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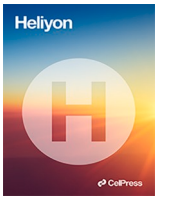

Research article

# Lipopolysaccharide promotes cancer cell migration and invasion through METTL3/PI3K/AKT signaling in human cholangiocarcinoma

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ABSTRACT

*Purpose:* As a major structural component of the outer membrane of Gram-negative bacteria, lipopolysaccharide (LPS) has been detected in the blood circulation and tissues in patients with chronic diseases and cancers, which plays a critical role in the tumor formation and progression. However, the biological role of LPS in human intrahepatic cholangiocarcinoma remains unclear. The aims of this study were to investigate the role of LPS in the malignant progression of intrahepatic cholangiocarcinoma.

*Methods:* The cell migration and invasion capacities of cholangiocarcinoma cell lines were evaluated by Boyden chamber assays. Expression levels of the key molecules involved in the PI3K/AKT signaling and METTL3 were detected by qPCR and western blot. The molecular mechanism by which LPS promotes the malignant behaviors was investigated by using siRNAs, plasmids and small molecule inhibitors.

*Results:* In vitro experiments showed that exogenous LPS treatment promoted cell migration and invasion capacities in both QBC939 and HUCCT1 cell lines, while did not affect cell proliferation and apoptosis. Mechanistically, exogenous LPS treatment had been proved to induce the increased expression of METTL3 and activate the downstream PI3K/AKTsignaling pathway. In addition, suppression of METTL3 expression reduced cell proliferation, migration and invasion capacities in both cell lines. Furthermore, inhibition of METTL3 expression or inhibition of PI3K/AKT signaling decreased LPS-induced cell migration and invasion capacities. Moreover, knockdown of METTL3 or inhibition of METTL3 significantly inhibited LPS-induced activation of the PI3K/AKT signaling.

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*Conclusion:* In general, these results suggest that the LPS-METTL3-PI3K/AKT signal axis promotes cell migration and invasion in ICC, which contributes to a reduced overall survival in patients with ICC. It may broaden the horizon of cancer therapy with potential therapeutic targets.

## 1. Introduction

Intrahepatic cholangiocarcinoma (ICC) is the second most common form of primary liver cancer. It develops in the epithelial cells of the distal branch of the intrahepatic bile duct. Although surgery remains the only curative treatment for patients with ICC, only one-third of patients are eligible for surgical resection [1]. Most patients are diagnosed at an advanced stage of the disease with nonspecific symptoms, and chemotherapy has been established as the only standard treatment choice [1]. Although significant progress has been made in the diagnosis and treatment of ICC in recent decades, the overall survival rate of patients with ICC has not changed significantly. The molecular mechanisms underlying carcinogenesis and tumor progression are still poorly characterized in ICC. Therefore, identifying the mechanisms involved in the initiation and progression of ICC has recently gained importance as a topic of research.

Lipopolysaccharide (LPS), a major structural component of the outer membrane of gram-negative bacteria, can be released into circulation and can further induce systemic inflammation and sepsis [2]. A small volume of LPS (1–5 μg/mL) can circulate in the bloodstream of a healthy person without causing any major side effect [3]. The levels of LPS in the bloodstream increase in several different conditions or diseases, such as Crohn's disease and diabetes [3]. LPS can bind directly to its cell surface receptor, Toll-like receptor 4 (TLR4), and initiate an intracellular signaling cascade in a MyD88-dependent or -independent manner. This eventually activates downstream signaling pathways, such as the PI3K/AKT and NF-KappaB signaling pathways [4]. Numerous studies have described the crucial role of LPS in different types of human cancers. For example, LPS has been shown to promote cell adhesion and invasion through activation of TLR4 signaling in colorectal cancer [5,6]. LPS was also shown to promote prostate cancer cell invasion and metastasis [7]. Recently, emerging evidence has shown that human tumors contain significant levels of viable commensal microbiota that play an important role in promoting cancer progression [8,9]. More than 20 % of cancer cases are associated with bacterial infection, and bacterial footprints like LPS can be detected in approximately 60%–70 % cases in some cancers [8]. Moreover, elevated levels of LPS have been detected in both the blood and tissues of patients with chronic liver diseases or cancers [10]. However, the biological role of LPS and the underlying regulatory mechanisms in ICC progression are yet to be determined.

N6-methyladenosin (m6A) is the most common and frequent post-transcriptional modification and occurs ubiquitously in eukaryotic RNA [11]. The methyltransferase complex is composed of several proteins, including methyltransferase-like protein 3 (METTL3), which is responsible for the installation of m6A. METTL3 is the most important component of the m6A methyltransferase complex and reportedly plays an essential role in both physiological and pathological conditions, especially in the initiation and progression of different types of human cancers [12]. Chronic inflammation and injury of bile duct cells is closely related to ICC progression. Recently, METTL3 was shown to be upregulated in patients with ICC, and its expression was correlated with poor prognosis [13]. However, the process by which METTL3 expression is upregulated in ICC remains elusive. Our recent study showed that METTL3 protein expression can be induced in human renal proximal tubule epithelial cell line HK-2 in response to different stimuli, including tumor necrosis factor-alpha (TNF- α) and LPS [14]. Based on this, we speculated that inflammatory mediators such as LPS may affect METTL3 expression in ICC.

In the present study, we attempted to evaluate the biological function of LPS in facilitating ICC progression. Exogenous LPS treatment promoted cell migration and invasion in ICC. Furthermore, we also investigated PI3K/AKT activation and its roles in regulating LPS-promoted cell migration and invasion in ICC cells. Additionally, we showed that LPS treatment can induce METTL3 expression, and that METTL3 enhances LPS-promoted cell migration and invasion. Interestingly, we found that METTL3 is involved in regulating LPS-induced PI3K/AKT activation. Collectively, our findings reveal that the LPS-METTL3-PI3K/AKT signaling axis plays an important role in cell migration and invasion in ICC.

## 2. Material and methods

### 2.1. Cell culture

Human cholangiocarcinoma cell lines QBC939 and HUCCT1 were given as a gift from Dr. Tan wenliang (Sun Yat-sen University, Guangzhou, China). Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Procell, Wuhan, China) supplemented with 10 % of fetal bovine serum (FBS) (WISENT CORPORATION, Montreal, Canada) and 1 % penicillin–streptomycin (Beyotime, Wuhan, China) in an incubator at 37 °C with 5 % $CO_2$.

### 2.2. siRNA interference assay

siRNA sequences targeting human METTL3 and TLR4 were designed and synthesized by Ribobio (Guangzhou, China). An ineffective siRNA was included as a negative control. METTL3 and TLR4 siRNA were transfected into ICC cells using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions. Total RNA and protein were isolated after transfection and assessed by quantitative qPCR and western blotting. The target siRNA sequences used were: METTL3 siRNA-1: 5′-GCACTTGGATCTACGGAAT-3`; METTL3 siRNA-2: 5′- GCAAGAATTCTGTGACTAT-3`.

### 2.3. Plasmid construction and overexpression

METTL3 overexpression plasmids and control were designed by HanBio Technology (Shanghai, China). QBC939 and HUCCT1 cells were plated in six-well plates and allowed to reach 50%–70 % confluence during transfection. METTL3 overexpression plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions. Total RNA and protein were isolated 24 h post transfection and assessed using quantitative qPCR and western blotting. The target sequence of the METTL3 overexpression plasmid is provided in Table S1.

### 2.4. Real-time quantitative PCR

Cells were seeded in six-well plates and treated with LPS (0, 1, 2.5, and 5 μg/mL) for 24 h. Following this, the cells were washed twice with PBS, and total RNA was extracted from them using TRIzol reagent from Invitrogen. The total RNA concentration was determined by measuring absorbance using a UV spectrophotometer (Thermo Scientific, CA, USA). One microgram of RNA was used to synthesize cDNA. cDNA synthesis was performed using Hifair® III 1st Strand cDNA Synthesis SuperMix for qPCR (Yeasen, Shanghai, China). qPCR mixtures were prepared using Hieff® qPCR SYBR Green Master Mix (Yeasen, Shanghai, China). The experiments were performed according to the manufacturer's instructions. The sequences of the primers used for RT-PCR analyses are as follows. GAPDH - forward primer: 5′-GAGTCAACGGATTTGGTCGT-3`; reverse primer: 5′-GACAAGCTTCCCGTTCTCAG-3`. METTL3 - forward primer: 5′-GCCTTTGCCAGTTCGTTAGT-3`; reverse primer: 5′-TGACCTTCTTGCTCTGTTGTTCC-3`.

### 2.5. Western blotting

Cells were seeded in a six-well plate and treated with different concentrations of LPS (0, 1, 2.5, and 5 μg/mL) for 24 h. The cells were washed twice with PBS and lysed with RIPA buffer (Beyotime, Wuhan, China) supplemented with protease and phosphatase inhibitors (Beyotime, Wuhan, China) at 4 °C for 30 min. The lysis mixture was centrifuged at 12000 rpm and 4 °C for 30 min, and the supernatant containing cellular proteins was used in subsequent experiments. The protein concentration of the samples was normalized after performing a bicinchoninic acid assay (BCA) (Beyotime, Wuhan, China), according to the manufacturer's instructions. Proteins were separated from the samples by 10 % SDS-PAGE (60 V for 30 min and 110 V for 1 h 30 min) (40 μg/lane). The separated proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA; 200 mA for 120 min). After blocking with 5 % non-fat milk at room temperature for 2 h, the membranes were treated overnight with primary antibodies at 4 °C. The membranes were then washed three times with Tris-buffered saline supplemented with Tween 20 and treated with HRP-conjugated secondary antibodies at room temperature for 2 h. Following this, the membranes were washed three times with Tris-buffered saline supplemented with Tween 20. Finally, a chemiluminescence substrate (ECL) (Abbkine, Wuhan, China) was added to the membranes, and the western blot was resolved using a ChemiDoc (Bio-Rad, CA, USA). The quantification of the protein bands was assessed using ImageJ.

### 2.6. Cell counting kit (CCK-8) assay

We used a CCK-8 kit (Biosharp, Anhui, China) to study cell proliferation. In brief, 1000 cells per well were seeded in a 96-well plate and treated with a gradient concentration of LPS (0, 1, 2.5, 5, and 10 μg/mL) for 24 h and 48 h. CCK-8 solution was then added to each well. After incubation with the CCK8 reagent for 1–3 h at 37 °C, absorption was measured at 450 nm using a microplate reader (BioTek, VT, USA).

### 2.7. Transwell migration and invasion assay

Cells were seeded in a six-well plate and treated with 1 μg/mL LPS for 24 h. Following this, an adequate volume of Matrigel (Invitrogen) was prepared for the invasion assay. The membrane of the upper compartment of the transwell chamber was coated with Matrigel, which was allowed to solidify by incubation at 37 °C for 1 h (the migration assay does not have this step). Two hundred microliters of cell suspension ($2 \times 10^5$ cells/mL) were added to the upper compartment of each chamber, and 600 μL of culture medium supplemented with 10 % FBS was added to the lower compartment. Cells were incubated at 37 °C for 24 h. Subsequently, 4 % paraformaldehyde was used to fix cells on the microporous membrane for 30 min. Cells on the lower side of the membrane were stained with 1 % crystal violet for 20 min and washed twice with PBS. Cells were then observed under a microscope (magnification, × 400). Images of five random field were recorded, and the number of cells that had transgressed through the membrane was counted. The experiments were repeated three times.

### 2.8. Flow cytometry

Cells were cultured in a six-well plate and treated with 1 μg/mL LPS for 24 h. The cells were washed twice with cold PBS after the supernatant was collected and digested with 0.125 % trypsin. The supernatant and cells were centrifuged at 800 rpm for 3 min. Following this, the cells were gently re-suspended twice in 1 mL of PBS and then stained. In brief, the cells were first re-suspended in 400 μL of 1 × Annexin V binding solution at a concentration of approximately $1 \times 10^6$ cells/mL. Next, 5 μL of Annexin V-FITC staining solution was added to the cell suspension and gently mixed. The cells were then incubated at 4 °C in the dark for 15 min. Following this,

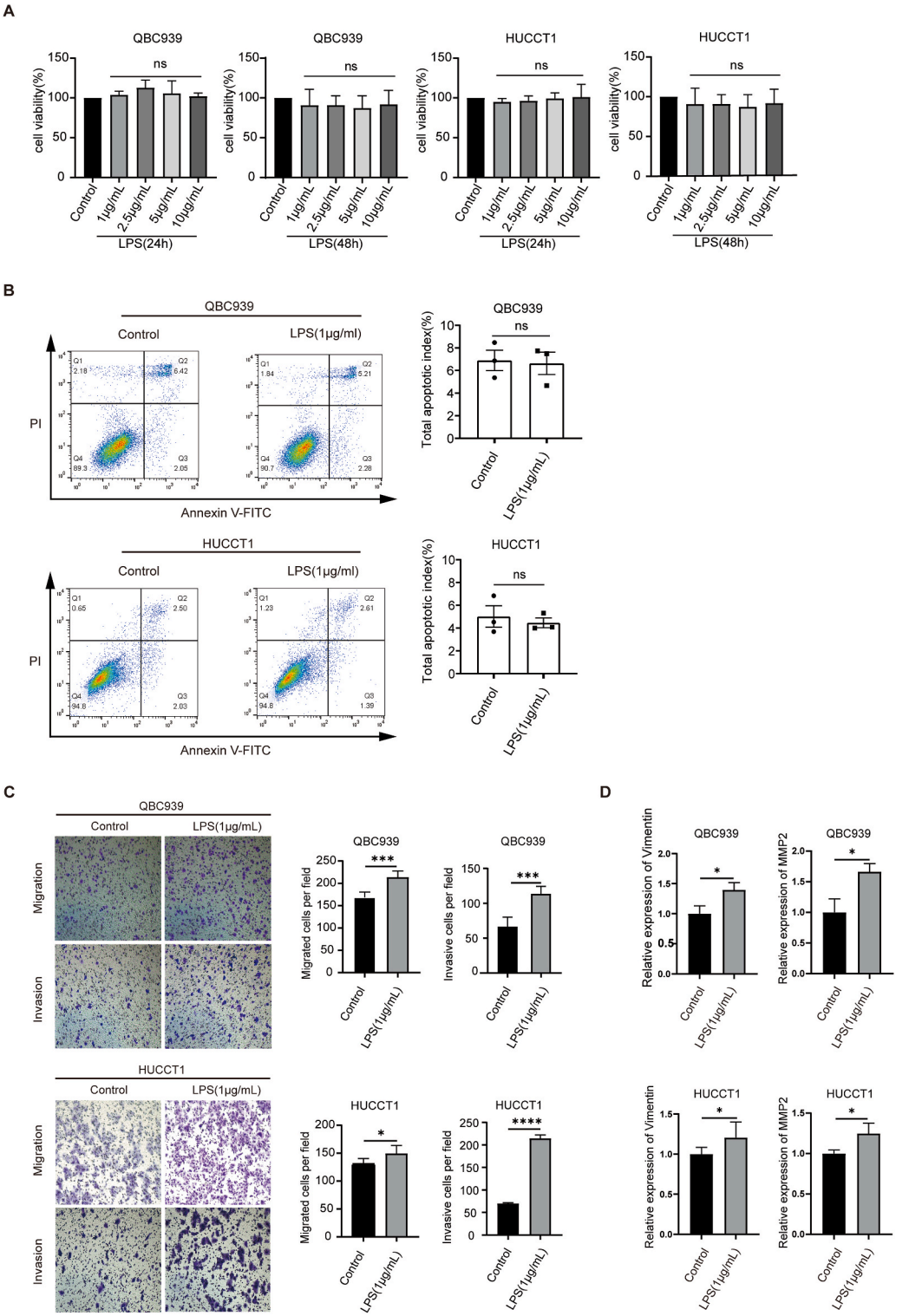


**Fig. 1. Exogenous LPS treatment promotes cell migration and invasion while does not affect cell proliferation and apoptosis in human cholangiocarcinoma cells** (**A**) QBC939 and HUCCT1 cells were treated with the indicated concentrations of LPS for 24 h and 48 h, cell growth was evaluated by CCK8 assay. Data represents means ± SD, n = 3. *p < 0.05, **p < 0.001, ***p < 0.001, n.s., non-significant (Student *t*-test). (**B**) QBC939 and HUCCT1 cells were treated with the low concentrations of LPS (1 μg/mL) for 24 h, cell apoptosis was detected by fluorescence activated cell sorting (FACS). Data represents means ± SD, n = 3. *p < 0.05, **p < 0.001, ***p < 0.001, n.s., non-significant (Student *t*-test). (**C**) Cell migration ability and cell invasion ability of QBC939 and HUCCT1 cells treated by LPS (1 μg/mL) or PBS control. Representative images (left

panel) and quantified analysis (right 2 panels) of transwell assays in QBC939 and HUCCT1 cells treated with and without LPS. Data represents means ± SD, n = 3. *p < 0.05, **p < 0.001, ***p < 0.001, n.s., non-significant (Student *t*-test). **(D)** QBC939 and HUCCT1 cells were treated with LPS (1 μg/mL) for 24 h and EMT related genes were detected by qPCR in both cell lines. Data represents means ± SD, n = 3. *p < 0.05, **p < 0.001, ***p < 0.001, n.s., non-significant (Student *t*-test).

5 μL Propidium Iodide (PI) dye was added to the mixture. The suspensions were incubated for 3 min at 4 °C in the dark. Lastly, apoptotic cells were evaluated by gating PI- and Annexin V-positive cells during fluorescence-activated cell-sorting (FACS) (BD Biosciences, NJ, USA). All the experiments were performed in triplicate.

### 2.9. Processing of public datasets

The ICC cohort and mRNA dataset were retrieved from a public website (https://www.biosino.org/node/project/detail/OEP001105). Data on LPS-related pathways were retrieved from the GSEA database (www.gseamsigdb.org/). The enrichment score of the two pathways was calculated on the Sangerbox website (http://sangerbox.com/) using GSVA [15]. The overall survival curve was prepared using R Studio (Version 4.30).

### 2.10. RNA-seq

QBC939 cells, treated with LPS (1 μg/mL, PBS as control group), were harvested at 24 h post-treatment, followed by RNA extraction using TRIzol solution (Invitrogen, CA, USA). The cDNA library was prepared by Novelgene (Hefei, China). The paired-end reads were generated by the Illumina® HiSeq 2500 platform supplied by Novelgene. An R package, DESeq, was used to quantify transcription levels and identify differentially expressed genes, using a cut-off of $P < 0.05$.

### 2.11. Statistical analysis

Data are expressed as the mean ± SD from at least three technical replicates. Statistical analysis was conducted using GraphPad Prism 8.0 (GraphPad Software, CA, USA). Data used for analysis were normally distributed. An unpaired Student's *t*-test was used for statistical analysis.

## 3. Results

### 3.1. LPS treatment does not affect cell proliferation and apoptosis in human cholangiocarcinoma cells

To determine the effects of exogenous LPS treatment on cell proliferation, we initially incubated two human cholangiocarcinoma cell lines QBC939 and HUCCT1 with gradient concentration of LPS (0, 1, 2.5, 5, and 10 μg/mL) for 24 h and 48 h. The results of the CCK8 assay showed that, in both cell lines, LPS treatment exerted no significant effect on cell proliferation compared with that in the control group after 24 h and 48 h (Fig. 1A). Similarly, LPS (1 μg/mL) exerted no significant effect on cell apoptosis compared with that in the control group (Fig. 1B).

### 3.2. LPS treatment promotes cell migration and invasion in human cholangiocarcinoma cells

Next, the effects of LPS on cell migration and invasion were determined by using transwell assays in QBC939 and HUCCT1 cells. Cells treated with LPS showed a significantly greater migration and invasive potential than control cells (Fig. 1C). Additionally, the effect of LPS on Epithelial-Mesenchymal Transition (EMT) related genes was detected by qPCR. Results showed that LPS can upregulate the mRNA level of Vimentin and Matrix Metallopeptidase 2 (MMP2) (Fig. 1D). These findings indicate that LPS plays important roles in promoting cell migration and invasion in human cholangiocarcinoma cells.

### 3.3. LPS treatment activates the PI3K/AKT signaling pathway in human cholangiocarcinoma cells

LPS has been shown to activate several kinases, including p38 MAPK, p42/44 (ERK1/2), MAPK, and AKT [16]. To study the molecular mechanisms underlying the promotion of cell migration and invasion in response to LPS treatment, transcriptome sequencing was performed on QBC939 cells treated with LPS (PBS as a control group). Through RNA-seq analysis, we found that exogenous LPS treatment lead to a large number of changes in gene expression levels (Fig. 2A), and the top 20 KEGG pathways of differentially expressed genes caused by LPS treatment are shown in Fig. 2B. Among them, the PI3K/AKT signaling pathway was included. The expression of proteins involved in the PI3K/Akt signaling pathway was detected using western blotting. PI3K and AKT phosphorylation were found to increase significantly upon treatment with different concentrations of LPS compared with that in the control group (Fig. 2C). Furthermore, the levels of AKT phosphorylation peaked at 6 h after LPS treatment and decreased thereafter (Fig. S1). These results suggest that LPS treatment induces the activation of PI3K/AKT signaling in both QBC939 and HUCCT1 cell lines.

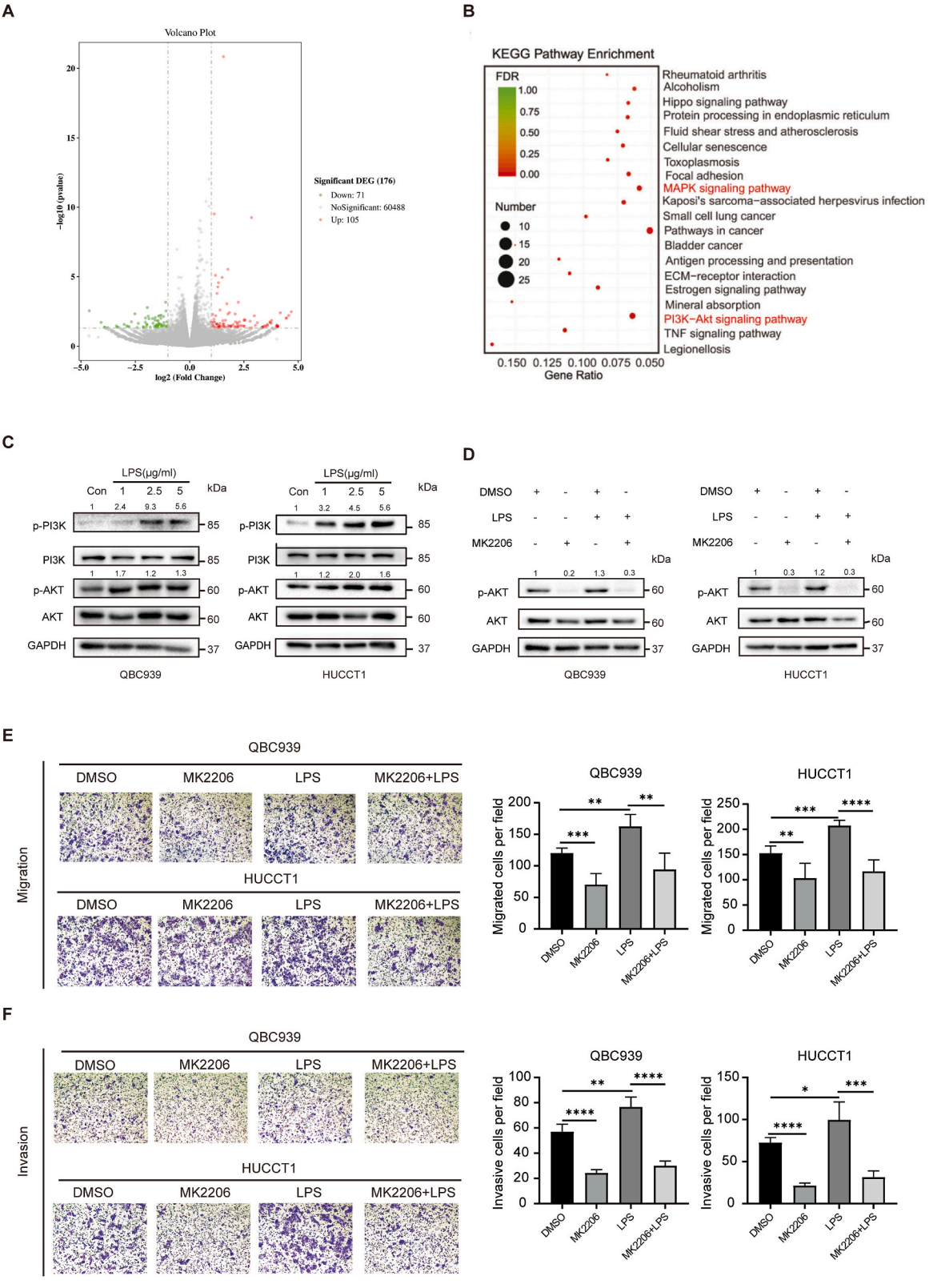


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**Fig. 2. LPS promotes cell migration and invasion through activation of PI3K/AKT signaling pathway in human cholangiocarcinoma cells** **(A)** Volcano plots of differential genes in QBC939 cells treated with LPS (1 μg/mL) for 24h (verse to control). **(B)** Results of KEGG enrichment analysis of differential genes. (**C**) QBC939 and HUCCT1 cells were treated with the indicated concentrations of LPS for 24 h, cell lysates were then analyzed for PI3K, p-PI3K, AKT and p-AKT expression by Western blot. GAPDH was used as loading control. Densitometric quantification is shown above the Western blots, representing the signal normalized to the loading control (GAPDH), relative to the untreated controls. (**D**)QBC939 and HUCCT1 cells were firstly incubated MK-2206 for 24 h, and then further treated with LPS (1 μg/mL) for another 24 h, cell lysates were then analyzed for AKT and p-AKT expression by Western blot. GAPDH was used as loading control. Densitometric quantification is shown above the Western blots, representing the signal normalized to the loading control (GAPDH), relative to the untreated controls. (**E** and **F**) QBC939 and HUCCT1 cells were firstly incubated MK-2206 for 24 h, and then further treated with LPS (1 μg/mL) for another 24 h, cell migration and invasion capacities were respectively evaluated by transwell migration and invasion assay. Representative images (left panel) and quantified analysis (right 2 panels) of transwell assays in QBC939 and HUCCT1 cells treated with and without LPS. Data represents means ± SD, n = 3. *p < 0.05, **p < 0.001, ***p < 0.001, n.s., non-significant (Student *t*-test).

### *3.4. PI3K/AKT signaling is involved in the regulation of cell migration and invasion by LPS in human cholangiocarcinoma cells*

To further investigate whether the activation of the PI3K/AKT pathway plays a role in LPS-induced cell migration and invasion, we used MK-2206, a pharmacological inhibitor of PI3K/AKT signaling, to inhibit the activation of AKT [17]. We treated QBC939 and HUCCT1 cells treated with a gradient concentration of MK-2206; 0.125 μM was selected as the final working concentration for both cell lines (Fig. S2). Next, QBC939 and HUCCT1 cells were incubated with MK-2206 and then treated with or without LPS. The pharmacological inhibition of the PI3K/AKT pathway with MK-2206 effectively blocked the phosphorylation of AKT (Fig. 2D). Next, we investigated cell migration and invasion potential using the transwell migration and invasion assay. Treatment with MK-2206 partially abrogated LPS-induced cell migration and invasion (Fig. 2E and F). The results suggest that the PI3K/AKT signaling pathway is involved in the LPS-induced regulation of cell migration and invasion.

### *3.5. TLR4 is involved in LPS-induced cell migration and invasion in human cholangiocarcinoma cells*

TLR4 is known to be the major receptor for LPS [18]. Indeed, activation of TLRs, including TLR-4, on tumour cells can not only promote cell proliferation and inhibit cell apoptosis, but also contribute to tumour invasion, metastasis and immune escape [19]. Our results showed that LPS treatment could upregulate the expression of TLR4 in QBC939 and HUCCT1 cells (Fig. 3A). To investigate the role of TLR4 in LPS-induced HCC cell migration, we knocked down TLR4 expression levels in the cell lines and verified the knockdown efficiency (Fig. 3B). Cell migration experiments showed that TLR4 knockdown could partially reverse LPS-induced migration of ICC cells (Fig. 3C and D). Consistently, TLR4 knockdown could partially reverse LPS-induced invasion of ICC cells (Fig. 3E and F). The results suggest that TLR4 is involved in the LPS-induced regulation of cell migration.

### *3.6. LPS treatment induces the expression of METTL3 in human cholangiocarcinoma cells*

Previous studies have shown that LPS induces the expression of METTL3 in immune cells and other types of human cells [14]. To investigate the mechanism of action of LPS in regulating cell migration and invasion, we hypothesized that exogenous LPS can induce METTL3 expression in ICC cell lines. Therefore, both QBC939 and HUCCT1 cells were treated with different concentrations (0, 1, 2.5, and 5 μg/mL) of LPS for 24 h. The results indicated that the gene and protein expression of METTL3 was induced in a dose-dependent manner upon LPS treatment in both cell lines, with maximum induction at 1 μg/ml (Fig. 4A). Therefore, 1 μg/ml LPS was selected as the ideal stimulation dose for the subsequent experiments.

### *3.7. METTL3 promotes cell proliferation in human cholangiocarcinoma cells*

METTL3 has been reported to be overexpressed in various human cancers and plays critical roles in human cancer development and progression [12]. The effects of METTL3 knockdown on cell proliferation and apoptosis were evaluated in these two cell lines. QBC939 and HUCCT1 cells were transfected with siRNA to knock down the expression of METTL3. As shown in Fig. 4B, knockdown efficiency was confirmed using both qPCR and western blotting. The results indicated that the cell proliferation capacity was significantly reduced upon METTL3 knockdown in both cell lines. (Fig. 4C). Following this, we transfected QBC939 and HUCCT1 cells with METTL3 overexpression vectors. METTL3 overexpression promoted proliferation in both cell lines (Fig. 4D). The results suggest that METTL3 plays a role in cell proliferation in human cholangiocarcinoma cells.

### *3.8. METTL3 knockdown partially reverses LPS-induced cell migration and invasion*

To investigate whether METTL3 is involved in LPS-induced ICC cell migration and invasion, we used siRNA to knock down the expression of METTL3 gene, and then further treated the cells with LPS. After transfection, we verified the knockdown efficiency at the transcription and protein levels. As shown in Fig. 5A–D, METTL3 knockdown significantly decreased the cell migration and invasion capacities, and the effect of LPS treatment on cell migration and invasion was considerably suppressed upon METTL3 knockdown. These data further confirmed the potential role of METTL3 in the regulation of LPS-induced cell migration and invasion.

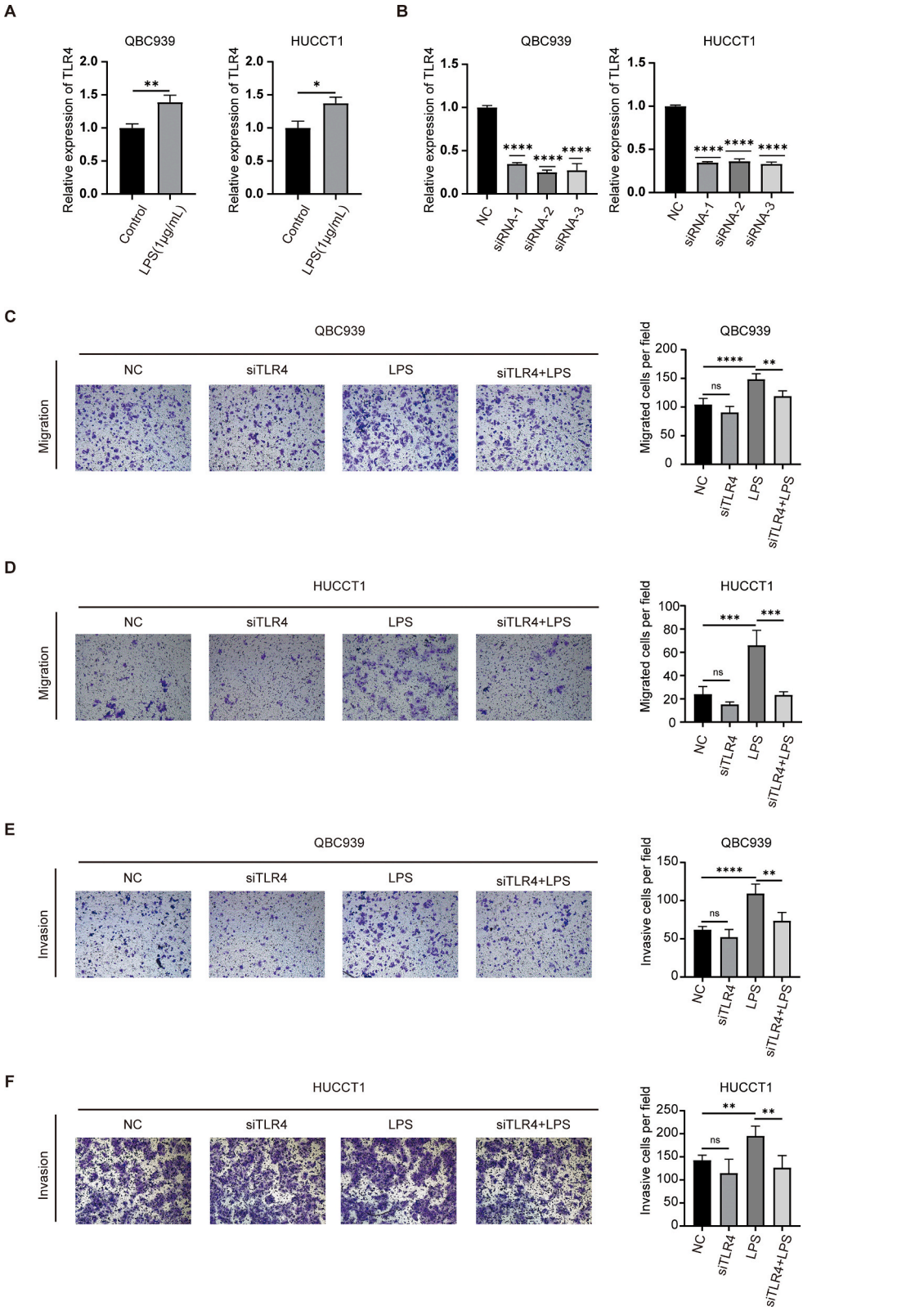


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**Fig. 3. TLR4 is involved in LPS-induced cell migration in human cholangiocarcinoma cells (A)** QBC939 and HUCCT1 cells were treated with LPS (1 μg/mL) for 24 h and TLR4 mRNA levels were detected by qPCR in both cell lines. Data represents means ± SD, n = 3. *p < 0.05, **p < 0.001, ***p < 0.001, n.s., non-significant (Student *t*-test). (B) QBC939 and HUCCT1 cells were transfected with three different TLR4 siRNA (siTLR4) and negative control for 24 h, RNA was extracted and analyzed for METTL3 mRNA expression by Real-Time Quantitative PCR. Data represents means ± SD, n = 3. *p < 0.05, **p < 0.001, ***p < 0.001, n.s., non-significant (Student *t*-test). (C and D). QBC939 and HUCCT1 cells were transfected with TLR4 siRNA (siTLR4) or scrambled siRNA (NC) for 24 h, then treated with LPS (1 μg/mL) for another 24 h, cell migration was evaluated by transwell migration assays. (E and F). QBC939 and HUCCT1 cells were transfected with TLR4 siRNA (siTLR4) or scrambled siRNA (NC) for 24 h, then treated with LPS (1 μg/mL) for another 24 h, cell invasion was evaluated by transwell invasion assays. Data represents means ± SD, n = 3. *p < 0.05, **p < 0.001, ***p < 0.001, n.s., non-significant (Student *t*-test).

### *3.9. METTL3 regulates the PI3K/AKT signaling pathway*

To investigate whether PI3K/AKT is a key downstream target of METTL3 in ICC, METTL3 was efficiently knocked down using siRNA, and then the cells were treated with LPS. METTL3 knockdown decreased the level of phosphorylated AKT (Fig. 6A and B). STM2457, a novel and specific METTL3 inhibitor, can suppress the enzymatic activity of METTL3 [20]. QBC939 and HUCCT1 cells were treated with gradient concentrations of STM2457; 5 μM and 0.625 μM were selected as the final working concentrations for each cell line (Fig. S3). STM2457 significantly inhibited the LPS-induced activation of PI3K/AKT signaling in both cell lines (Fig. 6C and D). Collectively, these results demonstrated that PI3K/AKT signaling is a downstream target of METTL3.

### *3.10. METTL3 or LPS upregulation predicts worse clinical outcomes in patients with ICC*

To determine whether our findings have clinical relevance for patients with ICC, we analyzed the mRNA expression data from a public dataset (https://www.biosino.org/node/project/detail/OEP001105). We found that patients with a high gene transcript level of METLL3 had a lower overall survival than patients with a low transcript level (Fig. 7A). We further calculated the enrichment score of LPS_mediated_signaling_pathway and LPS_immune_receptor_activity by GSVA. Our findings indicated that patients with a high enrichment score in LPS_mediated_signaling_pathway or LPS_immune_receptor_activity showed significant correlation with reduced overall survival (Fig. 7B and C). These data are consistent with the notion that LPS/METTL3/PI3K/AKT signaling plays a protumorigenic role in patients with ICC and reduces survival.

## 4. Discussion

The molecular mechanisms of cholangiocarcinogenesis and tumor progression are poorly understood. Increasing evidence suggests that ICC is associated with chronic liver inflammation [21]. Chronic inflammation is induced when the liver is persistently exposed to gut-derived microbes and associated products, such as LPS [21]. Moreover, increased intestinal permeabilization owing to a defective intestinal barrier has been shown to promote the translocation of the microbiota or LPS into the circulation and induce low-grade inflammation [22]. As a strong inflammatory mediator, LPS has been implicated in the promotion of tumorigenesis and metastasis in different types of human cancers. For example, previous studies have shown that LPS plays an important role in liver damage and tumor development in hepatocellular carcinoma [23]. However, the precise effect of LPS on ICC progression remains uninvestigated. In this study, we found that exogenous LPS treatment can promote cell migration and invasion in human ICC cell lines, whereas it does not affect cell proliferation and apoptosis.

In recent years, the molecular mechanism of action of LPS in promoting cancer formation and progression has been investigated in several types of human cancers. For example, LPS has been shown to enhance cancer metastasis through the activation of NF-KappaB signaling in prostate cancer [24]. However, the mechanisms by which exogenous LPS treatment promotes cell migration and invasion in ICC are yet to be investigated. LPS can bind directly to its cell surface receptor, such as TLR4, and initiate the intracellular signaling cascade [2]. The PI3K/AKT signaling pathway is a key signaling cascade that contributes to numerous physiological and pathological conditions. For example, the PI3K/AKT signaling was reported to activate epithelial-to-mesenchymal transition to promote malignant progression in cholangiocarcinoma [25]. In the present study, we found that exogenous LPS treatment activated PI3K/AKT signaling in ICC cells, and further inhibition of PI3K/AKT signaling by the AKT-specific inhibitor MK-2206 partially reduced the cell migration and invasion potential. These data suggest that LPS promotes cell migration and invasion by activating the PI3K/AKT signaling pathway in ICC.

m6A modification is the most prevalent RNA modification in eukaryotes. In recent years, emerging evidence has indicated the essential role of m6A modification/METTL3 in various cancers, including liver cancer [26]. The role of METTL3, as the critical m6A methyltransferase, in ICC is not fully understood. METTL3 was reported to be upregulated in ICC tissues, and its expression was found to be correlated with poor prognosis [13]. Here, our data demonstrated that METTL3 can promote cell proliferation, migration, and invasion in ICC *in vitro*, suggesting the oncogenic role of METTL3 this cancer. However, limited information is available about the regulation of METTL3 expression in ICC. We found that METTL3 expression can be induced by exogenous LPS treatment in a dose-dependent manner in ICC. These findings indicate a novel mechanism that increases METTL3 expression in ICC. Furthermore, we found that increased METTL3 expression or a high enrichment score of LPS_mediated_signaling_pathway or LPS_immune_receptor_activity is significantly associated with reduced overall survival in patients with ICC. We speculated that LPS promotes cell migration and invasion by inducing METTL3 expression. Our results showed that the increase in cell migration and invasion potential in response to LPS treatment was significantly suppressed when METTL3 expression was knocked down using siRNA. This confirmed that METTL3

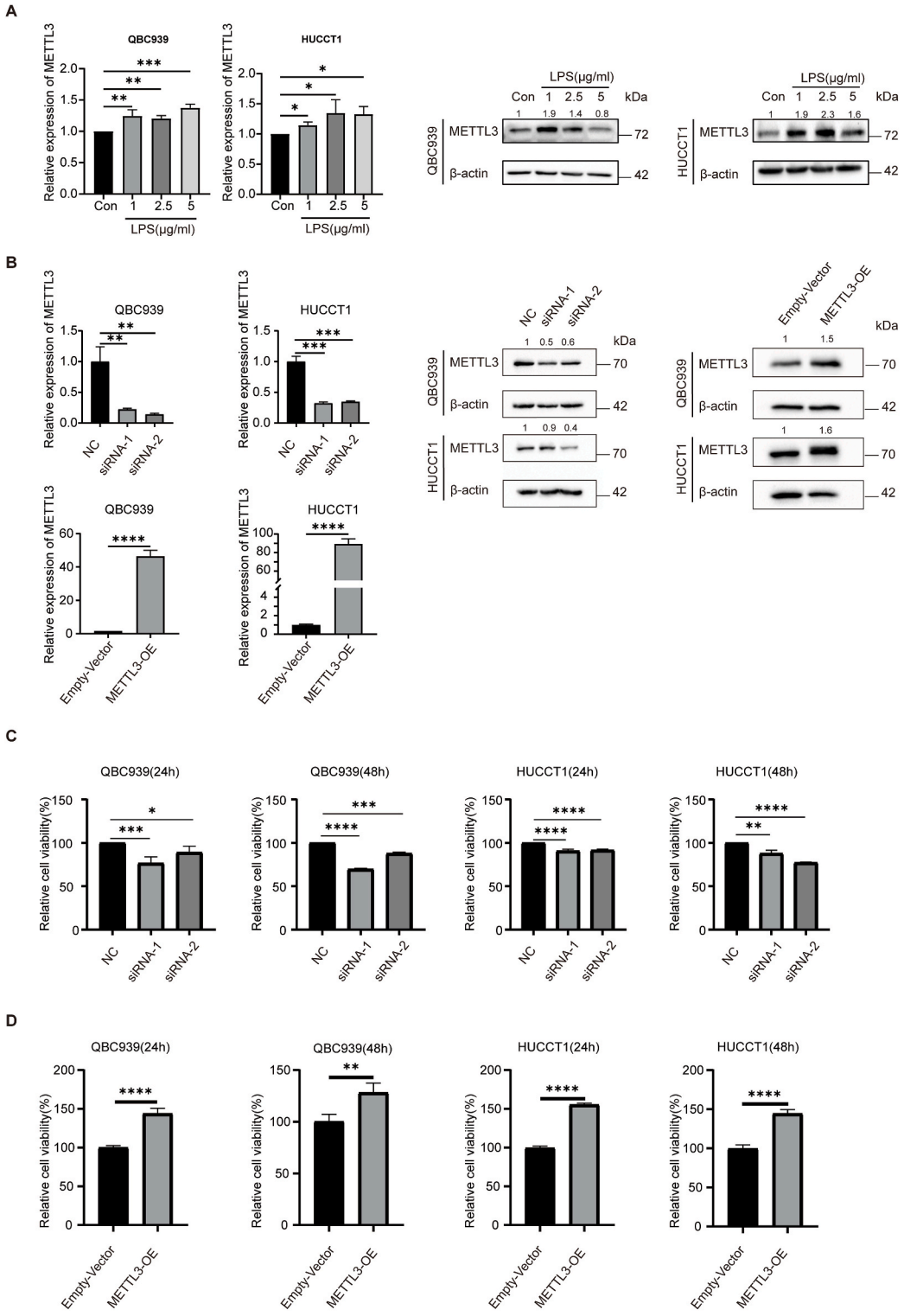


**Fig. 4. Exogenous LPS induces the expression of METTL3 which can regulate cell proliferation in human cholangiocarcinoma cells** (**A**) QBC939 and HUCCT1 cells were treated with the indicated concentrations of LPS, transfected with METTL3 siRNA (siMETTL3) or METTL3 overexpression plasmid for 24 h, RNA was extracted and analyzed for METTL3 mRNA expression by Real-Time Quantitative PCR. Data represents means ± SD, n = 3. *p < 0.05, **p < 0.001, ***p < 0.001, n.s., non-significant (Student *t*-test). (**B**) QBC939 and HUCCT1 cells were treated with the indicated concentrations of LPS, transfected with METTL3 siRNA (siMETTL3) or METTL3 overexpression plasmid for 24 h, cell lysates were then analyzed for METTL3 expression by Western blot. β-actin was used as loading control. Densitometric quantification is shown above the Western

blots, representing the signal normalized to the loading control (β-actin), relative to the untreated controls. (**C**) QBC939 and HUCCT1 cells were transfected with METTL3 siRNA (siMETTL3) or scrambled siRNA (siRNA) for 24 h, cell growth was evaluated by CCK8 assay. Data represents means ± SD, n = 3. *p < 0.05, **p < 0.001, ***p < 0.001, n.s., non-significant (Student *t*-test). (**D**) QBC939 and HUCCT1 cells were transfected with METTL3 overexpression plasmid or empty-vector for 24 h, cell growth was evaluated by CCK8 assay. Data represents means ± SD, n = 3. *p < 0.05, **p < 0.001, ***p < 0.001, n.s., non-significant (Student *t*-test).

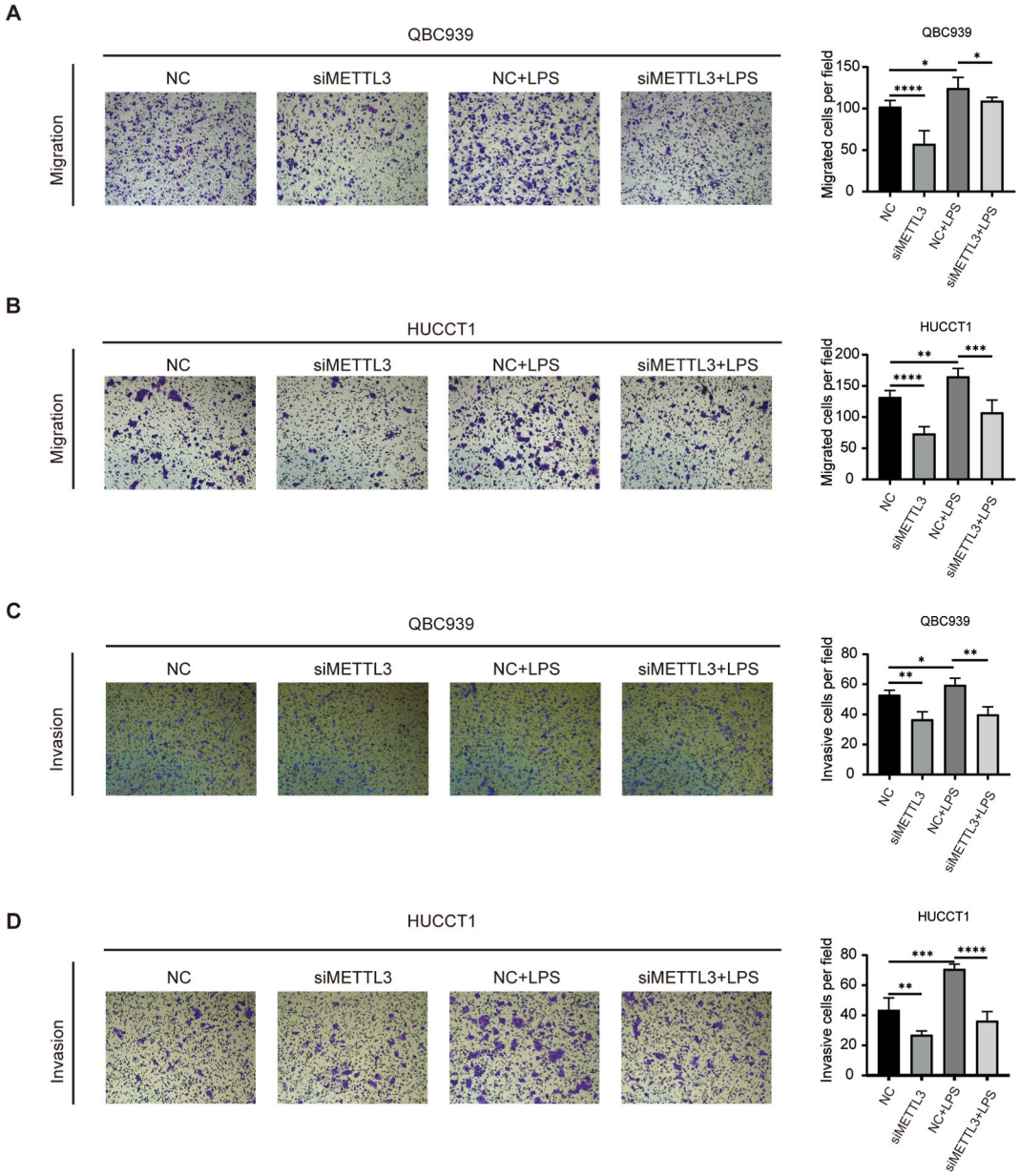


**Fig. 5. METTL3 is involved in LPS-promoted cell migration and invasion in human cholangiocarcinoma cells (A–D)** QBC939 and HUCCT1 cells were transfected with METTL3 siRNA (siMETTL3) or scrambled siRNA (siRNA) for 24 h, then treated with LPS for another 24 h, cell migration and invasion were evaluated by transwell migration and invasion assays. Data represents means ± SD, n = 3. *p < 0.05, **p < 0.001, ***p < 0.001, n.s., non-significant (Student *t*-test).

plays a key role in mediating LPS-induced cell migration and invasion. Our findings provide novel insights into the mechanism of action of LPS in the regulation of ICC progression and its potential as a therapeutic target in ICC.

METTL3 was shown to regulate mRNA translation in cancer cells. We investigated the role of METTL3 in the regulation of PI3K/AKT signaling in ICC. STM2457 is the catalytic inhibitor of METTL3 and has been shown to interfere with the level of m6A methylation

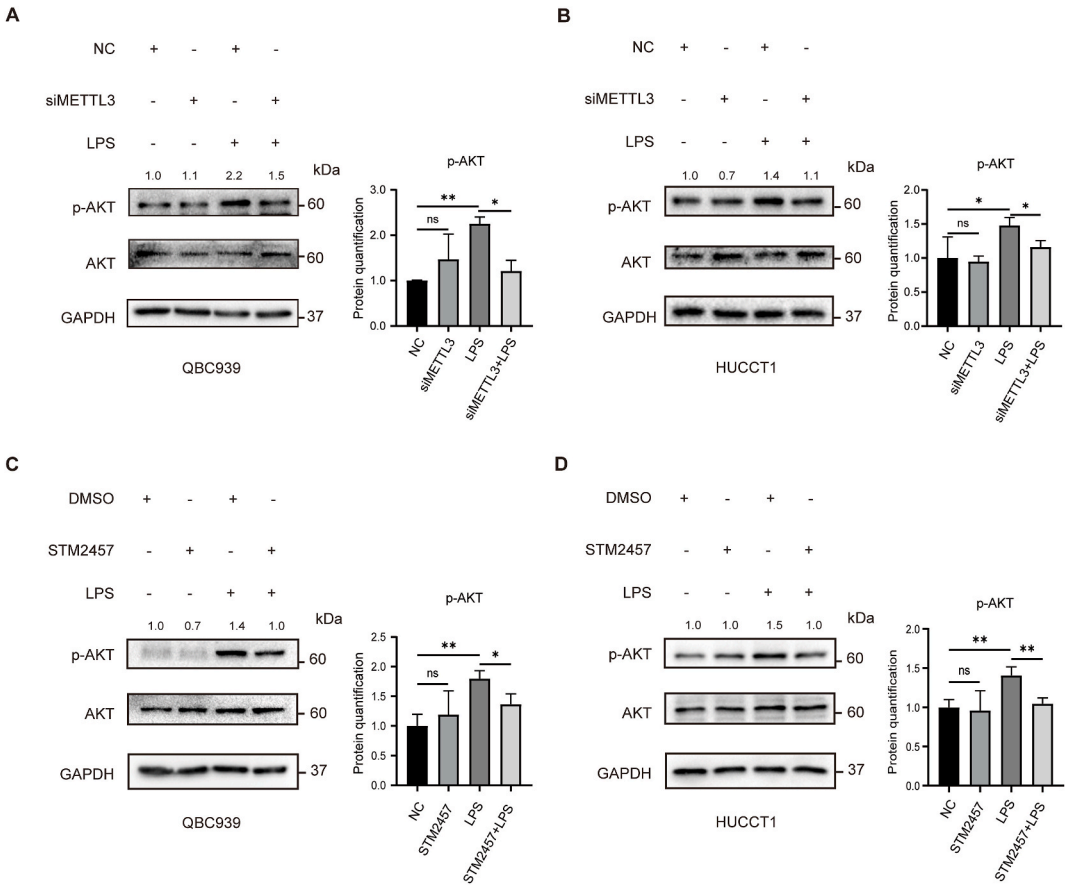


**Fig. 6. METTL3 regulates PI3K/AKT signaling pathway in human cholangiocarcinoma cells (A and B)** QBC939 and HUCCT1 cells were transfected with METTL3 siRNA (siMETTL3) or scrambled siRNA (siRNA) for 24 h, then treated with LPS for another 24 h. Cell lysates were then analyzed for AKT and p-AKT expression by Western blot. GAPDH was used as loading control. **(C and D)** QBC939 and HUCCT1 cells were treated with indicated concentrations of STM2457 or DMSO for 24 h, then treated with LPS for another 24 h, cell lysates were then analyzed for AKT and p-AKT expression by Western blot. GAPDH was used as loading control. Densitometric quantification is shown next to the Western blots, representing the signal normalized to total AKT, relative to the untreated controls. Data represents means ± SD, n = 3. *p < 0.05, **p < 0.001, ***p < 0.001, n.s., non-significant (Student *t*-test).

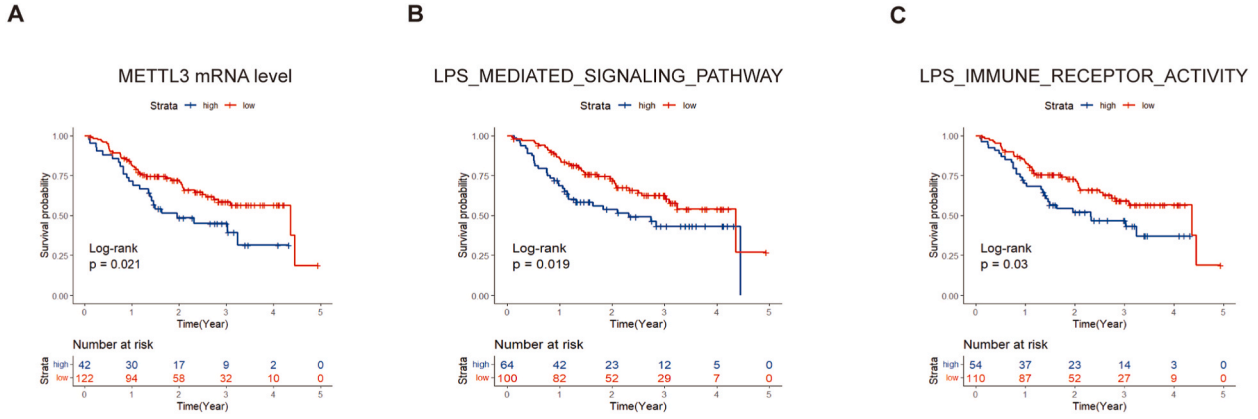


**Fig. 7. METTL3 and LPS upregulation predicts worse clinical outcomes in patients with ICC (A)** The overall survival curve stratified by METTL3 mRNA expression levels in the ICC cohort (OEP001105). The p value was calculated using the log-rank test. **(B)** The overall survival curve stratified by LPS_mediated_signaling_pathway enrich score in the CCA cohort (OEP001105). The P value was calculated using the log-rank test. **(C)** The overall survival curve stratified by LPS_immune_ receptor_activity enrich score in the CCA cohort (OEP001105). The P value was calculated using the log-rank test.

and the proliferation, migration, invasion, and other biological functions in leukemia cells, hepatocellular carcinoma cells, and small cell lung cancer cells [17,27,28]. Moreover, reportedly, STM2457 can inhibit proliferation, migration, and invasion and promote apoptosis in ICC [13]. When we inhibited METTL3 expression using STM2457, the phosphorylation level of AKT also reduced. These results suggest that METTL3 was partially responsible for the activation of PI3K/AKT signaling.

In summary, our findings confirmed that LPS can promote cancer progression and leads to a poor overall survival in ICC. We further indicated a novel mechanism by which LPS promotes ICC migration and invasion by inducing METTL3 expression and subsequently activating the PI3K/AKT signaling pathway.

## Ethics approval

Human cholangiocarcinoma cell lines QBC939 and HUCCT1 were given as a gift from Dr. Tan wenliang (Sun Yat-sen University, Guangzhou, China).

## Consent to participate

Not applicable.

## Consent to publish

Not applicable.

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## Data availability

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

## CRediT authorship contribution statement

**Jing Ke:** Writing – original draft, Visualization, Software, Investigation, Formal analysis, Data curation. **Chang-jiang Zhang:** Visualization, Software, Investigation, Formal analysis, Data curation. **Lian-zi Wang:** Visualization, Software, Investigation, Formal analysis, Data curation. **Feng-shuo Xie:** Visualization, Software, Data curation. **Hongyu Wu:** Visualization, Software, Data curation. **Tao Li:** Visualization, Software, Data curation. **Cong-Wen Bian:** Writing – review & editing, Resources, Methodology, Formal analysis, Conceptualization. **Ruo-Lin Wu:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e29683.

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