

Hsa_circ_0003998 promotes cell proliferation and invasion by targeting miR-326 in non-small cell lung cancer

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Background: Circular RNAs represent a new class of noncoding RNAs involved in the development of cancer. However, little is known about their role in non-small cell lung cancer (NSCLC).

Methods: We examined hsa_circ_0003998 levels in 60 NSCLC tissues by quantitative real-time polymerase chain reaction and analyzed the clinicopathologic significance of hsa_circ_0003998 expression. The effect of small interfering RNA-mediated hsa_circ_0003998 knockdown on proliferation and invasion was analyzed in A549 and H1299 cells in vitro. Moreover, the target genes of hsa_circ_0003998 were further explored by bioinformatic analysis, dual luciferase reporter assays, and rescue experiments.

Results: Hsa_circ_0003998 upregulation was associated with larger tumor size and lymph node metastasis and also correlated with shorter overall survival of NSCLC patients. Functional experiments showed knockdown of hsa_circ_0003998 restrained cell proliferation and invasion in NSCLC cells. In particular, hsa_circ_0003998 upregulated the expression of miR-326 target gene Notch1 through sponging miR-326. Furthermore, the tumor-inhibiting effect of hsa_circ_0003998 silencing was blocked by miR-326 inhibitor.

Conclusion: hsa_circ_0003998/miR-326/Notch1 pathway regulates the progression of NSCLC.

Keywords: circular RNA, hsa_circ_0003998, miR-326, Notch1, NSCLC

Introduction

Lung cancer has the highest mortality rate among malignant tumors, and ~85% of lung cancers are non-small cell lung cancers (NSCLCs).^{1,2} Despite advances in treatment strategies, including surgery, radiation therapy, chemotherapy, targeted therapy, and immunotherapy, the prognosis of these patients remains poor.^{3,4} Therefore, the mechanism of lung cancer must be explored to identify potential biomarkers and therapeutic targets.⁵

Circular RNAs (circRNAs) represent a novel class of noncoding RNAs formed by a covalently closed loop.^{6,7} Some studies revealed differential expression of circRNAs in gastric cancer, colorectal cancer, and esophageal squamous cancer, and these deregulated circRNAs have been suggested to play an important role in the regulation of cancer initiation and progression.^{8–10} However, the roles of circRNAs in NSCLC remain largely unknown.

Hsa_circ_0003998 is a circRNA that is 304 nt in spliced sequence length, and its gene is located at chr20:47570092–47580435. Based on CircBase database (<http://circbase.org/>) information, we examined the expression of hsa_circ_0003998 in

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NSCLC cells. Additionally, we further explored the biological function and mechanism of hsa_circ_0003998. Our data indicate that hsa_circ_0003998 exerts oncogenic functions in NSCLC.

Materials and methods

Tissue collection and cell culture

Sixty NSCLC and paired adjacent nontumorous tissue samples were obtained from patients during operation. Detailed clinical information is summarized in Table S1 and this study was approved by the Ethics Committee of Yinzhou People's Hospital (Ningbo, China). Written informed consent was obtained from every patient. All tissue specimens were stored at -80°C until use. The NSCLC cell lines H1299, H460, Calu1, and A549, and one cultured lung epithelial cell BEAS-2B were purchased from American Type Culture Collection and cultured in DMEM (GIBCO BRL, Grand Island, NY, USA) containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 g/mL streptomycin at 37°C in 5% CO_2 and 95% air.

Transient transfection

For the transfection of small interfering RNAs (siRNAs), A549 and H1299 cells (2×10^5) were seeded in 6- or 96-well plates. The following day, cells were transfected with 100 nM of siRNA using Lipofectamine 2000 Reagent (Invitrogen, Carlsband, CA, USA). The sequence of siRNA targeting hsa_circ_0003998 was as follows: 5'-UUAAGUUGCAG GAGGCCAGA-3'.

RNA isolation

Total RNA was isolated from tissues or cells 48 hours after transfection using a TRIzol kit (Invitrogen) in accordance with the manufacturer's instructions. Then chloroform was added to separate organic phase from inorganic phase. Iso-propanol was used for the precipitation of total RNA. RNA concentration was measured using NanoDrop ND-2000 (Thermo Fisher Scientific, Wilmington, DE, USA). Total RNA (2 μg) was reverse transcribed to complementary DNA using random primers under standard conditions with the PrimeScript RT Reagent Kit (TaKaRa, Dalian, China).

Quantitative real-time PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using TransStart Tip Green qPCRSuperMix (Transgen Biotech, Beijing, China) on the ABI 7500 system (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's instructions. Glyceraldehyde-3-phosphate

dehydrogenase mRNA was used to normalize the levels of circRNA and Notch1. U6 small nuclear RNA was used as an endogenous control for miR-326. All the values were standardized with $2^{-\Delta\Delta\text{CT}}$ method. The primers used were hsa_circ_0003998 forward, 5'-AAA GAG GCT CAT CAC TGT CAG G-3', reverse, 5'-GGA CTG GGG TTT TGA CTG GAT-3'; Notch1 forward, 5'-GAG GCG TGG CAG ACT ATG C-3', reverse, 5'-CTT GTA CTC CGT CAG CGT GA-3'; miR-326 forward, CCT CTG GGC CCT TCC TCC AG, reverse, 5'-GCG AGC ACA GAA TTA ATA CGA C-3'.

Cell proliferation and colony formation assays

Cell proliferation was quantified using the Cell Counting Kit-8 (CCK-8, Dojindo, Japan) according to the manufacturer's protocol. For colony formation assays, 24 hours after transfection, approximately 500 cells were plated in each well of six-well plates and cultured routinely for 2 weeks. Then, the colonies were fixed with methanol and stained with 0.5% crystal violet. Three independent experiments were performed for each assay.

Luciferase reporter assay

The wild-type and mutant hsa_circ_0003998 were cloned into pGL3-control vector. 5×10^4 lung cancer cells were seeded in 24-well plates. Forty-eight hours after cotransfection with corresponding plasmids and miR-326/negative control (NC), luciferase activity was measured using dual-luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Relative luciferase activity was normalized to the Renilla luciferase internal control.

Invasion assays

Invasion assays were performed using Matrigel invasion chambers (BD Bioscience, Billerica, MA, USA). 1×10^5 cells suspended in serum-free DMEM were seeded into the upper chamber, while medium containing 10% fetal bovine serum was placed into the lower chamber. After 24 hours of incubation, cells remaining on the upper membrane were discarded and the cells adherent to underside of the membrane were fixed and stained with 0.5% crystal violet. Ten random fields were counted under a microscope in the high-power field.

Western blot

Cancer cells were lysed using RIPA buffer, and 8% SDS-PAGE was used to separate protein extracts. Then immunodetection

was performed using standard techniques. Antibodies against Notch1 and β -actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). HRP-conjugated secondary antibodies were also used.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 software (SPSS Inc.). Data were presented as the mean \pm SD. Overall survival rates were calculated by Kaplan–Meier analysis. The relationship between hsa_circ_0003998 and miR-326/Notch1 expression was tested with two-tailed Pearson's correlation. Differences were considered significant when $P < 0.05$.

Results

Hsa_circ_0003998 expression was increased in NSCLC cell lines and tissues

To verify the bioinformatic analysis results for hsa_circ_0003998, we used qRT-PCR to measure hsa_circ_0003998 expression levels in NSCLC cell lines and tissues. Our results showed that hsa_circ_0003998 expression was increased in four lung cancer cell lines (A549, H1299, H460, and Calu1) compared with that in lung epithelial BEAS-2B cells (Figure 1A). We next assessed

hsa_circ_0003998 expression in NSCLC tissues. As shown in Figure 1B, hsa_circ_0003998 was significantly upregulated in NSCLC tissues compared with normal tissue. Examination of the correlation between hsa_circ_0003998 expression and clinical pathological features showed that hsa_circ_0003998 upregulation was correlated with larger tumor size and lymph-node metastasis (Figure 1C and D). Furthermore, Kaplan–Meier survival curves showed that patients with higher hsa_circ_0003998 expression in NSCLC had poorer prognoses (Figure 1E).

Hsa_circ_0003998 knockdown inhibited NSCLC cell proliferation and invasion

To confirm the functional role of hsa_circ_0003998 in NSCLC, siRNA for hsa_circ_0003998 was transfected into A549 and H1299 cells. A satisfactory transfection efficiency was obtained at 48 hours posttransfection (Figure 2A). CCK-8 and colony formation assays revealed that hsa_circ_0003998 knockdown dramatically reduced the proliferative capacities of A549 and H1299 cells (Figure 2B–D). In addition, invasion assays indicated that hsa_circ_0003998 knockdown significantly reduced the invasive capacity of A549 and H1299 cells (Figure 2E).

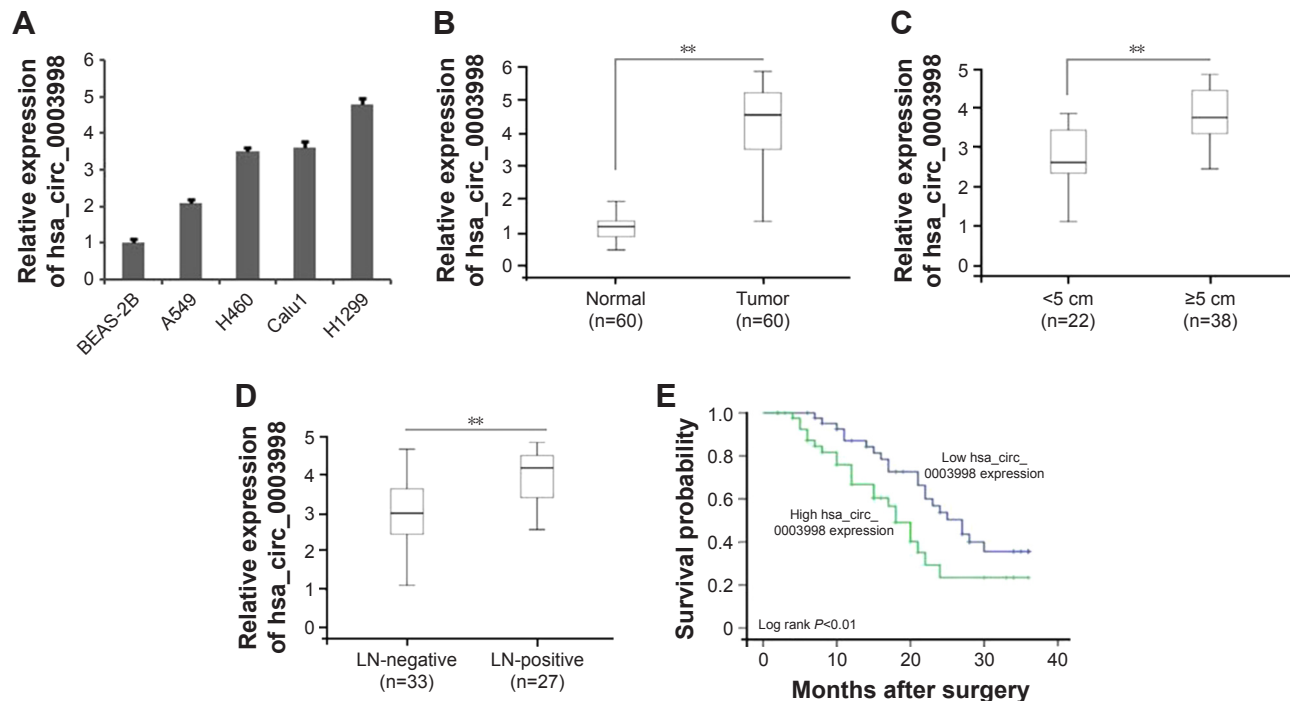


Figure 1 Hsa_circ_0003998 expression was upregulated in NSCLC cells and tissues.

Notes: Expression of hsa_circ_0003998 in four NSCLC cells and one cultured human lung epithelial cell line (**A**). Relative expression levels of hsa_circ_0003998 were determined in 60 paired NSCLC tissue samples and their corresponding normal samples (**B**). Upregulation of hsa_circ_0003998 in NSCLC was associated with tumor size (**C**) and lymph-node metastasis (**D**). NSCLC patients with higher expression of hsa_circ_0003998 had shorter overall survival time than those with lower expression of hsa_circ_0003998 (**E**). The values are the average of three independent experiments. ** $P < 0.01$.

Abbreviation: NSCLC, non-small cell lung cancer.

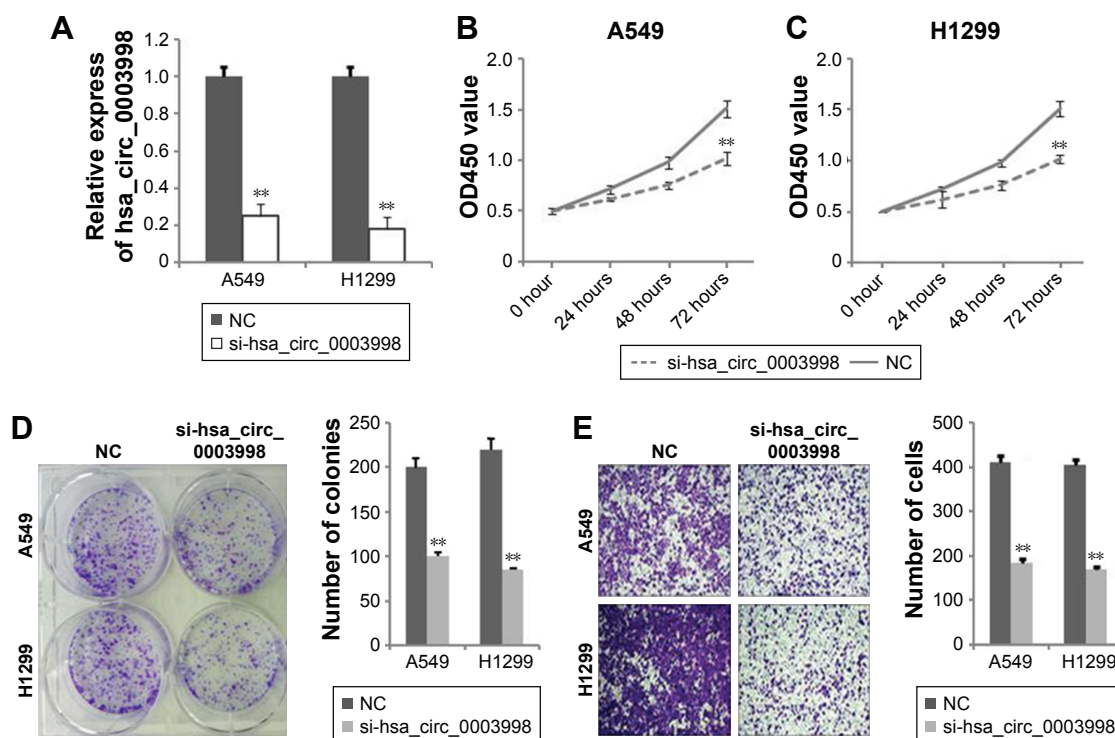


Figure 2 Hsa_circ_0003998 knockdown suppressed NSCLC cell proliferation and invasion in vitro.

Notes: Specific siRNA targeting hsa_circ_0003998 was transfected into A549 and H1299 cells (A). CCK-8 proliferation assay showed the proliferation in the hsa_circ_0003998 knockdown group and the control group (B, C). Colony formation assays showed the clone number in cells transfected with siRNA or control (D). Invasion assays were used to measure invasion ability (E). The values are the average of three independent experiments. ** $P<0.01$.

Abbreviations: NSCLC, non-small cell lung cancer; siRNA, small interfering RNA; NC, normal control.

Hsa_circ_0003998 sponged miR-326

Because circRNAs function mainly as miRNA sponges to regulate gene expression by binding functional miRNAs, we next examined the potential miRNAs associated with hsa_circ_0003998. By using bioinformatics program (Circular RNA Interactome, <https://circinteractome.nia.nih.gov/index.html>), we selected miR-326 as a possible target of hsa_circ_0003998 (Figure 3A). As illustrated in Figure 3B and C, miR-326 was dramatically downregulated in NSCLC tissues, and a significant negative correlation was also found between hsa_circ_0003998 and miR-326 expressions. After knockdown of hsa_circ_0003998, miR-326 expression was increased compared with control (Figure 3D). To further validate the regulatory relationship between hsa_circ_0003998 and miR-326, we performed dual-luciferase reporter assays. As shown in Figure 3E, miR-326 mimics reduced the luciferase activity of wild-type hsa_circ_0003998 reporter vector but not that of mutant reporter vector. These data suggest that hsa_circ_0003998 binds directly to miR-326.

An miR-326 inhibitor rescued the function of hsa_circ_0003998 knockdown

As miR-326 is downregulated in NSCLC (Figure 4A), we next performed rescue experiments to verify whether miR-326

is involved in hsa_circ_0003998-induced NSCLC cell proliferation and invasion. There was a significant decrease in miR-326 expression by the miR-326 inhibitor, compared with that in si-hsa_circ_0003998-transfected H1299 cells (Figure 4B). CCK-8, colony formation, and invasion assays showed that the miR-326 inhibitor rescued the suppressive roles of si-hsa_circ_0003998 (Figure 4C–E).

Hsa_circ_0003998 modulated the expression of the endogenous miR-326 target Notch1

We then detected one of the target genes of miR-326, Notch1,¹¹ which is expressed at significantly higher levels in the NSCLC cell lines than in BEAS-2B cells (Figure S1). As shown in Figure 5A and B, silencing of hsa_circ_0003998 significantly reduced the mRNA and protein expression levels of Notch1, while the miR-326 inhibitor increased the mRNA and protein expression levels of Notch1 in H1299 cells. Furthermore, the expressions of miR-326, hsa_circ_0003998, and Notch1 were examined in 30 tumor specimens used above. As shown in Figure 5C, Notch1 mRNA expression was negatively correlated with miR-326 expression. In contrast, Notch1 expression was positively correlated with hsa_circ_0003998 expression (Figure 5D).

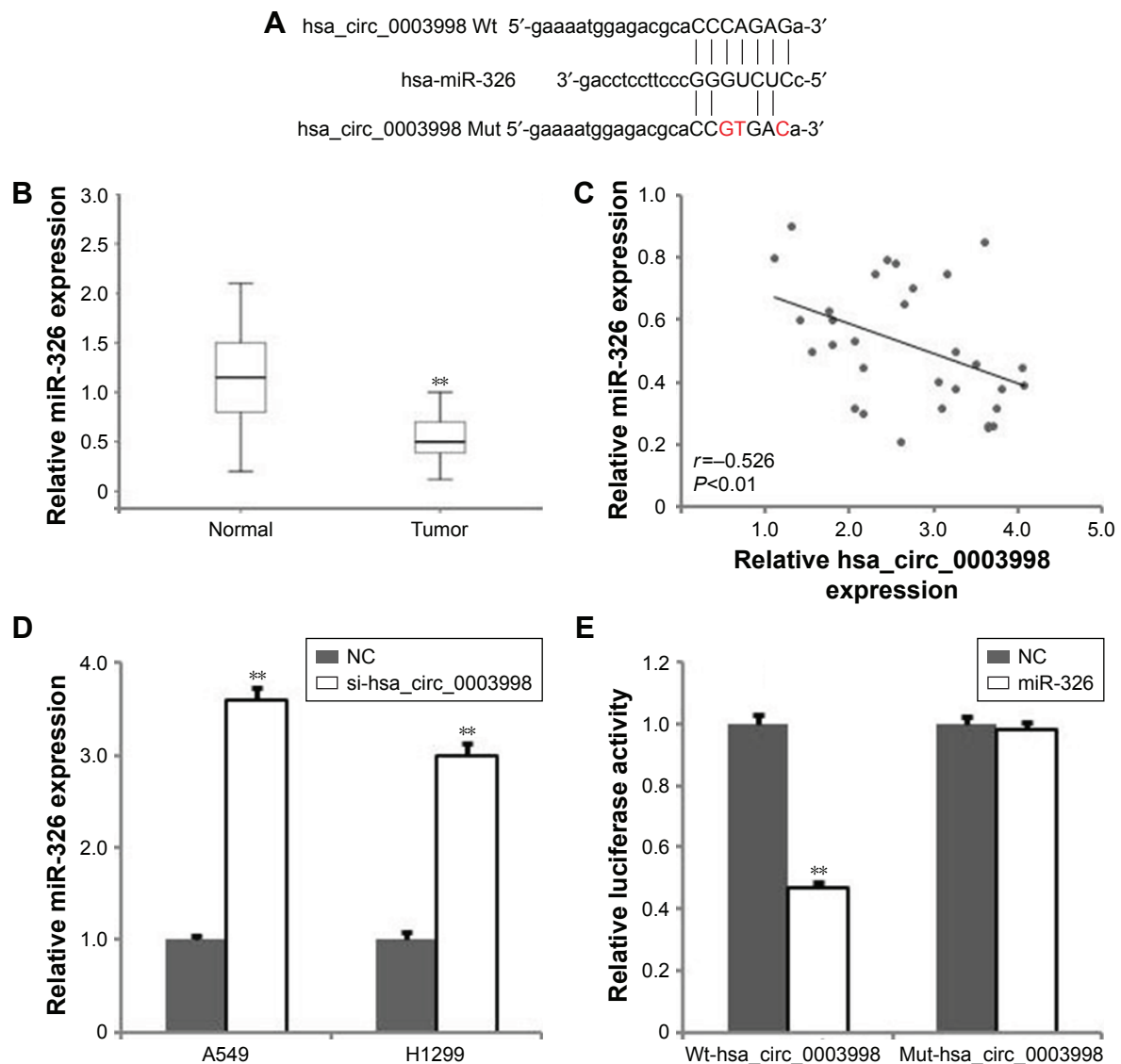


Figure 3 The interaction of hsa_circ_0003998 and miR-326.

Notes: The predicted miR-326/hsa_circ_0003998 binding site and its mutated version by site mutagenesis are as shown (A). The relative expression levels of miR-326 were detected in 30 paired NSCLC and their corresponding normal samples via qRT-PCR (B). Expression levels of miR-326 are negatively correlated with hsa_circ_0003998 among NSCLC samples, as indicated by two-tailed Pearson's correlation analysis ($r = -0.526$, $P < 0.01$) (C). Knockdown of hsa_circ_0003998 increased miR-326 expression (D). Luciferase assays were performed to determine the interaction between miR-326 and hsa_circ_0003998 in H1299 cells (E). The values are the average of three independent experiments. $**P < 0.01$.

Abbreviations: NSCLC, non-small cell lung cancer; qRT-PCR, quantitative real-time polymerase chain reaction.

These results suggest that hsa_circ_0003998 may exert oncogenic functions by modulating miR-326/Notch1.

Discussion

CircRNAs are a novel class of extensive endogenous RNAs. The two most important properties of circRNAs are that they are highly conserved and very stable;^{12,13} these advantages provide circRNAs with the potential to be ideal biomarkers for diagnosis of disease, including cancers.^{14–16} In this report, we demonstrated that a high expression level of hsa_circ_0003998 in NSCLC patients was associated with larger tumor size and lymph-node metastasis. Importantly,

high hsa_circ_0003998 levels had a significantly lower survival rate than those with low expression levels. These results suggest that hsa_circ_0003998 may be used as a potential predictor of prognosis in NSCLC.

The functions of circRNAs in cancer progression have not been elucidated very clearly. The most reported function pattern for circRNAs is acting as miRNA sponges; this “sponge” characteristic is similar to that of long noncoding RNAs (lncRNAs), which suggests that circRNAs exert their regulatory function by binding miRNAs. For example, Cdr1 as, also known as CiRS-7, was shown to harbor 76 miR-7 binding sites.¹⁷ The circRNA MTO1 suppresses

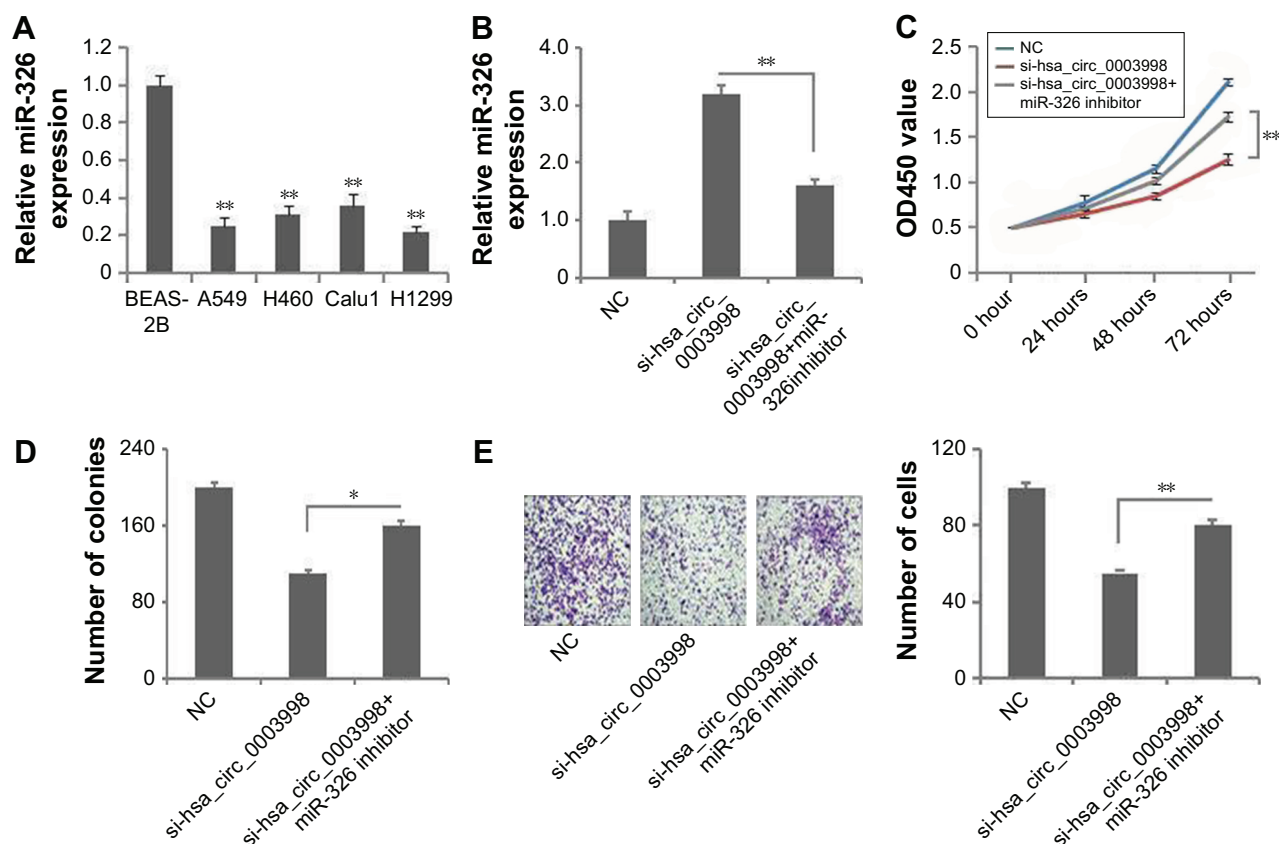


Figure 4 miR-326 inhibitor rescued the function of hsa_circ_0003998 knockdown in H1299 cells.

Notes: miR-326 expression in four NSCLC cell lines detected by RT-PCR (A). miR-326 expression in H1299 cells transfected with si-hsa_circ_0003998 and/or miR-326 inhibitor (B). CCK-8 assay (C). Colony formation assay (D). Invasion assay (E). The values are the average of three independent experiments. * $P < 0.05$, ** $P < 0.01$.

Abbreviations: NSCLC, non-small cell lung cancer; RT-PCR, real-time polymerase chain reaction; NC, normal control.

hepatocellular carcinoma progression by acting as a sponge of miR-9.¹⁸ In lung adenocarcinoma, hsa_circ_0013958 was identified as a sponge of miR-134, and hsa_circ_0013958 levels were associated with tumor node metastasis stage.¹⁹ Here, we employed bioinformatics analysis and provide

evidence that the hsa_circ_0003998 plays an oncogenic role in lung pathogenesis via the modulation of miR-326.

MiR-326 is downregulated in various types of cancers including colorectal cancer,²⁰ lung cancer,²¹ and glioma.²² Our previous studies showed that miR-326 involved in

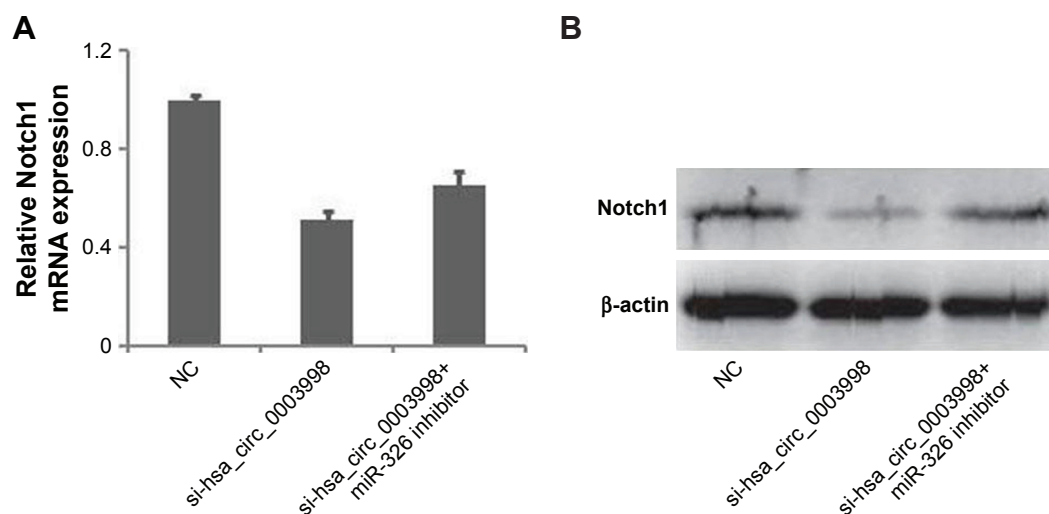


Figure 5 (Continued)

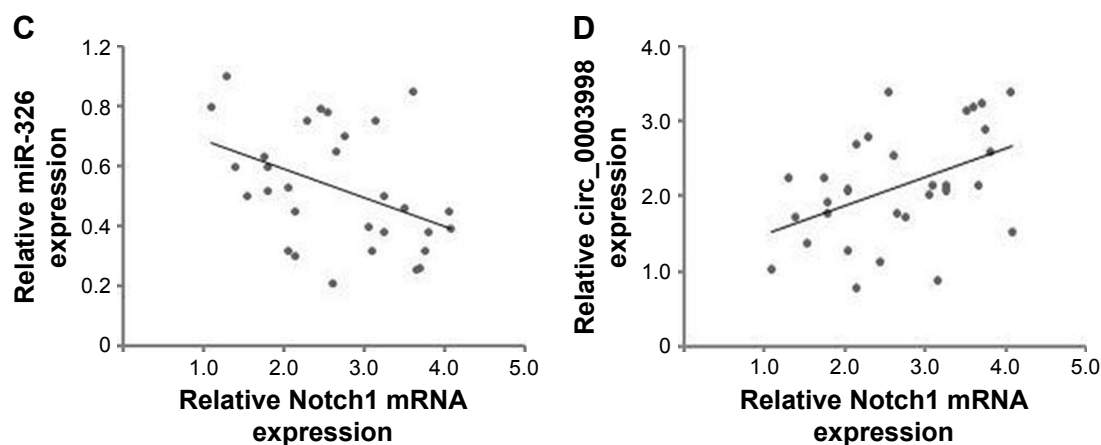


Figure 5 Hsa_circ_0003998 positively regulated the miR-326 target gene Notch1.

Notes: Notch1 mRNA (A) and protein (B) expressions in H1299 cells transfected with si-hsa_circ_0003998 and/or the miR-326 inhibitor. Notch1 mRNA levels were negatively correlated with miR-326 levels (C) and positively correlated with hsa_circ_0003998 levels (D) among NSCLC samples, as indicated by two-tailed Pearson's correlation analysis. The values are the average of three independent experiments.

Abbreviation: NSCLC, non-small cell lung cancer; NC, normal control.

chemotherapy resistance in lung cancer through modulating the expression of specificity protein 1.²³ In this study, we demonstrated miR-326/Notch1 axis mediated the effect of hsa_circ_0003998 on proliferation and invasion. Notch1 has been regarded as a candidate molecule responsible for the development of lung cancer.^{24–26} Therefore, hsa_circ_0003998/miR-326/Notch1 is a key pathway in the development of NSCLC.

In summary, our results show that hsa_circ_0003998 was upregulated in NSCLC and this elevated hsa_circ_0003998 expression was associated with aggressive clinicopathological features. Functional experiments showed hsa_circ_0003998 acted as an oncogene in NSCLC via modulating miR-326/Notch1. Our data suggest that hsa_circ_0003998 may have considerable potential as a prognostic predictor and therapeutic target in NSCLC.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

Table S1 Association between hsa_circ_0003998 expression and clinicopathological characteristics

Characteristics	circ_0003998 low no. case (%)	circ_0003998 high no. case (%)	P-value
Age (years)			0.793
<65	14 (42.4)	10 (37.0)	
≥65	19 (57.6)	17 (64.0)	
Gender			0.799
Male	16 (57.1)	17 (53.1)	
Female	12 (42.9)	15 (46.9)	
Smoking status			1.000
Yes	14 (45.2)	14 (48.3)	
No	17 (54.8)	15 (51.7)	
Tumor size (cm)			0.001 ^a
<5	17 (60.7)	5 (21.9)	
≥5	11 (39.3)	27 (78.1)	
Histological subtype			0.594
Squamous cell carcinoma	8 (34.8)	16 (43.2)	
Adenocarcinoma	15 (65.2)	21 (56.8)	
Lymph node metastasis			0.005 ^a
Yes	7 (25.0)	20 (62.5)	
No	21 (75.0)	12 (37.5)	

Note: ^aP<0.05.

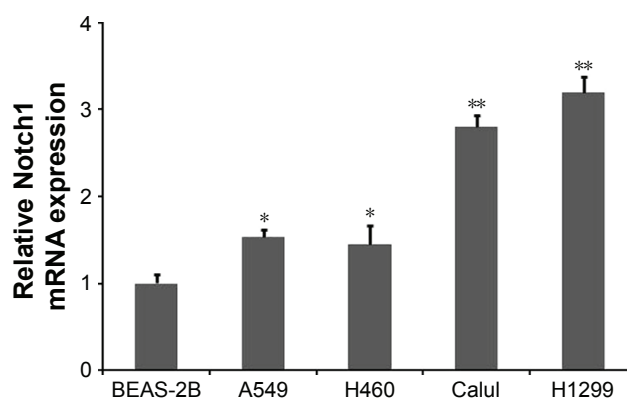


Figure S1 Relative expression levels of Notch1 mRNA were detected in four NSCLC cells and one cultured human lung epithelial cells via qRT-PCR.

Note: *P<0.05, **P<0.01.

Abbreviations: NSCLC, non-small cell lung cancer; qRT-PCR, quantitative real-time polymerase chain reaction.

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