

Original Article

Long noncoding RNA ADAMTS9-AS2 is regulated by DNA methyltransferase 1 and inhibits the malignant behaviors of non-small cell lung cancer cells

Feng Xue^{1,2}, Lin Zhu³, Sha Liu¹, Wei Liu¹, Chunyu Yang¹, Liyan Wang², Li Cai¹

Departments of ¹Medical Oncology, ³Radiotherapy, Harbin Medical University Cancer Hospital, Harbin, Heilongjiang, China; ²Department of Medical Oncology, Heilongjiang Provincial Hospital, Heilongjiang, China

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Abstract: We aimed to elucidate the potential role and possible regulatory mechanism of long noncoding RNA ADAMTS9-AS2 in the progression of non-small cell lung cancer (NSCLC). A total of 80 NSCLC patients were enrolled. ADAMTS9-AS2 expression in NSCLC tissues and its correlation with different clinicopathologic features of patients were investigated. Vectors pcDNA-ADAMTS9-AS2 was transfected into NSCLC cell lines (SPC-A1, H1299, and A549). Then the effects of ADAMTS9-AS2 overexpression on cell proliferation, migration, invasion and apoptosis were explored. Beside, DNA methyltransferase 1 (DNMT1) expression in H1299 and A549 cells was detected. DNMT1 was then knocked down and the effects of DNMT1 knockdown in cell migration and invasion were measured. ADAMTS9-AS2 was down-regulated in NSCLC tissues and was correlated with poor prognosis of NSCLC. In addition, ADAMTS9-AS2 overexpression resulted in proliferation inhibition, migration and invasion suppression, apoptosis induction of NSCLC cells (SPC-A1, H1299, and A549). Besides, ADAMTS9-AS2 expression was negatively correlated with expression of DNMT1, and the inhibitory effects of ADAMTS9-AS2 on the migration and invasion of H1299 and A549 cells were reversed after overexpression of DNMT1. Our findings indicate that down-regulation of ADAMTS9-AS2 may promote cell proliferation, migration and invasion, and inhibit cell apoptosis in NSCLC. Also, ADAMTS9-AS2 expression may be correlated with poor prognosis of NSCLC through interaction with DNMT1. The interplay between DNMT1 and ADAMTS9-AS2 may be exploited for NSCLC therapy.

Keywords: Non-small cell lung cancer, long noncoding RNA ADAMTS9-AS2, DNA methyltransferase 1, poor prognosis

Introduction

Lung cancer remains one of the leading causes of death worldwide [1]. Non-small-cell lung cancer (NSCLC) is the predominant form of lung cancer accounting for approximately 85% of lung cancer cases [2]. Despite recent advances in clinical oncology and early detection, NSCLC is always diagnosed at an advanced stage with a poor prognosis [3]. The 5-year overall survival rate of NSCLC is only 11% [4]. Therefore, elucidation of key molecular mechanism underlying SCLC is crucial to improve the prevention, diagnosis, and treatment of this disease.

Long noncoding RNAs (lncRNAs), ranging in length from 200 nt to 100 kb, are gaining prominence because their dysregulation has been

found to participate in a diverse range of human diseases, in particular cancers [5-8]. Accumulating evidences have highlighted the important roles of lncRNAs in the progression, prognosis and treatment of NSCLC. For instance, lncRNA X inactivate-specific transcript (XIST) is demonstrated to be up-regulated and acts as an oncogene in NSCLC [9]. lncRNA AK126698 can inhibit the cell proliferation and migration in NSCLC via targeting Frizzled-8 and suppressing Wnt/ β -catenin signaling pathway [10]. lncRNA CASC2 can regulate cell proliferation in NSCLC and its low expression indicates a poor prognosis [11]. Thereby, identification of key lncRNAs involved in the development of NSCLC is of great significance. A lncRNA, ADAM metalloproteinase with thrombospondin type 1 motif, 9 (ADAMTS9) antisense RNA 2 (ADAMTS9-

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AS2), the antisense transcript of tumor suppressor ADAMTS9, has been reported to function as a new tumor suppressor to inhibit the cell migration in glioma [12]. Interesting, a microarray analysis showed that ADAMTS9-AS2 was down-regulated in NSCLC tumors [13]. However, the potential roles of ADAMTS9-AS2 in the pathogenesis of NSCLC are still not well investigated.

In this study, whether ADAMTS9-AS2 was dysregulated in NSCLC tissues and the correlation between ADAMTS9-AS2 expression and different clinicopathologic features were investigated. Then the effects of ADAMTS9-AS2 overexpression on the proliferation, migration, invasion and apoptosis of 3 NSCLC cell lines (SPC-A1, H1299, and A549) were explored. Besides, the relationship between ADAMTS9-AS2 and DNA methyltransferase 1 (DNMT1) was detected. The objective of our study was to elucidate the potential role and possible regulatory mechanism of ADAMTS9-AS2 in the progression of NSCLC.

Materials and methods

Patients' samples

Between 2010 and 2011, a total of 80 NSCLC patients who underwent surgery at Harbin Medical University Cancer Hospital were enrolled in our study. NSCLC was diagnosed and confirmed according to histopathologic evaluation. None of these patients received local or systemic treatment before surgery. Clinicopathologic characteristics, including age, gender, TNM staging, tumor size and lymph node metastasis, were recorded. Then NSCLC tissues and their adjacent non-tumor lung tissues from these patients were collected, immediately snap-frozen in liquid nitrogen and then stored at -80°C until use. Our study was approved by the Research Ethics Committee of Harbin Medical University Cancer Hospital, China and all patients provided their written informed consent.

Cell lines and culture

Human normal bronchial epithelial cell line 16HBE and 3 NSCLC cell lines (SPC-A1, H1299, and A549) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). 16HBE

and SPC-A1 cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, USA); A549 and H1299 were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (HyClone, Logan, UT, USA) mixed with 10% fetal bovine serum (FBS, Gibco, USA) at a $37^{\circ}\text{C}/5\% \text{CO}_2$ incubator.

Cell transfection

Vector pcDNA-ADAMTS9-AS2 was constructed by introducing ADAMTS9-AS2 cDNA into the pcDNA3.1 at BamHI and EcoRI site. Then SPC-A1, H1299 and A549 cells were respectively transfected with pcDNA-ADAMTS9-AS2 and pcDNA-control using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Additionally, the pcDNA-DNMT1, pcDNA-control, si-DNMT1 or si-control was respectively transfected into A549 and H1299 cells using the same method.

RNA extraction and quantitative real-time PCR (qRT-PCR) assay

Total RNA was isolated from tissues and cells using TRizol reagent (Invitrogen) following the manufacturer's instructions. Equal amount of total RNA was then reverse transcribed to cDNA using the PrimeScript RT reagent Kit (TaKaRa, Dalian, China). qRT-PCR reaction was then performed using the SYBR Premix Ex Taq (TaKaRa, Dalian, China) on an ABI 7500 instrument to determine the expression levels of targets. The relative expression levels of each target were normalized to the expression of GAPDH and then calculated using $2^{-\Delta\Delta\text{CT}}$ method. Primers used for target amplification were as follows: ADAMTS9-AS2: forward, 5'-TCTGTTGCCCATTTCTACC-3'; reverse, 5'-CCCTTCCATCCTGTCTACTCTA-3', DNMT1: forward, 5'-CCTAGTTCGGTGCTACGAGGAGAA-3'; reverse, 5'-TCTCTCTCTCTGCAGCCGACTCA-3'.

Cell proliferation assays

Cell proliferation was monitored using MTT assay and colony formation assay. For MTT assay, the SPC-A1, H1299 or A549 cells transfected with pcDNA-ADAMTS9-AS2 and pcDNA-control at logarithmic stage were respectively grown in 96-well plates. Cell proliferation was then assessed every 24 h in accordance with the manufacturer's protocol. Briefly, cells grown in 96-well plates were collected by centrifuga-

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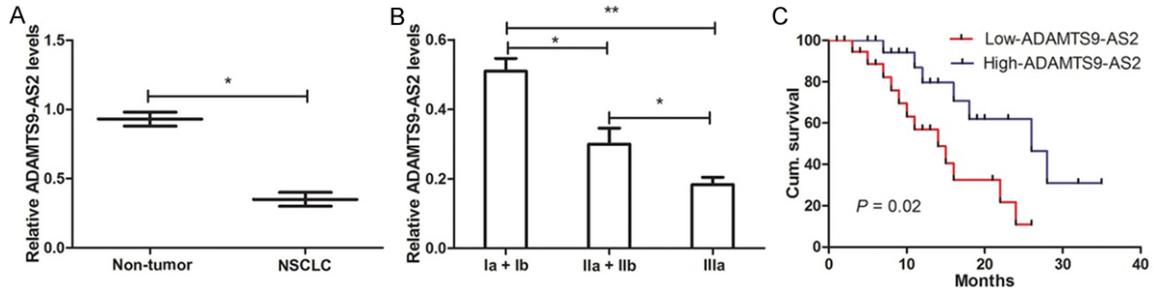


Figure 1. LncRNA ADAMTS9-AS2 was down-regulated in NSCLC tissues and was correlated with poor prognosis of NSCLC. A: qRT-PCR showed the expression of ADAMTS9-AS2 in NSCLC tissues and non-tumor tissues. B: qRT-PCR showed the expression of ADAMTS9-AS2 in NSCLC tissues with different TNM staging. C: Kaplan-Meier survival analysis showed the survival of NSCLC patients with high or low ADAMTS9-AS2 expression. Error bars indicate means \pm SD. *, $P < 0.05$, **, $P < 0.01$.

Table 1. Correlation between ADAMTS9-AS2 expression and different clinicopathological features of NSCLC patients

Characteristics	ADAMTS9-AS2		P value
	Low, N of cases (42)	High, N of cases (38)	
Age (years)			0.832
≤ 65	20	19	
> 65	22	19	
Gender			0.964
Male	23	21	
Female	19	17	
TNM stage			0.003
Ia + Ib	6	13	
IIa + IIb	17	11	
IIIa	19	4	
Tumor size			0.008
≤ 5 cm	12	22	
> 5 cm	30	16	
Lymph node metastasis			0.011
Negative	31	14	
Positive	17	24	

tion after being cultured for 24 h. A total of 20 μ L MTT was added into each well and cultured for another 4 h. After that, 150 μ L dimethylsulfoxide (DMSO) was added to mix with the cells for 10 min. Absorbance at 490 nm was finally measured under an absorption spectrophotometer (Olympus, Japan). Each experiment was performed in quadruplicate.

For colony formation assays, The SPC-A1, H1299 or A549 cells transfected with pcDNA-ADAMTS9-AS2 and pcDNA-control at logarithmic stage were placed in 6-well plates. Media

containing 10% FBS were added to culture these cells and replaced every 4 days. After 14 days, these cells were fixed with methanol and then stained with 0.1% crystal violet (Sigma-Aldrich). Finally, the visible colonies were counted. Each treatment was performed in triplicate.

Cell migration and invasion assays

Cell migration and invasion were assessed using Transwell assay. For the migration assay, the upper chamber of an insert (8-mm pore size; Millipore, Billerica, MA, USA) was not coated with Matrigel, while the upper chamber of insert was coated with Matrigel for invasion assay. Brief, at 48 h after transfection with pcDNA-ADAMTS9-AS2 and pcDNA-control, the SPC-A1, H1299 or A549 cells were added into the upper chamber of an insert containing serum-free medium. The lower chamber was added medium containing 10% FBS. After 24 h of incubation, cells remaining on the upper membrane were removed with a cotton swab. Cells that had migrated or invaded through the membrane were stained with methanol, stained with 0.1% crystal violet and counted using an I \times 71 inverted microscope (Olympus, Tokyo, Japan). Each experiment was repeated 3 times independently.

Cell apoptosis assay

At 48 h after transfection with pcDNA-ADAMTS9-AS2 and pcDNA-control, the SPC-A1, H1299 or A549 cells were harvested by trypsinization. After the double staining with 10 μ L FITC-Annexin V and 5 μ L Propidium iodide (PI)

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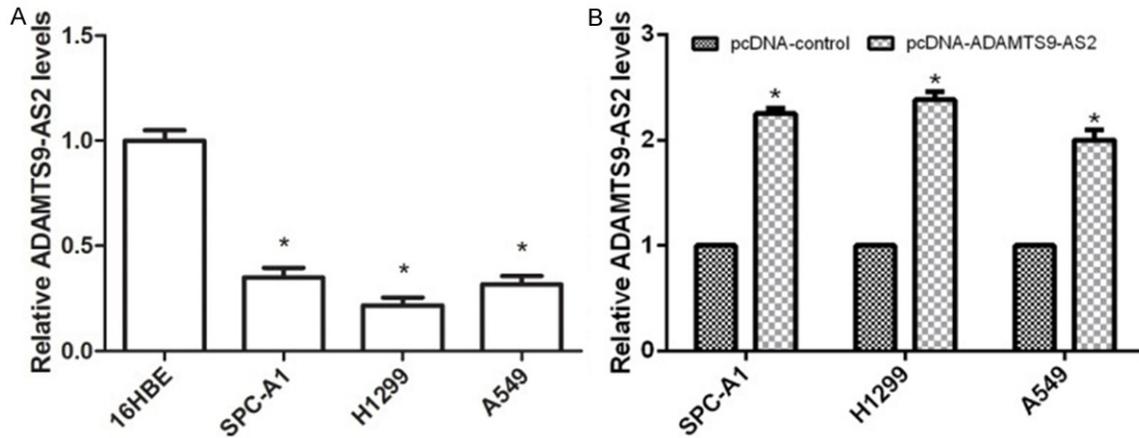


Figure 2. ADAMTS9-AS2 was down-regulated in NSCLC cells. A: qRT-PCR showed the expression of ADAMTS9-AS2 in NSCLC cell lines (SPC-A1, H1299 and A549) and normal bronchial epithelial cell line 16HBE. B: qRT-PCR showed the expression of ADAMTS9-AS2 in different transfected NSCLC cell lines (SPC-A1, H1299 and A549). Error bars indicate means \pm SD. *, $P < 0.05$.

using the FITC-Annexin V Apoptosis Detection Kit (BD Biosciences) following the manufacturer's recommendations, the apoptotic cells (Annexin V-positive and PI-negative) were analyzed with a flow cytometry (FACScan; BD Biosciences) equipped with a CellQuest software (BD Biosciences).

Statistical analysis

All measurement data were expressed as mean \pm SD. Differences between groups were analyzed using Student's t test or Chi-square test. Survival analysis was carried out using the Kaplan-Meier method, and the differences between patient groups were analyzed using the log-rank test. Pearson's correlation was used to assess the correlation between genes expression. All statistical analyses in this study were performed using SPSS version 18.0 (SPSS, Chicago, IL) and a value of $P < 0.05$ was considered statistically significant.

Results

ADAMTS9-AS2 was down-regulated in NSCLC tissues and was correlated with poor prognosis of NSCLC

The expression of ADAMTS9-AS2 in NSCLC tissues was firstly determined by qRT-PCR analysis in our study. As shown in **Figure 1A**, ADAMTS9-AS2 expression was significantly down-regulated in NSCLC tissues in comparison with their adjacent non-tumor lung tissues

($P < 0.05$). In addition, the correlation between ADAMTS9-AS2 expression and different clinicopathologic features were analyzed. As shown in **Table 1**, ADAMTS9-AS2 expression was significantly correlated with TNM staging ($P = 0.003$), tumor size ($P = 0.003$) and lymph node metastasis ($P = 0.011$). Furthermore, ADAMTS9-AS2 expression in IIa + IIb was significantly decreased compared with Ia + Ib, and further decreased in IIIa, indicating a negative correlation between ADAMTS9-AS2 expression and TNM staging (**Figure 1B**). However, there was no significantly difference between ADAMTS9-AS2 expression and age ($P = 0.832$) or gender ($P = 0.964$). Besides, we used Kaplan-Meier survival analysis and log-rank tests to detect the correlation between ADAMTS9-AS2 expression and the survival of NSCLC patients. The results showed that the survival of NSCLC patients with high ADAMTS9-AS2 expression was significantly higher than patients with low ADAMTS9-AS2 expression ($P = 0.02$, **Figure 1C**).

ADAMTS9-AS2 was down-regulated in NSCLC cells

As displayed in **Figure 2A**, ADAMTS9-AS2 expression in NSCLC cell lines (SPC-A1, H1299 and A549) was all significantly lower than normal bronchial epithelial cell line 16HBE ($P < 0.05$), while was in line with the ADAMTS9-AS2 expression in NSCLC tissues. In addition, we further overexpressed ADAMTS9-AS2 in the above 3 NSCLC cell lines to detect the effects

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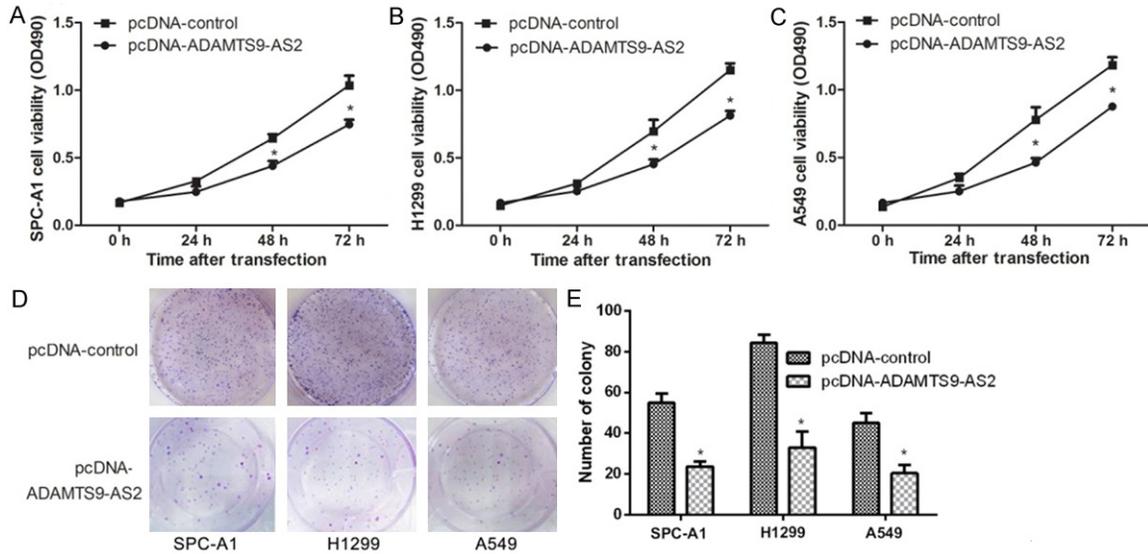


Figure 3. Cell proliferation was inhibited after ADAMTS9-AS2 overexpression. A-C: MTT assays showed the cell viabilities of SPC-A1, H1299 and A549 cells in pcDNA-ADAMTS9-AS2 group and pcDNA-control group in an experimental period of 72 h after transfection. D, E: Colony formation assay showed the number of colony of pcDNA-ADAMTS9-AS2 group and pcDNA-control group. Error bars indicate means \pm SD. *, $P < 0.05$.

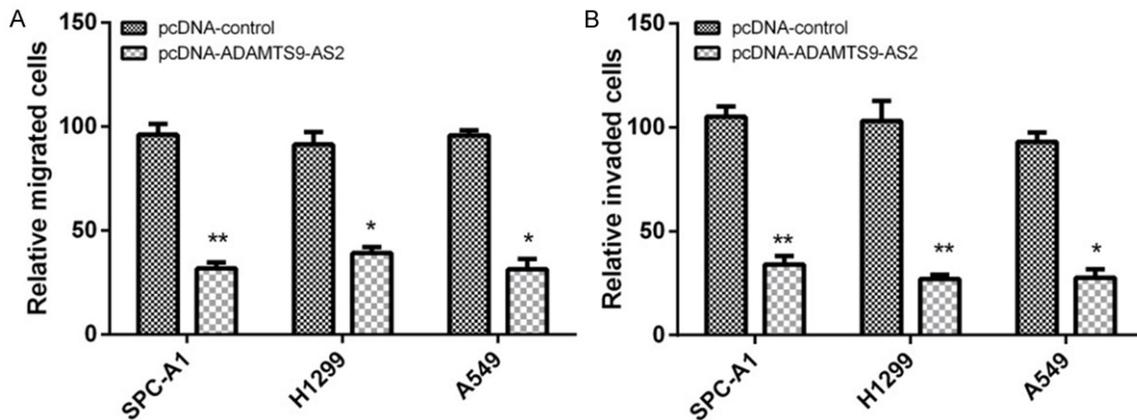


Figure 4. Cell migration (A) and invasion (B) was suppressed after ADAMTS9-AS2 overexpression. Error bars indicate means \pm SD. *, $P < 0.05$; **, $P < 0.01$.

of ADAMTS9-AS2. The results showed that ADAMTS9-AS2 expression was significantly increased in SPC-A1, H1299 and A549 cells transfected with pcDNA-ADAMTS9-AS2 compared with control group ($P < 0.05$, **Figure 2B**), indicating that ADAMTS9-AS2 was successfully overexpressed in NSCLC cells.

Cell proliferation was inhibited after ADAMTS9-AS2 overexpression

Figure 3 displayed the cell proliferation after ADAMTS9-AS2 overexpression. The results of MTT assays showed that, in an experimental

period of 72 h after transfection, cell viabilities of SPC-A1, H1299 and A549 cells in pcDNA-ADAMTS9-AS2 group were all significantly decreased in comparison with pcDNA-control group, especially at 48 h and 72 h after transfection ($P < 0.05$, **Figure 3A-C**). Moreover, expected results of colony formation assay were obtained that the number of colony of pcDNA-ADAMTS9-AS2 group was also markedly lower than pcDNA-control group ($P < 0.05$, **Figure 3D and 3E**). These data imply that ADAMTS9-AS2 could effectively inhibit cell proliferation of NSCLC cells.

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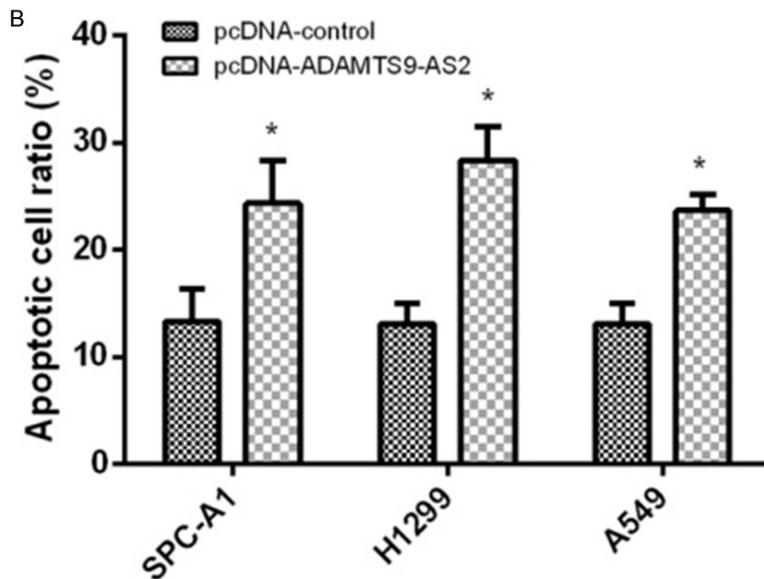
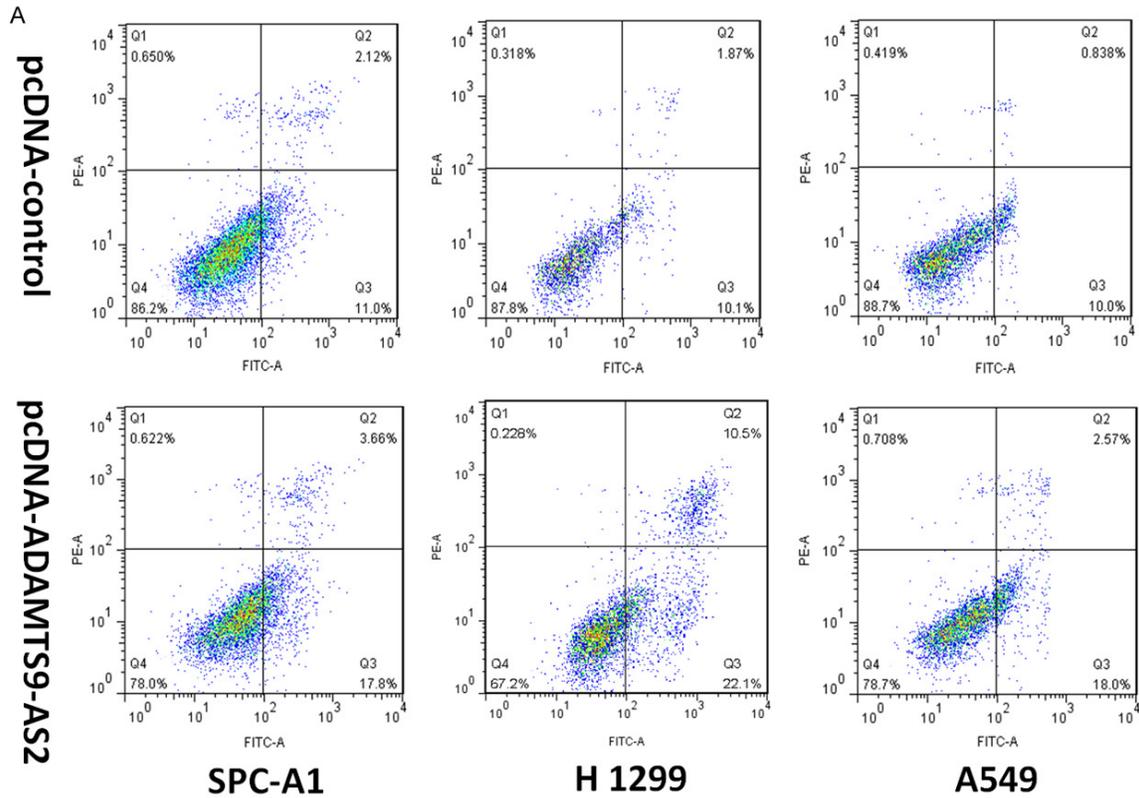


Figure 5. Flow cytometry showed that overexpression of ADAMTS9-AS2 induced the apoptosis of NSCLC cells. Error bars indicate means \pm SD. *, $P < 0.05$.

Cell migration and invasion was suppressed after ADAMTS9-AS2 overexpression

As shown in **Figure 4**, the results of Transwell assay showed that the migrated and invaded SPC-A1 cells in pcDNA-ADAMTS9-AS2 group were significantly lower than pcDNA-control group ($P < 0.05$). Similar results were found in

the migration and invasion of pcDNA-ADAMTS9-AS2 transfected H1299 and A549 cells.

Overexpression of ADAMTS9-AS2 induced the apoptosis of NSCLC cells

Cell apoptosis was also detected after ADAMTS9-AS2 overexpression by flow cytome-

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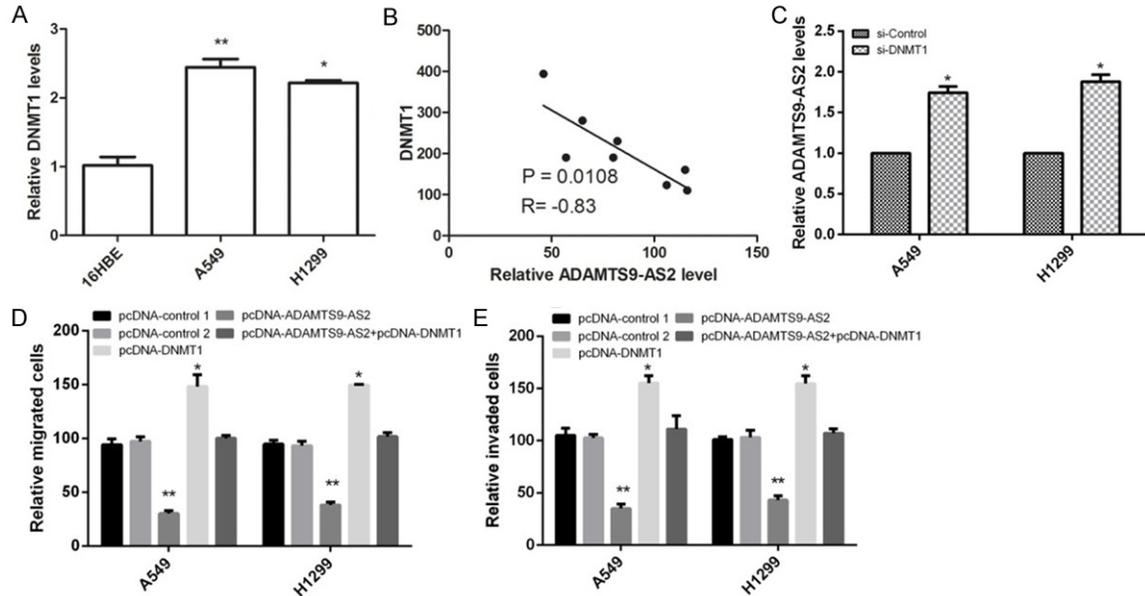


Figure 6. ADAMTS9-AS2 expression was negatively correlated with expression of DNMT1. A: qRT-PCR showed the expression of ADAMTS9-AS2 in DNMT1 in H1299 and A549 cells. B: Pearson's correlation analysis showed that ADAMTS9-AS2 expression was negatively correlated with expression of DNMT1. C: qRT-PCR showed the expression levels of ADAMTS9-AS2 in H1299 and A549 cells after knockdown of DNMT1. D: Transwell assay showed the number of migrated cells in different transfected groups. E: Transwell assay showed the number of invaded cells in different transfected groups. Error bars indicate means \pm SD. *, $P < 0.05$; **, $P < 0.01$.

try. The results showed that after ADAMTS9-AS2 overexpression, the apoptotic SPC-A1, H1299 and A549 cells were significantly increased compared with control ($P < 0.05$, **Figure 5A** and **5B**), indicating that overexpression of ADAMTS9-AS2 induced the apoptosis of NSCLC cells.

ADAMTS9-AS2 expression was negatively correlated with expression of DNMT1

We further selected H1299 and A549 cells to detect the relationship between ADAMTS9-AS2 and DNMT1. As shown in **Figure 6A**, the expression levels of DNMT1 in H1299 and A549 cells were significantly higher than normal bronchial epithelial cell line 16HBE ($P < 0.05$). Moreover, the results of Pearson's correlation analysis showed that ADAMTS9-AS2 expression was negatively correlated with expression of DNMT1 ($P = 0.0108$, **Figure 6B**). Furthermore, we knocked down DNMT1 using si-DNMT1 to explore the relationship between ADAMTS9-AS2 and DNMT1. As shown in **Figure 6C**, the expression levels of ADAMTS9-AS2 in H1299 and A549 cells were all significantly increased after knockdown of DNMT1 ($P < 0.05$). Besides, the

ADAMTS9-AS2 overexpression-decreased migrated and invaded NSCLC cells (H1299 and A549) were neutralized after cells were transfected with pcDNA-ADAMTS9-AS2 and pcDNA-DNMT1 simultaneously ($P < 0.05$, **Figure 6D** and **6E**), indicating that the inhibitory effects of ADAMTS9-AS2 on cell migration and invasion were reversed after overexpression of DNMT1 in NSCLC cell lines.

Discussion

It has now become widely accepted that many cancers are ascribed to lncRNAs dysregulation [14]. Nonetheless, the functional roles of key lncRNAs in cancer progression remain obscure. In the present study, ADAMTS9-AS2 was down-regulated in NSCLC tissues and was correlated with poor prognosis of NSCLC. In addition, ADAMTS9-AS2 overexpression resulted in proliferation inhibition, migration and invasion suppression, apoptosis induction of NSCLC cells (SPC-A1, H1299, and A549). Besides, ADAMTS9-AS2 expression was negatively correlated with expression of DNMT1, and the inhibitory effects of ADAMTS9-AS2 on the migration and invasion of H1299 and A549 cells were

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reversed after overexpression of DNMT1. These data imply that ADAMTS9-AS2 may act as a novel tumor suppressor in NSCLC and could be regulated by DNMT1.

LncRNA ADAMTS9-AS2 is an antisense transcript of protein coding gene ADAMTS9. ADAMTS9 has already been found to inhibit tumor progression in several cancers, such as nasopharyngeal cancer [15], gastric cancer [16] and head and neck cancer [17]. In addition, the antisense lncRNAs has been proved to modulate their sense mRNA partners [18, 19], thereby to participate in the progression of various malignant tumors. For instance, lncRNA C-terminal binding protein 1 antisense (CTBP1-AS) exerts its functions by inhibiting the sense transcript CTBP1 via the PSF-dependent mechanism, thus promotes cell cycle progression in prostate cancer [20]. LncRNA GAS6 antisense RNA 1 (GAS6-AS1) may be involved in the progression of NSCLC through influencing its host gene GAS6 [21]. In our study, ADAMTS9-AS2 was down-regulated in NSCLC tissues and NSCLC cells (SPC-A1, H1299, and A549). Moreover, ADAMTS9-AS2 overexpression resulted in the proliferation inhibition, migration and invasion suppression, apoptosis induction of these NSCLC cells. Considering the essential roles of ADAMTS9 in a variety of cancers, although the correlation between ADAMTS9-AS2 expression with its sense partner has not been investigated, our data prompt us to speculate that ADAMTS9-AS2 may be involved in NSCLC development possible via regulating its sense transcripts. However, some other lncRNA, like HNF1A-AS1, exerts their function without regulating its sense partner [22]. Thus, further studies are still needed to verify our speculation.

Furthermore, we further detected the relationship between ADAMTS9-AS2 and DNMT1. DNMT1 is required to maintain CpG methylation in human cancer cells and is essential for their proliferation and survival [23, 24]. Loss of DNMT1 expression is thought to abrogate tumor-associated promoter methylation in the pathogenesis of human lung and breast cancer [25]. A recent study indicates that the elevated expression of DNMT1 has been thought as a prognostic factor in NSCLC progression [26]. In addition, DNMT1-associated lncRNAs regulate global gene expression and DNA methylation in

the progression of colon cancer [27]. ADAMTS9-AS2 can be regulated by DNMT1 and inhibit cell migration in glioma [12]. The lncRNA H19/miR-148a-3p/DNMT1 axis is demonstrated to be key mechanism to regulate the progression of laryngeal squamous cell cancer [28]. Besides, the interplay between DNMT1 and miR-148a may have clinical prospect in the therapy of pancreatic cancer [29]. The DNMT1-targeted inhibition is shown to have potential advantages in cancer therapy [30]. In our study, ADAMTS9-AS2 expression was significantly correlated with TNM staging, tumor size and lymph node metastasis. Moreover, ADAMTS9-AS2 expression was negatively correlated with expression of DNMT1 in H1299 and A549 cells. Besides, the inhibitory effects of ADAMTS9-AS2 on cell migration and invasion were reversed after overexpression of DNMT1 in NSCLC cell lines. These data imply that the interplay between DNMT1 and ADAMTS9-AS2 may be associated with the poor prognosis of NSCLC and could be exploited for NSCLC therapy.

In summary, our findings indicate that down-regulation of ADAMTS9-AS2 may promote cell proliferation, migration and invasion, and inhibit cell apoptosis in NSCLC. Also, ADAMTS9-AS2 expression may be correlated with poor prognosis of NSCLC through interaction with DNMT1. The interplay between DNMT1 and ADAMTS9-AS2 may be exploited for NSCLC therapy.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Li Cai, Department of Medical Oncology, Harbin Medical University Cancer Hospital, 150 Haping Road, Harbin 150040, Heilongjiang, China. E-mail: licai052@126.com

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