features of CRC. The present study analyzed the association of G9A protein expression level with patient age, gender, size, TNM stages (primary tumor status (T1-T4), nodal metastasis (N0-N2), distant metastasis), Dukes stages (A-D), and histological grade (well, moderately, or poorly differentiated) in the CRC samples (Table I). There were significant differences in the G9A protein expression levels between later the TNM stage and the other TNM stages (P=0.035, P=0.020; IV compared with II and III, respectively; Table I, Fig. 2A). In addition, expression of G9A in tumor with distant metastasis was increased compared with that without distant metastasis (P=0.043, Table I, Fig. 2B). It also demonstrated a weak association with Dukes Stage that G9A expression in Dukes stage D tumor was significantly increased compared with that in Dukes stage C tumor (P=0.049; Table I, Fig. 2C). There was no significant correlation of G9A expression with other clinicopathological features, such as patient age, gender, tumor size, invasion and nodal metastasis.

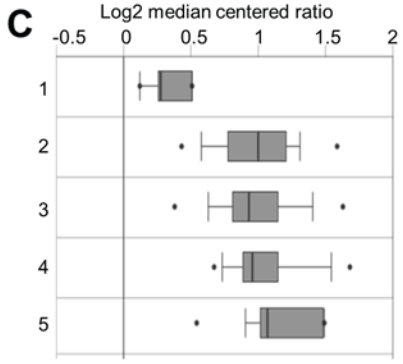
Expression of G9A using online analysis platform. The present study explored the mRNA expression level of G9A



B

G9A Expression in TCGA Colorectal (Reporter: A_24_P303389)

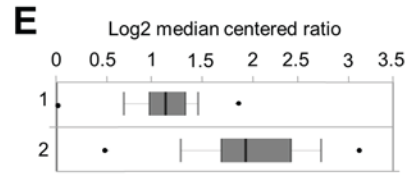
Legend code	Cancer/Normal type	Case number	P-value	Fold change
1	Normal rectum or colon	22	/	/
2	Cecum adenocarcinoma vs. Normal	22	9.40E-13	2.064
3	Colon adenocarcinoma vs. Normal	101	4.73E-13	1.955
4	Colon mucinous adenocarcinoma vs. Normal	22	1.14E-7	1.594
5	Rectal adenocarcinoma vs. Normal	60	1.74E-13	1.981
6	Rectal mucinous adenocarcinoma vs. Normal	6	5.29E-5	1.578



D

G9A Expression in Kaiser Colon (Reporter: 202326_at)

Legend code	Cancer/Normal type	Case number	P-value	Fold change
1	Normal	5	/	/
2	Cecum adenocarcinoma vs. Normal	17	2.99E-6	1.571
3	Colon adenocarcinoma vs. Normal	41	1.33E-5	1.572
4	Colon mucinous adenocarcinoma vs. Normal	13	2.99E-7	1.652
5	Rectosigmoid adenocarcinoma vs. Normal	10	3.66E-6	1.747



F

G9A Expression in Gaedcke Colorectal Colon (Reporter: A_23_P214638)

Legend code	Cancer/Normal type	Case number	P-value	Fold change
1	Normal	65	/	/
2	Rectal adenocarcinoma vs. Normal	65	4.90E-23	1.759

Figure 3. The box plots were downloaded from ONCOMINE. Integrative analysis of G9A overexpression in colorectal cancer vs. normal colorectal type. (A) Box plots exhibiting relative G9A expression levels in TCGA colorectal cancer datasets grouped by cancer and normal tissue. Numbers 1-6 represent different cancer/normal types described in (D). (B) Box plots exhibiting relative G9A expression levels in Kaiser Colon dataset grouped by cancer and normal tissue. Numbers 1-5 represent different cancer/normal types described in (E). (C) Box plots exhibiting relative G9A expression in Gaedcke Colorectal dataset grouped by cancer and normal tissue. Numbers 1 and 2 represent different cancer/normal types described in (F). (D) Comparison of G9A expression levels in different cancer types vs. normal colorectal tissue in the TCGA colorectal cancer datasets. (E) Comparison of G9A expression levels in different cancer types vs. normal colorectal tissue in the Kaiser Colon dataset. (F) Comparison of G9A expression levels in different cancer types vs. normal colorectal tissue in the Gaedcke Colorectal dataset. P-values represent the differences between two group comparisons. Fold change represents the fold difference in G9A overexpression in cancer tissue vs. normal tissue. TCGA, The Cancer Genome Atlas.

in CRC. Data mining and analysis of G9A mRNA expression level from the publicly available Oncomine database was performed. The threshold was set by the P-value (1E-6) and by fold change (1.5+). There were 4 data sets that met the requirements, which were the TCGA (The Cancer Genome Atlas) colorectal cancer dataset consisting of 237 clinical samples (37), the Kaiser colon dataset consisting of 105 clinical samples (38), the Gaedcke colorectal dataset consisting of 130 clinical samples (39) and the Hong colorectal dataset consisting of 82 samples (40). The results from Oncomine further confirmed the significantly higher expression level of G9A identified in various colorectal carcinomas, such as cecum adenocarcinoma, colon adenocarcinoma, colon mucinous adenocarcinoma and rectal adenocarcinoma (Fig. 3A-C). The corresponding P-values and fold changes are exhibited in Fig. 3D-F, respectively. These results indicated that G9A mRNA expression was increased in CRC tumor tissues.

Association between G9A mRNA expression and clinicopathological features of colorectal cancer. To figure out the relationships between elevated G9A expression level and the clinicopathological features of CRC, the present study chose 5 GEO datasets with corresponding clinical information

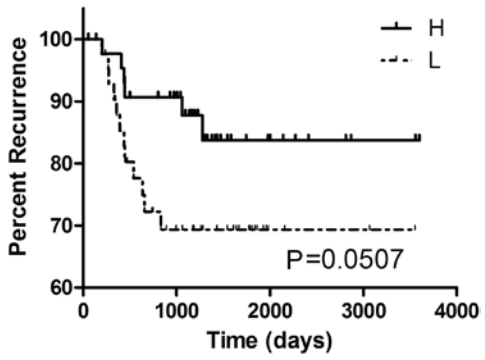


Figure 4. The Kaplan-Meier method was used to estimate survival curves for GSE33113. Log-rank test was used to compare the differences between curves. The top 50% samples with the higher expression were considered as the high-expression group (H), and the remaining 50% of the samples as the low-expression group (L). P=0.0507.

Table II. Correlation between G9A mRNA expression and the clinicopathological features of the CRC.

			G9 expression		
Datasets	Characteristic	Case size	High	Low	P-value[a]
GSE38832	Ajcc staging				0.259
Probe: 202326	1	18	7	11	
	2	35	17	18	
	3	39	25	14	
	4	30	14	16	
GSE28722	Duke's staging				0.618
Probe: 10023819278	A	3	1	2	
	B	83	38	45	
	C	34	12	22	
	D	5	3	2	
	Metastasis				0.916
	Y	33	14	19	
	N	92	40	52	
GSE17536	Ajcc staging				0.162
Probe: 202326	1	24	7	17	0.027[b]
	2	57	32	25	
	3	57	29	28	
	4	39	18	21	
	Differentiated				0.012
	Well	16	2	14	
	Moderately	134	69	65	
	Poorly	27	14	13	
	Recurrence				0.313
	Y	36	20	16	
	N	109	50	59	
GSE18088	Relapse				0.045
Probe: 202326	Y	13	10	3	
	N	40	18	22	
	Location				0.506
	Proximal	28	16	12	
	Distal	25	12	13	
	Differentiation				0.327
	Well	2	1	1	
	Moderate	35	21	14	
	Low	16	6	10	

Gene expression classification into either low or high groups was determined by whether the value was lower or higher than the mean of that dataset. [a]Chi-square test was used to statistically analyze the association between title category and G9A protein expression (all unmarked P-values); [b]AJCC stage 1 compared with stage 2. AJCC, American Joint Committee on Cancer.

for further analysis (Table II). The results demonstrated that G9A expression was associated with AJCC staging (P=0.027) and tumor cell differentiation (P=0.012) in GSE17536 (n=177), and it was also related with cancer relapse in GSE18088 (P=0.045, n=53). In GSE33113 (n=96), a marked association between G9A expression and the recurrence rate in CRC was found (P=0.0507, Fig. 4). It might suggest that CRC patients with high expression level of G9A were more likely to suffer relapse. However, cause the result based on GSE33113 was borederline signficant (P=0.0507), this result still requires further investigation to further verify, as the association in this dataset did not prove to be statistically significant. No other associations between G9A expression and clinicopathological features in any other CRC datasets we found, including metastasis, staging, location or survival rate.

G9A expression level associated with proliferation of CRC cells. The G9A expression level was found consistently increased in both public available datasets and in

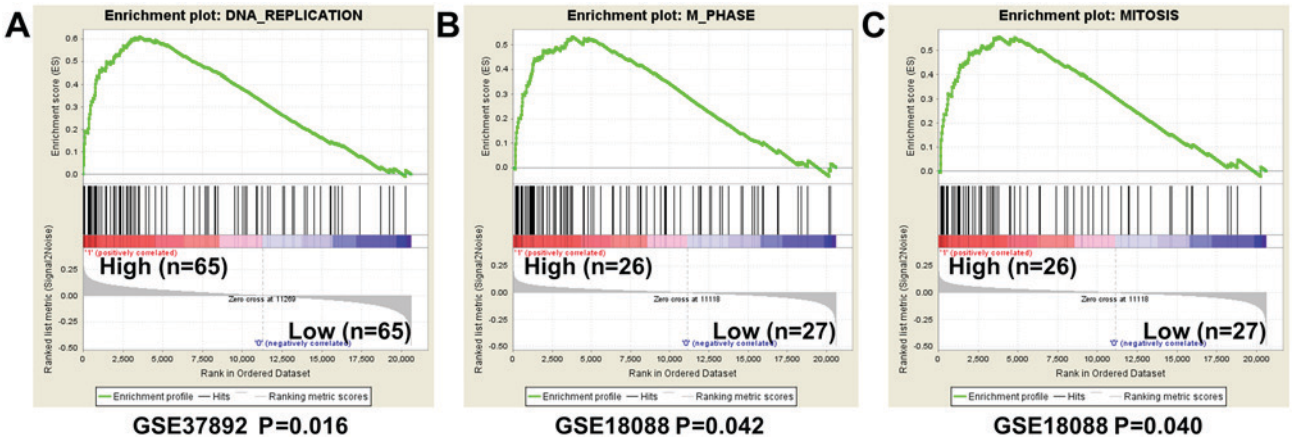


Figure 5. Gene set enrichment analysis demonstrates biological process modulated by G9A. GSEA analysis of GO terms showed G9A may regulate gene sets associated with (A) DNA replication, (B) M phase and (C) mitosis.

the present CRC tumor samples. A GSEA analysis was conducted to investigate the potential biological processes that G9A high expression may influence. The results from GSEA using GSE37892 demonstrated that gene sets differences in G9A high vs. low patients indicated that G9A regulated gene sets mainly associated with DNA replication (P=0.016, FDR=0.160, Fig. 5A). The result by analyzing GSE18088 also indicated that high expression of G9A regulated gene sets associated with mitosis, although the FDR value was weak (M phase, P=0.042, FDR=0.433; mitosis, P=0.040, FDR=0.442; Fig. 5B and C). The present study concluded that G9A may be important for proliferation of CRC cells.

Discussion

The present study investigated the clinicopathological significance of G9A expression in CRC progression. Immunohistochemistry was performed to explore G9A protein expression pattern in 100 pairs of CRC samples. Combined with the results of the bioinformatics analysis, it was demonstrated that G9A expression was increased in CRC and that it is therefore involved in the carcinogenesis of CRC.

G9A is the primary histone lysine methyltransferase of lysine 9 on histone H3. Various studies have demonstrated that G9A is critical for numerous biological progresses, such as embryo development, behavior plasticity, lymphocyte development, stem cell differentiation and tumor cell growth (13,16,17,41). It has been reported as overexpressed in numerous types of cancer, including hepatocellular carcinoma, bladder cancer, leukemia and lung cancer (17,18,42). Inhibition of G9A represses cellular proliferation by inducing autophagy related cell death in colon cancer, breast cancer and neuroblastoma cells (17,20). The present study investigated the potential functions of G9A in CRC progression.

The results of the present immunohistochemistry analysis were consistent with Oncomine datasets, demonstrating that G9A expression was significantly elevated in CRC tumor tissues. Fig. 1G exhibited clearly that G9A was strongly stained in the tumor region and weakly stained in the non-tumor region. The clinical significance of high G9A expression in CRC was further analyzed. It was found that G9A protein expression was significantly higher in TNM stage IV tumor than any other TNM stages in our 100 specimens. In addition, the G9A expression exhibited a higher expression in Dukes stage D tumor than that in stage C tumor, and also a higher expression in tumor with distant metastasis than that without distant metastasis. According to the principle of all these different types of staging methods, G9A expression levels may be important for the metastasis status of CRC. A previous study on lung cancer has stated that G9A has the ability to promote tumor invasion and metastasis in lung cancer (43). The present study provided the evidence that increased expression of G9A was associated with metastasis in CRC. In accordance, by using publicly available datasets from GEO, it was found that high G9A expression was associated with AJCC staging and differentiation of CRC in one of the datasets analyzed. However, since the results were not consistent among different datasets used, and the association between G9A expression and tumor relapse in CRC was borderline significant, the associations between G9A and tumor metastasis or tumor recurrence need to be further confirmed.

To find the potential function of G9A in CRC progression, GSEA analysis was performed. The results demonstrated that G9A was significantly associated with DNA replication and mitosis in CRC tumor cells in 2 datasets analyzed. These results strongly indicated that G9A was important for colorectal cancer cell proliferation, which was consistent with the previous research (20). It has been demonstrated that G9A inhibition represses proliferation in colon cancer HCT116 cells and numerous other types of cancer cell (20,21). The present study suggested that high expression level of G9A was critical for CRC tumorigenesis.

In the present study, the clinicopathological significance of G9A high expression in CRC was discussed. In conclusion, the results suggested that G9A expression was increased in CRC tumor samples and the high expression was important for tumorigenesis. Evidence that G9A high expression may be important for distant metastasis in CRC has been provided. The findings helped to further understand the crucial role of G9A in tumorigenesis, and also offered significant ideas for CRC therapy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JQ and ZZ analyzed and interpreted the patient data. ZZ, QL and YH collected the samples. TL contributed to data analysis and the manuscript drafting. JQ and LC contributed to the study design and manuscript writing.

Ethics approval and consent to participate

Ethical approval for the study was granted by the Renmin Hospital's ethics committee. Informed written consent was obtained from all participants involved in the study.

Consent for publication

Informed written consent for publication was obtained from all participants involved in the study.

Competing interests

The authors declare that they have no competing interests.

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