Increased expression of G9A contributes to carcinogenesis and indicates poor prognosis in hepatocellular carcinoma

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Received December 8, 2015; Accepted May 23, 2017

Abstract. Euchromatic histone-lysine N-methyltransferase (G9A), the primary histone methyltransferase for histone H3 Lys9, has been identified to be upregulated in numerous types of cancer. The aim of the present study was to analyze the clinical significance of G9A, and preliminarily explore its function in hepatocellular carcinoma (HCC). An increased expression level of G9A was demonstrated in the HCC samples and also in 5 publically available datasets. By analyzing GSE14520, it was revealed that its expression level was significantly associated with serum α-fetoprotein level of patients with HCC, and may serve as a potential prognostic indicator for patients with multinodular HCC. Bioinformatics tools were utilized to predict the potential function of G9A, and the results indicated that G9A may modulate gene sets involved in RNA processing and DNA replication. G9A inhibition may suppress cell proliferation by arresting cells in G1 phase and increasing the expression level of microtubule-associated protein light chain 3β (MAP1LC3B) in Huh7 and HepG2 cells. In addition, an inverse association between the expression of G9A and LC3B was demonstrated in HCC tumor samples in the publically available GSE14520 dataset, which indicated that G9A may also have the potential to regulate MAP1LC3B expression in HCC tumor tissues.

The results of the present study led to hypothesis that the G9A expression level may be of assistance in diagnosing HCC, and be a potential therapeutic target for HCC. The results provided novel evidence for additional understanding of the crucial role of G9A in tumorigenesis.

Introduction

Aberrant epigenetic regulations have been identified in various types of cancer and a number of types of human disease (1). The cancer epigenome is characterized by genome-wide changes in DNA methylation and altered histone modification patterns (1). The global pattern of histone modifications may serve as a predictor of the risk of recurrence of human cancer (2,3). Epigenetics has become a common focus in studies investigating the pathogenesis of many types of human diseases, particularly in cancer. Hepatocellular carcinoma (HCC) is one of the most common types of neoplasm, and the most frequent cause of cancer-associated mortality (4,5). Previous evidence has suggested that epigenetic alterations are important for the pathogenesis of HCC. A whole-genome sequencing analysis of 27 HCCs revealed that almost 50% of the tumors analyzed exhibited mutations in chromatin regulators, including histone methyltransferases (HMTs) (6). Previously, it has been demonstrated that histone H3 trimethylated at Lys27 and histone H3 trimethylated at Lys4 may serve as prognostic markers and therapeutic targets in HCC (7). These results indicate that aberrant histone methylation may be involved in the carcinogenesis of HCC, but the functions of HMT and histone demethylase (HDM) in the pathogenesis of HCC require additional study.

Euchromatic histone-lysine N-methyltransferase (G9A) is the primary HMT for mono- and dimethylation of histone H3 Lys9 (H3K9) in vivo (8). Among various well-studied histone methylations, H3K9 methylation is thought to be associated with gene repression (9). Previously, G9A has been identified to serve critical roles in various biological progresses such as behavior plasticity, lymphocyte development, stem cell differentiation and tumor cell growth (10-15). It has been suggested that the expression level of G9A is increased in numerous
types of cancer compared with their corresponding normal tissues, including melanoma, lung cancer, neuroblastoma, leukemia and HCC (15-17). Specifically decreasing G9A expression levels or inhibiting its activity may inhibit metastasis in lung cancer and decrease cell proliferation by inducing autophagy in colon cancer, breast cancer and neuroblastoma cells (15,18,19).

In the present study, the clinical significance of G9A expression and its potential function in HCC progression was investigated. The increased G9A expression level in HCC was additionally confirmed in 5 publicly available datasets and the HCC samples. By analyzing GSE14520, it was identified that increased expression of G9A may serve as an indicator for a poor prognosis in multinodular HCC. Gene set enrichment analysis (GSEA) was utilized to predict the potential function of G9A, and the results demonstrated that G9A may modulate gene sets involving RNA processing and DNA replication. G9A inhibition significantly decreased proliferation by arresting cells in G1 phase and increased microtubule-associated protein light chain 3β (MAP1LC3B) expression levels in Huh7 and HepG2 cell lines. An inverse association between the expression of G9A and MAP1LC3B was identified in HCC tumor samples in GSE14520, which indicated that G9A also had the potential to regulate MAP1LC3B expression in HCC tumor tissues. These results demonstrated that G9A was involved in the pathogenesis of HCC. These data may assist additional understanding of the crucial role of G9A in tumorigenesis.

Materials and methods

HCC specimens and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). HCC tumor specimens were obtained from the Department of Pathology, Renmin Hospital of Wuhan University (Wuhan, China). Informed consent was obtained from each patient prior to surgery. The research protocol was approved by Wuhan University School of Basic Medical Sciences Ethics Committee (Wuhan, China). The total RNA of each sample was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. RNA was reverse-transcribed into cDNA using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RT-qPCR was performed using the Takara Premix Ex Taq II 820 (Takara Bio, Inc., Woburn, MA, USA) and analyzed in the following experiments.

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Western blotting. Subsequent to treatment, the whole-cell extract was prepared by scraping the Huh7 and HepG2 cells in radioimmunoprecipitation buffer (cat. no. G2002, Servicebio Inc., Woburn, MA, USA). The protein concentrations were determined using a BCA protein assay kit (cat. no. P0011; Beyotime Institute of Biotechnology, Haimen, China). For every sample, 50 µg total proteins was used and separated by SDS-PAGE (12% gels) and transferred onto polyvinylidene fluoride membranes. The membranes were blocked at room temperature with non-fat milk for 1 h and incubated with primary antibodies (1:1,000) overnight at 4°C. Secondary antibodies [goat anti-rabbit (cat. no. 926-3221) or mouse (cat. no. 926-3220) IR Dye 800CW, LI-COR Biosciences, Lincoln, NE, USA] were used to detect binding of the primary antibodies by incubation at room temperature for 50 min. The bands were detected using the Odyssey CLx imaging system (LI-COR Biosciences) and analyzed by Image Studio Software for Odyssey CLx (LI-COR Biosciences). The primary antibodies used in the present study against the following were all
from Cell Signaling Technology, Inc. (Danvers, MA, USA): β-actin (cat. no. 4970), GAPDH (cat. no. 2118), MAP1LC3B (cat. no. 2775), G2/mitotic-specific cyclin B1 (cyclin B1; cat. no. 4135) and cyclin-dependent kinase inhibitor 1B (p27; cat. no. 2552).

Flow cytometry. Following BIX-01294 treatment, HuH7 and HepG2 cells were trypsinized and washed briefly with PBS once to remove the residual serum and trypsin. For cell cycle analysis, cells were fixed in 1 ml ice-cold 70% ethanol overnight at -20°C. Following fixation, cells were pelleted at 800 x g at 4°C for 5 min, the supernatant was aspirated carefully so as not to lose the pellet. The pellets were briefly washed twice with ice-cold PBS. The cells were resuspended with 500 µl propidium iodide (PI)/RNase A solution (50 µg/ml PI, 100 µg/ml RNase A and 0.1% Triton X-100 in PBS). The cells were incubated for 20 min at 37°C. DNA content was measured using a FACS Calibur instrument (BD Biosciences, Franklin Lakes, NJ, USA), and Modfit LT v3.3 (Verity Software House, Inc., Topsham, ME, USA) was used to analyze cell cycle phase distribution. For Annexin V/PI double staining analysis, following treatment with BIX-01294 as described above, HCC cells were double-stained with Annexin V-FITC and PI and incubated at room temperature for 5 min in the dark. A total of 10x10^4 cells from each group were analyzed using a FACS Calibur instrument.

Statistical analysis. Experiments were performed at least 3 times. Results are expressed as the mean ± standard deviation. Statistically significant differences among the groups were determined by one-way analysis of variance with Tukey's post hoc tests using SPSS 19.0 (IBM Corp., Armonk, NY, USA) and are presented as *P<0.05, **P<0.01 and ***P<0.001. P<0.05 was considered to indicate a statistically significant difference. Linear regression was used to determine the correlation between two variants with GraphPad Prism version 5.01 (GraphPad Software, Inc., La Jolla, CA, USA). The correlation between gene expression and the clinicopathologic features was analyzed by χ² test. Kaplan-Meier plots were constructed, and a log-rank test was used to evaluate differences in G9A expression levels for overall survival.

Results

G9A expression is increased in HCC tissues. It has been demonstrated that the expression level of G9A was increased in a number of types of cancer, including HCC (16). First, in the present study the relative mRNA expression level was examined in 14 pairs of HCC tissue samples using RT-qPCR, and identified a significant increase in G9A mRNA expression level in HCC tumor specimens compared with their adjacent non-tumorous liver tissue samples (P=0.0196; Fig. 1A). Subsequently, publicly available microarray datasets were analyzed to observe whether a similar phenomenon may be revealed. A total of four independent HCC microarray datasets containing HCC and normal liver tissues from the GEO were analyzed, including the GSE14520 training group (n=43) and testing group (n=445) (21,22); and GSE6764 (n=75) (20), GSE14323 (n=124) (23) and GSE50579 (n=80) (24). All four datasets exhibited significantly increased expression levels of G9A mRNA in HCC tumor tissues compared with their non-tumor liver tissues (P≤0.0001 in all datasets; Fig. 1B-F). Collectively, these data additionally confirmed that G9A expression level was significantly increased in HCC.

Association of G9A expression with the clinicopathological features of HCC. The association between G9A expression and clinicopathological features of HCC with GSE14520 were next analyzed to identify the potential roles of G9A in the pathogenesis of HCC. The samples pooled in the HCC dataset GSE14520 were divided into two groups according to the expression level of G9A in tumor tissues, and examined using a χ² test. The top 50% samples with the highest G9A expression were considered the high-expression group, and the other 50% as the low-expression group. As summarized in Table I, G9A expression was significantly associated with the serum α-fetoprotein (AFP) level (P=0.009). It appeared that patients with a lower expression level of G9A were more likely to exhibit a lower serum AFP level. With the exception of a moderate association between G9A expression and cirrhosis (P=0.054), there was no significant association between its expression level and the other clinicopathological features (P>0.05; Table I).

Association between G9A expression and survival of patients with HCC. To investigate the association between G9A expression and survival of patients with HCC, Kaplan-Meier estimator analysis was used to evaluate the effect of its expression level on the survival of patients with HCC by using the GSE14520 datasets stratified by clinical features included age, sex, active hepatitis B virus status (chronically active viral replication), AFP serum level, nodular type, cirrhosis, tumor size (>5 cm, large; ≤5 cm small) and various tumor staging methods such as Barcelona Clinic Liver Cancer, Cancer Liver Italian Program or Tumor Node Metastasis classification (21,22). The result of survival analysis demonstrated a weak difference in the survival between patients with multinodular HCC and patients with non-multinodular HCC (P=0.0548; Fig. 2A). Furthermore, the results of the survival analysis indicated that the survival rates of patients with multinodular HCC whose tumor tissues exhibited an increased G9A expression level was significantly decreased compared with the survival rates of patients with multinodular HCC with a decreased G9A expression level (P=0.0268; Fig. 2B), and also significantly decreased compared with that of the patients with non-multinodular HCC (P=0.0035; Fig. 2C). Additionally, there was no marked association between the survival and G9A expression level in patients with non-multinodular HCC (P=0.5124; Fig. 2D) or HCC patients with any other clinical features (data not shown).

G9A inhibitor represses HCC cell growth. Results of previous studies have suggested that suppression of G9A may inhibit proliferation in numerous types of cancer cell (15-17). To investigate the potential function of G9A in HCC pathogenesis, GSEA using HCC tumor gene profiling data from the GSE14520 group (n=225) was performed. There were 94 gene sets that were significantly upregulated in the ‘G9A high’ group compared with the ‘G9A low’ group (FDR <0.25 and nominal P<0.05), among which there were 10 gene sets associated with RNA and DNA processing. These results indicated that...
G9A-regulated gene sets that were clearly associated with RNA processing and DNA replication, and that G9A was involved in tumor cell proliferation in HCC (Fig. 3). DNA content analysis was performed and identified that the Huh7 and HepG2 cells were arrested in G1 phase by G9A-specific inhibition, and that HepG2 also exhibited a significant decrease in the population of cells in G2/M phase (Fig. 4A and B). In accordance with the results of the flow cytometry analysis, the expression levels of cyclin B1 were decreased following G9A inhibition, and the expression level of p27 was increased in Huh7 and HepG2 cells (Fig. 4C). Taken together, these results suggested that G9A dysfunction may suppress HCC cell proliferation.

G9A inhibitor increases MAP1LC3B expression in HCC cells. It has been suggested that G9A inhibition may induce autophagy and increase the expression level of a number of autophagy-associated genes in colon cancer, breast cancer and neuroblastoma cells (15,19). To assess whether apoptosis was also induced, Annexin V/PI double staining was performed and the results indicated that the number of PI+ cells, instead of Annexin V+, was significantly increased in Huh7 (Fig. 5A), which indicated that cell death induced by G9A inhibition was a result of necrosis rather than apoptosis. The expression of MAP1LC3B was examined by western blotting, and it was identified that its expression was triggered within 24 h following G9A inhibition in Huh7 and HepG2 cells, and it was processed into MAP1LC3B-II subsequent to 48 h treatment in Huh7 cells (Fig. 5B). A linear regression analysis was performed using HCC tumor samples of the GSE14520 dataset to determine whether the association between expression of G9A and MAP1LC3B existed in HCC tumor tissues. A significant negative association was identified between G9A and MAP1LC3B expression in HCC samples of GSE14520 (n=225; r=−0.1929; P=0.0037; Fig. 5C). The results of the present study...
indicated that G9A may also exhibit the potential to regulate MAP1LC3B expression in HCC tumor tissues.

Discussion

H3K9 methyltransferase G9A is involved in a number of important developmental events, such as the lineage commitment of stem cells and early embryonic stem cell differentiation (14,25). It has also been demonstrated to be overexpressed in numerous types of cancer, such as breast, prostate, colon, bladder, ovarian, melanoma, lung and liver cancer (16-18,26-28). Despite this, its functions in tumorigenesis require additional investigation. In the present study, its potential functions in the progression of HCC were preliminarily explored, and the association

Table I. Association between G9A expression and the clinicopathological features of the hepatocellular carcinoma cases from the GSE14520 dataset.

| Characteristics                      | Case size | G9A expression | χ² value | P-value |
|--------------------------------------|-----------|----------------|----------|---------|
|                                       |           | High | Low |           |          |
| Sex                                  |           |      |     | χ² | P       |
| Male                                 | 190       | 98   | 92  | 2.305 | 0.129   |
| Female                               | 30        | 11   | 19  |      |         |
| α-fetoprotein level, ng/ml           |           |      |     | χ² | P       |
| High (>300)                          | 99        | 58   | 41  | 6.909 | 0.009   |
| Low (≤300)                           | 118       | 48   | 70  |      |         |
| Age, years                           |           |      |     | χ² | P       |
| >50                                  | 108       | 51   | 57  | 0.456 | 0.500   |
| ≤50                                  | 114       | 59   | 55  |      |         |
| ALT level, U/l                       |           |      |     | χ² | P       |
| High (>50)                           | 91        | 46   | 45  | 0.063 | 0.802   |
| Low (≤50)                            | 129       | 63   | 66  |      |         |
| Tumor size, cm                       |           |      |     | χ² | P       |
| Large (>5)                           | 80        | 42   | 38  | 0.512 | 0.474   |
| Small (≤5)                           | 139       | 66   | 73  |      |         |
| Multinodular                         |           |      |     | χ² | P       |
| Yes                                  | 45        | 23   | 22  | 0.055 | 0.814   |
| No                                   | 175       | 86   | 89  |      |         |
| Cirrhosis                            |           |      |     | χ² | P       |
| Yes                                  | 202       | 104  | 98  | 3.716 | 0.054   |
| No                                   | 18        | 5    | 13  |      |         |
| Tumor-node-metastasis stage          |           |      |     | χ² | P       |
| I                                    | 93        | 46   | 47  | 0.345 | 0.841   |
| II                                   | 77        | 36   | 41  |      |         |
| III                                  | 48        | 25   | 23  |      |         |
| Barcelona clinic liver cancer staging|           |      |     | χ² | P       |
| A                                    | 148       | 69   | 79  | 0.535 | 0.765   |
| B                                    | 22        | 12   | 10  |      |         |
| C                                    | 28        | 14   | 14  |      |         |
| Cancer liver Italian program staging|           |      |     | χ² | P       |
| 0                                    | 97        | 42   | 55  | 4.601 | 0.467   |
| 1                                    | 74        | 38   | 36  |      |         |
| 2                                    | 35        | 20   | 14  |      |         |
| 3                                    | 9         | 5    | 4   |      |         |
| 4                                    | 3         | 2    | 1   |      |         |
| 5                                    | 1         | 0    | 1   |      |         |

aG9A expression was significantly associated with the serum α-fetoprotein level (P<0.05). G9A, euchromatic histone-lysine N-methyltransferase; ALT, alanine aminotransferase.
Figure 2. Survival analysis of G9A expression in HCC patients in the GSE14520 dataset. The Kaplan-Meier estimator method was used to estimate survival curves and the log-rank test was used to compare the differences between curves. The top 50% with the highest G9A expression were considered the 'high-expression' group and the remaining 50% of samples were the 'low-expression' group. (A) Survival curves between patients with multinodular HCC and patients with non-multinodular HCC. (B) Survival curves for patients with multinodular HCC. (C) Survival curves for patients with multinodular HCC compared with distinct G9A expression levels and patients with non-multinodular HCC. (D) Survival curves for patients with non-multinodular HCC. HCC, hepatocellular carcinoma; G9A, euchromatic histone-lysine N-methyltransferase.

| Gene sets name                                      | NES    | NOM p-value | FDR q-value |
|----------------------------------------------------|--------|-------------|-------------|
| Protein_RNA_complex_assembly                        | 1.9144 | 0.0020      | 0.0524      |
| RNA_splicing/transterification_reactions            | 1.7630 | 0.0019      | 0.0304      |
| RNA_processing                                     | 1.7710 | 0.0042      | 0.0395      |
| RNA_splicing                                      | 1.7616 | 0.0043      | 0.0395      |
| RNA_binding                                       | 1.7224 | 0.0150      | 0.1190      |
| DNA_directed_RNA_polymerase_Helicases              | 1.0888 | 0.0162      | 0.1461      |
| MRNA_processing_GO_0008307                         | 1.0884 | 0.0226      | 0.1401      |
| DNA_replication                                    | 1.6742 | 0.0101      | 0.1385      |
| MRNA_metabolic_process                             | 1.6639 | 0.0282      | 0.1393      |
| DNA_dependent_DNA_replication                      | 1.6397 | 0.0355      | 0.1382      |

Figure 3. GSEA demonstrates the biological processes modulated by G9A. (A) GSEA analysis of Gene Ontology terms indicated that G9A may regulate gene sets associated with RNA processing and DNA replication. Enrichment plots demonstrating (B) protein RNA complex assembly, (C) RNA processing and (D) DNA replication. GSEA, gene set enrichment analysis; G9A, euchromatic histone-lysine N-methyltransferase; NES, normalized enrichment score; NOM, nominal; FDR, false discovery rate.
between G9A expression and clinicopathological features of HCC were analyzed.

The results of the present study from RT-qPCR analysis of HCC samples and the publicly available microarray datasets, additionally confirmed that G9A was overexpressed in HCC tumor tissues. These results suggested that increased G9A expression was a biomarker of malignant cells. By analyzing the association between G9A expression and clinicopathological features using GSE14520, it was identified that its expression level was significantly associated with serum AFP level, and that the high expression level of G9A may indicate poor prognoses in patients with multinodular HCC. As serum AFP level is a primary tumor serological marker for HCC diagnosis, it was hypothesized that G9A expression level may be of assistance in diagnosing HCC, and may also have significant prognostic value in HCC.

The increased level of G9A expression in many types of tumor tissue clearly indicated that this molecule may promote the survival of tumor cells (15-17). This assumption had been confirmed by the GSEA analysis in the present study, which indicated that G9A may modulate genes that are associated with RNA processing and DNA replication, suggesting that G9A was involved in cell proliferation. The results of the present study, and those of a number of previous studies, have demonstrated that G9A inhibition may suppress cell proliferation in HCC cells and numerous other types of cancer cell (15,16,19). As G9A is important for cell proliferation, it is important to consider the crucial role of its primary target, H3K9me2. A previous study indicated that G9A and H3K9me2 were involved in R-loop formation, efficient transcriptional termination and gene post-transcriptional regulation (29). Therefore, it was hypothesized that G9A served a crucial role in cell proliferation, which may be involved in its role in tumorigenesis.

Autophagy is an evolutionarily conserved 'self-eating' process that is characterized by the formation of autophagosomes (30). MAP1LC3B is essential for membrane elongation and closure, which is the key molecular event in autophagy (30). It has been demonstrated experimentally that G9A is an epigenetic regulator for MAP1LC3B expression, and that G9A inhibition may induce autophagy-associated cell death (15,19,31). The present study demonstrated that G9A inhibition may increase MAP1LC3B expression in Huh7 and HepG2 cells. Furthermore, by analyzing the publicly available datasets in the GSE14520...
testing group, a significant inverse association between G9A and MAP1LC3B expression levels was identified. These results provide evidence that G9A may have the potential to regulate MAP1LC3B expression in HCC tumor tissues.

Despite increased G9A expression being demonstrated as being crucial for tumor cell proliferation and autophagy-induced cell death (15-17) its clinical significance in these types of cancer remains obscure. The present study provided results that the high expression of G9A may serve as an indicator for poor prognosis in multinodular HCC, and additionally confirmed an association between G9A and cell proliferation and MAP1LC3B expression in HCC cells. The results also demonstrated that G9A was involved in the pathogenesis of HCC, and may also serve as a potential target for HCC therapy. As aforementioned, its importance was not only confined to tumor cells. It is vital to disable G9A specifically in tumor cells without affecting normal cells during cancer therapy. The underlying molecular mechanisms of its expression remain obscure and require elucidation, which would assist in understanding the origin of G9A-associated cancer, and the developmental events in which G9A is involved.

In conclusion, the present study investigated the association between G9A expression level and the clinicopathological features of HCC by using publicly available datasets, and identified that G9A expression was associated with cell proliferation and autophagy in HCC cells. These results provide novel evidence for additional understanding of the crucial role of G9A in the tumorigenesis of HCC.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Natural Science Foundation of Hubei Province in China (grant nos. 2012FFB04316 and 2013CFB255), The Incubator Project of Renmin Hospital Wuhan University (grant no. 2013RMFH008), and the National Natural Science Foundation of China (grant no. 31300609).

Availability of data and materials

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

Author's contributions

JQ and QL conducted the majority of the experiments. ZZ, PW, YJ and YH collected the samples. TL contributed to data analysis and manuscript drafting. XJ and QZ contributed to Flow Cytometry. JQ and LC contribute to design and manuscript.

Ethics approval and consent to participate

The research protocol was approved by Wuhan University School of Basic Medical Sciences Ethics Committee. Informed written consent was obtained from all participants involved in the study.

Consent for publication

Informed written consent was obtained from all participants involved in the study.
Competing interests

The authors declare that they have no competing interests.

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