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m^{6}A methyltransferase METTL3 promotes the progression of prostate cancer *via* m^{6}A-modified LEF1

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Abstract. – OBJECTIVE: This study aims to uncover the regulatory effects of METTL3 on promoting the progression of prostate cancer (PCa) through N6-Methyladenosine (m6A) methylation on LEF1 mRNA.

PATIENTS AND METHODS: The relative levels of METTL3 and LEF1 in 48 paired PCa tissues and adjacent ones were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Their correlation in PCa tissues was analyzed by Spearman correlation test. The survival of PCa patients affected by METTL3 was assessed by introducing Kaplan-Meier method. Wound closure assay was performed to evaluate the potential influence of METTL3 on migratory ability in PC-3 cells. Protein level of LEF1 in PC-3 cells with METTL3 or IGF2BP2 knockdown was examined. The activity of the Wnt pathway was tested through TOP/FOP-Flash. Furthermore, the interaction between LEF1 with METTL3 or IGF2BP2 was verified through RIP (RNA-Binding Protein Immunoprecipitation) assay. At last, the regulatory effects of METTL3/LEF1 axis on the activity of the Wnt pathway and migratory ability in PC-3 cells were determined.

RESULTS: METTL3 and LEF1 were upregulated in PCa tissues, and they presented a positive correlation in PCa. A high level of METTL3 predicted poor prognosis in PCa patients. The knockdown of METTL3 suppressed the migratory ability in PC-3 cells. Meanwhile, the knockdown of METTL3 downregulated protein level of LEF1 and decreased the activity of the Wnt pathway. The results of RIP assay indicated that METTL3 methylation sites were present on LEF1 mRNA. Moreover, the silence of METTL3 decreased the enrichment abundance of LEF1 in anti-IGF2BP2. Rescue experiments demonstrated that the overexpression of LEF1 partially reversed the regulatory effects of METTL3 on the Wnt activity and migratory ability in PCa cells.

CONCLUSIONS: METTL3 is upregulated in PCa tissues. METTL3 influences the activity of the Wnt pathway through m6A methylation on LEF1 mRNA, thereafter, promoting the progression of PCa.

Key Words:
m6A, METTL3, LEF1, Wnt/β-catenin, Prostate cancer (PCa).

Introduction

Prostate cancer (PCa) is a prevalent malignant cancer throughout the world, which is the second fatal cancer in the United States[1]. Serum level of prostate-specific antigen (PSA) is an important marker for monitoring the progression or recurrence of PCa. However, elevated serum level of PCa could be seen in patients with prostatitis, benign prostatic hyperplasia and urinary tract infection[2,3]. Therefore, it is of significance to develop sensitive and effective hallmarks for PCa.

RNA N^6-Methyladenosine (m^{6}A) was first proposed in 1974. Later, m^{6}A methylation is identified to widely present in different tissues and organs. It is dynamically and reversibly altered during the development period[4-6]. m^{6}A RNA methylation is the most abundant modification in eukaryotes, which is highly conserved in many species[7]. m^{6}A methyltransferases and demethylases contribute to regulate m^{6}A methylation[8,9]. METTL3 maintains the homeostasis of m6A methylation by methylating its target mRNA, thus participating in diverse pathological processes[10-12].

The Wnt pathway is critical in tissue homeostasis, stem cell biology, organ develop, and tumor progression[13,14]. The activated Wnt pathway enhances drug resistance of osteosarcoma[15,16], which also triggers proliferation and migration in

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endothelial cells. Through nuclear translocation of β-catenin, β-catenin interacts with lymphotropic enhancer and T cell factor (Lef and Tcf), thus activating Wnt[14,17].

In this research, we clarified the regulatory effects of METTL3 on promoting the progression of PCa through m^{6}A methylation on LEF1 mRNA. Our findings may provide novel directions in clinical monitoring and treatment of PCa.

Patients and Methods

Sample Collection

PCa tissues and adjacent normal ones (3 cm away from the tumor tissues) were surgically resected from 48 PCa patients undergoing surgical resection in Nanjing First Hospital from April 2017 to December 2018. None of enrolled patients received preoperative anti-tumor therapy. Tissue samples were immediately frozen in liquid nitrogen and preserved at -80°C. Patients and their families have been fully informed. This study was approved by the Ethics Committee of the Nanjing First Hospital.

Cell Culture

Human prostate cells RWPE-2 and PCa cells PC-3 and LNCaP purchased from Cell Bank (Shanghai, China), were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 μg/mL streptomycin, and 100 IU/mL penicillin (Invitrogen, Carlsbad, CA, USA). They were maintained in an incubator with 5% CO_2 at 37°C. Culture medium was regularly replaced.

Cell Transfection

Cells were grown to 60% confluence, and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Fresh medium was replaced 6 hours later. The cells transfected for 48 hours were collected for functional experiments.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

TRIzol (Invitrogen, Carlsbad, CA, USA) was applied for isolating cellular RNA, which was quantified using a spectrometer. RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using the PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). SYBR Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) was utilized for qRT-PCR. Relative level was calculated using $2^{-\Delta\Delta Ct}$ method. The primer sequences are listed in Table I.

Western Blot

Cellular protein was isolated and electrophoresed. Protein samples were loaded on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 hours. The membranes were reacted with primary (LEF1, CST, 2230S; GAPDH, CST, 5174S) and secondary antibodies for indicated time. Band exposure and analyses were finally conducted.

Wound Healing Assay

The cells were seeded in a 24-well plate at 5×10^5 cells/well. An artificial wound was created in the confluent cell monolayer using a 200 μL pipette tip. Wound closure was captured at 0 and 24 h for reflecting the migratory ability in PCa.

TOP/FOP-Flash Luciferase Reporter Assay

TOP/FOP-Flash Luciferase reporter assay was conducted as previously reported[18]. The cells were inoculated in a 24-well plate and transfected with TOP/FOP plasmids (Simo Biomedical Technology, Shanghai, China). The cells were then lysed for determining the relative Luciferase activity.

RIP (RNA-Binding Protein Immunoprecipitation) Assay

RIP assay was performed following the procedures of Millipore Magna RIP Kit (Millipore, Billerica, MA, USA). The cells were incubated with the input, corresponding antibodies or anti-IgG at 4°C overnight. A protein-RNA complex was obtained after capturing intracellular specific proteins by the antibody. Subsequently, the proteins were digested by proteinase K and the RNAs were extracted. During the experiment, the

Table I. Primer sequence.

Gene	Primer sequence
LEF1	F: 5'-CACAGCGGAGCGGAGATTACA-3'
	R: 5'-AATGAGCTTCGTTTTCCACCATG-3'
METTL3	F: 5'-AGATGGGGTAGAAAGCCTCCT-3'
	R: 5'-TGGTCAGCATAGGTTACAAGAGT-3'
GAPDH	F: 5'-CGGAGTCAACGGATTTGGTCGT-3'
	R: 5'-GGGAAGGATCTGTCTCTGACC-3'

magnetic beads were repeatedly washed with RIP washing buffer to remove non-specific adsorption as much as possible. The immunoprecipitant RNAs were finally quantified by qRT-PCR.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 16.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Data were expressed as mean ± SD (standard deviation). The *t*-test was used for analyzing the differences between the two groups. Survival analysis was conducted by Kaplan-Meier method, followed by the Log-rank test. Spearman correlation test was applied for assessing the relationship between the expression levels of METTL3 and LEF1 in PCa tissues. $p<0.05$ indicated the statistically significant difference.

Results

Upregulated METTL3 in PCa

Compared with those in adjacent normal tissues, METTL3 and LEF1 were markedly upregulated in PCa tissues (Figure 1A, 1C). Spearman correlation test uncovered a positive correlation between expression levels of METTL3 and LEF1 in PCa tissues (Figure 1D). Meanwhile, Kaplan-Meier curves illustrated worse prognosis in PCa patients expressing a high level of METTL3 (Figure 1B). It is suggested that METTL3 is upregulated in PCa and predicts a poor prognosis.

Knockdown of METTL3 Inhibited Migratory Ability in PCa

In vitro level of METTL3 was upregulated in PCa cell lines in comparison with that of human

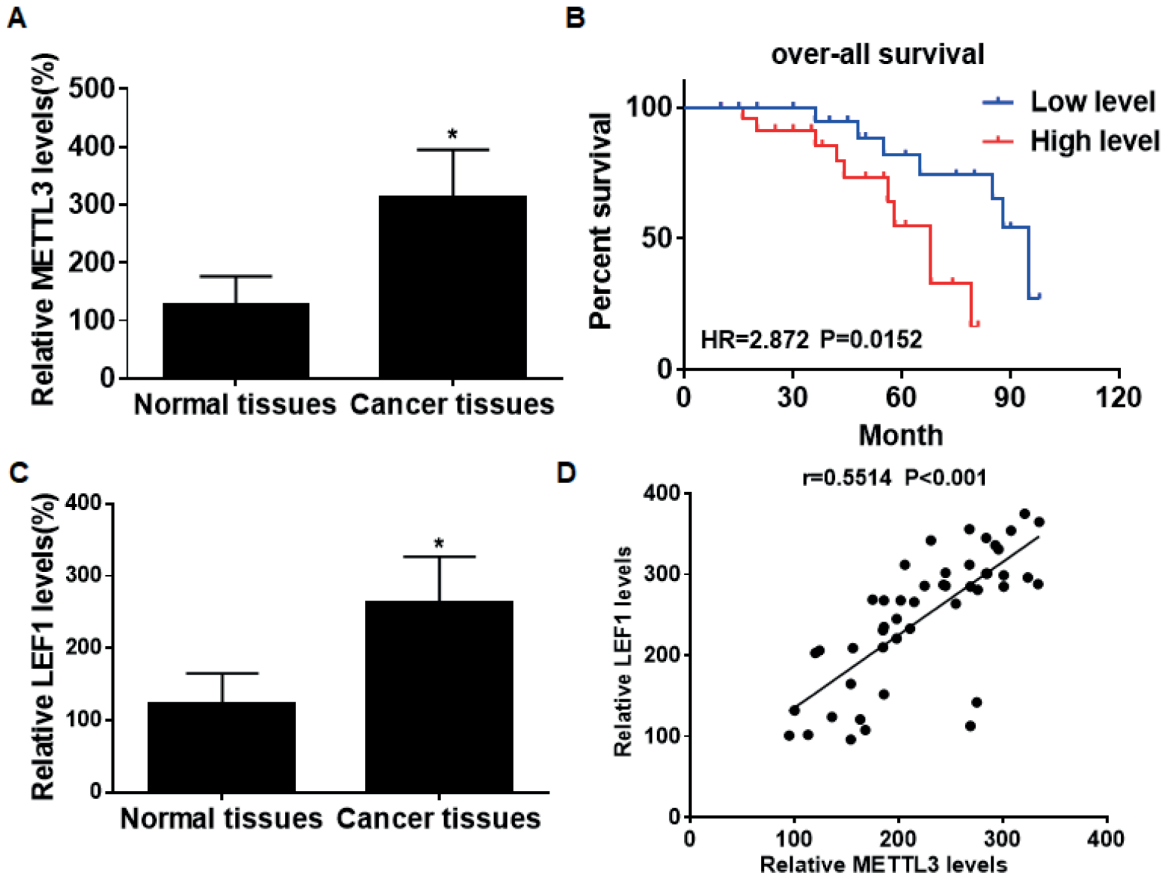

Figure 1. Upregulated METTL3 in PCa. **A,** METTL3 levels in paracancerous tissues and PCa tissues. **B,** Overall survival in PCa patients expressing a high or low level of METTL3. **C,** LEF1 levels in paracancerous tissues and PCa tissues. **D,** A positive correlation between expression levels of METTL3 and LEF1 in PCa tissues.

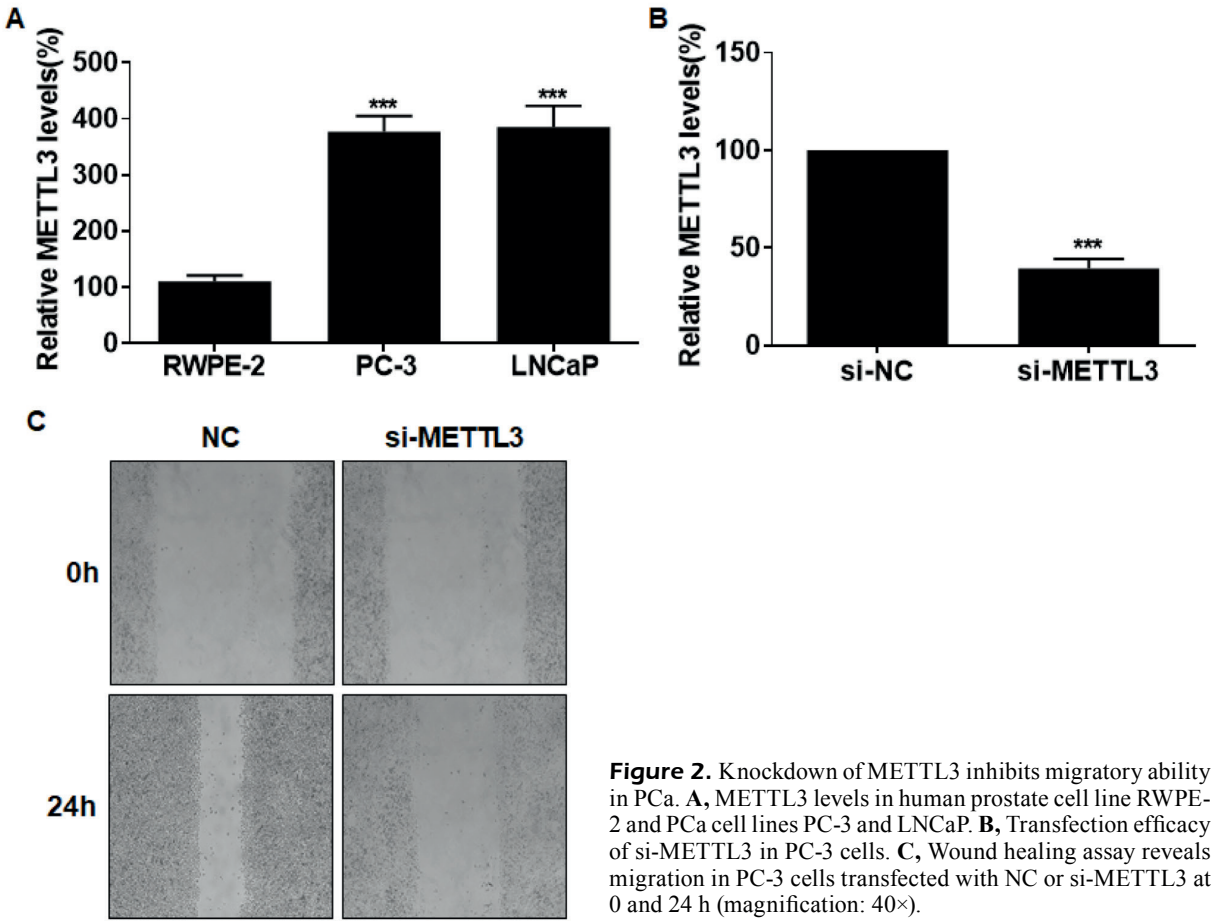

Figure 2. Knockdown of METTL3 inhibits migratory ability in PCa. **A,** METTL3 levels in human prostate cell line RWPE-2 and PCa cell lines PC-3 and LNCaP. **B,** Transfection efficacy of si-METTL3 in PC-3 cells. **C,** Wound healing assay reveals migration in PC-3 cells transfected with NC or si-METTL3 at 0 and 24 h (magnification: 40×).

prostate cell line (Figure 2A). To further clarify the biological function of METTL3, si-METTL3 was constructed. Transfection of si-METTL3 effectively downregulated METTL3 level in PC-3 cells (Figure 2B). In addition, wound healing assay revealed the attenuated migratory ability in PC-3 cells after the knockdown of METTL3 (Figure 2C).

METTL3 Induced m6A Methylation on LEF1

Western blot analysis illustrated that the protein level of LEF1 was downregulated in PC-3 cells transfected with si-METTL3 (Figure 3A). Besides, relative TOP/FOP ratio decreased after knockdown of METTL3, indicating the decreased activity of Wnt pathway (Figure 3B). RIP assay revealed the interaction between METTL3 and LEF1 (Figure 3C). Subsequently, the protein level of LEF1 was found to be declined after transfection of si-IGF2BP2 (Figure 3D). RIP assay not only proved the interaction between IGF2BP2 and LEF1, but also demonstrated their weakened interaction ability after the knockdown of METTL3 (Figure 3E, 3F). The above data indicated that METTL3 regulated the protein level of LEF1 through m^6A methylation on LEF1 mRNA.

METTL3 Activated the Wnt Pathway Through m6A-Modified LEF1 in PCa

Based on the above findings, we speculated that LEF1 was involved in METTL3-regulated phenotypes of PCa cells. First of all, pcDNA LEF1 was constructed, and its transfection efficacy was tested (Figure 4A). The decreased TOP/FOP ratio in PC-3 cells transfected with si-METTL3 was partially reversed by co-transfection of pcDNA LEF1 (Figure 4B). Furthermore, the knockdown of METTL3 suppressed migratory ability in PC-3

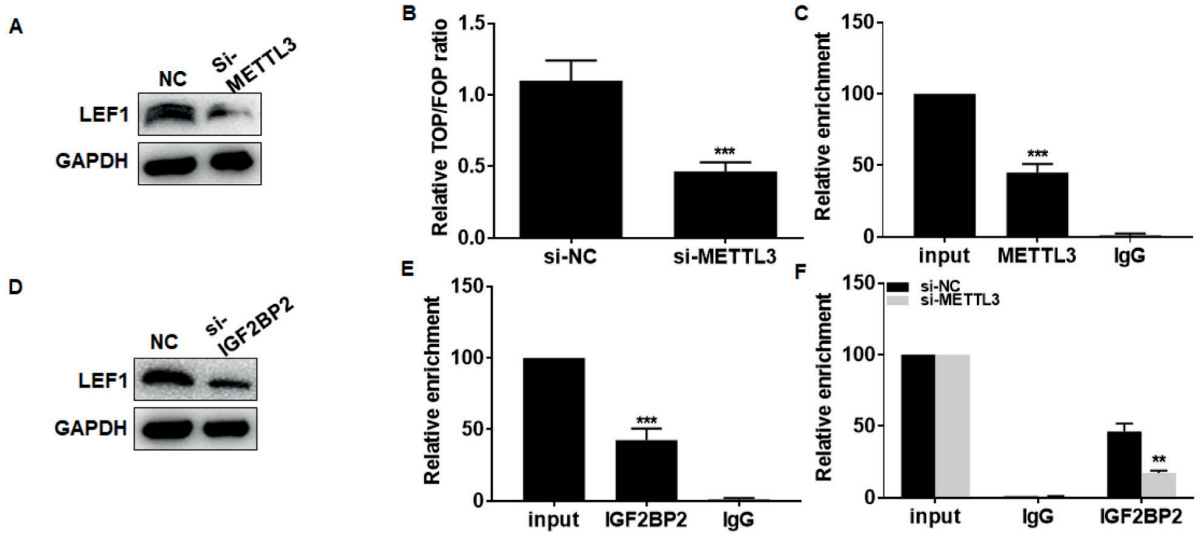

Figure 3. METTL3 induces m^{6}A modification on LEF1. **A,** Protein level of LEF1 in PC-3 cells transfected with NC or si-METTL3. **B,** Relative TOP/FOP ratio in PC-3 cells transfected with NC or si-METTL3. **C,** Relative enrichment of LEF1 in input, anti-METTL3 and anti-IgG. **D,** Protein level of LEF1 in PC-3 cells transfected with NC or si-IGF2BP2. **E,** Relative enrichment of LEF1 in input, anti-IGF2BP2 and anti-IgG. **F,** Relative enrichment of LEF1 in input, anti-IGF2BP2 and anti-IgG after transfection of NC or si-METTL3 in PC-3 cells.

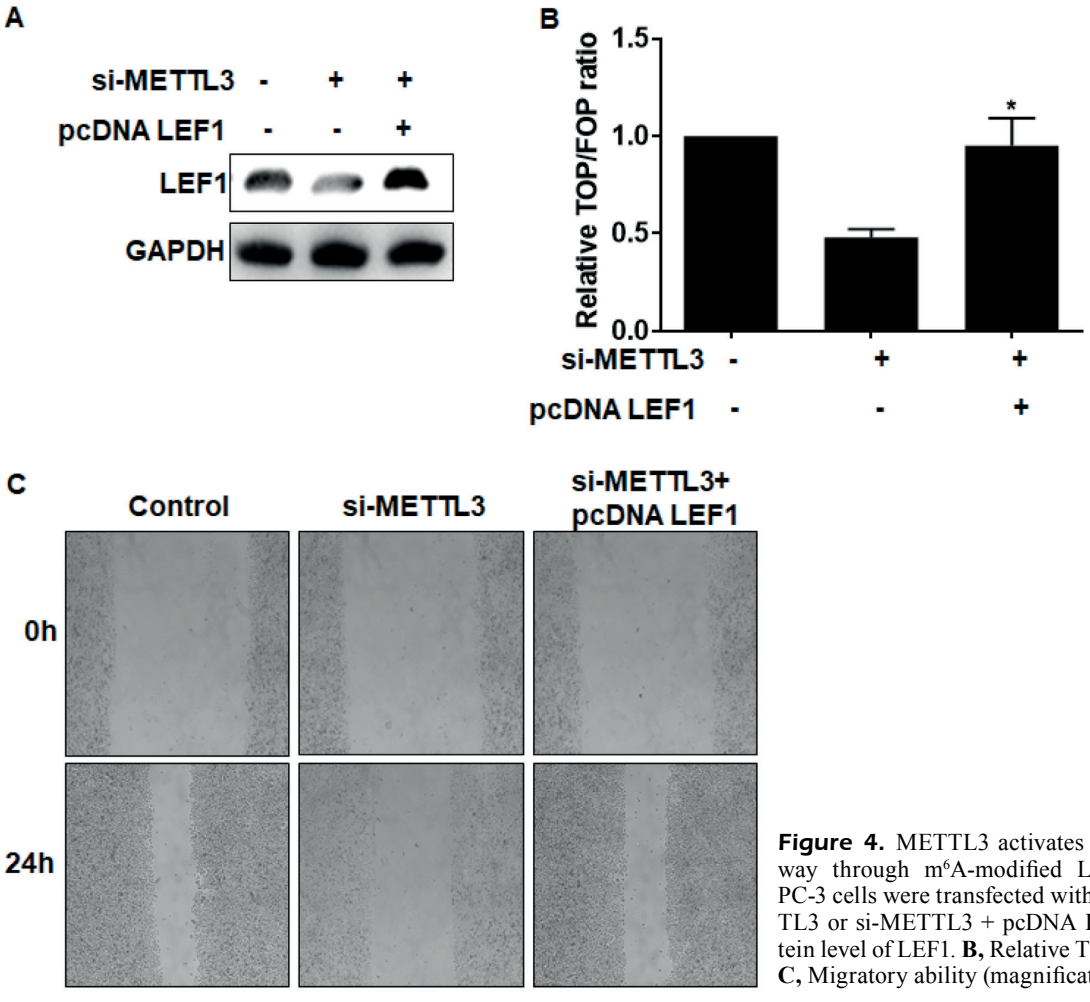

Figure 4. METTL3 activates the wnt pathway through m^{6}A-modified LEF1 in PCa. PC-3 cells were transfected with NC, si-METTL3 or si-METTL3 + pcDNA LEF1. **A,** Protein level of LEF1. **B,** Relative TOP/FOP ratio. **C,** Migratory ability (magnification: 40×).

cells, which was abolished by the overexpression of LEF1 (Figure 4C). Therefore, LEF1 was responsible for METTL3-regulated PCa progression.

Discussion

As the most common chemical methylation on human mRNAs, m^{6}A methylation is considered to be crucial during tumor progression[19]. METTL3 is an m^{6}A RNA methylase affecting m^{6}A methylation. Our findings uncovered that METTL3 was upregulated in PCa tissues and a high level of METTL3 predicted worse prognosis in PCa patients.

METTL3 is involved in tumor progression through m^{6}A-dependent or m^{6}A-independent way. It is reported that METTL3 promotes the progression of breast cancer by upregulating HBXIP *via* enhancing its m^{6}A methylation[20]. m^{6}A methylation contributes to determining mRNA fate. Meanwhile, IGF2BP 1-3 can prolong mRNA half-life by binding to methylated mRNA[21]. In this paper, METTL3 was able to conduct m^{6}A methylation on LEF1 mRNA. At the same time, IGF2BP2 could interact with m^{6}A-modified LEF1 mRNA prolong LEF1 mRNA half-life, thereafter upregulating protein level of LEF1.

Lymphoid enhancer-binding factor 1 (LEF1) is abnormally and differentially expressed under pathological conditions[22,23]. The interaction between LEF1/TCF protein with β-catenin triggers the activation of nuclear Wnt and its downstream genes[24,25]. In addition, LEF1 is able to stimulate cell proliferation and inhibit differentiation *via* activating the Wnt pathway[26]. In this paper, the activity of Wnt pathway was reflected by the relative TOP/FOP ratio. It is shown that the knockdown of METTL3 markedly decreased the relative TOP/FOP ratio. More importantly, the decreased activity of Wnt due to METTL3 knockdown was partially reversed by overexpression of LEF1.

Conclusions

In summary, METTL3 is upregulated in PCa tissues. METTL3 influences the activity of the Wnt/β-catenin pathway through m^{6}A methylation on LEF1 mRNA, thereafter, promoting the progression of PCa. We suggested that m^{6}A methylation on LEF1 induced by METTL3 could be a therapeutic target for PCa treatment.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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