LncRNA NR2F2-AS1 induces epithelial-mesenchymal transition of non-small cell lung cancer by modulating BVR/ATF-2 pathway via regulating miR-545-5p/c-Met axis

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Abstract: Non-small cell lung cancer (NSCLC) is one type of the most common cancers, which results in the major death worldwide. This study focuses on the understanding of the molecular mechanism of lncRNA NR2F2-AS1 and its regulation on epithelial-mesenchymal transition (EMT) in the development of NSCLC. Expressions of lncRNA NR2F2-AS1, miR-545-5p, c-Met, biliverdin reductase (BVR), ATF-2 and EMT-related markers in NSCLC tissues and cells were measured by western blotting and RT-qPCR assays. The impact of lncRNA NR2F2-AS1 and miR-545-5p on the cell proliferation, migration, invasion and EMT were analyzed by CCK-8, colony formation, wound healing and transwell assays. The interactions among lncRNA NR2F2-AS1, miR-545-5p and c-Met predicted by bioinformatic analysis were evaluated through dual luciferase reporter assay and fluorescence in situ hybridization (FISH). After generating tumor xenografts, immunohistochemistry was utilized to measure the expression of Ki-67 and EMT-related proteins in vivo. Our results showed that lncRNA NR2F2-AS1, c-Met, BVR and ATF-2 were overexpressed while miR-545-5p was silenced in NSCLC tissues and cells. Silencing of lncRNA NR2F2-AS1 or upregulating miR-545-5p significantly inhibited the cell proliferation, migration, invasion and EMT process. The EMT process could be inhibited by suppressing c-Met/BVR/ATF-2 axis. The tumor xenograft experiments demonstrated that the tumor growth and EMT process were significantly inhibited by silencing lncRNA NR2F2-AS1 or overexpression of miR-545-5p in vivo. LncRNA NR2F2-AS1 promoted the NSCLC development through suppressing miR-545-5p to activate EMT process through c-Met/BVR/ATF-2 axis. Our study indicated that lncRNA NR2F2-AS1 and miR-545-5p could be used as potential therapeutic targets to improve NSCLC treatment.

Keywords: Non-small cell lung cancer, LncRNA NR2F2-AS1, MiR-545-5p, c-Met, biliverdin reductase, epithelial-mesenchymal transition

Introduction

As the most common reported cancer worldwide, lung cancer was estimated to have 520,000 new diagnosed cases in China 2008, of which 85% were non-small-cell lung cancer (NSCLC) [1, 2]. Over the past few decades, even the therapeutic strategies have been continued to develop, including surgery, radiotherapy, chemotherapy and immunotherapy, the treatment of lung cancer has no obvious improvement [3]. The patients with NSCLC are usually identified at an advanced stage [4]. In the process of epithelial-to-mesenchymal transition (EMT), the epithelial cells loss the polarization and cell-cell contacts and then obtain the features of mesenchymal-like cells [5, 6]. EMT plays a crucial role in the development of migration and invasion of NSCLC, resulting in high morbidity and mortality [5, 7]. To demon-
Furthermore, how does the BVR regulate the EMT process is another important topic to illustrate the impact of lncRNA NR2F2-AS1 in NSCLC. Kravets et al. reported that BVR activated the activating transcription factor-2 (ATF-2) in 293A cells [23]. The activation of ATF-2 predicted the poor prognosis and boosted EMT process in lung cancer cells by controlling the genes related to the EMT process [24]. Therefore, we believe it is important to discover the important role of lncRNA NR2F2-AS1 in NSCLC through targeting miR-545-5p and the followed EMT regulation by c-Met/BVR/ATF-2 axis.

LncRNA NR2F2-AS1/miR-545-5p/c-Met axis in NSCLC

In this work, we found the roles of lncRNA NR2F2-AS1 and miR-545-5p during the development of NSCLC. Our results demonstrated that lncRNA NR2F2-AS1 was overexpressed in NSCLC patients, which was related to the NSCLC stage and metastasis [14]. Recently, only one report indicated that lncRNA NR2F2-AS1 promoted tumourigenesis by regulating miR-320b in NSCLC [4]. However, the underlying mechanism of lncRNA NR2F2-AS1 in regulation the EMT and development of NSCLC is not fully investigated. MicroRNAs (miRNAs) are also involved in the development of NSCLC [15, 16]. For instance, Cui et al. recently reported that the miRNA-545 suppressed the development of NSCLC by targeting ZEB2 to suppress Wnt/β-catenin pathway [17]. Through the bioinformatic analysis, we found that lncRNA NR2F2-AS1 could bind with miR-545-5p. However, the relationship between lncRNA NR2F2-AS1 and miR-545-5p has not been discovered and their functions in the development of NSCLC are not clear.

The c-mesenchymal-epidermal transition factor (c-Met) is important in the progression and invasion of NSCLC [18]. C-Met was overexpressed in NSCLC patients and NSCLC cell lines. In pre-clinic and clinic trails, inhibition of c-Met showed anti-tumor activities in NSCLC [19]. Human biliverdin reductase (BVR) catalyzed biliverdin to bilirubin in the heme metabolism pathway, which was reported to regulate the EMT process [20, 21]. Also, it was reported that the c-Met could regulate the expression of BVR to affect the EMT and the invasion ability of cancer cells [22]. Through the bioinformatic analysis, miR-545-5p might bind with c-Met. However, to our best knowledge, no study has been conducted to illustrate the interaction of between miR-545-5p and c-Met in cancer progression. We proposed that miR-545-5p regulated the EMT process through regulating the expression of c-Met and downstream BVR in NSCLC.

Materials and methods

Tissues and cell culture

Thirty pairs of NSCLC and normal samples were collected from thirty patients from Harbin Medical University Cancer Hospital with their signed statement of informed consent. All the tissue samples were stored at -80°C in liquid nitrogen till further analyses. This work was approved by the Ethics Committee of the Harbin Medical University Cancer Hospital.

Three NSCLC cell lines including A549, H1650, H1299 and normal human bronchial epithelial cells 16HBE were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). RPMI 1640 medium (Gibco Laboratories, Grand Island, NY, USA) with 10% fetal bovine serum (FBS, Hyclone, Logan City, UT, USA), containing 100 U/mL penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO, USA), were used to culture the cells at 37°C with a humidified atmosphere of 5% CO₂. The 16HBE cells cultured in the same medium worked as a control cell line.
Cell transfection

To transfect A549 cells, the pcDNA3.1-NR2F2-AS1, shNR2F2-AS1, miR-545-5p mimics, miR-545-5p inhibitor, pcDNA3.1-ATF-2, siBVR, and pcDNA3.1-ATF-2 were ordered from GenePharma (Shanghai, China). After culturing A549 cells in 6-well plates at 37°C, 5% CO2 incubator to reach 60% confluence, Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used for the transfections following the manufactural protocols. The sequences of shNR2F2-AS1, siBVR and their negative controls were provided in Supplementary Materials File.

Bioinformatical analysis

The relationships among the expression levels of NR2F2-AS1, miR-545-5p and the survival rate of lung adenocarcinoma (LUAD) patients were analyzed by the online GEPIA database (http://gepia.cancer-pku.cn/) or StarBase database (http://starbase.sysu.edu.cn/index.php) respectively. The binding sites of the miR-545-5p on c-Met were analyzed by TargetScan 7.2 website (http://www.targetscan.org/vert_72/). The binding sites of miR-545-5p on NR2F2-AS1 were predicted using LncBase v.2 website (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2%2Findex).

Luciferase reporter assay

The wide-type or mutant of NR2F2-AS1 (WT/MUT-NR2F2-AS1) and c-Met (WT/MUT-c-Met) were firstly incorporated into the pmiRGL0 luciferase vector (Promega, Madison, WI). A549 cells were co-transfected with the miR-545-5p mimics, miR-545-5p inhibitor or their negative controls (NC) and the constructed pmiRGL0 luciferase vectors by Lipofectamine 2000 (Invitrogen). The luciferase activities were determined by the Dual-Glo Luciferase Assay System (Promega) after 48 h of incubation.

Fluorescence in situ hybridization (FISH) assay

The FISH assay was conducted using the Ribo IncRNA FISH Kit (Ribobio, Guangzhou, China) according to its instruction. The fluorescent labelled IncRNA NR2F1-AS1 and miR-545-5p probes were purchased from Ribobio as well. Briefly, the cells were fixed using 4% paraformaldehyde followed by the treatment of 0.5% Triton X-100 in PBS. Cells were prehybridized and then hybridized with 100 nL NR2F1-AS1 and miR-545-5p probes (Ribobio) in standard solution at 37°C overnight. The nucleus was counterstained with DAPI and observed using Zeiss Axio microscope (Carl Zeiss, Jena, Germany).

CCK-8 assay

CCK-8 assay was performed to measure the cell proliferation of A549 cells. Briefly, at 24 h after transfection, the A549 cells were treated with CCK-8 reagent (Sigma-Aldrich, St. Louis, MO, USA) and then set at 37°C for another 4 h. Then, the cell proliferation was accessed by the measurement of the O.D. value at 450 nm with a multifunction microplate reader (SpectraMax M5, Molecular Devices, San Jose, CA).

Colony formation assay

A549 cells after transfection were cultured in a 6-well plate for another 15 days. The number of colony formation was investigated by fixing these cells with methanol, then stained with 0.1% crystal violet. We took the images with the microscopy and count the visible colonies to assess the colony formation ability.

Wound healing assay

A549 cells were cultured in a 6-well plate and followed by the different transfections. Then, a wound was produced by scratching the monolayer cells using a 100 µL pipette tip. After gently washing the floating cells with PBS, the cells left in the plates were added with serum-free medium to incubate for another 24 h. The migrated cells from the leading edge were imaged and counted with a light microscopy.

Transwell assay

The invasion assay of A549 cells was conducted using the 24-well transwell. For the invasion assay, the 100,000 A549 cells were firstly cultured into the upper chamber modified with Matrigel (BD Sciences) in the RPMI 1640 medium with 10% FBS for 24 hours. After removing cells in the top chamber, we treated the cells invaded into the lower chamber with methanol and then stained with 1% crystal violet, following manually counting.
Tumor xenografts experiments in vivo

The BALB/c female mice with 6-8 weeks of age were obtained from Shanghai SLAC Laboratory Animal Center (Shanghai, China). All of the animals were cultured in the pathogen-free facility in the Harbin Medical University Cancer Hospital. All the experiments involved animals were approved by the Animal Care and Use Committee at Harbin Medical University Cancer Hospital.

The A549 cells stably transfected with shNC, shNR2F2-AS1, mimics NC, or miR-545-5p mimics were injected into BALB/c nude mice subcutaneously. Six mice were included for each group. The volume of the tumor was measured during the 24 days and the volumes of tumors were quantified using the equation: \(0.5 \times \text{length} \times \text{width}^2\). After 24 days, the mice were sacrificed to collect the tumor samples, and then weighed and photographed. The RT-qPCR, immunohistochemistry and western blot analyses were performed for the collected tumor tissues.

Immunohistochemistry analysis

Immunohistochemistry was utilized to quantify the expression levels of Ki-67, BVR, N-cadherin, and E-cadherin in the NSCLC tissues with different treatments. The tumor tissues were prepared in 4-μm slices and then deparaffinized in xylene and hydrated in gradient alcohol. Specific primary antibodies followed by biotinylated goat anti-rabbit IgG secondary antibody were incubated with sections with the standard procedure. All the antibodies were purchased from Abcam (1:500 in dilution). The images were captured using a light microscopy.

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

The mRNA expression levels were analyzed by RT-qPCR. Briefly, the total RNA was collected from the tissues and cells with TRIzol reagent (Takara, Japan). Then, complementary DNA (cDNA) was obtained using RealMasterMix First Strand cDNA Synthesis Kit (Tiangen). SYBR Premix ExTagTM (Takara) was used to perform RT-qPCR following the manufacturer’s protocols by Applied Biosystems 7500 Real-time PCR system. The primers sequences were purchased from Sangon Biotech Co., Ltd. GAPDH and U6 small nuclear RNA (U6 snRNA) were utilized as internal references for mRNA and miRNA, respectively. The relative expressions of targets were calculated by \(2^{-\Delta\Delta C_t}\) method.

Western blot analysis

We collected proteins from cells and tissues using RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for further analysis. The concentration of protein was determined with a bicinchoninic acid assay Kit (Thermo Fisher Scientific, Inc.). Then, we separated 40 μg proteins using 10% SDS-PAGE and transferred to PVDF membranes (Millipore Corporation, USA). Then, the membranes were blocked with 5% non-fat milk (BD Bioscience), the primary antibodies, including antibodies for E-cadherin (1:1000, ab1416, Abcam), N-cadherin (1:1000, ab76011, Abcam), vimentin (1:1000, ab92547, Abcam), MMP-2 (1:2000, ab86607, Abcam), slug (1:1500, ab27568, Abcam), snail (1:1000, ab216347, Abcam), c-Met (1:1000, ab2165-74, Abcam), BVR (1:1500, ab192925, Abcam), p-ATF2 (1:1000, ab32019, Abcam), ATF-2 (1:3000, ab239361, Abcam) and GAPDH (1:2000, ab8245, Abcam) were incubated overnight with the membranes at 4°C. The PVDF membranes were incubated with the secondary antibodies (goat anti-rabbit, 1:5000, ab6721; goat anti-mouse, 1:5000, ab6789, Abcam) for 1 h and then quantified by the ECL detecting system (Thermo Scientific, USA). All the antibodies were obtained from Abcam. The detailed information of antibodies were provided in Supplementary Table 1 of Supplementary Materials File. GAPDH was used as a loading control. Finally, Quantity One version 4.2.1 (Bio-Rad Laboratories, Inc.) was used to analyze the separated proteins.

Statistical analysis

All experiments were performed at least for three times in triplicate, with one representative experiment shown. The data were presented as the mean ± standard deviation (SD) and quantified with Prism 6.0 (GraphPad Software, USA). The Student’s t test was used to distinguish the difference between two groups. For the analysis of three or more groups, one-way analysis of variance (ANOVA) followed by
Tukey’s post hoc test was utilized. Spearman correlation analysis was conducted to analyze the relationship between NR2F2-AS1, miR-545-5p and c-Met in NSCLC tissues. The difference was recognized as significant if P < 0.05.

Results

LncRNA NR2F2-AS1 was overexpressed and promoted the development of NSCLC

To discover the importance of lncRNA NR2F2-AS1 in NSCLC, we firstly examined the expression level of lncRNA NR2F2-AS1 in cancer tissues obtained from NSCLC patients. After the analysis by RT-qPCR, we demonstrated that lncRNA NR2F2-AS1 in 30 NSCLC tissues was markedly overexpressed than in the normal tissues (Figure 1A). We also found that the survival time and survival rate were significantly enhanced when the expression of lncRNA NR2F2-AS1 was low in the patients’ tissues (Figure 1B). The NSCLC cell lines, including A549, H1650 and H1299, also showed up-regulated expression levels of lncRNA NR2F2-AS1 compared with the normal 16HBE cells (Figure 1C). To discover the roles of lncRNA NR2F2-AS1, A549 cells were transfected with pcDNA3.1-NR2F2-AS1 to overexpress the NR2F2-AS1 and three shNR2F2-AS1 sequences (#1-3) to downregulate NR2F2-AS1 (Figure 1D and 1E). shNR2F2-AS1-#2 exerted the most significant transfection effect, so we used #2 to perform the following experiments (Figure 1E). We tested the proliferation, migration and invasion abilities in each group by clone formation (Figure 1F), CCK-8 (Figure 1J), wound healing (Figure 1G), and transwell (Figure 1H) assays, respectively. The results showed that silencing NR2F2-AS1 in the A549 cells could significantly enhance the expression level of miR-545-5p (Figure 2E), which indicated the important interaction between NR2F2-AS1 and miR-545-5p. Next, a luciferase reporter assay was used to study the relationship between IncRNA NR2F2-AS1 and miR-545-5p. MiR-545-5p mimics significantly inhibited while miR-545-5p inhibitor increased the luciferase activity of wide type of NR2F2-AS, but no changes were found in the mutated NR2F2-AS1 group (Figure 2F). We also investigated the co-localization of the NR2F2-AS1 and miR-545-5p in the A549 cells using FISH assay. The results showed that both of them localized in the cytoplasm (Figure 2G). Furthermore, the results from analyzing the interaction between lncRNA NR2F2-AS1 and miR-545-5p in NSCLC tissues showed that lncRNA NR2F2-AS1 was reversely related to miR-545-5p in NSCLC tissues (Figure 2H). Taken together, we concluded that miR-545-5p was decreased in the NSCLC tissues and cells. Moreover, miR-545-5p has a reverse correlation with the expression of lncRNA NR2F2-AS1 in NSCLC.

Knockdown of lncRNA NR2F2-AS1 inhibited the NSCLC cell proliferation, migration, invasion and EMT by regulating miR-545-5p

Then, we investigated the impact of lncRNA NR2F2-AS1/miR-545-5p axis on the NSCLC cells’ proliferation, migration, invasion and
LncRNA NR2F2-AS1/miR-545-5p/c-Met axis in NSCLC

A

Relative expression level of NR2F2-AS1

Normal  Tumor

B

Overall survival

Low NR2F2-AS1 TPM
High NR2F2-AS1 TPM
Logrank p=0.093
HR(high)=1.3
p(HR)=0.094
n(high)=239
n(low)=239

C

Relative expression level of NR2F2-AS1

iHHBE  A549  H1650  H1299

D

Relative expression level

Control  pcDNA3.1-NC  pcDNA3.1-NR2F2-AS1  shNC  shNR2F2-AS1

E

Relative expression level of NR2F2-AS1

Control  shNC  shNR2F2-AS1#1  shNR2F2-AS1#2  shNR2F2-AS1#3

F

Number of colony

0 h  24 h

G

Migration rate (%)

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Figure 1. LncRNA NR2F2-AS1 was overexpressed and promoted the development of NSCLC. (A) RT-qPCR analysis of lncRNA NR2F2-AS1 in NSCLC tissues and normal tissues (n=30). (B) The relationship between NR2F2-AS1 expression and the survival time of the LUAD patients from GEPIA database. (C) RT-qPCR analysis of lncRNA NR2F2-AS1 expression in A549, H1650, H1299 and 16HBE cells. (D) The expression levels of lncRNA NR2F2-AS1 were analyzed with the treatment of pcDNA3.1-NC and pcDNA3.1-NR2F2-AS1. (E) The expression levels of lncRNA NR2F2-AS1 were analyzed with the treatment of shNC, shNR2F2-AS1-#1, shNR2F2-AS1-#2, shNR2F2-AS1-#3, respectively. (F) The clone formation assay was conducted to measure the cell proliferation ability. (G, H) Wound healing assay (G) and transwell assay (H) were carried out to investigate the effect of lncRNA NR2F2-AS1 on cell migration and invasion abilities. (I) Morphologic changes in cells after the different treatments. Scale bar: 20 μm. (J) CCK-8 assay was conducted to determine the cell proliferation ability. (K) Western blotting analysis of E-cadherin, vimentin, slug, N-cadherin, MMP-2, and snail in A549 cells after different treatments. The lanes from gels or blots have been grouped or rearranged, and the original images were provided in original materials. *P < 0.05, **P < 0.01 and ***P < 0.001.
LncRNA NR2F2-AS1/miR-545-5p/c-Met axis in NSCLC

EMT. Firstly, after the transfection of shNR2F2-AS1, the expression of NR2F2-AS1 was significantly decreased, and the co-transfection of inhibitor NC or miR-545-5p inhibitor had no impact on NR2F2-AS1 expression (Figure 3A). Moreover, the expression of miR-545-5p in A549 cells with the transfection of shNR2F2-AS1 was significantly enhanced as well as in the group co-transfected with inhibitor NC (Figure 3B). The co-transfection of miR-545-5p inhibitor partially lessened miR-545-5p expression in A549 cells (Figure 3B). Thereafter, we tested the cell proliferation, invasion and migration in each group by CCK-8, clone formation, transwell and wound healing assays, respectively (Figure 3C-F). The results showed that silencing IncRNA NR2F2-AS1 in A549 cells significantly suppressed the cell proliferation, migration and invasion abilities. But downregulating the expression of miR-545-5p by its inhibitor compromised the effects of the silencing IncRNA NR2F2-AS1 on the above results (Figure 3C-F). A549 cells had shortened synapses and appeared round following the silence of IncRNA NR2F2-AS1, but miR-545-5p inhibitor reversed this effect (Figure 3G). As shown in Figure 3H, E-cadherin was enhanced significantly, but N-cadherin,
LncRNA NR2F2-AS1/miR-545-5p/c-Met axis in NSCLC

A

Relative expression level of NR2F2-AS1

B

Relative expression level of miR-545-5p

C

Cell viability (%)

D

Control  shNC  shNR2F2-AS1  shNR2F2-AS1+ inhibitor NC  shNR2F2-AS1+ miR-545-5p inhibitor

E

0 h  24 h

Number of colon

Migration rate (%)

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Figure 3. Knockdown of IncRNA NR2F2-AS1 inhibited the NSCLC cell proliferation, migration, invasion and EMT by regulating miR-545-5p. (A) Detection of NR2F2-AS1 in A549 cells with different treatments. (B) Detection of miR-545-5p in A549 cells with different treatments. (C) CCK-8 assay was performed to evaluate the cell proliferation ability after the treatments. (D) The clone formation assay was conducted to study the effect of IncRNA NR2F2-AS1 on cell proliferation ability. (E, F) Wound healing assay (E) and transwell assay (F) were carried out to investigate the effect of IncRNA NR2F2-AS1 on cell migration and invasion abilities. (G) Morphologic changes in cells after the different treatments. Scale bar: 20 μm. (H) Western blotting analysis of E-cadherin, N-cadherin, vimentin, MMP-2, slug, and snail in A549 cells after different treatments. The lanes from gels or blots have been grouped or rearranged, and the original images were provided in original materials. *P < 0.05 and **P < 0.01.
vimentin, MMP-2, and transcription factors slug and snail were significantly decreased when the lncRNA NR2F2-AS1 was silenced. In contrast, the inhibition of miR-545-5p partially reversed the above effects (Figure 3H). Taking together, we summarized that lncRNA NR2F2-AS1 in NSCLC cells promoted the development of NSCLC cells through controlling miR-545-5p.

C-Met regulated by miR-545-5p was upregulated in NSCLC tissues and cells

We further explored the interaction between miR-545-5p and c-Met in NSCLC. In the NSCLC cancer tissues, RT-qPCR analysis showed enhanced expression of c-Met than in normal tissues (Figure 4A). Also, c-Met was overexpressed in NSCLC cell lines than that in the normal cell line, 16HBE (Figure 4B). We further studied the interaction between miR-545-5p and c-Met. A549 cells were treated with miR-545-5p mimics, miR-545-5p inhibitor or their negative controls, respectively (Figure 4C). The analysis in these groups demonstrated that upregulated miR-545-5p significantly inhibited c-Met expression (Figure 4D). In contrast, silence of miR-545-5p promoted the expression of c-Met in A549 cells (Figure 4D). The analysis through western blotting demonstrated that c-Met protein was decreased in the miR-545-5p mimics-transfected A549 cells but increased in the miR-545-5p inhibitor-transfected A549 cells (Figure 4E). Through the bioinformatric analysis using Targetscan, we proposed that miR-545-5p could bind to c-Met (Figure 4F). Therefore, we proved the interaction by a luciferase reporter assay. We found that miR-545-5p mimics decreased but the inhibitor increased the luciferase activity of the wide type of c-Met but not the mutated c-Met (Figure 4G). Furthermore, spearman correlation analysis proved that miR-545-5p was reversely related to c-Met in NSCLC tissues (Figure 4H). All these results indicated that miR-545-5p negatively regulated c-Met in NSCLC tissues and cells.

Overexpression of miR-545-5p suppressed cell proliferation, invasion, migration, and EMT by targeting c-Met in NSCLC

We investigated the role of the miR-545-5p/c-Met axis in NSCLC cells. MiR-545-5p was successfully up-regulated by transfecting miR-545-5p mimics. Transfection of pcDNA3.1-c-Met had no impact on the expression of miR-545-5p (Figure 5A). However, the up-regulated miR-545-5p in A549 cells significantly suppressed the expression of c-Met, but pcDNA3.1-c-Met vector reversed the results, which were measured by both RT-qPCR and western blotting (Figure 5B, 5C). The group with up-regulated miR-545-5p showed the significantly decreased cell proliferation rate (Figure 5D) and clone formation (Figure 5E) along with the decreased cell migration and invasion (Figure 5F, 5G) compared with the control group. On the contrary, the overexpression of c-Met significantly reversed the effect of up-regulating miR-545-5p on the capacity of A549 cells for proliferation, migration and invasion (Figure 5D-G). The morphological imaging of the cells after different treatments showed that up-regulating miR-545-5p inhibited the cell expansion, indicating by the significant change from spindle-shape to round of the cells. Overexpression c-Met reversed this effect (Figure 5H). Furthermore, we examined the EMT-related proteins by western blotting in each group. As shown in Figure 6A, E-cadherin was enhanced significantly when the miR-545-5p was overexpressed, but the effect was partially reversed by co-transfection with pcDNA3.1-c-Met. In contrast, N-cadherin, MMP-2, vimentin, slug and snail, as well as the BVR were significantly decreased when the miR-545-5p was overexpressed. Similarly, the overexpression of c-Met partially reversed the above impacts (Figure 6A). Because the c-Met regulated the EMT process through controlling the expression of BVR, we also monitored the level of BVR in A549 cells after adjusting lncRNA NR2F2-AS1 and miR-545-5p. As demonstrated in Figure 6B, BVR was significantly down-regulated when lncRNA NR2F2-AS1 was suppressed. However, the inhibiting miR-545-5p could partially reverse this effect. Based on this, we therefore tested the expression level of BVR in normal and tumor tissues using RT-qPCR and immunohistochemistry methods. The results showed that BVR was up-regulated in tumor tissues than that in normal tissues (Figure 6C, 6D). Taking together, we found that overexpression of miR-545-5p might inhibit the cell proliferation, migration and invasion abilities of NSCLC cells through inhibiting EMT process by suppressing the expression of c-Met, along with suppressing the expression of BVR.
Silencing BVR inhibited cell proliferation, migration, invasion and EMT through controlling ATF-2 in NSCLC

Furthermore, we investigated the function of BVR in the A549 cells. As shown in Figure 7A, BVR was successfully down-regulated by the transfection of siBVR, and siBVR-#1 transfection most obviously decreased the BVR mRNA level. Therefore, siBVR-#1 transfection was applied in the further experiments. Down-regulation of BVR obviously inhibited the expression levels of ATF-2 and p-ATF-2 through western blotting analysis (Figure 7B). To discover

Figure 4. c-Met regulated by miR-545-5p was upregulated in NSCLC tissues and cells. (A) RT-qPCR analysis of c-Met in different tissues (n=30). (B) RT-qPCR analysis of c-Met expression in A549, H1650, H1299, and 16HBE. (C, D) The expression of miR-545-5p (C) and c-Met (D) were analyzed by RT-qPCR analysis after transfecting with mimics NC, miR-545-5p mimics, inhibitor NC, and miR-545-5p inhibitor. (E) The level of c-Met was analyzed by western blotting analysis after treatment of mimics NC, miR-545-5p mimics, inhibitor NC, and miR-545-5p inhibitor. The lanes from gels or blots have been grouped or rearranged, and the original images were provided in original materials. (F) The bioinformatics analysis of the binding sites between c-Met and miR-545-5p from Targetscan database. (G) Relative luciferase activities were measured by luciferase reporter assay. (H) Spearman correlation analysis indicated the negative correlation between c-Met and miR-545-5p in patients' tissue samples (n=30). *P < 0.05, **P < 0.01 and ***P < 0.001.
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LncRNA NR2F2-AS1/miR-545-5p/c-Met axis in NSCLC

Figure 5. Overexpression of miR-545-5p suppressed cell proliferation, invasion and migration by targeting c-Met in NSCLC. (A) Detection of miR-545-5p in A549 cells by RT-qPCR. (B) Detection of c-Met in A549 cells by RT-qPCR. (C) Western blotting analysis of c-Met in A549 cells with different treatments. The lanes from gels or blots have been grouped or rearranged, and the original images were provided in original materials. (D) CCK-8 assay was conducted to assess the cell proliferation ability. (E) The clone formation assay was conducted to study the impact of miR-545-5p on cell proliferation ability. (F, G) The effect of miR-545-5p on cell migration and invasion abilities were analyzed by wound healing assay (F) and transwell assay (G). (H) Morphologic changes in cells after the different treatments. Scale bar: 20 μm. *P < 0.05, **P < 0.01 and ***P < 0.001.
LncRNA NR2F2-AS1/miR-545-5p/c-Met axis in NSCLC

Figure 6. Overexpression of miR-545-5p inhibited EMT process by targeting c-Met and BVR in NSCLC. A. E-cadherin, N-cadherin, vimentin, MMP-2, slug, snail and BVR in A549 cells after different treatments were measured by western blotting. The lanes from gels or blots have been grouped or rearranged, and the original images were provided in original materials. B. BVR expression in A549 cells after different treatments were analyzed by western blotting analysis. The lanes from gels or blots have been grouped or rearranged, and the original images were provided in original materials. C. BVR expression in NSCLC tissues and normal tissues (n=30) quantified by RT-qPCR. D. Immunohistochemistry analysis of expression of BVR in NSCLC tissues and normal tissues. Scale bar: 50 μm. *P < 0.05 and **P < 0.01.
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A

Relative expression level of BVR

Control  siNC  siBVR-#1  siBVR-#2  siBVR-#3

B

Relative protein levels

BVR  p-ATF2  ATF-2

Control  siNC  siBVR

C

Cell viability (%)

Control  siBVR+  pcDNA3.1-NC  pcDNA3.1-ATF-2

D

Number of colonies

Control  siNC  siBVR  pcDNA3.1-NC  pcDNA3.1-ATF-2

E

Migration rate (%)

0 h  24 h

Control  siNC  siBVR  pcDNA3.1-NC  pcDNA3.1-ATF-2

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LncRNA NR2F2-AS1/miR-545-5p/c-Met axis in NSCLC

Figure 7. Silencing BVR inhibited cell proliferation, migration, invasion and EMT through controlling ATF-2 in NSCLC. (A) Detection of BVR in A549 cells after transfection with siNC or siBVR-#1, siBVR-#2, and siBVR-#3 by RT-qPCR. (B) Detection of BVR, p-ATF2, and ATF-2 in A549 cells with the treatment of siNC or siBVR by western blotting. The lanes from gels or blots have been grouped or rearranged, and the original images were provided in original materials. (C) Cell viability was analyzed by CCK-8 assay. (D) The clone formation assay was conducted to investigate the impact of BVR on cell proliferation ability. (E, F) The effect of BVR on cell migration and invasion abilities was investigated by wound healing assay (E) and transwell assay (F). (G) Morphologic changes in cells after the different treatments. Scale bar: 20 μm. (H) E-cadherin, N-cadherin, snail, slug, BVR, p-ATF-2, and ATF-2 in A549 cells after different treatments were analyzed by western blotting. The lanes from gels or blots have been grouped or rearranged, and the original images were provided in original materials. *P < 0.05 and **P < 0.01.
the role of BVR in A549 cells, the A549 cells were treated with siNC, siBVR, siBVR+pcDNA3.1-NC, siBVR+pcDNA3.1-ATF-2, respectively. Compared the control group, the group with silenced BVR showed the decreased cell proliferation (Figure 7C) and clone formation (Figure 7D) along with the decreased migration and invasion abilities (Figure 7E, 7F). In contrast, the overexpression of ATF-2 significantly reversed the effects of silencing of BVR in cell proliferation, migration and invasion abilities of A549 (Figure 7C, 7F). The morphological imaging after different treatments showed that silenced BVR caused the cell morphology from the spindle-shape to round shape with the shortened synapses, and overexpression ATF-2 reversed this effect (Figure 7G). Furthermore, we studied the EMT-related proteins by western blotting. The E-cadherin was significantly enhanced, but N-cadherin, slug and snail were significantly decreased as well as the decreased expression levels of BVR, p-ATF2 and ATF-2 when the BVR was knocked down (Figure 7H). Similarly, the overexpression of ATF-2 partially reversed the above effects. Importantly, as shown in Figure 7H, the phosphorylation of ATF-2 could be significantly enhanced when the ATF-2 was overexpressed by pcDNA3.1. All these results proved that knockdown of BVR in A549 cells could suppress the cell proliferation, migration and invasion abilities by suppressing EMT process, along with inhibition of ATF-2.

Silencing lncRNA NR2F2-AS1 or overexpression of miR-545-5p suppressed the growth of tumors and EMT process in vivo

The in vivo investigation about the function of lncRNA NR2F2-AS1 and miR-545-5p was further conducted to support the in vitro discovery. As shown in Figure 8A-C, the weight and volume of tumors were significantly decreased in the groups with silenced lncRNA NR2F2-AS1 or overexpressed miR-545-5p. Furthermore, the analysis of Ki-67 expression in these groups showed that the Ki-67 was significantly suppressed in groups with silenced lncRNA NR2F2-AS1 or overexpressed miR-545-5p (Figure 8D). Then, we conducted the immunohistochemistry analysis to quantify N-cadherin and E-cadherin in different tumor tissues (Figure 8E). The results showed that N-cadherin was suppressed in the tissues with silenced lncRNA NR2F2-AS1 or overexpressed miR-545-5p. In contrast, E-cadherin was overexpressed in the tissues with silenced lncRNA NR2F2-AS1 or overexpressed miR-545-5p. Furthermore, compared with control group, we demonstrated that the expression of lncRNA NR2F2-AS1, c-Met, BVR and ATF-2 were significantly decreased in the tissues of silenced lncRNA NR2F2-AS1 (Figure 8F), but the expression of miR-545-5p was higher in the tissues with silenced lncRNA NR2F2-AS1 (Figure 8F). The transfection of miR-545-5p also suppressed the expression of c-Met, BVR and ATF-2 in the tumor tissues (Figure 8G). We also investigated the expression levels of BVR, ATF-2 and p-ATF-2 in the tumor tissues using western blotting. The results showed that silencing NR2F2-AS1 or overexpression of miR-545-5p could significantly decrease the expression of BVR, ATF-2 and p-ATF-2 (Figure 8H). Taking together, we summarized that both knockdown of lncRNA NR2F2-AS1 or overexpression of miR-545-5p could suppress the tumor growth and EMT process in vivo.

Discussion

The metastasis and recurrence of NSCLC lead to the significant death of patients [4]. In order to identify the potential therapeutic target, it is significant to discover the mechanism of the NSCLC cancer metastasis and invasion through EMT. LncRNAs participate in varies of physiological processes, such as proliferation, apoptosis, migration and invasion, through different pathways [14, 25, 26]. This work was to investigate the role of lncRNA NR2F2-AS1, miR-545-5p, c-Met, BVR and ATF-2 in the cell proliferation, migration and invasion of NSCLC cells by adjusting EMT process. To the best of our knowledge, this was the first time to show that NR2F2-AS1 regulated the miR-545-5p and following c-Met and downstream expression of BVR and ATF-2 in the development of NSCLC.

In this work, we confirmed that lncRNA NR2F2-AS1 was overexpressed in the NSCLC tissues and cell lines. Also, we found the survival time and survival rate were significantly enhanced when the expression of lncRNA NR2F2-AS1 was low in the patients’ tissues. This was similar with the results from the past studies, indicating that lncRNA NR2F2-AS1 might involve in
Figure 8. Silencing IncRNA NR2F2-AS1 or overexpression of miR-545-5p inhibited the growth of tumors and EMT process in vivo. (A) The collected tumors from the mice with the treatment of shNR2F2-AS1, shNC, miR-545-5p mimics and mimics NC. (B, C) The tumor volume (B) and weight (C) in different groups. (D) The expression of Ki-67 in the tumor tissues of different groups. (E) The expression of E-cadherin and N-cadherin in tumor tissues of different groups analyzed by immunohistochemistry. (F) The IncNR2F2-AS1, miR-545-5p, c-Met, BVR and ATF-2 in tumor tissues with the transfection of shNC or shNR2F2-AS1 were analyzed by qRT-PCR. (G) The expression levels of miR-545-5p, c-Met, BVR and ATF-2 were analyzed by qRT-PCR in tumor tissues with the transfection of miR-545-5p mimics or mimics NC. (H) The expression levels of BVR, ATF-2 and p-ATF-2 in tumor tissues with the transfection of shNC, shNR2F2-AS1, miR-545-5p mimics, and mimics NC by western blotting. The lanes from gels or blots have been grouped or rearranged, and the original images were provided in original materials. *$P < 0.05$ and **$P < 0.01$. 

the development of NSCLC. Then, we focused on the investigation of the function of IncRNA NR2F2-AS1 on NSCLC. Firstly, we silenced and overexpressed the expression of NR2F2-AS1
LncRNA NR2F2-AS1/miR-545-5p/c-Met axis in NSCLC
to A549 cells, and the results demonstrated that silence of lncRNA NR2F2-AS1 inhibited the several processes in NSCLC cells such as cell proliferation, invasion, migration, and EMT process. However, overexpression of it exerted the opposite results. Current research findings indicated that IncRNA might inhibit the function of miRNA through serving as a natural miRNA “sponge” [27, 28]. For instance, the glioma cell migration and invasion could be significantly suppressed by silencing IncRNA NEAT1 through regulation of miR-132 [29]. In gastric cancer, IncRNA H19 was reported to target miR-141 to influence the downstream target genes [30]. In NSCLC patients, miR-107 could be suppressed by IncRNA H19 to promote cell cycle progression [31]. Recently, Zhang et al. demonstrated that IncRNA NR2F2-AS1 promoted the proliferation, invasion and inhibited apoptosis by regulating miR-320b via influencing BMI1 in NSCLC [4]. In this study, we proposed that IncRNA NR2F2-AS1 could work as a sponge to control miR-545-5p, concluded from the results that the expression levels of lncRNA NR2F2-AS1 and miR-545-5p had a reverse correlation in the NSCLC tissues and cells. We also found that IncRNA NR2F2-AS1 could reversely regulate miR-545-5p. Meanwhile, the FISH results indicated that both of IncRNA NR2F2-AS1 and miR-545-5p were localized in the cytoplasm. We also confirmed that IncRNA NR2F2-AS1 acted as a ceRNA via targeting to miR-545-5p. Moreover, inhibiting miR-545-5p partially reversed the effect of silencing IncRNA NR2F2-AS1 on the proliferation, migration, invasion and EMT of A549 cells. What’s more, the decreased expression of miR-545-5p corrected with the poor survival rate of NSCLC patients. Our present study firstly discovered that IncRNA NR2F2-AS1 promoted the cell proliferation, invasion, migration and EMT through silencing the expression of miR-545-5p in NSCLC.

As we known, miRNAs might be oncogenes or anti-oncogenes to regulate various target genes. MiR-545 was reported to play a tumor suppressing role in epithelial ovarian cancer [32], colorectal cancer [33] and NSCLC [17]. Du et al. demonstrated that miR-545 was down-expressed in the NSCLC tissues and cells, moreover, overexpression of miR-545 could suppress the cell proliferation through regulating cyclin D1 and CDK4 [34]. To discover the molecular mechanism of miR-545-5p in NSCLC, bioinformatics analysis was conducted by TargetScan and dual-luciferase reporter assay proved that miR-545-5p directly bind with c-Met. c-Met played an important role in the process of EMT, whose overexpression increased the cellular properties of proliferation, migration, and invasion [18]. For example, Zhu et al. found that c-Met signal pathway could be inhibited by miRNA-198 to induce cell apoptosis [35]. In this work, we reported that c-Met was over-expressed in the NSCLC tissues and cell lines. Uprogulation of miR-545-5p could significantly inhibited c-Met expression in A549 cells. Furthermore, the inhibited cell proliferation, migration, invasion and EMT process due to the overexpression of miR-545-5p in A549 cells could be significantly reversed by overexpression of c-Met, proving that miR-545-5p influenced the tumorigenesis through regulating c-Met. This was the first time to discover that miR-545-5p regulated c-Met in NSCLC.

During the investigation of the interaction between miR-545-5p and c-Met, we also found that the BVR was significantly inhibited by the overexpression of miR-545-5p, but overexpression of c-Met could reverse this impact. We also found that silence of NR2F2-AS1 could downregulate the expression of BVR through overexpression of miR-545-5p. BVR was found to promote EMT in breast cancer [20], lung and liver cancers [21]. In breast cancer, Zhang et al. found that EMT was promoted by BVR through the ERK1/2 signaling pathway [20]. However, BVR was also reported to regulate the ATF-2, which played important role in the process of EMT and radio-sensitivity of A549 cells [24]. In our study, we proved that silencing BVR could inhibit the cell proliferation, migration, invasion and EMT process through suppressing the activity of ATF-2 in A549 cells. Therefore, first of all, c-Met is involved in the regulation of BVR. Secondly, BVR is an important factor to regulate the expression of ATF-2. Both literature and our results indicated that silencing BVR decreased the expression of ATF-2 and its phosphorylation. Thirdly, the ATF-2 pathway has been found to regulate the EMT in cancers. Our results and literatures both showed that the silenced ATF2 regulated by BVR could significantly inhibit EMT process of NSCLC. Therefore, we studied
the c-Met/BVR/ATF-2 pathway in regulation of EMT in our project. We proposed that miR-545-5p affected by lncRNA NR2F2-AS1 could regulate the NSCLC cancer cell proliferation, migration, invasion and EMT process by regulating c-Met and the downstream target of BVR and ATF-2.

In conclusion, we discovered that up-regulated lncRNA NR2F2-AS1 expression and down-regulated miR-545-5p expression in NSCLC tissues and cell lines. The overexpressed lncRNA NR2F2-AS1 promoted cell proliferation, migration, invasion and EMT through regulating c-Met/BVR/ATF-2 axis. Considering the abnormal expression of lncRNA NR2F2-AS1 and miR-545-5p in NSCLC patients, they may be identified as potential therapeutic targets or biomarkers for the diagnosis and treatment of NSCLC.

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Disclosure of conflict of interest

None.

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References


[32] Huang X and Lu S. MicroR-545 mediates colorectal cancer cells proliferation through up-regulating epidermal growth factor receptor expression in HOTAIR long non-coding RNA dependent. Mol Cell Biochem 2017; 431: 45-54.


Homo sapiens NR2F2 antisense RNA 1 (NR2F2-AS1), transcript variant 1, long non-coding RNA

shNR2F2-AS1-#1: GGGAAACGACTTTGGACTCAC

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shNC: GTTCTCCGAACGTGTCACGT

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Homo sapiens biliverdin reductase A (BLVRA, also called BVR)

Gene ID: 644

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**Supplementary Table 1.** The detailed information of antibodies used

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