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AIMS AND SCOPE

The primary aim of *World Journal of Gastrointestinal Oncology* (*WJGO*, *World J Gastrointest Oncol*) is to provide scholars and readers from various fields of gastrointestinal oncology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

WJGO mainly publishes articles reporting research results and findings obtained in the field of gastrointestinal oncology and covering a wide range of topics including liver cell adenoma, gastric neoplasms, appendiceal neoplasms, biliary tract neoplasms, hepatocellular carcinoma, pancreatic carcinoma, cecal neoplasms, colonic neoplasms, colorectal neoplasms, duodenal neoplasms, esophageal neoplasms, gallbladder neoplasms, *etc*.

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ORIGINAL ARTICLE

Basic Study

N6-methyladenosine modification of hypoxia-inducible factor-1α regulates *Helicobacter pylori*-associated gastric cancer *via* the PI3K/AKT pathway

Tong-Yan An, Quan-Man Hu, Peng Ni, Yan-Qiao Hua, Di Wang, Guang-Cai Duan, Shuai-Yin Chen, Bin Jia

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Abstract

BACKGROUND

Helicobacter pylori (*H. pylori*) colonizes the human gastric mucosa and is implicated in the development of gastric cancer (GC). The tumor microenvironment is characterized by hypoxia, where hypoxia-inducible factor-1α (HIF-1α) plays a key role as a transcription factor, but the mechanisms underlying *H. pylori*-induced HIF-1α expression and carcinogenesis remain unclear.

AIM

To explore the underlying mechanism of *H. pylori*-induced HIF-1α expression in promoting the malignant biological behavior of gastric epithelial cells (GES-1).

METHODS

The study was conducted with human GES-1 cells *in vitro*. Relative protein levels of methyltransferase-like protein 14 (METTL14), HIF-1α, main proteins of the PI3K/AKT pathway, epithelial-mesenchymal transition (EMT) biomarkers, and invasion indicators were detected by Western blot. Relative mRNA levels of *METTL14* and *HIF-1α* were detected by quantitative reverse transcription-polymerase chain reaction. mRNA stability was evaluated using actinomycin D, and the interaction between METTL14 and HIF-1α was confirmed by immunofluorescence staining. Cell proliferation and migration were evaluated by cell counting kit-8 assay and wound healing assay, respectively.

RESULTS

H. pylori promoted HIF-1α expression and activated the PI3K/AKT pathway. Notably, METTL14 was downregulated in *H. pylori*-infected gastric mucosal epithelial cells and positively regulated HIF-1α expression. Functional experiments showed that the overexpression of HIF-1α or knockdown of METTL14 enhanced the activity of the PI3K/AKT pathway, thereby driving a series of malignant transformation, such as EMT and cell proliferation, migration, and invasion. By contrast, the knockdown of HIF-1α or overexpression of METTL14 had an opposite effect.

CONCLUSION

H. pylori-induced underexpression of METTL14 promotes the translation of HIF-1α and accelerates tumor progression by activating the PI3K/AKT pathway. These results provide novel insights into the carcinogenesis of GC.

Key Words: *Helicobacter pylori*; Gastric cancer; Methyltransferase-like protein 14; Hypoxia-inducible factor-1α; N6-methyladenosine; PI3K/AKT pathway

Core Tip: *Helicobacter pylori* (*H. pylori*) is the most significant risk factor for gastric cancer. Studies have shown that hypoxia-inducible factor-1α (HIF-1α) is highly expressed in tumors, enabling them to adapt to hypoxic microenvironment and promoting malignant behavior. However, the pathogenesis of HIF-1α in *H. pylori* infection remains unclear. We found that methyltransferase-like protein 14 acts as a molecular switch of *H. pylori*-induced HIF-1α expression and regulates the malignant behavior of gastric epithelial cells through the PI3K/AKT pathway. This study is the first to explore the mechanism by which *H. pylori* induces HIF-1α expression from the perspective of N6-methyladenosine modification.

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INTRODUCTION

Gastric cancer (GC) is one of the most common malignant tumors of the gastrointestinal tract. According to the global cancer statistics in 2020, its incidence and mortality rates rank fifth and fourth worldwide, respectively[1]. As the primary risk factor for GC, *Helicobacter pylori* (*H. pylori*) is classified as a class I carcinogen and has infected more than half of the world's population. Nearly 90% cases of distal GC are closely associated with *H. pylori* infection, probably depending on strain virulence and the genetic susceptibility of hosts and environmental factors[2-4].

Hypoxia is a typical characteristic of the tumor microenvironment with increased hypoxia-inducible factor (HIF)-1α expression and enhanced transcription of many downstream target genes. HIF-1 is composed of HIF-1α, which is the active subunit, and HIF-1β. HIF-1α is degraded *via* the ubiquitin-proteasome pathway under normoxia conditions, but hypoxia can stabilize its expression. Thus, HIF-1α is a crucial regulator in the hypoxic microenvironment of tumors, including GC. However, two studies have shown that HIF-1α can be induced by *H. pylori* under normoxic conditions through reactive oxygen species or the PI3K/AKT/mTOR pathway[5-7], and *H. pylori* strains isolated from high-risk patients exert strong effects[8]. In addition, the mechanisms underlying *H. pylori*-induced HIF-1α expression remains unclear. As an important transcription factor, HIF-1α can not only regulate target genes but also promote epithelial-mesenchymal transition (EMT), proliferation, migration, and invasion through the PI3K/AKT signaling pathway[9,10].

N6-methyladenosine (m6A) is a dynamic and reversible post-transcriptional modification of eukaryotic RNA. That is, the H on the sixth nitrogen atom of RNA adenylate is replaced by a methyl group, and this substitution is mainly regulated by methyltransferases (writers, such as METTL3, METTL14, and WTAP), demethylases (erasers, including FTO and ALKBH5), and RNA-binding proteins (readers, such as YTHDF1/2/3 and YTHDC1/2)[11]. m6A modification affects the metabolic processes of the RNA, for example, RNA stability, alternative splicing, translation, and RNA-protein interaction[12-15], thus playing a considerable role in the initiation and progression of tumors. m6A modifiers can act as oncogenes or tumor suppressor genes. For example, METTL3 mediates the upregulation of FBXO31 in a YTHDF1-dependent manner, promotes the proteasomal-dependent degradation of SIRT2, and facilitates the migration and invasion of pancreatic cancer cells[16]. Another study indicated that METTL14 interacts with the microprocessor protein DGCR8, promotes the processing and maturation of pri-miRNA126, and inhibits the metastasis of liver cancer[17]. Furthermore, m6A modification participates in the various aspects of tumor development, such as immunity, metabolism, and tumor stem cells' self-renewal[18,19], emerging as an important molecular biomarker in tumor initiation and development. m6A dysregulation exerts multiple regulatory effects in GC[20,21]. The pathogenic mechanism of

H.pylori as a significant risk factor for GC remains unclear. We have found that m6A modification has an important effect on *H. pylori*-associated GC. Therefore, we aimed to explore the specific mechanisms of *H. pylori*-associated GC from the perspective of m6A modification.

The mechanism of HIF-1α induction in *H. pylori*-infected gastric mucosal epithelial cells is not fully understood. Additionally, the regulatory network between HIF-1α and the PI3K/AKT pathway is complex, and METTL14 plays a potential role in GC. Therefore, we hypothesized that METTL14 may act as a molecular switch for *H. pylori*-induced HIF-1α expression, subsequently activate the PI3K/AKT pathway, and accelerate GC development. The results will provide a novel direction for research on the pathogenic mechanisms of *H. pylori* infection.

MATERIALS AND METHODS

Cells and H. pylori strain culture

Human gastric epithelial cells (GES-1, Bohui Biotechnology) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% fetal bovine serum (FBS, Biochannel) and 1% penicillin-streptomycin solution (100 ×, Gibco). *H. pylori* 27 strain (*Hp27*) was isolated from the tissues of patients with chronic atrophic gastritis in Zhengzhou, China, and cultured in Columbia blood agar plates with 5% off fiber sheep blood and four antibiotics (vancomycin, amphotericin, trimethoprim, and polymyxin; Hopebio). The cells and *H. pylori* were incubated at 37 °C with 5% CO_2 as previously described[22].

Cell infection with H. pylori

The cells were seeded in culture plates and cultivated to an appropriate density for infection. The well-grown *H. pylori* was collected into complete medium without antibiotics, and the concentration was adjusted. An OD_{600} = value of 1 was considered equivalent to 5.9×10^8 CFU/mL. The cells were infected at the corresponding multiplicity of infection (MOI) [23].

Analysis of mRNA levels by quantitative reverse transcription-polymerase chain reaction

The mRNA levels of *HIF-1α* and *METTL14* were detected by quantitative reverse transcription-polymerase chain reaction (RT-qPCR). Total RNA was isolated with RNAiso Plus (Takara) in accordance with the instructions of the manufacturer, and cDNA was synthesized with Hifair® III 1st Strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus, Yeasen). The primers for RT-qPCR are shown in Supplementary Table 1. We used Hieff® qPCR SYBR Green Master Mix (No Rox, Yeasen) and performed RT-qPCR in a CFX Opus 96 real-time PCR system (Bio-Rad). The relative mRNA levels were calculated by the $2^{-\Delta\Delta Ct}$ method[24].

Protein extraction and Western blot analysis

The protein levels of HIF-1α, m6A modifiers (METTL3, METTL14, FTO, YTHDF1, YTHDF2, YTHDF3, and YTHDC1), EMT biomarkers (N-cadherin, Vimentin, and ZEB1), and cell invasion indicators (MMP2 and MMP9) were detected through Western blot analysis. Total protein was collected using RIPA lysis buffer and protease-phosphatase inhibitor cocktail (50 ×; Beyotime). Protein concentration was measured with a BCA protein assay kit (Biomed). Subsequently, approximately 50 μg of proteins per lane were separated by SDS-PAGE and transferred to PVDF membranes (Millipore). The membranes were blocked with 5% skim milk (BD, Biosciences). The primary antibodies used are shown in Supplementary Table 2. The membranes were incubated with the primary antibodies and then with the corresponding secondary antibodies (HRP-conjugated Affinipure Goat Anti-Mouse/Rabbit IgG (H+L), 1:5000, Proteintech). Finally, the blots were detected with ECL chemiluminescence substrate (Biosharp) and scanned on Amersham Imager 600. Protein extraction and Western blot analysis were performed in accordance with our previous study[25].

Immunofluorescence staining

The interaction of METTL14 and HIF-1α after *H. pylori* infection was confirmed through immunofluorescence staining[26, 27]. Sterile coverslips (24 mm × 24 mm) were placed in a six-well plate and cell suspension (approximately 1-2 × 10^4 cells) was added onto them. After 5 h, the culture medium was replenished, and the plate was placed in an incubator with 5% CO_2 at 37 °C overnight. Then, the coverslips were washed with PBS and fixed with 4% paraformaldehyde (Beyotime) for 10-15 min before subsequent immunofluorescence analysis. Immunofluorescence staining and laser confocal microscopy scanning were provided by Servicebio.

Plasmids, small interfering RNAs, and transfection

Plasmids were constructed and transfected into GES-1 cells to overexpress METTL14 or HIF-1α. By contrast, small interfering RNA (siRNAs) were designed and transfected for METTL14 or HIF-1α knockdown. METTL14 plasmid was constructed by ligating linearized pcDNA3.1(+) vector and the full-length coding sequence of METTL14. The related primers are shown in Supplementary Table 3. HIF-1α plasmid was donated by Henan Provincial Center for Disease Control and Prevention. It was constructed by ligating a linearized pcDNA3 vector and the full-length coding sequence of HIF-1α. siRNA-negative control (NC), siRNA-METTL14, and siRNA-HIF-1α were purchased from OBiO Technology, and the related sequences are shown in Supplementary Table 3. The plasmids and siRNA were transfected into GES-1 cells by using Lipofectamine 2000 Reagent (Invitrogen) and opti-MEM (Gibco)[28].

Cell counting kit-8 assay

Cell proliferation in each treatment group was measured by cell counting kit-8 (CCK8) assay. Approximately 2 × 10^3 cells were seeded into a 96-well plate. After cells were adhered, the corresponding treatment measures were given to each group, and then 10 μL of CCK8 reagent (Yeasen) was added into the wells to be detected. After 2 h of incubation, the absorbance was detected at 450 nm[29].

Wound healing assay

The cell migration of each treatment group was evaluated by wound healing assay. The cells were seeded in 6-well plates, and a 200 μL pipette tip was used to scratch the surface after the treatment and cell confluence reached 100%. Then, we performed *H. pylori* infection and changed the medium to DMEM with 1% FBS. Finally, images were obtained at 0, 12, and 24 h[30].

Detection of mRNA stability

Actinomycin D (2.5 μg/mL, MedChemExpress) was used as the transcription inhibitor in the HIF-1α mRNA stability test after METTL14 overexpression. It was added to the METTL14-overexpressed GES-1 cells, and total RNA was extracted at 0, 6, 12, and 24 h. Finally, the half-life of HIF-1α mRNA was analyzed through RT-qPCR[31].

Statistical analysis

All analyses were conducted using GraphPad Prism 8.0. All data are presented as the mean ± SD, and at least three independent experiments were conducted. The results of Western blot and wound healing assay were assessed with Image J. Differences between treatments were analyzed by Student's *t*-test or one-way ANOVA. *P* values of < 0.05 indicated statistical significance.

RESULTS

H. pylori promotes HIF-1α and activates the PI3K/AKT pathway

As a crucial transcription factor, HIF-1α plays a significant role in hypoxia and is closely associated with signaling pathways. To investigate the effect of *H. pylori* on HIF-1α, we first examined the mRNA level of *HIF-1α* at different infection time points (3 h, 6 h, 12 h, and 24 h) and MOI (50, 100, and 200) (Figure 1A). The results indicated that *H. pylori*-induced HIF-1α has a certain time dependency but doesn't have this association with MOI. Therefore, the subsequent experiments will be conducted at t = 24 h and MOI = 100. And then we found that the protein level of HIF-1α was significantly increased in the *H. pylori*-infected group (Figure 1B and C). Moreover, we observed a substantial increase of p-PI3K and p-AKT expression after *H. pylori* infection, suggesting that *H. pylori* can activate the PI3K/AKT pathway (Figure 1B and C). These results strongly support the involvement of HIF-1α and the PI3K/AKT pathway in *H. pylori*-induced effects.

HIF-1α overexpression promotes activation of the PI3K/AKT pathway, EMT, and cell proliferation, migration, and invasion

In order to investigate the potential regulatory role of HIF-1α in the PI3K/AKT pathway and its influence on GC progression, we overexpressed HIF-1α in GES-1 cells and co-cultured them with *H. pylori*. The overexpression of HIF-1α was confirmed by RT-qPCR and Western blot analysis (Figure 2A-C). Subsequently, we observed a significant increase in the expression of p-PI3K and p-AKT in the HIF-1α-overexpressed GES-1 cells (Figure 2B and C). Additionally, the overexpression of HIF-1α led to an increase in the levels of N-cadherin, Vimentin, and ZEB1, which are established markers of EMT and indicators of mesenchymal cell characteristics (Figure 2B and C). Moreover, we observed elevated expression of MMP2 and MMP9, which are key members of the matrix metalloproteinase family closely associated with tumor invasion (Figure 2B and C). Consistent with these molecular changes, the CCK8 proliferation assay and wound healing assay demonstrated enhanced cell proliferation and migration abilities in the HIF-1α-overexpressed cells (Figure 2D-F). Overall, these findings indicate that *H. pylori*-induced HIF-1α expression may promote the malignant behaviors of GC through the PI3K/AKT pathway, suggesting a potential regulatory role for HIF-1α in GC progression.

HIF-1α knockdown suppresses the PI3K/AKT pathway, EMT, and cell proliferation, migration, and invasion

To further elucidate the functional role of HIF-1α, we utilized siRNA transfection to downregulate HIF-1α expression, which led to contrasting results. Initially, a reduction in mRNA levels was confirmed through RT-qPCR analysis (Figure 3A). Subsequent co-culture with *H. pylori* resulted in decreased levels of phosphorylated PI3K and AKT, as well as a concurrent decrease in the expression of EMT biomarkers, including N-cadherin, Vimentin, and ZEB1 (Figure 3B and C). Similarly, the expression of MMP2 and MMP9 was also found to be reduced (Figure 3B and C). Both CCK8 and wound healing assays denoted a decrease in cell proliferation and migration rates, respectively (Figure 3D-F). Collectively, these findings provide further evidence supporting the role of HIF-1α in promoting the PI3K/AKT pathway and GC progression.

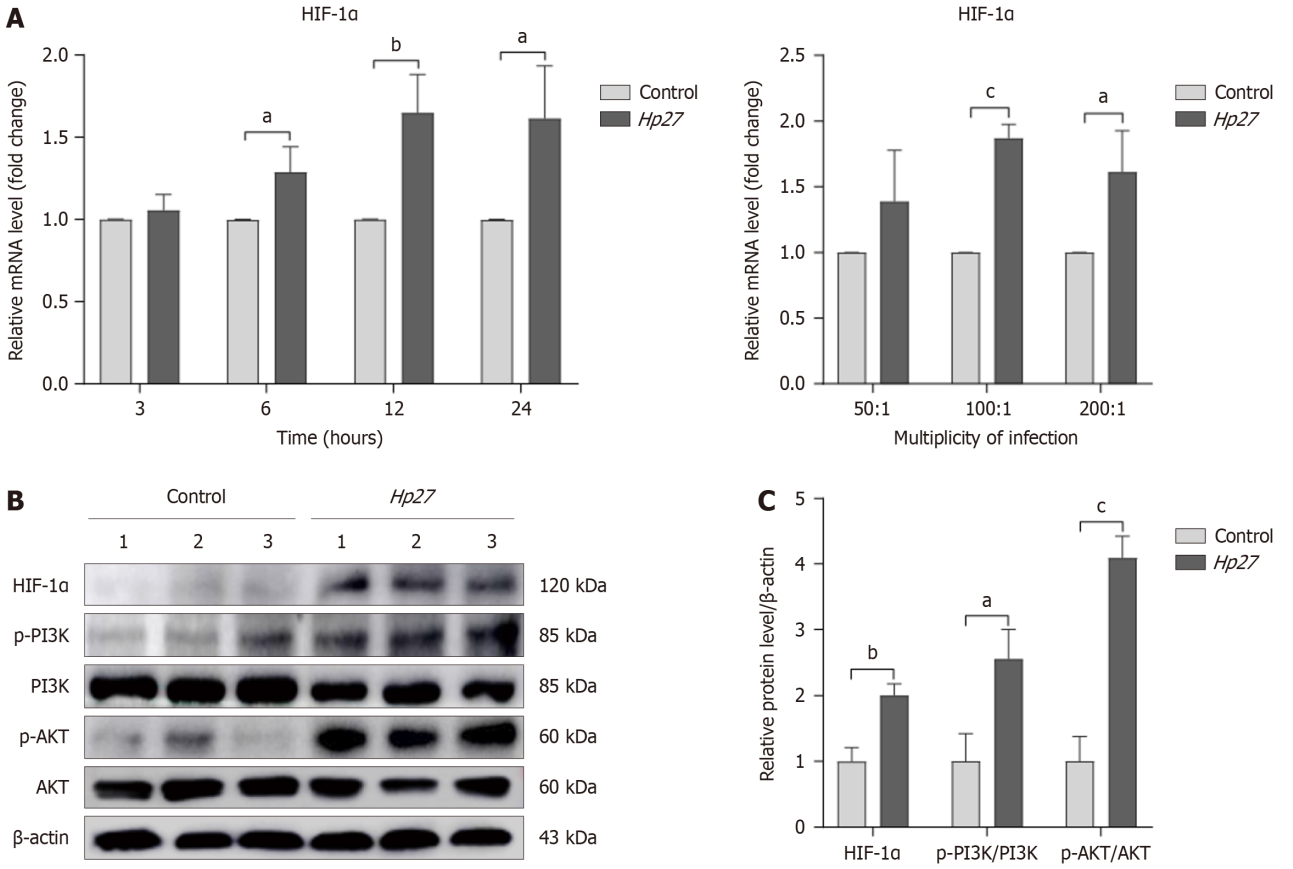


Figure 1 *Helicobacter pylori* induces hypoxia-inducible factor-1α expression and activates the PI3K/AKT pathway. A: Quantitative reverse transcription-polymerase chain reaction was performed to detect the relative mRNA level of hypoxia-inducible factor-1α (*HIF-1α*) at different infection time points (3 h, 6 h, 12 h, and 24 h) and multiplicity of infection (50, 100, and 200) in *Helicobacter pylori*-infected human gastric epithelial cells. β-actin was used as the internal reference; B and C: HIF-1α and PI3K/AKT pathway proteins were detected by Western blot analysis. β-actin was used as the internal reference. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, compared with control group. HIF-1α: Hypoxia-inducible factor-1α.

METTL14 is downregulated in H. pylori-infected GES-1 cells and involved in HIF-1α induction by H. pylori

Given the lack of understanding of the molecular mechanisms underlying *H. pylori*-induced HIF-1α expression and the impact of m6A dysregulation on GC biology, we investigated the expression of primary m6A modifiers, and found that there was a significant downward trend in the expression of METTL14 (Figure 4A). Subsequently, through overexpression and silencing of METTL14 (Figure 4B), we observed a negative correlation between METTL14 and *H. pylori*-induced HIF-1α (Figure 4D-G). Immunofluorescence staining also showed the obvious co-localization between METTL14 and HIF-1α after *H. pylori* infection (Figure 4H). Meanwhile, we assessed the stability of *HIF-1α* mRNA following transfection with the METTL14 plasmid and utilized actinomycin D. Notably, our findings showed no statistically significant difference between the *Hp27*+pcDNA3.1(+) and *Hp27*+pcDNA3.1(+)-METTL14 groups (Figure 4C). These observations suggest a potential regulatory role for METTL14 in HIF-1α translation efficiency, instead of mRNA level, warranting further investigation into its involvement in the context of *H. pylori*-induced GC.

METTL14 overexpression precludes activation of the PI3K/AKT pathway, EMT, and cell proliferation, migration, and invasion

Since our results revealed an increase of HIF-1α in GES-1 cells with METTL14 knockdown, we next evaluated whether METTL14 has the similar effect on the PI3K/AKT pathway and the biological behaviors of the cells. After METTL14-overexpressed GES-1 cells were infected by *H. pylori*, the PI3K/AKT pathway was inhibited, and N-cadherin, Vimentin, ZEB1, MMP2, and MMP9 were downreguated (Figure 5A and B). The phenotypic experiments also suggested that cell proliferation and migration rates were slow down (Figure 5C-E). To sum up, our observations implicated the inhibiting effect of METTL14 on PI3K/AKT pathway activation and GC progression in the presence of *H. pylori* infection.

METTL14 inhibition facilitates PI3K/AKT pathway activation and GC progression

To obtain more exact conclusions, we transfected cells with siRNA to inhibit METTL14 expression and detected the above indicators repeatedly. As predicted, GES-1 cells lacking METTL14 incubated with *H. pylori* exhibited increased expression of p-PI3K and p-AKT proteins, as well as enhanced expression of EMT and invasion biomarkers (Figure 6A and B). CCK8 and wound healing assay also indicated heightened cell proliferation and migration rates (Figure 6C-E).

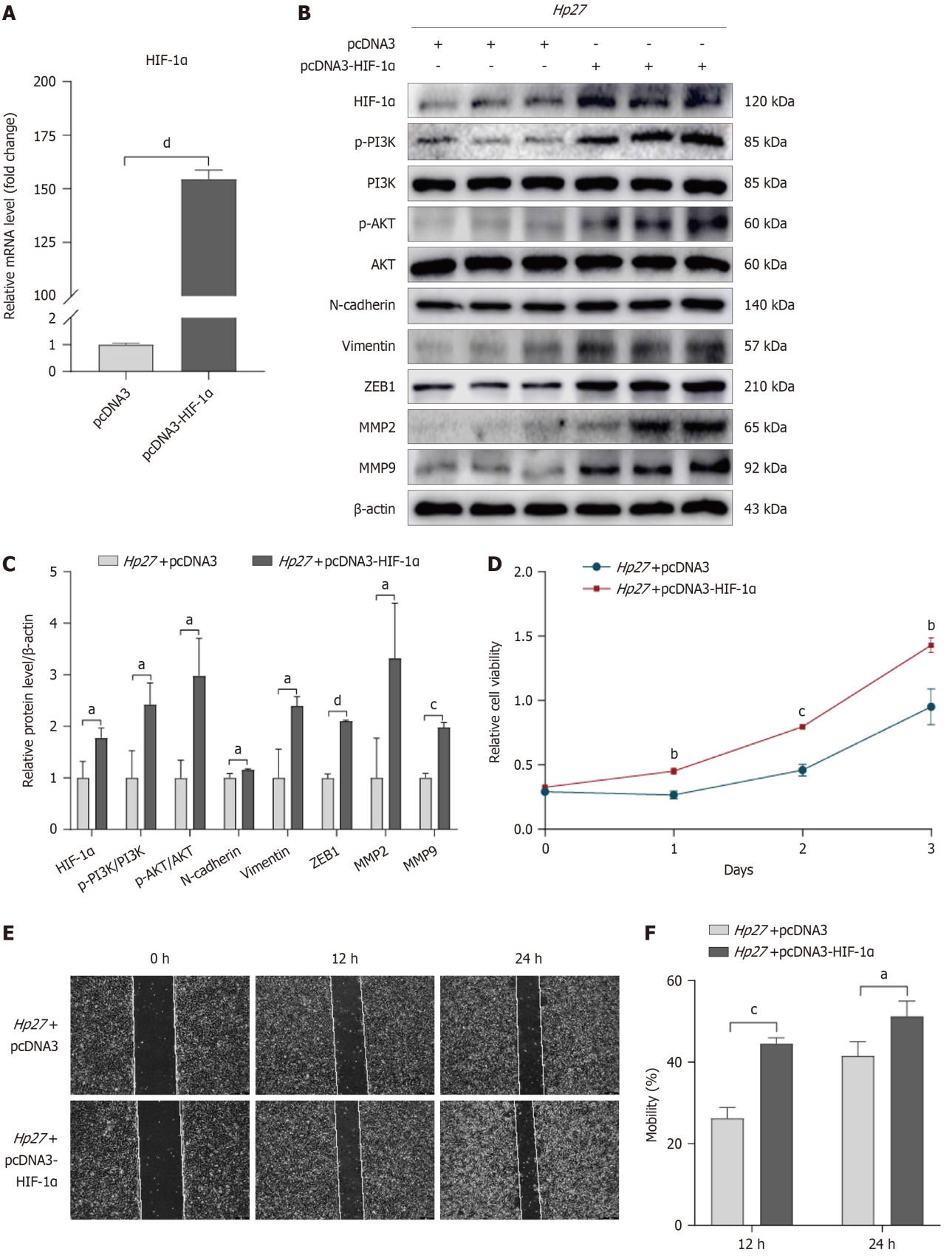


Figure 2 Hypoxia-inducible factor-1α overexpression promotes the activation of PI3K/AKT pathway and the malignant behaviors of cells.
A: Quantitative reverse transcription-polymerase chain reaction showed that hypoxia-inducible factor-1α (HIF-1α) plasmid was successfully transfected into gastric epithelial cells; B and C: Protein levels were analyzed by Western blot, including HIF-1α, p-PI3K, PI3K, p-AKT, AKT, N-cadherin, Vimentin, ZEB1, MMP2, and MMP9, all normalized to β-actin. These proteins respectively represented the PI3K/AKT pathway, epithelial-mesenchymal transition, and cell invasion ability; D: Cell counting kit-8 assay was utilized to evaluate cell viability; E and F: Wound healing assay was conducted to test the migration ability. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$, compared with pcDNA3 or *Hp27* + pcDNA3 group. *Hp27*: *H. pylori* 27 strain; HIF-1α: Hypoxia-inducible factor-1α.

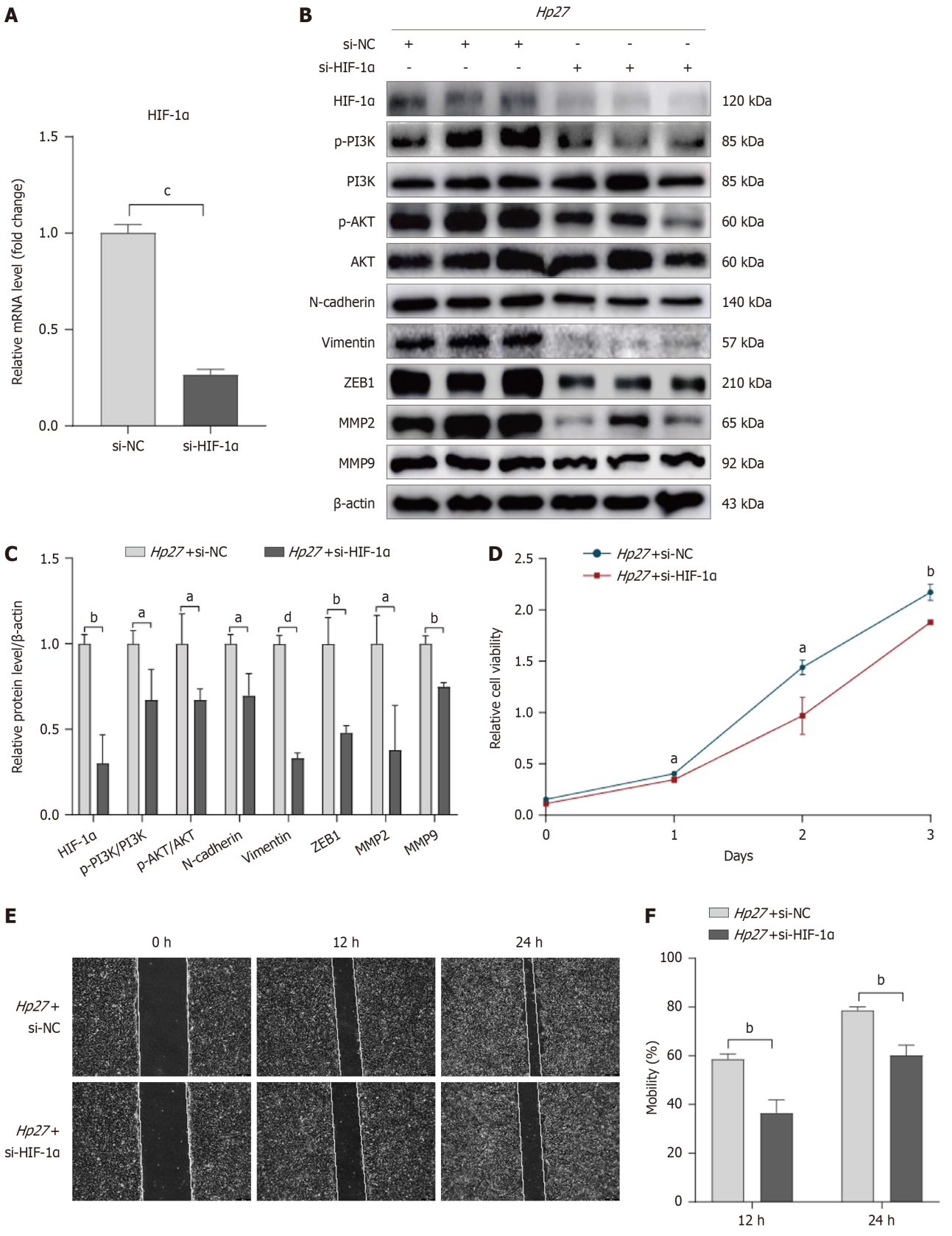
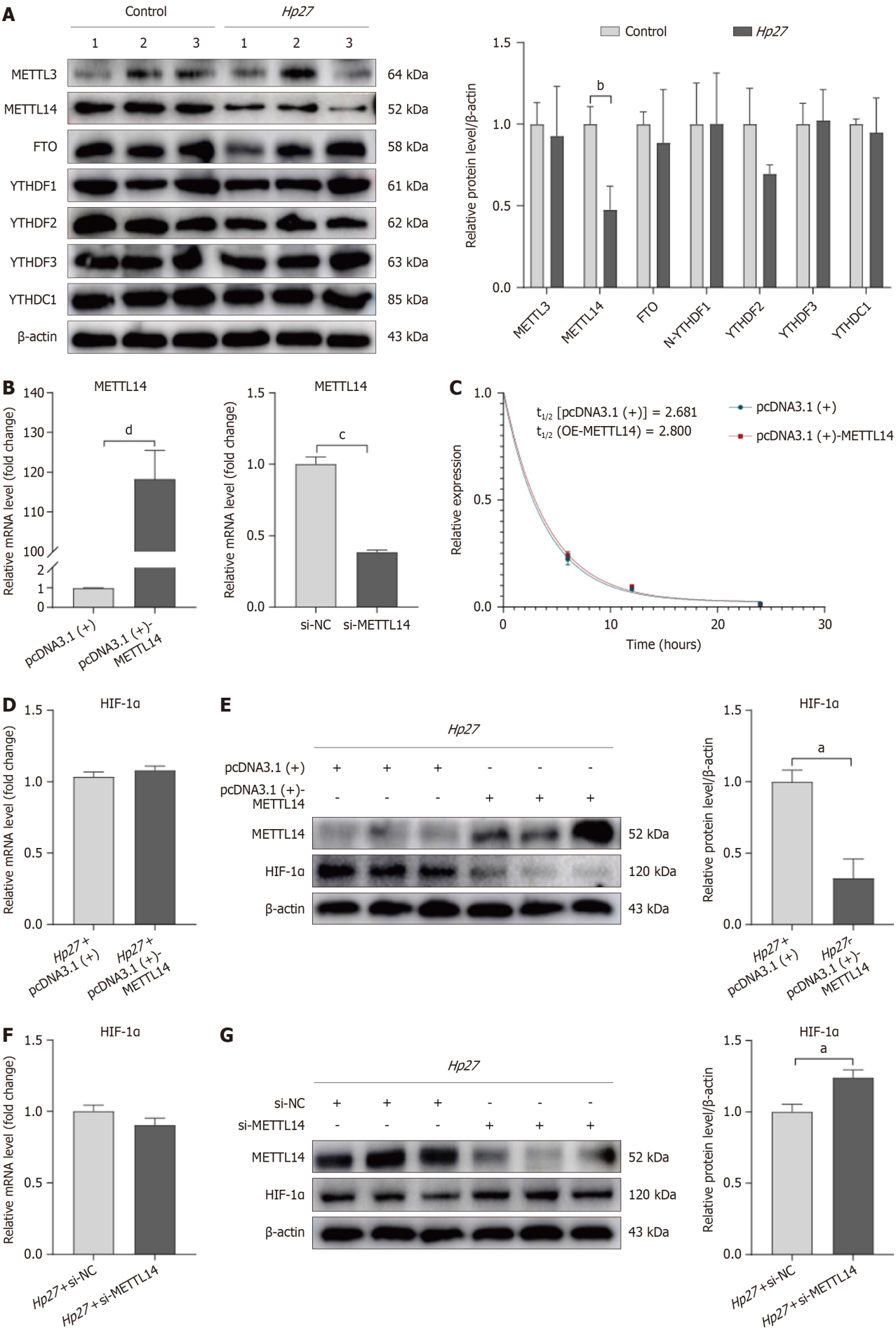


Figure 3 Knockdown of hypoxia-inducible factor-1α suppresses the PI3K/AKT pathway, epithelial-mesenchymal transition, and cell proliferation, migration, and invasion. A: Quantitative reverse transcription-polymerase chain reaction showed that hypoxia-inducible factor-1α (HIF-1α) small interfering RNA (siRNA) was successfully transfected into gastric epithelial cells (GES-1); B and C: The transfected GES-1 cells were co-cultured with *H. pylori* and Western blot analysis revealed the changes of HIF-1α, p-PI3K, p-AKT, epithelial-mesenchymal transition biomarkers, and invasion indicators; D: Cell counting kit-8 reagent was added into the cells with corresponding treatment and the absorbance at 450 nm was recorded; E and F: Detection of migration rate by wound healing assay. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$, compared with siRNA-negative control (NC) or *Hp27* + siRNA-NC group. siRNA-NC: Small interfering RNA-negative control; HIF-1α: Hypoxia-inducible factor-1α.


A
Control
Hp27
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METTL3 64 kDa
METTL14 52 kDa
FTO 58 kDa
YTHDF1 61 kDa
YTHDF2 62 kDa
YTHDF3 63 kDa
YTHDC1 85 kDa
β-actin 43 kDa
Control
Hp27
Relative protein level/β-actin
b
METTL3
METTL14
FTO
N-YTHDF1
YTHDF2
YTHDF3
YTHDC1
B
METTL14
Relative mRNA level (fold change)
d
pcDNA3.1 (+)
pcDNA3.1 (+)-METTL14
METTL14
c
si-NC
si-METTL14
C
Relative expression
t1/2 [pcDNA3.1 (+)] = 2.681
t1/2 (OE-METTL14) = 2.800
pcDNA3.1 (+)
pcDNA3.1 (+)-METTL14
Time (hours)
D
HIF-1α
Relative mRNA level (fold change)
Hp27 + pcDNA3.1 (+)
Hp27 + pcDNA3.1 (+)-METTL14
E
Hp27
pcDNA3.1 (+)
pcDNA3.1 (+)-METTL14
METTL14 52 kDa
HIF-1α 120 kDa
β-actin 43 kDa
HIF-1α
Relative protein level/β-actin
a
F
HIF-1α
Relative mRNA level (fold change)
Hp27+si-NC
Hp27+si-METTL14
G
Hp27
si-NC
si-METTL14
METTL14 52 kDa
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Hp27+si-METTL14

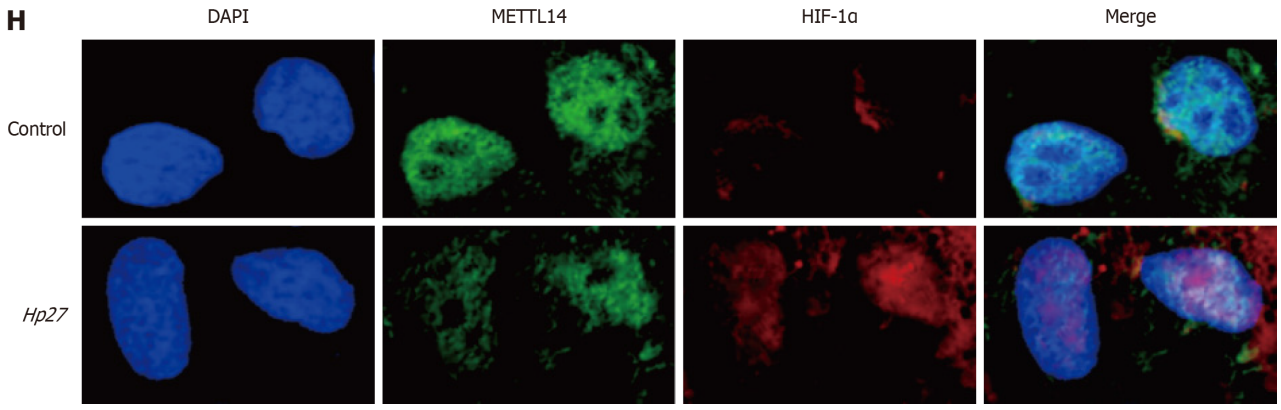

Figure 4 Methyltransferase-like protein 14 is downregulated by *Helicobacter pylori* and involved in hypoxia-inducible factor-1α expression. A: Detection of primary m6A modifiers under *Helicobacter pylori* (*H. pylori*) infection; B: Methyltransferase-like protein 14 (METTL14) plasmid and METTL14 small interfering RNAs (siRNAs) were successfully transfected into gastric epithelial cells (GES-1) and the mRNA expression was determined by quantitative reverse transcription polymerase chain reaction; C: Actinomycin D experiment was performed to test the stability of hypoxia-inducible factor-1α (HIF-1α) mRNA at 0 h, 6 h, 12 h, and 24 h. remaining (%) = 6/12/24 h HIF-1α mRNA level/0 h HIF-1α mRNA level; D and E: The mRNA and protein levels of HIF-1α were detected in cells that were transiently transfected with pcDNA3.1(+)-METTL14 and infected with *H. pylori*; F and G: Transiently transfected siRNA-METTL14 cells were infected with *H. pylori*, and then the mRNA and protein levels of HIF-1α were detected; H: Immunofluorescence staining and confocal microscopy images of control and *Hp27*-infected GES-1 cells. Nuclei (blue), METTL14 (green), and HIF-1α (Red). Scale bars = 20 μm. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$, compared with pcDNA3.1(+) or *Hp27* + pcDNA3.1(+), siRNA-NC or *Hp27* + siRNA-NC group, all normalized to β-actin. siRNAs: Small interfering RNA; METTL14: Methyltransferase-like protein 14; HIF-1α: Hypoxia-inducible factor-1α; NC: Negative control.

In summary, our findings suggested that METTL14-mediated *H. pylori*-induced HIF-1α expression suppresses GC progression by regulating the PI3K/AKT pathway.

DISCUSSION

Hypoxia is a crucial characteristic of the tumor microenvironment, and the role of HIF-1α as the crucial transcription factor in response to hypoxia in GC has been well-documented[32]. The m6A modification of *HIF-1α* mRNA has been implicated in various diseases. Hypoxia can affect the epigenetic regulatory mechanism of cells and subsequently increase the level of HIF-1α protein by promoting the binding of YTHDF2 to the m6A-modified HIF-1α mRNA[33]. In hepatocellular carcinoma and colorectal cancer, METTL3 mediates the m6A modification of *HIF-1α* mRNA, and then extends its half-life and improves stability and translation, subsequently interacting with target genes related to glycolysis [34,35]. However, in cells with METTL14 knockdown used for a psoriasis study, the m6A level of *HIF-1α* mRNA decreased, but the protein expression increased[36]. m6A modification directly affected the translation efficiency of HIF-1α instead of the mRNA level[37]. Therefore, the relation of m6A modification and HIF-1α remains controversial. In addition, the PI3K/AKT pathway is a typical intracellular signal transduction pathway and involved in various cellular biological processes[38]. Apart from PI3K/AKT pathway regulation[39], HIF-1α can promote GC progression through this pathway, providing logical evidence for our hypothesis[10,40]. A primary finding of our study was that after *H. pylori* infection, we verified that *H. pylori* can induce HIF-1α and activate the PI3K/AKT signaling pathway. This result is consistent with previous studies[41,42], and HIF-1α overexpression amplified the activation effect of *H. pylori* on the PI3K/AKT pathway, while the effect was reversed in cells with HIF-1α knockdown. These findings demonstrated that HIF-1α can accelerate GC progression *via* the PI3K/AKT pathway during *H. pylori* infection.

The dysregulation of m6A methylation engages in GC through various pathways, including PI3K/AKT signal transduction. METTL3 is highly expressed in GC tissues. It enhances the m6A modification of *ADAMTS9*, which is a tumor suppressor gene, and then inhibits *ADAMTS9* transcription in a YTHDF2-dependent manner. In addition, the decline of *ADAMTS9* activates the PI3K/AKT pathway[43]. Moreover, YTHDF3 can recognize m6A sites to promote translation. GO and KEGG analyses on stomach adenocarcinoma tissue samples with differential YTHDF3 showed that YTHDF3-related differential genes are enriched in the PI3K/AKT pathway and relevant to immune microenvironment and GC cell migration and invasion[44]. Given that the molecular mechanism of *H. pylori*-induced HIF-1α expression remains unclear and its m6A modification has potential functions in cancer, we endeavored to perform the study from the view of m6A modification. In the present study, we found that *H. pylori* inhibited METTL14 expression remarkably. m6A methyltransferase complex (MTC) consists of METTL3, METTL14, and other auxiliary subunits. However, METTL14 has no catalytic activity, and it is mainly responsible for identifying substrate RNAs and enhancing the catalytic activity and efficiency of METTL3. Meanwhile, compared with METTL3 isolated *in vitro*, METTL3-METTL14 complex has stronger catalytic activity[45,46]. Therefore, research about METTL14 is essential. We conducted a functional intervention with METTL14. METTL14 inhibited *H. pylori*-induced HIF-1α expression. Meanwhile, PI3K/AKT pathway activity decreased, and GC progression was delayed. Considering our previous results about ENO1 and HK2 and the metabolic disorder caused by *H. pylori*[23,25,47], we hold a view that low METTL14 expression mediates the upregulation

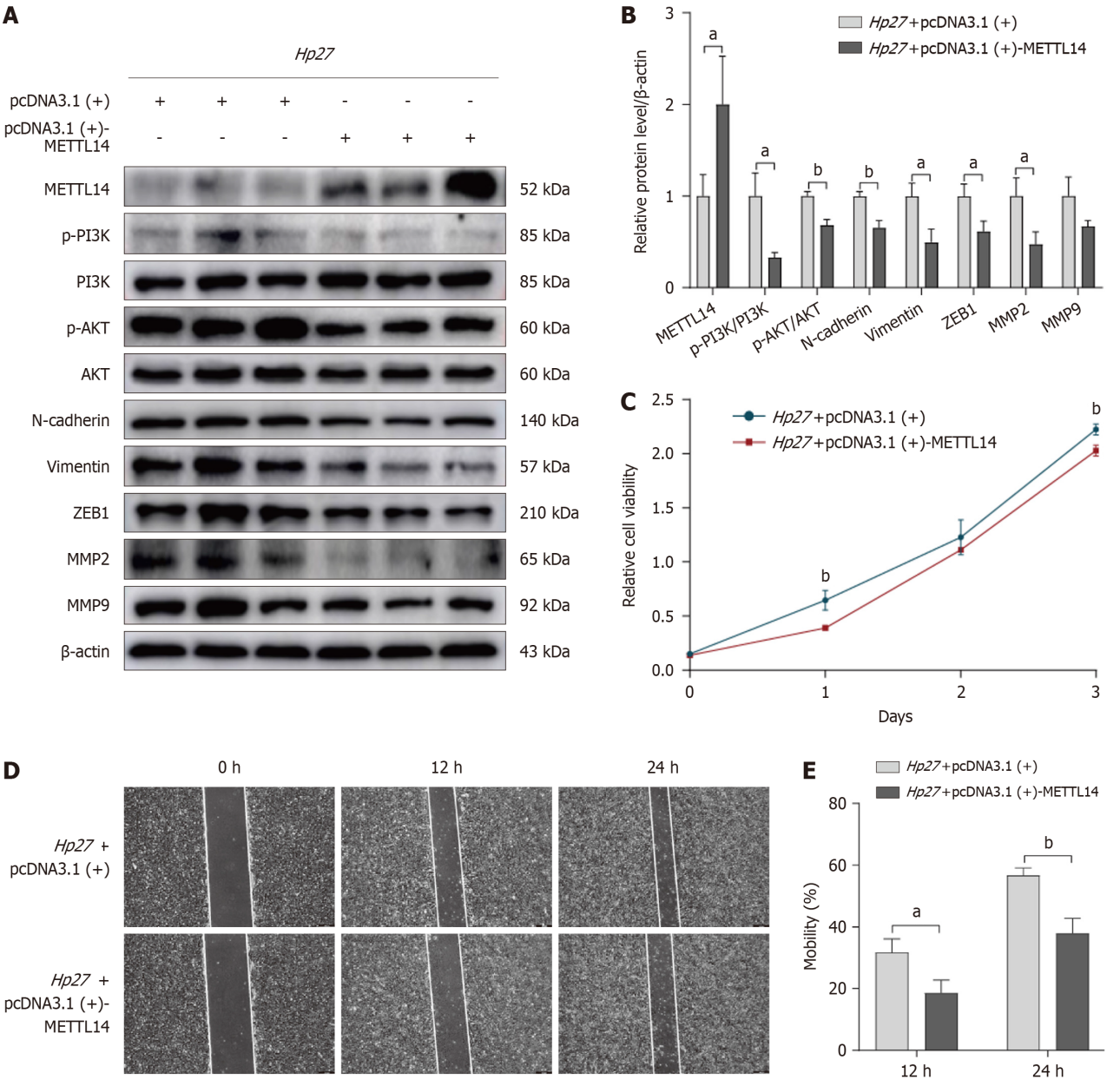

Figure 5 Overexpression of methyltransferase-like protein 14 inhibits the activation of PI3K/AKT pathway and gastric cancer progression.
A and B: After methyltransferase-like protein 14 (METTL14) overexpression, the related protein levels were checked by Western blot, including METTL14, p-PI3K, PI3K, p-AKT, AKT, N-cadherin, Vimentin, ZEB1, MMP2, and MMP9, all normalized to β-actin; C: Cell viability was detected by cell counting kit-8 assay; D and E: Cell migration was detected by wound healing assay. $^{a}P < 0.05$, $^{b}P < 0.01$, compared with *Hp27* + pcDNA3.1(+) group. METTL14: Methyltransferase-like protein 14.

of *H. pylori*-induced HIF-1α expression in a m6A-dependent manner, and then probably drives metabolic reprogramming and malignant transformation *via* the PI3K/AKT pathway.

METTL14 plays a dual role in various types of cancer, and progress in research on METTL14 in GC is limited and inconsistent. Previous studies have shown that low expression of METTL14 is associated with increased malignant activity in GC. This relationship is linked to proliferation and metastasis induced by the PI3K/AKT pathway and is negatively correlated with patient survival rates. These findings were based on comprehensive cohort analysis, database research, and *in vitro* experiments. Mechanistically, METTL14 inhibited circORC5 expression through m6A modification, leading to the upregulation of miR-30c-2-3p, subsequently reducing AKT1S1 and EIF4B (eukaryotic translation initiation factor 4B) expression, and ultimately suppressing GC progression[48]. In addition, METTL14 can exert a suppressing effect on GC by precluding the Wnt and PI3K/AKT signaling pathways[49,50]. Patients with low METTL14 expression levels have worse overall survival than those with high METTL14 expression levels[51]. These findings are highly consistent with our findings. Moreover, similar results were found in clear cell renal cell carcinoma. As an important component of MTC, METTL14 inhibited the PI3K/AKT pathway by improving PIK3R3 stability and p85 ubiquitination, and then impeded tumor growth[52]. Other studies hold an opposite viewpoint, that is, METTL14 mediated the m6A modification of LINC01320 and enhanced its stability, and then promoted malignant behavior in GC *via* the miR-495-5P/RAB19 axis[53]. *METTL14* mRNA was obviously higher in GC tissues than in adjacent tissues. This difference was correlated with the Laurén classification, and the mRNA and protein levels of METTL14 increased in the *H. pylori*-

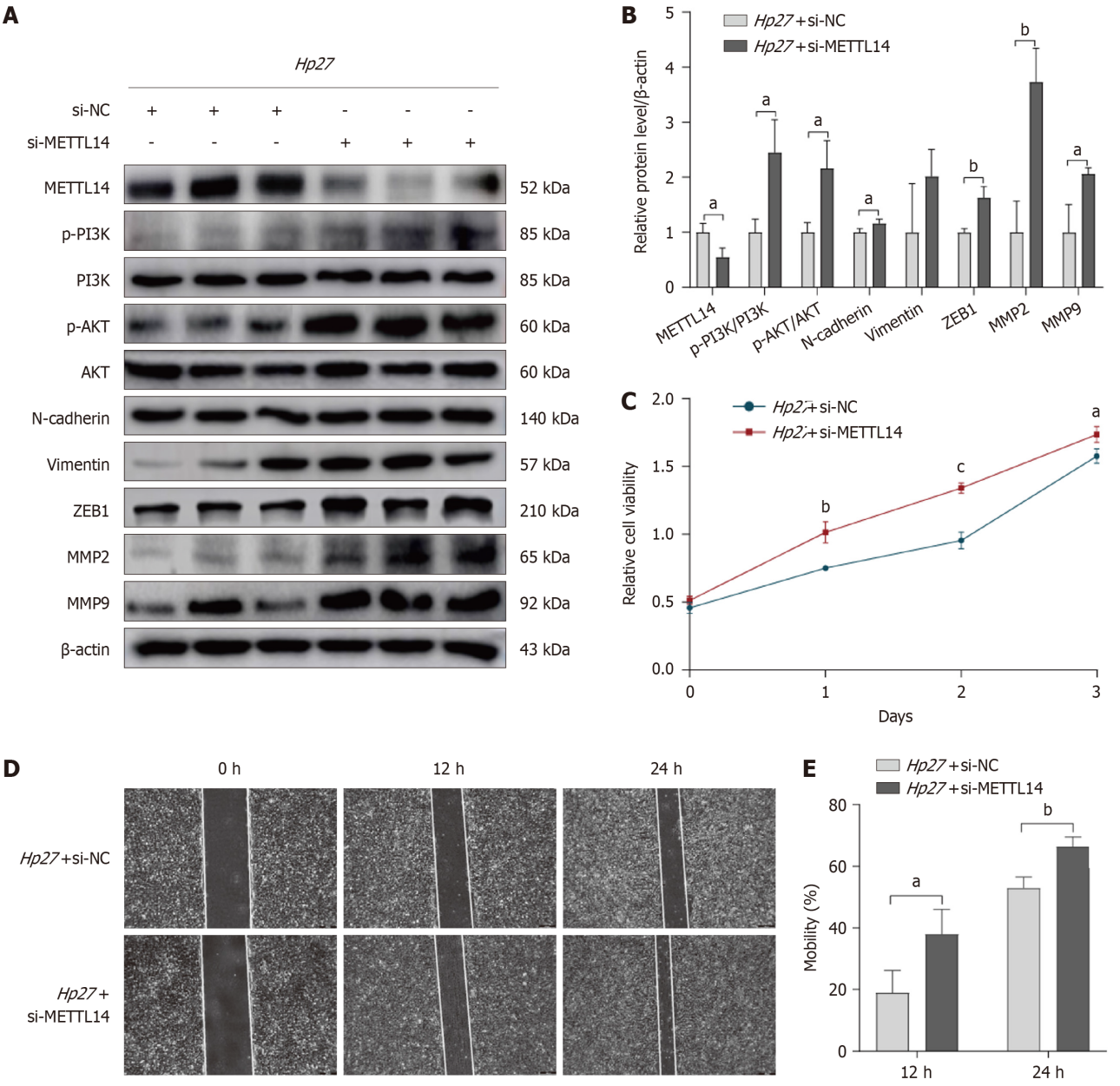

Figure 6 Knockdown of methyltransferase-like protein 14 cells promotes the activation of PI3K/AKT pathway and gastric cancer progression. A and B: Gastric epithelial cells transiently transfected with methyltransferase-like protein 14 small interfering RNA (siRNA) were infected with *Helicobacter pylori*, and then the protein levels of PI3K/AKT pathway proteins, epithelial-mesenchymal transition biomarkers, and invasion indicators (MMP2 and MMP9), were detected; C: Cell viability was assessed by cell counting kit-8 assay; D and E: Cell migration was detected by wound healing assay. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, compared with *Hp27* + siRNA-negative control group. METTL14: Methyltransferase-like protein 14; NC: Negative control.

infected group. Similarly, *METTL14* acts as an oncogene in other types of cancer[54]. These contradictory results in GC may stem from different experimental models and *H. pylori* strains. *H. pylori* strains have strong regional clustering and high diversity globally, and each of them has undergone relatively independent evolution, leading to genomic diversity [55,56]. These discrepancies indicate the complicated molecular mechanisms of METTL14 in GC.

Our study has some limitations. The research is relatively narrow and lacks *in vivo* validation. We intend to address these limitations by constructing *H. pylori*-infected animal models with corresponding interventions and by utilizing population data to confirm our current findings.

Moreover, considering the previous studies and our own findings, there are more aspects to explore the molecular mechanisms of *H. pylori* infection. For example, existing studies have shown inconsistent results regarding the specific contributions of *H. pylori* virulence factors to HIF-1α expression. Some studies indicated that the cagA-positive (cagA^{+}) strain has a stronger effect on HIF-1α than the cagA-negative (ΔcagA) strain[57], whereas others indicated that the *H. pylori* urease virulence factor is essential for HIF-1α induction[58]. After culturing cagA^{+} and ΔcagA strains with GES-1 cells, we found no considerable difference in HIF-1α between the groups (Supplementary Figure 1). Therefore, further investigation is required to clarify the specific *H. pylori* virulence factors that contribute to HIF-1α expression. Additionally, although *H. pylori* induces HIF-1α expression partly through METTL14, related m6A readers are still

unknown. IGF2BP3, which is an m6A reader, can upregulate HIF-1α in a m6A-dependent manner in GC[59]. However, as far as we know, this field has not been fully explored.

CONCLUSION

This study demonstrates that *H. pylori* infection downregulates the expression of METTL4 and subsequently promotes the malignant biological behavior of gastric mucosal epithelial cells through the HIF-1α/PI3K/AKT axis. The results reveal the potential mechanism of m6A modification in the pathogenesis of *H. pylori* and provide a theoretical basis for understanding the epigenetic regulatory mechanism underlying the adaptation of GC to hypoxia and for developing GC-related drugs.

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FOOTNOTES

Author contributions: An TY, Chen SY, and Jia B designed the study; An TY, Hu QM, and Ni P carried out the research and statistical analyses; An TY drafted the manuscript; Hua YQ, Wang D, Duan GC, and Chen SY contributed to the review and editing of the manuscript; all authors have read and approved the final manuscript.

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