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ORIGINAL ARTICLE

GATA6 activated long non-coding RNA PCAT1 maintains stemness of non-small cell lung cancer by mediating FRK

Qiwei Zang, Long Xu, Jiyao Li, Huijun Jia

Department of Cardiothoracic Surgery, Suqian First Hospital, Suquian 223 800, China.

Summary

Purpose: *Long non-coding RNA (lncRNA) prostate cancer-associated transcripts 1 (PCAT1) is a noticeable lncRNA involved in the tumorigenesis of various cancers. Nowadays, the biological function of PCAT1 on the stemness of non-small cell lung cancer (NSCLC) is still unclear. Our purpose was to explore the molecular mechanism of PCAT1 and its target protein in advanced NSCLC.*

Methods: *The levels of PCAT1 and Fyn-related kinase (FRK) in NSCLC tissues and cell lines were evaluated by quantitative polymerase chain reaction (qPCR). The log-rank test was applied to evaluate the role of high PCAT1 levels in shortening the overall survival of NSCLC patients. Chi-square test was to assess the relation between PCAT1 expression and clinicopathological features of NSCLC patients. CCK8 assay tested the cell proliferation of NSCLC cells with PCAT1 overexpression. The underlying regulatory mechanism between PCAT1 and Fyn-related kinase (FRK) was predicted by bioinformatics and verified by RNA transfection, qPCR, and western blotting. Chromatin immunoprecipitation (ChIP) assay was done to examine the relation between GATA binding protein 6 (GATA6) and the PACT1 gene. Mice xenografts were applied to examine the function of PACT1 on NSCLC development in vivo.*

Results: *PCAT1 was aberrantly elevated in tissues from patients with NSCLC. High levels of PCAT1 expression were more likely to present in patients with late-stage, positive CD133, and inferior overall survival. PCAT1 knockdown reduced cell proliferation, stem cell properties (Sox2 and Nanog expression) of H226 and A549 cells in vitro, and repressed tumor development in vivo. Furthermore, FRK in NSCLC cells was downregulated by silencing PCAT1. The tissue level of FRK was positively correlated with PCAT1 expression in patients with NSCLC. Furthermore, GATA6 was found to be promoter of PCAT1 and increased PCAT1 levels in NSCLC cells.*

Conclusions: *GATA6/PCAT1 may markedly maintain the stemness of NSCLC, resulting in late TNM stage and poor survival. These findings suggest that the GATA6-PCAT1-FRK axis may be a useful target for intervention in NSCLC.*

Key words: *GATA binding protein 6, long non-coding RNA, prostate cancer-associated transcripts 1, non-small cell lung cancer, Fyn-related kinase, stemness*

Introduction

The annual incidence of lung cancer and its associated deaths rank first among both men and women [1]. Compared to small-cell subtype (15%), non-small cell lung cancer (NSCLC) represents most of the lung cancer cases (85%) [2]. Nowadays, late diagnosis and consequent lack of therapeutic strategy are responsible for the poor prognosis of NSCLC [3]. Although one-third of NSCLC patients can be relieved (completely or partialy) after first-line chemotherapy and radiotherapy, most of them will suffer from relapse or metastasize within 6 to 12 months [4]. Cancer stem cells characterized by self-renewal and tenacious proliferative capacity are widely blamed for relapse and therapy resistance in NSCLC patients. An in-depth study of molecular markers that potentially affects tumor stemness in NSCLC tumorigenesis may properly improve patient therapy strategies.

Corresponding author: Qiwei Zang, MD, PhD. Department of Cardiothoracic Surgery, Suqian First Hospital, no.120 Suzhi Rd, Suqian 223800, China.
Tel and Fax: +86 0527 8342298, Email: zangqiweixq@163.com
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More and more long non-coding RNAs (lncRNAs) are involved in the development of diseases [5]. Long non-coding RNAs (lncRNAs) are long RNA (containing more than 200 nucleotides) with no protein-coding capacity [6,7]. Reports revealed that lncRNAs can participate in different behaviors of tumor cells and can act as an useful tool for prognosis prediction [8]. Li et al reported that lncMIR205HGPTN inhibited the apoptotic abilities of cervical cancer cells by inducing KRT17 level via targeting SRSF1 [9]. lncRNA TUG1/miR-197/TYMS axis in colorectal cancer not only promotes tumor growth and recurrence but enhances 5-fluorouracil resistance of tumor cells [10]. Besides, an investigation reported that lncRNA MALAT1 can accelerate the growth of tumor cells by sponging miR-199a [11]. Growing studies have shown that lncRNAs exhibit a substantial impact on cell proliferation [12], chemoresistance [13], and stemness [14] of NSCLC. Thus, identifying the significant lncRNAs related to stem cell features may have far-reaching value for understanding the molecular mechanism of NSCLC progression.

Although increasing numbers of lncRNAs are found to be involved in NSCLC, the role of prostate cancer-associated transcripts 1 (PCAT1) in NSCLC is still unclear. The purpose of our study was to explore the molecular mechanism of PCAT1 and its target protein in advanced NSCLC.

Methods

NSCLC tissues and patients

The Ethics Committee of Suqian First Hospital approved this study. All patients gave their written informed consent before study entry. After a transbronchial lung biopsy, a total of 93 NSCLC patients were collected from May 2014 to November 2014. All patients were diagnosed by biopsy and received no radiotherapy or chemotherapy. Any distant clinical metastasis (M1 stage, the 7th AJCC TNM staging system) was confirmed by magnetic resonance imaging (MRI) or bone scanning. Tissue specimens were frozen in -80°C for further experiments. Survival was followed up for 5 years.

RNA preparation and quantitative analysis

Total RNA of tissue and cell line was extracted using RNAiso Plus (TAKARA, Beijing, China) according to the instructions. The extracted RNA was synthesized to cDNA by the PrimeScript™ R.T. reagent Kit (TAKARA, Beijing, China). Quantitative PCR was done using SYBR® Green Realtime PCR Master Mix (TOYOBO, Shanghai, China) on the Applied Biosystems Veriti Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. The quantitation of the target RNA expression was assessed using the endogenous control by the $2^{-\Delta\Delta Ct}$ method (Glyceraldehyde-phosphate dehydrogenase, GAPDH). NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to evaluate the quality of the prepared RNA, and cDNA was measured. The corresponding sequences of primers are shown in Supplementary Table I.

Cell lines

The NSCLC cell lines H226, H520, SK-MES-1, A549, H1975, H1299, and normal human bronchial epithelium cell line BEAS-2B were purchased from the Chinese Academy of Sciences (Shanghai Institute of Cell Biology, Shanghai, China). All cell lines were cultured in 10% fetal bovine serum (FBS)-supplementing RPMI (Roswell Park Memorial Institute) 1640 Medium (Thermo Fisher Scientific, Waltham, MA, USA). All of them were in a 37°C atmosphere with 5% CO_2.

Gene knockdown

Short hairpin RNA (shRNA) and pcDNA3.1 expression vector was constructed to knockdown PCAT1 expression (sh-PCAT1 and sh-NC) (GenePharma, Shanghai, China). After NSCLC cells (1×10^6 per well) were seeded in 6-well plates overnight, each well was transfected with the knockdown vector or control using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) based on the protocol. PCR was applied to evaluate the efficacy of transfection.

Proliferation ability of tumor cells

Following the protocol, cell viability (5 days) of NSCLC cells was measured every 24 h utilizing Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Kyushu, Japan). The absorbance shaped the proliferation curves representing the proliferation ability at each time point.

Flow cytometry for apoptosis analysis

NSCLC cells with sh-PCAT1 or sh-NC were cultured 48 h for the apoptosis analysis. Tumor cells (5×10^4 cells) were incubated with Annexin V-propidium iodide (PI) antibodies (Beyotime Biotechnology, Shanghai, China) for 30 min. Cells were measured by CytoFLEX Flow Cytometry (Beckman, Brea, CA, USA).

Supplementary Table I. The primers for genes detected by real-time PCR

Gene	*Forward primer (5'-3')*	*Reverse primer (5'-3')*
PCAT1	TTGTGGAAGCCCCGCAAGGCCT	TGTGGGGCCTGCACTGGCACTT
PCAT1 shRNA	AUACAUAAGACCAUGGAAAU	
FRK	CTTTGGAGCAATCGGAAGATCA	TGTAGTGGCTCACAAATTCGTTC
GAPDH	CCCTTCATTGACCTCAACTACA	ATGACAAGCTTCCCGTTCTC

Tumor growth experiment in vivo

All the experiments attained approval from the Institutional Animal Care and Use Committee of Suqian First Hospital. 14-16g BALB/c-nude mice were purchased from the Laboratory Animal Center of Xuzhou Medical University. Transfected NSCLC cells were injected subcutaneously into mice (sh-PCAT1,n=5; sh-NC, n=5, 1×10^6 cells in 80 ul). The tumor volume of each mouse was recorded every week. All mice were fed in a specific pathogen-free condition with a 12-h light/dark cycle. All mice were sacrificed after 5 weeks, followed by tumor weight measurement.

Western blotting assay

RIPA buffer (Solarbio, Beijing, China) was applied to extract total proteins, supplementing with 1% phosphorylation and protease inhibitors (Thermo Fisher Scientific, Waltham, MA, USA). According to the manufacturer's protocol, the concentration of the protein samples was tested by the bicinchoninic acid (BCA) protein assay kit (Tiangen, Beijing, China). After denatured at 96°C for 10 min, 9% SDS-PAGE (Solarbio, Beijing, China) was used to divide the target proteins. PVDF membrane (Solarbio, Beijing, China) was used for transfer. After incubation with 5% non-fat milk for blockade of non-specific signals, PVDF membranes were incubated with primary antibodies against Nanog (1:4000), Sox2 (1:4000), FRK (1:2000), GATA6 (1:3000) (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. Then, the PVDF membrane was dealt with HRP conjugated secondary antibody (1:5000, Cell Signaling Technology, Danvers, MA, USA). The protein blots were photographed using a Western imaging system (General Electric Company, Boston, MA, USA). The density of bands was quantified by Image J software (Bio-Rad, Hercules, CA, USA).

Chromatin immunoprecipitation (ChIP) assay

According to the instruction, the ChIP Assay Kit (Beyotime, Shanghai, China) was applied to investigate the relation between GATA6 and PCAT1. Briefly, 1% formaldehyde was used to cross-link cells. After a sonication on ice for producing 200-500 bp DNA fragments, chromatin was incubated with primary antibodies against GATA6 or IgG (isotype control) (Abcam, San Francisco, CA, USA) overnight at 4°C. The antibody precipitated the target chromatin DNA. After the DNA was recovered, a qPCR assay was applied to evaluate it.

Statistics

All data were presented as the mean ± standard deviation. The Student's *t*-test made a comparison between the means from two groups. Survival analysis was performed using the Kaplan-Meier method, followed by comparison with the Log-rank test. Furthermore, Spearman's correlation analysis was applied to evaluate the correlation between FRK and PCAT1 levels in NSCLC tissues. Every experiment was repeated for at least three independent times. All statistical analyses were carried out by SPSS 22.0 software (International Business Machines Corporation, USA). Statistical significance was set at $p<0.05$.

Results

Elevated PCAT1 in NSCLC tissues is strongly associated with CD133⁺ and inferior survival

By analyzing 93 pairs of NSCLC tissues we found PCAT1 inclined mostly in NSCLC tissues relative to adjacent normal tissues (Figure 1A). Then, we observed that the expression of PCAT1 was higher in NSCLC cell lines (H226, H520, SK-MES-1, A549, H1975, and H1299) than in normal human bronchial epithelial cells (BEAS-2B) (Figure 1B). Based on Kaplan-Meier analysis, a higher expression of PCAT1 was associated with inferior overall survival (Figure 1C, $p<0.05$). Moreover, NSCLC patients with late TNM stages (III+IV) or positive CD133 exhibited significantly increased PCAT1 expression than NSCLC tissues with early TNM stages (I+II, Table 1, $p=0.008$; Figure 1D, $p<0.05$) or negative CD133 (Table 1, $p=0.026$; Figure 1E, $p<0.05$), respectively. Altogether, our results indicated that lncRNA PCAT1 is highly expressed in NSCLC tissues, and it may be a helpful biomarker for predicting poor prognosis in patients with NSCLC.

PCAT1 promotes NSCLC cell proliferation and stem cell property in vitro

Since elevated PCAT1 was associated with CD133 expression, a biomarker for NSCLC stem cells, we explored whether PCAT1 could mediate the stem cell properties of NSCLC *in vitro*. The level of PCAT1 expression in NSCLC cell lines (H226 and A549) was decreased by short hairpin RNA (shRNA) to verify the impact of lncRNA PCAT1 on the malignant behavior of NSCLC cells. Quantitative PCR confirmed the shRNA effect on PCAT1 knockdown (Figure 2A,B, $p<0.01$). PCAT1 silencing resulted in the limitation of proliferation of NSCLC cells (Figure 2C,D, $p<0.05$). Besides, apoptosis of NSCLC cells was strengthened after the PCAT1 level was restrained (Figure 2E,F, $p<0.05$). Also, the expression of Nanog, Sox2 (specific transcription factors of NSCLC stem cells) (Figure 2 G,H, $p<0.05$) were downregulated after the PCAT1 level was decreased. Thus, our results indicate that lncRNA PCAT1 may regulate the stem cell abilities of NSCLC *in vitro*, including proliferation and distinctive transcription behavior.

PCAT1 silencing inhibits tumor growth of NSCLC in BALB/C-N1 mice

A xenograft tumor model inoculated with PCAT1 knockdown H226 and A549 cells was induced to investigate the regulatory function of PCAT1 on NSCLC *in vivo*. The results revealed that H226 and A549 cells transfected with sh-PCAT1exhibited smaller tumor size (Figure 3A,B, $p<0.05$)

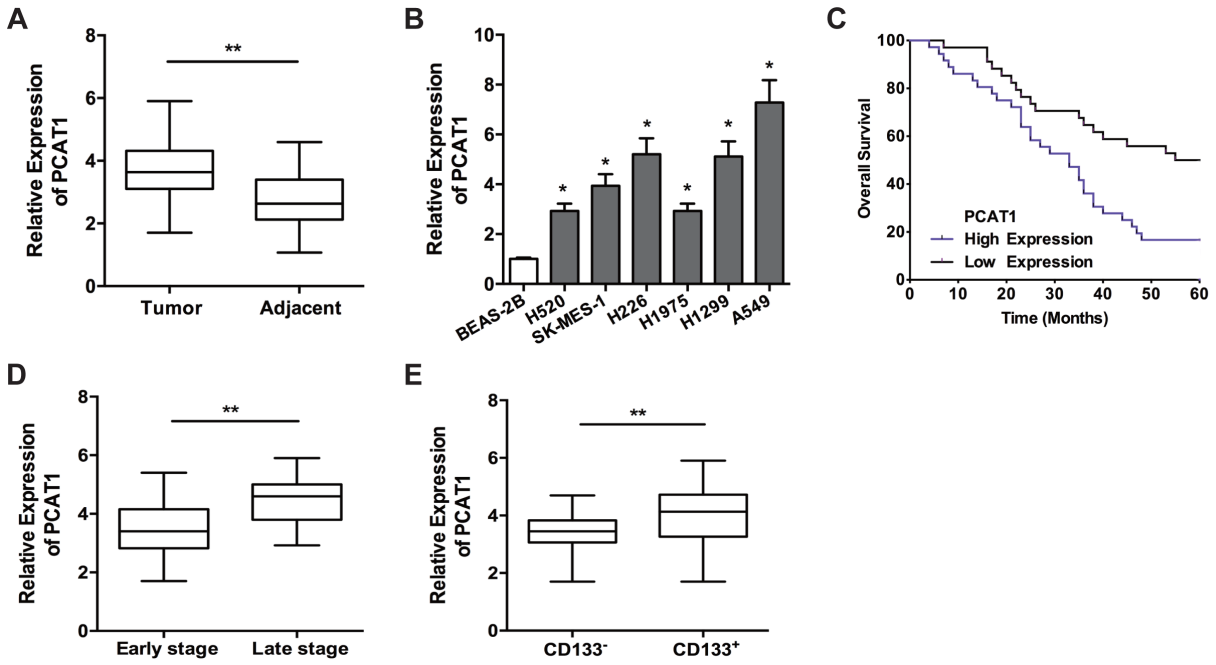

Figure 1. Elevated PCAT1 expression is highly related to CD133+ and inferior survival in NSCLC. **A:** Expression of lncRNA PCAT1 in NSCLC tissues and adjacent normal tissues shown by qPCR. **B:** PCAT1 expression in NSCLC cells (H226, H520, SK-MES-1, A549, H1975, and H1299) and BEAS-2B cells. **C:** Overall survival of NSCLC patients with high and low lncRNA PCAT1 expression. **D:** Expression of lncRNA PCAT1 in NSCLC tissues with different TMN stage (Early, n=39; Late, n=54). **E:** Expression of lncRNA PCAT1 in NSCLC tissues with different CD133 expression (Negative, n=59; Positive, n=34). **p<0.01, *p<0.05 compared to the control group.

Table 1. Relation between PCAT1 expression and clinicopathological features in NSCLC (n=93).

Features	*Total n*	*PCAT1 expression*		*p value*
		Low (n=47)	*High (n=46)*	
Gender				0.602
Male	45	24	21	
Female	48	23	25	
Age				0.611
≤60	40	19	21	
>60	53	28	25	
Tumor size				0.065
≤3 cm	35	22	13	
>3 cm	58	25	33	
Lymph node				0.105
Negative	36	22	14	
Positive	57	25	32	
Distant metastasis				0.076
No	55	32	23	
Yes	38	15	23	
TNM stage				0.008*
I+II	39	26	13	
III+IV	54	21	33	
Pathology				0.349
Squamous carcinoma	48	22	26	
Adenocarcinoma	45	25	20	
CD133				0.026*
Negative	59	35	24	
Positive	34	12	22	

*p < 0.05 shows statistically significant difference

and lower tumor weight (Figure 3C,D, p<0.05) than the ones with sh-NC. Together with the macroscopic comparison of tumor nodes (Figure 3E,F), the data suggest that aberrant PCAT1 promotes NSCLC development *in vivo*.

PCAT1 modulates NSCLC stemness by upregulating FRK

Using TargetScan for bioinformatics analysis, we predicted that Fyn-related kinase (FRK), which exhibits increased expression in lung cancer, maybe a downstream target for lncRNA PCAT1. This regulatory relation was verified based on a western blot assay (Figure 4A,B). FRK expression was declined in H226 and A549 cells after PCAT1was silenced (Figure 4A, p<0.01). Moreover, FRK levels in NSCLC cancer cells were notably higher than those in BEAS-2B cells (Figure 4C, p<0.01). Elevated FRK expression was more likely to be present in NSCLC tissues compared to adjacent normal tissues (Figure 4D, p<0.01). FRK expression was positively correlated to PCAT1 levels in NSCLC tissues (Figure 4E; r=0.4147, p<0.0001). Furthermore, NSCLC tissues with positive CD133 showed a higher FRK expression than those with negative CD133 (Figure 4F, p<0.05). Altogether, our data suggest that FRK is a downstream protein targeted by PCAT1 in NSCLC.

GATA6 activates PCAT1 expression in NSCLC cells

To further investigate the upstream regulatory mechanism of PCAT1 in NSCLC, we predicted the transcription factors which may target to the promoter of PCAT1 using the JASPAR (https:// jaspar.genereg.net/) databases. The transcription factor GATA binding protein 6 (GATA6) is recommended by JASPAR (Supplementary Table II), and the binding sites in the PCAT1 sequence are shown in

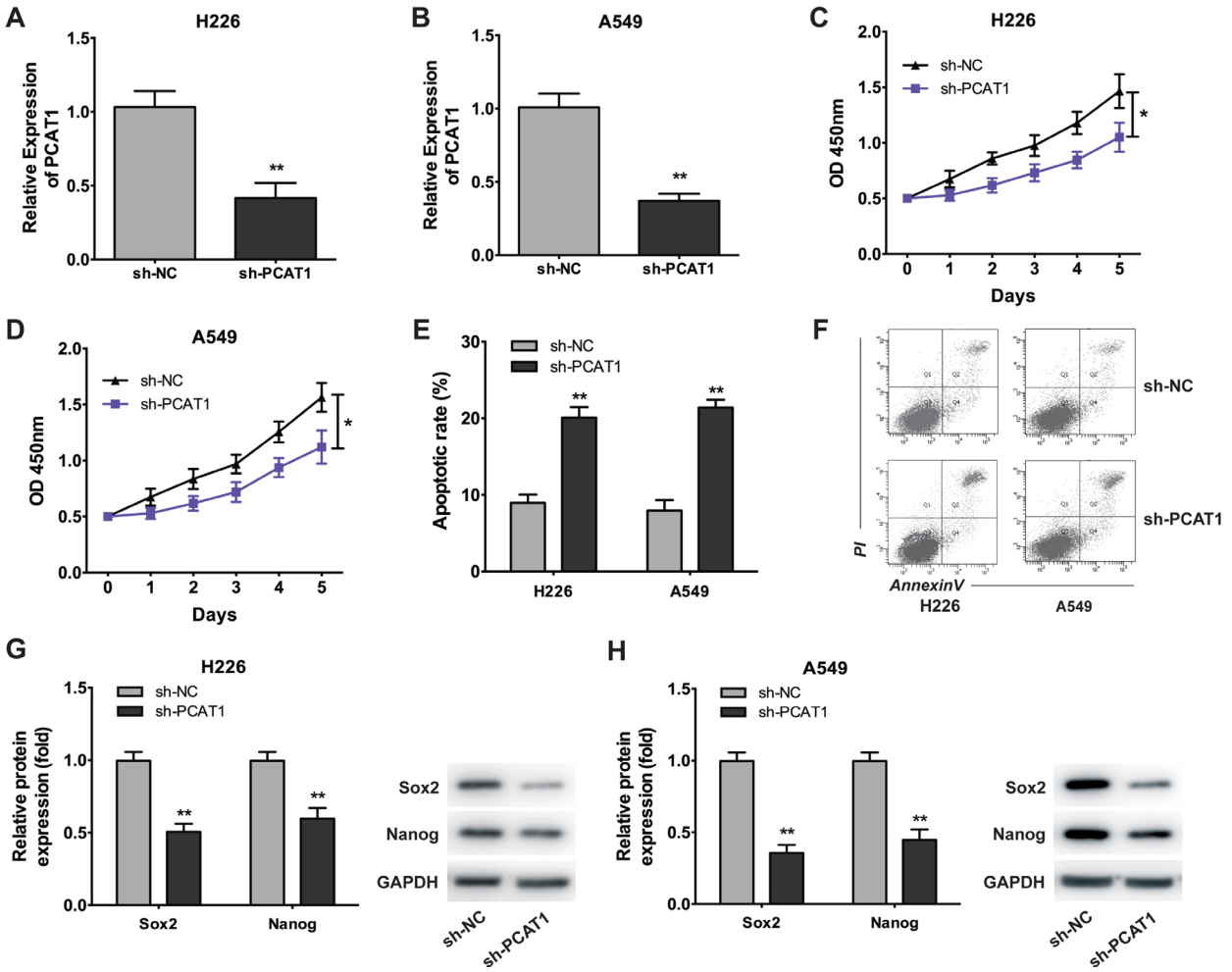

Figure 2. PCAT1 promotes NSCLC cell proliferation and stem cell property *in vitro*. **A, B:** PCAT1 expression was silenced by shRNA targeting PCAT1 in NSCLC cells. **C, D:** Proliferative ability of NSCLC cells transfected with shRNA or vector control measured by CCK-8 test. **E, F:** Flow cytometry showed the apoptotic rate of NSCLC cells transfected with overexpressing vector or vector control. **G, H:** Western blot assay showed the Sox2 and Nanog protein expression in NSCLC cells transfected with overexpressing vector or control in H226 **(G)** and A549 **(H)** **p<0.01, *p<0.05 compared to the control group.

Figure 5A. ChIP assays exhibited that the PCAT1 promoter was significantly pulled down by the antibody against GATA6 rather than the isotype antibody (Figure 5B). A synthesized lentiviral GATA6-overexpressing (oe-GATA6) vector was transfected into A549/DDP and H226/DDP cells to augment the GATA6 signal (Figure 5C). Furthermore, the overexpression of GATA6 strongly induced PCAT1 expression in A549 and H226 cells (Figure 5D). Therefore, these results indicate that GATA6 acts as a promotor for PCAT1 in NSCLC.

Discussion

Our study showed that the upregulation of lncRNA PCAT1 is strongly associated to malignant features in advanced NSCLC. Although PCAT1 will not function via a coding protein, it acts as a transcription mediator in the pathogenesis of several diseases. Originally, bioinformatics and cellular validation revealed that PCAT1 had an impact on the tumorigenesis in prostate cancer [15]. Zhang et al found that PCAT1 could modulate osteosarcoma cell migration by inducing epithelial-to-mesenchymal transition [16]. Moreover, Bi et al reported that polymorphisms of lncRNA PCAT1 (rs1026411 and rs710886) were strongly related to high susceptibility to NSCLC. In contrast, patients with rs16901904 polymorphism were more likely to develop lung squamous cell carcinoma [17]. However, few studies, if at all, have investigated the regulatory mechanism of lncRNA PCAT1 in the development of NSCLC. In accordance with the results presented in previous reports, our study also revealed that ectopic-inclined PCAT1 significantly induced the development of NSCLC cells both *in vitro* and *in vivo* (Figures 2 and 3). Besides, we also reported

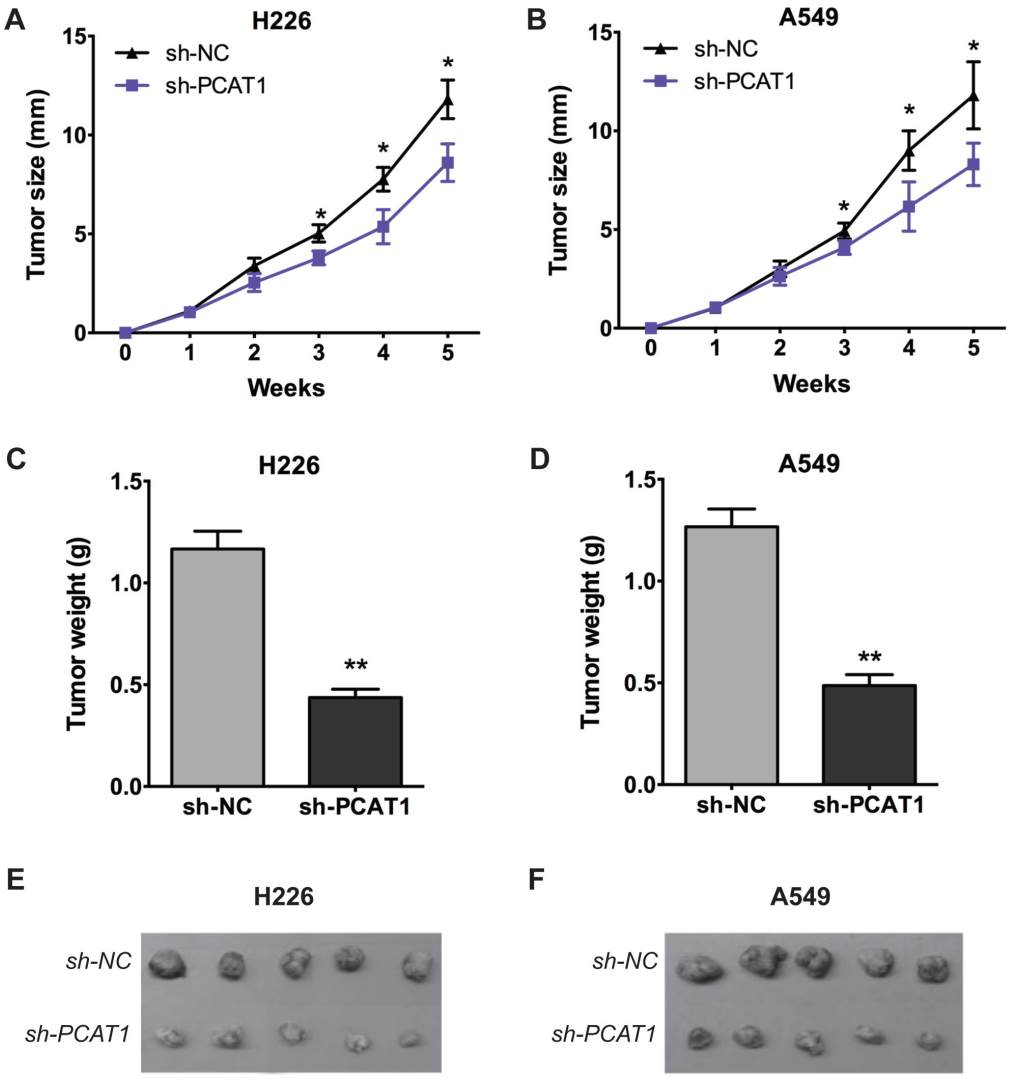

Figure 3. PCAT1 silencing inhibits NSCLC tumor growth *in vivo*. **A, B:** Tumor size between PCAT1 knockdown mice and the control mice. **C, D:** Tumor weight of tumor nudes after stable knockdown of PCAT1. **E, F:** The macroscopic observation of tumor nudes. **p<0.01, *p<0.05 compared to the control group.

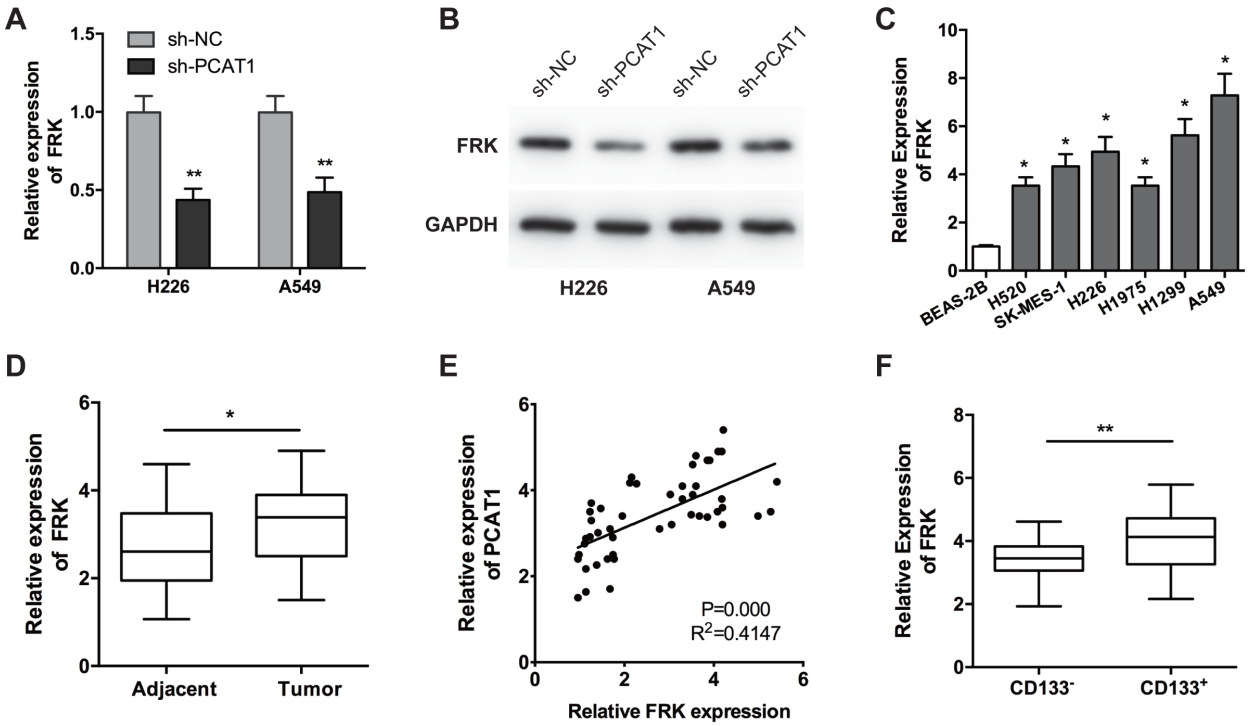

Figure 4. PCAT1 modulates NSCLC stemness by upregulating FRK. **A, B:** Western blot assay showed the FRK protein expression in NSCLC cells with or without PCAT1 silencing. **C:** FRK relative expression in NSCLC cells (H226, H520, SK-MES-1, A549, H1975, and H1299) and BEAS-2B cells. **D:** qRT-PCR showed the FRK expression in NSCLC tissues (n=93) and adjacent normal tissues (n=93). **E:** A positive correlation between the expression of FRK and PCAT1 in tumor tissues. **F:** qRT-PCR showed the FRK expression in $CD133^+$ NSCLC tissues (n=34) and $CD133^-$ NSCLC tissues (n=59). **p<0.01, *p<0.05 compared to the control group.

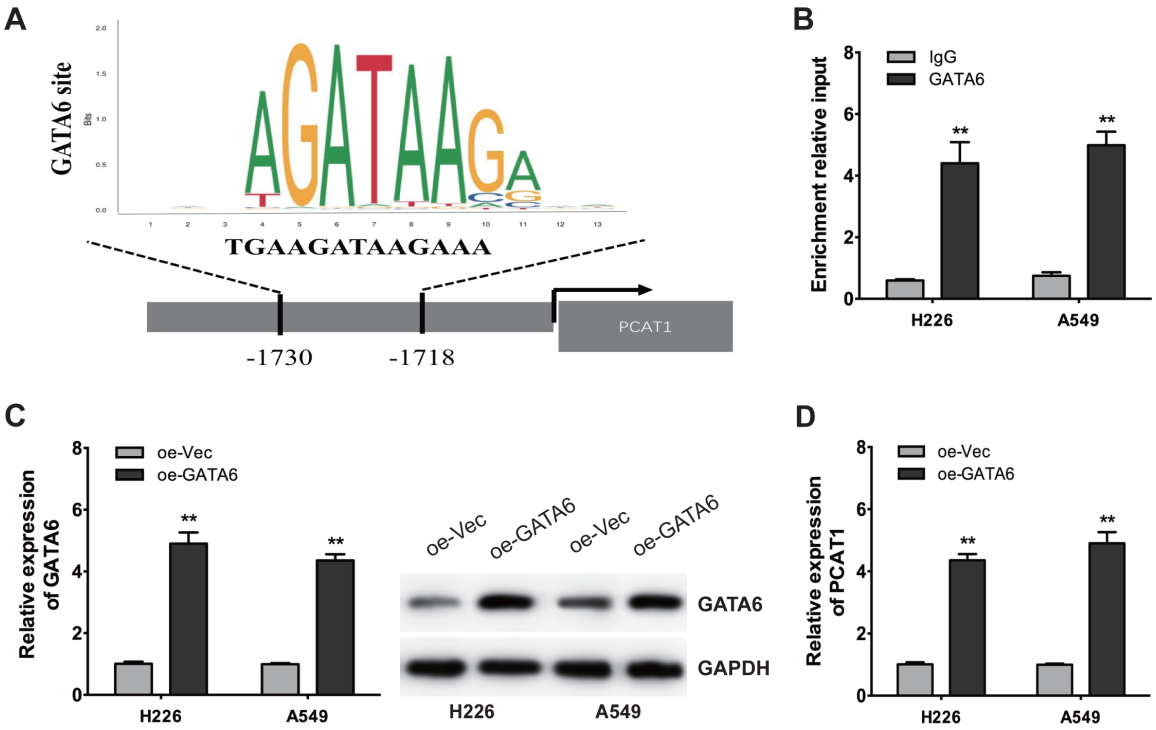

Figure 5. EFL1 activates PCAT1 expression in NSCLC cells. **A:** JASPAR database reveals the complementary binding sites within the promoter region of PCAT1. **B:** The targeting ability of GATA6 to the PCAT1 promoter validated by ChIP assay and qRT-PCR. **C:** Western blot assay showed the GATA6 protein expression after overexpression of GATA6. **D:** PCAT1 expression was increased by lentiviral vectors of GATA6 in H226/DDP and A549/DDP cells. **p<0.01, *p<0.05

Supplementary Table II. The transcription factor GATA binding protein 6 (GATA6) is recommended by JASPAR

Matrix ID	*Name*	*Score*	*Relative score*	*Sequence ID*	*Start*	*End*	*Strand*	*Predicted sequence*
MA1596.1	ZNF460	26.8261	1.000000009	NC_000008.11:127011154-127013253	1026	1041	+	GCCTCAGCCTCCCGAG
MA1596.1	ZNF460	18.3144	0.901237694	NC_000008.11:127011154-127013253	994	1009	+	ACCTCCACCTCCCAGG
MA1596.1	ZNF460	18.2879	0.900929959	NC_000008.11:127011154-127013253	1159	1174	+	GCCTCACCCTCCCAAA
MA0043.3	HLF	17.0121	0.985828331	NC_000008.11:127011154-127013253	1227	1240	-	AGGTTATGCAACTA
MA1124.1	ZNF24	16.6465	0.90632974	NC_000008.11:127011154-127013253	1440	1452	-	CTCTCATTCATTC
MA1104.1	GATA6	15.9031	0.991609781	NC_000008.11:127011154-127013253	1718	1730	+	TGAAGATAAGAAA
MA0508.3	PRDM1	15.6446	0.996369413	NC_000008.11:127011154-127013253	616	626	-	TTCTTTCTCTC
MA1104.2	GATA6	15.3313	0.966766656	NC_000008.11:127011154-127013253	1719	1731	-	ATTTCTTATCTTC
MA0508.3	PRDM1	15.3199	0.99013094	NC_000008.11:127011154-127013253	600	610	-	CTCTTTCTCTC
MA0079.3	SP1	14.3996	0.962303907	NC_000008.11:127011154-127013253	580	590	-	CCCCCACCCCC
MA0036.3	GATA2	14.1367	0.993423138	NC_000008.11:127011154-127013253	1719	1729	-	TTCTTATCTTC
MA0043.3	HLF	14.0758	0.941767219	NC_000008.11:127011154-127013253	39	52	+	TAGTTATGCAAAAG
MA0830.1	TCF4	13.879	0.99969233	NC_000008.11:127011154-127013253	1052	1061	-	CGCACCTGCA
MA0830.2	TCF4	13.5209	0.9453481	NC_000008.11:127011154-127013253	1050	1062	-	GCGCACCTGCAGT
MA0508.3	PRDM1	13.4769	0.954724311	NC_000008.11:127011154-127013253	1216	1226	+	TACTTTCTCAA
MA0036.2	GATA2	13.4161	0.934508304	NC_000008.11:127011154-127013253	1719	1732	-	GATTTCTTATCTTC
MA0079.3	SP1	12.687	0.940757285	NC_000008.11:127011154-127013253	873	883	+	TCCCCTCCTCT
MA0079.3	SP1	12.6172	0.939879405	NC_000008.11:127011154-127013253	586	596	-	CCTCCACCCCC
MA0079.2	SP1	12.3964	0.943489426	NC_000008.11:127011154-127013253	580	589	-	CCCCACCCCC
MA0508.2	PRDM1	12.321	0.931402074	NC_000008.11:127011154-127013253	1215	1224	+	CTACTTTCTC
MA0043.2	HLF	11.9254	0.937423731	NC_000008.11:127011154-127013253	1228	1239	+	AGTTGCATAACC
MA0508.2	PRDM1	11.2056	0.912082785	NC_000008.11:127011154-127013253	2073	2082	-	CCACTTTCCT
MA0079.3	SP1	10.9452	0.918844079	NC_000008.11:127011154-127013253	1978	1988	-	CTCCCTCCTCT
MA0079.3	SP1	10.928	0.918627066	NC_000008.11:127011154-127013253	995	1005	+	CCTCCACCTCC
MA0079.2	SP1	10.9156	0.90619608	NC_000008.11:127011154-127013253	579	588	-	CCCACCCCCC
MA0043.2	HLF	10.841	0.920416596	NC_000008.11:127011154-127013253	1228	1239	-	GGTTATGCAACT
MA0508.2	PRDM1	10.5768	0.901192214	NC_000008.11:127011154-127013253	160	169	-	GTACTTTCAA
MA0508.2	PRDM1	10.571	0.901092648	NC_000008.11:127011154-127013253	776	785	+	ATACTTTCTT
MA0036.3	GATA2	10.2472	0.913598031	NC_000008.11:127011154-127013253	1146	1156	+	TCATTATCTGC
MA0036.3	GATA2	9.88386	0.906141586	NC_000008.11:127011154-127013253	18	28	+	TTATTATCTTG
MA0830.1	TCF4	8.03284	0.910282666	NC_000008.11:127011154-127013253	2057	2066	-	CCCATCTGCA
MA0036.1	GATA2	6.65143	1.000000007	NC_000008.11:127011154-127013253	1313	1317	-	GGATA
MA0036.1	GATA2	6.65143	1.000000007	NC_000008.11:127011154-127013253	2007	2011	+	GGATA
MA0036.1	GATA2	6.65143	1.000000007	NC_000008.11:127011154-127013253	2014	2018	+	GGATA
MA0036.1	GATA2	6.23202	0.975650738	NC_000008.11:127011154-127013253	22	26	-	AGATA
MA0036.1	GATA2	6.23202	0.975650738	NC_000008.11:127011154-127013253	100	104	+	AGATA
MA0036.1	GATA2	6.23202	0.975650738	NC_000008.11:127011154-127013253	388	392	+	AGATA
MA0036.1	GATA2	6.23202	0.975650738	NC_000008.11:127011154-127013253	1150	1154	-	AGATA
MA0036.1	GATA2	6.23202	0.975650738	NC_000008.11:127011154-127013253	1254	1258	+	CGATA
MA0036.1	GATA2	6.23202	0.975650738	NC_000008.11:127011154-127013253	1304	1308	-	AGATA
MA0036.1	GATA2	6.23202	0.975650738	NC_000008.11:127011154-127013253	1721	1725	+	AGATA
MA0036.1	GATA2	6.23202	0.975650738	NC_000008.11:127011154-127013253	2017	2021	-	AGATA
MA0036.1	GATA2	5.9783	0.960920821	NC_000008.11:127011154-127013253	1124	1128	+	GGATG
MA0036.1	GATA2	5.9783	0.960920821	NC_000008.11:127011154-127013253	1420	1424	+	GGATG
MA0036.1	GATA2	5.77818	0.949302466	NC_000008.11:127011154-127013253	177	181	-	TGATA
MA0036.1	GATA2	5.77818	0.949302466	NC_000008.11:127011154-127013253	221	225	-	TGATA
MA0036.1	GATA2	5.77818	0.949302466	NC_000008.11:127011154-127013253	1860	1864	-	TGATA
MA0036.1	GATA2	5.55889	0.93657158	NC_000008.11:127011154-127013253	127	131	-	AGATG
MA0036.1	GATA2	5.55889	0.93657158	NC_000008.11:127011154-127013253	1371	1375	+	AGATG

Continued on the next page

Matrix ID	*Name*	*Score*	*Relative score*	*Sequence ID*	*Start*	*End*	*Strand*	*Predicted sequence*
MA0036.1	GATA2	5.55889	0.93657158	NC_000008.11:127011154-127013253	1747	1751	-	AGATG
MA0036.1	GATA2	5.55889	0.93657158	NC_000008.11:127011154-127013253	2060	2064	+	AGATG
MA0036.1	GATA2	5.10505	0.91022328	NC_000008.11:127011154-127013253	57	61	+	TGATG
MA0036.1	GATA2	5.10505	0.91022328	NC_000008.11:127011154-127013253	521	525	-	TGATG
MA0036.1	GATA2	5.10505	0.91022328	NC_000008.11:127011154-127013253	634	638	+	TGATG
MA0036.1	GATA2	5.10505	0.91022328	NC_000008.11:127011154-127013253	714	718	+	TGATG
MA0036.1	GATA2	5.10505	0.91022328	NC_000008.11:127011154-127013253	886	890	+	TGATG
MA0036.1	GATA2	5.10505	0.91022328	NC_000008.11:127011154-127013253	1110	1114	-	TGATG
MA0036.1	GATA2	5.04698	0.906851967	NC_000008.11:127011154-127013253	208	212	+	GGATC
MA0036.1	GATA2	5.04698	0.906851967	NC_000008.11:127011154-127013253	209	213	-	GGATC
MA0036.1	GATA2	5.04698	0.906851967	NC_000008.11:127011154-127013253	838	842	+	GGATC
MA0036.1	GATA2	5.04698	0.906851967	NC_000008.11:127011154-127013253	1272	1276	-	GGATC

that PCAT1 seems to be a significant promotor for the stem cell characteristics of NSCLC (Figure 1E, Figure 2G&H). Therefore, we proved that PCAT1 might be a predictor for stemness and poor survival in patients with NSCLC.

The transcription factor GATA6, located on human chromosome 18 (q11.1 ~ q11.2), is a member of the GATA family [18]. The GATA6 gene is essential for the early development of the human organs [19,20]. Aberrant expression of GATA6 is involved in the development of various human diseases, especially lung cancer [21]. Downregulated GATA6 promotes abnormal tumor differentiation, and relapse by regulating malignant transcription in the alveolar epithelium [22]. Retinoic acid-activated GATA6 increases the response sensitivity of NSCLC cells to tyrosine kinase inhibitors (TKIs) by activating Wnt signaling [23]. However, the role of GATA6 in lung cancer has been controversial. GATA6 inhibited by miR-200 can downregulate bone morphogenetic protein 4, thereby suppressing the malignant behavior of NSCLC [24]. Moreover, the GATA6 gene can promote autophagy of NSCLC cells [25], resulting in the sensitivity to TKI and EGFR-targeted chemotherapeutics [26,27]. Our study shows that GATA6 can target and bind the PCAT1 expression regulatory region (-1730 ~ -1718, Figure 5A&B). Function experiments have verified that overexpressed GATA6 can increase PCAT1 levels in H226 and A549 cells (Figure 5C&D). Collectively, GATA6 can increase PCAT1 levels in NSCLC cells as a promoter.

In 1993, when researchers studied breast cancer cell lines and primary breast cancer, FRK was identified as a new tyrosine kinase (then known as TK1) [28]. FRK was later called protein tyrosine kinase 5 (PTK5) and belonged to the BRK family kinase (BFK) [29]. It has been confirmed that FRK is involved in the development of several cancers [30], including breast cancer [31], cervical cancer [32], and glioma [33]. Bagu et al found that promoter methylation of FRK was activated by STAT5A and GATA3-binding protein FOG1 at 17 CpG sites, resulting in inhibition of the development of triple-negative breast cancer [34]. Over-expressed FRK in glioma cells can not only translocate β-catenin from nuclear fraction but also enrich N-cadherin protein to combine with β-catenin, repressing migration and invasion behaviors [35]. FRK seemed like a tumor-suppressive protein in previous cancers; however, it acts as an oncogene in the development of hepatocellular carcinoma and NSCLC. Bioinformatics assay on tumor-associated gene database revealed that an inclined expression of FRK was present in hepatocellular carcinoma [36]. Cell lines verification *in vitro* proved the regulatory function of FRK on the invasiveness of liver cancer [36]. Besides, a recent investigation reported that FRK promoted the proliferation, metastasis and stemness of NSCLC cells by reprogramming the metabolism via switching the energy resource [37]. FRK knockdown reduced the expression of CD44 and CD133, two indicators of stemness phenotype [37].

Although FRK is involved in the formation, metastasis, and even stemness of NSCLC, the molecular mechanism of FRK modulation via lncRNA in the development of NSCLC remains unknown. In our study, FRK expression could be repressed after PCAT1 was silenced (Figure 4A&B). Besides, the association between FRK and PCAT1 was confirmed in human tissues (Figure 4E). FRK increased in cancer tissues and showed a quantitative dependence on PCAT1 levels. However, the specific molecular mechanism of FRK regulation mediated by PCAT1 has not been investigated in our study. Interfering with the expression of downstream target proteins via binding miR as mRNA sponges is the molecular way lncRNAs mainly function [38,39]. When comparing the expression of 80 miRNAs in primary liver tumor tissues and adjacent tissues, the

investigators revealed that miR-330 was strongly down-regulated in cancer tissues, targeting to FRK [40]. FRK controls cell viability and growth of clear cell renal cell carcinoma, being inhibited by miR-19 [41]. All these indicate that PCAT1 can mediate the expression of FRK by modulating a certain miR in NSCLC. This requires further exploration in our next study.

Conclusion

We have demonstrated that high levels of PCAT1 expression are associated with advanced NSCLC with positive CD133 and inferior overall survival. PCAT1 may markedly promote the proliferation and stemness of NSCLC. FRK in NSCLC is downregulated by silencing PCAT1. Furthermore, GATA6 can increase PCAT1 levels in NSCLC cells as a promotor. These data suggest that the GATA6-PCAT1-FRK axis may be a novel therapeutic target in NSCLC.

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Conflict of interests

The authors declare no conflict of interests.

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