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JMJD2A predicts prognosis and regulates cell growth in human gastric cancer

Cheng-En Hu [a], Yong-Chao Liu [a], Hui-Dong Zhang [b], Guang-Jian Huang [a,*]

[a] Department of General Surgery, Huashan Hospital, Fudan University, Shanghai, China
[b] Department of General Surgery, Shanghai Children's Medical Center, Shanghai, China

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ABSTRACT

A number of JmjC domain-containing histone demethylases have been identified and biochemically characterized in mammalian. JMJD2A is a transcriptional cofactor and enzyme that catalyzes demethylation of histone H3 lysines 9 and 36. Here in this study, we aim to explore the role of JMJD2A in human gastric cancer. Quantitative real-time PCR, Western blot and immunohistochemistry analyses reveal higher expression of JMJD2A in clinical gastric cancer tissues than that in normal gastric mucosa. JMJD2A expression is associated with tumor stage and nodal status, and high level of JMJD2A predicts poor overall and disease-free survival. Univariate and multivariate survival analyses demonstrate that JMJD2A could serve as an independent prognostic factor. Furthermore, we show that inhibition the expression of JMJD2A attenuates the growth and transformation of three lines of gastric cancer cells. Mechanically, JMJD2A knockdown induces apoptosis of gastric cancer cells by up-regulating the expression of pro-apoptotic proteins and by down-regulating anti-apoptotic protein. Finally, we show that JMJD2A level is correlated with the level of the pro-apoptotic microRNA miR-34a in gastric cancer tissues and JMJD2A represses the expression of miR-34a by decreasing its promoter activity. Those findings demonstrate that JMJD2A regulates gastric cancer growth and serves as an independent prognostic factor, and implicate that JMJD2A may be a promising target for intervention.

1. Introduction

Lysine methylation is one of the most prominent histone post-translational modifications that regulate chromatin structure and gene expression. Changes in histone lysine methylation status have been observed during cancer formation and development, which is a consequence of the dysregulation of histone lysine methyltransferases or demethylases [1]. The JMJD (JmjC domain-containing) proteins, is composed of 30 members in humans based on the presence of the roughly 150 amino acid-long JmjC domain [2]. One of the largest JMJD subfamilies that has recently attracted much attention is the JMJD2 proteins (JMJD2A–JMJD2D), which are capable of recognizing di- and tri-methylated H3K9 and H3K36 as well as trimethylated H1.4K26 as substrates [1]. Various studies have shown that JMJD2A, JMJD2B, and/or JMJD2C are overexpressed in breast, colorectal, lung, prostate, and other tumors and are required for efficient cancer cell growth [1].

The most studied member of the JMJD2 family may be JMJD2A. A major study focus on JMJD2A has been in transcription regulation, where it may either stimulate or repress gene transcription. The latter function of JMJD2A involves association with the nuclear receptor co-repressor complex [3,4] or histone deacetylases or binding directly to a transcription factor such as the p53 tumor suppressor [5]. But it remains elusive whether this repressing function requires JMJD2A enzymatic activity. On the other hand, JMJD2A forms complexes with both the androgen and estrogen receptor (ER) and stimulates their activity, which relies largely upon JMJD2A catalytic activity [6,7]. Diverse physiological or pathological functions of JMJD2A have been identified. For instance, JMJD2A is demonstrated to function in human Wiskott–Aldrich syndrome [8], Kaposi's sarcoma-associated herpesvirus replication [9], cardiac hypertrophy [10], and DNA repair [11]. Importantly, JMJD2A has been reported to participate in several types of cancer, including duct carcinoma [12], breast cancer [7,13], lung cancer [14,15], colon cancer [5], bladder cancer [16], ovarian cancer, renal adenocarcinoma, and Head and Neck squamous cell carcinoma [17]. The aim of this study is to determine the role of JMJD2A in human gastric cancer and the underlying mechanism.

* Corresponding author. Address: Department of General Surgery, Huashan Hospital, Fudan University, 12 Wurumuqizhong Road, Shanghai 200040, China.
E-mail address: huanggjfdu@sina.com (G.-J. Huang).

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We identify JMJD2A as an oncogenic protein in human gastric cancer. JMJD2A expression is significantly up-regulated in human gastric cancer tissues. High JMJD2A level predicts poor overall and disease-free survival and serves as an independent prognostic factor for adverse outcome. JMJD2A facilitates gastric cancer cell growth and transformation. Mechanically, JMJD2A knockdown induces cellular apoptosis through, at least in part, by regulating miR-34a expression.

2. Materials and methods

2.1. Patients

One hundred and twenty-three cases of gastric cancer with full case history and paraffin-embedded tissue between January 1996 and December 2005 were collected at Huashan Hospital, Fudan University (Shanghai). The characteristics of the patients are shown in Supplementary Table 1. The diagnosis of gastric cancer was established using the *World Health Organization (WHO) morphological criteria* [18]. For the non-cancer normal gastric mucosa (NGM), 17 biopsy-tissue specimens were obtained from the antrum and the body of the normal stomach separated by a distance of 5 cm. Those normal samples were collected at Huashan Hospital, Fudan University (Shanghai) as well. A written form of informed consent was obtained from all patients and donors. The study was approved by the Clinical Research Ethics Committee of Huashan Hospital, Fudan University (Shanghai).

2.2. Cell proliferation assay

Cell proliferation was monitored by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation/Viability Assay kit (Sigma; #TOX1-1KT) according to the guidelines.

2.3. Soft sugar colony formation assay

Gastric cancer cells were suspended in 1.5 ml complete medium supplemented with 0.45% low melting point agarose (Gibco; #18300-012). The cells were placed in 35 mm tissue culture plates containing 1.5 ml complete medium and agarose (0.75%) on the bottom layer. The plates were incubated at 37 °C with 5% CO2 for 2 weeks. Cell colonies were stained with 0.005% crystal violet and analyzed using a microscope.

2.4. Apoptosis assay

Apoptosis and DNA damage were evaluated with fluorescence-activated cell sorting (FACS) assay and TUNEL staining, respectively. For apoptosis, FACS analysis was conducted with an Annexin V-FITC Apoptosis Detection Kit (Abcam; #ab14085) according to the manufacturer's protocol. A FACS Calibur flow cytometer was used for data analysis. For DNA damage, TUNEL staining was performed with TUNEL Apoptosis Detection Kit (Millipore; #17-141) according to the manufacturer's protocol. Percentage of TUNEL-positive cells was analyzed with an IPP software.

2.5. Luciferase assay

We designed the luciferase assay in according to the results of a previous report [19]. In detail, the human miR-34a (−1402 to +578 bp) promoter was cloned into the pGL4 reporter vector (Promega) to generate a miR-34a-luc reporter vector. 293T cells

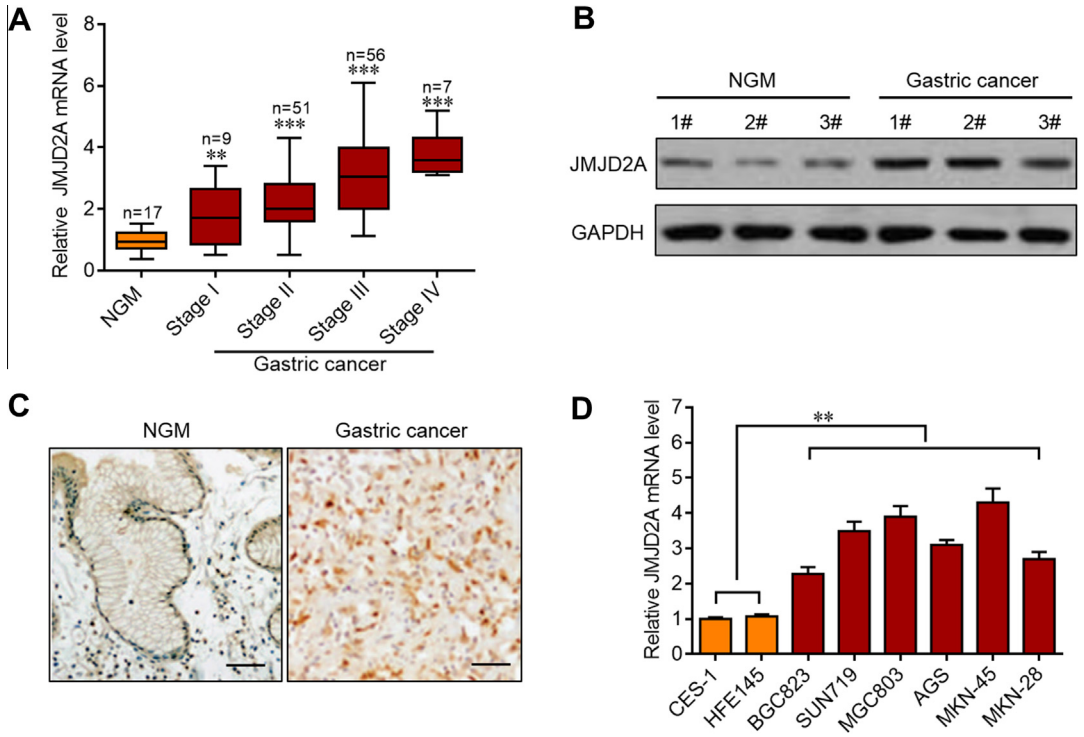

Fig. 1. JMJD2A is overexpressed in human gastric cancer tissues and cell lines. (A) JMJD2A mRNA level is up-regulated in human gastric cancer. RNA from normal gastric mucosa (NGM) and gastric cancer of different stages were subjected to cDNA synthesis and q-PCR analysis to test the mRNA levels of JMJD2A in these tissues. $^{**}p < 0.01$; $^{***}p < 0.0001$ *vs.* NGM. (B and C) JMJD2A protein level is up-regulated in human gastric cancer. (B) Protein from NGM and gastric cancer tissues were subjected to Western blot with anti-JMJD2A and anti-GAPDH antibodies. Representative Western blot result is shown. (C) Paraffin sections of NGM and gastric cancer tissues were subjected to immunohistochemistry analysis with anti-JMJD2A antibody. Representative immunohistochemical result is shown. (D) JMJD2A level is higher in gastric cancer cell lines than in epithelial cell lines. RNA from epithelial cell lines (GES-1 and HFE145) and gastric cancer cell lines (BGC823, SNU-719, MGC803, AGS, MKN-45 and MKN-28) was subjected to cDNA synthesis and q-PCR analysis to test the mRNA level of JMJD2A. $^{**}p < 0.01$.

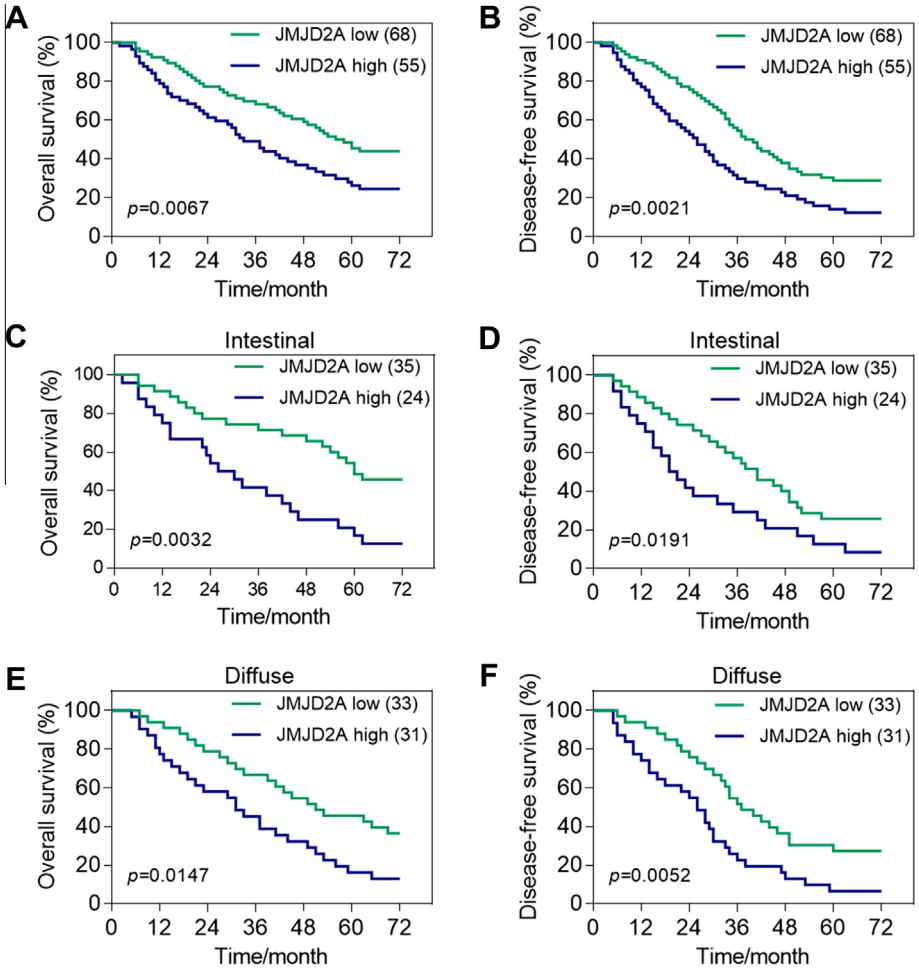

Fig. 2. High JMJD2A level predicts poor overall and disease-free survival. The immunohistochemical results were analyzed and the mean JMJD2A level was evaluated. The cases whose JMJD2A level was lower than the mean JMJD2A level (25% positive staining) were enrolled to the JMJD2A low group ($n = 68$), while the others were enrolled to the JMJD2A high group ($n = 55$). (A) Overall survival and (B) disease-free survival durations are worse in gastric cancer patients with higher JMJD2A expression. (C) Overall survival and (D) disease-free survival durations are worse in intestinal gastric cancer patients with higher JMJD2A expression. (E) Overall survival and (F) disease-free survival durations are worse in diffuse gastric cancer patients with higher JMJD2A expression.

were cultured in triplicate in 24-well plates until reaching 80% confluence. The cells were then co-transfected with miR-34a-Luc/p-RL-luc and were infected with sh-Ctrl or sh-JMJD2A virus. Dual luciferase assays (Promega) were performed as described by the manufacturer.

2.6. Xenograft mice experiment

Xenograft mice experiments were performed as described previously [20]. The tumor weight was evaluated at the terminal of experiments. $N = 10$ in each group.

2.7. Statistical analysis

Statistical differences between groups were determined using Student's t test (two groups) or two-way ANOVA test (more than two groups). The significance of the correlation between JMJD2A staining patterns and clinicopathological data was tested by Fisher's exact test and χ^2 test for trends. Probability of differences in overall and disease-free survival as a function of time was ascertained by use of the Kaplan–Meier method, with a log-rank test to probe for significance. Multivariate survival analysis was undertaken with the Cox model of proportional hazards. Linear regression analysis was performed to analyze the relation between JMJD2A and miR-34a expression in human gastric cancers. The statistical analysis was performed with GraphPad Prism 6 software and SPSS 19. p Values of less than 0.05 were considered statistically significant.

Other information on materials and methods are shown in Supplementary Information.

3. Results

3.1. JMJD2A is overexpressed in human gastric cancer

To determine the role of JMJD2A in human gastric cancer, we first test the expression levels of JMJD2A in 17 normal gastric mucosa and 123 gastric cancer tissues of different stages. The q-PCR results showed that JMJD2A mRNA level was significantly up-regulated in human gastric cancer tissues compared to normal gastric mucosa, and that JMJD2A mRNA level was higher in the cases with higher disease stage (Fig. 1A), which is consistent with a recent report [17]. We further investigated the protein level of JMJD2A in normal and cancer tissues with Western blot and immunohistochemistry assays. The results indicated that JMJD2A protein level was up-regulated in human gastric cancer tissues

compared to normal gastric mucosa (Fig. 1B, C). In addition, we compared the mRNA level of JMJD2A in normal epithelial cell lines (GES-1 and HFE145) and gastric cancer cell lines (BGC823, SNU-719, MGC803, AGS, MKN-45 and MKN-28). As shown in Fig. 1D, the mRNA level of JMJD2A was higher in gastric cancer cells compared to that in normal epithelial cells (Fig. 1D). Taken together, JMJD2A expression is up-regulated in human gastric cancer.

3.2. High JMJD2A level predicts poor prognosis

To further study the relationship between JMJD2A level and patients' prognosis, we analyzed the immunohistochemistry results. We divided the patients to JMJD2A low ($n = 68$) and JMJD2A high ($n = 55$) groups in according to immunohistochemistry scores. In detail, we calculated the JMJD2A-postive area using IPP software and the mean percentage of JMJD2A-positive area was 25%. The cases whose JMJD2A-postive area was lower than 25% were enrolled to the JMJD2A low group while the others were enrolled to the JMJD2A high group. The potential correlation between JMJD2A level and several clinical characteristics were analyzed with Fisher's exact test. High JMJD2A level was correlated with high disease stage, nodal status and metastasis (Suppl. Table 1). Furthermore, log-rank test was performed to determine the correlation between JMJD2A level and patients' survival. Patients with high expression of JMJD2A had a markedly worse overall and disease-free survival compared to those with low JMJD2A level (Fig. 2A, B). Since we did not find any significant difference in JMJD2A expression between intestinal and diffuse types of gastric cancer (Suppl. Table 1), we analyzed the correlations between JMJD2A level and intestinal or diffuse types of gastric cancer, respectively. The results demonstrated that JMJD2A level was markedly associated with overall and disease-free survival in patients with intestinal type of gastric cancer (Fig. 2C, D). Similar results were observed in diffuse type of gastric cancer (Fig. 2E, F). In addition, univariate and multivariate survival analyses indicated that JMJD2A could serve as an independent prognostic factor for outcome (Suppl. Tables 4 and 5). Taken together, JMJD2A predicts poor survival and serves as an independent prognostic factor.

3.3. JMJD2A knockdown represses growth and transformation of gastric cancer cells

We next wanted to know whether JMJD2A participates in growth and transformation of gastric cancer cells. We knocked down JMJD2A in human gastric cancer cell lines, MKN-45, SUV791, and MGC803 (Fig. 3A and Suppl. Fig. 1A). The cell proliferation assay showed that JMJD2A knockdown significantly attenuated the proliferation rate of MKN-45, SUV791, and MGC803 cells *in vitro* (Fig. 3B, C, Suppl. Fig 1B). To test whether JMJD2A knockdown affects the growth of gastric cancer cells *in vivo*, we performed xenograft mice experiments using control MKN-45 cells or MKN-45 cells with sh-JMJD2A transduction. The results revealed that JMJD2A knockdown significantly repressed gastric cancer MKN-45 cell growth *in vivo* (Fig. 3D, E). These evidence demonstrated that JMJD2A regulates *in vitro* and *in vivo* growth of gastric cancer cells. We next probed the potential contribution of JMJD2A to the transformative property of gastric cancer cells. Control gastric cancer cells or gastric cancer cells with sh-JMJD2A transduction were subjected to soft sugar colony formation assay. The results showed that JMJD2A knockdown markedly attenuated

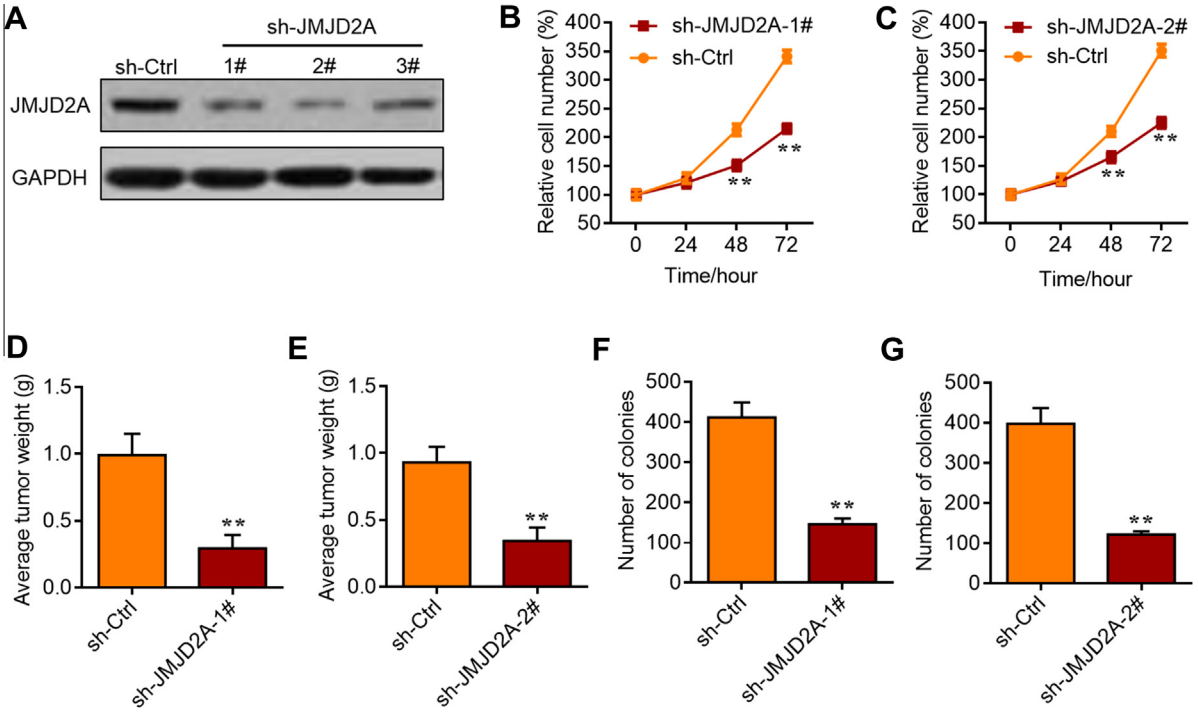

Fig. 3. JMJD2A knockdown attenuates gastric cancer growth and transformation. (A) Representative Western blot showing JMJD2A knockdown in MKN-45 gastric cancer cells. MKN-45 cells were infected with retrovirus carrying sh-Ctrl or three non-overlapping sh-JMJD2A sh-RNAs for 48 h. Then cells were lysed and were subjected to Western blot analysis with anti-JMJD2A and anti-GAPDH antibodies. (B and C) JMJD2A knockdown attenuates MKN-45 cell proliferation. MKN-45 cells were infected with retrovirus carrying sh-Ctrl, sh-JMJD2A-1# (B) or JMJD2A-2# (C) and the cell numbers were evaluated with MTT method at 24, 48, and 72 h post infection, respectively. (D and E) JMJD2A knockdown represses *in vivo* growth of MKN-45 cells. Control MKN-45 cells or those with JMJD2A-1# (D) or JMJD2A-2# (E) transduction were subjected to xenograft mice experiments. Tumor weight was evaluated at the terminal of the experiments. $N = 10$ in each group. (F and G) JMJD2A knockdown inhibits MKN-45 cell transformation. Control MKN-45 cells or those with JMJD2A-1# (F) or JMJD2A-2# (G) transduction were subjected to soft sugar colony formation assay. The number of colonies was analyzed two weeks later. $^{**}p < 0.01$ *vs.* sh-Ctrl.

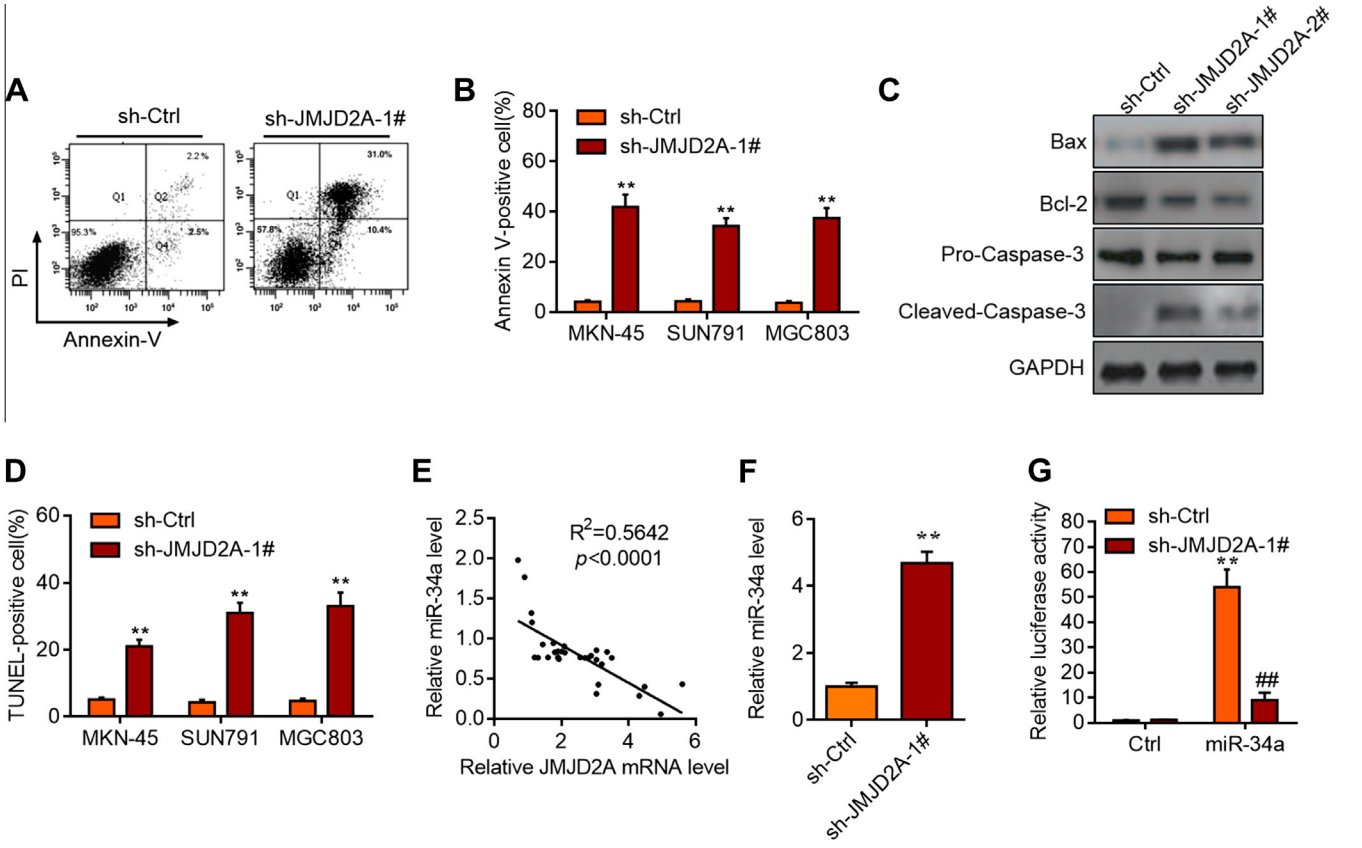

Fig. 4. JMJD2A knockdown induces gastric cancer cell apoptosis. (A and B) Apoptotic cells were detected by fluorescence-activated cell sorting (FACS) using Annexin V and propidium iodide (PI). (A) Representative FACS analysis of Annexin V and PI staining of MKN-45 cells infected with sh-Ctrl or sh-JMJD2A-1# retrovirus. (B) Quantitative analysis of Annexin V-positive cell of MKN-45, SUN791, and MGC803 cells. $^{**}p < 0.01$ *vs.* sh-Ctrl. (C) JMJD2A knockdown induces expression of pro-apoptotic proteins (cleaved caspase 3, Bax) and inhibits expression of anti-apoptotic proteins (Bcl-2) in MKN-45 cells. MKN-45 cells were infected with sh-Ctrl or sh-JMJD2A-1# or JMJD2A-2# retrovirus for 48 h, then protein was extracted and subjected to Western blot analysis with indicated antibodies. (D) JMJD2A knockdown induces DNA damage in gastric cancer cells. MKN-45, SUN791 and MGC803 cells were infected with sh-Ctrl or sh-JMJD2A-1# for 48 h. TUNEL assay was performed the detect DNA damage. Quantitative result is shown. $^{**}p < 0.01$ *vs.* sh-Ctrl. (E) miR-34a level is negatively correlated with JMJD2A level in human gastric cancer tissues. Relative mRNA level of miR-34a was assessed by q-PCR. Linear regression analysis was performed to analyze the correlation between miR-34a level and JMJD2A level. (F) JMJD2A knockdown up-regulates the expression of miR-34a. MKN-45 cells were infected with sh-Ctrl or sh-JMJD2A retrovirus for 48 h. Then the expression of miR-34a was evaluated with q-PCR. (G) JMJD2A knockdown enhances the promoter activity of miR-34a. Cells were transfected with either an empty pLuc reporter plasmid or a pLuc-miR-34a 3′UTR reporter plasmid, followed by infection with retrovirus carrying sh-Ctrl or sh-JMJD2A-1#. Firefly luciferase activity was determined relative to a co-transfected Renilla luciferase internal control and expressed as a percentage of the matching pLuc empty vector control. $^{**}p < 0.01$ *vs.* Ctrl + sh-Ctrl; $^{\#\#}p < 0.01$ *vs.* miR-34a + sh-Ctrl.

the colony formation capacity of MKN-45, SUN791, and MGC803 cells (Fig. 3F, G, Suppl. Fig. 1C). Accordingly, those findings indicated that JMJD2A regulates gastric cancer growth and transformation.

3.4. JMJD2A repression induces apoptosis of gastric cancer cells

Finally, we tried to examine the potential mechanism underlying the role of JMJD2A in human gastric cancer. We found that JMJD2A knockdown induced cellular apoptosis in MKN-45, SUN791, and MGC803 cells (Fig. 4A, B). The Western blot results showed that JMJD2A knockdown up-regulated the expression of pro-apoptotic proteins (Bax, cleaved caspase 3), whereas the level of the anti-apoptotic protein Bcl-2 was significantly down-regulated (Fig. 4C). In addition, a markedly increase in TUNEL-positive cells was observed in MKN-45, SUN791, and MGC803 cells when JMJD2A was knocked down (Fig. 4D), indicating that JMJD2A maintains genomic stability of gastric cancer cells.

miR-34a was reported to act as a pro-apoptotic microRNA and is downregulated in human gastric cancer [21–23]. We wanted to know whether JMJD2A regulates miR-34a in human gastric cancer. We found that miR-34a was indeed down-regulated in human gastric cancer (data not shown). Linear regression analysis was performed to test whether JMJD2A mRNA level is correlated with the level of miR-34a. Interestingly, our data showed that miR-34a level was significantly but negatively correlated with JMJD2A level (Fig. 4E), indicating JMJD2A may regulate miR-34a level in gastric cancer. We knocked down JMJD2A in MKN-45 cells and found that JMJD2A knockdown increased the level of miR-34a (Fig. 4F). Furthermore, the luciferase assay in 293T cells showed that JMJD2A knockdown up-regulated the promoter activity of miR-34a (Fig. 4G). These findings indicated that JMJD2A regulates gastric cancer apoptosis, at least in part, by modulating the expression of the pro-apoptotic microRNA miR-34a.

4. Discussion

Although JMJD2A has been evidenced to function in diverse types of cancer, its role in human gastric cancer remains unknown. In the present work, we showed that JMJD2A was overexpressed in human gastric cancer tissues, which is consistent with a recent work [17]. High expression of JMJD2A predicted overall and disease-free survival, and JMJD2A could serve as an independent prognostic factor. JMJD2A regulated gastric cancer growth and transformation. Finally, we showed that JMJD2A regulated gastric

cancer survival and genomic stability partly through targeting miR-34a.

JMJD2A is a histone demethylase that plays critical roles in diverse types of cancer. In human advanced bladder cancer, JMJD2A levels are significantly lower in malignant versus benign urothelium [16]. Lower JMJD2A intensity correlates with additional poor prognostic features and predicts significantly worse overall survival [16]. JMJD2A is overexpressed in human breast tumors both at the mRNA and protein level. In breast cancer cells, JMJD2A is capable of forming a complex with ERα *in vivo*. Consistently, the downregulation of JMJD2A in human T47D breast cancer cells leads to a decreased expression of Cyclin D1, a prominent ERα target gene and cell cycle regulator. The downregulation of JMJD2A induced a reduction in the growth of T47D cells [7]. Additionally, knockdown of JMJD2A significantly reduces proliferation rate and cell migration in human breast cancer cell line MDA-MB-231 [13]. A recent report shows that JMJD2A promotes cellular transformation by blocking cellular senescence through transcriptional repression of the tumor suppressor CHD5 [15]. Furthermore, JMJD2A is involved in human carcinogenesis through regulation of the G (1)/S transition in human bladder and lung cancers [14]. Recently, Black et al. [17] reported that JMJD2A was overexpressed in human breast cancer, head and neck cancer, lung cancer, ovarian cancer, renal adenocarcinoma, stomach adenocarcinoma and uterine and endometrial cancer. Consistently, we also found the overexpression of JMJD2A in human stomach/gastric cancer. We performed log-rank analyses and the results revealed that JMJD2A high expression predicted poor overall and disease-free survival. Additionally, the univariate and multivariate survival analyses demonstrated JMJD2A could serve as an independent prognostic factor. Altogether, those findings implicated that JMJD2A was overexpressed in human gastric cancer and may be a potential prognostic factor.

Furthermore, to determine the potential role of JMJD2A in human gastric cancer, we provided series of evidence. Firstly, we showed that JMJD2A could regulate the proliferation rate of human gastric cell lines *in vitro*. The *in vivo* xenograft mice experiments also revealed that JMJD2A promoted gastric cancer growth. As a previous work has reported that JMJD2A promotes cellular transformation in human lung cancer cells [15], we next performed soft sugar colony formation assay to test whether JMJD2A regulates transformation of human gastric cancer cells. The results indicated that JMJD2A was critically involved in the transformation of several human gastric cell lines. Finally, we investigated the potential mechanism underlying the roles of JMJD2A in human gastric cancer. We found that JMJD2A regulates gastric cancer cell survival and JMJD2A deficiency induces gastric cancer cell apoptosis by up-regulating pro-apoptotic proteins (Bax, cleaved caspase 3) and by down-regulating the anti-apoptotic protein Bcl-2. In addition, JMJD2A knockdown induces genomic instability, as evidenced by increased level of DNA damage, which is a previous finding that JMJD2A regulates genomic stability and DNA repair [24]. We found that miR-34a, which is reported to regulate apoptosis of gastric cancer cells, was negatively correlated with the expression of JMJD2A. JMJD2A knockdown significantly up-regulated the expression of miR-34a. Additionally, JMJD2A regulated the promoter activity of miR-34a. Taken together, JMJD2A participated in human gastric cancer growth and transformation. JMJD2A deficiency led to gastric cancer apoptosis partly due to the up-regulation of miR-34a.

However, several questions remain to study in our further work. The first one is how JMJD2A regulates cell transformation. Mallette et al. [15] showed that JMJD2A promotes cellular transformation by blocking cellular senescence through transcriptional repression of the tumor suppressor CHD5. Whether this pathway exists in human gastric cancer remains to determine. Furthermore, JMJD2A inhibits miR-34a expression in human gastric cancer cells. However, the underlying mechanism is not fully investigated in the present study. Two potential mechanisms may exist. The first one is that JMJD2A targets the histones of miR-34a promoter. The second one is that JMJD2A inhibits miR-34a through p53, which was reported to be repressed by JMJD2A and can regulate the transactivation of miR-34a [5,25]. Finally, whether miR-34a is the only target of JMJD2A in gastric cancer cell remains to determine, and further work is needed to explore whether miR-34a is critically essential for the role of JMJD2A in human gastric cancer.

In summary, we identify JMJD2A as an oncogenic protein in human gastric cancer. JMJD2A could serve as an independent prognostic factor and potential target for intervention.

Conflict of interests

None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.126.

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