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Simvastatin is beneficial to lung cancer progression by inducing METTL3-induced m6A modification on EZH2 mRNA

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Abstract. – OBJECTIVE: To elucidate the molecular mechanism of Simvastatin on inhibiting malignant progression of lung cancer.

PATIENTS AND METHODS: Relative levels of METTL3 and EZH2 in lung cancer tissues and adjacent normal ones were detected by quantitative real-time polymerase chain reaction (qRT-PCR). In addition, their levels in lung cancer patients with different pathological stages were determined as well. A549 cells were induced with different doses of Simvastatin for 24 h. Subsequently, relative levels of METTL3 and EZH2 in cells were detected. Proliferative and metastatic abilities in A549 cells were examined by cell counting kit-8 (CCK-8), 5-Ethynyl-2'- deoxyuridine (EdU) and transwell assay, respectively. RIP assay was conducted to detect the presence of m6A modification on EZH2 mRNA and the interaction between IGF2BP2 and EZH2. Relative levels of EZH2 and epithelial-mesenchymal transition (EMT)-associated genes (E-cadherin and N-cadherin), and metastatic abilities were detected in Simvastatin-induced A549 cells transfected with pcDNA-METTL3.

RESULTS: METTL3 and EZH2 levels were upregulated in lung cancer tissues, which were higher in advanced stage lung cancer patients. Their levels, as well as cell proliferative and metastatic abilities, were dose-dependently inhibited in Simvastatin-induced A549 cells. METTL3 positively regulated EZH2 level, and m6A modification on its mRNA. Moreover, the interaction between IGF2BP2 and EZH2 could be inhibited by knockdown of METTL3. Simvastatin could abolish the role of METTL3 in regulating relative levels of EZH2 and EMT-associated genes, as well as metastatic abilities in A549 cells.

CONCLUSIONS: Simvastatin induces METTL3 down-regulation in lung cancer tissues, which further influences EMT via m6A modification on EZH2 mRNA and thus inhibits the malignant progression of lung cancer.

Key Words:
Lung cancer (LCa); Simvastatin; METTL3; EZH2; EMT.

Introduction

Lung cancer is the most-common malignant tumor with a very high mortality[1]. Non-small-cell lung cancer (NSCLC) is the major histological subtype (80-85%). It is reported that the 5-year survival of NSCLC is as low as about 15%, and it can be attributed to atypical symptoms in the early phase and lack of effective treatment[2].

Simvastatin is an HMG-CoA reductase inhibitor that inhibits the mevalonate pathway and reduces endogenous production of cholesterol. It is clinically applied for lowering blood lipid and prevention of cardiovascular and cerebrovascular diseases[3]. Experimental evidences have confirmed the anti-cancer effect of Simvastatin on tumor cell proliferation[4,5]. Simvastatin also has significant cytotoxic activity on NSCLC by enhancing the apoptosis of NSCLC cells[6].

The m6A modification on RNAs is the most abundant RNA modification in eukaryotic cells,

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which is responsible for maintaining normal cell functions[7-9]. As the most critical component of the m6A modification, METTL3 participates in various pathological processes by maintaining the dynamic balance of m6A modification on mRNAs[10-12]. A recent study reported that METTL3 triggers TGF-β-induced EMT in lung cancer through regulating JUNB[13].

EZH2 is a vital component of the polycomb repressive complex 2 (PRC2). PRC2 silences the target gene by catalyzing H3K27me3[14]. A large number of studies have found that EZH2 is highly expressed in many cancers, and that the highly expressed EZH2 drives tumor progression through binding the promoter region of E-cadherin to silence its expression[14-16]. By upregulating CCL5, EZH2 contributes to macrophage recruitment and lung cancer invasion[17]. The relationship between METTL3, EZH2 and lung cancer has been rarely reported. In this paper, A549 cells were induced with Simvastatin. We explored whether simvastatin regulated EZH2 protein expression through METTL3-mediated m6A modification of EZH2 mRNA and promoted EMT level, thereby inhibiting the occurrence and development of lung cancer.

Patients and Methods

Patients and Clinical Samples

From October 2016 to December 2018, 36 lung cancer tissues and 30 normal lung tissues were collected from patients treated in the Yancheng Third People's Hospital. None of them had anti-cancer treatment history, nor history of other malignancies. Patients and their families in this study have been fully informed. This investigation was approved by the Ethics Committee of Yancheng Third People's Hospital.

Cell Culture

A549 cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA), 1% penicillin and 1% streptomycin. Until Cells were cultured to 60% confluence, they were induced with 0, 5, 10 or 20 μM Simvastatin for 24 h.

Transfection

Cells were cultured to 60% confluence and transfected with si-NC, si-METTL3 or pcDNA-METTL3 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Fresh medium was replaced at 6 h.

RNA Extraction and Quantitative Real Time Polymerase Chain Reaction (qPCR)

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, Kumatsu, Japan). SYBR Premix Ex Taq TM (TaKaRa, Kumatsu, Japan) was utilized for qRT-PCR. Primer sequences were listed in Table I.

Cell Proliferation Assay

Cells were inoculated in a 96-well plate with 2×103 cells per well. At the appointed time points, absorbance value at 450 nm of each sample was recorded using the cell counting kit-8 (CCK-8) kit (RIBOBIO, Guangzhou, China) for plotting the viability curves.

5-Ethynyl-2′-Deoxyuridine (EdU) Assay

Cells were pre-inoculated in a 24-well plate (4×104 cells/well). They were incubated in 4% methanol for 30 min, followed by 10-min permeabilization in 0.5% TritonX-100, and 30-min reaction in 400 μL of 1×ApollorR. Afterwards, cells were dyed in Hoechst3342 for another 30 min. Positive EdU-stained cells were counted for calculating EdU-positive rate (Sigma-Aldrich, St. Louis, MO, USA).

Transwell Assay

Transwell chambers (Millipore, Billerica, MA, USA) were inserted in each well of a 24-well plate. 3×104 cells were applied in the upper layer of the chamber with 500 μL of medium containing 20% FBS in the bottom. After 48-h incubation, migratory cells in the bottom were reacted with 15-min methanol, 20-min crystal violet and

Table I. Primer sequences.

Gene	Primer sequences
EZH2	F: 5'-TGCACATCCTGACTTCTGTG-3' R: 5'-AAGGGCATTCACCAACTCC-3'
METTL3	F: 5'-AGATGGGGTAGAAAGCCTCCT -3' R: 5'-TGGTCAGCATAGGTTACAAGAGT -3'
GAPDH	F: 5'-CGGAGTCAACGGATTTGGTCGT-3' R: 5'-GGGAAGGATCTGTCTCTGACC-3'

captured using a microscope. Migratory cells were counted in 10 random selected fields per sample. Invasion assay was conducted using transwell chambers pre-coated with diluted Matrigel overnight.

RNA-Binding Protein Immunoprecipitation (RIP) Assay

RIP assay was performed following the procedures of Millipore Magna RIP (RNA-Binding Protein Immunoprecipitation) Kit (Millipore, Billerica, MA, USA). 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, proteins were digested by proteinase K and the RNAs were extracted. During the experiment, the 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.

Western Blots

Cells were lysed for isolating proteins and electrophoresed. Protein samples were loaded on polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 hours. Primary and secondary antibodies were applied for indicated time. Band exposure and analyses were finally conducted.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 16.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Experimental data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Independent sample *t*-test was used to compare the differences between the two groups. $p<0.05$ was considered statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Results

Upregulation of METTL3 and EZH2 in Lung Cancer Tissues

We collected 36 lung cancer tissues and 30 normal lung tissues in this trial. QRT-PCR data showed higher level of METTL3 in lung cancer tissues than controls (Figure 1A). Recruited lung cancer patients were classified according to their tumor stage. It is found that METTL3 level in lung cancer tissues increased with the worsening of tumor stage (Figure 1B). Similar to the expression pattern of METTL3, EZH2 was upregulated in lung cancer tissues as well (Figure 1C). Its level also increased with the advance of tumor stage in lung cancer (Figure 1D).

Simvastatin Inhibited Proliferative and Metastatic Abilities in A549 cells

To uncover the impact of Simvastatin on lung cancer, A549 cells were induced with different doses of Simvastatin for 24 h. It is shown that both METTL3 and EZH2 were dose-dependently downregulated in A549 cells, suggesting that Simvastatin inhibited their expressions (Figure 2A, 2B). Moreover, CCK-8 results revealed a dose-dependent decline in A549 cell viability (Figure 2C). EdU assay obtained the similar trend that Simvastatin induction resulted in a dose-dependent decrease in proliferative cell number (Figure 2D). Transwell assay results, identically, showed a dose-dependent suppression on migratory and invasive potentials in A549 cells (Figure 2E).

METTL3 Induced m6A Modification on EZH2 mRNA

Transfection efficacy of si-METTL3 was first detected in A549 cells (Figure 3A). EZH2 level was downregulated after knockdown of METTL3 (Figure 3B). As RIP assay revealed, there was m6A modification on EZH2 mRNA, and notably, the m6A modification was reduced after knockdown of METTL3 (Figure 3C). Moreover, the interaction between IGF2BP2 and EZH2 mRNA was identified, and their interaction was much inhibited by knockdown of METTL3 (Figure 3D). It is concluded that METTL3 regulated EZH2 level in A549 cells by mediating m6A modification on its mRNA.

Simvastatin Regulated Migration and Invasion of A549 Cells Through METTL3-mediated m6A Modification of EZH2

To explore whether Simvastatin regulated EMT level, migration and invasion of A549 cells through METTL3-mediated m6A modification of EZH2 mRNA, cells were induced with 20 μM Simvastatin for 24 h with or without transfected with pcDNA-METTL3. Interestingly, the upregulated level of EZH2 in A549 cells with overexpression of METTL3 was reversed by

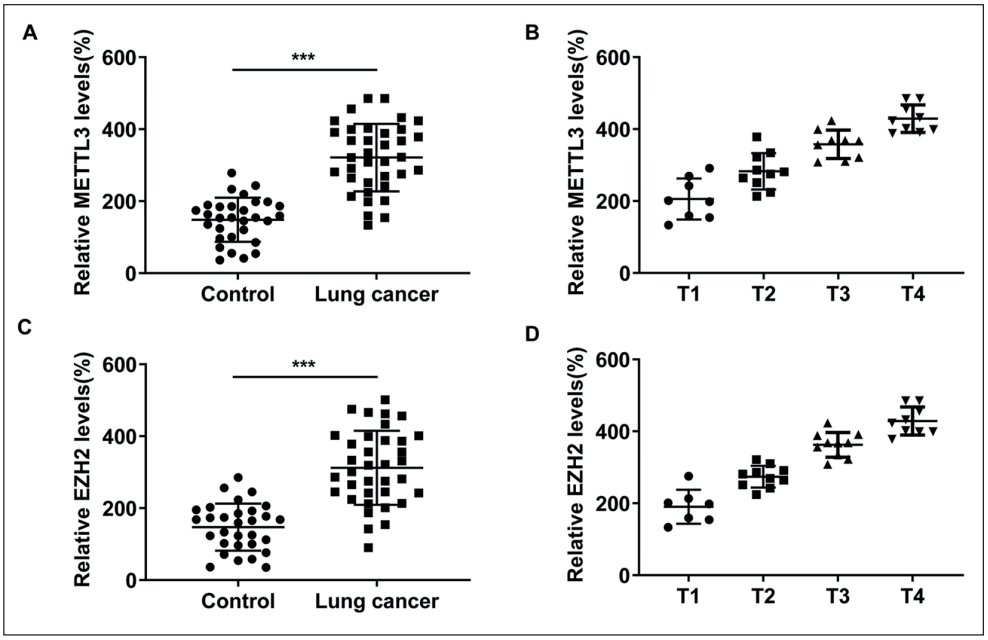

Figure 1. Upregulation of METTL3 and EZH2 in lung cancer tissues. **A,** METTL3 levels in 36 lung cancer tissues and 30 normal lung tissues. **B,** METTL3 levels in lung cancer patients with different tumor stages. **C,** EZH2 levels in 36 lung cancer tissues and 30 normal lung tissues. **D,** EZH2 levels in lung cancer patients with different tumor stages.

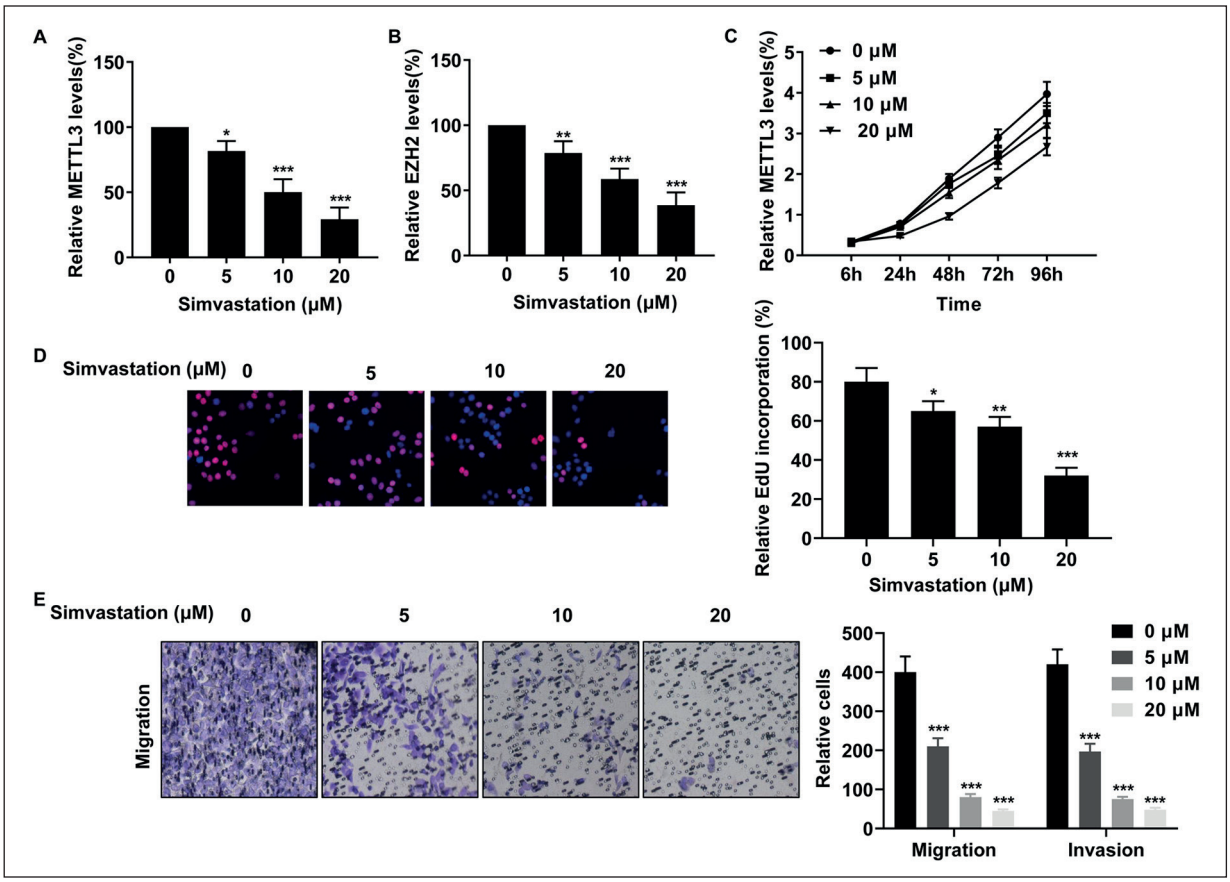

Figure 2. Simvastatin inhibited proliferative and metastatic abilities in A549 cells. **A,** METTL3 level in A549 cells induced with different doses of Simvastatin for 24 h. **B,** EZH2 level in A549 cells induced with different doses of Simvastatin for 24 h. **C,** Viability in A549 cells induced with different doses of Simvastatin for 24 h. **D,** EdU-positive rate in A549 cells induced with different doses of Simvastatin for 24 h. **E,** Migration and invasion in A549 cells induced with different doses of Simvastatin for 24 h (magnification: 40×).

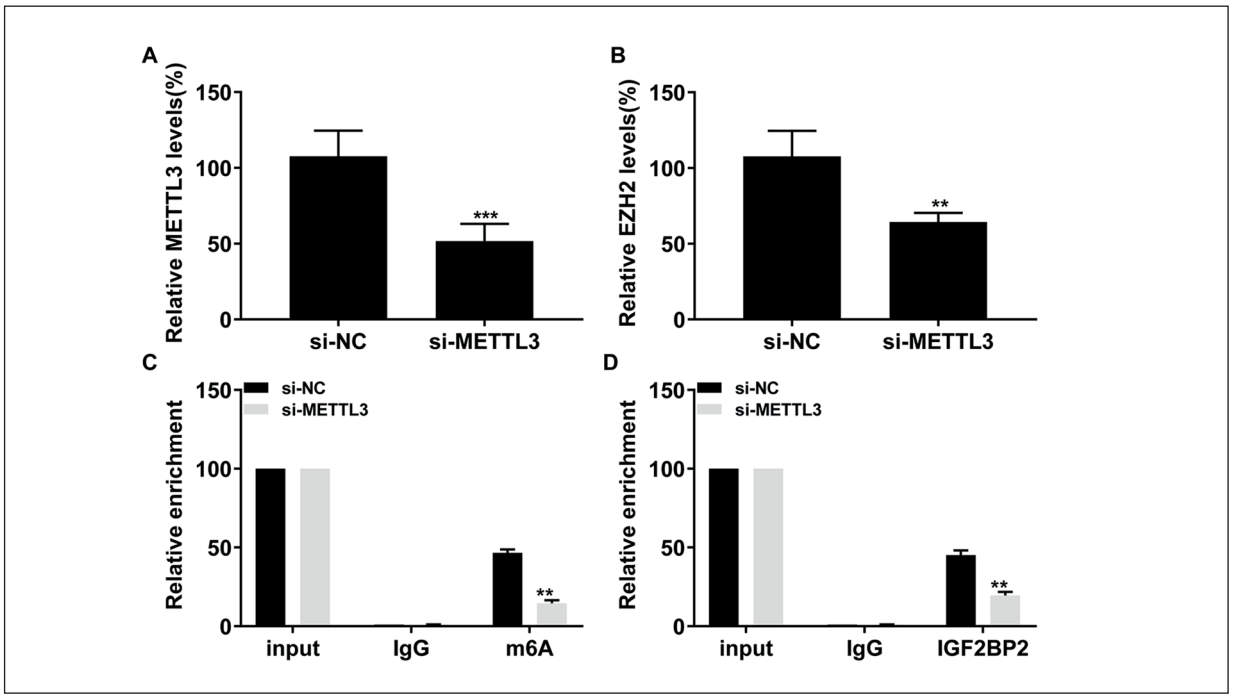

Figure 3. ETTL3 induced m6A modification on EZH2 mRNA. **A,** Transfection efficacy of si-METTL3 in A549 cells. **B,** EZH2 level in A549 cells transfected with si-NC or si-METTL3. **C,** RIP assay showed m6A modification on EZH2 mRNA. **D,** The enrichment of EZH2 in anti-IGF2BP2 in A549 cells transfected with si-NC or si-METTL3.

Simvastatin treatment (Figure 4A, 4B). Western blot results uncovered that Simvastatin reversed the downregulated E-cadherin (the epithelial cell marker) and the upregulated N-cadherin (the mesenchymal cell marker) in A549 cells with METTL3 overexpression (Figure 4B). Enhanced metastatic abilities in A549 cells transfected with pcDNA-METTL3 were also reversed by Simvastatin induction (Figure 4C). We believed that Simvastatin regulated EMT, migration and invasion level *via* METTL3 regulating EZH2, thus inhibited the malignant progression of lung cancer.

Discussion

As an extremely fatal disease, effective diagnosis and treatment of lung cancer are difficult in clinical practice[18, 19]. METTL3 is a vital component of m6A methylation complex, displaying important roles in tumor progression[20-23]. In our experiments, METTL3 was detected to be upregulated in lung cancer tissues we collected, and importantly, its level increased with the worsening of tumor stage.

The function of EZH2 is closely related to PCG nucleosome methylation and DNA methylation. Through acting on H3K27me3, EZH2 at position 21 is phosphorylated by serine/threonine protein kinase. Eventually, PRC2 histone methyltransferase activity is reduced and downstream tumor suppressors are silenced[24, 25]. In many types of tumor tissues, the upregulated EZH2 regulates tumor cell phenotypes through the DACT3, DKK1 and Wnt/β-catenin pathways[26, 27]. Coe et al[28] pointed out that EZH2 expression in lung cancer tissues is enhanced by increasing the level of transcription factor E2F. Here, similar to the expression pattern of METTL3, EZH2 was upregulated in lung cancer tissues, especially advanced stage ones. Knockdown of METTL3 downregulated EZH2 in A549 cells. It is suggested that METTL3 was involved in lung cancer progression through positively regulating EZH2 level.

Previous studies have shown that m6A modification affects RNA expression by regulating its half-life, and IGF2BP2 affects the stability of m6A-modified Mrna[29, 30]. In prostate cancer, IGF2BP2 contributes to extend the half-life of mRNA that is modified by m6A[31]. Our findings identified m6A modification site in EZH2 mRNA and importantly, knockdown of METTL3 could reduce the enrichment of EZH2 in anti-IGF2BP2.

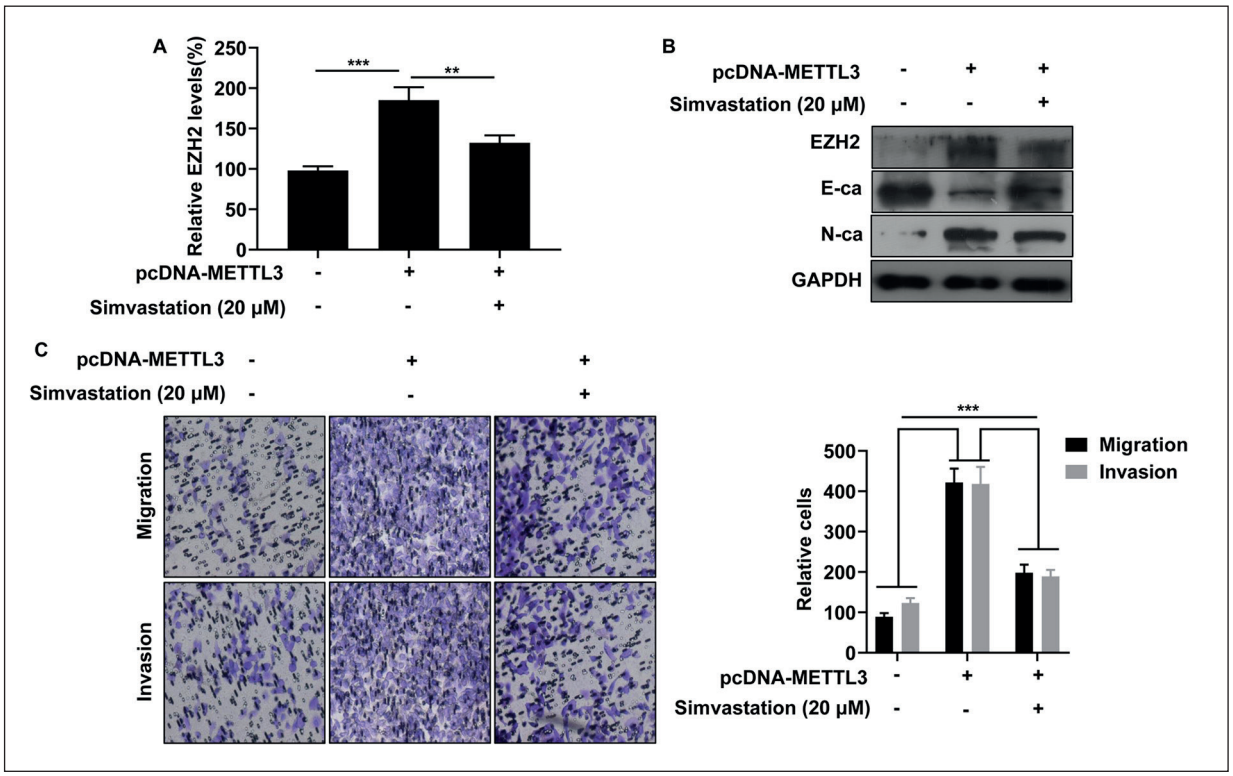

Figure 4. Simvastatin regulated migration and invasion of A549 cells through METTL3-mediated m6A modification of EZH2. **A,** EZH2 level in A549 cells induced with 20 μM Simvastatin or not for 24 h and transfected with pcDNA-METTL3. **B,** Protein levels of E-cadherin and N-cadherin in A549 cells induced with 20 μM Simvastatin or not for 24 h and transfected with pcDNA-METTL3. **C,** Migration and invasion in A549 cells induced with 20 μM Simvastatin or not for 24 h and transfected with pcDNA-METTL3 (magnification: 40×).

EMT is a critical event involved in metastasis of tumor cells. During the process of EMT, typical characteristics of epithelial cells, including cell polarity and cell-cell adhesion, are lost. Nevertheless, cells acquire the features of invasiveness and migration as mesenchymal stem cells[32, 33]. JUNB is an important transcriptional factor of EMT. The m6A-modified mRNA level and mRNA stability of JUNB are markedly reduced by silence of METTL3[13]. We found out that METTL3 was capable of regulating protein levels of EMT-associated genes, indicating the regulatory effect of METTL3 on EMT in lung cancer.

Simvastatin is the first-line drug for hypercholesterolemia treatment. It selectively inhibits the rate-limiting enzyme of the HMG-CoA reductase pathway and thus blocks the synthesis of mevalonic acid. Recent studies demonstrated the inhibitory effect of Simvastatin on tumor cell proliferation[34]. Through inhibiting p38 activity and p-p38 level, Simvastatin weakens proliferative ability in Lewis cells. In addition, their invasive and migratory potentials are suppressed owing to the effects of Simvastatin on downregulating RhoA and MMP-2. Consistently, our results identified that Simvastatin abolished the regulatory effects of METTL3 on EZH2 level, EMT-associated gene expressions and metastatic abilities in A549 cells. Collectively, Simvastatin was responsible for alleviating lung cancer progression by triggering METTL3-induced m6A modification on EZH2 mRNA, thus regulating cancer cell metastasis.

Conclusions

We showed that simvastatin induces METTL3 downregulation in lung cancer tissues, which further influences EMT *via* m6A modification on EZH2 mRNA and thus inhibites the malignant progression of lung cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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