Enhancement of Proliferation and Invasion of Gastric Cancer Cell by KDM5C Via Decrease in p53 Expression

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Abstract
Gastric cancer is a malignancy with high incidence and the second leading cause of cancer death worldwide. Development of efficient therapies against gastric cancer is urgent. Until now, the mechanisms of gastric cancer genesis remain elusive. The KDM5C is a histone demethylase that promotes cancer cell growth and is enriched in drug-resistant cancer cells. But the pathogenic breadth and mechanistic aspects of this effect relative to gastric cancer have not been defined. In present study, we found that KDM5C was overexpressed in gastric cancer cell lines and gastric cancer tissues but not in normal gastric tissues. The proliferation and invasive potential of gastric cancer cells was significantly increased by ectopic expression of KDM5C. Contrarily, RNA interference targeting KDM5C in gastric cancer cells significantly decreased the proliferation and invasive potential of cells. Moreover, we also found that the expression of p53 was modulated by KDM5C. Cells with overexpression of KDM5C exhibited greatly decreased p53 expression, whereas silencing of KDM5C expression dramatically increased p53 expression at both the messenger RNA and protein levels. Inhibition of p53 by small-interfering RNA reversed the shKDM5C-induced proliferation and invasion. Our results collectively suggested that KDM5C played a role in gastric cancer cells proliferation and invasion, which may be partly associated with the p53 expression.

Keywords
KDM5C, gastric cancer, p53, proliferation, invasion

Abbreviations
ER, estrogen receptor; HDM, histone demethylase; HIF, hypoxia inducible factor; PCNA, proliferating cell nuclear antigen; siRNA, small interfering RNA; cDNA, complementary DNA; qRT-PCR, quantitative reverse transcription polymerase chain reaction; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium; mRNA, messenger RNA; HIF, hypoxia inducible factor; shRNA, short hairpin RNA; ER, estrogen receptor

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Introduction
Gastric cancer is one of the most common and lethal human malignancies. Despite vital improvements in diagnostic and therapeutic techniques for gastric cancer, prognosis for patients remains poor.1 Despite a considerable amount of research, very few stable biomarkers have been identified for risk assessment or predication of clinical outcome, and further investigations are necessary.

Covalent histone modifications, including acetylation, methylation, phosphorylation, ubiquitination, glycosylation, and SUMOylation can modulate chromatin dynamics and affect multiple cellular functions.2 Histone lysine methylation is catalyzed by the large family of SET domain-containing histone methyltransferase, which is reversed by the activity of Jumonji (Jmj) and KDM histone demethylase (HDM) enzymes. Discriminate mono-, di-, and trimethylation and respective demethylation of lysine within histones H3 and H4 encompassing euchromatic domains acts as a genome-wide epigenetic switch that can either activate or repress transcription.3 More recently, 2 separate reports have suggested that the expression levels of KDM5C were significantly upregulated in

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cancer tissues, compared with those in corresponding normal tissues. KDM5C was identified in silicon and shown to be one of the demethylases capable of removing the trimethyl group from histone H3 lysine 9 (H3K9) on pericentric heterochromatin in mammalian cells. A line of recent reports indicated that hypoxic conditions can induce the expression of some JmjC family members, including KDM5C. In fact, KDM5C has been shown to harbor hypoxia inducible factor (HIF)-binding sites in their promoter sequences. However, the significance of KDM5C in gastric cancer oncogenesis and cancer progression is not fully understood so far. Here we revealed the potential role of KDM5C in gastric cancer proliferation, invasion, and metastasis.

Material and Methods

Patients and Tissue Samples

A total of 39 gastric cancer tissue samples, along with matched normal tissues, were used in this study. All of the samples were obtained from the second affiliated hospital of Soochow University between 2011 and 2013. For all of the patients who participated in this study, written informed consent was obtained, which was approved by the Ethical Committee of Soochow University School of Medicine.

Cell Culture and Reagents

The gastric cancer cell lines (NCI-N87, AGS, MKN45, and GES-1) were obtained from the American Type Culture Collection (Rockville, Maryland). Antibodies that had been raised against KDM5C, p53, p27, and p21 were purchased from Cell Signaling Technology (Beverly, Maryland), and a mouse antiproliferating cell nuclear antigen (PCNA) antibody was obtained from Abcam (Cambridge, Maryland). The signal silence p53 small-interfering short hairpin RNA (siRNA) and its control siRNA were purchased from Cell Signaling Technology. All of the remaining reagents were obtained from Sigma-Aldrich (St. Louis, Missouri), unless otherwise specified.

Plasmid Construction and Transfection

For overexpression, the complementary DNA (cDNA) representing the complete open reading frame of KDM5C was cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, California) to generate the KDM5C expression plasmid. The expression plasmid was verified by sequencing both strands and was used to transfect the NCI-N87 cells to establish the KDM5C overexpression cell line. For KDM5C RNA interference, the control and KDM5C shRNA plasmids were purchased from Santa Cruz Biotechnology (Santa Cruz, California) and was used to transfect the MKN45 cells to establish the KDM5C knockdown cell line. The transfection efficiency of KDM5C was confirmed by Western blotting and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium Assay

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay was used to assess cell proliferation. The cells
were seeded and 20 mL of the MTT solution (5 mg/mL) was then added to each well at the indicated time. The absorbance at 490 nm was measured using a microplate reader (Bio-Rad, Hercules, California).

**Colony Formation Assay**

The cells were seeded in 6-cm dishes at a density of 300 cells per dish. After incubation for 14 days, the colonies were fixed with methanol for 10 minutes and stained with Crystal violet for 15 minutes, after which point the number of colonies containing more than 50 cells was scored.

**Western Blot Assay**

Equal amounts of protein were separated using sodium dodecyl sulfate polyacrylamide gels and were electrotransferred to polyvinylidene fluoride membranes (Millipore, Bedford, Massachusetts). The membranes were immunoblotted overnight at 4 C with primary antibodies, followed by their respective secondary antibodies. β-Actin was used as the loading control.

**Quantitative Reverse Transcription PCR**

RNA was extracted using TRIzol reagent, according to the manufacturer’s recommended protocol (Invitrogen). Quantitative reverse transcription PCR was performed using Applied Biosystems (Foster City, California) StepOne and StepOne Plus Real-Time PCR Systems. Glyceraldehyde 3-phosphate dehydrogenase was used as a loading control. The experiments were repeated a minimum of 3 times to confirm the results.

**Immunofluorescence Staining**

The cells were grown on the sterile coverslips, and the cells were fixed with 4% paraformaldehyde and permeabilized using 0.1% Triton-X100. Cells were blocked with rabbit anti-PCNA antibody followed by rhodamine-conjugated antirabbit secondary antibody. Finally, the cells were further stained with 4, 6-diamidino-2-phenylindole.

**Chamber Assay**

Migration and invasion assays were performed as described previously. Invasion assays were performed in 24-well Transwell chambers (BD Biosciences, Bedford, Massachusetts) containing polycarbonate filters with 8 mm pores coated with Matrigel (BD Biosciences). First, the cells that were suspended in serum-free Dulbecco’s Modified Eagle Medium were added to the upper compartment of the chamber, and medium containing 10% fetal bovine serum was added to the lower compartment of the chamber. At the indicated time points, the number of cells that had migrated through the membrane and attached to the lower surface of the membrane was counted under a light microscope for a minimum of 10 random visual fields. The migration assay was similar to the migration assay except that the upper side of the membranes was not coated with the Matrigel.

**Tumor Formation Assay In Vivo**

The in vivo tumorigenesis and metastasis assays were performed, as described previously. Briefly, 1 \times 10^6 cells were injected subcutaneously into the right flanks of severe
combined immunodeficient mice. Tumor length (L) and width (W) were measured every 3 days, and tumor volume was calculated using the following equation: volume = \frac{W^2 \times L}{2}.

After 6 weeks, the mice were killed, and the tumor volume and weight were measured. All of the animal experiments were performed with the approval of the Zhenjiang University School of Medicine Animal Care and Use Committee.

**Statistical Analysis**

The results were analyzed using SPSS 18.0 software (Chicago, Illinois). Each experiment was repeated a minimum of 3 times. A 2-tailed t test was used to determine statistical significance. The results were presented as the means ± standard deviation. P values < .05 were considered to be statistically significant.

**Results**

**Expression of KDM5C Was Upregulated in Gastric Cancer Tissues**

To test whether KDM5C is overexpressed in gastric cancer, we first compared the expression levels of KDM5C in 39 gastric cancer tissue samples to those in the adjacent normal tissues using Western blotting. The protein levels of KDM5C were found to be increased in the tumor lesions compared with the matched normal tissue lesions in all of the samples (Figure 1A). Next, we assayed the messenger RNA (mRNA) expression of KDM5C by qRT-PCR in these tissues, and the results showed that the KDM5C mRNA was also upregulated in gastric cancer tissues compared with the matched normal gastric tissues (Figure 1B). These results suggest that a possible role for KDM5C in the development or progression of gastric cancer.

**Establishment of Stable KDM5C Transfectants in Gastric Cancer Cell Line**

We measured the KDM5C expression levels in 4 gastric cancer cell lines (NCI-N87, AGS, MKN45, and GES-1) and one normal gastric tissue by Western blot (Figure 2A). The results show that high levels of KDM5C were expressed in nearly all tumor cell lines compared with the normal gastric tissue. The NCI-N87 cell line was chosen for establishing a stable cell line because it has the lowest expression of KDM5C in the 4 gastric cancer cell lines. We also used shRNA to generate a stable KDM5C knockdown in the MKN45 gastric cancer cell line.
The transfection efficiency was confirmed using Western blotting analyses. As shown in Figures 2B and C, the NCI-N87 cells that had been transfected with the KDM5C expression plasmid displayed significantly increased KDM5C expression at both the mRNA and protein levels compared with the vector cell lines. In addition, the MKN45 cells that had been transfected with the KDM5C shRNA plasmid displayed significantly decreased KDM5C expression at both the mRNA and protein levels compared with the control cells (Figures 2B and C).

**KDM5C Promoted Gastric Cancer Cell Proliferation**

We first explored the effects of KDM5C expression on cell growth using the MTT assay. As shown in Figure 3A, overexpression of KDM5C significantly enhanced the growth of NCI-N87 cells, whereas KDM5C knockdown significantly inhibited the growth of MKN45 cells (Figure 3B). Next, we performed a clonogenic assay to confirm the effects of KDM5C on proliferation. We found that overexpression of KDM5C dramatically increased the colony formation efficiency of
NCI-N87 cells, whereas the colony formation efficiency was dramatically reduced in the MKN45 shRNA cell lines (Figures 3C and D). As PCNA is an important marker of cell proliferation, we next examined the PCNA by immunofluorescence staining. As shown in Figure 4, we found that the overexpression of KDM5C in NCI-N87 cells significantly upregulated PCNA staining (Figure 4A). Also, knockdown of KDM5C in MKN45 cells dramatically downregulated the staining of PCNA (Figure 4B). These results suggested that KDM5C could significantly promote the proliferation of gastric cancer cells.

KDM5C Enhanced Gastric Cancer Cell Mobility

We next assessed whether KDM5C could affect the ability of gastric cancer cells to migrate and invade using a transwell assay. Overexpression of KDM5C enhanced both migration and invasion in NCI-N87 cells (Figure 5A and B). In addition, KDM5C knockdown in MKN45 cells significantly inhibited cell migration and invasion (Figure 5C and D). These results indicated that KDM5C significantly promoted the invasion and migration of gastric cancer cells.

KDM5C Promoted Tumorigenesis in Vivo

To explore the effects of KDM5C on tumorigenesis in vivo, different cells were injected subcutaneously into the flanks of nude mice. The diameters of the tumors were measured every 3 days. The tumors in the KDM5C overexpression group grew largely compared with those in the mice that had been injected with the control cells during the subsequent days (Figure 6A). Similar results were observed in the MKN45 cells. We found that the KDM5C knockdown cells formed tumors earlier and that the tumor volumes were much smaller in those that were formed from the knockdown cells than in those that were formed from the control cells (Figure 6B). We also analyzed the weight of the tumors (Figure 6C and D). These results suggested that KDM5C promotes gastric cancer cell xenograft formation and growth in vivo.

KDM5C Enhanced Tumor Proliferation and Metastasis Partly Via p53

The p53 has important roles in the proliferation, migration, and invasion of various cancer types, including gastric cancer.13,14
Thus, we determined whether the p53 was involved in KDM5C-mediated tumor proliferation and metastasis. We evaluated the effects of KDM5C on the p53 and its downstream molecules p27 and p21 in NCI-N87 and MKN45 cells by Western blot. As shown in Figure 7A, upregulation of KDM5C significantly inhibited the expression of p53, p27, and p21 in NCI-N87 cells. Knocking down of KDM5C dramatically upregulated the expression of p53, p27, and p21. To further define a causal relationship between p53 induction and defective foci formation, we transfected MKN45 cells with both KDM5C shRNA and p53 siRNA (Figure 7B). p53 inhibition significantly restored the proliferation potential of MKN45 cells treated with KDM5C shRNA (Figure 7C). We further tested the role of p53 in KDM5C-induced migration by knocking down p53 expression using siRNA. As shown in Figure 7D, the migration ability that was induced by shKDM5C was obviously attenuated following p53 knockdown using siRNA. Thus, KDM5C siRNA knockdown impaired the proliferation and migration potential of gastric cancer cells at least in part through p53 induction.

**Discussion**

Gastric cancer is one of the most common cancers in the world. Despite the decline in mortality, a number of patients with gastric cancer develop metastatic tumors even after surgical removal of the primary tumors. Therefore, metastasis continues to be the main obstacle to the effective treatment of gastric cancer, and there is an urgent need to identify novel molecular factors that lead to the invasiveness and metastasis of gastric cancer. In the present article, we identified KDM5C as a candidate target gene for the promotion of gastric cancer growth and metastasis.

The KDM5 family of HDMs has 4 members, KDM5A, B, C, and D, that trimethylated histone H3K9. KDM5 family proteins promote transcriptional activation, thereby affecting important processes such as the hormone response, stem cell renewal, germ cell development, and cellular proliferation and differentiation. The involvement of KDM5C in tumorigenesis has been supported by recent findings that high expression of KDM5C in ER-positive primary gastric cancers and regulated by both ER-α and HIF1-α in normoxia and hypoxia. However, the mechanisms underlying KDM5C involved carcinogenesis, and the roles of KDM5C in gastric cancer are largely unknown.
In present study, we found that KDM5C was overexpressed in gastric cancer cell lines and gastric cancer tissues but not in normal gastric tissues. The proliferation and invasive potential of gastric cancer cells was significantly increased by ectopic expression of KDM5C. Contrarily, RNA interference targeting KDM5C in gastric cancer cells significantly decreased the proliferation and invasive potential of cells. Moreover, we also found that the expression of p53 was modulated by KDM5C. Overexpressed KDM5C cell exhibited greatly decreased p53 expression, whereas silencing of KDM5C expression dramatically increased p53 expression at both the mRNA and protein levels. Inhibition of p53 by siRNA reversed the shKDM5C-induced proliferation and invasion. Our results collectively suggested that KDM5C expressed in gastric cancer played a role in gastric cancer cells proliferation and invasion, which may be partly associated with the p53 expression.

Oncogene can enhance tumor growth and invasive and metastatic potential.\textsuperscript{21,22} Overexpression of oncogenes may lead to a malignant cancer phenotype. Previous studies have reported that the expression levels of oncogenes were increased in tumors compared with normal tissues.\textsuperscript{23,24} To confirm the tumor promoter function of KDM5C, we first examined the levels of KDM5C in gastric cancer samples and matched normal gastric tissue samples using Western blotting. We found that KDM5C was significantly increased in cancers, which suggested that KDM5C was a candidate tumor oncogene in gastric cancer. To further explore the role of KDM5C in gastric cancer, we transfected gastric cancer cells either to ectopically express KDM5C or to inhibit its expression using RNA interference. Overexpression of KDM5C in vitro significantly enhanced the proliferation, migration, and invasion of gastric cancer cells, while knockdown of KDM5C inhibited cell growth and mobility. Our in vivo experiments also demonstrated that KDM5C markedly enhanced tumorigenesis and growth. These data further supported the tumor promoter role of KDM5C in gastric cancer.

The p53 protein has been demonstrated to have an essential role in gastric cancer proliferation, motility, and invasion.\textsuperscript{23} Lower expression of p53 was observed in gastric cancer tissues, and the lower level of p53 was associated with both an increased risk of lymph node metastases and poor prognoses.\textsuperscript{23,25,26} Therefore, we investigated whether KDM5C enhanced tumorigenesis and metastasis in gastric cancer via p53. Our results indicated that the levels of p53 and its downstream molecules p27 and p21 were significantly decreased in
KDM5C-overexpressing cells, and p53, p27, and p21 were upregulated in KDM5C knockdown cells. When we pretreated the KDM5C knockdown cells with p53 siRNA, the decreased proliferation, migration, and invasion ability of KDM5C knockdown cells was also reversed. All of these data revealed that KDM5C suppresses the proliferation, migration, and invasion activities of gastric cancer cells partially via p53.

In conclusion, we found that KDM5C expression was generally lower in cancer lesions compared with matched nontumor tissues. Our in vitro and in vivo data demonstrate that KDM5C has a vital function in inhibiting cell mobility, which is at least partially controlled by the p53. Thus, we propose that the candidate tumor oncogene KDM5C may be an effective novel therapeutic target in the management of gastric cancer.

**Authors’ Note**
The authors Liming Xu and Wei Wu contributed equally to this article.

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