Received: 4 April 2023 | Revised: 20 July 2023 | Accepted: 26 July 2023

DOI: 10.1111/cas.15946

ORIGINAL ARTICLE

Cancer Science WILEY

N-acetyltransferase 10 promotes the progression of oral squamous cell carcinoma through N4-acetylcytidine RNA acetylation of MMP1 mRNA

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Abstract

The pathogenesis of oral squamous cell carcinoma (OSCC) remains unclear. Therefore, clarifying its pathogenesis and molecular-level development mechanism has become the focus of OSCC research. N-acetyltransferase 10 (NAT10) is a crucial enzyme involved in mRNA acetylation, regulating target gene expression and biological functions of various diseases through mediating N4-acetylcytidine (ac4C) acetylation. However, its role in OSCC progression is not well understood. In this study, we showed that NAT10 was significantly upregulated in OSCC tissues compared to normal oral tissues. Moreover, lentivirus-mediated NAT10 knockdown markedly suppressed cell proliferation, migration, and invasion in two OSCC cell lines (SCC-9 and SCC-15). Interestingly, MMP1 was found to be significantly upregulated in OSCC tissues and was a potential target of NAT10. N-acetyltransferase 10 knockdown significantly reduced both the total and ac4C acetylated levels of MMP1 mRNA and decreased its mRNA stability. Xenograft experiments further confirmed the inhibitory effect of NAT10 knockdown on the tumorigenesis and metastasis ability of OSCC cells and decreased MMP1 expression in vivo. Additionally, NAT10 knockdown impaired the proliferation, migration, and invasion abilities in OSCC cell lines in an MMP1-dependent manner. Our results suggest that NAT10 acts as an oncogene in OSCC, and targeting ac4C acetylation could be a promising therapeutic strategy for OSCC treatment.

KEYWORDS
MMP1, N4-acetylcytidine (ac4C), N-acetyltransferase 10 (NAT10), Oral squamous cell carcinoma

Abbreviations: ac4C, N4-acetylcytidine; NAT10, N-acetyltransferase 10; OSCC, oral squamous cell carcinoma; qPCR, quantitative PCR; RIP, RNA immunoprecipitation.

1 | INTRODUCTION

Oral squamous cell carcinoma is a common head and neck malignant tumor, accounting for more than 90% of oral cancer.[1] The treatment methods mainly include surgery, radiotherapy, chemotherapy, and immunotherapy. For patients with recurrent, metastatic, and advanced OSCC, the treatment methods are mainly radiotherapy and chemotherapy, with poor therapeutic effects.[2] Oral squamous cell carcinoma is highly invasive and prone to early lymph node metastasis, which affects the treatment effect. Early diagnosis of OSCC is significant for improving the prognosis of patients.[3] The study of genes involved in the occurrence and development of OSCC to find gene expression markers for the diagnosis of OSCC is a hot topic, which is conducive to clarifying the molecular mechanism of cancer.

Posttranscriptional modification of RNA plays an important role in regulating its structure and function, and there are currently more than 150 types of natural RNA modification known.[4] N4-acetylcytidine (ac4C) acetylation is a conserved chemical modification found in eukaryotes and prokaryotes.[5] As a new type of RNA modification, its mechanism and function have received more and more attention. Early studies suggested that ac4C mainly exists in tRNA and 18SrRNA, while recent studies have shown that ac4C can also promote its expression by improving the mRNA stability and translation efficiency of target genes.[6,7] N-acetyltransferase 10 is considered to be the critical RNA ac4C modification enzyme, which is one of the current appraisal domain structures both acetylation enzyme proteins and RNA structure domain.[8–10] Emerging studies have shown that NAT10-mediated acetylation modification is associated with various diseases, including cancer, osteogenesis, and aging.[10–14] However, it is not clear whether NAT10 is involved in the progress of OSCC.

In this study, we found that NAT10 was significantly upregulated in OSCC tissues compared with normal oral tissues. Lentivirus-mediated knockdown of NAT10 significantly suppressed cell proliferation, migration, and invasion in two OSCC cell lines (SCC-9 and SCC-15). We next found that MMP1 was significantly upregulated in OSCC tissues, which was a potential target of NAT10. Knockdown of NAT10 dramatically decreased the total and ac4C acetylated levels of MMP1 mRNA. Furthermore, NAT10 knockdown significantly decreased the MMP1 mRNA stability. Xenograft results further verified that NAT10 knockdown dramatically inhibited the tumorigenesis and metastasis ability of the OSCC cell line and decreased the MMP1 expression in vivo. Additionally, NAT10 knockdown impaired the proliferation, migration, and invasion abilities in OSCC cell lines in an MMP1-dependent manner.

Collectively, our results suggest an oncogene role of NAT10, and targeting ac4C acetylation could be a potential and attractive therapeutic target in the treatment of OSCC.

2 | MATERIALS AND METHODS

2.1 | Patients and tissue samples

Normal oral tissues and OSCC tissues were obtained from 10 patients undergoing surgical resections between 2015 and 2020 at the First Affiliated Hospital of Nanchang University. Liquid nitrogen was used to freeze the samples, and they were stored at −80°C until use. Informed consent was obtained from each participant prior to surgery by the Ethical Committee of the First Affiliated Hospital of Nanchang University.

2.2 | Cell lines and cell culture

SCC-9 and SCC-15 human OSCC cell lines were obtained from the Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences. Dulbecco's modified Eagle's medium supplemented with 10% FBS was used for all cell lines under standard culture conditions (5% CO_2, 37°C).

2.3 | Immunohistochemistry

Immunohistochemical assay was carried out to evaluate NAT10 expression in OSCC tissues and tissue array in accordance with a previously described protocol. Briefly, after drying for 4 h at 90°C, dewaxing in xylene, and rehydrating in graded ethanol solutions, paraffin-embedded tissue slides were prepared. Sections were incubated with the primary Ab (anti-NAT10 Ab purchased from Abcam (MA, USA), used in 1:200) at 4°C overnight. Following a PBS wash, sections were incubated with HRP-labeled secondary Ab at 37°C for 30 min. Dehydrated sections were then cleared, mounted, and dehydrated again. The nuclear counterstain was hematoxylin, and the chromogen was diaminobenzene.

2.4 | RNA isolation and qPCR

The RNA was isolated by chloroform-isopropanol-ethanol protocol from tissue and cells using TRIzol (Thermo Fisher Scientific). SYBR Green for qPCR was used to carry out real-time RT-qPCR using 1 g RNA input. Primers were synthesized as follows: human NAT10 forward primer, 5′- TCACTCCCCGGAAGGACCTG-3′ and reverse primer, 5′- AGCCTGGGGGTCAAGCCATA-3′; MMP1 forward, 5′- GGGGTGTGGTGTCTCACAGC-3′ and reverse, 5′- GTTTGCTCCCA GCGAGGGTT-3′; and GAPDH forward, 5′- TGACTTCAACAGCG ACACCCA-3′ and reverse, 5’- CACCCTGTTGCTGTAGCCAAA-3′. mRNA quantification was normalized to the housekeeping gene *GAPDH* and represented as fold change ($2^{-\Delta\Delta C_t}$).

2.5 | Western blot analysis

Tissues or cells lysates were prepared by digesting the tissues in 1× lysis buffer, protease, and phosphatase inhibitor and PMSF (Sangon Biotech) in a Qiagen Tissue Lyser for 8 min at 50 rpm at 4°C, rocked for 1 h at 4°C, and sonicated. For 10% SDS-PAGE, 20 μg of each lysate was processed using the Pierce BCA protein assay (Thermo Fisher Scientific). The SDS-PAGE gels were transferred to PVDF membranes (Millipore) and blocked with 5% BSA (Sigma-Aldrich) for 1 h at 25°C. At a concentration of 1:1000, primary Abs (ab139468; Abcam) were incubated at 4°C with rocking. A secondary HRP Ab was incubated for 1 h at room temperature under rocking conditions. High-signature ECL Western Blotting Substrate (Tanon) was used to develop the blots.

2.6 | Cell proliferation, migration, and invasion assays

A CCK-8 was used to determine the ability of cells to proliferate. A wound healing assay was used to assess cell migration, and a Matrigel (BD Biosciences) coated Transwell chamber (8 μm pore size; BD Biosciences) was used to examine cell invasion.

2.7 | Global ac4C acetylation quantification

Using the total RNA from each sample, we undertook global ac4C acetylation quantification using a Total Ac4C Acetylation Quantification kit (Colorimetric, Genelily Biotech) in accordance with the manufacturer's instructions.

2.8 | Immunofluorescence staining

Immunofluorescence staining with anti-NAT10 Ab (sc-271,770, 1:200; Santa Cruz Biotechnology) and anti-MMP1 Ab (ab137332, 1:300; Abcam) Ab or anti-α-SMA Ab (ab5694, 1:300; Abcam) was carried out overnight at 4°C, followed by 2 h of incubation at room temperature with the secondary Ab (1:400), and mounted with DAPI containing mounting media (P36935; Thermo Fisher Scientific). Images were taken with a Zeiss LSM800 confocal microscope.

2.9 | N4-acetylcytidine-RIP-qPCR

An RNA immunoprecipitation kit (Genelily Biotech) was utilized to carry out ac4c-RIP, in accordance with the manufacturer's guidelines. In brief, cells were lysed using 1 mL RIP lysis buffer for 10 min and preserved at −80°C. Protein A/G beads were mixed with either anti-ac4c Ab (1:50, ab252215; Abcam) or normal rabbit IgG (1:50, 2729S; Cell Signaling Technology) at 4°C for 2 h. Next, the beads were incubated in 450 μL lysate for 2 h at 4°C. After washing the beads with buffer and extracting RNA, a quantitative real-time PCR analysis was carried out.

2.10 | RNA stability assay

In six-well plates, actinomycin D (5 μg/mL; MedChemExpress) was applied to SCC-9 cells for 0, 1, 3, and 6 h. Based on linear regression analysis of total RNA analyzed with the above-mentioned quantitative real-time PCR method, the MMP1 mRNA half-life was estimated.

2.11 | Tumor xenografts

For the establishment of xenograft tumor models, 5-week-old BALB/c nude mice were purchased from the Changzhou Kawensi Laboratory Animal Centre. Several animal experiments have been approved by Nanchang University's First Affiliated Hospital Animal Care Use Committee. As a result of measuring tumor volume every 5 days, the following dimensions were estimated: tumor volume = (length × width^2)/2. After 30 days of injection of cells, the mice were killed and tumors were removed, photographed, and weighed.

2.12 | Statistical analysis

Three replicates were carried out for each experiment, and GraphPad Prism 9.0 software was used for statistical analyses. Data are shown as mean ± SD. Student's *t*-test, one-way ANOVA, and two-factor ANOVA were used to compare differences among groups. In all tests, $p < 0.05$ (bilateral) was considered statistically significant.

3 | RESULTS

3.1 | N-acetyltransferase 10 significantly upregulated in OSCC tissues

To understand the potential role of NAT10 in the progress of OSCC, we first screened the GEO database (GSE37991) and found that NAT10 was upregulated in OSCC tissues ($n = 40$) compared with normal oral tissues ($n = 40$) (Figure 1A). We then carried out immunoblotting in an OSCC tissue array, comprising of 70 human OSCC tissues and 10 paired normal oral tissues. Figure 1B of the immunohistochemistry results revealed that NAT10 protein levels were predominantly located in the nucleus of OSCC and significantly higher than those observed in normal oral tissues. The correlation between NAT10 expression in OSCC tissues and the clinical characteristics is shown in Table 1. Moreover, 10 pairs of fresh OSCC tissues and

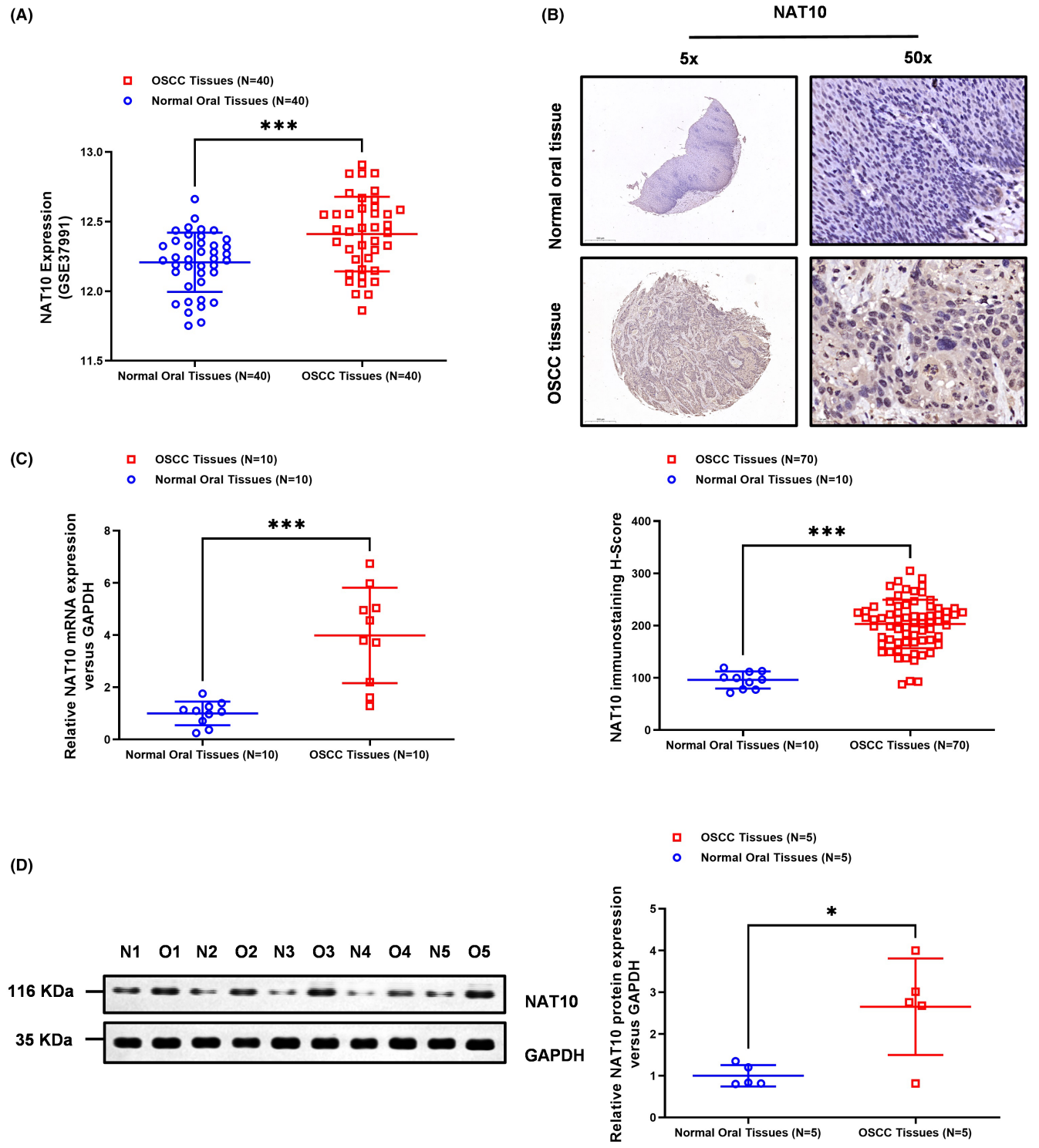

FIGURE 1 N-acetyltransferase 10 (NAT10) was significantly upregulated in oral squamous cell carcinoma (OSCC) tissues. (A) Expression levels of NAT10 in OSCC were analyzed in the GEO database (GSE37991). (B) Protein levels of NAT10 in an OSCC tissue array were analyzed by immunohistochemistry. (C) NAT10 mRNA levels in 10 paired fresh OSCC tissues and adjacent normal oral tissues were analyzed by real-time PCR. (D) NAT10 protein levels in 10 paired fresh OSCC tissues (O1–O5) and adjacent normal oral tissues (N1–N5) were analyzed by western blotting. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared with indicated group.

adjacent normal oral tissues were analyzed for NAT10 mRNA and protein levels. As displayed in Figure 1C, NAT10 mRNA levels in OSCC tissues were significantly higher than their paired adjacent normal oral tissues, which was confirmed by the western blot analysis (Figure 1D). These findings conclusively indicate that NAT10 is significantly overexpressed in OSCC tissues.

TABLE 1 Correlation between N-acetyltransferase 10 (NAT10) expression in oral squamous cell carcinoma tissues and clinical characteristics

Clinical parameter	Cases	NAT10 expression level		χ^2	*p* value
		Low	High		
Gender				0.057	1.000
Female	37	18	20	-	-
Male	33	17	16	-	-
Age (years)				6.937	**0.016**
<64	37	13	24	-	-
≥64	33	22	11	-	-
Tumor size (cm)				0.560	0.618
≤3	45	21	24	-	-
>3	25	14	11	-	-
Pathological stage				6.248	**0.028**
I+II	61	34	27	-	-
III	9	1	8	-	-
Lymph node metastasis				0.979	0.458
Negative	44	20	24	-	-
Positive	26	15	11	-	-

Note: Bold signifies $p < 0.05$.

3.2 | N-acetyltransferase 10 knockdown or inhibition impaired proliferation, migration, and invasion abilities in OSCC cell lines

The NAT10-knockdown cell lines (SCC-9 and SCC-15) were first established by the lentivirus method in order to determine the function of NAT10 in OSCC. In SCC-9 and SCC-15 cells, the Lv-sh-NAT10 groups expressed significantly less NAT10 mRNA than the Lv-sh-NC groups, according to real-time PCR results (Figure S1A). The decreased NAT10 protein level in the Lv-sh-NAT10 groups of SCC-9 and SCC-15 cells was then verified using western blot analysis (Figure S1B) and immunofluorescence staining (Figure S1C). Consistently, the total ac4C acetylated RNA levels were significantly suppressed by NAT10 knockdown in SCC-9 and SCC-15 cells (Figure 2A). CCK-8 assay results showed that the cell proliferation ability in NAT10-knockdown OSCC cells was significantly suppressed compared to the vector control groups (Figure 2B). Moreover, the impaired cell proliferation ability in NAT10-knockdown SCC-9 and SCC-15 cells was verified by the colony formation assay, which showed that the formatted colonies in NAT10-knockdown OSCC cells were significantly lower than in the vector control groups (Figure 2C). Furthermore, EdU assay showed that the EdU-positive ratios in the NAT10-knockdown SCC-9 and SCC-15 cells were significantly reduced, indicating a decreased proliferation ability of SCC-9 and SCC-15 cells (Figure 2D). By using wound healing assays and invasion assays, NAT10 was evaluated for its effect on OSCC metastasis ability. A significant reduction in migration of SCC-9 and SCC-15 cells was observed with NAT10 knockdown (Figure 2E), as was a decrease in the number of invasive cells (Figure 2F). Consistently, the specific NAT10 inhibitor remodelin significantly inhibited the total ac4C acetylated RNA levels (Figure 3A) and the biological function including cell proliferation (Figure 3B,C), migration (Figure 3D), and invasion (Figure 3E) of SCC-9 and SCC-15 cells. Based on these results, it appears that NAT10 knockdown impairs the proliferation, migration, and invasion abilities of OSCC cells.

3.3 | N-acetyltransferase 10 knockdown impaired mRNA stability of MMP1 and decreased its expression in OSCC cells

To further explore the underlying mechanisms through which NAT10 knockdown impaired the proliferation and metastasis ability of OSCC cells, we downloaded microarray data (GSE37991, Figure 4A) from the GEO database (http://www.ncbi.nlm.nih.gov/geo/). We found that the levels of MMP1 were significantly higher in OSCC tissues than in normal oral tissues, as shown in the volcano plot (Figure 4B), heatmap (Figure 4C), and statistical analysis (Figure 4D). Notably, the level of NAT10 and MMP1 showed a positive correlation in OSCC tissues (Figure 4E). A functional enrichment analysis was carried out to confirm the underlying function of potential targets. The ECM and structure organization were obviously enriched in the upregulated genes in OSCC (Figure S2). The PI3K-Akt signaling pathway was dramatically correlated in the upregulated genes in OSCC (Figure S2). Interestingly, in the NAT10-knockdown SCC-9 and SCC-15 cells, the expression level of MMP1 mRNA was significantly decreased (Figure 5A). Consistently, the protein levels of MMP1 were also inhibited in the NAT10-knockdown SCC-9 and SCC-15 cells (Figure 5B), which were further verified by immunofluorescence staining (Figure 5C). Furthermore, the ac4C acetylated MMP1 mRNA levels were also suppressed by NAT10 knockdown (Figure 5D). In a stability analysis of mRNA, values represented the number of remaining mRNA compared to mRNA levels before

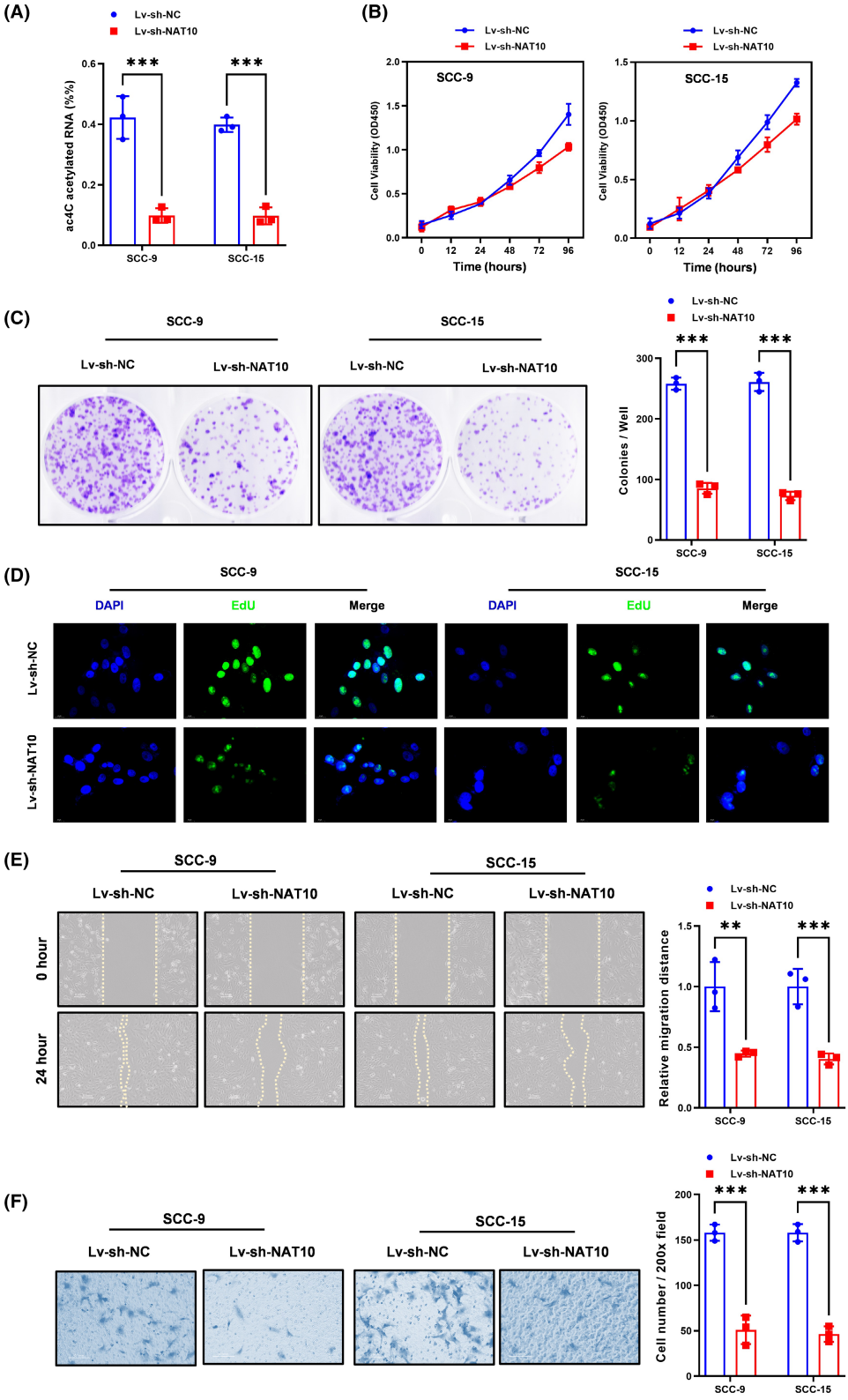

FIGURE 2 N-acetyltransferase 10 (NAT10) knockdown impaired proliferation, migration, and invasion abilities in oral squamous cell carcinoma cell lines. (A) Total N4-acetylcytidine (ac4C) acetylated RNA levels were analyzed by colorimetric method. (B) CCK-8 assay was used to analyze the effect of NAT10 knockdown on the cell proliferation ability in SCC-9 and SCC-15 cells. (C) Colony formation assay and (D) EdU assay were used to verify the effect of NAT10 knockdown on the cell proliferation ability in SCC-9 and SCC-15 cells. (E) Effect of NAT10 knockdown on the migration ability of SCC-9 and SCC-15 cells was evaluated using wound healing assay. (F) Role of NAT10 knockdown on invasion abilities of SCC-9 and SCC-15 cells was analyzed by Transwell invasion assay. $^{**}p < 0.01$, $^{***}p < 0.001$. Lv-sh-NC, letivirus negative control group; Lv-sh-NAT10, NAT10 knockdown group.

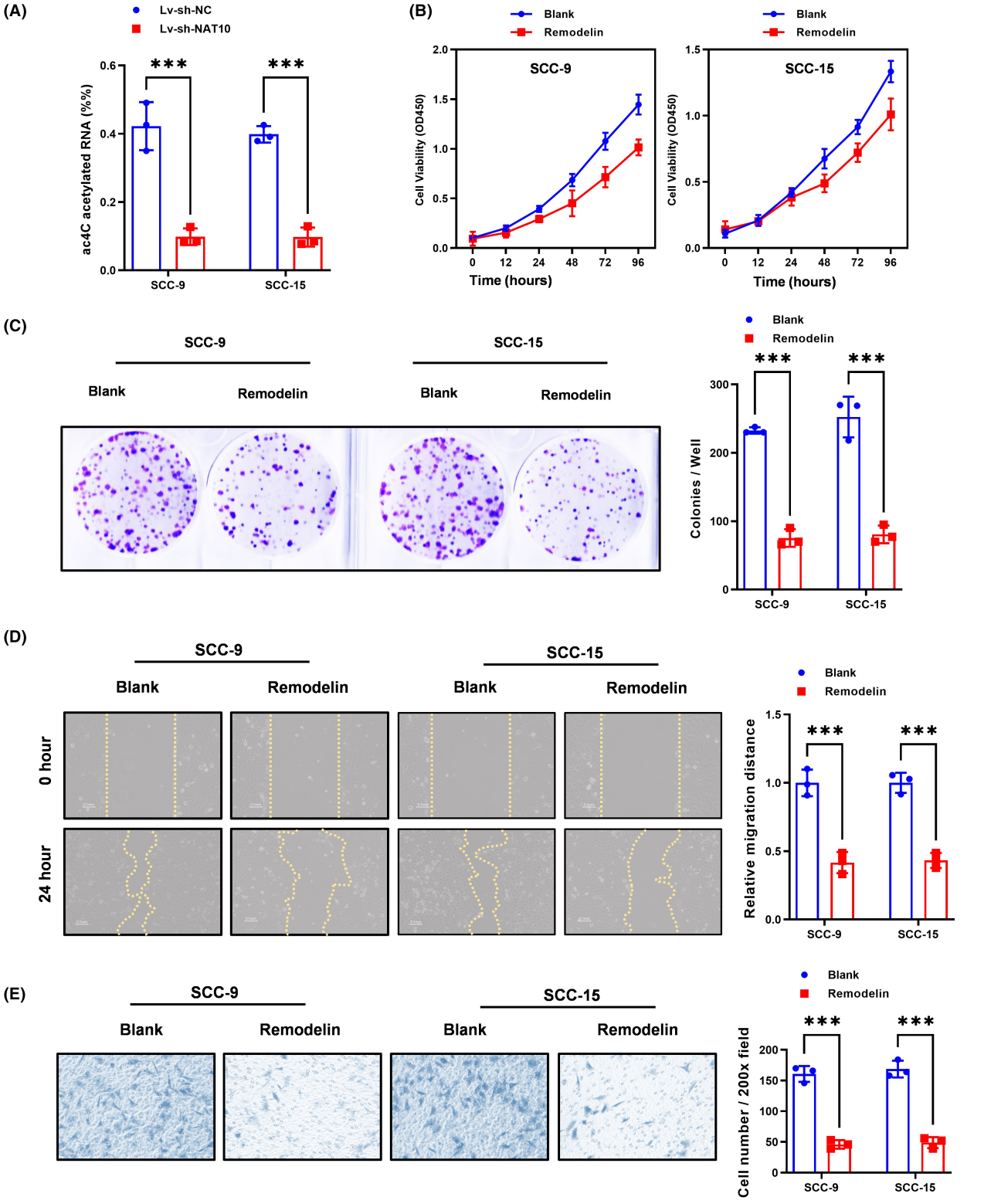

FIGURE 3 N-acetyltransferase 10 (NAT10) inhibition with remodelin impaired the proliferation, migration, and invasion abilities in oral squamous cell carcinoma cell lines. (A) Total N4-acetylcytidine (ac4C) acetylated RNA levels were analyzed by colorimetric method. (B) CCK-8 assay was used to analyze the effect of NAT10 inhibition on cell proliferation ability in SCC-9 and SCC-15 cells. (C) Colony formation assay was used to verify the effect of NAT10 inhibition on cell proliferation ability in SCC-9 and SCC-15 cells. (D) Effect of NAT10 inhibition on the migration ability of SCC-9 and SCC-15 cells was evaluated using wound healing assay. (E) Role of NAT10 inhibition on invasion abilities of SCC-9 and SCC-15 cells was analyzed by Transwell invasion assay. ***$p < 0.001$. Lv-sh-NC, letivirus negative control group; Lv-sh-NAT10, NAT10 knockdown group; OD450, optical density at 450 nm.

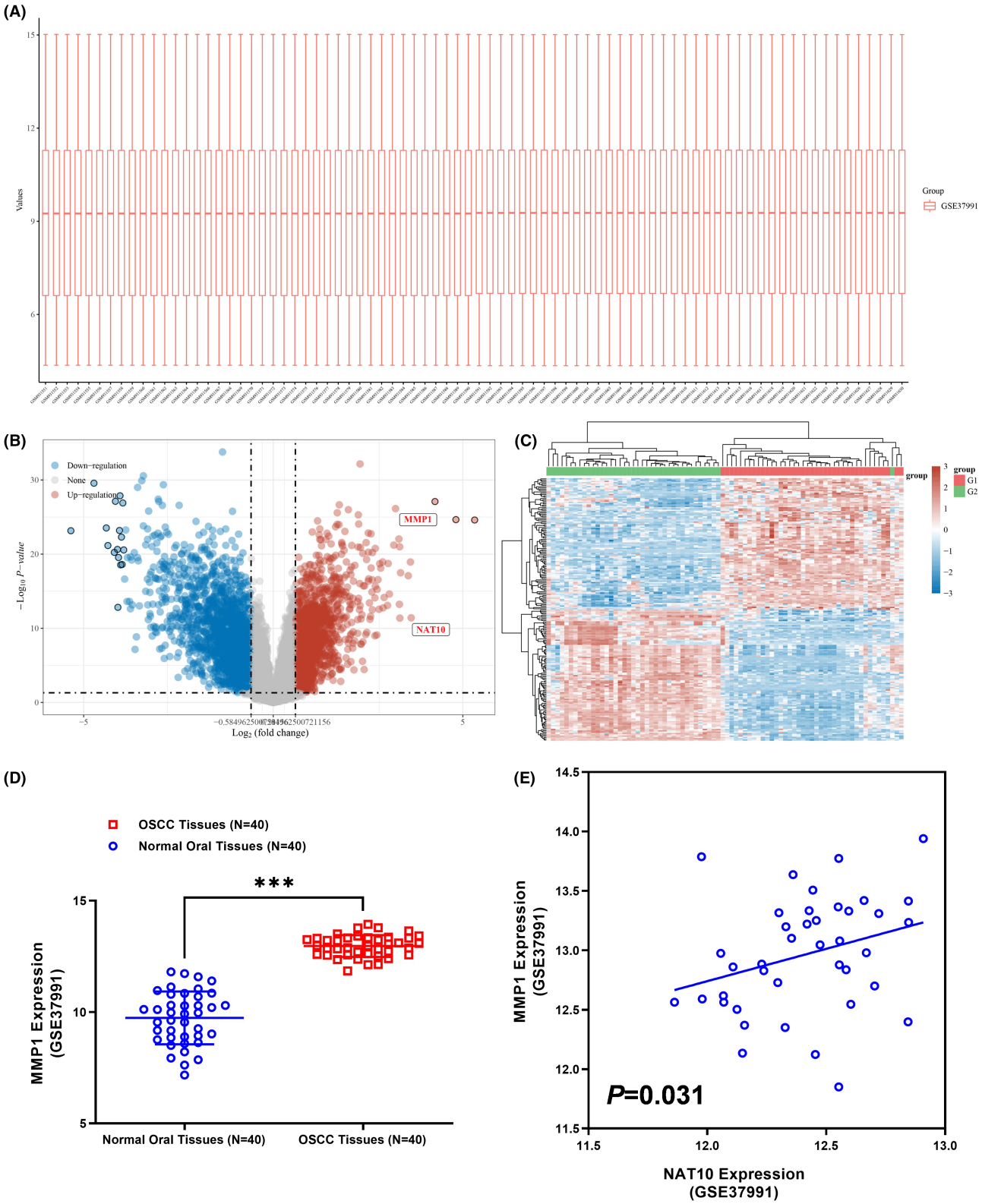

FIGURE 4 Analysis of dysregulated genes in oral squamous cell carcinoma (OSCC) tissues from the GEO database (GSE37991). (A) Boxplot of the normalized data. Different colors represent different datasets. Rows represent samples, and columns represent the gene expression values in the samples. (B) Volcano plot constructed using the fold change values and *p*-adjust. Red dots indicate upregulated genes; blue dots indicate downregulated genes. (C) Heatmap of the differential gene expression; different colors represent the trend of gene expression in different tissues. Top 50 upregulated genes and top 50 downregulated genes are shown. (D) Expression levels of MMP1 in OSCC were analyzed in the GEO database (GSE37991). (E) Correlation of expression levels of NAT10 and MMP1 in OSCC were analyzed in the GEO database (GSE37991). ***$p < 0.001$.

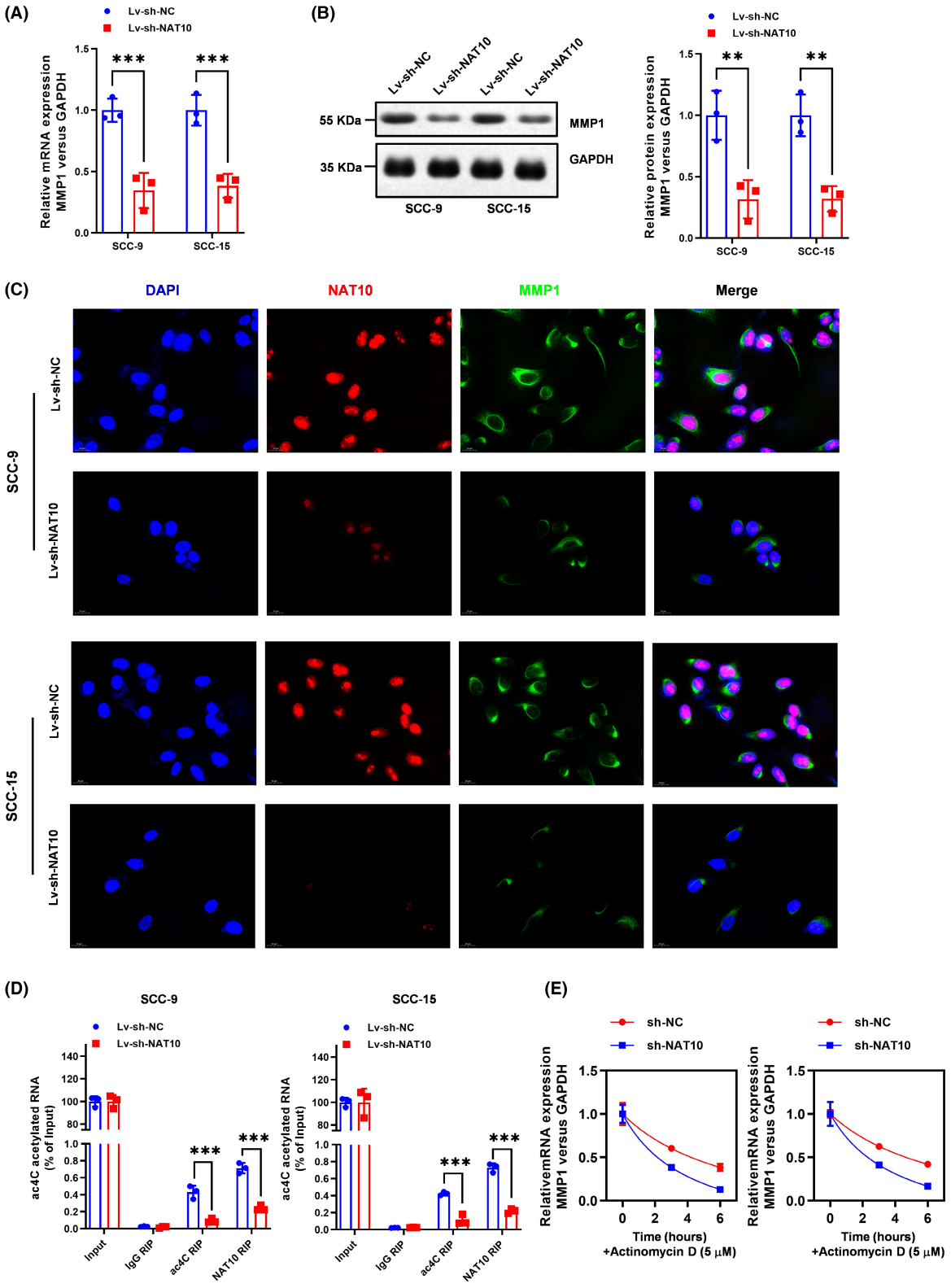

FIGURE 5 N-acetyltransferase 10 (NAT10) knockdown impaired the mRNA stability of MMP1 and decreased its expression in oral squamous cell carcinoma cells. (A) Real-time PCR was used to analysis the expression of MMP1 in the Lv-sh-NAT10 groups of SCC-9 and SCC-15 cells compared to the sh-NC groups. (B) Decreased protein levels of MMP1 in the Lv-sh-NAT10 groups of SCC-9 and SCC-15 cells were then verified using western blot analysis. (C) Decreased protein levels of MMP1 in the Lv-sh-NAT10 groups of SCC-9 and SCC-15 cells were then verified using immunofluorescence staining. (D) Total N4-acetylcytidine (ac4C) acetylated MMP1 mRNA levels were analyzed by ac4C RNA immunoprecipitation (RIP). (E) Percentages of remaining mRNA versus mRNA levels after the addition of actinomycin D (0, 1, 3, and 6 h) were quantified to analyze the mRNA stability of MMP1 mRNA. $^{**}p < 0.01$, $^{***}p < 0.001$. Lv-sh-NC, letivirus negative control group; Lv-sh-NAT10, NAT10 knockdown group.

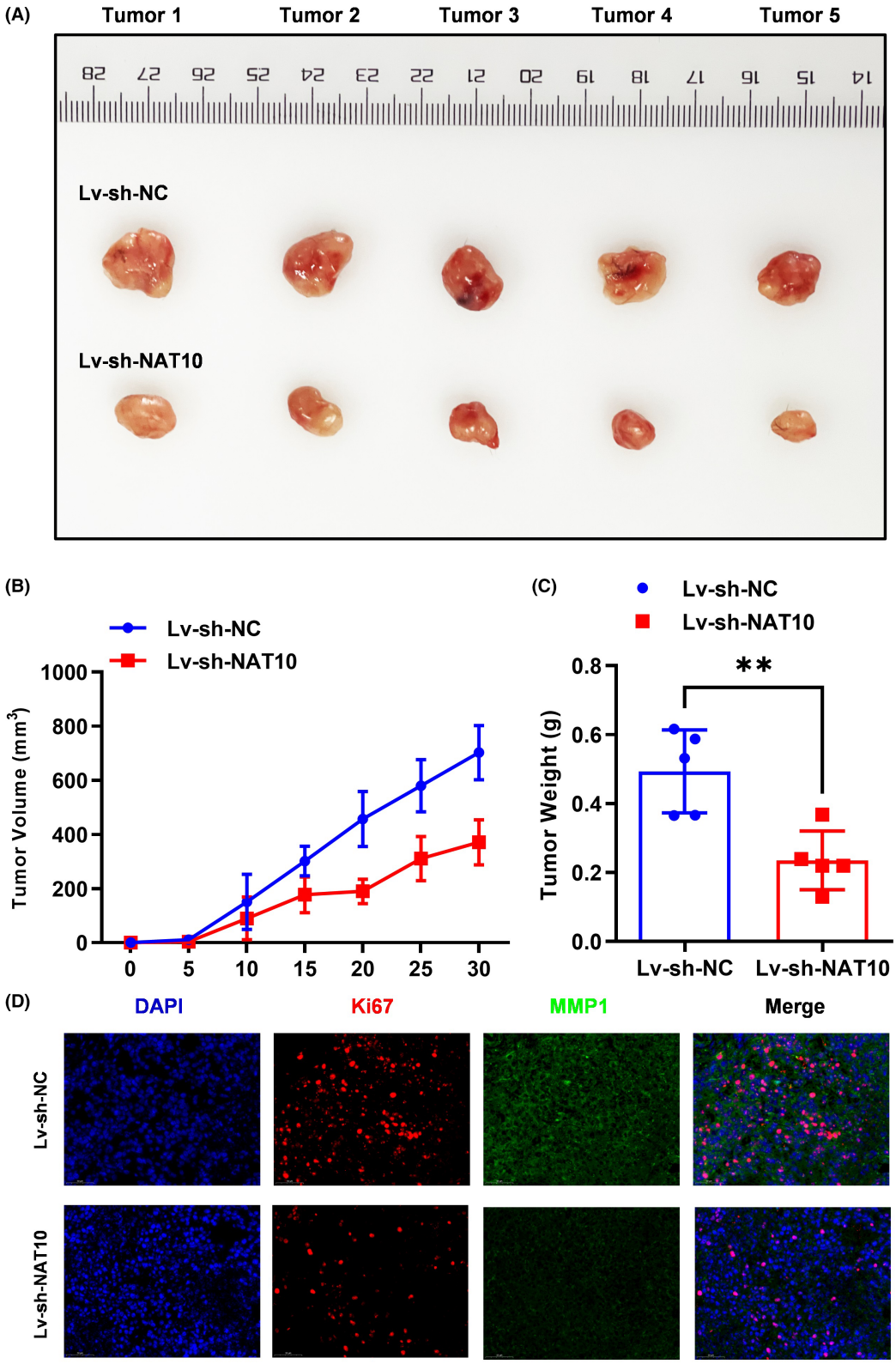

FIGURE 6 N-acetyltransferase 10 (NAT10) knockdown impaired the tumorigenesis ability of oral squamous cell carcinoma cells in the xenograft model. (A) Subcutaneous transplantation tumor model was used to evaluate NAT10 knockdown on the tumorigenesis of CC cells. The generated tumors from SCC-9 (Lv-sh-NC and Lv-sh-NAT10) cells were isolated and pictured. (B) A growth curve of tumors generated from SCC-9 cells was plotted every 5 days after subcutaneous injection. (C) Weight of tumors generated from SCC-9 cells was analyzed. (N) Double immunofluorescence staining showed that NAT10 knockdown significantly reduced the Ki-67-positive cells (red signal) in the generated tumors, accompanied by decreased MMP1 expression (green signal). $^{**}p < 0.01$. Lv-sh-NC, letivirus negative control group; Lv-sh-NAT10, NAT10 knockdown group.

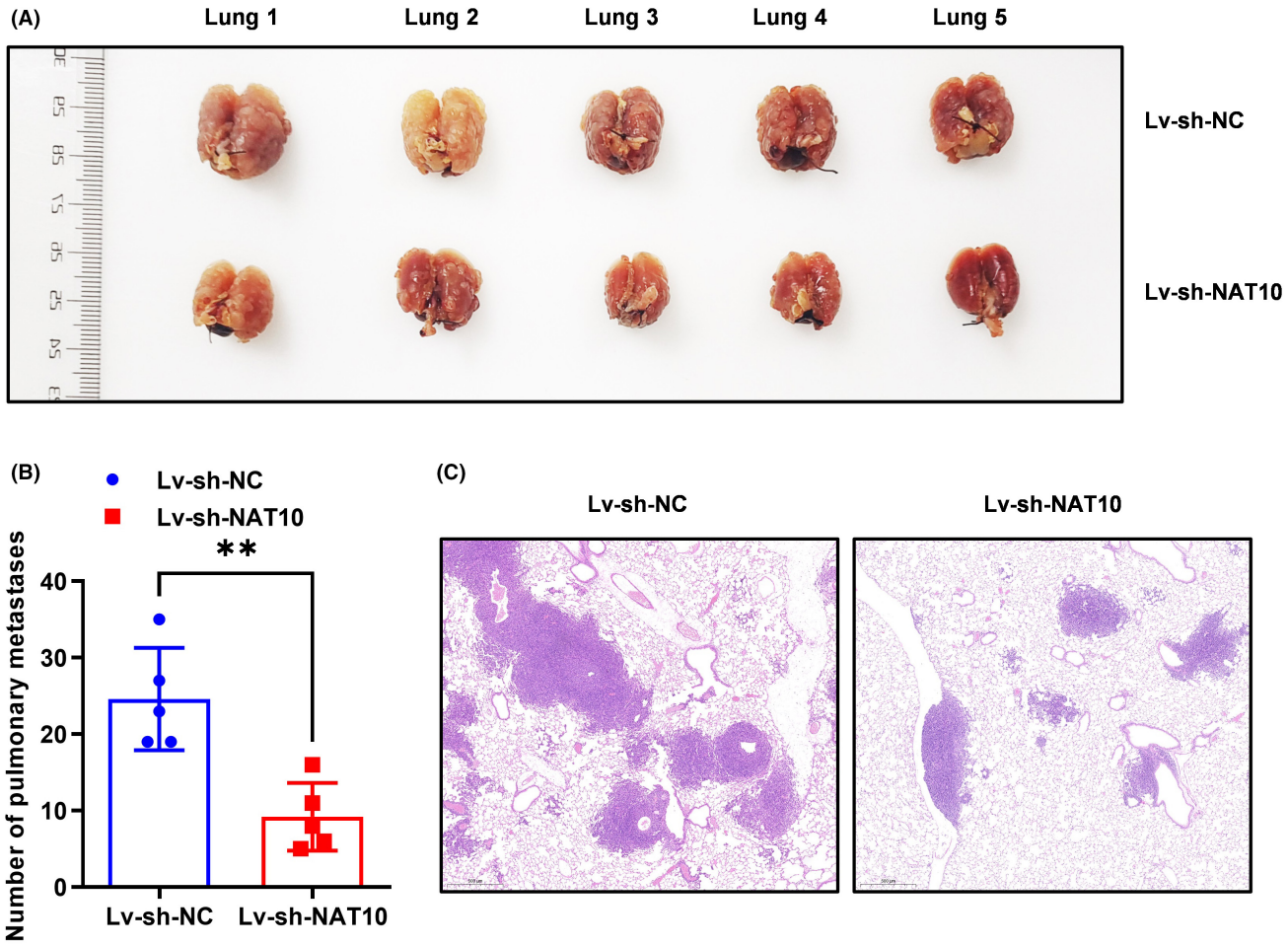

FIGURE 7 N-acetyltransferase 10 (NAT10) knockdown impaired the tumor metastasis ability of oral squamous cell carcinoma cells in the xenograft model. (A) Subcutaneous lung metastasis tumor model was used to evaluate NAT10 knockdown on the metastasis of SCC-9 cells. The generated pulmonary metastases from SCC-9 (Lv-sh-NC and Lv-sh-NAT10) cells were isolated and pictured. (B) Number of pulmonary metastases is shown. (C) Histopathological diagnosis was carried out using H&E staining to show pulmonary metastases. $^{**}p < 0.01$. Lv-sh-NC, letivirus negative control group; Lv-sh-NAT10, NAT10 knockdown group.

adding actinomycin D (0, 1, 3, and 6 h). After quantification, it was found that the mRNA stability of MMP1 was significantly reduced in NAT10-knockdown SCC-9 and SCC-15 cells (Figure 5E). These results suggest that NAT10 knockdown impaired MMP1 mRNA stability and decreased its expression in OSCC cells.

3.4 | N-acetyltransferase 10 knockdown impaired tumorigenesis and metastasis ability of OSCC cells in xenograft model

To investigate the impact of NAT10 on the tumorigenic potential of OSCC cells in vivo, a subcutaneous transplantation tumor model was established. Notably, NAT10-knockdown SCC-9 cells were used to create the subcutaneous transplantation tumor model in nude mice. The generated tumors are shown in Figure 6A, which shows a smaller tumor in the NAT10-knockdown group. It was also found that NAT10 knockdown resulted in significant decreases in tumor growth (Figure 6B) and tumor weight (Figure 6C) in OSCC cells. Consistently, the double immunofluorescence staining showed that NAT10 knockdown significantly reduced the Ki-67-positive cells (red signal) in the generated tumors, accompanied by decreased MMP1 expression (green signal), indicating a decreased tumorigenesis ability of SCC-9 cells (Figure 6D). Like the tumorigenesis results (Figure 7A), NAT10 knockdown also significantly inhibited the number of pulmonary metastases generated by SCC-9 cells (Figure 7B,C) in the lung metastasis xenograft model. Collectively, these results suggested that NAT10 knockdown impaired the tumorigenesis and metastasis ability of OSCC cells in the xenograft model.

3.5 | N-acetyltransferase 10 knockdown impaired proliferation, migration, and invasion abilities in OSCC cell lines in an MMP1-dependent manner

To identify the critical role of MMP1 in the NAT10-knockdown impaired cellular biological functions in OSCC cells, we rescued the expression of MMP1 in the vector control and NAT10-knockdown

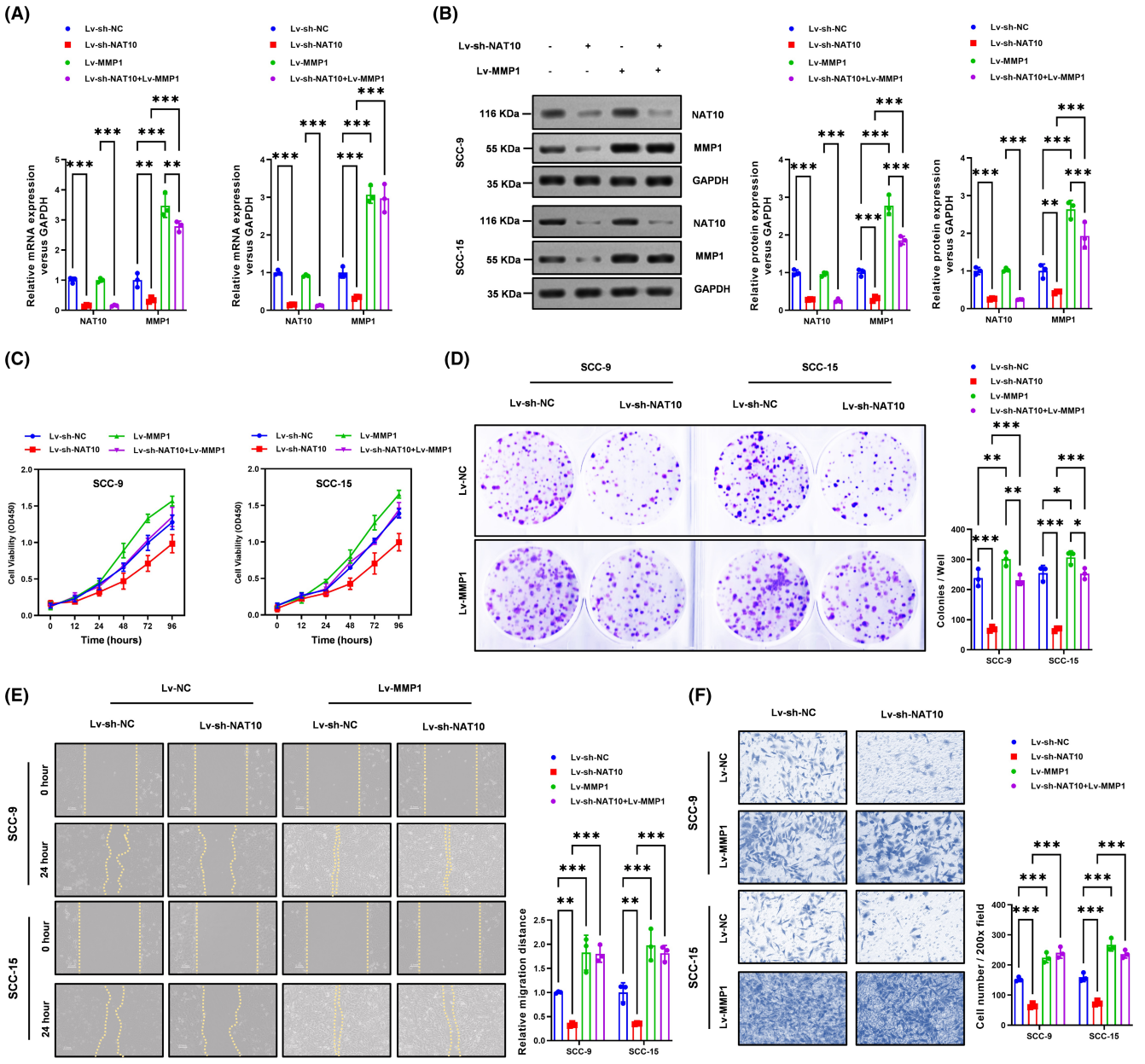

FIGURE 8 N-acetyltransferase 10 (NAT10) knockdown impaired the proliferation, migration, and invasion abilities in oral squamous cell carcinoma cell lines in an MMP1-dependent manner. (A) Real-time PCR was used to verify the rescue efficiency of MMP1 in vector or NAT10-knockdown SCC-9 and SCC-15 cells. (B) Rescue efficiency of MMP1 in vector or NAT10-knockdown SCC-9 and SCC-15 cells was then verified using western blot analysis. (C) CCK-8 assay was used to analyze the effect of MMP1 overexpression on the cell proliferation ability in vector or NAT10-knockdown SCC-9 and SCC-15 cells. (D) Colony formation assays were used to verify the effect of MMP1 overexpression on the cell proliferation ability in vector or NAT10-knockdown SCC-9 and SCC-15 cells. (E) Effect of MMP1 overexpression on the cell migration ability in vector or NAT10-knockdown SCC-9 and SCC-15 cells was evaluated using wound healing assay. (F) Effect of MMP1 overexpression on the cell invasion ability in vector or NAT10-knockdown SCC-9 and SCC-15 cells was analyzed by Transwell invasion assay. $^{**}p < 0.01$, $^{***}p < 0.001$. Lv-sh-NC, letivirus negative control group; Lv-sh-NAT10, NAT10 knockdown group; OD450, optical density at 450 nm.

OSCC cell lines (SCC-9 and SCC-15). The rescue efficiency of MMP1 expression was confirmed by real-time PCR (Figure 8A) and western blot analysis (Figure 8B). Interestingly, MMP1 overexpression promoted cell proliferation (Figure 8C,D), migration (Figure 8E), and invasion abilities in OSCC cells. Furthermore, MMP1 overexpression reversed the NAT10 impaired cell proliferation (Figure 8C,D), migration (Figure 8E), and invasion abilities in OSCC cells. These results indicated that NAT10 knockdown impaired the proliferation, migration, and invasion abilities in OSCC cell lines in an MMP1-dependent manner.

4 | DISCUSSION

Oral squamous cell carcinoma is a primary intraoral tumor that is prone to early distant metastasis and recurrence, with a relatively

short overall survival period. The treatment of OSCC includes surgery, chemotherapy, and radiotherapy, but most patients have poor prognoses.[15] The pathogenesis of OSCC is not clear.[16] It is the focus of OSCC research to clarify its pathogenesis and development mechanism at the molecular level.

Emerging evidence indicates that epigenetic modification is closely related to the pathological progress of cancers, such as m6A methylation, histone modification, and DNA methylation.[17,18] Similar to the regulation of m6A methylation modification,[19] ac4C acetylation modification is a conserved chemical modification in which the N4 site of cytosine is acetylation under the action of RNA acetyl transferase.[4] N-acetyltransferase 10, a lysine acetyltransferase, is a member of the GCN5-related N-acetyltransferase (GNAT) family.[20] In recent years, it has been found that NAT10 is the only enzyme known to mediate ac4C acetylation, whose mediated acetylation modification has been confirmed to be involved in the occurrence and development of a variety of diseases.[21] Previous studies have shown that NAT10 is associated with Hutchinson–Gilford progeria, and inhibition of NAT10 expression can restore the nuclear/plasma ratio of nonclassical nuclear import transportin-1 (TNPO1), thereby improving the symptoms of patients with progeria.[22] N-acetyltransferase 10 participates in the DNA damage repair process by mediating histone acetylation of P53 and the DNA damage reaction protein MORC2.[6] Recently, it has been reported that NAT10 can catalyze the acetylation modification of mRNA and participate in the occurrence and development of a variety of malignant tumors.[12] For example, the expression level of NAT10 in gastric cancer is significantly upregulated by acetylation of COL5A1, which significantly promotes the metastasis and epithelial–mesenchymal transition process of gastric cancer cells.[13] N-acetyltransferase 10 drives cisplatin chemoresistance by enhancing ac4C-associated DNA repair in bladder cancer.[14] Increased ac4C acetylation levels also promote osteogenic differentiation of bone marrow mesenchymal stem cells, and NAT10 could be a molecular target for the treatment of osteoporosis.[23] Although the effect of NAT10-mediated ac4C RNA acetylation on the development of OSCC is not well understood, we found that NAT10 expression was markedly elevated in OSCC tissues compared to normal oral tissues. By using lentivirus-mediated knockdown of NAT10, we observed a significant reduction in cell proliferation, migration, and invasion of two OSCC cell lines (SCC-9 and SCC-15). Moreover, NAT10 knockdown impaired the tumorigenesis and metastasis ability of OSCC cells in a xenograft model. These results suggested an oncogene role of NAT10 in OSCC.

The degradation of the basement membrane and the invasion of the underlying connective tissue by tumor cells are the key steps for the local invasion and distant metastasis of epithelial malignant tumors.[24] A variety of proteolytic enzymes participate in this process, and MMPs play an important role in this process.[25] The *MMP1* gene is the main proteolytic enzyme in the MMP family and can specifically degrade collagen fibers of types I, II, III, VII, X, and XI.[26] It has been confirmed that the *MMP1* gene is overexpressed in a variety of malignant tumors and predicts a worse prognosis. It has been confirmed that *MMP1* is overexpressed in esophageal squamous cell carcinoma, colorectal carcinoma, cervical carcinoma, bladder cancer carcinoma, lung adenocarcinoma, laryngeal squamous cell carcinoma, pancreatic cancer, ovarian cancer, lung squamous cell carcinoma and OSCC, and predicts a worse prognosis, which could be an important molecular marker of these tumors.[27–31] However, the regulation mechanism of MMP1 expression in tumors, especially in OSCC, largely remain unclear. In the present study, we found that MMP1 was significantly upregulated in OSCC tissues, which was a potential target of NAT10. Knockdown of NAT10 dramatically decreased the total and ac4C acetylated levels of MMP1 mRNA. Furthermore, NAT10 knockdown significantly decreased MMP1 mRNA stability and impaired the proliferation, migration, and invasion abilities in OSCC cell lines in an MMP1-dependent manner.

In conclusion, our results suggest an oncogene role of NAT10, and targeting ac4C acetylation could be a potential and attractive therapeutic target in the treatment of OSCC.

ACKNOWLEDGMENTS

Not applicable.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ETHICS STATEMENT

Approval of the research protocol by an institutional review board: Approved by the Ethical Committee of the First Affiliated Hospital of Nanchang University.

Informed consent: Informed consent was obtained from each participant prior to surgery.

Registry and the registration no. of the study/trial: N/A.

Animal studies: Approved by the Ethical Committee of the First Affiliated Hospital of Nanchang University.

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REFERENCES

1. Siegel RL, Miller KD, Wagle NS, Jemal A. Cancer statistics, 2023. *CA Cancer J Clin*. 2023;73(1):17-48.
2. Shanmugam DK, Anitha SC, Najimudeen RA, Saravanan M, Arockiaraj J, Belete MA. Conspectus on nanodiagnostics as an incipient platform for detection of oral potentially malignant disorders and oral squamous cell carcinoma. *Int J Surg*. 2023;109:542-544.
3. Sun J, Tang Q, Yu S, et al. F. Nucleatum facilitates oral squamous cell carcinoma progression via GLUT1-driven lactate production. *EBioMedicine*. 2023;88:104444.
4. Thomas JM, Briney CA, Nance KD, et al. A chemical signature for cytidine acetylation in RNA. *J Am Chem Soc*. 2018;140(40):12667-12670.
5. Tsai K, Jaguva Vasudevan AA, Martinez Campos C, Emery A, Swanstrom R, Cullen BR. Acetylation of cytidine residues boosts HIV-1 gene expression by increasing viral RNA stability. *Cell Host Microbe*. 2020;28(2):306-312.e306.
6. Liu HY, Liu YY, Yang F, et al. Acetylation of MORC2 by NAT10 regulates cell-cycle checkpoint control and resistance to DNA-damaging

chemotherapy and radiotherapy in breast cancer. *Nucleic Acids Res*. 2020;48(7):3638-3656.
7. Liu X, Cai S, Zhang C, et al. Deacetylation of NAT10 by Sirt1 promotes the transition from rRNA biogenesis to autophagy upon energy stress. *Nucleic Acids Res*. 2018;46(18):9601-9616.
8. Cao Y, Yao M, Wu Y, Ma N, Liu H, Zhang B. N-acetyltransferase 10 promotes micronuclei formation to activate the senescence-associated secretory phenotype machinery in colorectal cancer cells. *Transl Oncol*. 2020;13(8):100783.
9. Liu X, Tan Y, Zhang C, et al. NAT10 regulates p53 activation through acetylating p53 at K120 and ubiquitinating Mdm2. *EMBO Rep*. 2016;17(3):349-366.
10. Tao W, Tian G, Xu S, Li J, Zhang Z, Li J. NAT10 as a potential prognostic biomarker and therapeutic target for HNSCC. *Cancer Cell Int*. 2021;21(1):413.
11. Balmus G, Larrieu D, Barros AC, et al. Targeting of NAT10 enhances healthspan in a mouse model of human accelerated aging syndrome. *Nat Commun*. 2018;9(1):1700.
12. Zhang X, Chen J, Jiang S, et al. N-acetyltransferase 10 enhances doxorubicin resistance in human hepatocellular carcinoma cell lines by promoting the epithelial-to-mesenchymal transition. *Oxid Med Cell Longev*. 2019;2019:7561879.
13. Zhang Y, Jing Y, Wang Y, et al. NAT10 promotes gastric cancer metastasis via N4-acetylated COL5A1. *Signal Transduct Target Ther*. 2021;6(1):173.
14. Xie R, Cheng L, Huang M, et al. NAT10 drives cisplatin Chemoresistance by enhancing ac4C-associated DNA repair in bladder cancer. *Cancer Res*. 2023;83(10):1666-1683.
15. Sun L, Kang X, Wang C, et al. Single-cell and spatial dissection of precancerous lesions underlying the initiation process of oral squamous cell carcinoma. *Cell Discov*. 2023;9(1):28.
16. Li X, Wang C, Zhang H, et al. circFNDC3B accelerates vasculature formation and metastasis in oral squamous cell carcinoma. *Cancer Res*. 2023;83:1459-1475.
17. Li T, Zhuang Y, Yang W, et al. Silencing of METTL3 attenuates cardiac fibrosis induced by myocardial infarction via inhibiting the activation of cardiac fibroblasts. *FASEB J*. 2021;35(2):e21162.
18. Mathiyalagan P, Adamiak M, Mayourian J, et al. FTO-dependent N(6)-Methyladenosine regulates cardiac function during remodeling and repair. *Circulation*. 2019;139(4):518-532.
19. Li X, Yang Y, Chen S, Zhou J, Li J, Cheng Y. Epigenetics-based therapeutics for myocardial fibrosis. *Life Sci*. 2021;271:119186.
20. Larrieu D, Britton S, Demir M, Rodriguez R, Jackson SP. Chemical inhibition of NAT10 corrects defects of laminopathic cells. *Science*. 2014;344(6183):527-532.
21. Arango D, Sturgill D, Alhusaini N, et al. Acetylation of cytidine in mRNA promotes translation efficiency. *Cell*. 2018;175(7):1872-1886.e1824.
22. Larrieu D, Vire E, Robson S, Breusegem SY, Kouzarides T, Jackson SP. Inhibition of the acetyltransferase NAT10 normalizes progeric and aging cells by rebalancing the Transportin-1 nuclear import pathway. *Sci Signal*. 2018;11(537):eaar5401.
23. Yang W, Li HY, Wu YF, et al. ac4C acetylation of RUNX2 catalyzed by NAT10 spurs osteogenesis of BMSCs and prevents ovariectomy-induced bone loss. *Mol Ther Nucleic Acids*. 2021;26:135-147.
24. Niranjan R, Kishor S, Kumar A. Matrix metalloproteinases in the pathogenesis of dengue viral disease: involvement of immune system and newer therapeutic strategies. *J Med Virol*. 2021;93(8):4629-4637.
25. Zinter MS, Delucchi KL, Kong MY, et al. Early plasma matrix metalloproteinase profiles. A novel pathway in pediatric acute respiratory distress syndrome. *Am J Respir Crit Care Med*. 2019;199(2):181-189.
26. Naveed SU, Clements D, Jackson DJ, et al. Matrix Metalloproteinase-1 activation contributes to airway smooth muscle growth and asthma severity. *Am J Respir Crit Care Med*. 2017;195(8):1000-1009.
27. Li F, Fang Z, Zhang J, et al. Identification of TRA2B-DNAH5 fusion as a novel oncogenic driver in human lung squamous cell carcinoma. *Cell Res*. 2016;26(10):1149-1164.
28. Lodge W, Zavortink M, Golenkina S, et al. Tumor-derived MMPs regulate cachexia in a Drosophila cancer model. *Dev Cell*. 2021;56(18):2664-2680.e2666.
29. Pajuelo-Lozano N, Alcala S, Sainz B Jr, Perona R, Sanchez-Perez I. Targeting MAD2 modulates stemness and tumorigenesis in human gastric cancer cell lines. *Theranostics*. 2020;10(21):9601-9618.
30. Wada Y, Shimada M, Murano T, et al. A liquid biopsy assay for non-invasive identification of lymph node metastases in T1 colorectal cancer. *Gastroenterology*. 2021;161(1):151-162.e151.
31. Zhu S, Yang N, Niu C, et al. The miR-145-MMP1 axis is a critical regulator for imiquimod-induced cancer stemness and chemoresistance. *Pharmacol Res*. 2022;179:106196.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Liu Y, Huang H, Zhang C-b, Fan H-n. N-acetyltransferase 10 promotes the progression of oral squamous cell carcinoma through N4-acetylcytidine RNA acetylation of MMP1 mRNA. *Cancer Sci*. 2023;114:4202-4215. doi:10.1111/cas.15946