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Original Article

Tumor suppressor genes are reactivated by miR-26A1 via enhancer reprogramming in NSCLC

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Abstract

Non-small cell lung cancer (NSCLC) is one of the most malignant epithelial tumors. Studies have suggested that DNA hypermethylation of promoters and abnormal histone modifications could induce tumor suppressor genes (TSGs) downregulation in NSCLC. However, the exact mechanism of TSGs downregulation remains unclear. In this study, we found that there is no difference in the regions of most TSGs promoters in NSCLC. Moreover, we found that there is no DNA methylation difference in the region of VILL promoter in NSCLC compared with adjacent tissue samples by pyrosequencing. We further demonstrated that VILL was markedly reactivated in A549 and H1703 cells infected with miR-26A1 lentivirus while this activation was inhibited by JQ1, an enhancer inhibitor. In addition, we identified that miR-26A1 could function as a tumor suppressor to inhibit proliferation and metastasis of NSCLC cells. Chromatin immunoprecipitation assays revealed that overexpression of miR-26A1 could significantly induce the enrichment of H3K27ac at the enhancer regions in A549 cells. To sum up, our findings revealed that enhancer-mediated TSGs regulation occured in NSCLC, suggesting that miR-26A1 could serve as a key regulator and may be a potential therapeutic target for NSCLC.

Introduction

Lung cancer, a heterogeneous tumor with complex clinical characteristics, is one of the most common cancers in the world (1). Lung cancer is usually classified into two major subtypes: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), among which NSCLC accounts for 85% (2). Environmental pollution, smoking and a bad lifestyle have been considered as the main factors causing the high incidence of lung cancer in China (3). Surgery is a mainstream treatment for early-stage NSCLC. Radiotherapy and chemotherapy are commonly applied to NSCLC patients at the advanced-stage. Nevertheless, the global 5-year survival rate is only 10–20% and the exact tumorigenesis mechanism of NSCLC is still poorly understood (4). Therefore, a novel biomarker or target molecule for early detection and treatment for NSCLC is urgently needed.

Enhancers are DNA sequences that function as logic gate of the genetic regulatory elements (5,6). Given that enhancers played crucial roles in cell differentiation, body development and tumorigenesis (7). Moreover, enhancers are enriched with histone-modified H3K27ac and H3K4me1, representing activated or restrained states, respectively (8,9). What determines the enhancer activation or inactivation? The study has shown that an ocean of miRNAs overlapped with enhancer genome locus switched the activity of enhancer via changing the enrichment of H3K27ac, including miR-26A1 (10). However, the association between miR-26A1 and enhancer activity is rarely discussed in tumors. Considering the crucial role of enhancers in the process of tumorigenesis, we assumed that the enhancers reactivated by miR-26A1 is an effective treatment for NSCLC.

microRNAs play an essential role in tumor proliferation and metastasis. Studies have showed that microRNAs could regulate metabolic, cellular pathways and gene transcription both in the cell cytoplasm and nucleus (11,12). Moreover, one of the NamiRNAs, miR-339 promoted GPER1 transcription by interacting with enhancers in cell nucleus. In addition, it is demonstrated that lots of miRNAs could regulate the enhancer activity via changing the state of H3K27ac enrichment (10,13). miR-26A1 played as a tumor suppressor via targeting mRNAs in the cytoplasm, but its epigenetic functions in the nucleus have not been studied yet (14–16). Thus, it is of great significance to explore the mechanism

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of enhancer reactivation by miR-26A1 in nuclear for a supplement of its anti-cancer role.

TSGs are genes that protect normal cells from malignant transformation. Since the discovery of the first TSG termed Rb, a large number of TSGs have been found (17–19). These TSGs participate in cell cycle control, DNA damage repair and cell senescence (20–22). Besides, a loss of function or inactivation of TSGs has been recognized as a common factor contributing to carcinogenesis in most cancers. Thus, it is important to define the unique property of VILL for inhibiting proliferation and metastasis of NSCLC. In this paper, we aimed to illustrate the function of VILL and the potential regulatory mechanism in NSCLC from a different perspective.

Results

Low expression pattern of TSGs in NSCLC without promoter DNA hypermethylation

Epigenetic dysfunction and DNA hypermethylation of gene promoters are considered critical in the inactivation or loss function of TSGs (23–27). In this study, we performed a comprehensive gene profiling on 1037 NSCLC and 108 normal lung tissue samples (obtained from TCGA) to acquire their expression patterns by bioinformatic analysis. We discovered that 1217 TSGs may be involved in NSCLC, includingHNF4A, GKN1 and VILL. Furthermore, we found that that 96 (Fig. 1A) and 122 (Fig. 1B) TSGs were significantly down-regulated in LUAD and LUSC, respectively.

To explore the potential mechanisms of these TSGs downregulation in NSCLC, we comprehensively analyzed the methylation status of TSGs promoters. There were 31 down-regulated TSGs with available DNA methylation datasets in TCGA, based on a rank-sum test by profiling 535 LUAD and 59 normal tissues. Moreover, there are 43 down-regulated TSGs with available DNA methylation datasets in TCGA, based on a rank-sum test by profiling 502 LUSC and 49 normal tissues. The differential ratio within ±5% indicated no methylation difference. The analysis in Cluster 1revealed that only 12 downregulated TSGs exhibited relative DNA hypermethylation status in promoter regions, yet 20 downregulated TSGs such as BIK, CDKN2A and DSP showed hypomethylation with no methylation difference in promoter regions, and 10 downregulated TSGs like HRG, AMH and VILL showed DNA hypermethylation in promoter regions in both LUAD and normal lung tissue samples but without methylation difference (Fig. 1C**)**. There are 12 TSGs with hypermethylation, 28 TSGs with hypomethylation and 3 TSGs are hypermethylation in DNA promoter region of LUSC but without methylation difference (±5%). Moreover, the analysis result of Cluster 1 revealed that only 12 downregulated TSGs exhibited relative DNA hypermethylation status in promoter regions. In the meantime, 28 downregulated TSGs like E2F1, BIK and CDKN2A showed hypomethylation but with no methylation difference in promoter regions in LUSC. As the Remaining 3 downregulated TSGs such as MIA, CXCL14, and VILL displayed DNA hypermethylation in promoter regions in both LUAD and normal lung tissue samples but without methylation difference (Fig. 1D**)**.

To elucidate these abnormal regulation patterns, we focused on another cis-element, the enhancers. By collecting the ChIP-Seq profile from ENCODE database, we analyzed the enrichment of H3K27ac, an active enhancer marker around the TSGs. As predicted, we found that 13 TSGs with significantly decreased H3K27ac peaks such as VILL, supporting that the inactivation of enhancers was involved in TSGs downregulation in NSCLC (Fig. 1E and F**)**.

Down-regulation of TSGs and miR-26A1 in NSCLC

Knowing that miR-26A1 is downregulated and positively upregulates TSGs through enhancer reprogramming process in NSCLC, we explored the potential reactivated genes by miR-26A1 in TCGA database. Firstly, we predicted potential target genes of miR-26A1 according to the seed sequence which was overlapped with enhancer elements. A total of 30 related target genes were positively reactivated by miR-26A1 genome-wide in LUAD (Fig. 2A). Further GO analysis of these potential reactivated TSGs revealed that these genes were involved in cell proliferation, cell cycle, and gene transcription in LUAD (Fig. 2B). Accordingly, we found that 36 TSGs downregulated in LUSC were positively related to miR-26A1 downregulation in LUSC. We analyzed that these target genes are involved in cell proliferation and transcription (Fig. 2C and D).

VILL and miR-26A1 are decreased in NSCLC without promoter hypermethylation

Subsequently, we evaluated the expression of miR-26A1 and VILL in a cohort of LUAD and LUSC patients from archived tissues. A total of 11 NSCLC tissue samples and adjacent tissues were collected from Gansu Provincial Hospital, including 7 LUAD and 4 LUSC tissue samples. The detailed clinical information of NSCLC patients is shown in Table 1. As shown above, miR-26A1 and VILL were decreased in LUAD and LUSC tissues (Fig. 3A and B). In addition to evaluating LUAD and LUSC tumors tissues, a set of human LUAD and LUSC cell lines and normal lung cells such as MRC5 and BEAS-2B were detected and analyzed for their expression. miR-26A1 and VILL expression are also decreased in A549 and H1703, compared with MRC5 and BEAS-2B cell lines (Fig. 3C and D). ISH and IHC results showed that miR-26A1 and VILL were downregulated in LUAD and LUSC (Fig. 3E and F). Next, we further detected the methylation level of VILL promoter in LUAD and LUSC tissue samples. There was no difference in the methylation level of TSG VILL promoter in LUAD and LUSC tissue samples and cells ($P > 0.05$, Fig. 3 G and I). These results confirmed that expression of VILL in LUAD and LUSC was associated with enhancers enrichment of H3K27ac induced by miR-26A1.

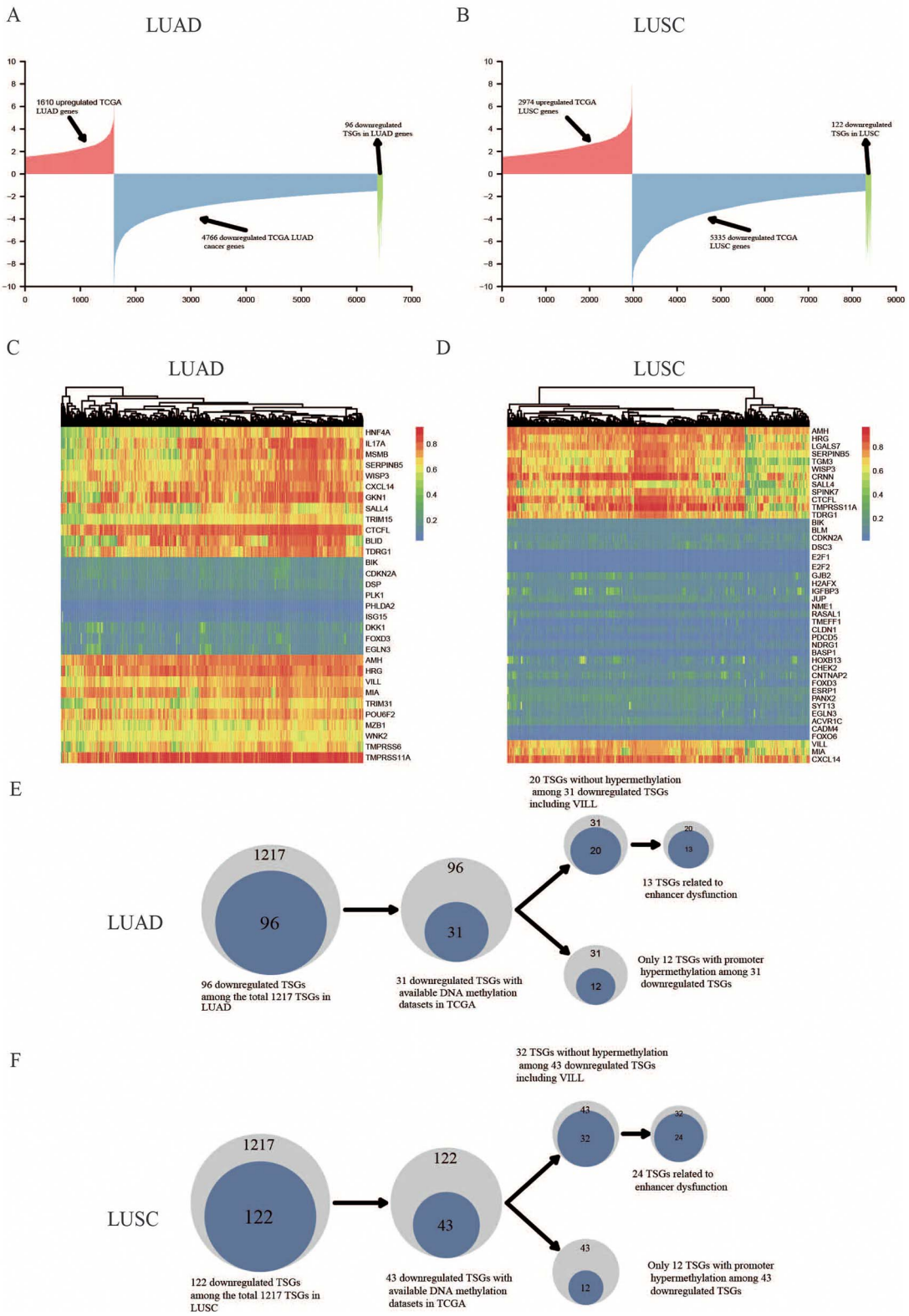

Figure 1. Low expression pattern of TSGs in NSCLC without promoter DNA hypermethylation. (**A**) Differential expression genes in LUAD were analyzed. (**B**) Disordered genes in LUSC were showed via TCGA database. (**C**) The DNA promoter methylation status of downregulated TSGs in LUAD was shown by heatmap. (**D**) Similarly, the promoter methylation status of downregulated TSGs in LUSC samples was analyzed. (**E**) The schematic diagram for the profiling process of TSGs promoter methylation and TSGs reactivated by enhancer in LUAD were analyzed. (**F**) At the same time, the status of TSGs DNA promoter methylation and TSGs reactivated via enhancer was analyzed in LUSC.

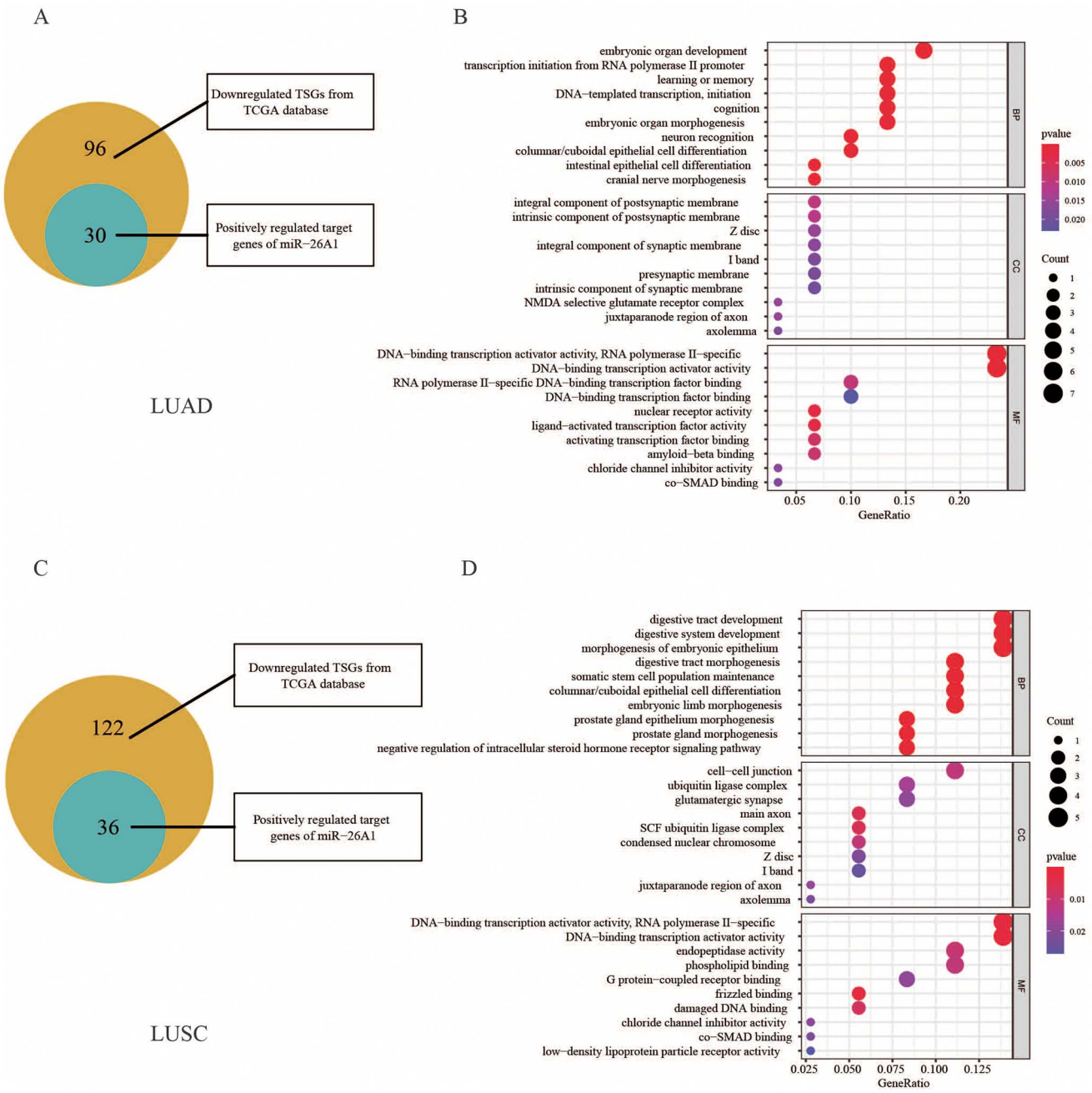

Figure 2. Down-regulation of TSGs and miR-26A1 in NSCLC. (**A**) Among 96 downregulated genes in LUAD, TSGs positively regulated by miR-26A1 were analyzed. (**B**) The pathways targeted genes involved in were shown. (**C**) Among122 downregulated genes in LUSC, TSGs which are positively regulated by miR-26A1 were performed. (**D**) The reactivated TSGs by miR-26A1 are displayed in LUSC.

MiR-26A1 reactivates VILL by targeting its enhancer in NSCLC

In order to verify the regulation role of miR-26A1 on VILL through enhancers, we established stable expression cell lines of A549 and H1703 transfected with miR-26A1 (Fig. 4A). Overexpression of miR-26A1 increased VILL expression in A549 cell lines. However, block of miR-26A1 with its inhibitors decreased the expression of VILL (Fig. 4B). These data were further confirmed by western blot assays (Fig. 4C). miR-26A1 was also overexpressed in H1703 via qPCR assay qPCR (Fig. 4D). Similarly, treatment with miR-26A1 inhibitors caused the downregulation of VILL in H1703 (Fig. 4E). Western blot assay was also demonstrated that VILL was adjusted by miR-26A1 via enhancers (Fig. 4F). On the other hand, when the enhancers inhibitor JQ1 was added to A549 and H1703 transfected with miR-26A1, VILL was significantly down-regulated in A549 (Fig. 4G) and H1703 (Fig. 4I). These data suggested that miR-26A1 upregulated VILL through reactivating enhancers.

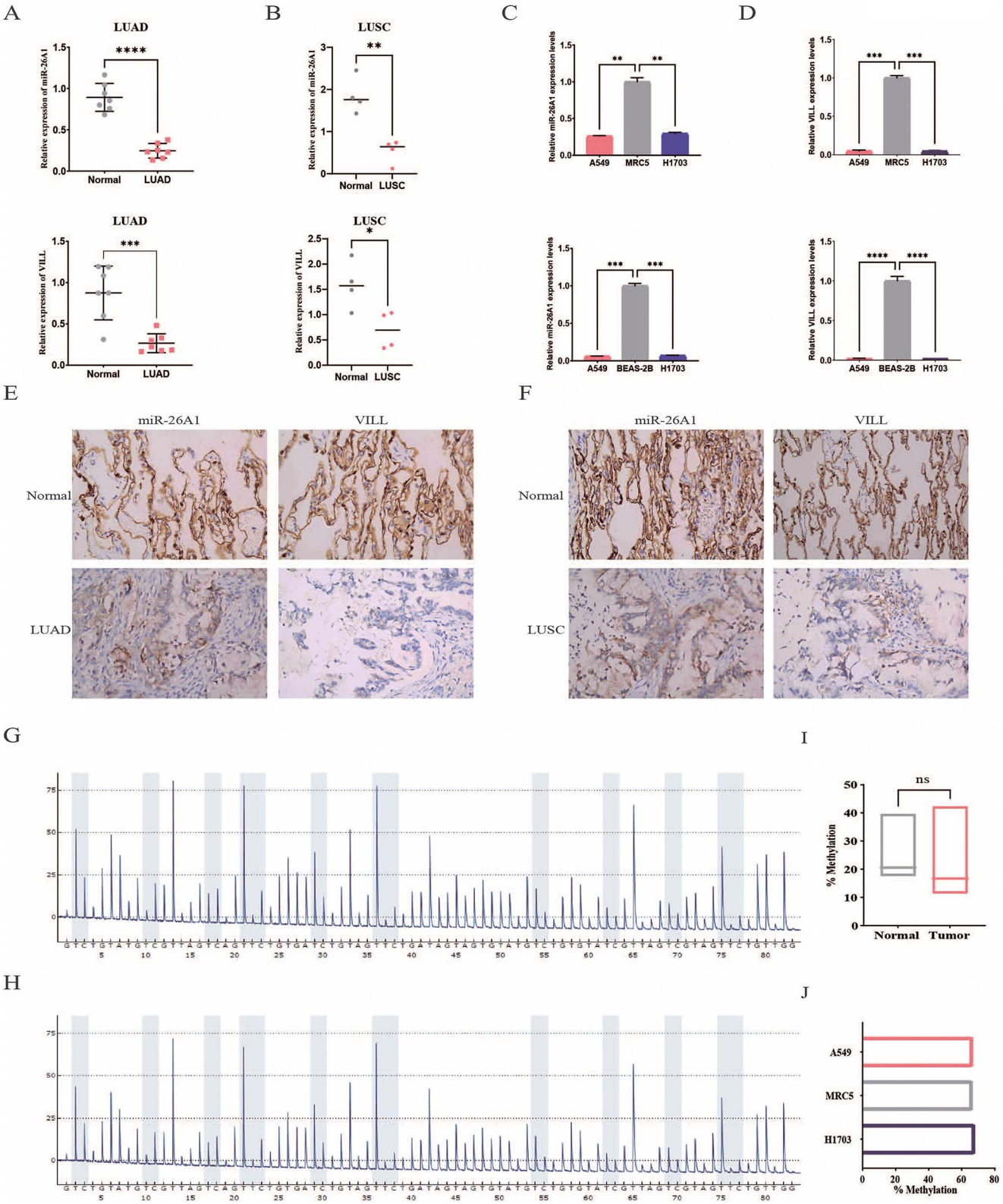

Figure 3. Expression of VILL and miR-26A1 are decreased in NSCLC without promoter hypermethylation. (**A**-**B**) Expression of miR-26A1 and VILL are detected in primary LUAD (A), LUSC (B) specimens and their corresponding adjacent tissues via qPCR assays. (**C**-**D**) QPCR assays were used to measure miR-26A1 and VILL in A549 and H1703. (**E**-**F**) Representative programs of DNA methylation levels for the VILL promoter. (**G**-**H**) Methylation of VILL promoter was detected in NSCLC primary tumor tissues and cell lines via pyrosequencing.

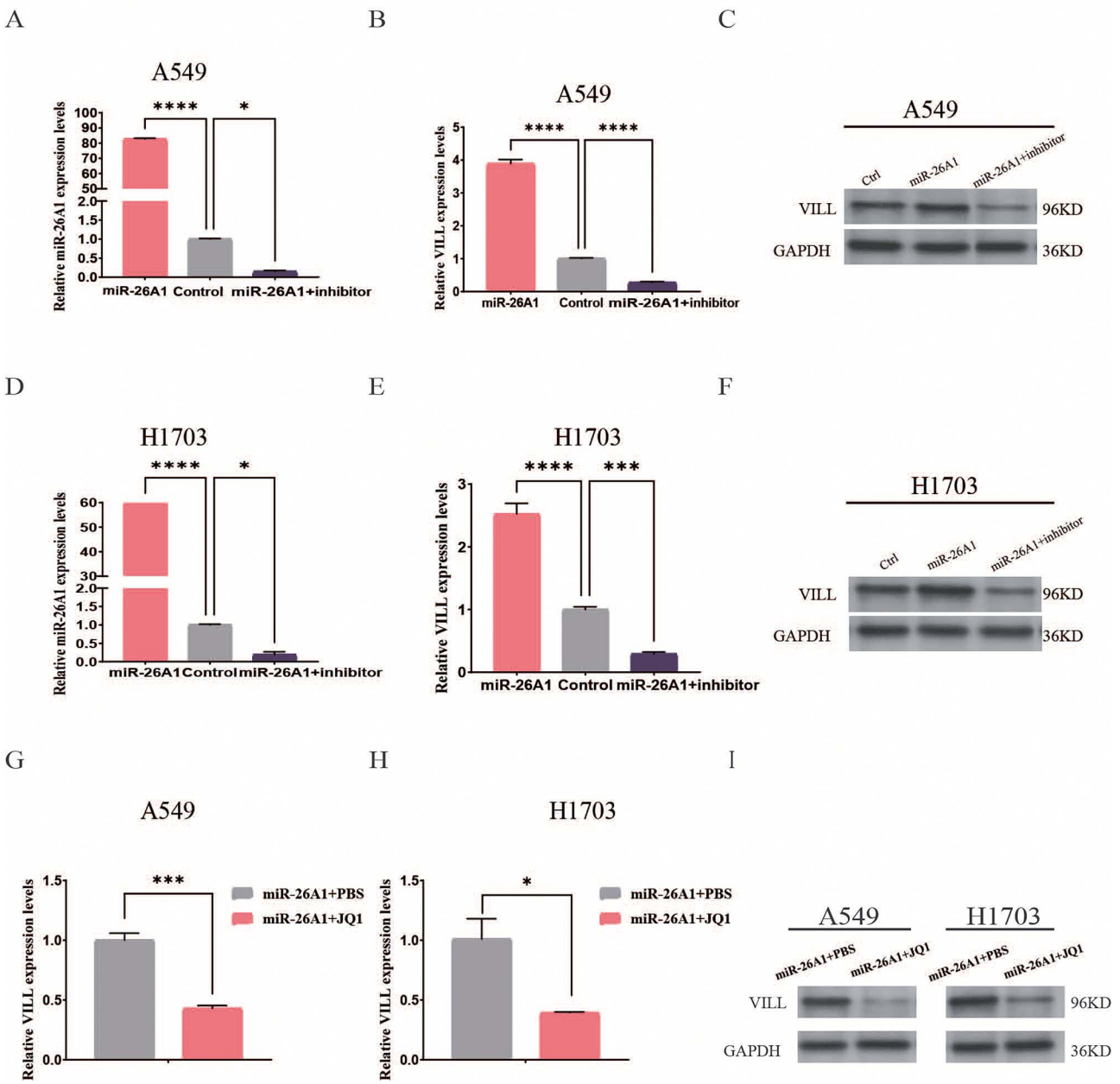

Figure 4. MiR-26A1 reactivates VILL by targeting its enhancer in NSCLC. (**A**) Expression of miR-26A1 was examined in A549 cell lines infected with miR-26A1 lentivirus or stable cell lines inhibited by JQ1. (**B**-**C**) VILL was detected when we overexpressed miR-26A1 via qPCR (B) and western blot (C). (**D**) miR-26A1 was detected in H1703 stable cell lines infected with miR-26A1. (**E**-**F**) VILL was examined in overexpressed miR-26A1 H1703 stable cell lines by qPCR (E) and Western blot (F). (**G**-**H**) Expression levels of VILL were detected in A549 (G) and H1703 (H) transfected with miR-26A1 after JQ1 was added via qPCR assays. (**I**) Meanwhile, the Western blot assays were also detected in cell lines of A549 and H1703 infected with miR-26A1.

MiR-26A1 inhibits proliferation and metastasis of NSCLC cells

To assess whether miR-26A1 could inhibit the proliferation and metastasis of A549 and H1703 cell lines, a serious of cell biological behavior assays were conducted. The CCK-8 result showed that miR-26A1 repressed the proliferation of A549 cells significantly (Fig. 5A). For colony forming assay, the result demonstrated that miR-26A1 can inhibit proliferation ability (Fig. 5B). Moreover, miR-26A1 inhibited A549 migration ability via transwell assay (Fig. 5C). We further investigated the function of miR-26A1 via CCK-8 (Fig. 5D) and colony formation assays (Fig. 5E) in LUSC, and the results suggested that H1703 infected with miR-26A1 lentivirus repressed cell proliferation. Transwell assays demonstrated that miR-26A1 could inhibit H1703 migration ability (Fig. 5F). In response to overexpression of miR-26A1, the ability of cell migration was decreased in A549 (Fig. 5G). Similar results were also found in H1703 (Fig. 5H). Thus, these results suggested that miR-26A1 is potential tumor suppressor in NSCLC.

VILL inhibits the malignant behavior of NSCLC *in vitro*

To further evaluate the effects of VILL on proliferation and metastasis of A549 and H1703 cell lines,

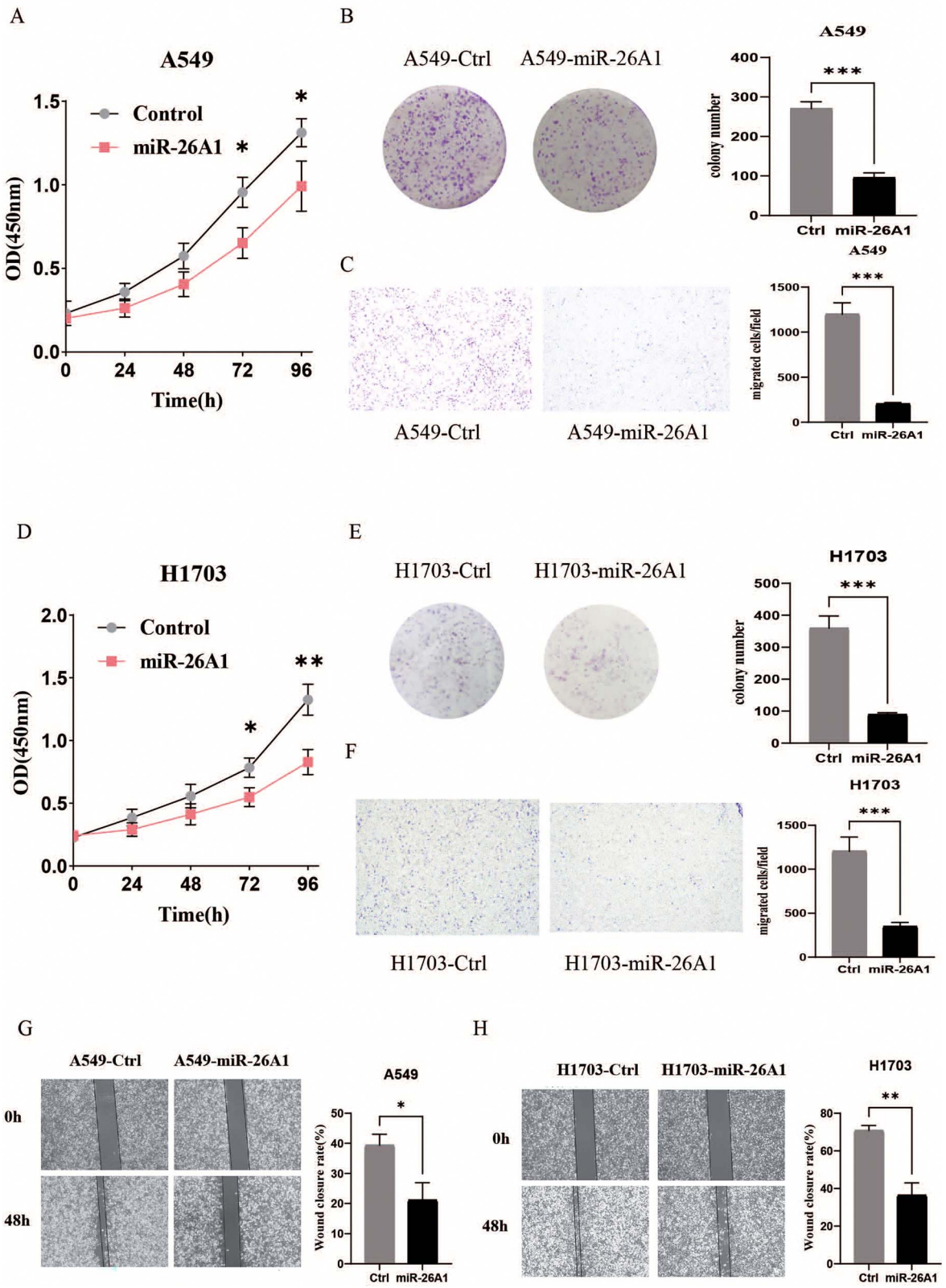

Figure 5. MiR-26A1 inhibits proliferation and metastasis of NSCLC cells. (**A**-**B**) The function of miR-26A1 were detected by CCK-8 (A) and colony forming assays (B). (**C**) The cell migration ability of miR-26A1 was shown via transwell assays. (**D**-**E**) The proliferation ability of miR-26A1 infected with miR-26A1 was examined via CCK-8 assays (D) and colony forming assays (E). (**F**) H1703 cells transfected with miR-26A1 was performed via transwell assays. (**G**) The role of miR-26A1 in A549 cells was conducted via wounding healing assays. (**H**) Similarly, the role of miR-26A1 in H1703 was detected in H1703.

Table 1. Baseline Clinical characteristics of NSCLC patients

Total	11
Age, years	
≥60	5
<60	6
Sex	
Male	6
Female	5
Histology	
LUAD	7
LUSC	4
Smoking history	
Yes	3
No	8
EGFR mutations	
Yes	1
No	2
Unknown	8
Tumor size	
≤3	4
>3	7
N status	
N0	6
N1	2
N2	3
Stage	
I-II	8
III-IV	3

LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma EGFR, epidermal growth factor receptor

CCK-8, colony formation and transwell assays were conducted. In the CCK-8 and transwell assays, the results showed that overexpression of VILL repressed the cell proliferation of A549 significantly (Fig. 6A and B). Moreover, transwell assays showed that VILL suppressed cell migration ability in A549 (Fig. 6C). Besides, CCK-8 and colony forming assays demonstrated that overexpression of VILL repressed cell proliferation in H1703 (Fig. 6D and E). VILL repressed H1703 migration ability via wound healing assay (Fig. 6F). Accordingly, wound healing assays showed that overexpressed VILL markedly decreased the cell migration ability in both A549 and H1703 (Fig. 6G and H).

MiR-26A1 reactivates VILL via enhancer elements

The activity of reporter gene was activated by pGL3-miR-26A1 (Fig. 7A). The activity of reporter gene was activated in co-transfecting with pGL3-miR-26A1 and miR-26A1, compared with co-transfecting pGL3-miR-26A1 and PCDH empty vectors (Fig. 7B). The enrichment of H3K27ac in enhancer regions were detected containing miR-26A1 locus by ChIP-Seq (Fig. 7C). The enrichment of H3K27ac was upregulated in enhancer region containing miR-26A1 locus by ChIP-qPCR after transfecting with miR-26A1 (Fig. 7D). However, it i's not reactivated in HEK293T cells transfected with mut-miR-26A1 (Fig. 7E).

This study illustrated that how TSG VILL is silenced in NSCLC. In normal lung cells, miR-26A1 binds to the enhancer region to activate VILL, which protects the normal lung cells from transformation into malignant ones (Fig. 8). However, when miR-26A1 is downregulated to reduce enhancer activity, the corresponding TSG VILL would be silenced and failed to play its repressive functions on tumorigenesis.

Discussion

Deregulation of TSGs is commonly observed in malignant cells, including lung cancer cells (28). Previous studies have demonstrated that the main epigenetic reason for TSGs downregulation was ascribed to hypermethylation of their promoters, and sometimes, to aberrant histone modifications and non-coding RNA dysregulations (29,30). Studies have also indicated that TSGs downregulation is associated with hypermethylation of CpG island in the promoter region (23). Herein, we found that ∼62.5% of the downregulated TSGs in LUAD and ∼72.7% downregulated TSGs in LUSC had no significant DNA hypermethylation in promoter regions compared with the normal group. This conclusion is consistent with a previous study, which reported that promoter methylation is not an important cause of TSGs low expression in tumor tissues (31). For instance, the downregulation of p16 is regulated by genome disorder and homozygosity deletion (32,33). Moreover, homozygosity deletion also resulted in APC and PTEN down-regulation (34,35). The evidence suggested that promoter hypermethylation is not essential for TSG dysregulation. It i's reported that VILL is a novel TSG (36). Lower expression of VILL in tumor has been related to epigenetic dysregulation and loss of gene homology (37). However, our research suggested that the lower expression of VILL in NSCLC was not caused by sequence mutations or DNA promoter hypermethylation.

miRNAs are ∼22 nt non-coding RNA located in the cytoplasm or nucleus that regulate mRNA expression at the post-transcriptional level (38). Over the years, miRNA-mediate transcriptional gene activation (TGA) has also been gradually reported. For instance, the first miRNA identified as a TGA was human miR-373, which induced transcription of both E-cadherin (CDH1) and cold-shock domain-containing protein 2 (CSDC2) (39). Moreover, miR-223 drives TGS NFI-A interacting with the NFI-A promoter region complementary to the miR-223 seed sequence (40). miR-26A1 is involved in the degradation of an oncogene in the cytoplasm and is dysregulated in a variety of tumors (41). Yet, the function of miR-26A1 in the nucleus remains unclear (42–44). In this study, we collected NSCLC tissues and confirmed that the expression of miR-26A1 was downregulated in NSCLC. In NSCLC cells, miR-26A1 can activate VILL and repress their proliferation. These results suggested that miR-26A1 could act as a potential inhibitor and provide a new clinical approach for NSCLC treatment.

In consistent with our conclusion, previous studies have shown that many miRNAs on DNA locus overlap with enhancers, and these miRNAs interact with

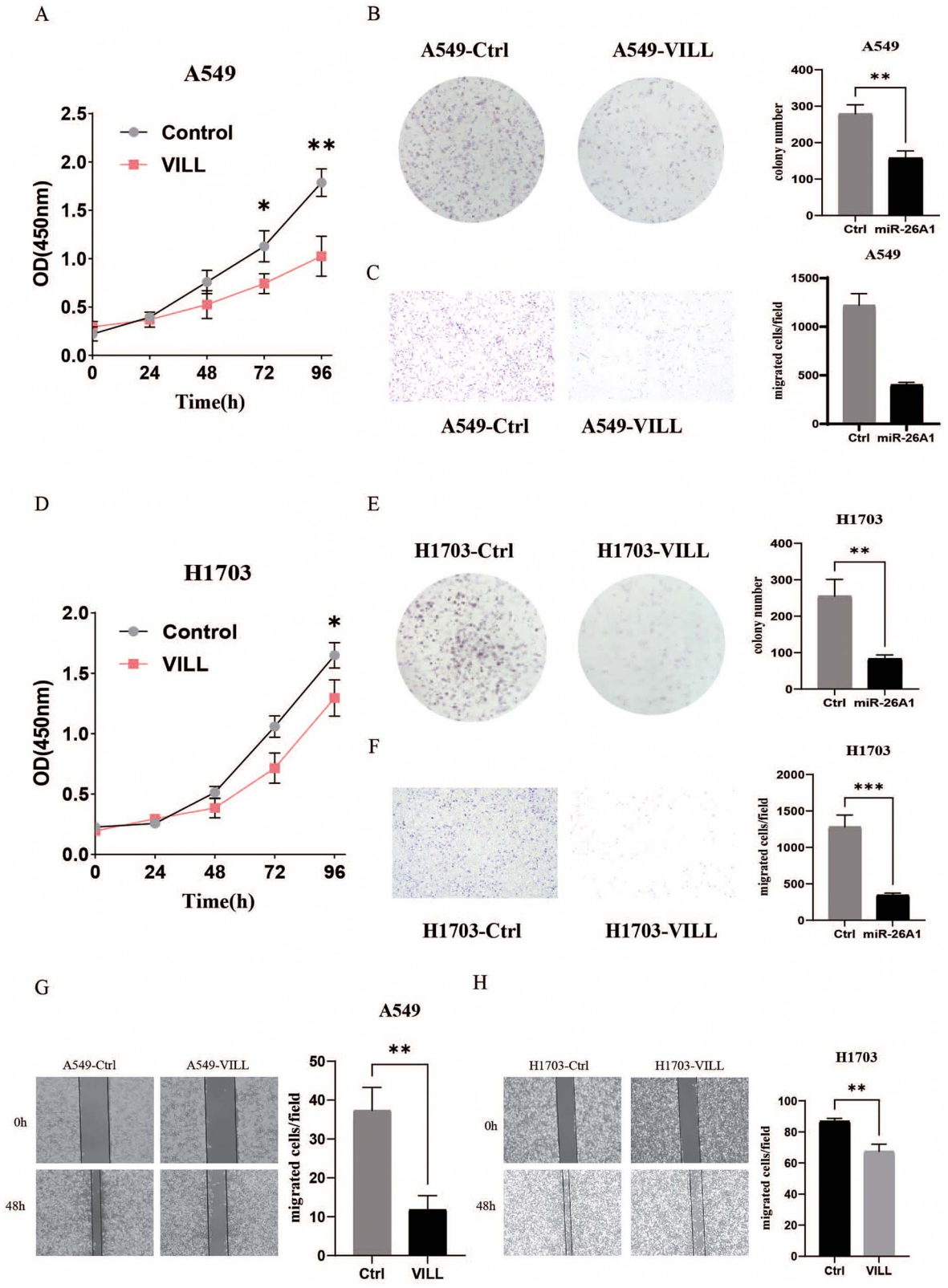

Figure 6. VILL inhibits the malignant behavior of NSCLC *in vitro*. (**A**-**B**) The function of VILL in A549 cells transfected with VILL was tested by CCK-8 (A) and colony formation assays (B). (**C**) The function of VILL was shown in A549 via transwell assays. (**D**-**E**) The proliferation ability of H1703 infected with VILL was detected via CCK-8 assay (D) and colony forming assays (E). (**F**) The migration ability was examined in H1703 infected with VILL via wound healing assays. (**G**-**H**) Similarly, the migration ability of A549 (G) and H1703 (H) transfected with VILL were conducted by wound healing assays.

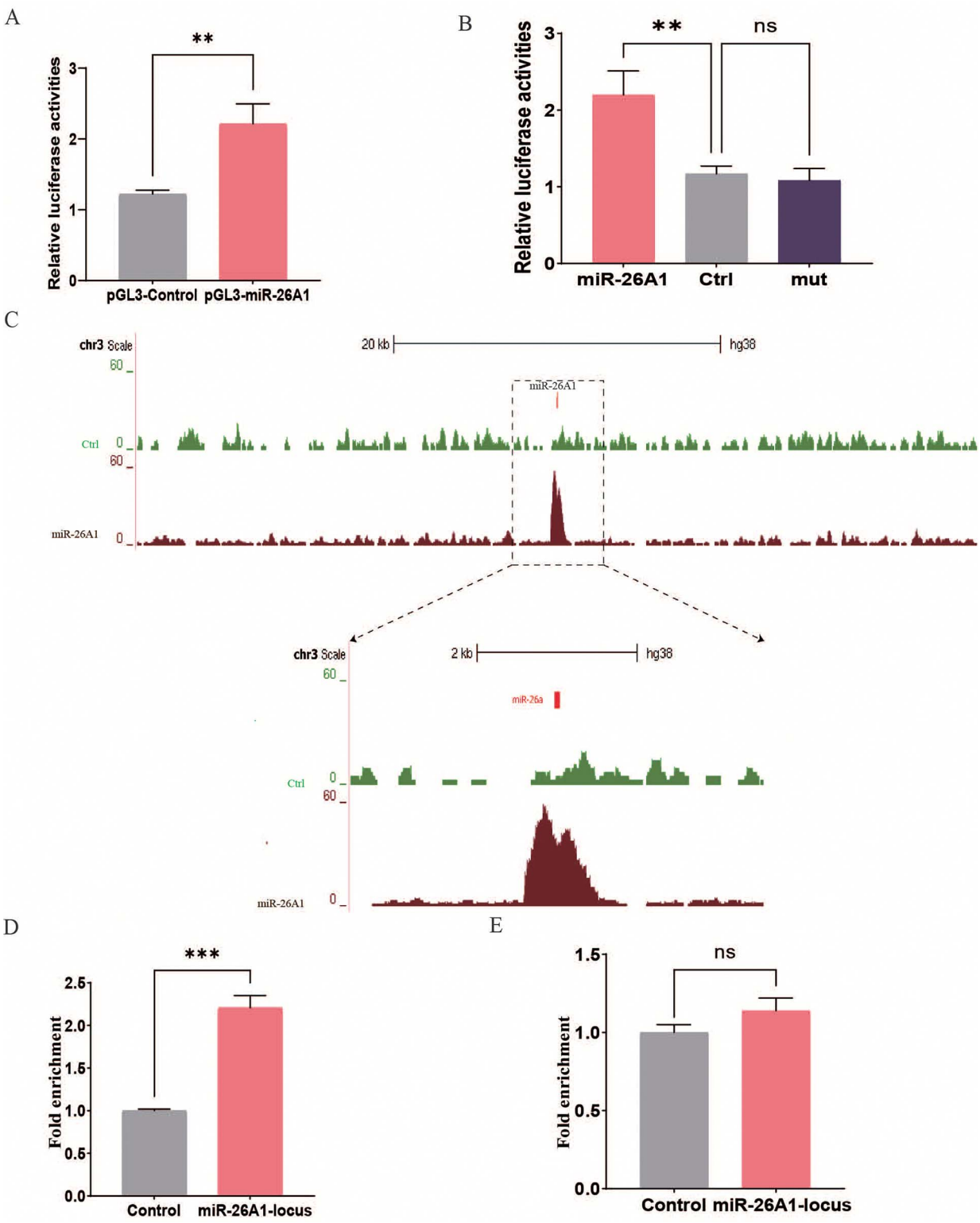

Figure 7. MiR-26A1 reactivates VILL via enhancer elements. (**A**) Luciferase reporter gene activity was observed in HEK293T transfected with pGL3-miR-26A1 or pGL3-promoter. (**B**) The reporter gene activity co-transfected with pGL3-miR-26A1 and PCDH-miR-26A1 was shown. (**C**) ChIP-Seq was used to examine the enrichment of enhancer region H3K27ac in A549. (**D**-**E**) The statement of H3K27ac in the enhancer region transfected with miR-26A1 or mut-miR-26A1 was demonstrated by the ChIP-qPCR assay.

enhancers to up-regulate TSGs expression, such as miR-339 (10,45). Our results showed that enhancer was reactivated in A549 and H1703 infected with miR-26A1 lentivirus. At the same time, miR-26A1 could not reactivate TSGs after inhibiting the enhancer activity via JQ1. These results suggested that miR-26A1 could regulate TSGs expression through the enhancers. The enhancers possess a remote regulation function, and

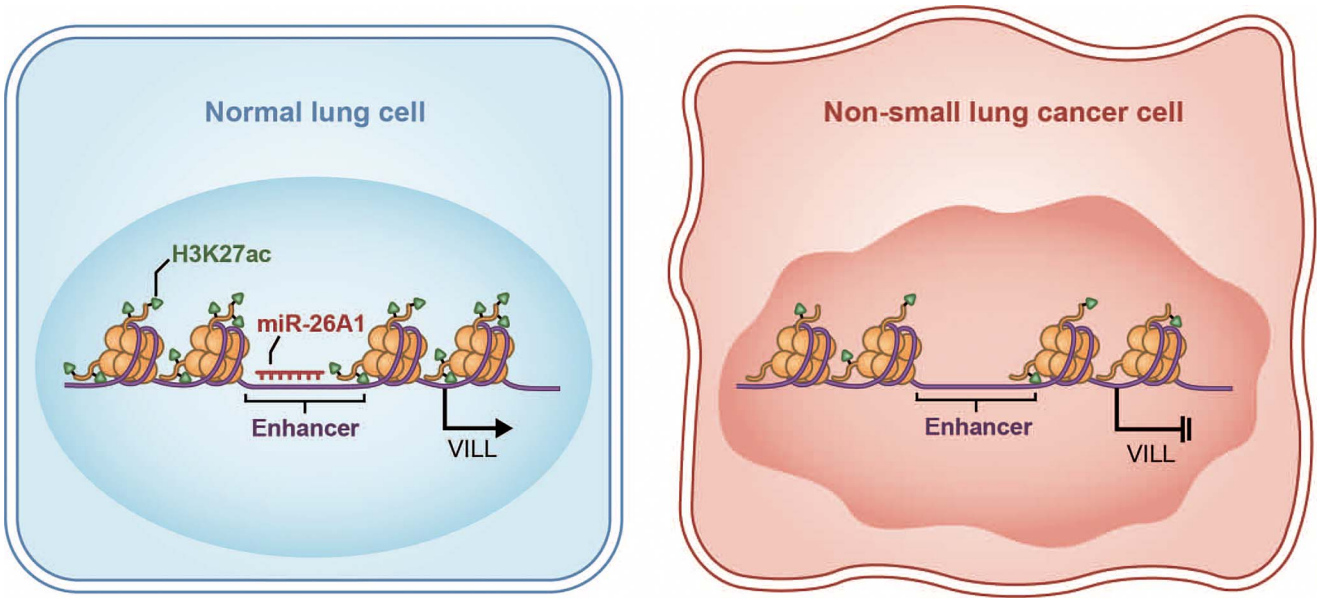

Figure 8. Tumor Suppressor Gene VILL is reactivated by miR-26A1 via enhancer reprogramming in NSCLC. miR-26A1 binds to the enhancer to reactivate VILL transcription in a normal lung cell. On the other hand, when the expression of miR-26A1 is reduced, the corresponding enhancer will be restrained, after which VILL is downregulated in NSCLC.

TSGs in NSCLC may be interpreted by enhancers dysregulation (46–48). We will further investigate the potential TSGs regulated by miR-26A1 in NSCLC by conducting high-throughput sequencing.

Taken together, our results suggested that miR-26A1 could inhibit the proliferation of NSCLC cells by upregulating the expression of VILL through reactivating enhancers. These results implied that miR-26A1 can be used as a potential therapeutic target for NSCLC. Additionally, it is believed that the inactivation of enhancers caused by miR-26A1 downregulation can also be used to explain the molecular mechanism of NSCLC development. Further research should focus on developing a novel nucleic acid drug targeting miR-26A1to treat NSCLC patients.

Materials and Methods

Tissue samples

NSCLC tissues and adjacent lung tissues were obtained from Gansu Provincial Hospital (Gansu, China). Upon collection, samples were immediately stored in the RNAwait (Biosharp) and frozen at −80°C. These clinical data of NSCLC patients are shown in Table 1. Informed consent was obtained from all patients, and the Ethics Committee of Gansu Provincial Hospital approved this study.

Bisulfite pyrosequencing

DNA was extracted using the phenol-chloroform method. Briefly, 400 ng DNA was used for DNA sulfite conversion (ZYMO EZ DNA Methylation-Gold Kit, D5005) according to the manufacturer's instruction. Primers were processed for DNA amplification and sequencing (Sunny). The primers used for real-time PCR are shown in Supplementary Material, Table S1. If the single purpose of banding was found and dimers were rare, then the primers designed were carried out for regular pyrosequencing.

Cell culture

The cell line of human embryonic kidney 293 T (HEK293T), human small cell lung cancer cell lines A549 and H1703, and normal lung fibroblast cell line MRC5 were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS (fetal calf serum) and 1% penicillin–streptomycin mixture (Hyclone) in a humidified atmosphere containing 5% CO_2 at 37°C.

Plasmid construction

To establish the miR-26A1 expression vectors, the fragment (770 bp) of pri-miR-26A1 was amplified from genomic DNAs of normal liver tissues from Baolong Zhang and then cloned into the BamHI/EcoRI site of the pCDH-CMV-MCS-EF1-copGFP lentiviral vector. Moreover, protein-coding regions of VILL were cloned into the XbaI/NotI site of the pCDH-CMV-MCS-EF1-copGFP lentiviral vector. The primers used in PCR are shown in Supplementary Material, Table S1. miR-26A1 inhibitors were purchased from RiboBio (Guangzhou, China). The process of miR-26A1 inhibitors transfection was conducted according to the manufacturer's protocol.

Transfection

DNA transfection in A549 and H1703 cells was performed using the Hieff Trans™ Liposomal Transfection Reagent (Yeasen, China) according to the manufacturer's protocol. Overexpression or knock-down vectors were transfected into HEK293T cells with packaging plasmids psPAX2 and pMD 2.G. The recombinant lentiviruses in the supernatant were collected after 48 h transfection and

directly used to infect A549 and H1703. The expression of miR-26A1 and VILL was verified by quantitative real-time PCR.

qPCR

RNA was extracted with Trizol reagent (Invitrogen) and then reversely transcribed using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa) following the manufacturer's instructions. The expression of miR-26A1 and VILL was examined by RT-PCR using an SYBR Green qRT-PCR (master mix kit (TIANGEN) on LightCycler 480II Real-time PCR system (Roche)) according to the instructions. GAPDH was used as an endogenous control. Fold changes were calculated by relative quantification ($2^{-\Delta\Delta Ct}$). The primers used in real-time PCR are shown in Supplementary Material, Table S1.

Western blot

Cells were cultured and lysed in SDS buffer contained with protease inhibitors (Roche). Next, cell lysates were centrifuged at 12,000 rpm at 4°C for 15 min. Proteins were then loaded on 10% gradient SDS-polyacrylamide gel (EpiZyme) and transferred to PVDF membranes (Millipore, Billerica, MA). The immunoblots were probed with a primary antibody against VILL (1/500 dilution), and GAPDH (1/5000 dilution) in 3% BSA, followed by washing in TBST three times. Then, secondary antibodies including horseradish peroxide (HRP)-conjugated donkey anti-rabbit or anti-mouse, were probed for 2 h (1/5000 dilution; Amersham). Immunostaining was detected with an enhanced chemiluminescent system (ECL).

CCK-8, cell counting and colony-forming assays

Cell proliferation was measured by cell counting and CCK-8 assays. A total of 4000 cells transfected with overexpression of miR-26A1 or control vectors were plated in the 96-well plates for 24, 48 and 72 h. At each time point, 10 μL of sterile CCK-8 was added to each well and incubated for another 2 h at 37°C. The absorbance at 450 nm was determined using a microplate reader (SpectraMax Plus 384). In the colony-forming assays, 1000 cells were spread in 6-well plates and incubated with DMEM containing 10% fetal bovine serum for 14 days. The colonies were then washed with PBS two times, and fixed with methanol, and stained with a crystal violet dye. Finally, the colonies were photographed and analyzed.

Transwell assay

For transwell assays, 8 μm transwell chambers (Corning) were used for cell migration assays. Briefly, 50,000 cells containing 1% fetal bovine serum were seeded in 24-well plates upper chambers, while DMEM containing 20% fetal bovine serum was added into a lower chamber. After 48 h incubation, cells were washed with PBS stained with a 0.1% crystal violet solution.

ISH and IHC

ISH and IHC staining in tissue samples were conducted as described previously (49).

Luciferase reporter assay

The genomic sequence of the miR-26A1 locus was amplified from the DNA genome of normal liver tissue, and the primer sequence was synthesized by SunnyBio (Shanghai, China), which was directly inserted into the pGL3 vector. The primers used in the construction of the pGL3-26A1 vector are shown in Supplementary Material, Table S1.

HEK293T cells were seeded in a six-well plate and cultured for 24 h. pGL3-26A1 were co-transfected with liposome 2000 and overexpressed miR-26A1 vectors, respectively. Luciferase and Renilla activity were measured at 48 h after transfection using a dual-luciferase reporting kit (Promega) according to the kit instructions.

Chromatin immunoprecipitation assay

In order to identify the upregulated expression of VILL caused by overexpression of miR-26A1, we conducted an immunoprecipitation experiment to detect the H3K27ac enrichment of the enhancer. Overexpression miR-26A1 of A549 was harvested, washed with PBS 3 times, and fixed with 1% formaldehyde at room temperature for 10 min. After the supernatant was removed, formaldehyde was neutralized with 0.125 M glycine solution for 10 min. Cells were then collected on a cell scraper and transferred to a 1.5 mL centrifuge tube. Samples were then mixed with lysate (10 mM HEPES pH 7.9, 1.5 mM MgCl2, 0.5% NP-40,10 mM KCl) containing protease K (Roche). Chromatin was dissolved with nuclear lyse solution (50 mM Tris–HCl pH 8.1, 0.3% SDS, 10 mM EDTA, and 1× Cocktail). After ultrasound treatment, cell extracts were treated with H3K27ac antibody and Protein A Dynabeads (Invitrogen) overnight at 4°C. Chromatin was then further collected using the QIAGEN purification kit. First, the template DNA was detected with specific primers by real-time PCR. The primers used in real-time PCR are shown in Supplementary Material, Table S1. Next, a 20 ng DNA template was used to construct the ChIP library, and Agilent Technologies Inc performed further sequencing.

Statistical analysis

All data were representative, and three independent experiments were conducted. All values are presented as calculated means ± standard deviations following triplicated experiments unless otherwise noted in the figure. $P < 0.05$ indicated statistical significance (*** means $P < 0.001$, ** means $P < 0.01$ *and* * means $P < 0.05$). All data were analyzed by GraphPad Prism and Student t-test (Version 9.0, GraphPad Software, Inc).

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Data Availability

The accession number for the sequencing data generated in this article is shown (SRA accession no. PRGNA808239). For LUAD and LUSC samples, expression data were downloaded from The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/).

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