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Original Article

METTL14-Mediate the Biological Effects of EMT in Bladder Cancer Cells by Methylating SOX4 mRNA with m6A

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ABSTRACT

Objectives: Bladder Cancer (BC) is one of the most common malignant tumours of urinary system, with high rates of metastasis and mortality. Methyltransferase-like 14 (METTL14) is an RNA N6-adenosine methyltransferase that can affect the development of tumours by modifying RNA expression. The study aims to uncover the molecular mechanism and biological function of METTL14 in BC. **Materials and Methods:** qRT-PCR assays were employed METTL14 in BC cell lines. MeRIP-qPCR and RIP were used to verify that SRY-related high-mobility-group box 4 (SOX4) modification by m6A is a downstream target of METTL14. **Results:** Biological experiments *in vitro* have demonstrated the biological function of METTL14 on BC cells. In the MGH-U3 cell lines, the expression of METTL14 was significantly down-regulated ($p < 0.001$), while the expression of SOX4 was significantly up-regulated ($p < 0.01$). Intervention of METTL14 expression can affect cell proliferation, migration, invasion, apoptosis, as well as the expression of EMT proteins in BC cell lines ($p < 0.05$). In addition, METTL14 can affect the biological functions of MGH-U3 cells and the expression of EMT proteins through the m6A modification of SOX4. **Conclusion:** METTL14 overexpression inhibited the proliferation, migration, invasion, and promoted apoptosis in BC cells, and also downregulated the expression of EMT-related proteins, while these effects were abolished by silencing SOX2. This suggests that METTL14, through its influence on SOX4 m6A modification, plays a crucial role in regulating the biological functions of bladder cancer cells, indicating its potential as a therapeutic target for BC.

Keywords: METTL14, EMT, Bladder cancer, SOX4 mRNA, m6A.

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INTRODUCTION

Bladder Cancer (BC) is one of the most common malignant tumours of the urinary system and one of the most common cancers worldwide.[1] Since BC has a high metastasis rate and mortality, with an average of about 550,000 new cases and 200,000 deaths every year.[2] The potential molecular mechanism of BC is still unclear, which inhibits the progress of clinical treatment. Therefore, exploring the mechanism of BC progression will accelerate the search for the development of effective therapeutic targets.

*N*6-methyladenine (m6A) is a common form of RNA modification, mainly referring to the addition of a methyl group to adenine in RNA molecules. m6A modification is one of the most common RNA modifications in eukaryotes, widely present in various RNA molecules such as mRNA, long non-coding RNA, and microRNA.[3] m6A modification is a dynamic process involving three main functional modules: "writing" (m6A methylase), "erasing" (m6A demethylase), and "reading" (m6A binding protein).[4] m6A is the most common type of mRNA modification, involved in various physiological processes. The m6A RNA modification process is regulated by the dynamic interactions of proteins such as "writers", "targets", and "readers".[5-7] m6A modification plays an important role in many biological processes, participating in the regulation of RNA stability, post-transcriptional modification, translation regulation, RNA splicing, and RNA interaction.[8]

Increasing research has found that m6A modification plays a key role in the pathogenesis of tumours, including Hepatocellular Carcinoma (HCC),[9] Endocrine Cancer (EC),[10] Renal Clear Cell Carcinoma (RCC),[11] Lung Cancer (LC),[12] and Gastric Cancer (GC).[13] In recent years, a large number of studies have shown that the relationship between m6A and BC is of great clinical significance for clinical diagnosis, treatment, and prognosis.[14,15] However, the effect of METTL14 in BC has not been fully elucidated.

In this study, we unveiled the abnormally low expression of METTL14 in BC cell lines and proved that intervention METTL14 expression has an impact on the biological function and EMT protein expression level of BC cell line (MGH-U3).

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In addition, this study proves that METTL14 promote the biological function of BC cells through m6A epigenetic modification to promote SRY-related high-mobility-group box 4 (SOX4)-mediated EMT process. Therefore, we have identified the downstream target genes of METTL14, and also uncover molecular mechanisms underlying BC.

MATERIALS AND METHODS

Cell culture

Human BC cells MGH-U3, and human normal bladder epithelial cells SV-HUC-1 were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA); The sediment was mixed with complete culture medium (fetal bovine serum with 1% cyanine/streptomycin in DMEM/F12 solution (Gibco, Rockville, MD, USA)) and transferred to a culture bottle for cultivation. All these cells were cultured at 37°C with 5% CO_2.

Gene silencing and transduction

shRNAs targeting METTL14 and the scramble Negative Control (sh-NC) were purchased from Suzhou Gemma Genetics (Suzhou, China). PcDNA3.1RNAs targeting METTL14 and the scramble negative control (pcDNA3.1-NC) were purchased from Suzhou Gemma Genetics (Suzhou, China). 3.75 μL of diluted Lipofectamine 3000 reagent was added to 250 μl of diluted plasmid vector of each group and incubated for 15 min at room temperature. The viral solution of lentiviral pLKO.1-METTL14 shRNA and pLKO.1-METTL14 pcDNA-RNA expression vector were added into MGH-U3 cells in six-well plates according to the grouping for infection. MGH-U3 cells were infected with lentivirus. Infected cells were selected with puromycin (MedChem Express, Monmouth Junction, NJ, USA) (10 μg/mL) for 7-14 days after infection to generate stable cells.

qRT-PCR

To extract total RNA from cells using TRIzol reagent (Vazyme, Nanjing, China), the absorbance of the sample was determined using a UV spectrophotometer to calculate the concentration of RNA. The cDNA was synthesized using HiScript III 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). qRT-PCR conditions were as follows: 94°C for 2 min, 94°C for 30 sec, 60°C for 30 sec, and 72°C for 2 min, for 40 cycles. The relative expression of the experimental results was analyzed using the $2^{-\Delta\Delta Ct}$ method compared to GAPDH or U6 expression. The primers used in this study were purchased from Sangon Biotech (Shanghai, China) and the primer sequences are shown in Table 1.

Western blot

The total protein was extracted, which was subsequently transferred membrane and blocked membrane. The membranes were added primary antibody and secondary antibody. The chemiluminescence reagents were mixed in equal volumes of liquid A and liquid B (NCM Biotech, Suzhou, China). The membranes were incubated for 5 min and then detected by JP-K6000 chemiluminescence imager (Jiapeng, Shanghai, China). Protein expression was analyzed using Image J software for optical density values.

Cell viability assay

The cell viability was evaluated by the CCK-8 assay. In brief, cells were seeded into a 96-well plate at a concentration of 2000 cells per well. After the cells adhered, they were starved in a serum-free medium for 12 hr. Then, a fresh complete medium with CCK-8 (1:10) was added to each well, and the cells were incubated at 37°C with 5% CO_2 for 1 hr. Finally, the Optical Density (OD) value at 450 nm was measured using an RT-6000 enzyme microplate reader from Rayto (Norcross, GA, USA).

Cell apoptosis assessment

We employed the BD Pharmingen FITC Annexin V Apoptosis Detection Kit (BD Biosciences, 0027279, Franklin Lakes, NJ, USA), a valid method, for apoptosis evaluation. The MGCs/OECs were first dyed with Annexin V-FITC/Propidium Iodide (PI) and then analyzed by flow cytometry. Here's how: washed and chopped MGCs/OECs in PBS at 37°C for 3 to 5 min with 0.25% trypsin. Next, we added DMEM/F12 medium with 10% fetal bovine serum to quench the residual trypsin. Then, we mixed each sample's cells in 100 μL binding buffer, containing 5 μL Annexin V-FITC and 5 μL PI, and let it sit in the dark for 15 min before analysis on an Attune NxT (Invitrogen, Carlsbad, CA, USA).

Transwell

For transwell migration and invasion experiments, consider seeding MGH-U3 cells in the upper chamber, either with or without matrigel (BD Biosciences, Franklin Lakes, NJ, USA), respectively. Following a 24-hr incubation period, gently remove non-migrated or invaded MGH-U3 cells using a cotton swab, then fix the remaining cells on the bottom of the chamber with 4% polyformaldehyde. Apply a 0.2%~0.5% crystal violet stain and randomly capture images from 5 fields (×200 magnification) under an inverted microscope (Shanghai Optical Instrument Factory, Shanghai, China).

MeRIP-qPCR

The informative MeRIP procedure (Bes5203, BersinBio, Guangzhou, China) utilized m6A RNA immunoprecipitation (MeRIP). Primarily, total RNA is extracted, chemically fragmented, exposed to m6A antibody (ab208577, abcam, Cambridge, MA, USA), or negative Immunoglobulin G (IgG) antibody, and then was separated into magnetic beads.

RIP

MGH-U3 bladder cancer cells were lysed using a cell lysis buffer. The lysates were then incubated with anti-METTL14 antibodies (abcam, Cambridge, MA, USA) bound to protein A/G beads to capture RNA-protein complexes. Following incubation and washing steps to remove non-specific RNA, RNA molecules specifically binding to METTL14 were isolated and subjected to downstream analyses such as reverse transcription PCR or sequencing to identify interacting proteins. Rabbit IgG was used as a negative control to ensure the specificity of the RIP assay results.

Statistical analysis

In this study, GraphPad Prism 9 statistical software (La Jolla, CA, USA) was used for data analysis. Descriptive statistics for continuous variables are presented as mean ± standard deviation (x ± s). The comparisons between groups were conducted using a *t*-test and one-way ANOVA. A statistical significance level of $p < 0.05$ was used to determine if there were significant differences.

RESULTS

METTL14 is low expressed and SOX4 is highly expressed in MGH-U3 cells

To investigate and detect the expression of METTL14 and SOX4 in BC cell (MGH-U3), this study cultured SV-HUC-1 and MGH-U3, and used qRT-PCR to detect the expression levels of METTL14 and SOX4 in MGH-U3 and SV-HUC-1. The results revealed that METTL14 was lowly expressed in MGH-U3 (Figure 1A, $p<0.01$), while SOX4 was highly expressed in MGH-U3 (Figure 1B, $p<0.001$).

Downregulation of METTL14 can inhibit the proliferation, migration, and invasion of BC cells, and promote cell apoptosis

To investigate the functional role of METTL14 in BC cells, METTL14 was knocked down or overexpressed in MGH-U3 cells. The CCK-8 assay showed that the cell proliferation ability increased in cells with METTL14 expression intervention, while the cell proliferation ability decreased in cells with METTL14 overexpression (Figure 2A, $p<0.001$). Flow cytometry results showed that intervention of METTL14 expression could inhibit cell apoptosis, while overexpression of METTL14 could promote cell apoptosis (Figure 2B, $p<0.001$). The Transwell migration and invasion experiments showed that intervention of METTL14 could promote the increase of cell migration and invasion, while overexpression of METTL14 could inhibit cell migration and invasion (Figure 2C-D, $p<0.05$).

METTL14 can target SOX4, promote the expression of EMT proteins, and inhibit the expression of SOX4

To prove the role of SOX4 on the expression of METTL14, and to verify that METTL14 can affect the expression of EMT proteins in BC cells, we conducted RIP assay in BC cell lines. Results showed that METTL14 can interact with SOX4 and can target binding (Figure 3A, $p<0.05$). The MeRIP-qPCR results showed that m6A is enriched on SOX4 protein (Figure 3B, $p<0.001$). The Western blot results showed that overexpression of METTL14 can promote the expression of EMT proteins (N-cadherin and Vimentin), and

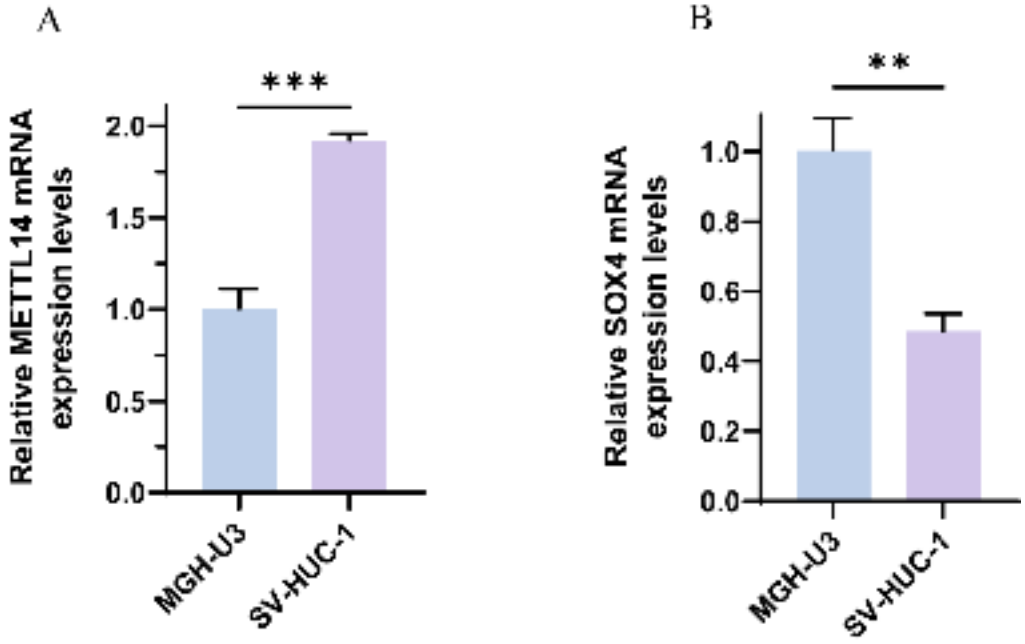

Figure 1: The expression of METTL14 and SOX4 in BC cells and human normal bladder epithelial cells. (A) qRT-PCR was used to detect the mRNA expression of METTL14 in MGH-U3 and SV-HUC-1. (B) qRT-PCR was used to detect the mRNA expression of SOX4 in SV-HUC-1 and MGH-U3. **$p<0.01$, ***$p<0.001$. $N=3$.

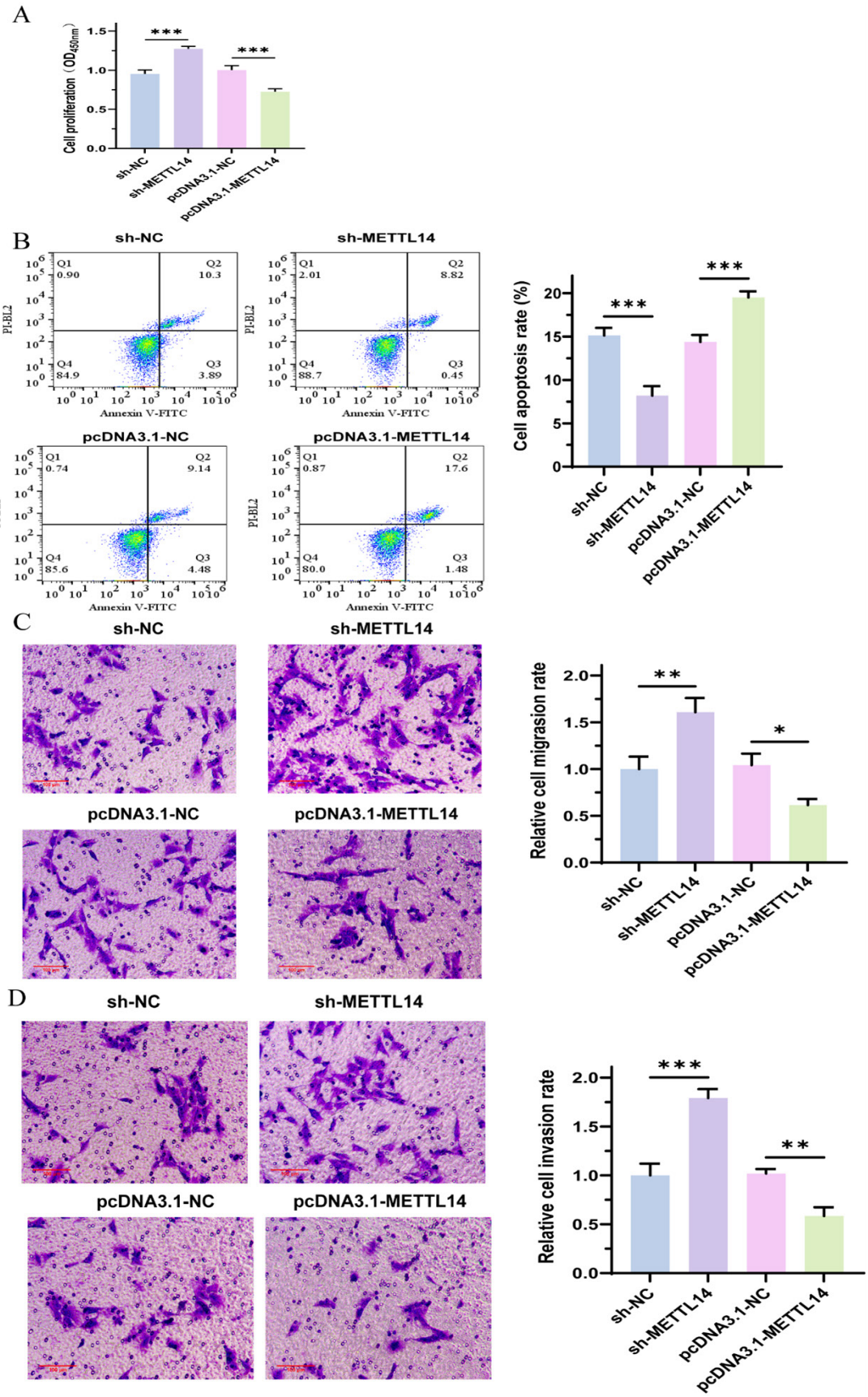

Figure 2: Biological effects of METTL14 on BC cells. (A) Cell proliferation was detected by CCK-8. (B) Cell apoptosis was detected by flow cytometry. (C) Cell migration was detected by Transwell migration assay. (D) Cell invasion was detected by Transwell invasion assay. *p<0.05, **p<0.01, ***p<0.001. N=3.

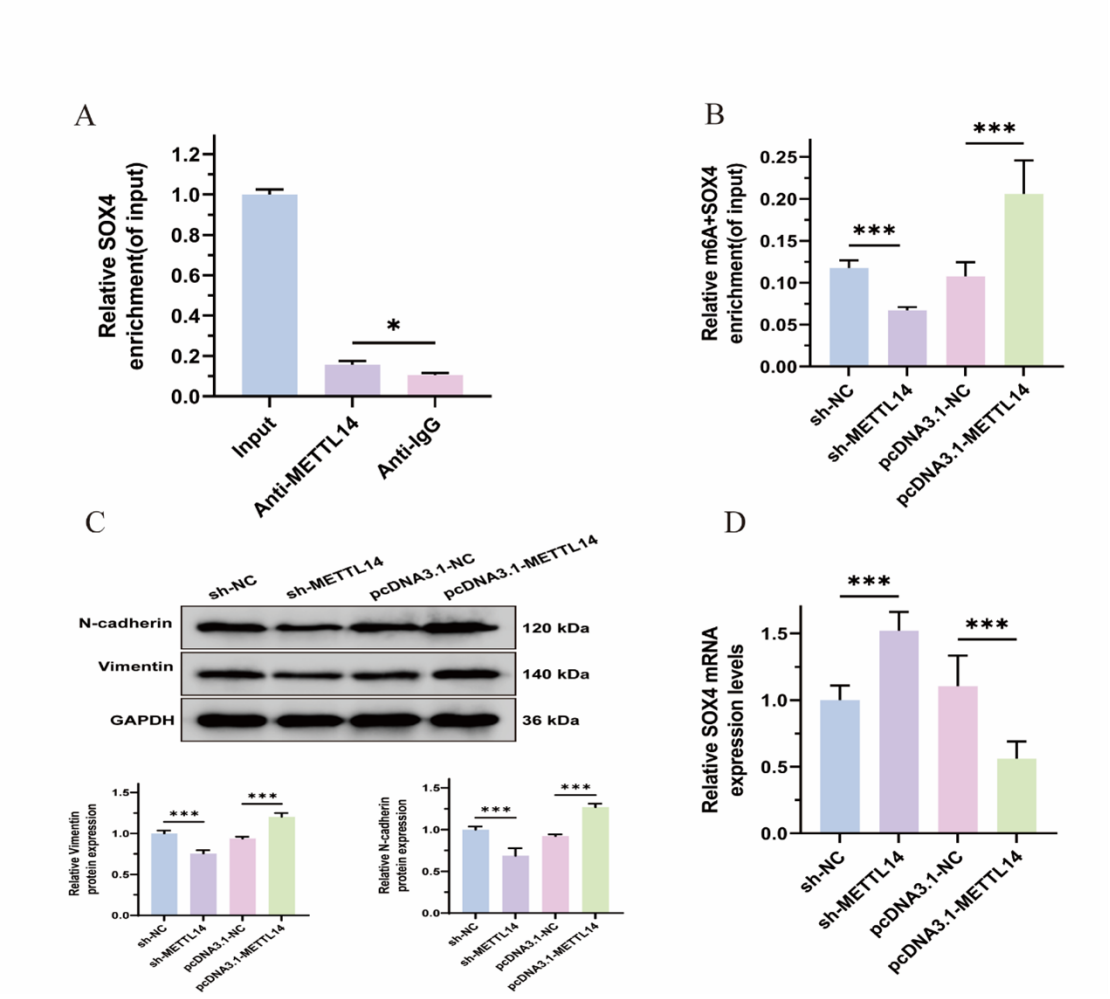

Figure 3: SOX4 is a downstream target of METTL14. (A) RIP was used to detect the targeted relationship between METTL14 and SOX4. (B) MeRIP-qPCR was used to detect the enrichment of m6A in SOX4. (C) Western blot was used to detect the expression of EMT-related proteins (N-cadherin and Vimentin). (D) qRT-PCR was used to detect the expression of SOX4 mRNA. *p<0.05, **p<0.01, ***p<0.001, ns: no significance. N=3.

intervention of METTL14 can inhibit the expression of EMT proteins (Figure 3C, p<0.001). qRT-PCR results showed that the expression of SOX4 increased when the expression of METTL14 was intervened, and the expression of SOX4 decreased when METTL14 was overexpressed (Figure 3D, p<0.001).

SOX4 reverses the effects of METTL14 in BC

To investigate the role of SOX4 in BC, this study used shRNA targeting SOX4 to downregulate the expression of SOX4 in MGH-U3 cells, and while overexpressing METTL14. qRT-PCR results showed that overexpression of METTL14 significantly upregulated METTL14 expression in MGH-U3 cells. When intervening SOX4 expression, it significantly inhibited SOX4 expression in MGH-U3 cells (Figure 4A, p<0.001). CCK-8 proliferation experiments showed that intervening SOX4 could reverse the inhibitory effect of METTL14 on cell proliferation (Figure 4B, p<0.01). Transwell migration and invasion experiments showed that METTL14 overexpression inhibited the migration and invasion abilities of MGH-U3 cells, which could be reversed by SOX4 (Figure 4C-D, p<0.01). Flow cytometry experiments showed that METTL14 overexpression promoted cell apoptosis while intervening SOX4 expression inhibited apoptosis of MGH-U3 cells (Figure 4E, p<0.001). Western blot experiments showed that METTL14 overexpression increased the expression of Vimentin and N-cadherin in MGH-U3 cells, while intervening SOX4 expression decreased their expression (Figure 4F, p<0.05).

Table 1: Oligonucleotide sequences used for qRT-PCR.

Primers		Sequence (5′→3′)
METTL14	Forward	TTCCACAGACTCGGAAGAAAGG
	Reverse	TGGAGCAGAGGTATCATAGG
SOX4	Forward	AAGCTGCAGCAAGAGAAACTG
	Reverse	AATCGGCACTAAGGAGTTGG
GAPDH	Forward	GGTCTCCTCTGACTTCAACA
	Reverse	GTGAGGGTCTCTCTCTTCCT

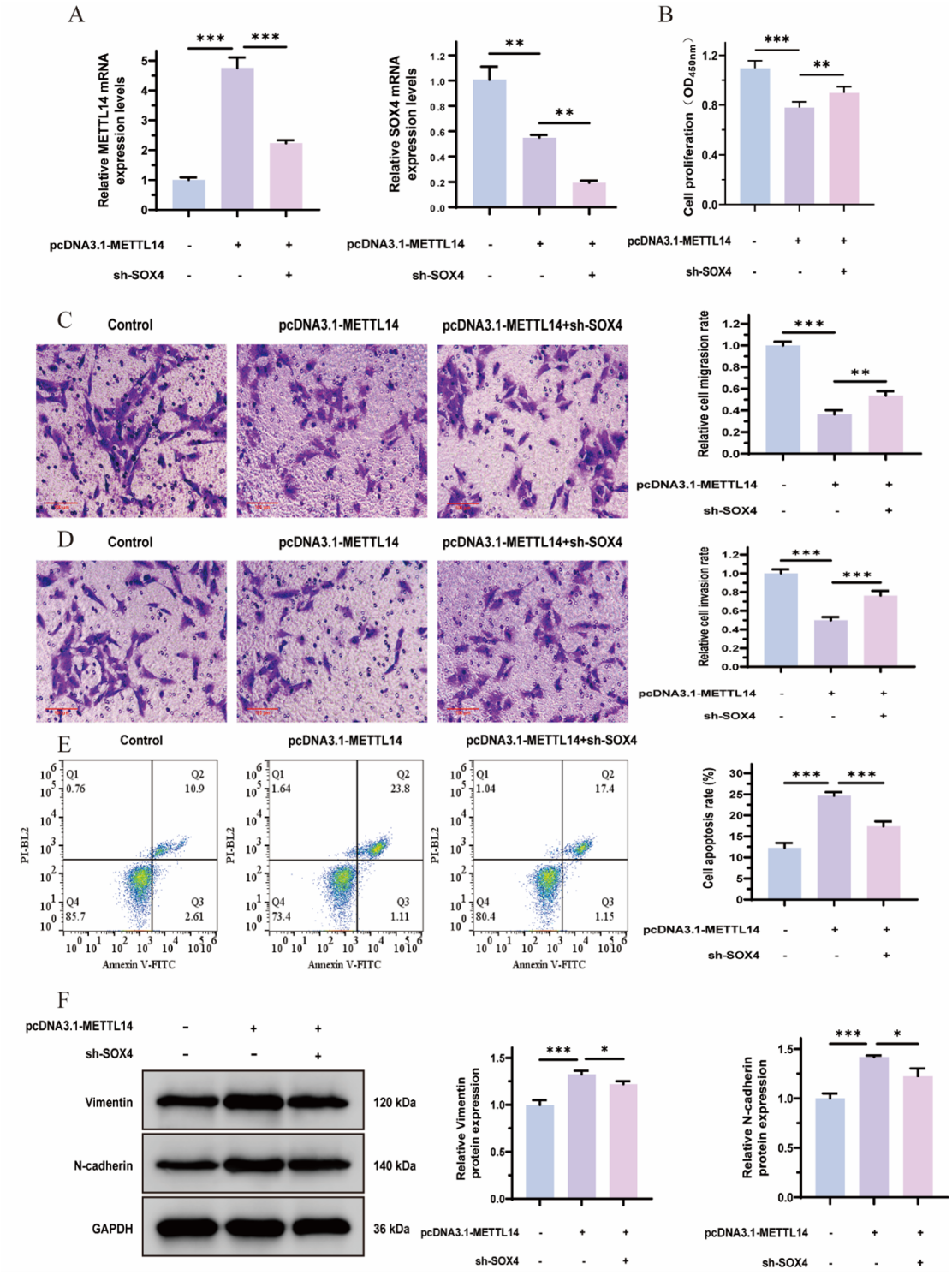

Figure 4: SOX4 reversed the effects of METTL14 in BC. (A) When METTL14 was overexpressed and SOX4 was silenced, qRT-PCR proved the presence of METTL14 and SOX4 mRNA levels in MGH-U3 cells. (B) CCK-8 was used to detected cell proliferation. (C-D) Migration and invasion were detected by Transwell migration assay and Transwell invasion assay. (E) Apoptosis was detected by flow cytometry. (F) Western blot was used to detect protein levels of Vimentin and N-cadherin in MGH-U3. *p<0.05, **p<0.01, ***p<0.001. N=3.

DISCUSSION

BC is a malignant tumour that occurs on the bladder mucosa.[1] In recent years, a large number of studies have shown that the occurrence and development of BC were closely related to changes in key genes, but there is currently no effective treatment in clinical practice. Previous studies have shown that m6A modification plays an important role in cancer development.[6,16] This study reveals a significant decrease in METTL14 expression

and a marked increase in SOX4 protein levels in MGH-U3 cells compared to SV-HUC-1 cells, indicating distinct gene expression profiles between the two cell lines.

M6A is a recognition protein for N6-methyladenosine (m6A), which is involved in post-transcriptional modification of RNA as a recognition protein for mRNA, snRNA, rRNA.[17,18] Dysregulation of m6A modification can lead to many diseases, especially cancer. At the same time, M6A reader proteins can indirectly or directly bind to m6A-modified motifs to affect RNA function.[19-21] In addition, m6A affects tumour expression, immunotherapy, and targeted therapy by altering programmed cell death, metabolism, drug resistance, and oncogenes.[22,23] Here, to further verify the targeted relationship between METTL14 and SOX4, this study used RIP and MeRIP-qPCR to demonstrate that m6A-modified SOX4 was the downstream target of METTL14. The experimental findings revealed that METTL14 exerts a negative regulatory effect on SOX4 expression, with SOX4 expression being modulated by METTL14-mediated m6A methylation. Additionally, METTL14 expression influences the levels of N-cadherin and Vimentin proteins in MGH-U3 cells. Further investigations demonstrated that METTL14 inhibits the proliferation, migration, and invasion of MGH-U3 cells while promoting apoptosis, following previous research.[24]

In mechanism, the imbalance of METTL14 expression leads to the increase of m6A modification in SOX4, promotes the proliferation, migration and invasion of BC cells, and inhibits cell apoptosis. This study further found that SOX4, which was the m6A-dependent manner in BC, was a downstream target gene of METTL14. The abnormal expression of SOX4 in tumours is associated with different regulatory mechanisms. Studies have found that miR-133b affects the biological effects of BC cells through its influence on SOX4 levels.[25] At the same time, SOX4 can mediate the differentiation of neuroblastoma induced by ATRA.[26] SOX4 is an important downstream molecule of METTL14-targeted m6A modification.[6,11] This study unveiled the role of METTL14-modified SOX4 in BC, thus expanding our understanding of the diverse molecular mechanisms governing METTL14 expression and enriching the current knowledge base on N6-methylation. These discoveries unveil a novel layer of epigenetic alterations influencing BC progression, offering a fresh therapeutic target for precision treatment. Specifically, by modulating METTL14 expression, heightened m6A modifications on SOX4 were observed, fostering BC cell proliferation, migration, and invasion while suppressing apoptosis.

CONCLUSION

In summary, our study work revealed the important role of METTL14-driven m6A-dependent regulatory mechanism in BC progression. We show that METTL14 regulates SOX4 transcription by m6A-dependent process. The understanding of the METTL14-SOX4 pathway and its involvement in BC metastasis could contribute to future BC studies and potential therapeutic approaches.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

BC: Bladder cancer; **METTL14:** Methyltransferase-like 14; **SOX4:** SRY-related high-mobility-group box 4.

SUMMARY

Bladder Cancer (BC) is a prevalent malignancy of the urinary system with high metastasis and mortality rates. METTL14, an RNA N6-adenosine methyltransferase, impacts tumor development by modifying RNA expression. This study elucidates the molecular mechanisms and biological functions of METTL14 in BC. METTL14 expression was inhibited in BC cell lines. METTL14 overexpression inhibited proliferation, migration, and invasion while promoting apoptosis in BC cells. SOX4 was identified as a downstream target of METTL14, which abolished the inhibitory effects of METTL14 overexpression. This suggests that METTL14 plays a crucial role in regulating the biological functions of BC cells through SOX4 m6A modification, indicating its potential as a therapeutic target for BC.

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