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Research Article

METTL14 inhibits malignant progression of oral squamous cell carcinoma by targeting the autophagy-related gene RB1CC1 in an m6A-IGF2BP2-dependent manner

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N6-methyladenosine (m6A) plays crucial roles in tumorigenesis and autophagy. However, the underlying mechanisms mediated by m6A and autophagy in the malignant progression of oral squamous cell carcinoma (OSCC) remain unclear. In the present study, we revealed that down-regulated expression of METTL14 was correlated with advanced clinicopathological characteristics and poor prognosis in OSCC. METTL14 knockdown significantly inhibited autophagy and facilitated malignant progression *in vitro*, and promoted tumor growth and metastasis *in vivo*. A cell model of rapamycin-induced autophagy was established to identify RB1CC1 as a potential target gene involved in m6A-regulated autophagy in OSCC, through RNA sequencing and methylated RNA immunoprecipitation sequencing (meRIP-seq) analysis. Mechanistically, we confirmed that METTL14 posttranscriptionally enhanced RB1CC1 expression in an m6A-IGF2BP2-dependent manner, thereby affecting autophagy and progression in OSCC, through methylated RNA immunoprecipitation qRT-PCR (meRIP-qPCR), RNA stability assays, mutagenesis assays and dual-luciferase reporter. Collectively, our findings demonstrated that METTL14 serves as an OSCC suppressor by regulating the autophagy-related gene RB1CC1 through m6A modification, which may provide a new insight for the diagnosis and therapy of OSCC.

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most prevalent types of head and neck cancer, and was the seventh most common malignant tumor worldwide in 2020 [1,2]. OSCC is prone to local recurrence and early cervical lymph node metastasis, leading to a low 5-year overall survival (OS) rate, despite significant advances in the diagnosis and treatment of OSCC in recent years [3,4]. Thus, it is crucial to illuminate the molecular mechanisms underlying the tumorigenesis and progression of OSCC with the purpose of diagnostic and therapeutic intervention [5].

Currently, researchers have proposed a number of molecular mechanisms involved in oncogenesis and progression, including epigenetics, genetic alterations and transcriptional cascades [6,7]. Accumulating evidence has shown that epigenetic is critically related to tumorigenesis and progression, including methylation modification of RNA, which is essential for gene expression [8,9]. N6-methyladenosine (m6A) is the most common and abundant posttranscriptional modification in eukaryotes [10]. RNA m6A modification has been reported to play important roles in regulating RNA splicing, translation, and stability

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[11,12]. Recently, m6A modification has been shown to mediate the biological processes of cancer cells, and aberrant m6A modification underlies tumorigenesis [13–17]. Moreover, studies have shown that the effect of m6A modification could be different among various tumors [18]. However, few reports have focused on the relationship between m6A methylation and OSCC.

Macroautophagy (hereinafter referred to as autophagy) is a fundamental cellular pathway to degrade endogenous substrates and intracellular macromolecules and remove damaged organelles [19,20]. Numerous studies have linked autophagy to tumorigenesis and tumor metastasis [21]. In our previous studies, rapamycin induced autophagy and inhibited malignant progression in OSCC cells [22,23]. Moreover, our preliminary research demonstrated the cross-talk between autophagy and m6A modification [24,25]. All these findings expand our understanding of the relationship between autophagy and m6A modification in OSCC. However, we found another pathway by which METTL14 affects autophagy and progression in OSCC.

In the present study, we demonstrated that loss of METTL14 expression was significantly correlated with clinicopathological characteristics and poor overall survival in OSCC patients. METTL14 knockdown resulted in impaired autophagic flux and tumor progression by inhibiting the expression of the autophagy-related gene RB1CC1 in an m6A-IGF2BP2-dependent manner. Our findings imply that METTL14 may serve as a new potential biomarker or molecular target in diagnostic and therapeutic strategies for OSCC.

Materials and methods

Cell lines and cell culture

Two human OSCC cell lines (CAL33 and HSC3) obtained from the American Type Culture Collection were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM) basic (Gibco, U.S.A.) supplemented with 10% fetal bovine serum (FBS; WISENT, Canada). All of these cells were incubated in a 5% CO_2 environment at 37°C.

Patients and specimens

A total of 106 OSCC specimens and 73 adjacent noncancerous tissues (ANCTs) were collected from the Hospital of Stomatology, Sun Yat-Sen University, between 2011 and 2018, with available 5-year follow-up data. Patients with preoperative radiotherapy or chemotherapy were excluded. The pathological diagnosis and clinicopathological parameters were determined according to the 8th American Joint Committee on Cancer Staging System. All human samples were obtained with informed consent before enrollment and conducted according to the Declaration of Helsinki consent principles. The present study was approved by the hospital's ethics committee (KQEC-2020-16-03).

Immunohistochemistry (IHC)

IHC was performed according to our previous protocol [23]. The primary antibodies were anti-METTL14 (1:1000, ab220030, Abcam, U.S.A.), anti-RB1CC1 (1:100, 17250-1-AP, Proteintech, U.S.A.), and anti-pankeratin (1:1000, #4545, Cell Signaling Technology, U.S.A.). The IHC score was evaluated according to the proportion of positively stained cells (0–100) and the staining intensity (0–3). The final IHC score was calculated by multiplying the proportion of positively stained cells and staining intensity, and then the tissues were divided into high or low expression groups using the median score.

Lentiviral transfection and transient transfection

METTL14 cDNA (GeneChem, Shanghai, China) was cloned into the pLVoeRNA lentiviral vector (oeMETTL14) and the pcDNA3.1 vector (Jiangsu Saisofi Biotechnology Co., Ltd, Wuxi, China) was employed to construct the RB1CC1-overexpressing plasmid (oeRB1CC1). siRNAs were applied for RB1CC1 and IGF2BP2 silencing. Two shRNA sequences targeting METTL14 were cloned into the pLKO.1-puro lentiviral vector (Jiangsu Saisofi Biotechnology Co., Ltd). Lipofectamine 3000 (Invitrogen, Carlsbad, California, U.S.A.) was used for plasmid transfection and siRNA transfection. Stably transfected cells were selected with puromycin for 2 weeks. The sequences of shMETTL14 and siRNAs are listed in Supplementary Table S1.

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed according to our previously described protocol [26]. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, with the level normalized to GAPDH. The specific primer sequences against targeted genes are listed in Supplementary Table S2.

Western blotting

Western blotting was performed according to our previous protocol [26]. The primary antibodies were anti-METTL14 (#51104S), anti-RB1CC1 (#12436S), anti-LC3B (#3868T) (1:1000, Cell Signaling Technology), and anti-GAPDH (1:2000, 30202ES60, Yeasen, China).

Cell proliferation and colony formation assay

Cell proliferation was detected with Cell Counting Kit-8 (Yeasen), which was employed according to our previous protocol [24]. For colony formation assay, the cells were seeded into 6-well plates at a density of 2×10^3 cells per well (CAL33) or 5×10^3 cells per well (HSC3) and cultured for 10 days. After fixation with 4% paraformaldehyde for 25 min, the cells were stained with 0.1% crystal violet for 30 min.

Cell migration and invasion assay

Cell migration and invasion assays were performed according to our previously described protocol [24]. Images were captured using an inverted microscope in five random fields per chamber.

Transmission electron microscopy (TEM)

TEM was performed according to our previous protocol [24]. Images were captured using a Philips CM10 transmission electron microscope.

Dual-luciferase reporter assay

Cells were cotransfected with plasmids containing wild-type or mutant RB1CC1 and siIGF2BP2 using Lipofectamine 3000 following the manufacturer's protocol. In addition, shMETTL14 cells were transfected with plasmids containing wild-type or mutant RB1CC1 using Lipofectamine 3000. The ratios of firefly and Renilla luciferase activities were evaluated at 48 h after transfection using a Dual-Luciferase Assay kit (Yeasen).

mRFP-GFP-LC3 adenovirus transfection assay

Cells with lentiviral transfection or transient transfection were seeded into confocal dishs and transfected with mRFP-GFP-LC3 adenovirus (Dongzebio Co., Ltd, China) according to the manufacturer's instructions. After 48 h, cells were fixed with 100% methanol for 15 min at 4°C, and nuclei were stained with DAPI for 15 min at room temperature. Images were captured by inverted laser confocal microscopy.

RNA stability assays

To measure RNA stability in METTL14 stable knockdown cells, we treated CAL33 and HSC3 cells with actinomycin D (ActD, 5 μg/ml) and collected total RNA for qRT-PCR after incubation at the indicated times (0, 2, 4, and 8 h). The half-life ($t_{1/2}$) reflecting the time required for mRNA expression to decrease by half was calculated.

MeRIP qRT-PCR

Total RNA from cells transfected with shMETTL14 and shNC was fragmented by sonication for 10 min, incubated with anti-m6A antibody (Abcam) for 4 h at 4°C and then with protein A/G magnetic beads (BersinBio, China) at 4°C for 1 h. The RNA was eluted with elution buffer and proteinase K in immunoprecipitations and extracted by phenol:chloroform:isoamylalcohol, and the purified RNA was quantified using qRT-PCR. The primers used in the MeRIP-qPCR are listed in Supplementary Table S2.

Tumor xenografts

Thirty-six specific pathogen-free male BALB/c-nude mice (age, 5–6 weeks) were randomly assigned to the groups: CAL33/shMETTL14#2, CAL33/shMETTL14#3, CAL33/shNC and HSC3/shMETTL14#2, HSC3/shMETTL14#3, HSC3/shNC ($n = 6$ per group). Fifty microliters of PBS buffer containing approximately 1×10^6 cells was injected into the left tongue under 2% pentobarbital sodium intraperitoneal injection anesthesia to establish a tumor xenograft. The weight of the mice was measured every 3 days after one week until they lost more than 15% of their body weight in a short period of time. Then, the mice were killed by cervical dislocation under 2% pentobarbital sodium anesthesia to harvest the tongues, and the xenograft volumes were calculated using the formula: $V = (\text{length} \times \text{width} \times \text{width})/2$. The tongues and cervical lymph nodes were embedded, sectioned, and stained. All animal experiments were performed in the Laboratory Animal Center of Sun Yat-sen University and all procedures were approved by the Sun Yat-Sen University Institutional Animal Care and Use Committee (Approval No. SYSU-IACUC-2022-001843).

Clinical Science (2023) **137** 1373–1389
https://doi.org/10.1042/CS20230219

Table 1 Correlation between METTL14 expression and clinicopathological features of OSCC patients

Characteristic	Number of cases	METTL14 expression		*P*-value
		Low (*n*=54)	High (*n*=52)	
Age				
<60	77	36	41	0.1597
≥60	29	18	11	
Sex				
Man	74	34	40	0.1176
Woman	32	20	12	
Tumor location				
Tongue	65	27	38	0.0956
Gingiva	16	11	5	
Buccal mucosa	13	9	4	
Floor of the mouth	12	7	5	
T stage (UICC/AJCC)				
T1	15	2	13	**<0.0001***
T2	38	9	29	
T3	25	19	6	
T4	28	24	4	
Cervical lymph node metastasis				
Negative	64	24	40	**0.0006***
Positive	42	30	12	
Differentiation				
Poorly	10	9	1	**<0.0001***
Moderately	45	37	8	
Well	51	8	43	

*$P<0.05$ indicates a significant relationship among the variables.
T stage was determined according to the 8th edition of the UICC-AJCC TNM staging system.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.0. All experiments were repeated at least three times and values are presented as the mean ± SEM. Statistical differences between two groups were analyzed by unpaired two-tailed Student's *t* tests and one-way ANOVA was applied for multiple groups. Survival analysis curves were plotted based on the Kaplan–Meier method, and statisticant differences were analyzed by the log-rank test. A chi-squared test was performed to evaluate the association between METTL14 expression and clinicopathological factors. A *P*-value less than 0.05 was considered statistically significant.

Results

METTL14 expression was down-regulated in OSCC and correlated with poor prognosis in OSCC patients

To explore the expression and clinical significance of METTL14 in OSCC, 106 OSCC tissues and 73 ANCTs were included in our study. Immunohistochemistry staining analysis showed that METTL14 was significantly down-regulated in OSCC tissues compared with ANCTs (Figure 1A). Moreover, METTL14 expression was significantly associated with tumor differentiation, with a higher METTL14 staining score in well differentiated OSCC tissues than in poorly and moderately differentiated tissues (Figure 1B). Furthermore, down-regulated METTL14 was positively associated with lymph node metastasis and advanced tumor stage. However, no statistically significant correlation was observed between METTL14 expression and age, sex, or tumor location (Table 1). Kaplan–Meier survival analysis demonstrated that low METTL14 expression was significantly associated with a shorter overall survival time in OSCC patients (Figure 1C). Thus, METTL14 deficiency may serve as a prognostic factor in OSCC.

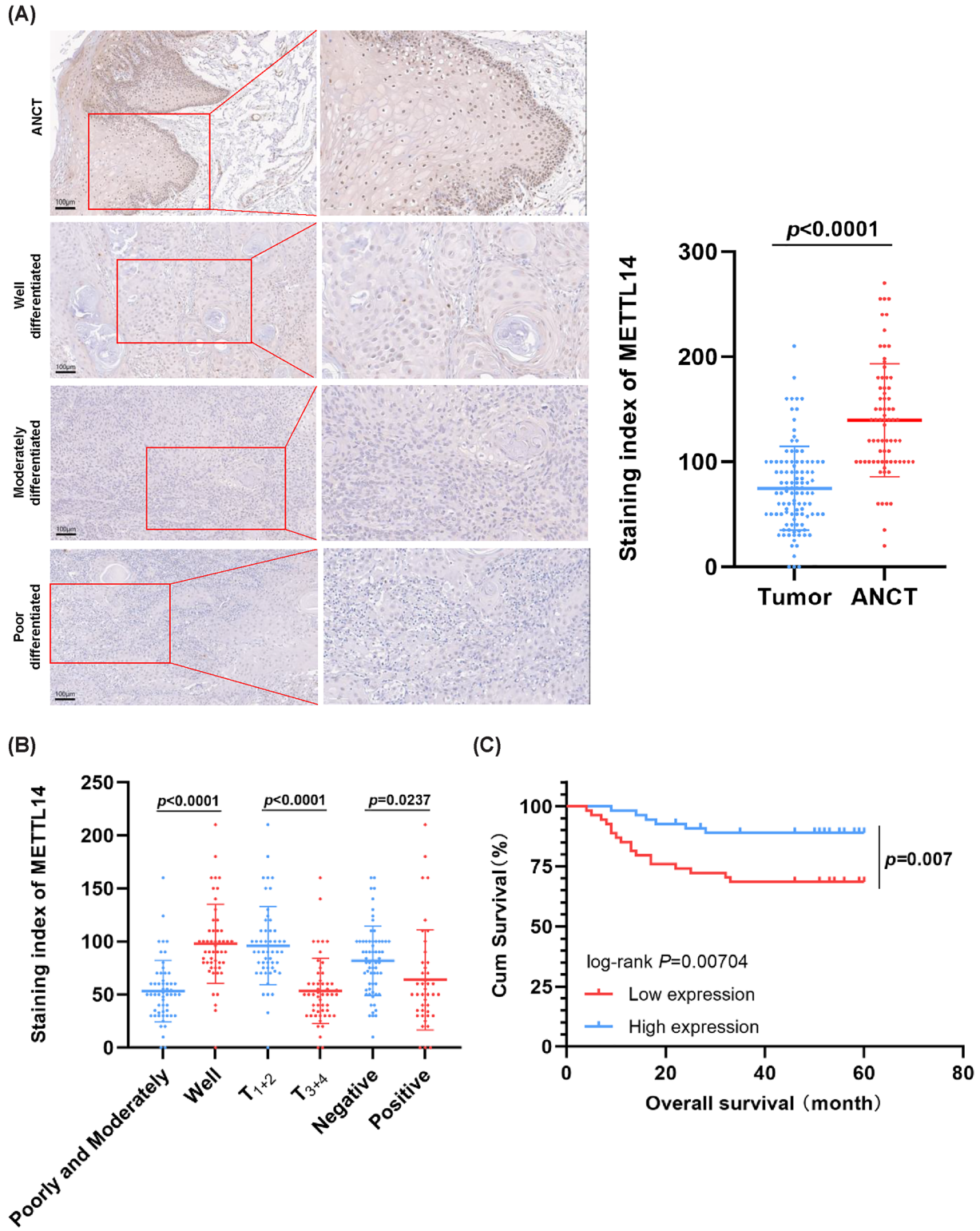

Figure 1. Decreased METTL14 expression was associated with poor prognosis in OSCC patients

(**A**) Representative image of IHC staining in OSCC tissues and ANCT, and statistical analysis of METTL14 IHC staining scores (bar = 100 μm). (**B**) Statistical analysis of METTL14 IHC staining scores in OSCC tissues stratified by advanced tumor stage, differentiation, and lymph node metastasis. (**C**) Kaplan–Meier OS analysis based on METTL14 expression in OSCC patients. *P*-values are indicated in the figure.

Clinical Science (2023) **137** 1373–1389
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METTL14 knockdown inhibited autophagic flux and promoted the proliferation, migration, and invasion of OSCC cells

To explore the role of METTL14 in autophagy, we silenced or overexpressed METTL14 (Supplementary Figure S1A–D), and mRFP-GFP-LC3 adenovirus was transfected into shMETTL14 OSCC cells to evaluate autophagic flux. Immunofluorescence assays showed that the red puncta (autolysosomes) and yellow puncta (autophagosomes) were significantly decreased upon silencing METTL14 compared with the control group, indicating that the fusion of autophagosomes and lysosomes was hampered and autophagic flux was inhibited (Figure 2A). In contrast, METTL14 overexpression promoted the maturation of autophagosomes into autolysosomes, with significantly increased red puncta (Figure 2A). In addition, increased autolysosomes and autophagosomes were observed in the METTL14 overexpression group using TEM, while METTL14 knockdown led to the opposite results (Figure 2B). To assess autophagic flux, accumulation of the microtubule-associated protein 1 light-chain 3 (LC3)-II, a most widely monitored autophagy-related protein, was obtained after autophagy inhibitors bafilomycin A1 (Baf A1, a vacuolar-type H^+-translocating ATPase inhibitor) treatment. As expected, METTL14 knockdown treatment markedly decreased LC3-II levels induced by Baf A1 (Supplementary Figure S1E), indicating an impaired autophagic flux in shMETTL14 OSCC cells. All of these results suggested that METTL14 promoted autolysosome formation and facilitated autophagic flux.

To investigate the biological functional role of METTL14 in OSCC, we performed transwell assays, Cell Counting Kit-8 (CCK-8) proliferation assays and colony formation assays. METTL14 silencing promoted the proliferation of CAL33 and HSC3 cells, as evaluated by CCK-8 proliferation and colony formation assays (Figure 2C,D). Furthermore, transwell assays demonstrated markedly enhanced migration and invasion ability in the METTL14-silenced group (Figure 2E,F). Conversely, METTL14 overexpression inhibited the proliferation, invasion and migration of CAL33 and HSC3 cells (Supplementary Figure S1F, G).

METTL14 targeted RB1CC1 in autophagy regulation

In our previous studies, we revealed that METTL14 regulated autophagy and malignant progression of OSCC by mediating eIF4G1 expression in an m6A-YTHDF2-dependent manner [25]. Through meRIP-seq and RNA-seq analysis (GEO accession: GSE186581) [24], we found that RB1CC1 mRNA exhibited multiple m6A peaks enriched in the CDS during autophagy induction. Peak calling analysis distinguished m6A peak enrichment in exon 15 of RB1CC1 mRNA that was heightened in both autophagy-induced cell models (Figure 3A). Moreover, a mutual peak in RB1CC1 mRNA was identified in autophagy-induced cell models. In addition, METTL14 expression was up-regulated and FTO expression was down-regulated during autophagy induction, while the expression of other major m6A demethyltransferase and methyltransferase remained constant [25]. Thus, considering the function of METTL14 and FTO, we hypothesized that the m6A methyltransferase METTL14 regulates RB1CC1 mRNA. To verify this, qRT-PCR and Western blotting revealed that both the mRNA (Figure 3B) and protein levels (Figure 3C) of RB1CC1 were significantly decreased owing to METTL14 depletion. Moreover, METTL14 depletion significantly decreased the microtubule-associated protein 1 light chain 3-β (LC3B)-II/LC3B-I ratio, an autophagy marker in mammals, indicating that autophagy was inhibited (Figure 3C). Interestingly, we detected the stability of RB1CC1 mRNA in shMETTL14 cells, and an RNA stability assay revealed that the half-life of RB1CC1 mRNA in METTL14 knockdown cells was markedly shorter than that in control cells (Figure 3D). To further characterize METTL14-mediated m6A methylation in RB1CC1 mRNA, meRIP-qPCR was applied to confirm that the level of m6A methylation in RB1CC1 mRNA significantly decreased in the METTL14 knockdown group compared with the control group (Figure 3E). To further clarify whether METTL14 targeted RB1CC1 mRNA through the m6A consensus sequence in exon 15, we constructed luciferase reporter plasmids including wild-type (WT-RB1CC1) and mutant RB1CC1 (Mut-RB1CC1) by replacing adenosine (A) with thymine (T) in the m6A sequence of exon 15 (Supplementary Figure S2). Dual-luciferase analysis and mutagenesis assays revealed that METTL14 depletion resulted in a significant decrease in luciferase activity in cells transfected with WT-RB1CC1, while cells transfected with Mut-RB1CC1 remained constant (Figure 3F). Collectively, these results demonstrated that exon 15 of RB1CC1 mRNA was the crucial target of METTL14-mediated regulation of RB1CC1 mRNA.

METTL14 regulated RB1CC1 mRNA levels in an m6A-IGF2BP2-dependent manner

The regulatory effects of m6A modification on biological processes of mRNA rely on m6A readers, a group of various proteins recognizing and binding to m6A modifications [27]. IGF2BPs (including IGF2BP1/2/3), a family of m6A

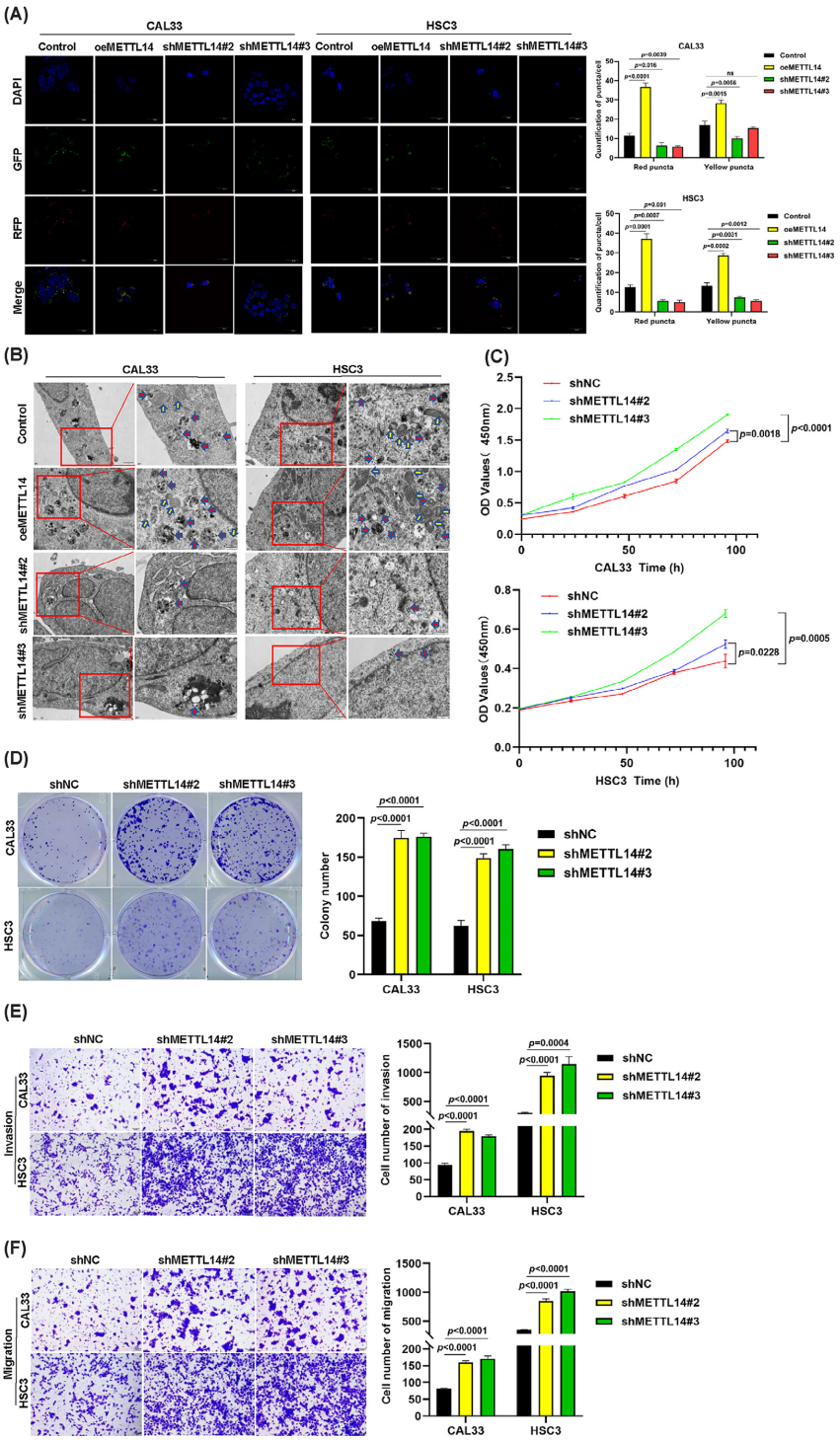

Figure 2. METTL14 expression affected autophagic flux and migration, invasion, and proliferation of OSCC cells

(**A**) CAL33 and HSC3 cells were transfected with mRFP-GFP-LC3 adenovirus vector and treated with shMETTL14, oeMETTL14. The quantification of red dots (autolysosomes) and yellow dots (autophagosomes) per cell was calculated (bars = 40 μm). (**B**) TEM detected the change of autophagosome (yellow arrow) and autolysosomes (red arrow) in CAL33 and HSC3 cells with METTL14 knockdown or overexpression, respectively (bar = 1 μm). (**C,D**) CCK-8 (C) and colony formation assay (D) were performed to measure the proliferation of cells transfected with shMETTL14. (**E,F**) Transwell invasion assay (E) and migration assay (F) were performed to detect the invasive and migratory abilities of cells transfected with shMETTL14 (bar = 100 μm). *P*-values are indicated in the figure, ns non-significant.

Clinical Science (2023) **137** 1373–1389
https://doi.org/10.1042/CS20230219

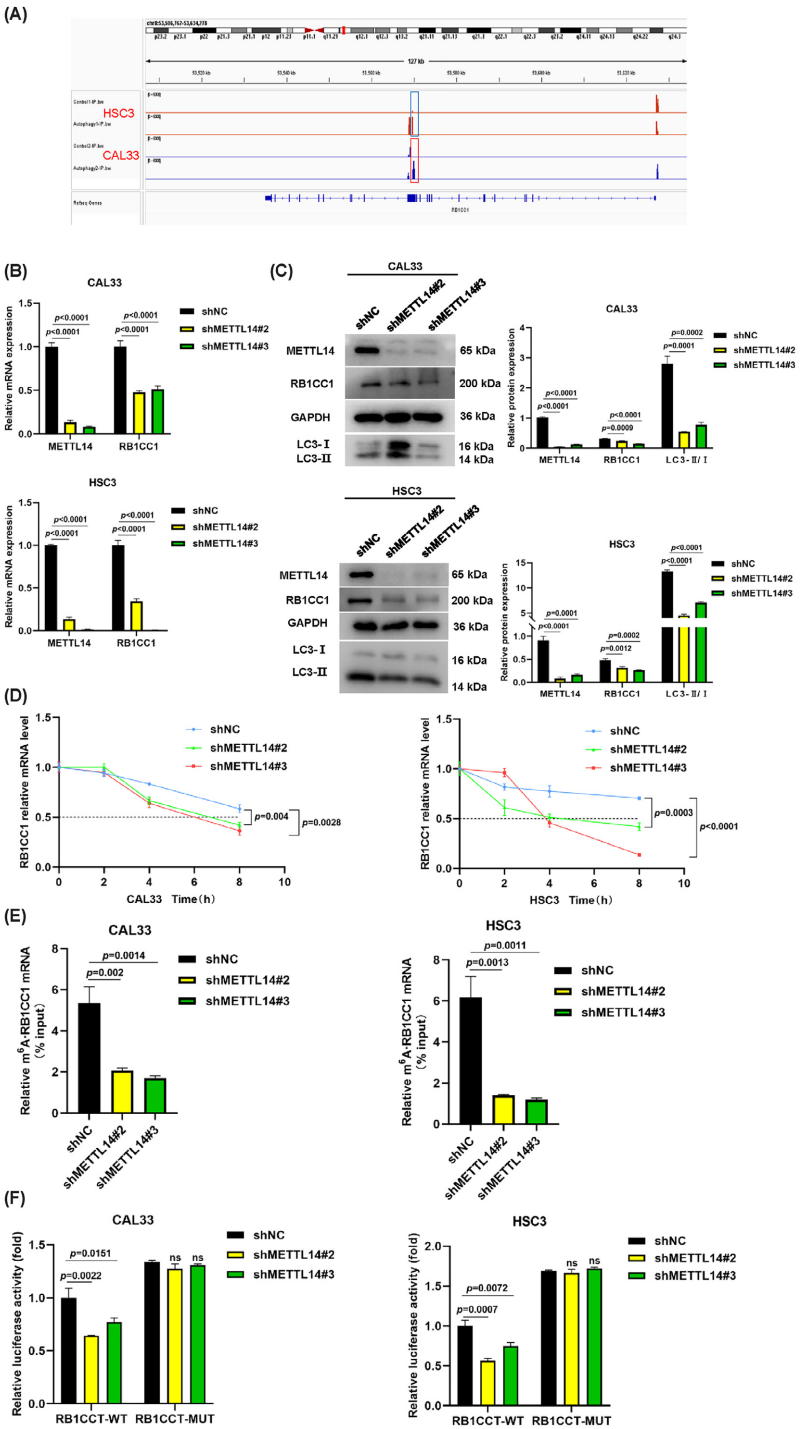

Figure 3. METTL14 recognized m6A residues on the RB1CC1 mRNA and enhanced its stability and affected autophagy in CAL33 and HSC3 cells

(**A**) m6A peaks were enriched in the exon 15 of RB1CC1 mRNA. Squares showed collective increases in m6A peaks in autophagy-induced OSCC cells. (**B**) METTL14 knockdown decreased the level of RB1CC1 mRNA. (**C**) Western blot analysis of RB1CC1 and LC3 in control, shMETTL4 CAL33 and HSC3 cells. (**D**) RNA stability assay showed the RB1CC1 mRNA half-life ($t_{1/2}$) in shMETTL14 and control cells. (**E**) MeRIP-qPCR analysis confirmed that METTL14 knockdown depleted the m6A modification of RB1CC1 mRNA. (**F**) Relative luciferase activities of CAL33 and HSC3 cell co-transfected wild-type or mutant RB1CC1 luciferase reporter and shMETTL14. Firefly luciferase activity was measured and normalized to Renilla luciferase activity. *P*-values are indicated in the figure, ns non-significant.

readers recognizing the consensus GG(m6A)C motif, have been reported to facilitate mRNA translation by enhancing the stability or inhibiting the degradation of mRNAs [28]. In our previous study, elevated IGF2BP2 expression was observed in rapamycin-induced autophagy cell models, through RNA-seq analysis (Figure 4A). Considering that IGF2BP2 is the major m6A reader facilitating m6A-methylated mRNA translation [29,30], we suspected that METTL14 regulated RB1CC1 mRNA levels in a manner dependent on IGF2BP2. To verify our suspicions, we first investigated whether the expression of RB1CC1 mRNA was regulated by IGF2BP2. qRT-PCR analysis indicated that IGF2BP2 knockdown significantly inhibited RB1CC1 expression (Figure 4B and Supplementary Figure S3A). Next, we detected whether m6A modifications on RB1CC1 mRNA were necessary for IGF2BP2-mediated gene recognition. The dual-luciferase assays showed that IGF2BP2 knockdown significantly down-regulated luciferase activity in cells transfected with wild-type RB1CC1, while the decrease was completely abolished by mutant-RB1CC1 (Figure 4C). In addition, METTL14 overexpression up-regulated RB1CC1 mRNA, which could be partially reversed by IGF2BP2 knockdown (Figure 4D). Overall, METTL14 regulates RB1CC1 expression in an m6A-IGF2BP2-dependent manner.

METTL14 regulates autophagic flux and malignant progression of OSCC by mediating RB1CC1 expression

To further investigate the biological role of RB1CC1 in OSCC, endogenous expression of RB1CC1 was silenced with small interfering RNA (siRNA), and exogenous overexpression of RB1CC1 was achieved with a plasmid (Supplementary Figure S4A). Silencing of RB1CC1 significantly decreased the LC3B-II/I ratio as illustrated by Western blotting, while RB1CC1 overexpression yielded the opposite result, indicating that autophagy was regulated by RB1CC1 (Figure 5A and Supplementary Figure S4B). Additionally, METTL14 overexpression partially recovered the expression of RB1CC1 and increased the LC3B-II/I ratio in RB1CC1 knockdown cells (Figure 5B). Moreover, immunofluorescence assays validated that silencing RB1CC1 significantly decreased yellow puncta and red puncta in merged images (Figure 5C and Supplementary Figure S4C), suggesting the inhibition of autolysosome and autophagosome formation. Interestingly, METTL14 overexpression increased yellow and red puncta, which could be partially reversed upon RB1CC1 silencing (Figure 5C and Supplementary Figure S4C). Furthermore, RB1CC1 overexpression increased the number of autophagosomes and autolysosomes as observed by TEM, while silencing RB1CC1 had the opposite result (Figure 5D). All these results manifested that silencing or overexpressing RB1CC1 could correspondingly inhibit or promote autophagic flux in OSCC respectively, but this process was affected by METTL14.

In addition, the CCK-8 assay showed that silencing RB1CC1 led to a significantly increased cell proliferation rate in CAL33 and HSC3 cells compared with the control group (Figure 5E). Colony formation assays showed that silencing RB1CC1 increased the colony formation number of OSCC cells after 10 days (Figure 5F). Moreover, RB1CC1 silencing also promoted cell invasion and migration (Figure 5G,H and Supplementary Figure S4D). Importantly, METTL14 overexpression partially reversed the enhanced proliferation, invasion, and migration induced by RB1CC1 silencing (Supplementary Figure S4E–G). Taken together, these results suggested that METTL14 promoted autophagic flux and inhibited malignant progression in OSCC cells by regulating the expression of RB1CC1.

METTL14 deficiency promoted OSCC growth and cervical lymph node metastasis *in vivo*

To verify the tumor suppressor role of METTL14 in OSCC *in vivo*, we constructed shMETTL14 cells and then injected them into the left tongue of nude mice to establish a tumor xenograft model. Consistent with our observations *in vitro*, METTL14 depletion significantly increased the volume of xenograft tumors and dramatically promoted the tumor growth compared with the control group (Figure 6A,B and Supplementary Figure S5A). We used pankeratin, which is mainly expressed in epithelial cells, an an indicator to evaluate lymph node metastasis. IHC staining revealed that METTL14 knockdown resulted in a significant increase in cervical lymph node metastasis (Figure 6C). Moreover, METTL14 knockdown reduced RB1CC1 abundance in xenograft tumors (Figure 6D). Collectively, these results proved that METTL14 knockdown promoted OSCC growth and cervical lymph node metastasis *in vivo*.

Discussion

Autophagy is a complex self-digesting process induced by multiple factors and regulated by various signaling pathways that is been deeply involved in the occurrence and development of tumors [31–34]. Loss of autophagy can lead to environmental instability and DNA damage, which promotes the activation of oncogenes and malignant progression of cancer cells [34–36]. Elevated levels of autophagy have been observed in some tumor types, such as colorectal cancer [37], gastric cancer [38], liver cancer [39], breast cancer [40], and cervical cancer [41]. Hypoxia increased the level of autophagy and facilitated the migration and invasion abilities of tongue squamous cell carcinoma cells [22]. In

Clinical Science (2023) **137** 1373–1389
https://doi.org/10.1042/CS20230219

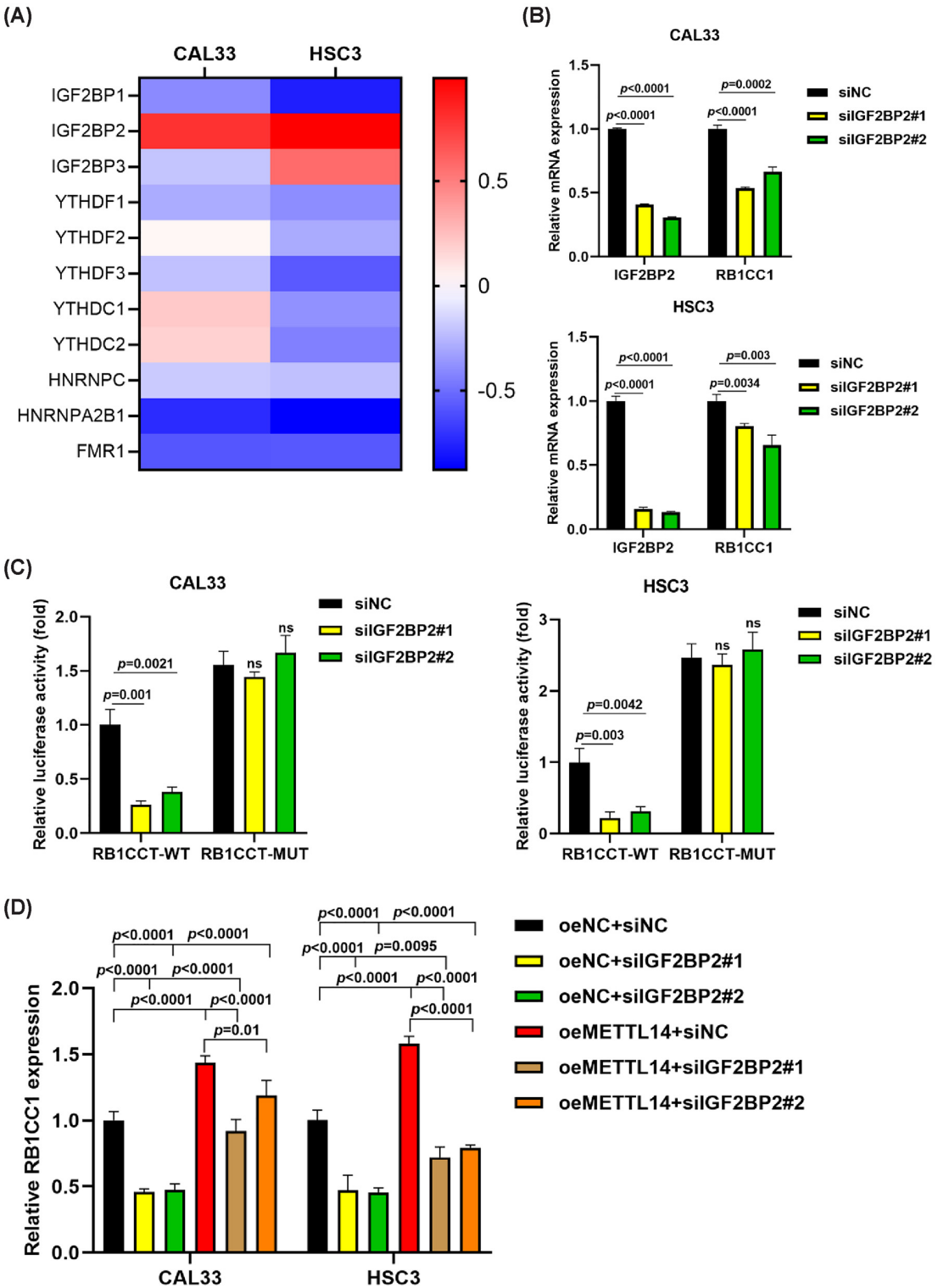

Figure 4. METTL14 modulated RB1CC1 expression in IGF2BP2-mediated manner

(**A**) Heatmap based to RNA-seq data revealed the IGF2BP2 up-regulation in both autophagy-induced OSCC cells. (**B**) IGF2BP2 knockdown down-regulated the mRNA level of RB1CC1. (**C**) Relative luciferase activities of cells co-transfected with wild-type or mutant RB1CC1 luciferase reporter and siIGF2BP2. Firefly luciferase activity was measured and normalized to Renilla luciferase activity. (**D**) METTL14 overexpression resulted in the RB1CC1 up-regulation, while the up-regulation could be partially reversed in IGF2BP2 silencing. *P*-values are indicated in the figure, ns non-significant.

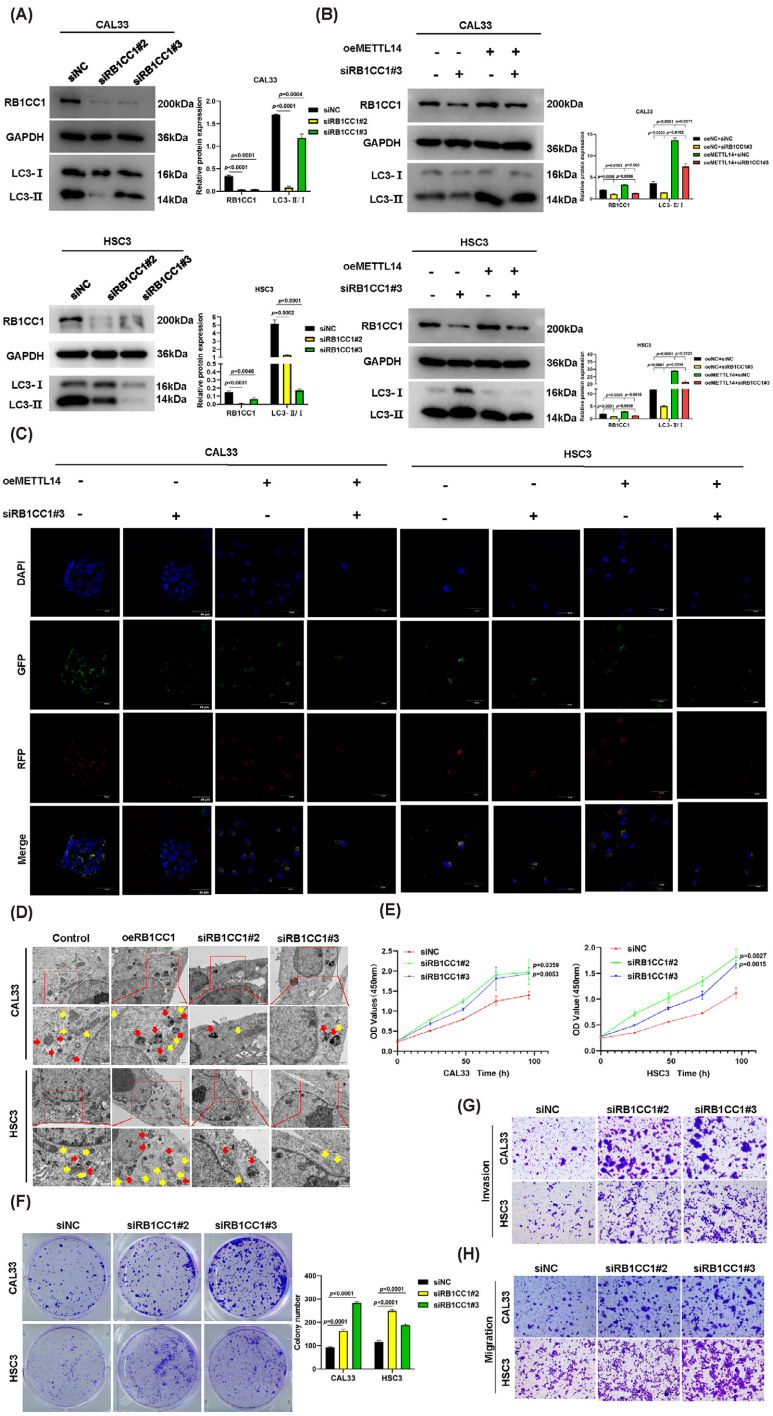

Figure 5. RB1CC1 regulation had impact on autophagic flux and proliferation, migration, invasion in OSCC cells.

(**A**) RB1CC1 silencing decreased the LC3B-II/I ratio in CAL33 and HSC3 cells. (**B**) RB1CC1 silencing resulted in decreased LC3-II/I ratio, whereas METTL14 overexpression partially rescued the down-regulation of RB1CC1 and the reduction of LC3B-II/I ratio. (**C**) HSC3 and CAL33 cells were transfected with mRFR-GFP-LC3 vector and treated with oeMETTL14, siRB1CC1#3 for 48 h. The changes were observed using a confocal microscope (bars = 40 μm). (**D**) TEM analysis of autophagosomes (yellow arrow) and autolysosomes (red arrow) in CAL33 and HSC3 cells with RB1CC1 overexpression or knockdown, respectively (bars = 1 μm). (**E,F**) CCK-8 (E) and colony formation assays (F) revealed that RB1CC1 silencing significantly promoted the proliferation of OSCC cells. (**G,H**) Transwell assays showed the accelerating invasion (G) and migration (**H**) in HSC3 and CAL33 cells, followed RB1CC1 silencing (bar = 100 μm). *P*-values are indicated in the figure.

Clinical Science (2023) **137** 1373–1389
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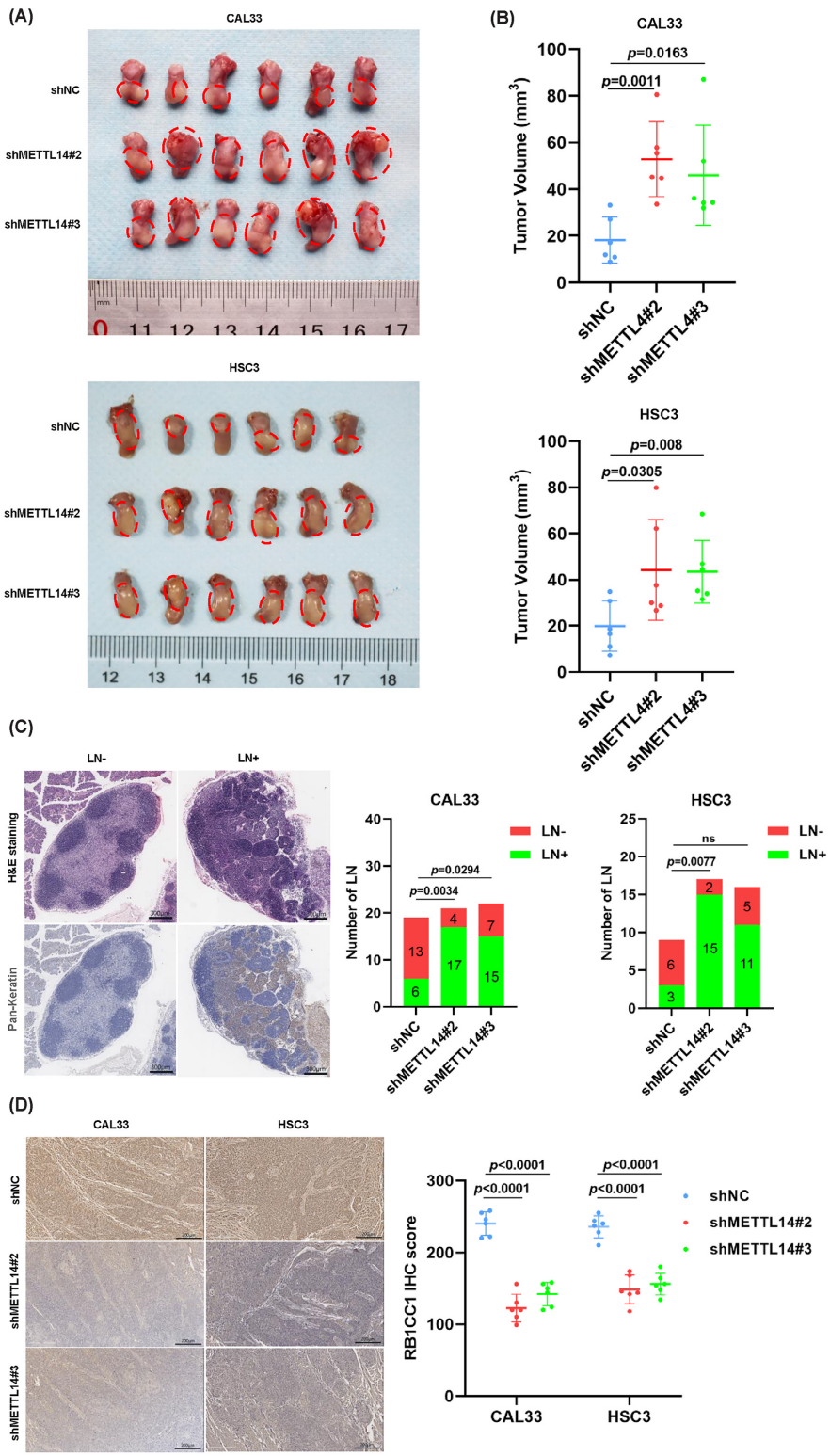

Figure 6. METTL14 knockdown promoted OSCC growth and metastasis *in vivo*

(**A**) Xenograft tumor formed by shMETTL14 or shNC OSCC cells in nude mice. (**B**) Quantitative analysis of xenografts tumors volume. (**C**) Representative images of IHC-stained cervical lymph node and statistical results of lymph node metastasis in METTL14 knockdown (bar = 300 μm). (**D**) Representative images of IHC staining in xenograft tumors and statistical results of RB1CC1 protein abundance in shMETTL14 and shNC groups (bar = 200 μm). *P*-values are indicated in the figure, ns non-significant.

our previous study, we found that activation of autophagy by rapamycin suppressed the progression of OSCC, while the autophagy inhibitor 3-MA produced the opposite results. m6A modifications are the most common internal modification of mRNA and have emerged as a widespread regulatory mechanism controlling a variety of cellular and biological processes in the majority of eukaryotes. With the recent development of high-throughput sequencing technology, it has been confirmed that specific mRNAs are regulated by m6A through specific m6A sites [6,10]. Increasing evidence suggests that m6A modifications are extensively involved in the development of a variety of solid cancers [42–45]. In our previous study, we confirmed that both m6A modification and METTL14 expression were up-regulated in an OSCC cell model of rapamycin-induced autophagy. Moreover, meRIP-seq and RNA-seq analysis revealed the increased expression of RB1CC1, with multiple m6A peaks. Thus, we hypothesized that RB1CC1 may be a potential target of METTL14-regulated autophagy in OSCC.

In this study, we verified a decrease in METTL14 expression in OSCC tissues compared with ANCTs by IHC staining. Low METTL14 expression significantly correlated with advanced T-stage, poor tumor differentiation and lymph node metastasis, suggesting that METTL14 deficiency was associated with unfavorable prognosis in OSCC. Further, Kaplan–Meier survival analysis revealed that high expression of METTL14 was positively correlated with the OS of OSCC patients, which was consistent with the findings in colorectal cancer [46,47]. In the present study, we observed that METTL14 down-regulation enhanced OSCC growth both *in vitro* and *in vivo*. Furthermore, METTL14 knockdown promoted cervical lymph node metastasis in a tumor xenograft model. To determine the mechanisms of METTL14 in the OSCC cell model of rapamycin-induced autophagy, we applied meRIP-seq and found strong enrichment of m6A peaks in exon 15 of RB1CC1 mRNA. RB1CC1 has been identified as a component of the ULK1/2 and Atg13 complex, exerting a crucial role in autophagy and tumor suppression [48–50]. We verified that METTL14 knockdown significantly inhibited autophagy and down-regulated RB1CC1 expression, which was modified by METTL14-mediated m6A methylation, as detected using meRIP-qPCR and dual-luciferase reporter assays. Emerging evidence has indicated that the regulation of mRNA fate by m6A ultimately relies on m6A readers, a group of proteins that recognize and bind to m6A-modified transcripts to modulate gene expression, influencing functions such as mRNA stability [51], mRNA splicing [52], mRNA structure [53], mRNA export [54], translation efficiency and miRNA biogenesis [55]. IGF2BP2 can enhance mRNA stability by binding to target mRNAs in an m6A-dependent manner under normal and stress conditions, while YTHDF1 promotes mRNA translation and RNA-seq analysis revealed its down-regulation. Comprehensive analysis of public databases demonstrated that all three IGF2BPs (IGF2BP1/2/3) are highly expressed in various human cancers, which implies oncogenic roles of IGF2BPs as m6A readers [56]. Similar to IGF2BP proteins, the main role of YTHDF1 is to promote translation efficiency by binding to m6A sites in mRNAs. However, in the present study, only IGF2BP2 was identified to be up-regulated among the common readers in the rapamycin-induced autophagy model of OSCC cells through RNA-seq. Here, we observed that IGF2BP2 knockdown inhibited RB1CC1 expression. Furthermore, luciferase/mutation assays also revealed that IGF2BP2 bound to the CDS of RB1CC1 mRNA. Collectively, our findings suggested that METTL14 knockdown inhibited RB1CC1 expression in an m6A-IGF2BP2-dependent manner. Similarly, our luciferase/mutation assay demonstrated that m6A sites located in the CDS of RB1CCI mediated the translation of RB1CC1 mRNA in OSCC cells. In addition, several studies verified that m6A was primarily enriched in the 3'-UTR and the stop codon [57]. However, a recent study revealed that m6A enriched in the Snail CDS region, rather than the 3'-UTR, triggers polysome-mediated translation in cancer cells [16].

To gain deeper insight into the influence of the METTL14-m6A-IGF2BP2-RB1CC1 axis in OSCC, we further investigated the role of RB1CC1 in OSCC. RB1CC1, as an indispensable constituent of autophagy vesicles, forms a complex with ATG13, ULK1 and ATG101 to induce autophagy [32,58]. RB1CC1 plays a fundamental role in autophagosome formation, which is extensively involved in protein synthesis, proliferation, migration, differentiation, and cycle progression [59,60]. Recent studies have showed that RB1CC1 expression deficiency is significantly associated with a worse prognosis in breast cancer [61,62]. Moreover, RB1CC1 has been reported to function as a tumor-suppressing gene in renal cell carcinoma [63]. RB1CC1-associated signaling sensitizes tumor cells to ferroptosis, which may provide novel therapeutic opportunities for cancer treatment. All these studies indicate that RB1CC1 may play an essential role in autophagy and malignant progression in OSCC. Our study revealed that silencing RB1CC1 significantly impaired autophagic flux and promoted invasion, migration, and proliferation in OSCC cells. Furthermore, the rescue experiments verified an RB1CC1-dependent role of METTL14 in autophagy regulation and malignant progression of OSCC.

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Conclusion

We identified a METTL14-m6A-IGF2BP2-RB1CC1 axis in OSCC, which provided a novel vision of the interactions between m6A modification and autophagy. METTL14 promoted the expression of the autophagy-related gene RB1CC1 in an m6A-IGF2BP2-dependent manner, thereby affecting autophagy and malignant progression in OSCC. Combined with our previous identification of the METTL14-m6A-YTHDF2-eIF4G1 axis in OSCC, we suggest that m6A modification is an extremely complex process with targeting specificity that may regulate autophagy and tumorigenesis in OSCC through multiple pathways.

Clinical perspectives

- Oral squamous cell carcinoma (OSCC) is one of the most prevalent types of head and neck cancer and is prone to local recurrence and early cervical lymph node metastasis, leading to a low 5-year overall survival (OS) rate. It is crucial to illuminate the molecular mechanisms underlying the tumorigenesis and metastasis of OSCC.

- In this study, we found that down-regulated METTL14 expression was correlated with advanced clinicopathological characteristics and poor prognosis in OSCC patients. METTL14 knockdown significantly inhibited autophagy, facilitated malignant progression *in vitro*, and promoted tumor growth and metastasis *in vivo*. METTL14 posttranscriptionally enhanced RB1CC1 expression in an m6A-IGF2BP2-dependent manner, thereby affecting autophagy and progression in OSCC.

- Our finding illustrates how m6A methylation affects autophagy and malignant progression in OSCC, which may provide new insight for the diagnosis and therapy of OSCC.

Data Availability

The meRIP-seq and RNA-seq data that support the findings of this study are openly available in Gene Expression Omnibus (GEO accession: GSE186581), other data underlying this article will be shared on reasonable request to the corresponding authors.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRediT Author Contribution

Jianfeng Liang: Data curation, Software, Formal analysis, Investigation, Visualization, Methodology, Writing—original draft, Writing—review & editing. **Hongshi Cai:** Data curation, Software, Formal analysis, Investigation, Visualization, Methodology. **Chen Hou:** Data curation, Formal analysis, Methodology, Writing—original draft. **Fan Song:** Data curation, Formal analysis, Methodology. **Yaoqi Jiang:** Data curation, Methodology. **Ziyi Wang:** Data curation, Methodology. **Danqi Qiu:** Data curation, Software. **Yue Zhu:** Data curation, Formal analysis. **Fang Wang:** Conceptualization. **Dongsheng Yu:** Conceptualization, Supervision, Project administration. **Jinsong Hou:** Conceptualization, Resources, Supervision, Funding acquisition, Writing—original draft, Project administration, Writing—review & editing.

Abbreviations

ATG, autophagy-related gene; CDS, coding sequence; IGF2BP2, insulin like growth factor 2 mRNA binding protein 2; METTL14, methyltransferase 14; RB1CC1, Rb1-inducible coiled-coil 1.

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