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ORIGINAL ARTICLE

# METTL3 participates in glioma development by regulating the methylation level of COL4A1

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## Summary

***Purpose:*** *The role of RNA methylation in human cancers has emerged. Its biological function in glioma development is explored in the present study.*

***Methods:*** *Differential levels and prognostic potentials of COL4A1 and METTL3 in glioma were analyzed by bioinformatic method. The regulatory effect of METTL3 on COL4A1 was assessed through qRT-PCR, MeRIP and dual-luciferase reporter assay. Their biological functions in influencing proliferative and metastatic capacities of glioma cells were examined by EdU, colony formation and Transwell assay, respectively.*

***Results:*** *COL4A1 was upregulated in glioma tissues, and METTL3 was downregulated. Knockdown of METTL3 in U87 and U251 cells could reduce the methylation level of COL4A1 and upregulate its expression level. Intervention of COL4A1 suppressed proliferative and metastatic capacities of glioma cells, while intervention of METTL3 yielded the opposite results.*

***Conclusions:*** *METTL3 reduces the methylation level of COL4A1 and upregulates its expression level, which further stimulates the malignant development of glioma. METTL3/COL4A1 can be potential therapeutic targets of glioma.*

***Key words:*** *glioma, COL4A1, METTL3, RNA methylation*

## Introduction

Glioma is a primary malignant tumor in the brain, which is characterized by poor prognosis and high recurrent rate [1,2]. According to the origins, glioma is classified to medulloblastoma, astrocytoma, ependymoma, oligodendroglioma and glioblastoma [3,4]. Therapeutic strategies of glioma have been gradually progressed. However, the survival of glioma, especially stage IV glioma is extremely poor owing to the invasive growth and strong proliferation of glioma cells [5-8]. Exploring molecular mechanism of glioma is of great significance to improve its clinical prognosis.

RNA methylation is a typical RNA modification, accounting for more than 60% [9]. In particular, m6A modification is extensively distributed in more than 7000 mRNAs and 300 noncoding RNAs, which is the most popular and abundant RNA modification. The function of m6A modification is determined by the writer, reader and eraser [10]. Methyltransferases METTL3, METTL14 and WTAP are the writer, which are responsible for writing methylation information into RNAs. The reader consists of the YTH domain protein and HNRNP family. It reads methylation modification information and participates in the translation and degradation of downstream RNAs. Demethylases ALKBH5 and FTO, as the eraser, reverse or eliminate the process of RNA modifications [11]. m6A

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modification participates in every step of RNA metabolism, including the stability, translation efficacy, alternative splicing and positioning. Abnormal m6A modification may lead to carcinogenesis, neurological diseases, metabolic diseases and growth retardation [12-14].

Through online prediction, several m6A modification sites were identified in COL4A1. Moreover, COL4A1 was upregulated in glioma. We speculated that COL4A1 may be involved in the malignant development of glioma.

## Methods

*Online data processing*

A GEPIA dataset containing 163 glioma tissues and 207 normal ones was downloaded from http://gepiacancer-pku.cn/index.html. Prognostic potentials of COL4A1 and METTL3 in overall survival and disease-free survival of glioma patients were assessed.

*Clinical tissues of glioma*

Fifty glioma and normal control tissues were collected. None of recruited patients had preoperative chemotherapy or radiotherapy. Tumor grade of glioma was defined based on UICC guidelines. This study was approved by the research ethics committee of Shenzhen University General Hospital and complied with the Helsinki Declaration. Informed consent was obtained from patients.

*Cell culture and transfection*

Human astrocytes (NHA) and glioma cell lines (U251, U87, LN229 and T98G) were obtained from Shanghai Institutes for Biological Sciences (Shanghai, China). Cells were cultivated in dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C, 5% $CO_2$. Transfection plasmids were synthesized by RiboBio (Guangzhou, China). Cells were seeded in a 6-well plate ($3\times10^4$ cells/ml) and cultured to 60% density. After 48-h transfection using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), cells were collected for verifying transfection efficacy and functional experiments.

*q real-time polymerase chain reaction (qRT-PCR)*

Tissue or cell samples were processed by TRIzol (Invitrogen, Carlsbad, CA, USA) for isolating RNAs. After purification, qualified RNAs were reversely transcribed to complementary deoxyribose nucleic acids (cDNAs) and subjected to qRT-PCR using SYBR®Premix Ex Taq™(TaKaRa, Tokyo, Japan). Relative levels of PCR products were calculated by $2^{-\Delta\Delta Ct}$ and normalized to that of glyceraldheyde 3-phosphate dehydrogenase (GAPDH).

Primer sequences (5'→3') were as follows. COL4A1 forward: GGACTACCTGGAACAAAAGGG, COL4A1 reverse: GCCAAGTATCTCACCTGGATCA; METTL3 forward: TTGTCTCCAACCTTCCGTAGT, METTL3 reverse: CCAGATCAGAGAGGTGGTGTAG; GAPDH forward: GGAGCGAGATCCCTCCAAAAT, GAPDH reverse: GGCTGTTGTCATACTTCTCATGG.

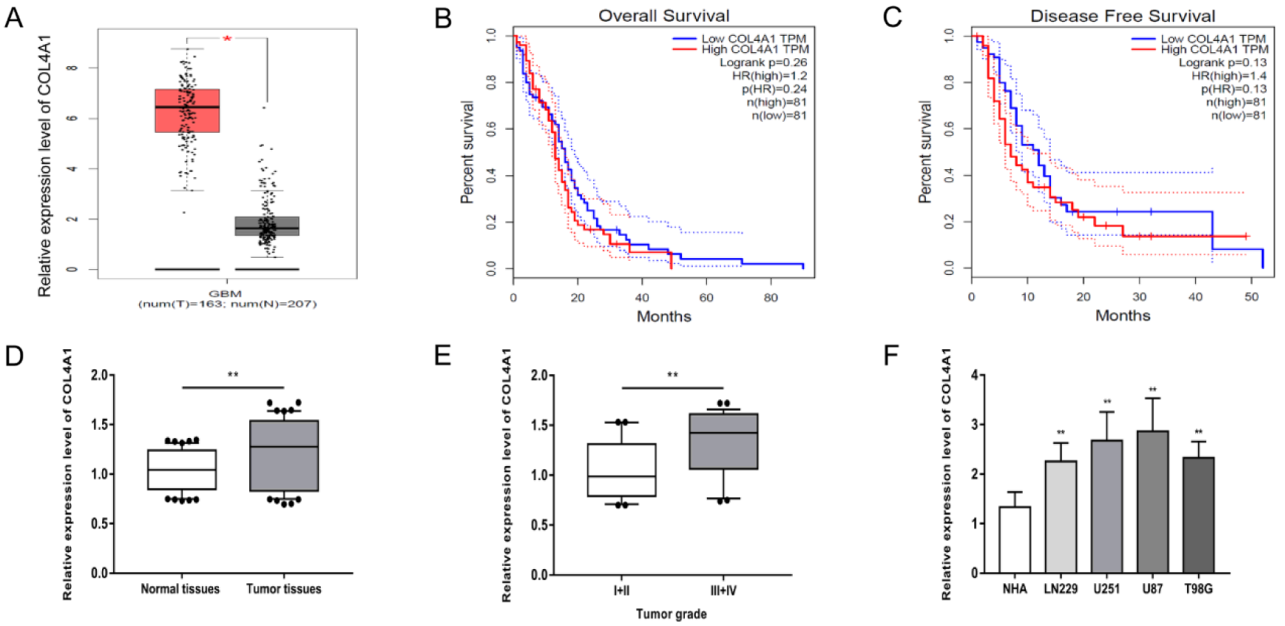


**Figure 1.** COL4A1 was upregulated in glioma. **A:** Analysis of the GEPIA database revealed that COL4A1 was upregulated in glioma tissues; **B:** Analysis of the TCGA database revealed that COL4A1 was unrelated to overall survival of glioma patients (Log-rank p=0.26); **C:** Analysis of the TCGA database revealed that COL4A1 was unrelated to disease-free survival of glioma patients (Log-rank p=0.13); **D:** qRT-PCR data showed that COL4A1 was upregulated in glioma tissues; **E:** COL4A1 was correlated to tumor grade of glioma; **F:** qRT-PCR data showed that COL4A1 was upregulated in glioma cell lines.

*5-Ethynyl-2'- deoxyuridine (EdU) assay*

Cells were seeded in a 96-well plate ($3\times10^3$cells/well), and induced with 50 μM EdU (RiboBio, Guangzhou, China) for 2 h, and dyed using AdoLo and DAPI in the dark. EdU-labeled cells were captured for calculating EdU-stained cell ratio.

*Colony formation assay*

Cells seeded in 12-well plates were cultured for 2 weeks. Visible colonies were stained with crystal violet and captured for counting.

*Transwell assay*

$4\times10^4$ cells suspended in 100 μL of serum-free DMEM were applied on the top of a Transwell chamber (8 μm), which was placed in 6-well plates containing 600 μL of medium and 10% FBS per well. Cells were allowed to migrate for 48 h, and they were fixed, dyed and captured for counting in five random fields per sample. Invasion assay was conducted using a Transwell chamber pre-coated with 50 μg Matrigel on the bottom.

*MeRIP*

Total cellular RNA was collected using TRIzol and incubated with DNase R (Qiagen, Hilden, Germany). RNA was immunoprecipitated using anti-m6A antibody (Abcam, Cambridge, MA, USA), followed by washing in IP buffer for three times. Subsequently, RNA was eluted from beads in elution buffer at 4°C for 1 h, centrifuged and subjected to qRT-PCR.

*Dual-luciferase reporter assay*

Wild-type and mutant-type COL4A1 vectors were co-transfected in cells with si-METTL3 or si-NC, respectively for 48 h. Luciferase activity was measured three times and the average value was calculated.

*Statistics*

GraphPad Prism 6 (La Jolla, CA, USA) was used for statistical analyses. Differences between groups were compared by the *t*-test. Survival analysis was conducted by Kaplan-Meier method. Correlation between expression levels of two genes was assessed by Pearson correlation test. P<0.05 was considered as statistically significant.

## Results

*COL4A1 was upregulated in glioma*

In a GEPIA dataset containing 163 glioma tissues and 207 normal ones, COL4A1 was analyzed to be upregulated in the former group (Figure 1A). COL4A1 level was unrelated to overall survival and disease-free survival of glioma patients (Figure 1B, 1C). QRT-PCR was conducted to validate the expression pattern of COL4A1 in clinical tissues of glioma, which was upregulated as well (Figure 1D). Classified by tumor grade, higher level of COL4A1 was detected in grade III+IV glioma cases in comparison to grade I+II ones (Figure 1E). COL4A1 was identically upregulated in glioma cell lines (Figure 1F).

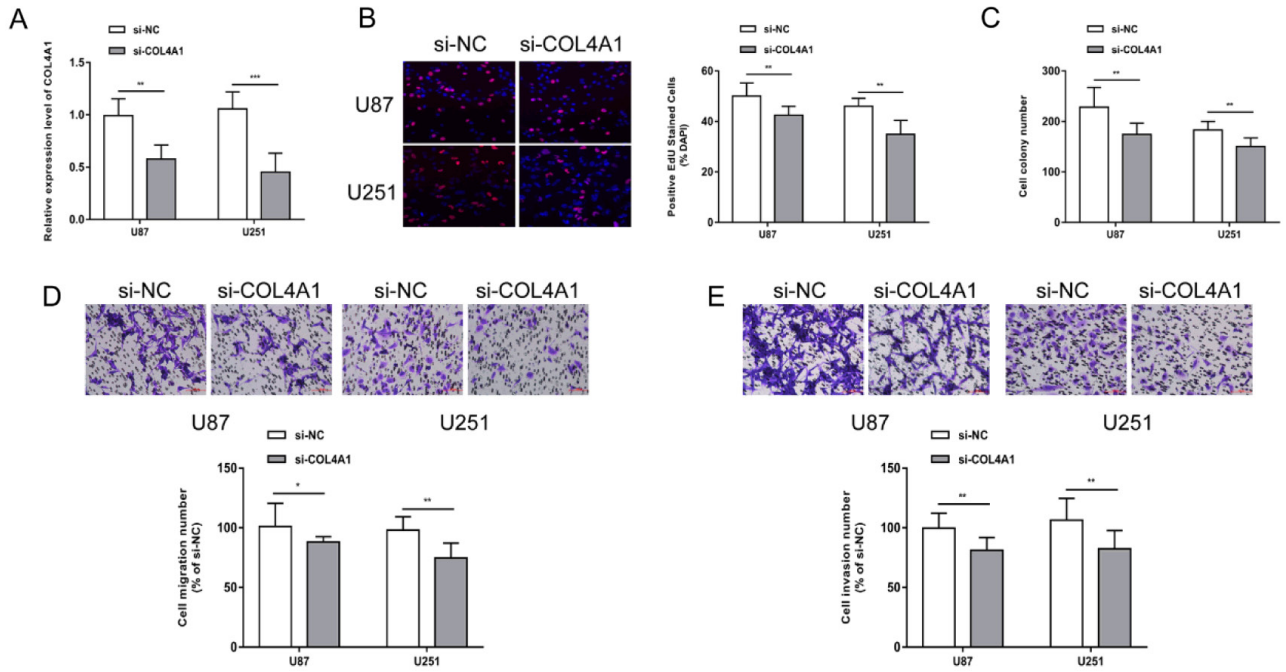


**Figure 2.** Knockdown of COL4A1 suppressed proliferative and metastatic capacities of glioma. **A:** qRT-PCR data showed that transfection of si-COL4A1 significantly downregulated COL4A1 in U87 and U251 cells; **B:** EdU assay showed that knockdown of COL4A1 inhibited proliferation of glioma cells (magnification: 200×); **C:** Colony formation assay showed that knockdown of COL4A1 inhibited proliferation of glioma cells; **D,E:** Transwell assay showed that knockdown of COL4A1 inhibited migration and invasion of glioma cells (magnification: 200×); *p<0.05; **p<0.01.

| m6A | m$^6$A source | Species | Gene | Variant(s) | Database | Transcriptome Regulation |
|---|---|---|---|---|---|---|
| chr8:11241170(-) | MeRIP-Seq (Medium) | Mouse | Col4a1 | rs36592557 | dbSNP146 | RBP 1 Splice 2 |
| chr13:110801541(-) | Prediction (Low) | Human | COL4A1 | rs372541562 | dbSNP147 | RBP 6 miRNA 1 |
| chr13:110801562(-) | Prediction (Low) | Human | COL4A1 | rs372541562 | dbSNP147 | RBP 6 miRNA 1 |
| chr13:110801562(-) | Prediction (Low) | Human | COL4A1 | rs372541562 | dbSNP147 | RBP 6 miRNA 1 |
| chr13:110801562(-) | Prediction (Low) | Human | COL4A1 | rs7273 | dbSNP147 | RBP 6 miRNA 1 |
| chr13:110822926(-) | Prediction (Low) | Human | COL4A1 | rs148801165 | dbSNP147 | RBP 1 Splice 1 |
| chr13:110822979(-) | Prediction (Low) | Human | COL4A1 | rs771875556 | dbSNP147 | RBP 1 Splice 1 |
| chr13:110822979(-) | Prediction (Low) | Human | COL4A1 | rs745478987 | dbSNP147 | RBP 1 Splice 1 |

**Figure 3.** Several m6A methylation sites in COL4A1 (http://m6avar.renlab.org/index.html).

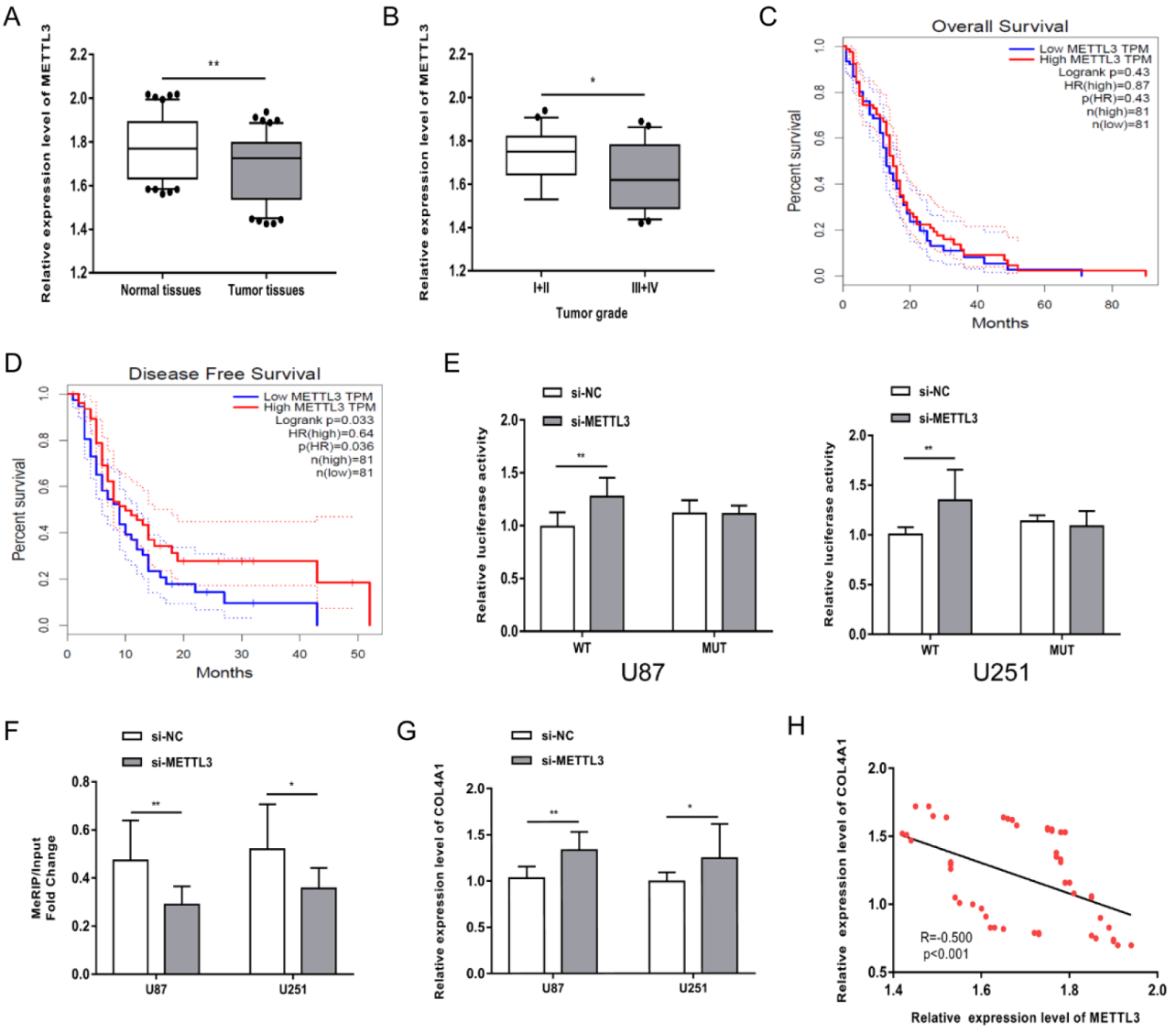


**Figure 4.** Knockdown of METTL3 reduced methylation level of COL4A1. **A:** qRT-PCR data showed that METTL3 was downregulated in glioma tissues; **B:** METTL3 was correlated to tumor grade of glioma; **C:** Analysis of the TCGA database revealed that METTL3 was unrelated to overall survival of glioma patients (Log-rank p=0.43); **D:** Analysis of the TCGA database revealed that METTL3 was correlated to disease-free survival of glioma patients (Log-rank p=0.033); **E:** Dual-luciferase reporter assay confirmed the binding between COL4A1 and METTL3; **F:** MeRIP revealed that knockdown of METTL3 reduced m6A methylation level of COL4A1 mRNA 3'UTR in U87 and U251 cells; **G:** qRT-PCR data showed that COL4A1 was upregulated by knockdown of METTL3; **H:** A linear negative correlation between COL4A1 and METTL3 in glioma tissues (R=-0.500, p<0.001). *p<0.05; **p<0.01.

*Knockdown of COL4A1 suppressed proliferative and metastatic capacities of glioma*

COL4A1 level was intervened in U87 and U251 cells by transfection of si-COL4A1 and the intervention efficacy was verified by qRT-PCR (Figure 2A). EdU assay and colony formation assay obtained the conclusion that knockdown of COL4A1 decreased proliferative rate of glioma cells (Figures 2B,C). Furthermore, Transwell assay uncovered that the migratory and invasive capacities of glioma cells were suppressed by transfection of si-COL4A1 (Figures 2D, E).

*Knockdown of METTL3 reduced methylation level of COL4A1*

Multiple m6A modification sites were predicted in COL4A1 using online tool (http://m6avar.renlab.org/index.html) (Figure 3). We speculated that RNA methylation of COL4A1 may be the reason for its upregulation in glioma tissues. It was found that METTL3 was downregulated in glioma tissues, especially advanced grade cases (Figures 4A,B). Although METTL3 level was unrelated to overall survival of glioma, it was correlated to the disease-free survival (Figures 4C,4D). Furthermore, dual-luciferase reporter assay demonstrated that knockdown of METTL3 could only affect luciferase activity in the wild-type COL4A1 vector, verifying the binding between METTL3 and COL4A1 (Figure 4E). By intervention of METTL3 in U87 and U251 cells, m6A modification level in COL4A1 3'UTR was markedly declined as MeRIP data showed (Figure 4F). Knockdown of METTL3 could upregulate COL4A1 in glioma cells (Figure 4G). A linear negative correlation was discovered between expression levels of METTL3 and COL4A1 in glioma tissues (Figure 4H).

*Knockdown of COL4A1 partially abolished the promotive effects of intervened METTL3 on proliferative and metastatic capacities of glioma*

Co-intervention of COL4A1 and METTL3 was performed in glioma cells. Transfection of si-METTL3 resulted in a higher level of COL4A1 in U87 and U251 cells compared with those co-transfected with si-METTL3 and si-COL4A1 (Figure 5A). Knockdown of METTL3 stimulated growth of glioma cells, which was further inhibited by intervention of COL4A1 (Figure 5B, 5C). In addition, migratory and invasive capacities of glioma cells were enhanced by transfection of si-METTL3, which were then inhibited by co-transfection of si-COL4A1 (Figures 5D, E).

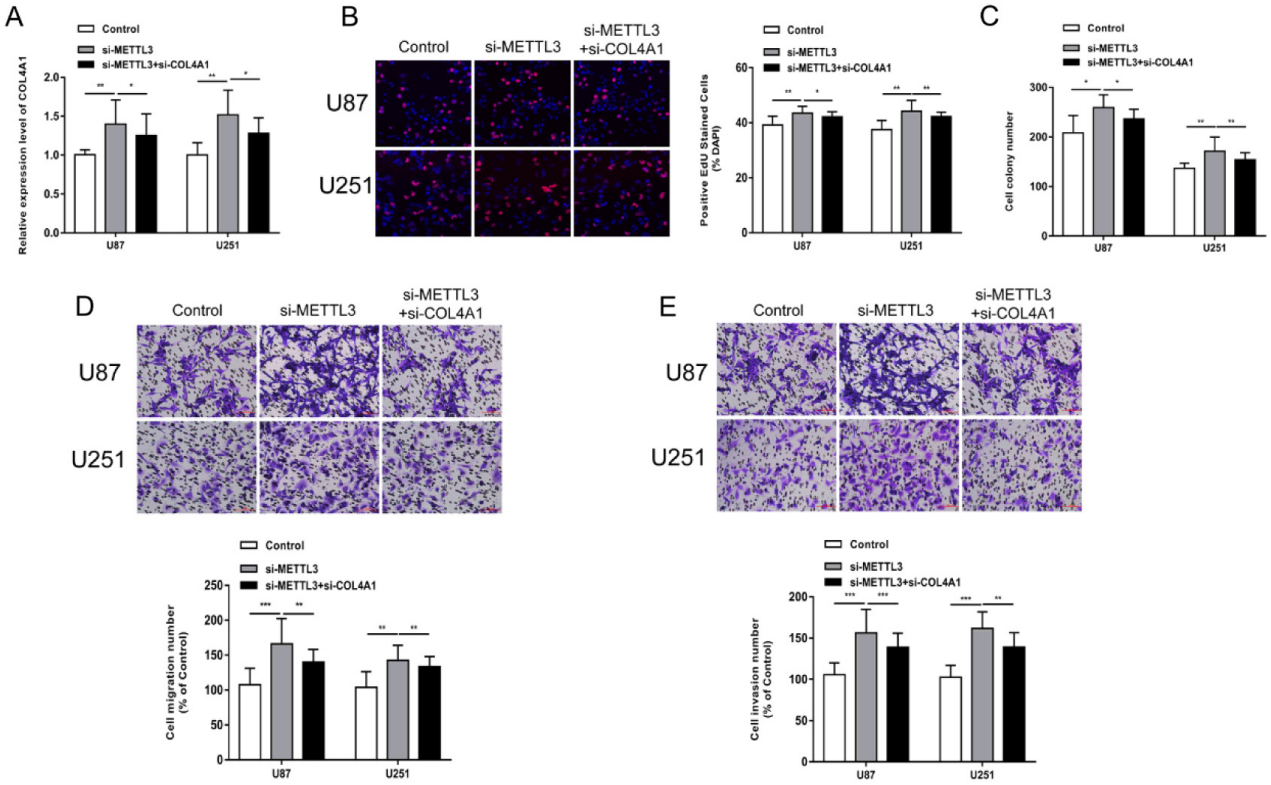


**Figure 5.** Knockdown of COL4A1 partially abolished the promotive effects of intervened METTL3 on proliferative, migratory and invasive capacities of glioma. **A:** COL4A1 level in U87 and U251 cells co-intervened by COL4A1 and METTL3; **B:** EdU-stained cell ratio in U87 and U251 cells co-intervened by COL4A1 and METTL3 (magnification: 200×); **C:** Colony number in U87 and U251 cells co-intervened by COL4A1 and METTL3; **D,E:** Migration and invasion in U87 and U251 cells co-intervened by COL4A1 and METTL3 (magnification: 200×). *p<0.05; **p<0.01. *p<0.05; **p<0.01.

## Discussion

COL4A1 (collagentype IV α1 chain) is composed of an amino-terminal 7S domain, a triple-helix forming collagenous domain and a carboxy-terminal non-collagenous domain [15-17]. As analyzed in the GEPIA dataset, COL4A1 was upregulated in glioma tissues. *In vitro* data showed that knockdown of COL4A1 remarkably suppressed proliferative and metastatic capacities of glioma cells, suggesting its oncogenic role.

Multiple m6A methylation sites were predicted in COL4A1. We therefore speculated that the abnormally upregulated COL4A1 in glioma may be attributed to the presence of RNA methylation. Experimental evidences have demonstrated that m6A modification is involved in human cancers. It is reported that LNC942 drives breast cancer development through METTL14-induced m6A modification [18]. ALKBH5 promotes the proliferation of renal cell carcinoma by regulating AURKB in a m6A-dependent way [19]. SUMO1-modified METTL3 mediates the stability of Snail mRNA and thus drives cancer development [20]. Interestingly, the same component of m6A modification in different types of cancers, or different components of m6A modification in one type of cancer may differentially function. METTL3 is the key component that induces the writing of m6A methylation, which displays either an oncogenic or anti-cancer role [21-23].

Our findings showed that METTL3 was downregulated in glioma tissues. Moreover, knockdown of METTL3 was able to reduce the methylation level of COL4A1 and therefore upregulated its level. *In vitro* knockdown of METTL3 remarkably strengthened proliferative and metastatic capacities of glioma cells. Taken together, METTL3-induced RNA methylation determines the upregulation of COL4A1, which further aggravates the development of glioma. Our findings highlighted the important role of RNA methylation in glioma and provided a new reference for clinical management of glioma.

There are some limitations in the present study. First of all, downstream proteins of COL4A1 are not explored. Secondly, the possibility of other RNA methyltransferases (METTL14/16) or demethyltransferases (ALKBH5/FTO) to recognize RNA methylation site in COL4A1 is unknown. Thirdly, the biological function of METTL3/COL4A1 needs to be validated in *in vivo* models.

## Conclusion

METTL3 reduces the methylation level of COL4A1 and upregulates its expression level, which further stimulates the malignant development of glioma. METTL3/COL4A1 can be potential therapeutic targets of glioma.

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## Conflict of interests

The authors declare no conflict of interests.

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