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METTL3 promotes the progression of nasopharyngeal carcinoma through mediating M^{6}A modification of EZH2

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Abstract. – OBJECTIVE: The aim of this study was to investigate whether METTL3 promoted the progression of nasopharyngeal carcinoma (NPC) by silencing CDKN1C through EZH2.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to examine the expression level of METTL3 in 48 pairs of NPC tissues and adjacent normal tissues. METTL3 expression in patients with different tumor lymph node metastasis (TNM) stages was detected by qRT-PCR as well. The Kaplan-Meier method was used to analyze the interplay between METTL3 expression and the prognosis of patients with NPC. At the same time, METTL3 expression in normal epithelial cell line (BEAS-2B) and NPC cell lines (SUNE-1 and C666-1) was examined using qRT-PCR. After METTL3 was knocked down in SUNE-1 cells, cell viability and migration abilities were analyzed by cell counting kit-8 (CCK8) test and wound healing assay, respectively. The mRNA and protein expression of EZH2 were detected by qRT-PCR and Western blot, respectively. RNA immunoprecipitation (RIP) assay was applied to detect the binding of METTL3 to EZH2 mRNA and the m6A modification on EZH2 mRNA. After knockdown of EZH2 in SUNE-1 cells, qRT-PCR was used to detect the mRNA expression of CDKN1C. Meanwhile, chromatin immunoprecipitation (ChIP) assay was conducted to analyze the binding of EZH2 to the CDKN1C promoter region. After down-regulation of METTL3 in SUNE-1 cells, the protein expressions of EZH2 and CDKN1C were detected using Western blot. After simultaneous knockdown of METTL3 and CDKN1C in SUNE-1 cells, CCK8 assay and wound healing assay were applied to examine cell viability and migration abilities.

RESULTS: METTL3 expression in NPC tissues was remarkably higher than that of adjacent normal tissues. Meanwhile, METTL3 expression in T3 and T4 tumors was significantly higher than that of T1 and T2 tumors. In patients with lymph node metastasis, the expression of METTL3 was remarkably higher than those without metastasis. Survival analysis demonstrated that patients with higher expression of METTL3 exhibited significantly longer overall survival time than those with lower METTL3 expression. QRT-PCR revealed that METTL3 was highly expressed in NPC cell lines, including SUNE-1 and C666-1. After knock-down of METTL3 in SUNE-1 cells, cell viability and migration abilities were both markedly weakened. Meanwhile, the protein expression of EZH2 was remarkably reduced. However, no significant changes were observed in mRNA level. RIP assay revealed that METTL3 could bind to EZH2 mRNA, and a m6A modification was verified on EZH2 mRNA. After knockdown of EZH2, the mRNA level of CDKN1C in SUNE-1 cells was significantly up-regulated. CHIP assay indicated that EZH2 could bind to CDKN1C. Western blot showed that, after interfering with METTL3 in SUNE-1 cells, the protein expression of EZH2 decreased significantly, while CDKN1C was up-regulated. In addition, simultaneous downregulation of METTL3 and CDKN1C in SUNE-1 cells reversed the influence of METTL3 on cell viability and migration abilities.

CONCLUSIONS: METTL3 was highly expressed in NPC tissues, which might inhibit EZH2 expression by mediating M6A modification of EZH2 mRNA. Furthermore, CDKN1C could increase the malignancy of NPC cells and promote the progression of NPC.

Key Words:
M^{6}A, METTL3, EZH2, CDKN1C, Nasopharyngeal carcinoma (NPC).

Introduction

Nasopharyngeal carcinoma (NPC) is a rare disease in the United States, with an incidence of 0.2-0.5 cases per 100,000 people. However, the incidence of NPC is relatively higher in sev-

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eral defined groups, including southern China and Hong Kong, with an incidence of 25-50 per 100,000 people[1]. Although chemotherapy and radiotherapy can remarkably improve the survival of NPC patients, metastasis or recurrence still occurs in about 30% of these patients. Once metastasis develops, the prognosis of NPC patients is extremely poor[2-4]. Currently, great progress has been made in molecular targeting methods for NPC therapy. However, the prognosis of NPC patients has not achieved the expected improvement[5]. Therefore, exploring the pathogenesis of NPC can contribute to improve the survival rate and treatment strategies of NPC patients.

N^6-methyl adenosine (m^6A) is the most common and reversible internal modification in mammalian messenger and non-coding RNAs. m^6A accounts for more than 80% of total RNA base methylation, which has also been observed in many species[6-9]. Deletion of m^6A results in increased half-life of mRNA of transcription factors, such as NANOG[9,10]. This may block the ability of pluripotent cells to progress through differentiation. Indeed, multiple researches[11,12] have suggested that m^6A level seriously affects the develop[illegible] of tumors.

Cell division cycle is directly controll[illegible]y positive regulators [cyclin and cyclin deper[illegible] kinase (Cdks) and negative regulators (cyc[illegible] dependent kinase inhibitors, C[illegible]3,14]. Cycl[illegible] dependent kinase inhibitors [illegible]re large protein families that can r[illegible]ate cel[illegible]cle progression, cell proliferati[illegible]d diff[illegible]15]. Meanwhile, they hav[illegible]een [illegible] be c[illegible]sively involved in [illegible]or inh[illegible]n and to be dysregulated in [illegible] types of [illegible]n cancer through genet[illegible]enetic chan[illegible],17]. CDKN1C (encod[illegible]g tumo[illegible]pressor p57 KIP2) is a cyclin d[illegible]ndent kinase [illegible]K) inhibitor[18] that is inact[illegible]d by promoter D[illegible]methylation in a varie[illegible]l huma[illegible]ancers[19,20].

[illegible] stud[illegible]PC cells were selected to investigate [illegible] METT[illegible]could regulate the ex[illegible]sion [illegible]ZH2 [illegible]n by mediating the m6A [illegible]cation [illegible] mRNA, thereby inhibiting CD[illegible]1C, imp[illegible]ing the malignancy of NPC ce[illegible]promoting the development of NPC.

Patients and Methods

Patients and Clinical Samples

From December 2017 to October 2018 in Linyi Cancer Hospital, 48 NPC patients who received surgical resection were enrolled in this study. NPC tissue samples and adjacent normal tissues were collected and rapidly frozen in liquid nitrogen until use. All adjacent [illegible]al tissue samples were separated at least [illegible] the tumor boundary. No patient [illegible]ved any radiotherapy or chemotherapy b[illegible]operation. This investigation was approved [illegible]Ethics Committee of Linyi Cancer [illegible]spital[illegible]rmed consent was obtained fr[illegible]patients a[illegible] families before the stud[illegible]

Cell Culture

BEAS-2B, S[illegible]-1, a[illegible]666-1[illegible] lines were purchas[illegible]om the S[illegible]ell Bank of the Chin[illegible]emy of Sc[illegible] (Shanghai, China). A[illegible]ells [illegible]cultured in Roswell Park Memorial Institute-[illegible]RPMI-1640; HyClone, Sou[illegible], UT, USA)[illegible]ium containing 10% fe[illegible]ovine serum (FBS; HyClone, South Logan, [illegible] USA) and 1[illegible] streptomycin and penicillin [illegible] 37°C, 5% C[illegible]incubator. Cells in the expon[illegible]l growth [illegible]e were digested and seeded into [illegible]pri[illegible]ll dishes. Then, the cells were culture[illegible]ncubator until 60% of cell density.

[illegible]ransfection

C[illegible] were first plated into 6-well plates (Corning, Corning, NY, USA) and grown to a cell density of 60%. Transfection of si-METTL3, si-EZH2, si-CDKN1C, and si-NC was performed according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h, the cells were collected for quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), Western Blot, and cell function experiments.

RNA Extraction and QRT-PCR

Total RNA in cells and tissues was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) reagent. Subsequently, RNA concentration was measured by an ultraviolet spectrophotometer. Extracted RNA samples were stored at –80°C until use. The complementary deoxyribonucleic acids (cDNAs) were obtained by reverse transcription, and the SYBR Green method was used for PCR detection. Primer sequences used in this study were shown in Table I.

Western Blot

Total protein in tissues and cells was extracted from each group. 50 μg of protein was separated by dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE)

Table I. The Primer sequences of gene.

Gene	Primer sequence
EZH2	F: 5'-TGCACATCCTGACTTCTGTG-3' R: 5'-AAGGGCATTCACCAACTCC-3'
CDNK1C	F: 5'-GCGGCGATCAAGAAGCTGT-3' R: 5'-GCTTGGCGAAGAAATCGGAGA-3'
METTL3	F: 5'-AGATGGGGTAGAAAGCCTCCT-3' R: 5'-TGGTCAGCATAGGTTACAAGAGT-3'
GAPDH	F: 5'-CGGAGTCAACGGATTTGGTCGT-3' R: 5'-GGGAAGGATCTGTCTCTGACC-3'

and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk powder for 1 h at room temperature, the membranes were incubated with primary antibodies overnight at 4°C. On the next day, the membranes were rinsed 3 times with Tris-Buffered Saline and Tween-20 (TBST-20) and incubated with the corresponding secondary antibody for 1 h at room temperature. Immuno-reactive bands were finally developed and analyzed with enhanced chemiluminescence (ECL) luminescence kit.

Cell Viability

48 h after transfection, the cells were collected and plated into 96-well plates at 2000 cells per well. After culture for 6 h, 24 h, 48 h, and 96 h, respectively, 10 μL of CCK-8 (Dojindo Laboratories, Kumamoto, Japan) reagent was added to each well, followed by incubation for 1 h in the dark. Optical density (OD) value of each well at the absorption wavelength of 450 nm was measured by a microplate reader. 5 replicates were set in each group.

Wound Healing Assay

Transfected cells after 48 h were digested, centrifuged, and resuspended in FBS-free medium. The density of cells was adjusted to 5 × 10⁵ cells/mL. The density of plated cells was determined according to the size of cells (the majority of the number of cells plated was set to 3000-5000 cells/well). Meanwhile, the confluence of cells reached 90% or more the next day. After stroke, the cells were rinsed gently with phosphate-buffered saline (PBS) for 2-3 times. Next, low-concentration serum medium (such as 1% FBS) was added in, and the cells were observed again after 24 h.

RNA Binding Protein Immunoprecipitation (RIP) Assay

RIP assay was performed according to the manufacturer's instructions of Magna RIP RNA Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA). After cell lysates were obtained, the magnetic beads were prepared, re-suspended in Wash Buffer, and placed on ice. RNA-binding protein immunoprecipitation was then performed. RNA purification was carried out by phenol, chloroform, Proteinase K Solution II, Precipitate RNA Binder, absolute ethanol (no RNAse), dissolved in 10-20 μL of diethyl pyrocarbonate (DEPC) water and stored at -80°C. Finally, the expression of METTL3 mRNA and m6A modification and EZH2 in the RNA protein precipitates were detected by qRT-PCR.

Chromatin Immunoprecipitation (CHIP)

Chromatin immunoprecipitation was performed in accordance with Magna ChIP A/G One-Color Chromatin Immunoprecipitation Kit (Millipore, Bedford, MA, USA). Chromatin immunoprecipitated DNA was eluted, reverse X-linked, purified, and qRT-PCR analyzed.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 16.0 (SPSS, Chicago, IL, USA) statistical software was used for all statistical analysis. Measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Independent sample *t*-test was used to compare quantitative data of two groups. The cumulative survival rate was assessed by the Kaplan-Meier method and the difference was determined by log-rank test. $p<0.05$ was considered statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Results

METTL3 Was Highly Expressed in NPC Tissues and Negatively Correlated With Prognosis of NPC Patients

To explore the interplay between METTL3 expression and the occurrence and the development of NPC, qRT-PCR was first used to detect the expression of METTL3 in NPC tissues and adjacent normal tissues. The results showed that METTL3 expression in NPC tissues was remarkably higher than that of adjacent normal tissues (Figure 1A). After paired analysis of tissue samples, the expression level of METTL3 in T3 and T4 stage tumors was remarkably higher than that

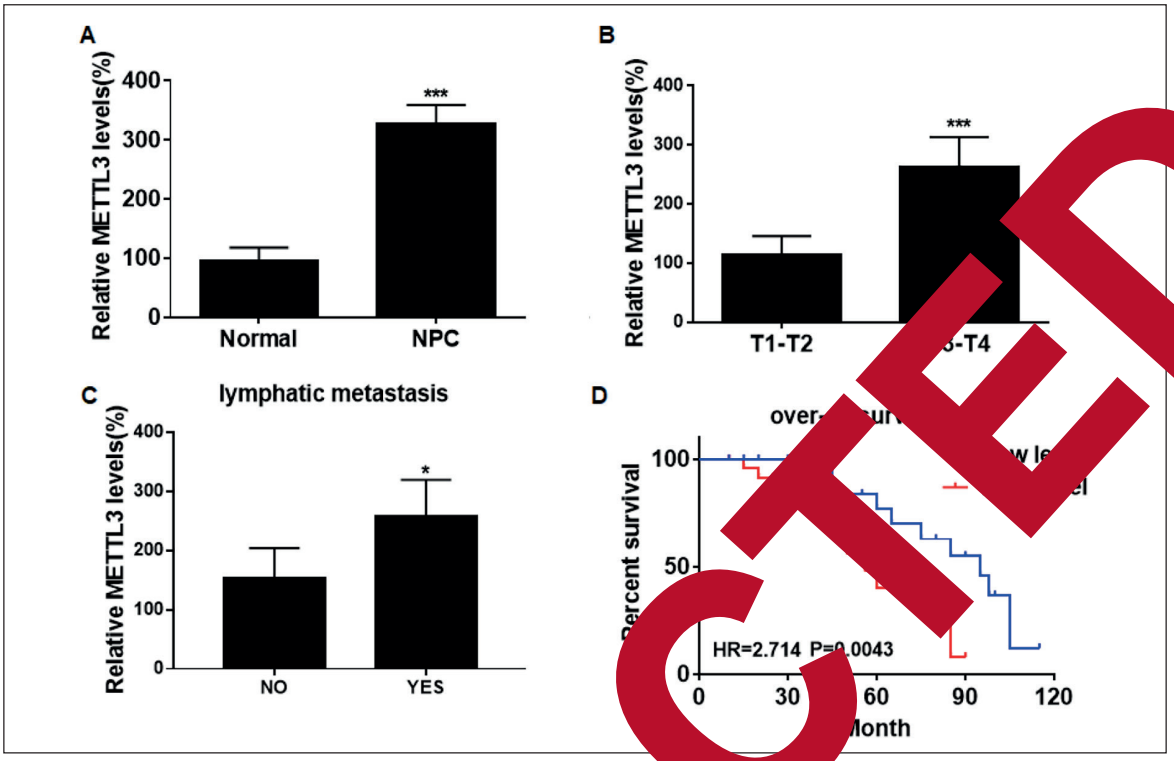

Figure 1. METTL3 was highly expressed in NP[illegible]was negatively [illegible]ated with prognosis of NPC patients. **A,** METTL3 was highly expressed in NPC tissues [illegible]xpression level was significantly higher in T1 and T2 phases than in T3 and T4 phases. **C,** METTL3 ex[illegible]ion w[illegible]ably higher in the metastatic group than the non-transferred group. **D,** Overall survival of patients w[illegible]high[illegible] of METTL3 was significantly lower than that of patients with lower expression.

in T1 and T2 tumors (Fi[illegible]1B). [illegible]nwhile, METTL3 level was rem[illegible]ly hig[illegible] static group than no[illegible]eta[illegible]up ([illegible]e 1C). Kaplan-Meier [illegible]alysis [illegible]ed that patients with highe[illegible]TTL3 expre[illegible]exhibited significantly w[illegible]p[illegible]osis and sho[illegible]survival time when co[illegible]pared [illegible]patients with lower expressio[illegible]gure 1D). T[illegible]ata indicated that METTL[illegible]as highly expres[illegible]in NPC tissues and [illegible]negati[illegible]correlated with the prognosis of p[illegible]ts.

[illegible]ck[illegible] of M[illegible]TL3 Weakened the [illegible]ferati[illegible]Migration Abilities of NP[illegible]Cells

[illegible]rve the role of METTL3 in the de-[illegible]pm[illegible]NPC, we detected the expression [illegible]METTL3 in normal epithelial cell line (BE-[illegible]2b) and NPC cell lines (SUNE-1 and C666-1) [illegible]qRT-PCR as well. The results showed that METTL3 was highly expressed in NPC cells (Figure 2A). Next, we knocked down METTL3 expression in SUNE-1 cells (Figure 2B). CCK-8 and wound healing assay results revealed that cell viability and migration abilities remarkably decreased (Figures 2C, 2D). The above findings suggested that downregulation of METTL3 could inhibit the viability and migration abilities of SUNE-1 cells.

METTL3 Mediated the Regulation of EZH2 Expression by M^{6}A

To further explore the mechanism of METTL3 in NPC development, we knocked down METTL3 expression in SUNE-1 cells. Subsequent Western blot findings showed that the protein expression of EZH2 was remarkably reduced. However, qRT-PCR showed no significant changes in the mRNA expression level of EZH2 after interference with METTL3 (Figures 3A and 3D). Further RIP assay revealed that METTL3 could bind to EZH2 mRNA in SUNE-1 cells (Figure 3B). Meanwhile, RIP data indicated that EZH2 mRNA in SUNE-1 cells was modified with m^{6}A (Figure 3C). The above results indicated that METTL3 could affect the protein expression of

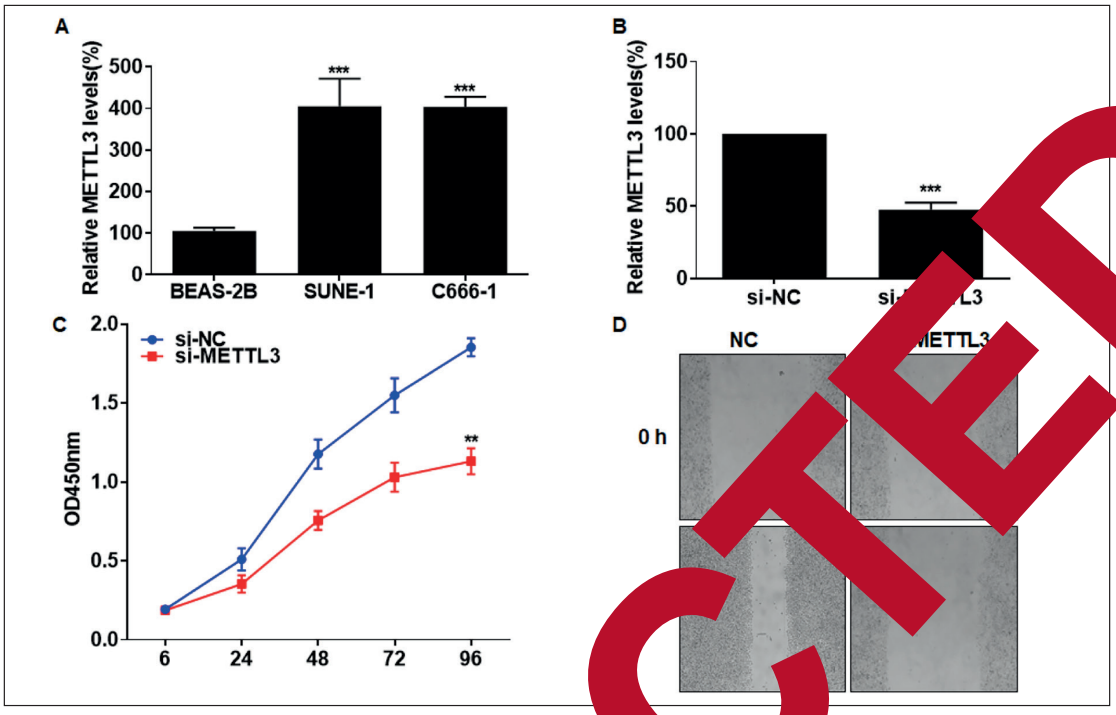

Figure 2 Interference with METTL3 blocked the proliferation a ration o C cells. **A,** METTL3 expression in normal epithelial cell (BEAS-2B) and NPC cell lines (SUNE-1 and C struction of the interference sequence to knockdown the expression level of METTL3. **C** experimental re howed that the viability of SUNE-1 cells decreased after interfering with METTL3. **D,** W say showed that the migration ability of SUNE-1 cells decreased after knockdown of METTL3 (magnificat 40 ×).

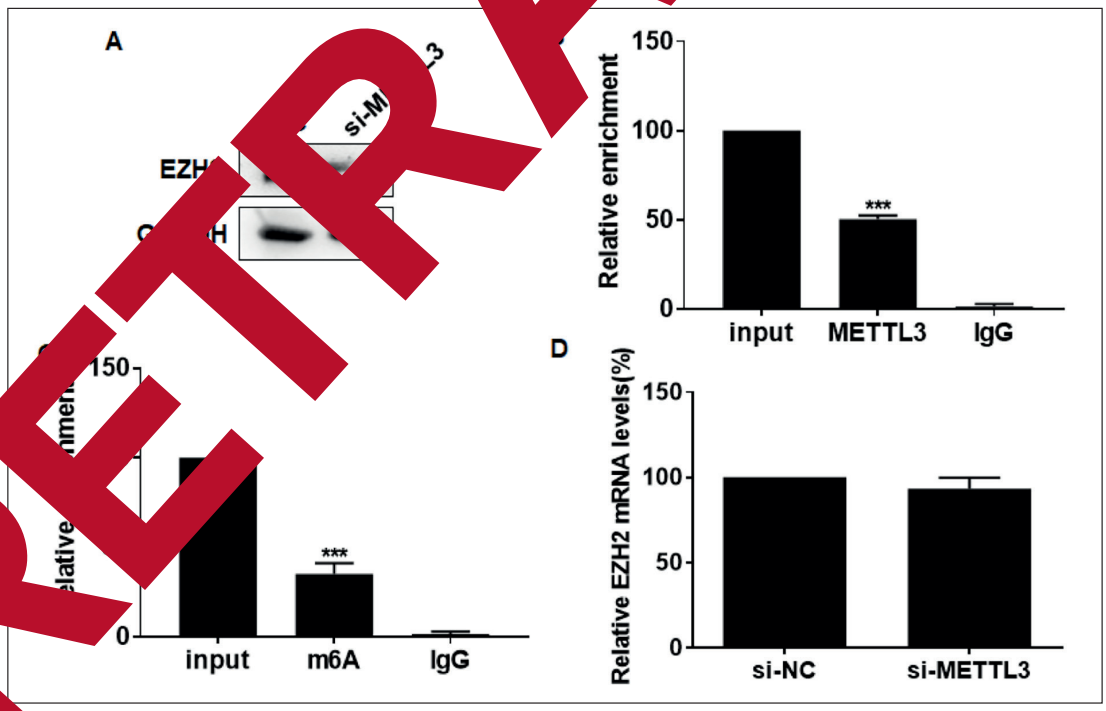

Figure 3. METTL3 mediated m6A regulation of EZH2 expression. **A,** The protein expression level of EZH2 decreased after knockdown of METTL3 in SUNE-1 cells. **B,** RIP experimental results indicated that METTL3 could bind to EZH2 mRNA. **C,** RIP results indicated that m^{6}A modification occurred on EZH2 mRNA. **D,** After knockdown of METTL3 in SUNE-1 cells, the mRNA expression level of EZH2 was not significantly changed.

EZH2 in NPC cells by mediating the m^{6}A modification of EZH2 mRNA.

EZH2 Inhibited the Expression of CDKN1C in SUNE-1 Cells

To observe the roles of METTL3 and EZH2 in tumor progression, we constructed and transfected interference sequences to knock down EZH2 in SUNE-1 cells (Figure 4A). After transfection, CDKN1C mRNA expression was found significantly up-regulated (Figure 4B). Subsequently, CHIP assay suggested that EZH2 could bind to the CDKN1C promoter region to inhibit the expression of CDKN1C (Figure 4C). In addition, Western blot data demonstrated that the protein expressions of EZH2 and CDKN1C in SUNE-1 cells decreased significantly after knockdown of METTL3 (Figure 4D). The above results suggested that METTL3 might regulate the expression of CDKN1C *via* regulating EZH2.

METTL3 Mediated EZH2 Inhibition of CDKN1C and Promoted the Malignancy of NPC

To investigate whether the inhibition of [illegible] KN1 through EZH2 mediated by METTL[illegible] promote NPC progression, we knocked [illegible]n METTL3 in SUNE-1 cells. QRT-PCR results indicated that CDKN1C expression was remarkably up-regulated. After simultaneous downregulation of CDKN1C and METTL3 (Figu[illegible]5A), CCK-8 data demonstrated that interfe[illegible] CDKN1C could reverse decreased [illegible] activity caused by inhibition of METTL[illegible]igure 5B). Similarly, the results of wound [illegible]g assay showed that interference wit[illegible]DKN[illegible]rsed the decreased migration a[illegible]y of SUNE[illegible] caused by knockdown [illegible]ETTL[illegible](Figure [illegible]). The above findings r[illegible]ed tha[illegible]TTL3 could mediate EZH2 inhibit[illegible]f [illegible]KN1C, thereby improving the [illegible]ity a[illegible]igratio[illegible] NPC cells.

Di[illegible]sion

[illegible]A post-transcriptiona[illegible] modification, the ef[illegible] of m^{6}A modi[illegible]cation on transcription, splic[illegible] mRNA stab[illegible], and translation rate, regula[illegible]he basic ch[illegible]cteristics of cells. Meanwhile, the [illegible]f these pathways is associated with [illegible]human diseases[21,22]. However, the molecular mechanism of METTL3 in the devel[illegible] of NPC has not been fully elucidated.

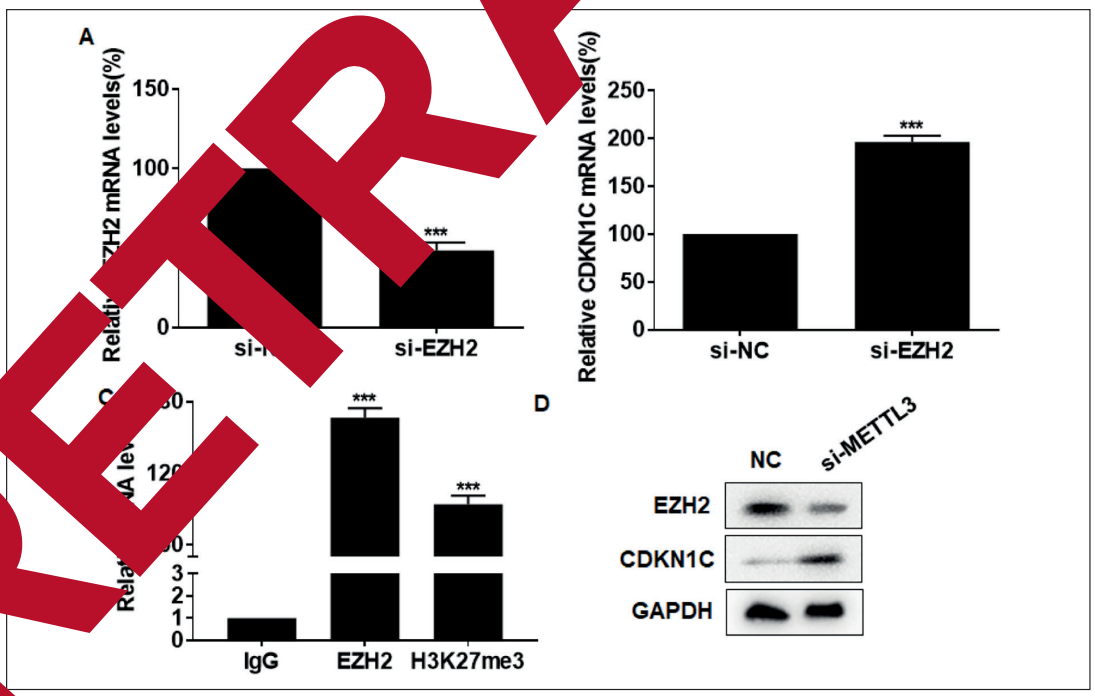

Figure 4. EZH2 inhibited CDKN1C expression in SUNE-1 cells. **A,** The interference sequence was constructed in SUNE-1 cells to inhibite the expression level of EZH2. **B,** QRT-PCR showed that the mRNA expression level of CDKN1C in SUNE-1 cells increased after interference with EZH2. **C,** CHIP assay showed that EZH2 could bind to CDKN1C. **D,** Western blot showed that the protein expression levels of EZH2 and CDKN1C decreased after METTL3 downregulation.

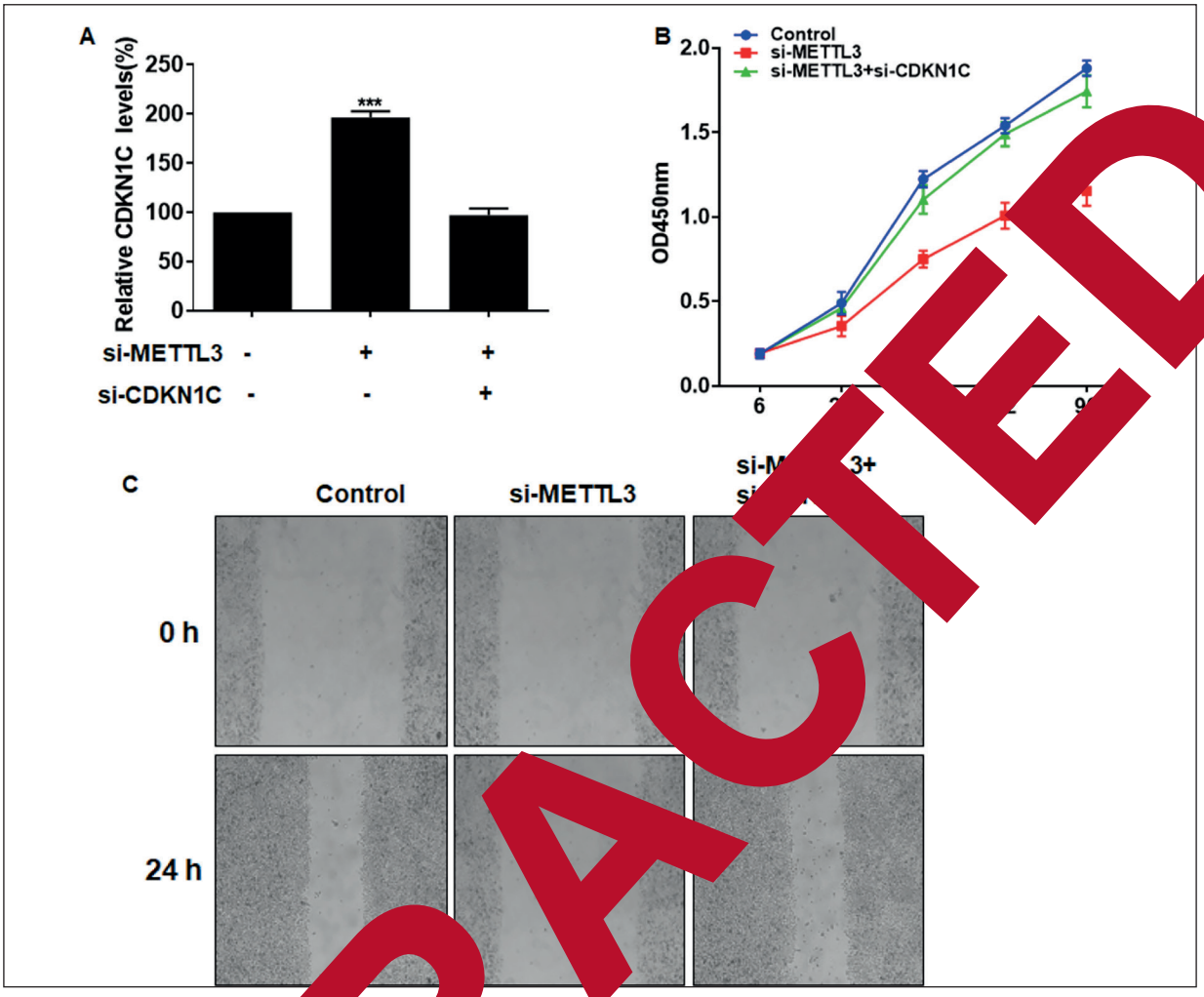

Figure 5. METTL3-mediated [illegible]2 in[illegible]N1C promoted malignant progression of NPC. **A,** Interfering sequences were transfect[illegible]SU[illegible]to knockdown the expression level of CDKN1C. **B,** CCK8 assay showed that knockdown of CDKN1[illegible]uld rever[illegible]decrease in the activity of SUNE-1 cells caused by knockdown of METTL3. **C,** Wound healing assa[illegible]ed that knoc[illegible]of CDKN1C could reverse the decreased migration ability of SUNE-1 cells induced by downr[illegible]METTL3 (m[illegible]ation: 40×).

Our st[illegible]emonstrated that [illegible]TTL3 was highly ex[illegible]sed in[illegible]C tissues. The expression of M[illegible]3 in[illegible]nced NPC tissues was remarkably l[illegible]n that[illegible]arly ones. Meanwhile, [illegible]ival [illegible]is re[illegible]ed that the overall survival[illegible]patien[illegible]igher METTL3 expression wa[illegible]markabl[illegible]wer than that of patients with lo[illegible]ETTL3 expression. These findings suggested that METTL3 might play a vital role in the process of NPC.

In eukaryotes, m^{6}A modification mediated by METTL3 and METTL14 is necessary for normal cell activity, and their disorder may cause a variety of diseases[10]. Current studies on the crystal structure of the heterodimer show that only METTL3 exerts catalytic activity. However, METTL14 stabilizes METTL3 and its interaction with RNA molecules[23,24]. This work revealed that METTL3 could affect EZH2 expression by mediating the m6A modification of EZH2 mRNA. After knockdown of METTL3 in NPC cells, the protein expression level of EZH2 decreased. Furthermore, the viability and migration abilities of NPC cells were significantly reduced.

EZH2 is an important component of the PRC2 complex, which catalyzes the methylation silencing of target gene lysine 27 of histone 3[25-27]. In this study, we found that EZH2 could bind to the CDKN1C promoter region to silence the expression of CDKN1C. When METTL3 was down-

regulated in SUNE-1 cells, CDKN1C expression was also inhibited. Meanwhile, knockdown of CDKN1C could reverse the decreased viability and migration ability of SUNE-1 cells caused by interference with METTL3.

Conclusions

In summary, this report shows that METTL3 was highly expressed in NPC tissues and affected the survival of patients with NPC. *In vitro* experiments indicated that METTL3 could promote the protein expression of EZH2 by mediating the m6A modification of EZH2 mRNA. In addition, EZH2 could increase the malignancy of NPC cells by silencing CDKN1C, thereby promoting the development of NPC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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