**Hsa_circ_0006677 regulates special AT-rich binding protein-2-mediated tumor-suppressive effect via functioning as a miR-1245a sponge in non-small cell lung cancer**

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**ABSTRACT**
Non-small cell lung cancer (NSCLC) is still one of the most challenging malignant tumors. Deregulation of circular RNAs (circRNAs) is associated with NSCLC progression. However, the regulatory mechanism of circRNAs in NSCLC still needs to be studied. We selected a differentially expressed hsa_circ_0006677 (circ_0006677) in NSCLC through analyzing the GSE158695 and GSE112214 datasets. Expression of circ_0006677 was evaluated by real-time quantitative polymerase-chain reaction (RT-qPCR). Effects of circ_0006677 overexpression on NSCLC cell proliferation, apoptosis, migration, invasion, and stemness were determined by clonogenic, 5-ethynyl-2'-deoxyuridine (EdU), flow cytometry, transwell, and sphere formation assays. The regulatory mechanism of circ_0006677 was predicted by bioinformatics analysis and verified by dual-luciferase reporter and RIP assays. Animal experiments were carried out to validate the function of circ_0006677 in vivo. We observed the downregulation of circ_0006677 in NSCLC samples and cells. Functionally, circ_0006677 overexpression decreased xenograft tumor growth and restrained NSCLC cell proliferation, invasion, migration, stemness, and induced NSCLC cell apoptosis in vitro. Molecular mechanism experiments exhibited that circ_0006677 functioned as a miR-1245a sponge and mediated SATB2 expression through adsorbing miR-1245a. Either miR-1245a overexpression or SATB2 knockdown weakened circ_0006677 overexpression-mediated repression on proliferation, invasion, migration, and stemness. In conclusion, circ_0006677 regulated SATB2-mediated tumor-suppressive effect via acting as a miR-1245a sponge in NSCLC, providing a new mechanism for understanding the progression of NSCLC.

**Introduction**
Non-small cell lung cancer (NSCLC), which makes up to 80% of lung cancer cases, is an aggressive disease with a poor prognosis [1]. Approximately 25% of patients are diagnosed with NSCLC disease amenable to potentially radical surgery [2]. Despite this, the five-year survival rate for stage I NSCLC patients undergoing radical resection is 50% to 70%, while that for stage IIIA patients is 10% to 30% [3]. Agents targeting NSCLC-driven mutations have revolutionized the approach to patients with metastatic disease [4]. Therefore, exploring the molecular mechanisms that drive NSCLC progression is essential for the development of new targeted therapeutic agents [5].

Recent studies have shown the contribution of circular RNAs (circRNAs) in the developmental and pathological processes [6]. CircRNAs, a novel class of transcripts, are characterized by covalently linked 3’-5’ ends. The biogenesis of circRNAs via the back-splicing mechanism differs from the standard splicing mechanism used to produce linear RNA [7]. This closed structure endows them with resistance to exonuclease degradation and attracts researchers to them because of their potential to become targets for the treatment of diseases [8,9]. An important feature of circRNA is its microRNA (miRNA) sponge function, which can effectively inhibit the activity of miRNA by binding to miRNA, resulting in affecting the expression of downstream genes and ultimately
participating in various diseases [10]. For instance, circRNA-cRAPGEF5 exerts a suppressive effect on renal cell carcinoma growth and metastasis through elevating TXNIP expression via sequestering miR-27a-3p [11]. Another example is circulatory carrier family 8 member A1, which acts as an endogenous miR-130b/miR-494 sponge to inhibit bladder cancer progression by increasing phosphatase and tensin homolog expression [12]. Researchers now recognize that circRNAs act as pivotal molecular regulators in NSCLC [13]. Nevertheless, the regulatory mechanism of circRNAs in NSCLC needs to be further elucidated.

Here, circ_0006677, derived from the WD repeat domain 78 (WDR78) gene on chr1:67,356,836–67,371,058, was selected as a differentially expressed circRNA in NSCLC based on bioinformatics analysis (the GSE158695 and GSE112214 datasets). This study aims to explore whether circ_0006677 is involved in the progression of NSCLC by functioning as a sponge for miRNA. We identified a novel mechanism by which circ_0006677 repressed NSCLC cell malignancy and stemness via regulating Special AT-rich binding protein-2 (SATB2) expression through function as a miR-1245a sponge.

Materials and methods

**Patient specimens**

The NSCLC tumor samples and corresponding non-tumor samples (n = 55) obtained during tumor resection surgery in the Civil Aviation General Hospital were stored at −80°C until use. All sample collections were done following a protocol approved by the Ethics Committee of the Civil Aviation General Hospital. All the participants gave their written informed consent before they entered the study.

**Cell culture**

Human bronchial epithelial cell line 16 HBE (#CL-0249) and lung cancer cell lines H1299 (#CL-0165), A549 (#CL-0016), HCC827 (#CL-0094), and H460 (#CL-0299) were obtained from Procell (Wuhan, China). Except the A549 cell line was maintained in Ham’s F-12 K medium (Thermo, Waltham, MA, USA), other cell lines were cultured in RPMI-1640 medium (Thermo). The growth conditions for these cells were in an incubator with 5% carbon dioxide at 37°C. To maintain cell growth, these media need to be supplemented with 10% FBS (Thermo) and 1% Penicillin–Streptomycin (Sigma-Aldrich, St. Louis, MO, USA).

**RNA preparation**

The total RNA extraction kit (Solarbio, Beijing, China) was utilized for the extraction of total RNA from collected samples and cultured cells as per manufacturer’s instructions. The active motif’s nuclear extract kit (Carlsbad, CA, USA) was used for the fractionation of nuclear and cytoplasmic RNA from cultured cells following the manufacturer’s instructions.

**RNase R digestion**

RNase R digestion was performed as previously described [14] using RNase R (Epicenter Technologies, USA) at 37°C for 30 min. Three independent experiments were done in triplicate.

**Real-time quantitative polymerase chain reaction (RT-qPCR)**

Generation of cDNA was conducted using the script RT reagent kit (Takara, Tokyo, Japan) for circRNA and mRNA. For miRNA, reverse transcription was done using the miRCURY LNA™ Universal RT microRNA PCR system (Exiqon, Aarhus, Denmark). Amplifications were carried out using the Lightcycler 480 (Roche, Basel, Switzerland) in triplicate with an SYBR Premix Ex Taq II (Takara). Relative fold change was evaluated using the equation 2−ΔΔCt [15], and all genes were normalized to the housekeeping gene β-actin or U6. Primer sequences were listed in Table 1.

**Plasmid construction**

To construct the circ_0006677 overexpression plasmid, the cDNA of circ_0006677 was cloned into the pLO5-ciR vector (Geneseed, Guangzhou,
China). The lentivirus-containing short hairpin RNA (shRNA) targeting SATB2 (sh-SATB2) was generated using the pLKO.1 vector (Addgene, Cambridge, MA, USA), and sh-NC was used as a control. Production of lentiviral particles and transduction of NSCLC cells (H1299 and A549) were performed as described [16,17].

**miRNA mimic and inhibitor**

The Lipofectamine RNAiMAX Reagent (Thermo) was utilized for the transfection of NSCLC cells with miR-1245a mimic (5'-AAGUGAUUAAAGGCCUACAU-3') and miR-NC (5'-CCGUAGAACGTGACUGCAAC-3') as per manufacturer's instructions.

**Clonogenic assay**

Clonogenic assay was conducted as the report of Mehta et al. [18]. The transfected NSCLC cells (5 × 10^2 cells/well) were allowed to grow on 6-well plates for 14 days, followed by washing with PBS (Thermo). After staining with 0.25% crystal violet (Solarbio), the colonies (more than 50 cells) were captured and counted by a microscope (Olympus, Tokyo, Japan).

**5-ethyl-2′-deoxyuridine (EdU) assay**

The EdU incorporation assay was carried out using Yefluor 488 EdU Imaging Kit (Yesen, Shanghai, China) following the manufacturer's instructions. The EdU-positive cells were captured using an Olympus fluorescence microscope.

**Measurement of the caspase 3 activity**

Detection of caspase-3 activity was performed using the caspase-3/CPP32 colorimetric analysis kit (Bio Vision, USA) following the manufacturer's steps. In brief, the supernatant of the lysed cells was diluted and added to the reaction tube, followed by the addition of reaction buffer and cetyl-Asp-Glu-Val-Asp p-nitroanilide substrate (DEVD-pNA). After incubating for 1 h, the optical density at 405 nm was measured in a microtiter plate reader (BioTek, Winooski, VT, USA).

**Flow cytometry assay**

Detection of cell apoptosis was conducted using the Annexin V-fluorescein isothiocyanate/Propidium Iodide kit (Keygen, Nanjing, China) according to the producer's guidelines. The flow cytometer (Becton Dickinson, NJ, USA) was used to detect the cells.

**Transwell assay**

Analysis of cell migration and invasion was performed using transwell assays as previously described [19]. Harvested NSCLC cells (1 × 10^5/chamber) re-suspended in a 200 μL of cell medium-serum free were added into the upper compartment of 24-well transwell chambers (Corning, NY, USA) without or with Matrigel (Corning), and 600 μL of complete medium was added to the bottom compartment. The cells were allowed to grow for 24 h. Migrating and invading cells fixed with 4% paraformaldehyde (Solarbio) and stained with 0.5% crystal violet (Solarbio) were captured and counted with a microscope (Olympus).

**Sphere formation assay**

The experiment was performed as previously described [20]. Single-cell suspensions (2.5 × 10^3/well) were seeded onto a 24-well ultra-low attachment plate (Corning) in serum-free medium supplemented with 20 ng/mL epidermal growth factor (Sigma-Aldrich), 20 ng/mL fibroblast growth factor (Sigma-Aldrich), 4 mg/mL heparin (Sigma-Aldrich), and 2% B27 (Sigma-Aldrich). Fourteen

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**Table 1. Primers for RT-qPCR used in the study.**

| Genes     | Primer sequences (5'-3')                                                                 |
|-----------|------------------------------------------------------------------------------------------|
| circ_000677 | Forward (F): 5’-AGACCTGAAGAAGACATCTC-3’  
               Reverse (R): 5’-CTGGTTGTGATTGCACGGT-3’  |
| β-actin   | F: 5’-CGGCTGATGATCTGCTG-3’  
               R: 5’-AGGAAGAGGCGAAGCG-3’  |
| U6        | F: 5’-CTGGCTGACGCGACA-3’  
               R: 5’-AACGCTGACGCGACA-3’  |
| GAPDH     | F: 5’-GAGATCGACAGCGACTTCTTCT-3’  
               R: 5’-GCGCCAATATCGACACCAATC-3’  |
| SATB2     | F: 5’-CTGGCTCTCTCCTGGCTTCTG-3’  
               R: 5’-CTGCTTTGATTTGCACCAGT-3’  |
| miR-1245a | F: 5’-CCGGAAAGATGCATCTAAAGGC-3’  
               R: 5’-CTCAACTGGTGTCGTGGAGT-3’  |
days later, the number of sphere formations was counted under a microscope (Olympus).

**Western blotting**

Isolation of total protein from collected samples and cultured cells was performed using radioimmunoprecipitation assay lysis with protease and phosphates inhibitor (Sigma-Aldrich). Western blotting was carried out as previously described [21]. Equal amount of protein extracts was loaded into 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by transferring onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA), which was then blocked with 5% skim milk powder. The membrane was probed with antibodies against proliferating cell nuclear antigen (PCNA) (ab92552, Abcam, Cambridge, MA, USA), E-cadherin (E-ca) (ab40772, Abcam), N-cadherin (N-ca) (ab18203, Abcam), SATB2 (ab92446, Abcam), matrix metalloproteinase (MMP)-2 (ab92536, Abcam), MMP-9 (ab76003, Abcam), t-MEK (#4694, Cell Signaling Technology, MA, USA), p-MEK (#3958, Cell Signaling Technology), t-ERK (#9102, Cell Signaling Technology), p-ERK (#8544, Cell Signaling Technology), and β-actin (ab8224, Abcam). Membranes were then incubated with a secondary antibody (Abcam). Protein bands were viewed using an enhanced chemiluminescence detection system with a chemiluminescence horseradish peroxidase (HRP) Substrate (Millipore).

**Dual-luciferase reporter assay**

The experiment was done according to the report by Wei et al. [22]. The recombinant luciferase reporters circ_0006677 wild type (WT), circ_0006677 mutant (MUT), SATB2 3’ untranslated region (UTR) WT, and SATB2 3’UTR MUT were constructed using the pmirGLO vector (Promega, Madison, WI, USA), respectively. NSCLC cells were co-transfected with pRL-TK, recombinant luciferase reporter, and miR-NC or miR-1245a mimic. Luciferase activities of firefly luciferase and Renilla luciferase were measured using the dual-luciferase reporter system (Promega) after 48 h of transfection.

**RIP assay**

The RIP assay was used to analyze the binding between circ_0006677 and miR-1245a using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) according to the manufacturer’s instructions. The co-precipitated RNA complex was extracted and purified with the RNeasy kit (Qiagen, Valencia, CA, USA), followed by subjecting to RT-qPCR analysis.

**Xenograft assay**

Animal experiments were approved by the Animal Ethics Committee of the Civil Aviation General Hospital. Xenograft assay was done as previously depicted [23]. Twelve BALB/c male nude mice (4 weeks old, 15–20 g) (Vital River Laboratory, Beijing, China) were reared in a highly clean SPF environment. A549 cells (5 × 10^6) stably overexpression of circ_0006677 in 100 μL of PBS were subcutaneously injected into the back of each nude mouse (n = 6 mice per group), and A549 cells carrying sh-NC were used as controls. Tumor volume was recorded once a week [(length × width^2)/2]. After 28 days, the mice were sacrificed and their xenograft tumors were stripped.

**Immunohistochemical (IHC) staining**

IHC assay was conducted based on the report of Xu et al. [24]. Xenograft tumors fixed with 4% formaldehyde were embedded in paraffin and cut into 4 μm sections. The sections were deparaffinized, rehydrated, antigen retrieval, and blocked, followed by incubation of antibodies against SATB2 (ab92446, Abcam), PCNA (ab92552, Abcam), and Bax (ab32503, Abcam). After incubation with HRP-conjugated secondary antibody, the sections were stained with 3,3’-Diaminobenzidine and hematoxylin, followed by viewing under a microscope (Olympus).

**Statistical analysis**

All experiments were repeated at least three times. Results, which were presented as mean ± standard deviation, were analyzed using GraphPad Prism
version 8 software (GraphPad, La Jolla, CA, USA). Comparisons of numerical values were performed using Student’s *t*-test or analysis of variance. A difference was considered statistically significant when *P* < 0.05.

**Results**

**Circ_0006677 was prominently underexpressed in NSCLC**

To explore the role of circRNAs in NSCLC, we first searched for differentially expressed circRNAs in NSCLC through analyzing the GSE158695 and GSE112214 datasets. Venn diagram showed that circ_0006677 was the only downregulated differentially expressed circRNA with a change greater than 4 in both two datasets (Figure 1(a)). And the expression of circ_0006677 in the GSE158695 and GSE112214 datasets was shown in Figure 1(b, c). Circ_0006677 is derived from the WDR78 gene on chr1:67,356,836–67,371,058, resulting from back-splicing of exons 2, 3, and 4 (473 bp) (Figure 1(d)). We then verified that circ_0006677 was prominently underexpressed in NSCLC via detection of circ_0006677 expression in 55 NSCLC samples (Figure 1(e)). Compared to the 16HBE cell line, NSCLC cell lines exhibited an overt reduction in circ_0006677 expression (Figure 1(f)). Moreover, linear WDR78 was severely degraded after RNase R incubation, but circ_0006677 was hardly affected by this enzyme (Figure 1(g,h)). The subcellular localization of circ_0006677 in NSCLC cells was analyzed. Data in Figure 1(i,j) displayed that the majority of circ_0006677 localized to the cytoplasm. Collectively, low circ_0006677 expression might be related to NSCLC tumorigenesis.

**Circ_0006677 overexpression decreased NSCLC cell malignancy and stemness**

Subsequently, we constructed the circ_0006677 overexpression plasmid to further analyze the function of circ_0006677 in NSCLC. RT-qPCR demonstrated the transfection efficiency of the circ_0006677-overexpressing plasmid (Figure 2(a)). Clonogenic and EdU assays showed that circ_0006677 overexpression decreased the proliferative capability of NSCLC cells (Figure 2(b,c)). In contrast, circ_0006677 overexpression significantly increased the caspase 3 activity in NSCLC cells and promoted the apoptosis of NSCLC cells (Figure 2(d,e)). Transwell assays exhibited that exogenetic expression of circ_0006677 restrained the migrating and invading capabilities of NSCLC cells (Figure 2(f,g)). Sphere formation assay displayed that the number of spheroids in the circ_0006677-overexpressing group was reduced significantly (Figure 2(h)). Overexpression of circ_0006677 reduced N-cad and PCNA protein levels and increased E-cad protein levels in NSCLC cells (Figure 2(i,j)). In addition, there were lower protein levels of MMP-2 and MMP-9 in circ_0006677-overexpressed NSCLC cells than the control cells (Figure 2(k,l)). Together, these results suggested that circ_0006677 overexpression decreased NSCLC cell malignancy and stemness.

**Circ_0006677 served as a miR-1245a sponge**

Considering the importance of circRNAs as miRNA sponges, we conducted bioinformatics analysis (Circular RNA Interactome) [25]. By consulting the literature [26], it was found that circ_0006677 may be combined with miR-1245a (Figure 3(a)). To validate this prediction, we used miR-1245a mimic to overexpress miR-1245a in NSCLC (Figure 3(b)). Luciferase reporter assays showed that a remarkable reduction in luciferase reporter activity was detected in NSCLC cells co-transfected with miR-1245a mimic and the circ_0006677 WT reporter, but not that of the circ_0006677 MUT reporter (Figure 3(c,d)). RIP assay exhibited that circ_0006677 and miR-1245a could be amplified from the complex precipitated by the anti-Ago2 antibody, suggesting that circ_0006677 bound with miR-1245a via Ago2 (Figure 3(e,f)). Additionally, we also detected miR-1245a expression in NSCLC samples, and the results exhibited a prominent elevation in miR-1245a expression in NSCLC samples compared to corresponding non-tumor samples (Figure 3(g)). And Pearson’s correlation coefficient analysis exhibited a negative correlation between circ_0006677 and miR-1245a expression in NSCLC samples (Figure 3(h)). Homoplastically,
Figure 1. The expression and characteristics of circ_0006677 in NSCLC. (a) Venn diagram showing the overlap of downregulated differentially expressed circRNA in the GSE158695 and GSE112214 datasets with a fold change of more than 4. (b and c) Relative expression of circ_0006677 in the GSE158695 and GSE112214 datasets. (d) Schematic illustration of circ_0006677. (e and f) RT-qPCR analysis of circ_0006677 expression in NSCLC samples and cell lines. (g and h) RT-qPCR analysis of the levels of circ_0006677 and WDR78 mRNA in total RNA with or without RNase R incubation. (i and j) The subcellular localization of circ_0006677 in NSCLC cells. *P < 0.05.
Figure 2. Impacts of circ_0006677 overexpression on NSCLC cell malignancy and stemness. (a) RT-qPCR exhibiting the transfection efficiency of the circ_0006677 overexpression plasmid. (b-i) NCCLC cells transfected with circ_0006677 or vector were utilized. (b and c) Clonogenic and EdU assays detection of the proliferation of NCCLC cells. (d) Analysis of the caspase 3 activity in NSCLC cells. (e) Flow cytometry assay evaluation of the apoptosis of NCCLC cells. (f and g) Transwell assays analysis of the migration and invasion of NCCLC cells. (h) Sphere formation assay detection of the stemness of NCCLC cells. (i and j) Western blotting showing N-ca, PCNA, and E-ca protein levels in NSCLC cells. (k and l) Western blotting showing MMP-2 and MMP-9 protein levels in NSCLC cells. *P < 0.05.
miR-1245a was overexpressed in NSCLC cells (Figure 3(i)). Also, circ_0006677 overexpression decreased miR-1245a expression, but this decrease was weakened by co-transfection with circ_0006677 and miR-1245a mimic (Figure 3(j)). Together, these results suggested that circ_0006677 functioned as a miR-1245a sponge.

**Circ_0006677 exerted its anti-tumor effect via repressing miR-1245a**

Whether circ_0006677 functions as a tumor suppressor gene through sequestering miR-1245a was further validated. We co-transfected the circ_0006677 overexpression plasmid and miR-1245a mimic into NSCLC to determine whether the tumor-inhibiting effect of circ_0006677 could be impaired by miR-1245a overexpression. We observed that introduction of miR-1245a mimic partly overturned the decreased proliferation of NSCLC cells mediated by circ_0006677 overexpression (Figure 4(a,b)). Moreover, the elevated caspase 3 activity and apoptosis rate in circ_0006677-overexpressing NSCLC cells were partly antagonized by introduction of miR-1245a mimic (Figure 4(c,d)). Ectopic expression of miR-1245a also attenuated the inhibiting effects of circ_0006677 upregulation on NSCLC cell migration, invasion, and spheroid formation (Figure 4(e-g)). In addition, the decreased N-cad and PCNA protein levels and the elevated E-cad protein levels in circ_0006677-overexpressing cells were reversed by exogenous expression of miR-1245a (Figure 4(h,i)). In addition, the downregulated protein levels of MMP-2 and MMP-9 in circ_0006677-overexpressed NSCLC cells were impaired after miR-1245a upregulation (Figure 4(j,k)). Altogether, circ_0006677 repressed NSCLC cell malignancy and stemness via sequestering miR-1245a.
Validation of SATB2 as a target of miR-1245a

To gain further insight into the mechanism by which miR-1245a regulates NSCLC cell malignancy and stemness, we predicted SATB2 was a target of miR-1245a using online prediction databases (TargetScan, miRDB, and miRWalk). Their binding sites were exhibited in Figure 5(a). Luciferase assays exhibited that co-transfection of the SATB2 3'UTR WT reporter with miR-1245a mimic caused an overt decrease in luciferase activity, while co-transfecting the SATB2 3'UTR MUT reporter and miR-1245a mimic did not change (Figure 5(b,c)). We also observed an obvious reduction in SATB2 mRNA expression in NSCLC samples (Figure 5(d)), and its expression was negatively correlated with miR-1245a (Figure 5(e)) and positively correlated with circ_0006677 (Figure 5(f)). Moreover, downregulated SATB2 protein levels were also downregulated in miR-1245a mimic-transfected NSCLC cells (Figure 5(i)). Additionally, circ_0006677 overexpression led to a distinct elevation in SATB2 protein levels, but this effect was overturned by co-transfecting...
with miR-1245a mimic (Figure 5(j)). Collectively, these results suggested that miR-1245a directly targeted SATB2.

**Circ_0006677 played its anti-tumor effect via upregulating SATB2**

To confirm the SATB2-dependent effect of circ_0006677 on NSCLC cell malignancy and stemness, we designed a short hairpin RNA targeting SATB2 and its interference efficiency was demonstrated by Western blotting (Figure 6(a)). Indeed, SATB2 knockdown reversed circ_0006677 overexpression-mediated repression on cell proliferation (Figure 6(b,c)), elevation on cell caspase 3 activity (Figure 6(d)), and promotion on cell apoptosis in NSCLC cells (Figure 6(e)). Silencing of SATB2 was indeed able to re-establish the migrating and invading capacities (Figure 6(f,g)), as well as the spheroid formation capacity of circ_0006677-overexpressing NSCLC cells (Figure 6(h)). In addition, SATB2 inhibition overturned the changes in N-ca, PCNA, and E-ca protein levels in NSCLC cells mediated by overexpression of circ_0006677 (Figure 6(i, j)). And SATB2 silencing partly overturned the decreased protein levels of MMP-2 and MMP-9 in NSCLC cells prompted by circ_0006677 overexpression (Figure 6(k,l)). In sum, circ_0006677 restrained NSCLC cell malignancy and stemness by SATB2.

**Circ_0006677 mediated the MEK/ERK via the miR-1245a/SATB2 axis**

Because oncogenic KRAS mutations often occur in NSCLC and cause the activation of the MEK/ERK pathway, we explored whether circ_0006677 mediates the MEK/ERK pathway through the miR-1245a/SATB2 axis. Data in Figure 7(a,b) exhibited that circ_0006677 overexpression resulted in a reduction in the activity of MEK and ERK, but
Figure 6. Circ_0006677 elevated SATB2 expression to restrain NSCLC cell malignancy and stemness. (a) Western blotting analysis of the interference efficiency of sh-SATB2. (b-h) The proliferation (b and c), caspase 3 activity (d), apoptosis (e), migration (f), invasion (g), and sphere formation (h) of NSCLC cells after transfection with circ_0006677 combined with or without sh-SATB2. (i and j) Relative protein levels of E-cà, PCNA, and N-ca in NSCLC cells after transfection with circ_0006677 combined with or without sh-SATB2. (k and l) Western blotting analysis of the protein levels of MMP-2 and MMP-9 in the above NSCLC cells. *P < 0.05.

Figure 7. Circ_0006677 mediated the MEK/ERK via the miR-1245a/SATB2 axis. (a and b) Western blotting analysis of the protein levels of t-MEK, p-MEK, t-ERK, and p-ERK in NSCLC cells with vector, circ_0006677, circ_0006677+ miR-NC, circ_0006677+ miR-1245a, miR-1245a+sh-NC, or circ_0006677+ SATB2. *P < 0.05.
these changes were weakened by miR-1245a overexpression or SATB2 silencing. These results suggested that circ_0006677 overexpression could block the MEK/ERK pathway through the miR-1245a/SATB2 axis.

**Circ_0006677 decreased xenograft tumor growth in vivo**

We further ascertained the cancer-repressing role of circ_0006677 in NSCLC through constructing xenograft models in vivo. Mice injected with A549 cells stably overexpressing circ_0006677 had smaller tumor volume and lighter tumor weight compared with the control group (Figure 8(a-c)). Tumors derived from mice injected with A549 cells stably overexpressing circ_0006677 had higher levels of circ_0006677 and SATB2 and lower levels of miR-1245a compared with the control group (Figure 8(d)). Also, the tumors in the circ_0006677 group had higher SATB2 and E-cad protein levels and lower PCNA and N-cad protein levels compared with the vector group (Figure 8(e)). Furthermore, the protein levels of MMP-2 and MMP-9 were lower in tumor samples of the circ_0006677-overexpression group than those from the vector group (Figure 8(f)). IHC analysis showed a significant decrease in PCNA expression and an overt increase in SATB2 and Bax expression in the circ_0006677 group compared with the vector group (Figure 8(g)). Collectively, circ_0006677 decreased xenograft tumor growth in vivo.

**Discussion**

It is becoming increasingly clear that circRNAs play significant roles in NSCLC. Nevertheless, their roles in NSCLC remain largely unknown. In this research, our findings demonstrated that reduced expression of circ_0006677 released miR-1245a with concomitant downstream downregulation of SATB2, resulting in the facilitation of NSCLC cell proliferation, migration, invasion, and stemness. Thus, circ_0006677 upregulation might represent a potential therapeutic strategy for NSCLC.

*Figure 8. Circ_0006677 reduced NSCLC cell growth in vivo. (a) Growth curves of tumor volume in different groups. (b) Representative images of xenograft tumors in different groups. (c) The tumor weight of xenograft tumors in different groups. (d) Relative levels of circ_0006677, miR-1245a, and SATB2 mRNA in xenograft tumors. (e) Relative protein levels of SATB2, E-cad, PCNA, and N-cad in xenograft tumors. (f) Relative protein levels of MMP-2 and MMP-9 in xenograft tumors. (g) IHC analysis of the expression of PCNA, SATB2, and Bax in xenograft tumors. *P < 0.05.*
Here, this study selected a downregulated differentially expressed circRNA circ_0006677 with a change greater than 4 in both GSE158695 and GSE112214 datasets. A recent study reported the downregulation of circ_0006677 in NSCLC, and circ_0006677 could repress NSCLC cell glycolysis, proliferation, migration, and invasion via sequestering miR-578 with concomitant downstream upregulation of SOS2 [27]. Analogously, a distinct downregulation in circ_0006677 expression was observed in NSCLC samples and cells in this study. In line with the report of Yang et al. [27], overexpression of circ_0006677 repressed NSCLC cell proliferation, migration, and invasion in vitro. In addition, our data also exhibited the repressive effect of circ_0006677 overexpression on NSCLC cell stemness. Furthermore, circ_0006677 overexpression decreased xenograft tumor growth, and PCNA and N-ca levels were lower in xenograft tumors derived from circ_0006677-overexpressing NSCLC cells, but E-cad and Bax levels were higher, suggesting that circ_0006677 could repress NSCLC cell growth in vivo. These results primarily suggested the inhibiting function of circ_0006677 in NSCLC.

Up to now, a series of studies have demonstrated that circRNAs can exert their function by acting as competing endogenous RNAs (ceRNAs) [28]. miR-1245a has been reported to exert an oncogenic role in breast cancer [29] and colon adenocarcinoma [30] progression by downregulating encoded by breast-cancer susceptibility gene 2 (BRCA2). In NSCLC, circ-4-hydroxylase subunit beta downregulation reduced BRCA2 expression via releasing miR-1245a, thus promoting NSCLC progression [26]. By extracting the cytoplasm and nucleus of NSCLC cells, circ_0006677 was validated to be mainly distributed in the cytoplasm, indicating that circ_0006677 might be a ceRNA. Importantly, we identified that circ_0006677 could interact with miR-1245a in NSCLC through luciferase and RIP assays. Ectogenic expression of miR-1245a weakened the inhibiting effects of circ_0006677 overexpression on NSCLC cell proliferation, migration, invasion, and stemness. Our findings demonstrated that circ_0006677 exerted an anti-tumor function in NSCLC by repressing miR-1245a activity.

SATB2, a transcription factor, can regulate gene expression through serving as a transcriptional cofactor and modulating chromatin architecture [31]. SATB2 has been uncovered as a tumor-inhibiting gene in colorectal cancer [32], and clear cell renal cell cancer [33], but it exerts an oncogenic effect on glioblastoma [34], hepatocellular cancer [35], oral squamous cell cancer [36], and osteosarcoma [37]. Also, SATB2 could repress epithelial-mesenchymal transition (EMT) through decreasing G9a and histone methylation in NSCLC [38]. Moreover, SATB2 could inhibit NSCLC initiation by suppressing stemness marker genes such as SRY-Box Transcription Factor 2 [39]. Our results exhibited low SATB2 levels in NSCLC samples and cells, and SATB2 was identified as a miR-1245a target. Interestingly, SATB2 could be regulated by circ_0006677, which functioned as a miR-1245a decoy. Importantly, SATB2 silencing whittled circ_0006677 overexpression mediated suppression on NSCCL cell proliferation, migration, invasion, and stemness. These findings prompted us to conclude that circ_0006677 exerts an anti-tumor effect in NSCLC at least in part through the ceRNA network, that is, circ_0006677 adsorbs miR-1245a to increase the expression of the anti-tumor gene SATB2.

Conclusion

This study demonstrated that circ_0006677 acts as an endogenous miR-1245a sponge and elevated SATB2 expression via adsorbing miR-1245a, leading to suppressing NSCLC cell malignancy and stemness. These findings provide a better understanding of the mechanism involved in NSCLC progression.

Disclosure statement

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