MicroRNA-338-3p suppresses cell proliferation and induces apoptosis of non-small-cell lung cancer by targeting sphingosine kinase 2

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Abstract

Background: Lung cancer is the major cause of cancer-related death worldwide, and 80% patients of lung cancer are non-small-cell lung cancer (NSCLC) cases. MicroRNAs are important gene regulators with critical roles in diverse biological processes, including tumorigenesis. Studies indicate that sphingosine kinase 2 (SphK2) promotes tumor progression in NSCLC, but how this occurs is unclear. Thus, we explored the effect of miR-338-3p targeting SphK2 on proliferation and apoptosis of NSCLC cells.

Methods: Expression of miR-338-3p and SphK2 in NSCLC A549 and H1299 cell lines was measured using qRT-PCR and Western blot. CCK-8 and colony formation assays were used to assess the effect of miR-338-3p on NSCLC cell line proliferation. Flow cytometry was used to study the effect of miR-338-3p on NSCLC apoptosis. Luciferase reporter assay and Western blot were used to confirm targeting of SphK2 by miR-338-3p. Finally, in vivo tumorigenesis studies were used to demonstrate subcutaneous tumor growth.

Results: miR-338-3p expression in 34 NSCLC clinical samples was downregulated and this was correlated with TNM stage. miR-338-3p significantly suppressed proliferation and induced apoptosis of NSCLC A549 and H1299 cells in vitro. SphK2 was a direct target of miR-338-3p. Overexpression of miR-338-3p significantly inhibited SphK2 expression and reduced luciferase reporter activity containing the SphK2 3′-untranslated region (3′-UTR) through the first binding site. SphK2 lacking 3′-UTR restored the effects of miR-338-3p on cell proliferation inhibition. miR-338-3p significantly inhibited tumorigenicity of NSCLC A549 and H1299 cells in a nude mouse xenograft model.

Conclusions: Collectively, miR-338-3p inhibited cell proliferation and induced apoptosis of NSCLC cells by targeting and down-regulating SphK2, and miR-338-3p could inhibit NSCLC cells A549 and H1299 growth in vivo, suggesting a potential mechanism of NSCLC progression. Therapeutically, miR-338-3p may serve as a potential target in the treatment of human lung cancer.

Keywords: MicroRNA-338-3p, Sphingosine kinase 2, Non-small-cell lung carcinoma, Cell proliferation, Apoptosis
MicroRNAs (miRNAs) are small, endogenous, non-coding RNAs of approximately 22 nt that regulate the expression of target mRNA by binding to 3′-untranslated regions (3′-UTRs), resulting in target mRNA degradation or silencing [7, 8]. Recent studies indicate that microRNAs (miRNAs) are important subtypes of noncoding RNAs in the regulation of diverse biological processes, especially those involved in critical pathways linked to cancer cell proliferation, apoptosis, and metastasis [9–11]. One target gene may be regulated by multiple miRNAs and one miRNA may regulate multiple target genes, which results in the formation of complex regulation networks in tumorigenesis [12]. Studies show that miRNAs exert oncogenic or tumor suppressor roles in the etiology and pathogenesis of cancer by targeting tumor suppressors or oncogenes [13, 14].

miR-338-3p is mapped to the seventh intron of the apoptosis-associated tyrosine kinase (AATK) gene and miR-338-3p regulates gene AATK expression in rat neurons [15]. miR-338-3p was first reported in prion-induced neurodegeneration: expression of miR-338-3p is reduced in mouse brains infected with mouse-adapted scrape [16]. In tumorigenesis, miR-338-3p is down-regulated in multiple cancers, including gastric, colorectal, and lung cancers [17–19]. However, little is known about the role of miR-338-3p in NSCLC proliferation and apoptosis so we investigated NSCLC progression and development by identifying miRNA targets.

Sphingolipids are a diverse group of water-insoluble molecules including ceramides, sphingoid bases, ceramide phosphates and sphingoid-based phosphates [20], all of which contribute to cell proliferation, invasion and apoptosis [21]. Sphingosine kinases (SphKs) are the rate-limiting enzymes for cellular sphingoid-base phosphates and have two distinct isoforms, SphK1 and SphK2 [22, 23]. SphK1, which is an oncogenic kinase, is involved in tumor development and progression of various human cancers but biological functions of SphK2 in NSCLC remain unknown. Thus, we studied the regulation of miR-338-3p on SphK2 and the consequent effects on proliferation and apoptosis of human NSCLC cells.

Methods
Ethics statement
This study was approved by the Ethics Committee of Zhengzhou University (Zhengzhou, China) and full informed consent was provided by all of the patients involved prior to sample collection.

Patients and tissue samples
A total of 34 patients diagnosed with primary NSCLC at the Henan Tumor Hospital (Zhengzhou, China) between August of 2015 and June of 2016 were included in this study. No patient received chemotherapy or radiotherapy prior to surgery. Tumor and corresponding non-tumor lung tissue samples were collected and rapidly frozen in liquid nitrogen and stored at −80 °C. Tumors were classified according to World Health Organization classification. Data for patient age, gender, smoking history, differentiation, and TNM stage were obtained from patient records (see Table 1).

Cell lines and cell culture
Normal human bronchial epithelial cell line NHBE and human lung cancer cell lines H460, H1299, A549, SPC-A-1 and Calu-3 were purchased from the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified cell incubator with 5% CO₂.

RNA isolation and qRT-PCR
Total RNA was isolated from tissue samples and cell lines using the Qiagen RNeasy kit (Valencia, CA) according to the manufacturer’s instructions. RNA quality and quantity were assessed by standard electrophoretic and spectrophotometric methods. Mature miR-338-3p expression was measured by qRT-PCR according to the Taqman MicroRNA Assays protocol (Applied Biosystems, Carlsbad, CA) and normalized using U6 small nuclear RNA (RNU6B; Applied Biosystems, Carlsbad, CA) with the 2−ΔΔCt method.

Table 1 Expression of SphK2 and miR-338-3p in tissues of 34 lung adenocarcinoma cases

| Parameter               | n | SphK2      | p value | miR-338-3p | p value |
|-------------------------|---|------------|---------|------------|---------|
| Gender                  |   |            |         |            |         |
| Male                    | 21| 0.539 ± 0.105 | 0.428 | 0.330 ± 0.201 | 0.485 |
| Female                  | 13| 0.504 ± 0.148 | 0.385 | 0.385 ± 0.255 | 0.243 |
| Age                     |   |            |         |            |         |
| <60                     | 16| 0.534 ± 0.133 | 0.689 | 0.304 ± 0.160 | 0.260 |
| ≥60                     | 18| 0.517 ± 0.114 | 0.393 | 0.393 ± 0.260 | 0.243 |
| Differentiation         |   |            |         |            |         |
| Well                    | 14| 0.551 ± 0.120 | 0.433 | 0.290 ± 0.121 | 0.404 |
| Moderate                | 13| 0.491 ± 0.140 | 0.401 | 0.401 ± 0.239 | 0.243 |
| Poor                    |  7| 0.539 ± 0.104 | 0.380 | 0.380 ± 0.306 | 0.243 |
| TNM stage               |   |            |         |            |         |
| I + II                  | 24| 0.495 ± 0.108 | 0.017* | 0.406 ± 0.228 | 0.023* |
| III                     | 10| 0.598 ± 0.127 | 0.220 | 0.220 ± 0.132 | 0.220 |
| Smoking history         |   |            |         |            |         |
| Smoker                  | 18| 0.523 ± 0.096 | 0.710 | 0.299 ± 0.108 | 0.145 |
| No smoker               | 16| 0.512 ± 0.149 | 0.410 | 0.410 ± 0.294 | 0.410 |

*p < 0.05
Western blot
Total protein was extracted from tissue samples and cell lines using RIPA buffer containing PMSF. A BCA protein assay kit (Beyotime, Haimen, China) was used to measure total protein. Proteins were electrophoresed via SDS-PAGE and transferred onto PVDF membranes. After blocking, membranes were washed four times with TBST at room temperature and then incubated overnight at 4 °C with diluted primary antibody. Following extensive washing, the membranes were incubated with secondary antibody (HRP-conjugated goat anti-rabbit IgG, 1:3000; Santa Cruz Biotechnology, Santa Cruz, CA). Signals were visualized using a chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ). Antibody against GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) served as an endogenous reference. Protein intensity was scanned on Typhoon PhosphorImager (GE Healthcare, Pittsburgh, PA) for fluorescent signal. Experiments were performed in triplicate.

RNA oligoribonucleotides
The miR-338-3p mimics (GMR-miR MicroRNA-338-3p mimics) used in this study were synthesized by Shanghai GenePharma Co. Ltd. Human NSCLC cells A549 and H1299 were seeded into six-well plates (2 × 10⁵ cells/well) and allowed to settle overnight until they were 50–80% confluent. Cells were transfected with miR-338-3p mimics using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Three groups were generated for the ensuing experiments: non-transfected group (blank control), scrambled miRNA transfected group (negative control, NC) and miR-338-3p mimics transfected group (inhibitor). Then, 24–48 h after the initial transfection, the cells were harvested for further experiments.

Plasmid construction and cell transfection
SphK2 coding sequences lacking the 3′-UTR were cloned into the pcDNA3.1 vector (Invitrogen) to generate the pcDNA3.1-SphK2 expression vector. Cell lines were grown at 37 °C in a humidified atmosphere with 5% CO₂. For transfection, cells were cultured to 70% confluence and transfected with plasmids using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations.

Cell proliferation assay
For growth curve experiments, different experimental groups of A549 and H1299 cells were plated in 96-well plates at 1 × 10⁴ cells per well and incubated for 48 h after transfection. Optical density (OD) was measured using water-soluble tetrazolium salt assay and microplate computer software (Bio-Rad Laboratories, Hercules, CA) according to Cell Counting Kit-8 (CCK-8) assay kit instructions (Dojindo Laboratories, Japan). Absorbance at 450 nm was read on a microplate reader (168–1000 Model 680, Bio-Rad), and proliferation curves were plotted. Cell proliferation was measured using the ratio of OD of transfected cells in each group to ODs of blank control cells in each group. Data were expressed as percents of control.

Colony formation assay
To measure colony-forming activity, three groups of A549 and H1299 cells were counted and seeded into 12-well plates (100 cells/well). Culture medium was replaced every 3 days. Twelve days after seeding, colonies containing more than 50 cells were counted.

Construction of 3′-UTR-luciferase plasmid and reporter assays
The 3′-UTR of the SphK2 fragment was PCR-amplified from human genomic DNA and inserted into the pmiR-GLO control vector (Promega, Madison, WI) at the XhoI and XbaI sites to the luciferase gene. Primer sequences used for PCR amplification were as follows: forward 5′-AU GGGACCAGAGUGAUGCUGGA-3′, reverse 5′-GUUG UUUAGUGACUACGACC-3′. The 3′-UTR of SphK2 was confirmed with sequencing and named pmiR-GLO-WT. Site-directed mutagenesis of the miR-338-3p target site in the SphK2 3′-UTR (pmiR-GLO-mut) was carried out using a Quikchange site-directed mutagenesis kit (Promega, Madison, WI), with pmiR-GLO-WT as the template. For the luciferase reporter assay, A549 and H1299 cells were cultured in 96-well plates. Then, using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA), they were each cotransfected with wildtype or mutant reporter plasmid (100 nM) and microRNA (100 nM). At 48 h after transfection, luciferase activity was measured using the dual-luciferase assay system (Promega, Madison, WI).

Apoptosis measurement using flow cytometry
A549 and H1299 cells were harvested 48 h after transfection and cell concentration was adjusted to 1 × 10⁶ cells. Annexin V-FITC/PI Apoptosis Detection Kit I (Best-Bio, Shanghai, China) was used to measure Annexin V. Results were obtained using FACScan Flow Cytometer (BD Biosciences, San Jose, CA). Tests were repeated in triplicate. Data were analyzed with Cell Quest software.

Animals and subcutaneous tumor growth
Male athymic nude mice (6–8 weeks-of-age) were obtained from the Animal Experimental Center of ZhengZhou University and were acclimated for 2 weeks. This study was conducted in accordance with the recommendations of the Guide for the Care and Use of
miR-338-3p expression is significantly reduced and SphK2 expression is significantly increased in NSCLC tissues

Analysis of patient data indicated that SphK2 expression was significantly correlated with NSCLC TNM stage ($p = 0.017$), and expression of miR-338-3p was also significantly correlated with NSCLC TNM stage ($p = 0.023$). Western blot confirmed that, compared to the distant normal tissues, SphK2 protein expression in NSCLC cancer tissues were higher (Fig. 1a). The results of half quantitative analysis for protein by Western blot indicated that the relative expression of SphK2 in NSCLC tumor tissues was much higher than in normal tissues ($p < 0.001$; Fig. 1b), and the relative expression of SphK2 in NSCLC I + II stage tumor tissues was lower than in NSCLC III stage tumor tissues ($p = 0.017$; Fig. 1c). qRT-PCR results showed that the relative expression of miR-338-3p in NSCLC tumor tissues was much lower than in normal tissues ($p < 0.001$; Fig. 1d), and the relative expression of miR-338-3p in I + II stage tumor tissues was higher than in III stage tumor tissues of NSCLC ($p = 0.023$; Fig. 1e). Thus, relative expression of SphK2 and miR-338-3p in NSCLC was negatively correlated (Fig. 1f).

Results of western blot indicated that protein expression level of SphK2 in human lung cancer cell lines H460, H1299, A549, SPC-A-1, Calu-3 was much higher than that in NHBE, and more obvious in H1299 and A549 cell lines (Fig. 1g). qRT-PCR of the relative expression of miR-338-3p showed the opposite results, in human lung cancer cell lines H460, H1299, A549, SPC-A-1 and Calu-3, it was much lower than that in NHBE, and also more obvious in H1299 and A549 cell lines (Fig. 1h). Thus, the lung cancer cell lines used in the following experiments are H1299 and A549.

miR-338-3p suppresses SphK2 expression by directly targeting the SphK2 3′-UTR

Bioinformatic analysis using TargetScan and miRanda indicated that the 3′-UTR of SphK2 contains a predicted binding site for miR-338-3p (Fig. 2a). Western blot and luciferase reporter assays were used to determine whether SphK2 is regulated by miR-338-3p. Western blot indicated that SphK2 expression was downregulated in A549 and H1299 cells after transfection with miR-338-3p (Fig. 2b). A dual-luciferase reporter system with luciferase reporter vectors containing either the wild-type or the mutant 3′-UTR of SphK2 verified whether SphK2 is a direct target of miR-338-3p. Co-transfection of miR-338-3p significantly decreased luciferase activity of the reporter containing wild-type 3′-UTR, but did not decrease that of the mutant 3′-UTR reporter ($p < 0.05$) in A549 cells (Fig. 2c). Also, co-transfection of miR-338-3p significantly decreased luciferase activity of the reporter containing the wild-type 3′-UTR reporter in H1299 cells ($p < 0.05$; Fig. 2d). Thus, SphK2 is a direct functional target of miR-338-3p, which negatively regulates SphK2 expression by directly binding to its putative binding site in the 3′-UTR sequence.

Overexpression of miR-338-3p suppresses proliferation of A549 and H1299 cells

CCK-8 assays and colony formation assays were performed in NSCLC cells to evaluate the effect of miR-338-3p on cell proliferation. Data show that compared with NCs cell viability of the miR-338-3p group decreased from 24 h onwards in A549 (Fig. 3a) and H1299 (Fig. 3b) cells. Also, colony formation in the miR-338-3p group was much lower than that of NCs in A549 and H1299 cells (Fig. 3c). Thus, miR-338-3p suppresses viability of NSCLC A549 and H1299 cells.

miR-338-3p induces apoptosis of A549 and H1299 NSCLC

Flow cytometry assay indicate that, after A549 and H1299 cells were transfected with miR-338-3p for 48 h, Annexin-FITC positive A549 cells were significantly increased compared with NCs, and results of H1299 cells were similar (Fig. 4a). TUNEL staining to measure NSCLC apoptosis indicated that apoptosis increased compared to NCs (Fig. 4b).

miR-338-3p inhibits NSCLC cells A549 and H1299 growth in vivo

Studies with human NSCLC xenografts in nude mice indicated that bioluminescence in the miR-338-3p group
less than in NCs and tumor growth curves for mice in the miR-338-3p group was less than NCs (Fig. 5a). H1299 cells were similar (Fig. 5b). Thus, miR-338-3p inhibited tumorigenicity of NSCLC A549 and H1299 cells in a nude mouse xenograft model.

Inhibitory effect of miR-338-3p on NSCLC A549 and H1299 cells is mediated by down-regulating SphK2
Western blot indicated that transfection of SphK2-siRNA and miR-338-3p inhibited expression of SphK2, respectively (Fig. 6a, b). CCK-8 assay showed that SphK2-siRNA inhibited proliferation of A549 and H1299 cells compared to NCs, and this was similar to cells transfected with miR-338-3p (Fig. 6c, d). A colony formation assay indicated that SphK2-siRNA reduced colonies of A549 and H1299 cells compared to NCs, and these reductions were similar to cells transfected with miR-338-3p (Fig. 6e). Flow cytometry confirmed that SphK2-siRNA induced apoptosis of A549 and H1299 cells compared to NCs, and activation was similar to cells transfected with miR-338-3p (Fig. 6f). Thus, miR-338-3p inhibited NSCLC biological effects by down-regulating SphK2.

Restoration of SphK2 rescues tumor suppression by miR-338-3p
To investigate whether the effects of miR-338-3p on the cell proliferation and apoptosis of NSCLC cells was mediated by SphK2 repression, we overexpressed SphK2 lacking the 3’-UTR in NSCLC cell lines and co-transfected with miR-338-3p. Results of western blot showed that expression level of SphK2 protein was downregulated in A549 cells after transfected with miR-338-3p, and
overexpressed both in cells transfected with pcDNA3.1-SphK2 (without the 3′-UTR) alone and co-transfected with miR-338-3p. In addition, expression level of SphK2 protein showed no significant difference between the latter two groups (Fig. 7a). Results of CCK-8 assay and colony formation assay showed that the proliferation inhibitory effects of miR-338-3p on A549 cells were partly restored by pcDNA3.1-SphK2 lacking the 3′-UTR (Fig. 7b, c), and the apoptosis promoted effects of miR-338-3p were also partly restored (Fig. 7d). These results indicated that the effects of miR-338-3p on NSCLC cell proliferation and apoptosis were restored by SphK2 lacking the 3′-UTR, suggesting that miR-338-3p suppress NSCLC cell proliferation and induce apoptosis by targeting the 3′-UTR of SphK2.

Discussion
miRNAs are small, noncoding RNAs, 21–25 nucleotides in length, which are master gene mediators that form the miRNA-induced silencing complex (miRISC) and lead to
mRNA instability or degradation [24]. Aberrant miRNA
expression occurs in many biological processes such as
cell proliferation, the cell cycle, apoptosis, invasion, and
migration. Depending on the cellular function of certain
miRNA targets, miRNAs can behave as oncogenes or
tumor suppressor genes.

The apoptosis-associated tyrosine kinase (AATK) gene
is located on chromosome 17 (17q25.3) [25]. Studies
indicate that the role of AATK in anti-tumorigenesis and
aberrant Aatk expression depends on methylation in the
CpG island promoter of Aatk [26, 27]. miR-338-3p sup-
presses the translation of a select group of cellular mRNA
whose protein products are negative regulators of neur-
ite growth. Previously, miR-338-3p was shown to act as a
tumor suppressor in some cancers [28, 29].

Previous studies indicate that miR-338-3p is down-
regulated in colorectal and hepatocellular carcinomas
and gastric cancer [30–32]. However, the expression
pattern of miR-338-3p in lung cancer, particularly in
NSCLC, is unreported. Data from miRNA arrays indi-
cate that miR-338-3p is downregulated in NSCLC tissues
[33, 34] and miR-338-3p may exert a tumor suppressor
role in NSCLC. Using various approaches, we observed
that overexpression of miR-338-3p suppressed NSCLC
A549 and H1299 cell proliferation and induced apoptosis
in vitro and in vivo.

Identification of targets of miR-338-3p in NSCLC is
necessary for understanding the underlying regulatory
mechanisms so we used bioinformatics for target gene
prediction. Considering overlap of the genes identified
by TargetScan, miRBase targets and PicTarget, SphK2 was selected to be a potential target for validation. Using luciferase reporter assays, Western blot, and qRT-PCR assays we verified that miR-338-3p directly targets SphK2 by interacting with the first binding site in the 3’-UTR.

**Conclusions**
We found that miR-338-3p was downregulated in NSCLC tissues, and was significantly correlated with NSCLC pathological stage. miR-338-3p overexpression suppressed NSCLC cell proliferation and induced apoptosis as well as directly targeted SphK2 and inhibited
effect of miR-338-3p on NSCLC A549 and H1299 cells by down-regulating SphK2. And miR-338-3p inhibited NSCLC cell growth in vivo. However, more studies are needed with more clinical samples to determine the clinical significance and prognostic value.

Abbreviations
NSCLC: non-small-cell lung cancer; miR-338-3p: miRNA-338-3p; SphK2: sphingosine kinase 2; CCK-8: cell counting kit 8; 3´-UTR: 3´-untranslated region; AATK: apoptosis-associated tyrosine kinase; FBS: fetal bovine serum; DMEM: Dulbecco's minimum essential medium; NC: negative control.

Authors' contributions
GJZ, GWZ, and GQZ: conceived of the study, and participated in its design and coordination and helped to draft the manuscript. GJZ, RRC, and CYL: collected the samples. GWZ, HZ, RRC, CYL, and YJG: carried out part of experiments and wrote the manuscript. GWZ, HZ, GJZ, and GQZ: performed the statistical analysis. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
The datasets supporting the conclusion of this study are included in this published article.

Ethics approval and consent to participate
The handling of the patient's tissues adhered to the tenets of the Declaration of Helsinki of 1975 and its 1983 revision in protecting patient's confidentiality. All patients were informed in advance, and signed explicit informed consent. This study was approved by the ethics committee of the First Affiliated Hospital of Zhengzhou University. All patients were informed in advance, and signed explicit informed consent. This study was approved by the ethics committee of the First Affiliated Hospital of Zhengzhou University.

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