Introduction

Non-small cell lung cancer (NSCLC) comprises approximately 80% of lung cancer cases (1). NSCLC has long been the predominant reason for global cancer-related mortality, and its 5-year survival rate is pessimistic (2). Although progress has been achieved in the study of NSCLC during past decades, the molecular mechanism behind the initiation and development of NSCLC remains largely unknown (3).

Long non-coding RNAs (lncRNAs) are acknowledged as a cluster of non-coding RNAs (ncRNAs) with a length of over 200 bp (4). LncRNAs are multifunctional in cancer progression and can regulate proliferation, migration, invasion, and apoptosis (5-8). Also, several lncRNAs are identified to possess tumor-promoting or tumor-suppressing properties in NSCLC, such as SNHG1 (9), HOXD-AS1 (10), and PICART1 (11). RAMP2 antisense RNA 1 (RAMP2-AS1) is a novel lncRNA proven to present downregulation and inhibit cell proliferation in glioblastoma (12). However, no study has related RAMP2-AS1 to NSCLC.

Receptor activity-modifying protein 2 (RAMP2) belongs to the family of single-transmembrane-domain proteins named RAMP, which is responsible for the transport of calcitonin receptor-like receptors to the plasma membrane (13). RAMP family can determine the specificity of the calcitonin-receptor-like receptor (CLR), a G protein-coupled seven transmembrane domain receptor that is required for the function of Adrenomedullin (AM), a member of the calcitonin superfamily (13,14). Although several studies have stated that AM exerted oncogenic functions in certain types of diseases, such as colorectal cancer as well as pancreatic cancer (15,16). Some studies argued that AM-RAMP2 signaling possesses tumor-suppressive roles. For example, Abasolo et al. (17) uncovered that AM curbed the growth of prostate cancer cells both in vitro and in vivo. The endothelial adrenomedullin-RAMP2 system modulated vascular integrity and suppressed tumor metastasis (18). Importantly, a study pointed out that RAMP2 was low expressed in lung cancer and its overexpression inhibited the proliferation of lung cancer cells (19). Nonetheless, the specific mechanism of RAMP2 in NSCLC and its relation with RAMP2-AS1 remains elusive.

Hence, our study planned to figure out the role of RAMP2-AS1 in NSCLC.
Materials and Methods

Cell lines and plasmid transfection
Human bronchial epithelial cell line (16HBE) and four accepted NSCLC cell lines (H23, H1299, SPC-A1, A549) were utilized in this study and kept in RPMI-1640 culture medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco). All cells were preserved in 5% CO₂ and 95% air at 37°C, and bought from American Type Culture Collection (ATCC, Rockville, MD, USA). Human RAMP2-AS1cDNA was amplified and cloned into the pcDNA3.1 vector, which was procured from Invitrogen (Carlsbad, CA, USA). The empty pcDNA3.1 vector was taken as a negative control. For silencing RAMP2-AS1, TIA1 or RAMP2, the duplex-specific shRNAs (GenePharma, Shanghai, China) were used. Plasmid transfection into SPC-A1 or A549 cells was implemented using Lipofectamine 2000 reagent, which was procured from Invitrogen.

Quantitative real-time polymerase chain reaction (RT-qPCR)
Using RNAiso Plus (TaKaRaBioTechnology, Tokyo, Japan), total cellular RNAs were isolated from SPC-A1 and A549 cells and transcribed in strict accordance with the specification of the Prime Script™ RT Master Mix (TaKaRaBio Technology). SYBR Premix Ex Taq II procured from TaKaRaBio Technology was applied for real-time PCR with Applied Biosystems 7900 Real-Time PCR System, which was procured from Applied Biosystems (Foster City, CA, USA). Relative expressions of genes were determined via the threshold cycle. The equation 2^{-ΔΔCT} was used for the measurement of fold change.

Cell proliferation assays
SPC-A1 and A549 cells were cultivated with 10 µl of CCK8 reagent which was procured from Dojindo (Kumamoto, Japan) at different time points at 37°C in 96-well plates (3000 cells/well). After 2 hrs of incubation, cell viability was monitored by the microplate reader procured from Bio-Rad Laboratories (Hercules, CA, USA) via the absorbance at 450 nm. Furthermore, transfected cells were collected for colony formation assay in 6-well plates with 500 cells/well. 14 days later, viable colonies with more than 50 cells were subjected to fixation and the treatment of 0.1% crystal violet (Beyotime, Shanghai, China).

Cell invasion assay
The invasive ability of transfected NSCLC cells was measured using the Transwell chambers with Matrigel and the Corning Kit (Corning, Cambridge, MA, USA) in light of the instruction. 2×10⁴ cells were placed into the upper well, and the complete culture medium with 10% FBS was added into the lower chamber. 48 hrs later, invasive cells were subjected to fixation in 4% paraformaldehyde and washed thrice, following the treatment of 0.1% crystal violet. The final image of cell invasion was observed and captured under a microscope (CX41, Olympus, Tokyo, Japan).

Cell apoptosis assay
Caspase-3 activity was determined in transfected cells to assess apoptotic changes by use of a Caspase-3 activity kit (Beyotime). Firstly, total cellular proteins were obtained and treated with caspase-3 substrate and buffer for 4 hrs. At length, caspase-3 activity was evaluated with the microplate reader at a wavelength of 405 nm.

Western blotting
Radioimmunoprecipitation assay buffer (Beyotime) was first applied for the extraction of total cellular protein. Protein samples were separated on 10% SDS-PAGE, followed by being transferred onto PVDF membranes, which were procured from Millipore (Billerica, MA, USA). Afterward, primary antibodies and HRP-conjugated secondary antibodies were subjected to incubation with the blocked membranes in 5% BSA. Anti-GAPDH (ab9484) and anti-RAMP2 (ab96546) were procured from Abcam (Cambridge, MA, USA). Immune complexes were detected by chemiluminescence (Pierce Biotechnology, Waltham, MA, USA).

Luciferase reporter assay
Human 293T cells from ATCC were prepared and plated into 24-well culture plates. RAMP2 promoter was sub-cloned into pGL3 Basic vector, which was procured from Promega (Madison, WI, USA), and subjected to the co-transfection with pcDNA3.1/RAMP2-AS1 or control. At last, the Dual-Luciferase Reporter Assay System (Promega) was utilized as per the guidebook.

Actinomycin D assay
Actinomycin D (ActD, 2 mg/ml) procured from Sigma-Aldrich (St. Louis, MO, USA) was added into the culture medium for blocking mRNA transcription. After treatment, RT-qPCR analysis was conducted.

RNA-binding protein immunoprecipitation (RIP) assay
In strict accordance with the protocol of the EZ-Magna RIP kit procured from Millipore, RIP assay was performed in NSCLC cells. Cellular lysates were cultured in RIP buffer with magnetic beads, which were conjugated to TIA1 antibody, Ago2 antibody, IgG antibody (negative control) or SNRNP70 antibody (positive control). The precipitated RNAs were extracted for RT-qPCR analysis.

RNA pull-down assay
Purified total cellular RNAs from A549 or SPC-A1 cells were subjected to biotin labeling using Pierce RNA 3’EndDeSthiobiotinylation Kit, which was procured from Thermo Fisher Scientific (Waltham, MA, USA). The cell lysates incubated with biotinylated RNAs were named as RAMP2 biotin, with RAMP2 non-biotin as the control. The biotinylated RNA pull-down assay was performed using the Thermo Scientific Pierce RNA Pull-down Kit. Immune complexes were detected by chemiluminescence.

Statistical analyses
In this study, triplicates were required for each assay. All statistical difference was assessed with the application of SPSS statistics 19.0 (SPSS, Chicago, IL, USA) as well as the GraphPad Prism 7.0 Software, which was procured from GraphPad Software (La Jolla, CA, USA). Data analyses were conducted by applying one-way ANOVA with Tukey’s multiple comparison test or sample-independent t-test. Data were exhibited as the mean ± standard deviation.
Results

RAMP2-AS1 served as a tumor suppressor in NSCLC

First of all, RAMP2-AS1 expression in NSCLC was analyzed through the bioinformatics tool GEPIA in order to evaluate the relation of RAMP2-AS1 with NSCLC. As presented in Figure 1A, RAMP2-AS1 was markedly low-expressed in lung adenocarcinoma (LUAD) specimens versus normal specimens. Concertedly, the reduced RAMP2-AS1 expression in NSCLC cell lines was confirmed through RT-qPCR data (Figure 1B). Then, gain-of-function experiments were designed to find out the underlying biological performance of RAMP2-AS1 in NSCLC. A549 and SPC-A1 cells were applied for the overexpression of RAMP2-AS1 because they presented the lowest RAMP2-AS1 level among 4 NSCLC cell lines. The overt induction of RAMP2-AS1 level upon pcDNA3.1/RAMP2-AS1 transfection in both cell lines was confirmed by RT-qPCR (Figure 1C). Ectopic expression of RAMP2-AS1 reduced the proliferation of two NSCLC cell lines (Figures 1D and 1E). The caspase-3 activity of NSCLC cells transfected with pcDNA3.1/RAMP2-AS1 was increased (Figure 1F), indicating that RAMP2-AS1 prompted NSCLC cell apoptosis. Additionally, the invasive ability of NSCLC cells was attenuated by RAMP2-AS1 upregulation (Figure 1G). As abovementioned, RAMP2-AS1 was markedly downregulated in NSCLC and served as a tumor-suppressor by restraining cell proliferative and invasive phenotypes, whereas facilitating cell apoptosis in NSCLC.

RAMP2-AS1 post-transcriptionally upregulated RAMP2 by stabilizing its mRNA

Next, the downstream mechanism of RAMP2-AS1 was exploited. LncRNAs are postulated to possess the potential to correlate with and regulate their nearby genes (20,21). Through UCSC (http://genome.ucsc.edu/), we found that the genomic position of RAMP2-AS1 was neighboring to RAMP2 (Figure 2A). RAMP2 has been documented to show downregulation in lung cancer tissues and play a tumor-suppressor role in lung cancer progression and the development of prostate cancer (17,19). The positive correlation of RAMP2-AS1 and RAMP2 levels in LUAD samples was revealed via the starBase pan-cancer tool (Figure 2B). Moreover, by analyzing the expression of RAMP2 through GEPIA, we uncovered the reduced level of RAMP2 in LUAD samples (Figure 2C). Accordingly, we validated the significantly low expression of RAMP2 in NSCLC cells (Figure 2D). Regulation of RAMP2-AS1 on RAMP2 expression was later evaluated. The levels of RAMP2 were increased in the presence of RAMP2-AS1 overexpression in NSCLC cells (Figure 2E). Furtherly, we detected at which level RAMP2-AS1 regulated RAMP2. It turned out that RAMP2-AS1 caused no pronounced change in the activity of the RAMP2 promoter (Figure 2F), suggesting that RAMP2-AS1 might regulate RAMP2 post-transcriptionally. As largely reported, lncRNAs could regulate the mRNA stability of target genes at the post-transcriptional level (22,23). Hence, we detected the impact of RAMP2-AS1 on RAMP2 mRNA stability. RAMP2-AS1 was silenced in H1299 and H23 cells expressing relatively high RAMP2-AS1 and RAMP2 levels (Figure 2G). Consequently, the half-life of RAMP2 mRNA was curbed after the depletion of RAMP2-AS1 (Figure 2H). Collectively, RAMP2-AS1 post-transcriptionally upregulated RAMP2 by stabilizing its mRNA.

RAMP2-AS1 interacted with TIA1 to stabilize RAMP2 mRNA

Subsequently, we investigated how RAMP2-AS1 re-
lated RAMP2 mRNA stability. To our knowledge, lncRNAs could regulate the stability of target mRNAs through the interplay with RNA binding proteins (RBPs) (22,23). Hence, we searched the starBase 3.0 database for the common RBPs of RAMP2-AS1 and RAMP2. As a result, we found 6 RBPs potentially interacting with both RAMP2-AS1 and RAMP2, which were CSTF2T, FBL, IGF2BP1, LIN28, PCBP2, and TIA1 (Figure 3A). To narrow the selection, we examined the levels of the 6 genes by RT-qPCR in cell lines. As presented in Figure 3B, only TIA1 was significantly downregulated in all NSCLC cell lines, indicating the involvement of TIA1 in NSCLC. TIA1 has been revealed to be implicated in the development of a variety of malignancies (24,25), and can regulate mRNA stability of tumor-suppressor genes such as PDCD4 (26). Hence, we conjectured that RAMP2-AS1 regulated RAMP2 mRNA stabilization via TIA1. RIP analysis depicted the abundance of RAMP2-AS1 and RAMP2 mRNA in the immunoprecipitated products of TIA1 antibody (Figure 3C). Pulldown assay showed that TIA1 protein was enriched by the pulldown of RAMP2 biotin probe group rather than RAMP2 non-biotin probe (Figure 3D). Additionally, we demonstrated that RAMP2-AS1 could be pulled down by RAMP2 biotin probe instead of RAMP2 non-biotin probe, and the enrichment of RAMP2-AS1 in RAMP2 biotin pulldown was diminished by the addition of proteinase K (Figure 3E), indicating that TIA1 was required for the interplay between RAMP2-AS1 and RAMP2. Moreover, the knockdown efficiency of TIA1 in H1299 as well as H23 cells was verified (Figure 3F). After the addition of ActD to block mRNA transcription, we found through RT-qPCR that the decrease of remaining RAMP2 mRNA was sharpened by TIA1 silence (Figure 3G). Besides, the knockdown of TIA1 reversed the upregulation of RAMP2 mRNA and protein caused by RAMP2-AS1 overexpression in NSCLC cells (Figure 3H). Altogether, RAMP2-AS1 interacted with TIA1 to stabilize RAMP2 mRNA.

RAMP2-AS1 reduced proliferation, increased apoptosis, and impeded invasion of NSCLC through RAMP2

Thereafter, to investigate whether RAMP2-AS1 repressed NSCLC progression through RAMP2, rescue assays were conducted in A549 cells. The expression of RAMP2 was elevated after RAMP2-AS1 overexpression, and the induction was abrogated by the transfection of sh-RAMP2 (Figure 4A). The decreased proliferation of A549 cells upon RAMP2-AS1 upregulation could be recovered by the silence of RAMP2 (Figures 4B and 4C). The apoptosis level of A549 cells was improved by forced expression of RAMP2-AS1, and silencing RAMP2 counteracted the effect (Figure 4D). The invasive capacity of A549 cells was hindered by RAMP2-AS1 overexpression and was restored by the co-transfection of pcDNA3.1/RAMP2 (Figure 4E). In sum, it was suggested that RAMP2-AS1 reduced proliferation, increased apoptosis, and impeded the invasion of NSCLC through RAMP2.

Discussion

During past decades, a great number of lncRNAs have been characterized to be functional in NSCLC initiation and development (27,28). A previous study stated that RAMP2-AS1 was low-expressed in glioblastoma and served as a tumor suppressor to suppress cell proliferative ability (12). The present study firstly showed that RAMP2-AS1 was downregulated in LUAD samples through bioinformatics analysis, and confirmed that RAMP2-AS1 level was significantly diminished in NSCLC cells, indicating the implication of RAMP2-AS1 in the progression of NSCLC. Functional experiments indicated that RAMP2-AS1 exhibited anti-tumor properties in NSCLC by hampering proliferation and invasion and facilitating apoptosis.

The role of RAMP2 in cancer progression is controversial as reported by previous studies. RAMP2 can determine the specificity of G protein-coupled seven transmembrane domain receptor CLR and CLR enables the function of AM (13,14). Some works suggested that AM-RAMP2

Figure 3. RAMP2-AS1 stabilized RAMP2 mRNA through TIA1. RAMP2-AS1 inhibited NSCLC progression through RAMP2. (A) Venn pattern of the shared RBPs for RAMP2-AS1 and RAMP2 from starBase 3.0. (B) Heat map of the level of 6 RBPs in NSCLC cells and normal cells. (C) The interaction of TIA1 with RAMP2-AS1 and RAMP2 mRNA was assessed by RIP assay. (D-E) The interaction of RAMP2 with TIA1 protein and RAMP2-AS1 was evaluated by pulldown assays. Proteinase K was added to digest TIA1 protein. (F) The efficiency of TIA1 silencing in H1299 and H23 cells was verified. (G) RT-qPCR results of remaining RAMP2 mRNA level after ActD addition at indicated time points. (H) RT-qPCR as well as western blot analyses of RAMP2 expression in A549 and SPC-A1 cells. "P<0.05, **P<0.01.

Figure 4. RAMP2-AS1 repressed NSCLC progression through RAMP2. (A) RT-qPCR data of RAMP2 expression in A549 cells with indicated transfections. (B-C) Proliferation assays revealed the proliferative phenotype of A549 cells with indicated transfections. (D) Caspase-3 level reflected the apoptosis of A549 cells under different transfections. (E) The invasive phenotype of A549 cells was depicted by Transwell-invasion assay under different transfections. **P<0.01.
signaling could promote the progression of certain types of cancers by regulating proliferation, metastasis and angiogenesis (15,16), while others pointed out that RAMP2 can suppress tumor progression. For example, AM was proven to repress the growth of prostate cancer cells in vivo and in vitro (17). The endothelial adrenomedullin-RAMP2 system modulated vascular integrity and suppressed tumor metastasis (18). Notably, a study demonstrated that RAMP2 presented low expression in lung cancer specimens and overexpressing RAMP2 inhibited cell proliferation in lung cancer (19). Herein, we first found that RAMP2 was a neighbor to RAMP2-AS1, indicating the potential correlation between RAMP2-AS1 and RAMP2. We revealed the positive correlation of RAMP2-AS1 and RAMP2 in LUAD samples. Through TCGA data, we revealed the low expression of RAMP2 in LUAD samples. Mechanistically, we revealed that RAMP2-AS1 upregulated RAMP2 at the post-transcriptional level by stabilizing its mRNA. Former works have illustrated the regulation of lncRNA-RBP interaction on mRNA stability (22,23). Herein, we first identified that TIA1 interacted with both RAMP2-AS1 and RAMP2. TIA1 is reputed as an RBP participating in multiple biologic processes related to RNA metabolism in both cytoplasm and nucleus (29). Notably, TIA1 is suggested as a novel tumor suppressor gene by regulating genes responsible for tumor-related malignant biological activities, including apoptosis, proliferation, angiogenesis, metastasis as well as immune evasion (30-34). Also, the mRNA-stabilizing function of TIA1 has been revealed by previous studies (26,35). Accordingly, we first revealed that TIA1 was downregulated in NSCLC cells and that RAMP2-AS1 stabilized RAMP2 mRNA through recruiting TIA1. Finally, the rescue assays indicated that RAMP2-AS1 inhibited NSCLC progression through RAMP2-AS1.

In conclusion, we first suggested lncRNA RAMP2-AS1 as a tumor-suppressor in NSCLC by interacting with TIA1 to stabilize RAMP2, offering new insights for the therapy development of NSCLC.

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Conflict of interest
The authors state that there are no conflicts of interest.

References


