

Article

A DNA Methylation-Dependent NOP56/MYC Positive Feedback Loop Promotes the Proliferation and Migration of Non-Small Cell Lung Cancer Through Regulating Ribosome Biogenesis

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Simple Summary

Non-small cell lung cancer is a leading cause of cancer mortality, underscoring the need for new therapeutic concepts. Cellular proliferation depends on the construction of ribosomes, the protein-synthesizing machinery, and malignant cells often intensify this process. We investigated whether NOP56, a factor in ribosome assembly, is pathologically elevated in this disease, how it influences tumor growth and dissemination, and which molecular events drive its increase. Integrating cohorts with mechanistic studies in vitro and in vivo, we show that NOP56 is upregulated, associates with unfavorable outcomes, and accelerates proliferation, invasion, and ribosome production. We further demonstrate that it activates a pro-growth gene program through a specialized mode of translation, which in turn augments NOP56, establishing a self-amplifying loop; reduced DNA methylation also contributes. These findings identify NOP56 as a tractable therapeutic node and inform biomarker development and rational combinations targeting ribosome biogenesis.

Abstract

Background: Recent findings underscore the importance of ribosome biogenesis, a complex molecular machinery, in cancer biology, highlighting opportunities for targeted treatment strategies. Here, we revealed that dysregulation of ribosome biogenesis is a distinctive feature of non-small lung cancer (NSCLC). However, further investigation is required to pinpoint which specific processes within this complex pathway are aberrant in this malignancy. **Methods:** The expression levels and clinical significance of NOP56 in NSCLC were investigated by microarray analysis, qPCR, TCGA and GEO datasets. Function assays were conducted to explore the biological role of NOP56 in NSCLC cells. The mechanisms that mediate the upregulation of NOP56 were investigated by bisulfite DNA sequencing, luciferase reporter assay, chromatin immunoprecipitation and TCGA datasets. The downstream pathway of NOP56 was explored by RNA sequencing, qPCR, Western blot and luciferase reporter assay. **Results:** High expression of NOP56 was detected in NSCLC tissues and was associated with poor prognosis. Functional assays revealed that overexpression of NOP56 promoted NSCLC cellular proliferation, metastasis and ribosome biogenesis in vitro, and further accelerated tumorigenesis in vivo. Mechanistically, NOP56 activates MYC signaling by regulating IRES-dependent translation, which in turn transcriptionally upregulated NOP56 expression, creating a positive feedback loop. Additionally, hypomethylation also contributed to the upregulation of NOP56 in NSCLC. **Conclusions:** Our study demonstrated that NOP56/MYC forms a positive feedback loop that enhances



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ribosome biogenesis and drives the progression of NSCLC, positioning NOP56 a promising therapeutic target for this malignancy.

Keywords: non-small cell lung cancer; ribosome biogenesis; NOP56; MYC; DNA methylation

1. Introduction

Lung cancer remains a significant challenge in terms of both its prevalence and mortality rate worldwide [1]. Non-small cell lung cancer (NSCLC), comprising mainly lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC), represents the primary type of lung cancer. Despite recent advancements in diagnostic and therapeutic modalities, the 5-year survival rate for NSCLC remains relatively low at 17.7%. The disheartening treatment outcomes in lung cancer largely stem from the fact that nearly 85% of NSCLC patients receive their diagnosis at an advanced stage [2]. Consequently, it is imperative to undertake further research to unravel the intricate molecular mechanisms underlying NSCLC, identify novel therapeutic targets and elucidate reliable prognostic biomarkers.

Ribosomes are intricate assemblies composed of ribosomal proteins and ribosomal RNA (rRNA) that function as molecular machines for messenger RNA (mRNA) translation, which is essential for cell proliferation and survival. The process of ribosome biogenesis encompasses various steps, including the synthesis and processing of rRNA, assembly of ribosome proteins, and the association of ribosomal subunits for protein synthesis. The hyperactivation of ribosome biogenesis, facilitating protein synthesis and supporting cell growth, is demanded in cancer initiation and progression [3]. Otherwise, activation of oncogenes or oncogenic signaling pathways contribute to the initiation of this hyperactivation [4]. Though the association between dysregulated ribosome biogenesis and elevated cancer risk has been established in recent decades, the broad role and the molecular basis of ribosome biogenesis in cancer require in-depth investigation [5]. A better interpretation of these will provide new insights into the design of potential target cancer treatment.

NOP56 is a core component of small nucleolar ribonucleoprotein complex (snoRNP), which mediates the site-specific 2'-O-methylation of rRNA, participates in the synthesis of mature rRNA, and regulates ribosome biogenesis [6,7]. Recent studies highlight the complex role of NOP56 in various tumor types. Elevated NOP56 expression in tumor tissues has been observed in colorectal cancer, diffuse large B-cell lymphoma, Burkitt's lymphoma, and in acute lymphoblastic leukemia [8–11]. Conversely, NOP56 expression is downregulated in HPV-positive cervical cancer [12]. However, the specific role of NOP56 in NSCLC remains largely unknown.

Therefore, we focused on NOP56 to define its functional relevance in NSCLC and to delineate the mechanisms involved. Our results indicate that NOP56 promotes NSCLC progression and may do so by influencing IRES-dependent translation initiation and MYC-associated signaling. We also assessed upstream regulation of NOP56 expression, including DNA methylation and MYC-mediated transcriptional control, which together suggest a potential positive regulatory loop between NOP56 and MYC. These observations nominate the NOP56/MYC axis as a candidate target for therapeutic intervention in NSCLC.

2. Materials and Methods

2.1. Clinical Samples

A total of 50 paired tumor and nontumor tissues were collected from well informed NSCLC patients in the Shanghai Changhai Hospital (Naval Medical University, Shanghai, China). Clinical and demographic variables were recorded at the time of surgery and are summarized in Table S2. The study involving human tissues was conducted in accordance with the Declaration of Helsinki and was approved by the Committee on Ethics of Biomedicine, Second Military Medical University (approval no. SMMU-2017, 1 March 2017). Written informed consent was obtained from all patients who donated tissues. The animal study was performed in accordance with the Basel Declaration and was approved by the Committee on Ethics of Biomedicine, Second Military Medical University (approval no. SMMU-2017, 1 March 2017).

2.2. Cell Culture

Human NSCLC cell lines (NCI-H1299 and A549) and the 293T embryonic kidney cell line were sourced from the Chinese Academy of Sciences' National Collection of Authenticated Cell Cultures. Cells were propagated in DMEM containing 10% FBS under standard conditions (37 °C, 5% CO₂, humidified incubator). As applicable, cells were treated with 2.5 µM 5-Aza (cat# ab120842, Abcam, Cambridge, UK) for a duration of 72 h.

2.3. Microarray Analysis

Five paired tumor and nontumor tissue samples were utilized for RNA profiling via microarray analysis. Tissue selection was based on predefined quality-control criteria, including adequate tissue availability, pathological confirmation of tumor content, and RNA quality. Briefly, total RNA isolation, cRNA labeling, and hybridization were carried out on the Human LncRNA 3.0 array (8 × 60 k, Arraystar, Rockville, MD, USA) by Aksonomics (Shanghai, China). Raw array data were acquired via Agilent Feature Extraction software and processed using GeneSpring GX v11.5.1 (Agilent Technologies, Santa Clara, CA, USA).

2.4. RNA Sequencing

RNA was isolated from control and NOP56-depleted A549 cell pellets, derived from biological triplicates (cells from different passages), using the Trizol reagent kit (Invitrogen, Carlsbad, CA, USA). Enriched mRNA was fragmented and reverse-transcribed into cDNA using the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB #7530, New England Biolabs, Ipswich, MA, USA). Double-stranded cDNA was subjected to end repair, A-tailing, and adapter ligation, followed by purification with AMPure XP Beads (1.0×) and PCR amplification. Libraries were sequenced on the Illumina NovaSeq 6000 platform by Gene Denovo Biotechnology Co. (Guangzhou, China).

2.5. RNA Extraction, Reverse Transcription and Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was extracted from clinical samples and cells using TRIzol (Thermo, Waltham, MA, USA). First-strand cDNA was synthesized with the PrimeScript™ RT Master Mix (Takara Bio, Dalian, China). qPCR reactions were performed using TB Green® Premix Ex Taq™ (Takara Bio, Dalian, China) according to the manufacturer's instructions. β-actin served as the endogenous control to normalize the total RNA in each sample. Results were calculated using the $2^{-\Delta\Delta CT}$ method. The sequences of all primers used are listed in Supplementary Table S1.

2.6. Lentivirus Infection and Transient Cell Transfection

NOP56 overexpression and control lentiviruses, as well as NOP56 depletion and control lentiviruses, were obtained from GenePharma (Shanghai, China). To establish cell lines with overexpression and knockdown of NOP56, cells were cultured in media supplemented with 5 µg/mL puromycin following infection. Cells were harvested for further experiments two weeks post-infection. The sequences of all short hairpin RNA (shRNA) used are listed in Supplementary Table S1.

All small interfering RNAs (siRNAs) used in this study were purchased from GenePharma (Shanghai, China). Transfection of siRNA was performed using Lipofectamine 3000 (Thermo, Waltham, MA, USA) according to the manufacturer's instructions. The sequences of all siRNAs utilized are listed in Supplementary Table S1.

2.7. Western Blot

Total protein was harvested in RIPA lysis buffer (Beyotime, Shanghai, China), resolved by SDS-PAGE, and electroblotted onto NC membranes (Millipore, Darmstadt, Germany). Membranes were blocked with 5% nonfat milk and probed with primary antibodies at 4 °C overnight with primary antibodies against MYC (49 kDa, 1:2000, 10828-1-AP, Proteintech, Rosemont, IL, USA), NOP56 (66 kDa, 1:1000, A302-720A-T, Thermo, Waltham, MA, USA), or GAPDH (36 kDa, 1:5000, 60004-1-Ig, Proteintech, Rosemont, IL, USA). Following three washes, the membranes were incubated for 40 min at room temperature with IRDye 700-conjugated goat anti-mouse IgG and IRDye 800-conjugated goat anti-rabbit IgG. Signals were detected using the Odyssey infrared scanner (Li-Cor Biosciences, Lincoln, NE, USA), with GAPDH serving as the endogenous control.

2.8. Cell Counting Kit-8 (CCK-8) Assay

Next, 3×10^3 cells were seeded into each well of 96-well plates containing 100 µL of normal culture medium. Cell viability was assessed using the CCK-8 reagent (Dojindo Laboratories, Mashiki, Japan) according to the manufacturer's instructions. The optical density (OD) at 450 nm was normalized to plot the cell growth curve. All experiments were conducted in triplicate.

2.9. Colony Formation Assay

For colony formation assays, 3×10^3 cells per well were plated in 6-well plates containing 2 mL complete medium and cultured for 10 days. Colonies were fixed in 4% paraformaldehyde (30 min), stained with crystal violet (Beyotime, China) for 30 min, imaged, and quantified.

2.10. Transwell Assays

Cell migration was evaluated using Transwell inserts (24-well, 8 µm pore; Merck Millipore (Burlington, MA, USA)) per the manufacturer's protocol. Briefly, 4×10^4 cells suspended in 200 µL serum-free DMEM were seeded into the upper compartment, while 0.5 mL DMEM containing 10% FBS was added to the lower compartment as a chemoattractant. Following 24 h of incubation, migrated cells on the lower membrane surface were stained and enumerated across five randomly selected high-power fields.

2.11. Wound Healing Assays

Wound healing migration assays were conducted as previously described [13]. Cells were plated in 6-well chamber slides with DMEM and grown to confluence. A linear wound was created using a sterile 10 µL pipette tip. Images were captured at 0 and 48 h post-scratching, and the healing rate was determined as wound closure (%) = $(\text{gap area}_{0\text{h}} - \text{gap area}_{48\text{h}}) / \text{gap area}_{0\text{h}} \times 100\%$.

2.12. Animal Studies

Male BALB/c nude mice (5 weeks old) were purchased and housed under conditions described previously [14]. Ethical approval was granted by the Committee on Ethics of Biomedicine at Naval Medical University. Subcutaneous tumor growth assays and tail vein injection models were conducted as outlined in prior studies.

2.13. Bisulfite DNA Sequencing

Genomic DNA was isolated from the indicated tissues and cells using the TIANamp Genomic DNA Kit (TIANGEN, Beijing, China) and bisulfite-converted with the EZ DNA Methylation-Gold™ Kit (Zymo Research, Irvine, CA, USA). CpG island regions were amplified by PCR with site-specific primers. Amplicons were gel-purified, cloned into pMD-19T vectors (Takara, China), and introduced into *E. coli*. Positive clones were sequenced by Jieli Science and Technology Ltd. (Shanghai, China). The sequences of all primers utilized are provided in Supplementary Table S1.

2.14. Reverse Transcription at Low dNTP Concentrations Followed by PCR (RTL-P)

The RTL-P approach was carried out as previously described [11], based on the principle that 2'-O-methylated nucleotides impede reverse transcription under limiting dNTP conditions. Extracted total RNA was reverse-transcribed with site-specific primers targeting upstream of the methylation site at low (1 μM) or high (1 mM) dNTP concentrations (Thermo). Products were quantified by qPCR, and methylation levels were derived using the formula $2^{(CT_{low} - CT_{high})}$. Primer sequences are provided in Supplementary Table S1.

2.15. OP-Puro Incorporation Assay

Global protein synthesis was assessed using the Click-iT Plus OPP Protein Synthesis Assay Kit (Thermo, Waltham, MA, USA) following the manufacturer's instructions. In brief, cells were incubated with 20 μM Click-iT OPP reagent for 30 min, then fixed in 4% formaldehyde/PBS (15 min) and permeabilized with 0.5% Triton X-100/PBS (15 min). Subsequently, cells were treated with the Click-iT Plus OPP reaction cocktail at room temperature for 30 min, followed by DAPI counterstaining (30 min). After two PBS washes, samples were prepared for fluorescence imaging.

2.16. Luciferase Reporter Assay

The promoter region of NOP56 was ligated into a pGL3 vector via PCR, with the empty pGL3 vector serving as a negative control. The pGL3 vector and siRNA targeting MYC were co-transfected into 293T cells using Lipofectamine 3000. Luciferase reporter assays were conducted according to the manufacturer's instructions (Promega, Madison, WI, USA). The pcDNA3-RLuc-PolIRES-FLuc reporter construct was a gift from Nahum Soneberg. IRES elements of IGF1R and MYC were amplified from A549 cells and inserted into the vector. Dual-luciferase activity was measured according to the manufacturer's protocol. All experiments were conducted in triplicate.

2.17. Chromatin Immunoprecipitation (ChIP)

ChIP was carried out with a ChIP assay kit (Millipore, Darmstadt, Germany) as per the manufacturer's protocol. In brief, A549 cells were crosslinked, lysed, and sonicated to yield 200–500 bp chromatin fragments. Precleared lysates were immunoprecipitated using anti-MYC antibody (10828-1-AP, Proteintech, Rosemont, IL, USA). After reversing the cross-linking, DNA was isolated for qPCR analysis. The relevant primer sequences are provided in Table S1.

2.18. Public Datasets

Publicly available transcriptomic datasets were used in this study. TCGA NSCLC RNA-seq expression profiles and corresponding clinical information were obtained from the TCGA repository. In addition, two independent NSCLC microarray datasets, GSE41271 and GSE30219, were downloaded from the Gene Expression Omnibus (GEO) database. These datasets were analyzed to evaluate the expression pattern of NOP56 and its clinical relevance in NSCLC.

2.19. Statistical Analysis

All statistical analyses were conducted using SPSS 16.0 and Graphpad Prism 8.0. Statistical comparisons were made using two-tailed Student's *t*-test, one-way analysis of variance (ANOVA), and chi-square tests as appropriate. Pearson's correlation coefficients was employed for correlation analyses. Survival curves were evaluated using the Kaplan–Meier method and log-rank test. *p*-values were two-sided, with a significance threshold set at $p < 0.05$. All data are presented as mean $\pm$ standard deviation (SD).

3. Results

3.1. Ribonuclear Protein NOP56 Is a Potential Target in NSCLC

With microarray analysis, we identified 1532 differently expressed genes between 5 NSCLC tissues and paired nontumor tissues (Figure 1A). KEGG pathway analysis indicated that hyperactivation of ribosome biogenesis might play vital roles in NSCLC development (Figure 1B). Among the top-ranking upregulated genes in ribosome biogenesis progression, NOP56 is a core component of box C/D snoRNPs (Figure 1C). Analysis of the TCGA database revealed that NOP56 expression was significantly increased in both LUSC and LUAD patients (Figure 1D,E). Additionally, GSE30219 datasets showed that the expression of NOP56 was upregulated in NSCLC tissues compared with normal lung tissues (Figure 1F). Kaplan–Meier analysis based on GSE30219 and GSE41271 datasets showed the NOP56 expression level was negatively correlated with prognosis (Figure 1G,H). To further explore the clinical significance of NOP56, we detected NOP56 expression in a cohort containing 50 pairs of NSCLC and adjacent nontumor tissues by qPCR. Consistent with the publicly available databases, NOP56 expression was upregulated in NSCLC tissues and elevated NOP56 level was associated with poor overall survival (Figure 1I,J). Thus, our study specifically focused on NOP56 to elucidate its role in NSCLC.

3.2. DNA Methylation Mediates the Upregulation of NOP56 in NSCLC

Intriguingly, we found a large CpG island near the promoter region of NOP56 with the algorithm described by Dahiya (Figure 2A). With bisulfite sequencing analysis, we detected the methylation levels of 11 CpG sites in the NOP56 promoter in five pairs of NSCLC tissues and adjacent nontumor tissues. The results showed the NOP56 promoter was hypomethylated in NSCLC tissues compared with paired nontumor tissues (Figure 2B). To determine whether hypomethylation contributes to NOP56 upregulation in NSCLC, four methylation probes located on the promoter of NOP56 with available methylation data were retrieved from the TCGA database. Downregulation of the methylation level of the NOP56 promoter was also confirmed in LUSC and LUAD tissues in TCGA datasets ($p < 0.01$, Figure 2C,D). Pearson correlation analysis showed that probes in the promoter of NOP56 were inversely correlated with NOP56 expression in TCGA-LUSC and LUAD samples (Figure 2E,F). Furthermore, we treated A549 and NCI-H1299 cells with demethylation reagent 5-Aza. The results showed that 5-Aza treatment could induce the reactivation of NOP56 in both cells (Figure 2G).

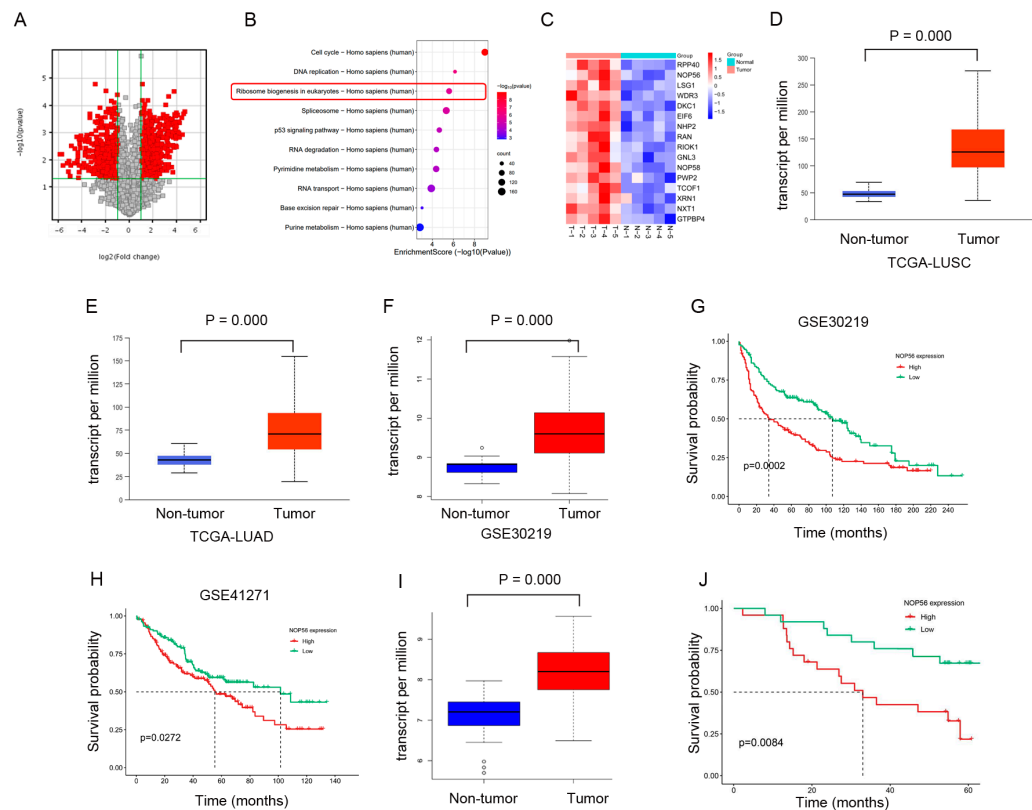


Figure 1. Upregulation of NOP56 is detected in NSCLC tissues and correlated with poor prognosis. (A) Volcano plot for mRNA that were differentially expressed in paired NSCLC and normal tissues (Log2fold-change > 1 and $p < 0.05$). (B) KEGG pathway enrichment analysis for top ten enriched pathways of differentially expressed genes. (C) Heatmaps for mRNA in ribosome biogenesis progression that were differentially expressed. (D–F) NOP56 expression in tumor and nontumor tissues in TCGA-LUAD cohort, TCGA-LUSC cohort and GSE30219 datasets. (G,H) Kaplan–Meier’s survival curves of OS in GSE30219 and GSE41271 datasets. (I) qPCR analysis of NOP56 expression in NSCLC and paired nontumor tissues ($n = 50$). (J) Kaplan–Meier’s survival curves for OS of NSCLC patients.

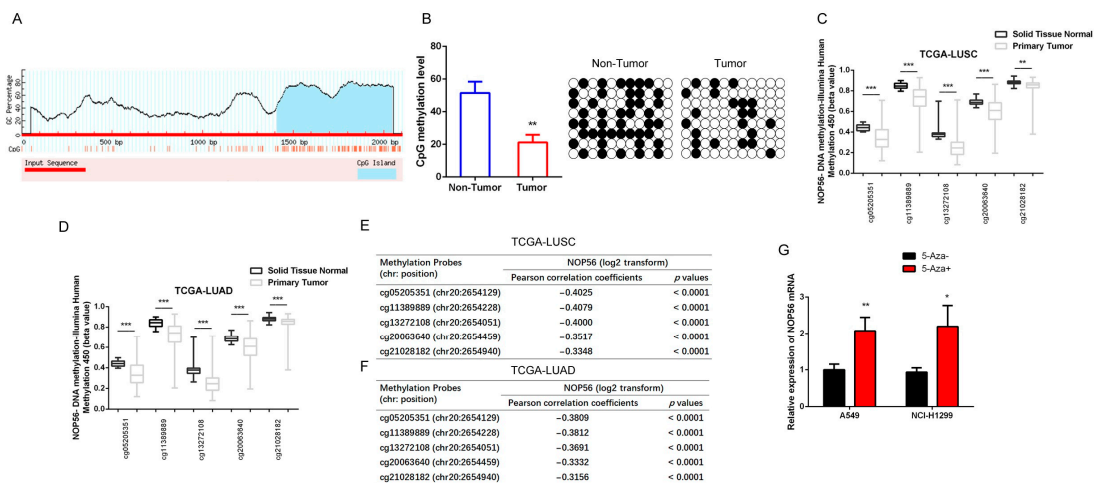


Figure 2. DNA methylation mediates the upregulation of NOP56 in NSCLC. (A) Predicted methylation sites in the promoter region of NOP56 with the algorithm described by Dahiya. (B) Bisulfate DNA sequencing of CpG islands from 3 pairs of NSCLC nontumor tissues. (C,D) Methylation levels of NOP56 promoter region from TCGA-LUSC and TCGA-LUAD datasets. (E,F) Correlation between NOP56 expression methylation probes from TCGA-LUSC and TCGA-LUAD datasets. (G) qPCR analysis of NOP56 expression in A549 and NCI-H1299 cells after treatment with 5-Aza. The data are shown as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Student’s t test.

3.3. NOP56 Promotes the Proliferation and Metastasis of NSCLC Cells

To investigate the potential roles of NOP56 in NSCLC, A549 and NCI-H1299 cells with stable overexpressed NOP56 were constructed with lentivirus infection (Figure 3A). CCK-8 assays showed that overexpression of NOP56 remarkably promoted A549 and NCI-H1299 cellular proliferation ($p < 0.001$, Figure 3B). Colony formation assays revealed that NOP56-overexpressed cells formed higher numbers of clones than control cells formed (Figure 3C). Transwell migration and wound healing assay showed that overexpression of NOP56 significantly promoted A549 and NCI-H1299 cellular migration (Figure 3D,E). Conversely, we constructed NOP56 depleted cells with NOP56-specific shRNA lentivirus (Figure S1A). CCK-8 assays, colony formation assays, migration assays and wound healing assays revealed that NOP56 depletion inhibited cellular proliferation, decreased the number of clones formed and repressed cellular migration in A549 and NCI-H1299 cells (Figure S1B–E). Collectively, the above findings demonstrated that NOP56 might promote the proliferation and migration of NSCLC cells in vitro.

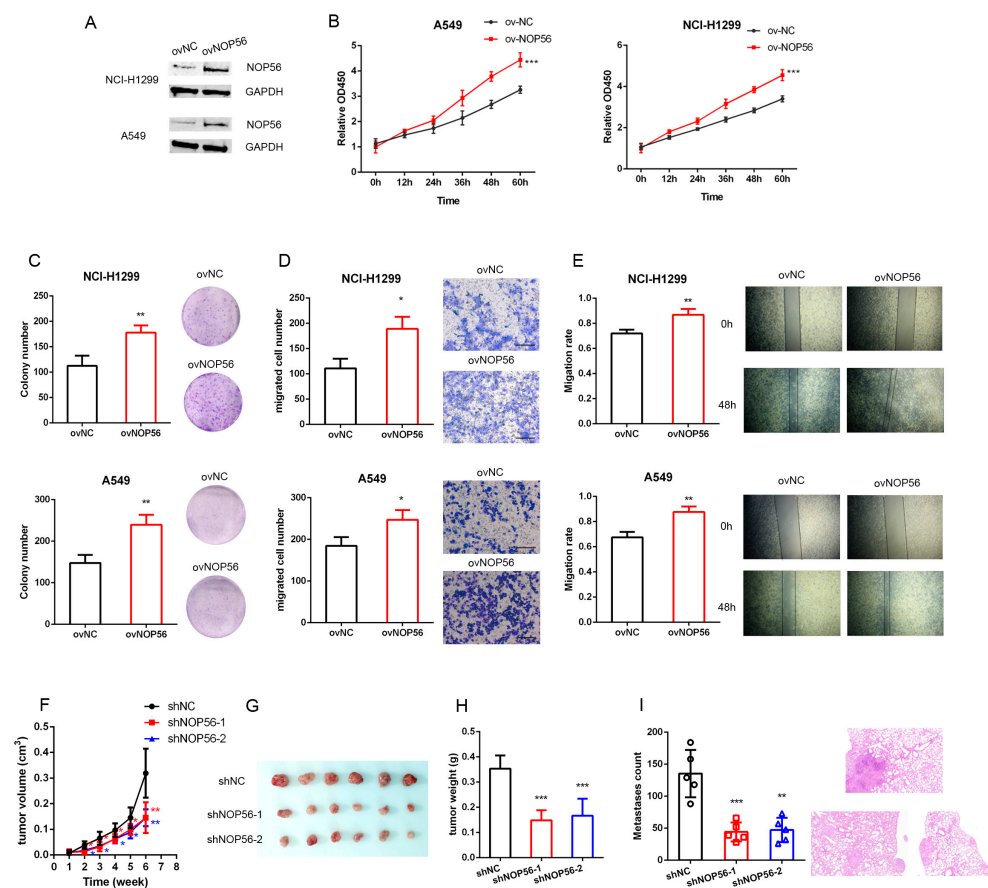


Figure 3. Overexpression of NOP56 exerts oncogenic roles in NSCLC. (A) Western blot analysis of NOP56 expression in NCI-H1299 and A549 cells with stable overexpressed NOP56 or control. (B) CCK-8 assays of NSCLC cells with stable overexpressed NOP56 or control. (C) Colony formation assays of NSCLC cells with stable overexpressed NOP56 or control. (D) Transwell assays of NSCLC cells with stable overexpressed NOP56 or control. (E) Wound healing assays of NSCLC cells with stable overexpressed NOP56 or control. (F–H) Volumes, photo and weight of subcutaneous tumors formed by A549 cells with stable depleted NOP56 or control (6 mice in each group). (I) Metastases formed in lung by A549 cells with stable depleted NOP56 or control (n = 6 mice in each group). The data are shown as mean $\pm$ SD. Scale bar, 100 μ M, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Student's *t* test.

To further verify the role of NOP56 in NSCLC in vivo, A549 cells with depleted NOP56 or control were subcutaneously injected into the armpit of athymic nude mice. A six-week

observation period showed that depletion of NOP56 could decrease tumor growth ($p < 0.01$, Figure 3F). Further measurements revealed that A549 cells with NOP56-stable knockdown formed much smaller tumors than the control ($p < 0.001$, Figure 3G,H). Meanwhile, we injected NOP56-depleted and control A549 cells into the veils of athymic nude mice. After six weeks, A549 cells with depleted NOP56 developed less detectable lung metastases than the control group (Figure 3I). Altogether, these results showed that NOP56 depletion has oncogenic roles in NSCLC.

3.4. Oncogenic Effects of NOP56 Depend on MYC Activation

Then, we explored the mechanism underlying the oncogenic role of NOP56 in NSCLC. We profiled gene expression in NOP56-depleted or control A549 cells using RNA sequencing. Gene set enrichment analysis (GSEA) of RNA sequencing data showed that $n =$ knockdown of NOP56 repressed multiple MYC-regulated genes (Figure 4A), which was confirmed by qPCR analysis (Figure 4B). Furthermore, we detected the expression of MYC in NOP56-depletion NSCLC cells using Western blot and qPCR. The results showed that NOP56 knockdown significantly decreased the MYC protein levels but not mRNA levels (Figure 4C,D).

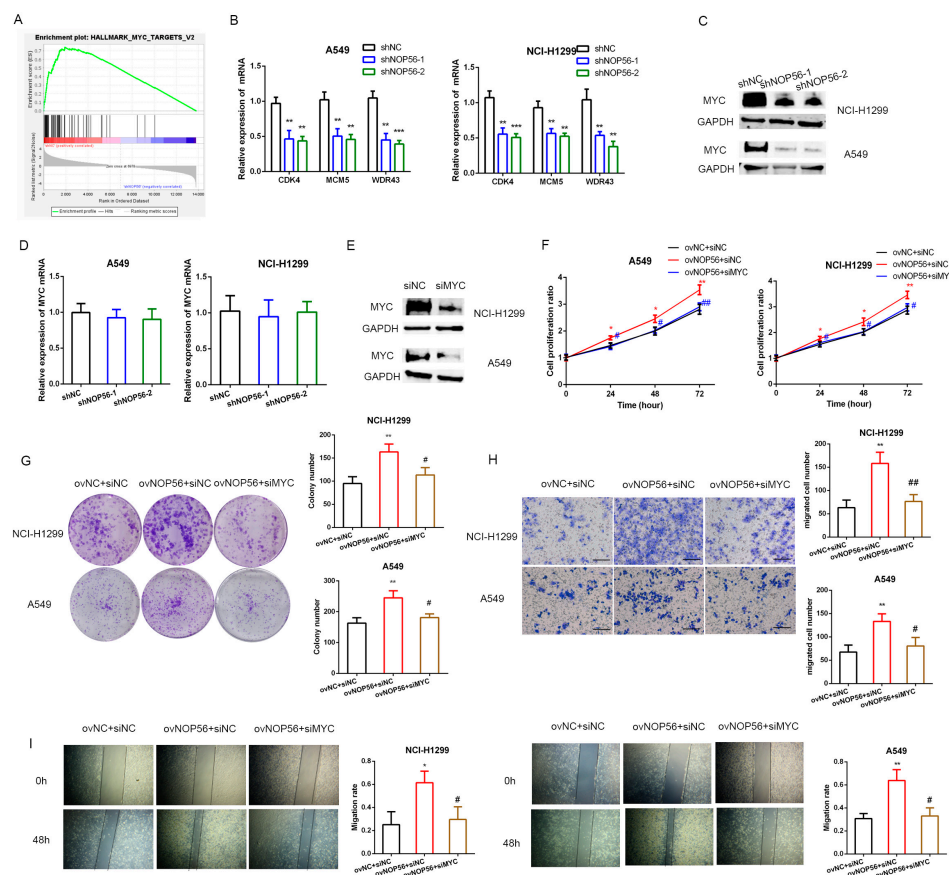


Figure 4. MYC mediates oncogenic role on NOP56 in NSCLC. (A) GSEA plot of MYC targets in NOP56-depleted A549 cells compared with control A549 cells. (B) qPCR analysis of MYC target gene expression in NOP56-depleted NSCLC cells or control. (C) Western blot analysis of MYC expression in NOP56-depleted NSCLC cells or control. (D) qPCR analysis of MYC expression in NOP56-depleted NSCLC cells or control. (E) Western blot analysis of MYC expression in MYC-depleted NSCLC cells or control. (F–I) CCK-8 assays, colony formation assays, transwell assays and wound healing assays in NSCLC cells with NOP56 overexpression or MYC knockdown. The data are shown as mean $\pm$ SD. Scale bar, 100 μ M, * means ovNCS + siNC vs. ovNOP56 + siNC, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Student's t test, # means ovNOP56 + siNC vs. ovNOP56 + siMYC, # $p < 0.05$, ## $p < 0.01$, by Student's t test.

MYC was a widely recognized driver in lung cancer progression, indicating the important role of MYC in NOP56-mediated carcinogenesis. Thus, we decreased MYC expression in NOP56-overexpression cells with siRNA (Figure 4E). CCK-8 assays showed that depletion of MYC reversed the accelerated cellular proliferation caused by NOP56 overexpression (Figure 4F). As colony formation assays indicated, MYC knockdown reversed the increased number of clones caused by NOP56 overexpression (Figure 4G). Transwell and wound healing assays showed that inhibition of MYC partially reversed the effects of NOP56 overexpression on cellular migration (Figure 4H,I). All the results suggested that MYC mediated the oncogenic roles of NOP56 in NSCLC.

3.5. NOP56 Regulates MYC Through IRES-Dependent Translation

Given that NOP56 is a core component of snoRNPs, we proposed that NOP56 might regulate rRNA 2'-O-Me and protein synthesis in lung cancer carcinogenesis. With RTL-P assays, we found that overexpression of NOP56 significantly increased the 2'-O-Me levels in rRNA (Figure 5A). OP-Puro incorporation assays showed that NOP56 overexpression promoted protein synthesis (Figure 5B). As a previous study indicated, 2'-O-Me participates in IRES-dependent modulation of translation. Thus, we constructed dual-luciferase reporter with Poliovirus (PV) IRES-driven firefly luciferase (Fluc). The Fluc/Rluc ratio, which indicates IRES-dependent translation, was significantly increased by NOP56 overexpression ($p < 0.01$, Figure 5C). Interestingly, IRES was identified in 5'UTR of MYC. Thus, we further proposed that NOP56 might regulate the protein translation of MYC through IRES-dependent translation. Then, IRES sequences of MYC in A549 cells were cloned and inserted into the bi-cistronic luciferase vector. The dual-luciferase reporter showed that ectopic expression of NOP56 significantly increased the MYC IRES-driven Fluc/Rluc ratio ($p < 0.01$, Figure 5D). Collectively, these data suggested that NOP56 regulated MYC expression through IRES-dependent translation.

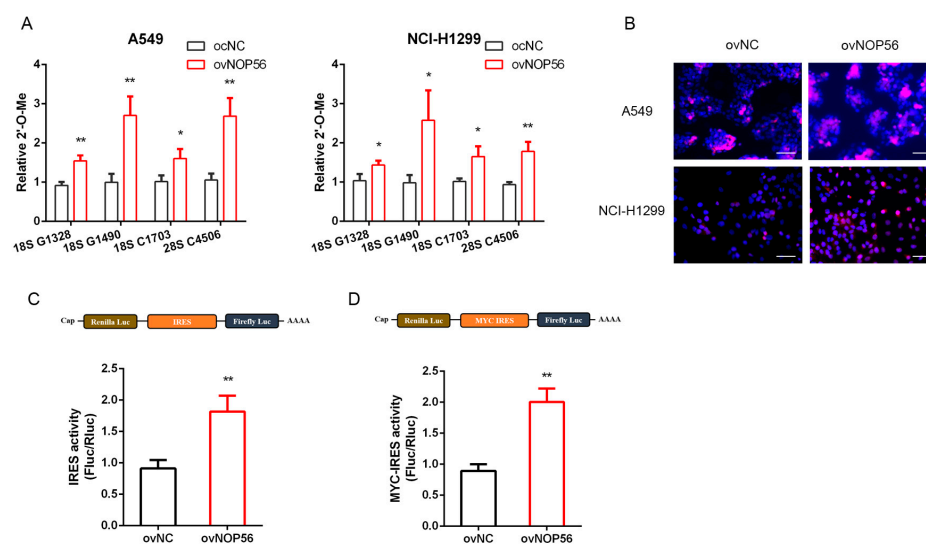


Figure 5. NOP56 regulates MYC through IRES-dependent translation. (A) RTL-P assays were conducted to detect the 2'-O methylation level of rRNA in NSCLC cells with NOP56 overexpression or control. (B) OP-Puro incorporation assay was conducted to detect the protein synthesis rate. (C) The bi-cistronic luciferase reporter was constructed as above and the Poliovirus (PV) IRES activity was calculated as the ratio of firefly luciferase activity over renilla luciferase activity. (D) The bi-cistronic luciferase reporter was constructed as above and the IRES-dependent translation (Fluc/Rluc) from IRES elements of MYC was measured. The data are shown as mean $\pm$ SD. Scale bar, 100 μ M, * $p < 0.05$, ** $p < 0.01$ by Student's t test.

3.6. MYC Is Involved in NOP56 Transcriptional Regulation

Though NOP56 does not affect MYC expression in mRNA level, a significant correlation between NOP56 mRNA and MYC mRNA was detected in TCGA-LUSC, TCGA-LUAD and GSE30219 datasets (Figure 6A–C). Moreover, putative MYC binding sites were found within the NOP56 promoter region using the transcription factor binding profile database (JASPAR), indicating that MYC might regulate NOP56 expression (Figure 6D). Then, qPCR analysis showed that NOP56 expression was significantly decreased with MYC knockdown in A549 and NCI-H1299 cells ($p < 0.01$, Figure 6E). Then, the putative binding sequence fragments and corresponding mutant fragments were inserted into the promoter region of pGL3 luciferase reporter vector. As expected, ectopic depression of MYC decreased the luciferase activity from the reporter containing wild-type binding sites, while mutation of the predicted binding sites abrogated the suppression of luciferase activity induced by MYC depletion (Figure 6F). Moreover, ChIP-PCR analysis further confirmed that MYC directly binds to the promoter region of NOP56 in A549 cells (Figure 6G). Altogether, MYC serves as a translational regulator in the upregulation of NOP56 in NSCLC progression.

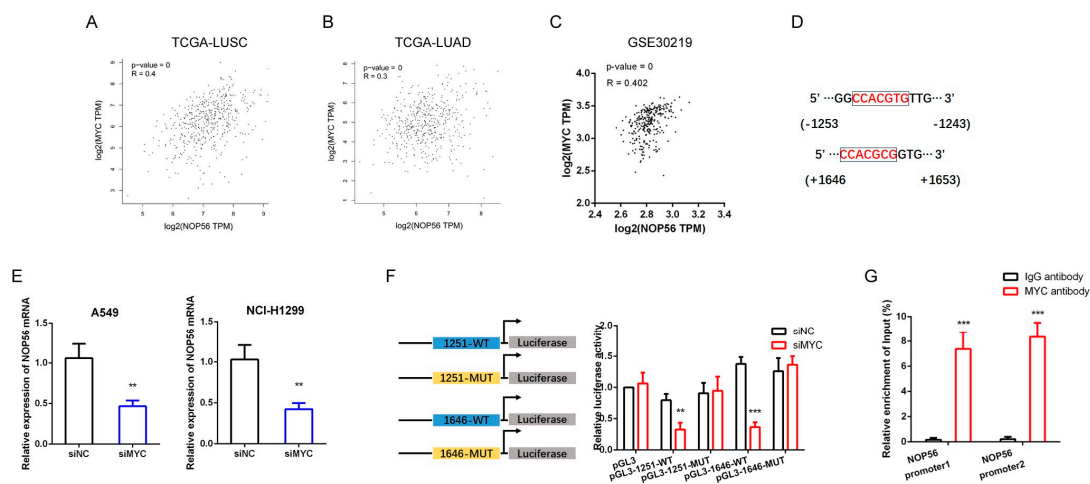


Figure 6. MYC contributes to NOP56 transcriptional regulation. (A–C) Correlation between NOP56 mRNA and MYC mRNA expression in TCGA-LUSC, TCGA-LUAD and GSE30219 datasets. (D) The predicted MYC binding sites in the NOP56 promoter region. (E) qPCR analysis of NOP56 expression in MYC-depleted NSCLC cells or control. (F) The luciferase reporter was constructed as above and the transcription (Fluc) from WT/MUT promoter elements of MYC was measured. (G) ChIP assay was conducted to confirm the MYC binding sites in NOP56 promoter. The data are shown as mean $\pm$ SD. ** $p < 0.01$, *** $p < 0.001$ by Student's t test.

4. Discussion

In this study, with microarray analysis we found that hyperactivated ribosome biogenesis is a distinguishing feature of lung cancer carcinogenesis. The heightened activity of ribosome biogenesis enables increased protein synthesis and supports rapid cell growth [3,15–17]. Among the genes involved in ribosome biogenesis, NOP56 is upregulated in NSCLC and associated with cancer-related death of NSCLC patients. Functionally, we also found that elevated expression of NOP56 contributed to the proliferation and metastasis of NSCLC cells in vitro and in vivo.

NOP56, together with fibrillarin (FBL), NOP58 and SNU13, assemble to form box C/D snoRNP, which mediates 2'-O methylation (2'-O-Me) of rRNA. 2'-O-Me is the most prevalent modification in rRNA and contributes to the function fidelity and translation capacity of ribosomes [18–20]. Alterations in 2'-O-Me modification may modulate cellular phenotypes and increase susceptibility to cancer [21]. In fact, overexpression of the FBL has been observed across multiple cancer types and has been shown to contribute to tu-

morigenesis through dysregulation of translation [22,23]. The formation of box snoRNP complexes is critically important for the self-renewal of leukemic stem cells and the pathogenesis of leukemia [24]. In our study, we also confirmed that upregulation of NOP56 contributed to the elevated 2'-O-Me levels of rRNA and activated IRES-dependent translation in NSCLC. Recent studies revealed that NOP56 is involved in multiple oncogenic signaling pathways, including p38/MAPK, JAK/STAT and mTOR, and plays important roles in tumorigenesis [25]. With RNA-seq analysis, we found that depletion of NOP56 influences MYC target genes, including CDK, cyclins and cycle inhibitors. Treatment with MYC siRNA partially counteracts the influence of NOP56 on cell proliferation and metastasis. Consistent with the previous findings that the promoter region of MYC contains an IRES, we observed that NOP56 regulates MYC translation through IRES activity.

Our work also demonstrated that MYC directly binds to NOP56 loci and regulates NOP56 translation conversely. Thus, we proposed that NOP56 and MYC constituted a positive feedback loop to promote the proliferation and metastasis of NSCLC cells. As the most frequently altered oncogenes in cancer, the cooperative interplay between MYC amplification and KRAS mutation constitutes an intricate and multi-dimensional phenomenon, exerting a significant impact on tumor development [26]. A previous study also pointed out that NOP56 is involved in KRAS-mutant lung cancer [27,28]. Thus, we cannot help proposing that NOP56 might serve as a pivotal nexus in the molecular programs driven by the interplay between MYC and KRAS. Given their complex nature, MYC and KRAS have long been considered “undruggable”. Our study might shed light on their intricate partnership and promote NOP56 as an appealing target in lung cancer therapy. However, the present study is mechanistic and preclinical, and prospective clinical cohorts will be required to determine whether NOP56 levels are associated with response or resistance to specific regimens.

Though MYC signaling contributes to the ribosome biogenesis that was confirmed in our study, the precise mechanisms underlying hyperactivation of ribosome biogenesis in tumorigenesis requires further exploration. DNA methylation is a well-studied epigenetic mechanism that plays a crucial role in various biological processes. Aberrant DNA methylation patterns, which have been consistently observed in lung cancer, lead to the dysregulation of genes involved in cell cycle control, apoptosis, angiogenesis, and epithelial–mesenchymal transition (EMT), all of which contribute to the hallmarks of cancer [29]. Though ribosome DNA methylation changes have been discussed in both pathological and physiological contexts, the relationship between ribosomal proteins and DNA methylation remains largely unknown [30]. In this study, we found that the promoter region of NOP56 bears a large area of CpG islands. Meanwhile, hypomethylation of NOP56 promoter was detected in NSCLC tissues in both TCGA database and clinical samples. Moreover, the observed hypomethylation of NOP56 promoter results in the upregulation of NOP56 in NSCLC. Interestingly, beyond NOP56, the promoter methylation levels of other top-ranking upregulated genes in ribosome biogenesis progression, including RPP40, WDR3 and DKC1, were also dysregulated in the TCGA-LUAD cohort (Figure S2A–C). This observation suggests that the dysregulation of ribosome synthesis pathways in lung cancer is closely linked to aberrant DNA methylation patterns. The connection between DNA methylation status and the dysregulation of ribosome biogenesis has important implications for both the understanding and clinical management of lung cancer.

5. Conclusions

In summary, we found that NOP56 was significantly upregulated in NSCLC. High expression of NOP56 is associated with advanced clinical staging and worse prognosis in NSCLC. The upregulation of NOP56 expression was facilitated by the hypomethylation

of its promoter. NOP56 exerts pro-tumorigenic effects through regulating rRNA 2'-O-Me and activating IRES-dependent translation of MYC. The elevated MYC levels, in turn, promote the transcription of NOP56. Thus, a positive feedback loop between NOP56 and MYC creates a self-sustaining and reinforcing regulatory axis that drives the aberrant proliferation and metastasis of NSCLC cells. These insights provide new avenues for exploring NOP56 as a potential biomarker and therapeutic target for lung cancer.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/cancers18050751/s1>, Figure S1: Depletion of NOP56 exerts tumor suppressive roles in NSCLC; Figure S2: The promoter methylation levels of other top-ranking upregulated genes in ribosome biogenesis progression; Figure S3: Age-dependent Kaplan–Meier survival curves of OS in GSE41271 datasets; Figure S4: Treatment of 5-AZA exerts tumor suppressive roles in NSCLC.; Figure S5: Western blot analysis of NOP56 expression in MYC-depleted NSCLC cells or control; Table S1: Sequences of primers for qPCR, RTL-P, Bisulfite DNA Sequencing, siRNA, shRNA and ChIP-PCR used in this study; Table S2: Clinicopathological characteristics of NSCLC patients.

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Institutional Review Board Statement: Ethical consent was granted from the Committee on Ethics of Biomedicine, Naval Medical University, for studies involving animals.

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Data Availability Statement: The bioinformatic data were deposited in the Cancer Genome Atlas (TCGA) for LUAD and LUSC, as well as in the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>, accessed on 17 February 2026) under accession numbers GSE98929, GSE30219 and GSE41271. Additional data generated or analyzed are included in this article or can be obtained from the corresponding author upon reasonable request.

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Conflicts of Interest: The authors declare no potential conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ChIP	Chromatin immunoprecipitation
cDNA	Complementary DNA
DMEM	Dulbecco’s Modified Eagle’s Medium
EMT	epithelial–mesenchymal transition
FBL	fibrillar
FBS	fetal bovine serum
Fluc	Firefly luciferase

GSEA	Gene set enrichment analysis
IRES	Internal ribosome entry site
LUAD	lung adenocarcinoma
LUSC	lung squamous cell carcinoma
mRNA	messenger RNA
NSCLC	non-small lung cancer
PV	Poliovirus
qPCR	quantitative polymerase chain reaction
RTL-P	Reverse transcription at low dNTP concentrations followed by PCR
rRNA	ribosomal RNA
2'-O-Me	2'-O-methylation

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