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Original Article

DNASE2B silencing suppresses proliferation and induces cell cycle arrest in non-small cell lung cancer cells

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Abstract: DNASE2B exists as the primary acid DNase that has an essential role in the degradation of nuclear DNA during lens cell differentiation. Previous reports showed DNASE2B might involve in tumorigenesis. However, its role in lung cancer remains unclear. Here we reported that DNASE2B might be associated with tumor genesis of NSCLC. We found knockdown of DNASE2B suppressed cell growth and proliferation in A549 cells with Celigo Cell Counting application, MTT and colonies formation assay. Flow cytometry results showed knock down of DNASE2B induced significant S depletion with G_1 and G_2/M accumulation in A549 cells. Therefore, DNASE2B serves an important role in the growth of lung cancer cells, and may be considered as a potential biomarker and therapeutic target for lung cancer.

Keywords: Lung cancer, NSCLC, DNASE2B, cell growth, cell cycle

Introduction

Lung cancer is one of the most common malignancies in the world, with 2.2 million new cases diagnosed annually and is also the leading cause of cancer deaths worldwide, causing 1.6 million deaths annually [1]. Non-small cell lung cancer (NSCLC) accounts for the majority type in lung cancer which comprises three different subtypes: squamous-cell carcinoma, large-cell carcinoma, and adenocarcinoma [2]. Standard platinum-based doublet chemotherapeutic treatment of advanced NSCLC seems to have reached a plateau in terms of efficacy, while more and more therapies were identified as effective strategies with potential. One promising treatment strategy involves the further subdivision of NSCLC into clinically relevant molecular subsets, according to a classification schema based on specific genes, such as EGFR, ALK, HER2, BRAF, KRAS etc. [3, 4]. Despite therapeutic advances, the overall 5-year survival remains under 20% [5]. Novel tumor related genes and therapeutic approaches are still therefore needed.

Some understanding of the molecular composition of tumors has led to the development of targeted agents [6, 7]. Those so-called driver mutations including in EGFR, ALK, HER2 (also known as ERBB2), BRAF, PIK3CA, AKT1, MAP2K1, and MET. More and more data from genomic expression, mutational and proteomic profiling studies have led to the identification of additional molecular driver mutations in lung cancer [4]. Those identification of multiple genes in lung cancer especially in NSCLC has improved the understanding of lung cancer pathogenesis, which benefits clinical diagnosis and treatment a lot. Genes belonging to EGFR signalling pathway such as EGFR, ALK, HER2 etc. contribute to practical significance nowadays. Meanwhile other genes that contribute to lung tumorigenesis still continually emerging and need exploring as promising new target.

DNases have been classified into DNase I enzymes and DNase II enzymes. DNase I enzymes have an activity optimum at approximately pH 7.0 and require magnesium ions. DNase II enzymes have a magnesium-independent activity optimum at approximately pH 5.0

[8]. Three DNase II enzymes have been identified in mammals so far. DNase 2a, usually referred to as DNase 2, is a ubiquitous lysosomal enzyme which degrades DNA of phagocytosed apoptotic bodies or DNA entering the cell via endocytosis. DNASE2B, DNase 2b, also known as DNase 2-like acid DNase, DLAD or DNase 2b [9, 10]. Previous reports showed DNASE2B is the most characteristic lysosomal endonuclease in the avian cells. However, in mammals the main lysosomal Dnase is DNase IIA but not DNASE2B. A report suggested DNASE2B had a limited function in the elimination of the nuclei of the differentiating lens fibers in the mouse eye [11, 12]. A genomewide SNP screening with a human SNP array results showed *DNASE2B rs3738573* was chemosensitive to docetaxel in gastric cancer patients which indicated the involvement of DNASE2B in tumorigenesis [13].

DNASE2B (NM_021233), which is also called DNase 2β or DNase IIβ, is encoded by *DLAD* (DNase II-like acid Dnase) gene and is found to exist as the primary acid DNase in the lens [14]. DNASE2B located in lysosomes that has an essential role in the degradation of nuclear DNA during lens cell differentiation which expelled from erythroid precursor cells [12]. Previous studies showed knockout of the DLAD gene in mice retarded the nuclear DNA degradation of lens and caused cataract formation of nucleus lentis [11, 12, 15]. Recently, more information was found about DNASE2B. Cui et al found positive regulation of DLAD mRNA level and protein expression (DNASE2B) by HSF4 thus facilitating de-nucleation of lens fiber cells [14]. Results reported by Fischer H and collegues identified DNase 2 as the predominant DNase on the mammalian skin surface and indicated that its activity was primarily targeted to exogenous DNA [8]. Other research suggested DNASE2B was expressed human salivary glands and lungs associated with disease such as autoimmune diseases, haematological cancers and hepatologicaldisease [16, 17]. However, whether if DNASE2B involved and the physiological role of DNASE2B in lung cancer especially in NSCLC remained unidentified.

In this study, we investigated the expression of DNASE2B in NSCLC cell lines. Then taking advantage of lentivirus mediated shDNASE2B, the expression of DNASE2B was knocked down in A549 cells. The inhibited effects of DNASE-2B on cell proliferation was investigated with Celigo system, MTT assay and colony formation. Furthermore, flow cytometry results indicated down regulation of DNASE2B arresting cell cycle in A549 cells. Together, our results may reveal a novel function and mechanism towards better understanding the role of DNASE2B in lung cancer.

Methods and materials

Cell lines and cell culture

Human lung cancer cell lines A549, NCI-H1299, 95D and NCI-H460 were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were cultured either in in F12K medium (A549 cells) or RPMI-1640 medium (NCI-H1299, 95D and NCI-H469 cells) supplemented with 10% bovine serum. Penicillin (100 U/ml) and streptomycin (100 U/ml) were added to all kinds of medium. Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Construction of DNASE2B knockdown lentivirus

The DNA sequence (TTACCATGTCTACAATATA) of DNASE2B was the target which were selected from the full-length DNASE2B sequence (NM_021233) by GeneChem Co. Ltd. (Shanghai, China). According to the sequence of DNASE2B, two vectors shRNA S1 and shRNA S2 were designed. The sequences are as follows: shRNAS1: 5'-CCG GCC TTA CCA TGT CTA CAA TAT ACT CGA GTA TAT TGT AGA CAT GGT AAG GTT TTT G-3'; shRNA S2: 5'-AAT TCA AAA ACC TTA CCA TGT CTA CAA TAT ACT CGA GTA TAT TGT AGA CAT GGT AAG G-3; The shRNAs were annealed and ligated to the linearized GV115 lentivirus vector to transform DH5α competent cells. The plasmid was extracted and verified by enzymatic digestion and sequencing. A549 cells were infected with the lentivirus. Cells infected with a lentivirus carrying an empty vector were used as a control. Fluorescence expression was measured after 72 hours when the achieved infection efficiency was 80%. The expression of DNASE2B was analyzed by RT-qPCR and western blotting.

Quantitative RT-PCR

Total RNA from the 4 cell lines, A549, NCI-H1299, 95D and NCI-H460, was extracted

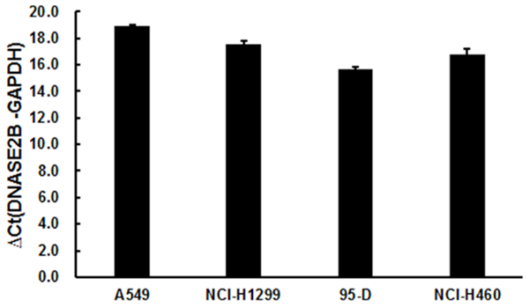

Figure 1. DNASE2B expressed in four cell lines including A549, NCI-H1299, 95-D and NCI-H460.

using the TRIzol reagent (Invitrogen, Shanghai, China), according to the manufacturer's instructions and was then used for RT reaction. Briefly, 2 μg of total RNA from each sample was reverse transcribed to single-stranded cDNA. One microliter of cDNA was used as a template for the following PCR. The primers used were as follows: for DNASE2B forward, 5'-AAG TCG GAT TCT TTT CTT GAC G-3' and reverse, 5'-GGT AAG GAA GGG AGC AGT TTG-3'; and for GAPDH forward, 5'-TGA CTT CAA CAG CGA CAC CCA-3' and 5'-CAC CCT GTT GCT GTA GCC AAA-3'. The quantitative RT-PCR comprised an initial denaturation at 95°C for 15 sec, then 45 cycles at 95°C for 5 sec and 60°C for 30 sec. The PCR products of DNASE2B and GAPDH were 241 and 121 bp, respectively. All samples were examined in triplicates.

Colony formation assay

DNASE2B-shRNA transfected and control A549 cells were trypsin-digested and resuspended in standard medium after achieving logarithmic growth phase. Cells were seeded into six-well plates at a density of 500 cells/well. The cells were incubated and observed over a period of 10 days with half of the medium being changed every 3 days. The cells were washed with PBS and then fixed with paraformaldehyde (1 ml/well; Shanghai Sangon, China) for 30-60 min. All cell wells were washed with PBS and then stained with 500 μL Giemsa (ECM550 Chemicon) for 20 min. Then, the cells were washed with ddH_2O for three times. Cell colonies were photographed by fluorescence microscopy (Micro Publisher 3.3RTV; Olympus, Japan).

Cell growth assay

3-(4,5-Dimethyl-thiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT, Sigma) assay was performed to assess cell proliferation after drug exposure as described previously [18]. Briefly, Lentiviral infected A549 cells were seeded in 96-well plates at an inoculation density of 3,000 cells/well. At different time points after incubation (1, 2, 3, 4 and 5 days), MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) solution was added to each well and incubated at 37°C for 4 h. Then 100 μL acidic isopropanol (10% SDS, 5% isopropanol and 0.01 mol/L HCl) was added into each well after the medium were carefully removed. Plates were then read with an Automated Microplate Reader (Molecular Device, US) at 490 nm.

Plate analysis with the adherent cell cytometry system Celigo®

In brief, shRNA DNASE2B transfected A549 cells were trypsin-digested and resuspended in standard medium after achieving logarithmic growth phase. Cells were seeded into 96-well-plates at a density of 2000 cells/well. The fluorescence expression was collected everyday with the Celigo® system for 5 days continually. With adjustments in the paraments in Analysis Setting, the fluorescence expression were converted to cell numbers.

Flow cytometric analysis of cellular DNA content

Cells were transfected with shDNASE2B or control shRNA. Then harvested, treated and untreated cells were fixed with 70% ice-cold ethanol and were kept at -20°C overnight. Fixed cells were centrifuged, washed and resuspended in PBS containing in concentration of 50 μg/mL of propidiumiodide (PI, P4170, Sigma) and 100 μg/mL of DNase-free RNase at 37°C for 30 min as previously reported. Cell cycle was analyzed by using cell cytometer (FACScan, Becton Dickinson) and Cell Quest Pro software.

Western blot analysis

Cells were transfected with shDNASE2B or control shRNA. Protein was abstracted and western blotting were performed as previously described [14, 19]. Protein concentrations of cell lysates were determined using the Bradford method. Briefly, certain quantized proteins were separated in SDS-PAGE and then were transferred to PVDF membrane. After blocked at room temperature, membranes were incubated with different primary antibodies before visualized and photographed. The antibodies

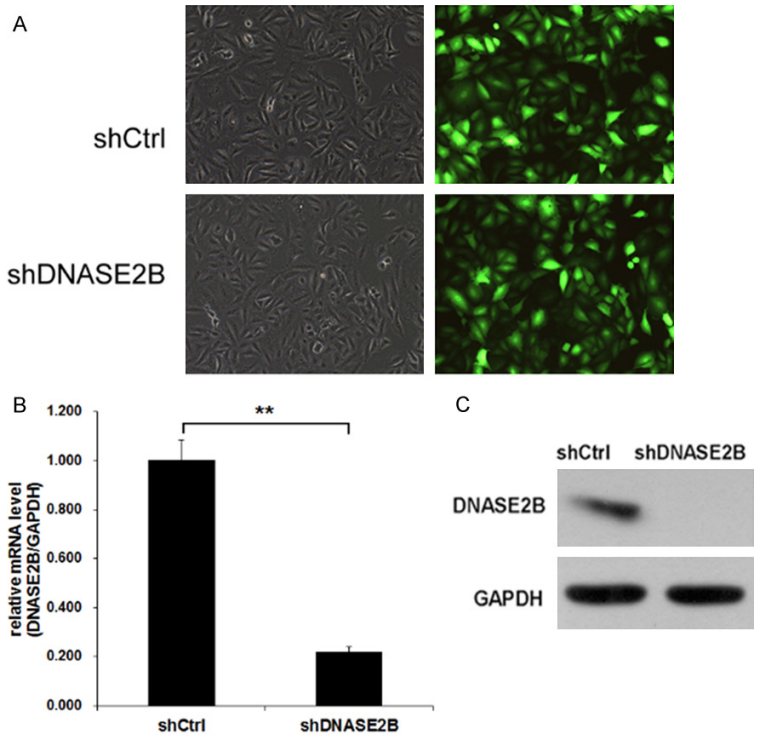

Figure 2. DNASE2B was effectively knocked down with lentivirus-mediated shDNASE2B in non-small cell lung cancer A549 cells. A. The white light and fluorescence photos of shCtrl and shDNASE2B lentivirus transfected A549 cells. B. The suppressed DNASE2B in mRNA level with lentivirus-mediated shDNASE2B in A549 cells. C. The suppression of DNASE2B protein with lentivirus-mediated shDNASE2B in A549 cells. All experiments were performed at least thrice and independently. Significant differences from untreated control were indicated as *P < 0.05; **P < 0.01; ***P < 0.001.

used in the experiments were: anti-DNASE2B and anti-GAPDH which were obtained from Epitomics (US). The protein expression was quantified with densitometry analysis by calculating the relative band OD values of target protein on that of GAPDH.

Statistical analysis

All experiments were performed in triplicate and were repeated at least three times. The results presented as mean values ± SD. Statistical significance was performed by using Student t test. The significance level was set as $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

Results

DNASE2B mRNA level is expressed highest in A549 among four lung cancer cell lines

We used Q-PCR to investigate the transcriptional level of DNASE2B in four lung cancer celllines. The results indicated the DNASE2B were expressed in four cellines including A549, NCI-H1299, 95-D and NCI-H460 (**Figure 1**). Compared with the GAPDH expression, the highest mRNA level is improved in A549 cells as 18.88 fold. The mRNA level is prolonged in NCI-H1299 cells as 17.50 fold and in 95-D cells as 15.70 fold. The mRNA level was up-regulated 16.81 fold in NCI-H460 cells consistently with A549, NCI-H1299 and 95-D cells.

Lentivirus-mediated knockdown of DNASE2B in non-small cell lung cancer A549 cells

To further explore the function of DNASE2B in lung cancer, we knocked down DNASE2B in non-small cell lung cancer A549 cells using lentivirus-mediated gene transfection. As shown in **Figure 2A**, most A549 cells presented EGFP positive signals after infected by lentivirus recombined with shRNA targeting DNASE2B (shDNASE2B) or control scrambled shRNA (shCtrl), indicating that the recombinant lentivirus we got could infect A549 cells with high efficiency. Further Real-time PCR and western-blot analysis suggested that the mRNA and protein levels of DNASE2B were both down-regulated significantly in shDNASE2B infected A549 cells (**Figure 2B** and **2C**). The Q-PCR results showed that the expression of DNASE2B gene in the shDNASE2B group was inhibited vs the shCtrl group significantly ($P < 0.05$), which indicated the reduction efficiency reached 77.9% after the infection of shRNA lentivirus. The western blotting results showed no DNASE2B protein band was detected in shDNASE2B infected cells. The above results indicated that recombinant lentivirus taking shDNASE2B could effectively suppress the expression of endogenous DNASE2B in lung cancer cells.

Knock down of DNASE2B inhibited cell proliferation in A549 cells

As the cells infected d with shDNASE2B or shCtrl could be identified with EGFP positive sig-

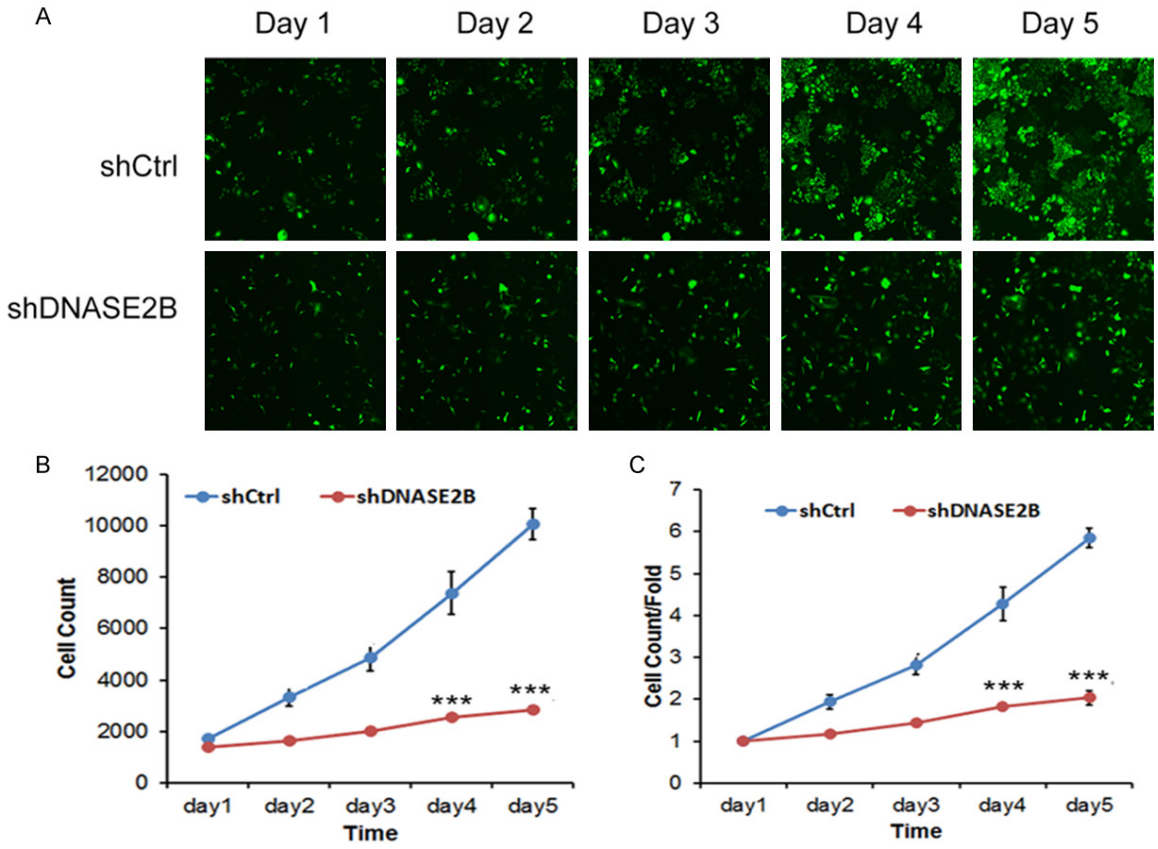

Figure 3. The inhibited effects of down-regulated DNASE2B in A549 cell growth with shDNASE2B transfection. A. Unprocessed raw images of cell growth with Celigo Cell Counting application assay for 5 days. B. Cell counts of A549 cells expressing shCtrl lentivirus and shDNASE2B lentivirus were seeded in 96-well plates and cell growth was assayed every day for 5 days. C. Cell counts fold of A549 cells expressing shCtrl lentivirus and shDNASE2B lentivirus on 1st day of 2nd, 3rd, 4th and 5th days by Celigo Cell Counting application assay. All experiments were performed at least thrice and independently. Significant differences from untreated control were indicated as ***P < 0.001.

nals, we used the Celigo Cell Counting application which to directly image and counted A549 cells continually for 5 days. As indicated in **Figure 3A**, the green signals in cells infected with shRNA targeting DNASE2B (shDNASE2B) were inhibited compared with those cells infected with control scrambled shRNA (shCtrl) significantly ($P < 0.05$). The calculated cell counting result also supported the inhibition in down-regulation of DNASE2B using shDNASE2B (**Figure 3B** and **3C**). On day 5, the number of cells in shDNASE2B treated group were just slightly increased than day 1, which was far below that of cells in shCtrl treated group accordingly.

Then we used MTT assay and colony formation to further investigated the potential effects of DNASE2B on cell proliferation in A549 cells. 2000 cells were seed at day 1 and the counts were investigated with MTT assay every day for 5 days. As indicated in **Figure 4A** and **4B**, the growth was much slower in cells transfected with shDNASE2B than that in cells transfected with shCtrl ($P < 0.05$), which showed the proliferation rate was significantly inhibited.

The colony formation assay showed inhibition of cell proliferation in shDNASE2B transfected A549 cells consistently. As shown in **Figure 5A**, the size was relatively smaller and colonies were fewer in shDNASE2B groups compared with shCtrl groups in A549 cells. Statistical analysis further confirmed that knockdown of DNASE2B significantly reduced colonies formed in A549 cells (**Figure 5B**, $P < 0.001$). Furthermore, we detected the protein expression of PCNA and Ki67, which are important molecules underlie proliferation regulatory function. These two marker proteins expression

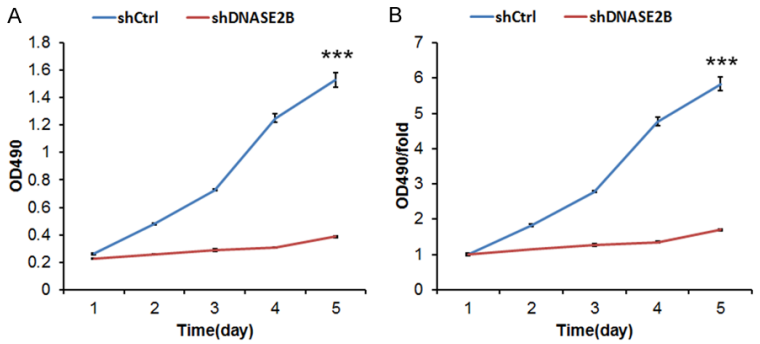

Figure 4. Downregulation of DNASE2B inhibits the proliferation in A549 cells identified by MTT assay. A. Cell viability of A549 cells expressing shCtrl lentivirus and shDNASE2B lentivirus were seeded in 96-well plates and cell growth was assayed every day for 5 days. B. Cell viability fold of A549 cells expressing shCtrl lentivirus and shDNASE2B lentivirus on 1st day of 2nd, 3rd, 4th and 5th days by Celigo Cell Counting application assay. All experiments were performed at least thrice and independently. Significant differences from untreated control were indicated as ***P < 0.001.

reduced in DNASE2B inhibited A549 cells (**Figure 5C**). The densitometry results (**Figure 5D**) was consistent with the protein expression results. Overall, these results suggested that suppression of DNASE2B could inhibit cell proliferation of A549 cells.

Knock down of DNASE2B impaired cell cycle progression of A549 cells

As flow cytometry results showed knock down of DNASE2B induced significant S depletion with G_1 and G_2/M accumulation in A549 cells (**Figure 6A**). The proportion at G_1 phase was 64.05% in the control whereas the proportion was 76.93% in the cells infected with shDNASE2B (**Figure 6B**). 20.33% cells were blocked in G_2/Mphase infected with shDNASE2B, which made significant difference compared to the control ($P < 0.01$) (**Figure 6B**). The proportions of S as the G_1 phase and G_2/Mphase increased. The cells gated% with shDNASE2B in S-phase was 2.75%, which made significant difference compared to the shCtrl group (17.375) ($P < 0.001$). Furthermore, we detected the protein expression of CCNE1 and CCND1, which are important marker molecules underlie G_1 phase checkpoint. These two marker proteins expression reduced in DNASE2B inhibited A549 cells (**Figure 6C**). The densitometry results (**Figure 6D**) was consistent with the protein expression results. These findings suggested that suppression of DNASE2B blocked the cell cycle at G_1 and G_2/Mphase in A549 cells.

Discussion

Multiple malignant cancers including lung cancer relates to mutations occur in genes that encode proteins crucial for cellular proliferation and survival. One promising treatment strategy involves the further subdivision of NSCLC into clinically relevant molecular subsets according to different genes. In this study, we reported DNASE2B, a gene which has an essential role in the degradation of nuclear DNA during lens cell differentiation which expelled from erythroid precursor cells, might be closely associated with tumorigenesis of NSCLC. We found the close relationship between DNASE2B and NSCLC in different NSCLC cell lines. Functional analysis demonstrated that knockdown of DNASE2B suppressed NSCLC cancer cell growth and proliferation. Flow cytometry results showed knock down of DNASE2B induced significant S depletion with G_1 and G_2/M accumulation in A549 cells. In our study, we also found DNASE2B is expressed in lung cancer cells. As **Figure 1** showed, different mRNA level was found in 4 lung cancer cells including A549, NCI-H1299, 95-D and NCI-H460. These data firstly suggested DNASE2B might be associated with the lung cancer lung tumorigenesis.

Functional studies indicated that lysosomal Dnases involved in programmed cell death in the Drosophila melanogaster ovary and in differentiating lens fibers in the mouse eye [11, 12, 14]. Regardless of isoform differences between species, lysosomal DNases have mainly been associated with DNA degradation by macrophages [20]. As previously reported, DNASE2B is a mammalian endonuclease that functions optimally at acid pH in the absence of divalent cations. It was found lysosomal localization and ubiquitous tissue distribution, which indicated this enzyme played a role in the degradation of exogenous DNA encountered by phagocytosis. Subsequent investigations showed that this enzyme was also important for DNA fragmentation and degradation during cell death [21]. As cell death is abnormal in cancer

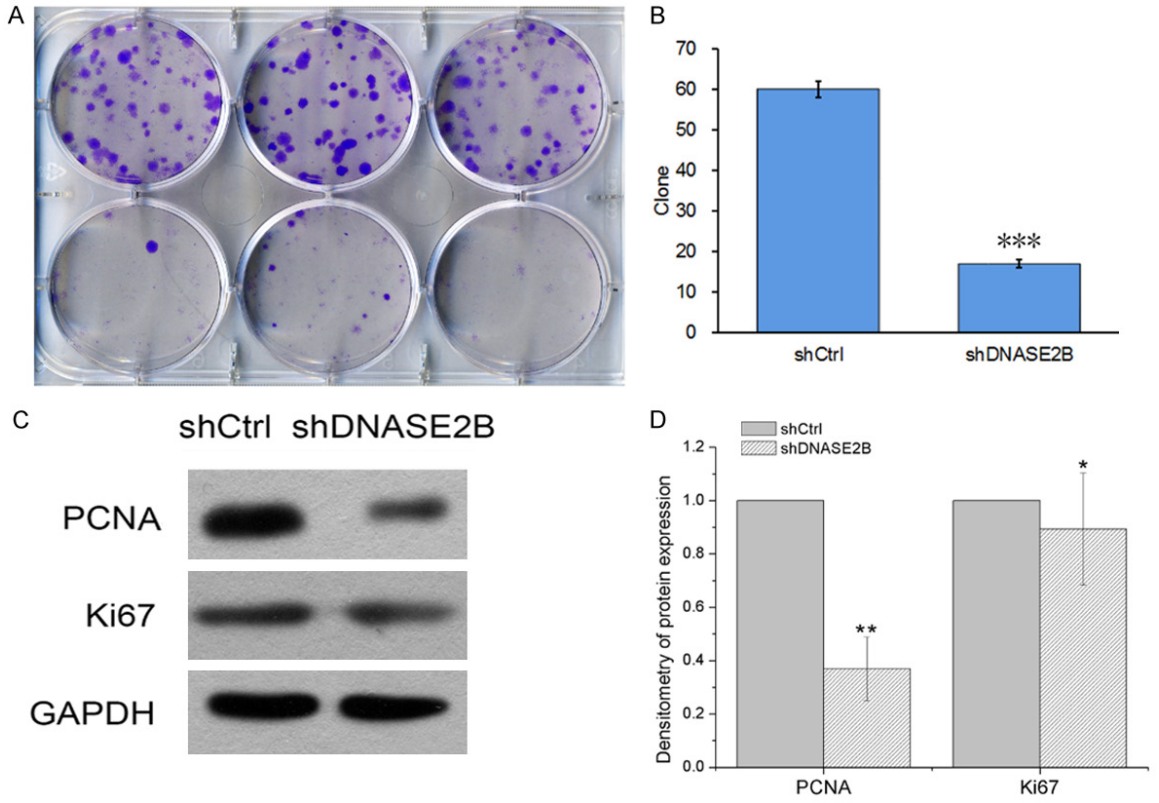

Figure 5. The colony proliferation abilities of A549 lung cancer cells were inhibited with downregulation of DNASE2B. A. Representative microscopic images of colonies were stained by crystal violet in A549 cells. B. Statistical analysis of the number of colonies in A549 cells. C. The expression of Ki-67 and PCNA protein with lentivirus-mediated shDNASE2B in A549 cells. D. Densitometry analyzed on density of the bands. All experiments were performed at least thrice and independently. Significant differences from untreated control were indicated as *P < 0.05; **P < 0.01; ***P < 0.001.

cells, it is rational to believe DNASE2B might involve in cancer cell growth and proliferation. Previous reports suggested gastric cancer cells such as MKN1 and SNU638 carrying the substitution allele (C) of *DNASE2B rs3738573* exhibited enhanced cell death in response to docetaxel as compared to those with the reference allele (G) [13]. After effectively knocking down of DNASE2B with shDNESE2B lentivirus (**Figure 2A-C**), Celigo Cell Counting application, MTT and colony formation assay results further confirmed that knock down of DNASE2B with shDNASE2B lentivirus transfection effectively inhibited the growth and proliferation in non-small cell lung cancer A549 cells. Compared to the control, the growth was much slower in cells transfected with shDNASE2B ($P < 0.05$) using Celigo Cell Counting application (**Figure 3A-C**). MTT assay also supported the inhibited effects with shDNASE2B consistently (**Figure 4A** and **4B**). Knockdown of DNASE2B significantly reduced colonies formed in A549 cells (**Figure 5A** and **5B**). Our study supported the involvement of DNASE2B in cancer cell function and indicated that suppression of DNASE2B could inhibit cell proliferation of A549 cells.

One feature in the malignant proliferation of tumor cells is aberrant cell cycle regulation. Cell cycle impairment always led to cell death along with apoptosis and DNA double strand breaks. Both DNase I and DNase II are involved in cell cycle regulation. DNase I is a secretory glycoprotein with endonuclease activity that cleaves DNA to yield 5'-phosphorylated and 3'-hydroxylated polynucleotides. When DNase I bound with actin, it was shown to decrease the activity of DNA breaks [22, 23]. A previous study about DNase II showed a two to seven-fold increase in DNase II activity at those times when DNA synthesis is taking place in HeLa S3 cells. The

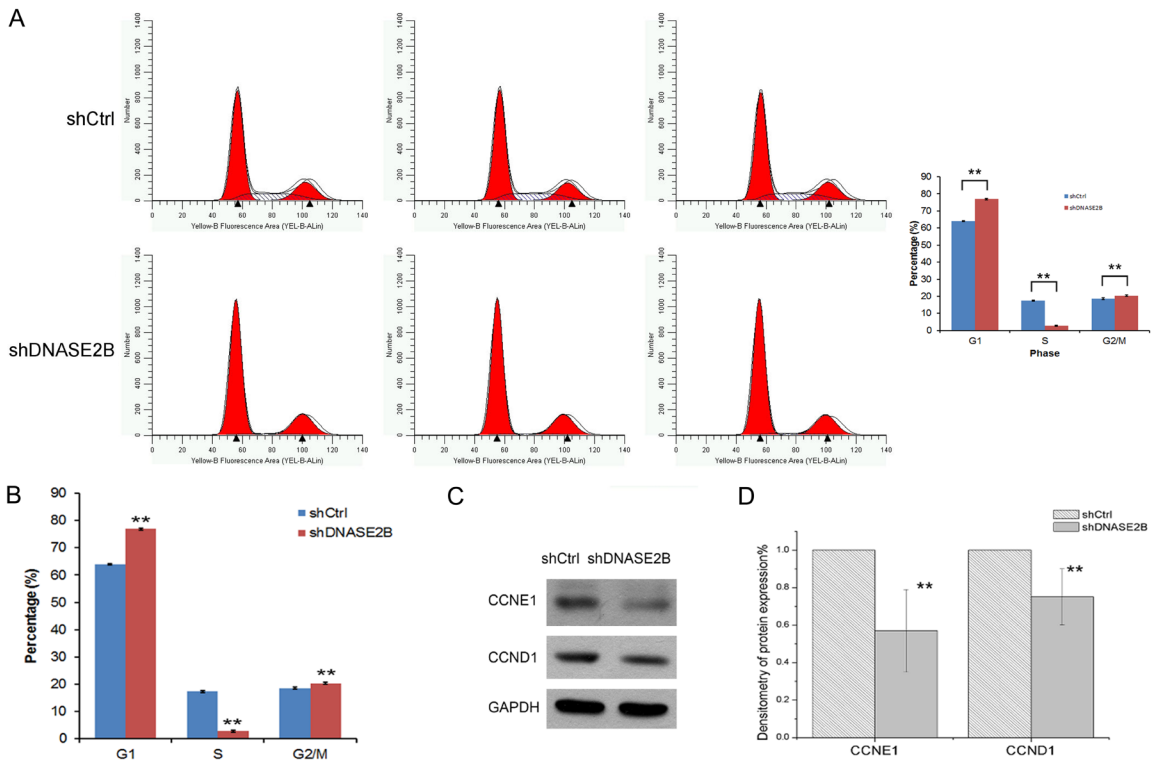

Figure 6. Depletion of DNASE2B arrested cell cycle progression in A549 lung cancer cells. A. Cell cycle progression was analyzed by flow cytometry. B. Statistical analysis of the percentage of cells in G_0/G_1, S and G_2/M phases in A549 cells after DNASE2B knockdown. C. The expression of CCNE1 and CCND1 protein with lentivirus-mediated shDNASE2B in A549 cells. D. Densitometry analyzed on density of the bands. All experiments were performed at least thrice and independently. Significant differences from untreated control were indicated as **P < 0.01.

peaks of DNase II activity coincide with the peaks of DNA synthesis, which suggesting that the enzyme activity increased at the S phase was caused by synthesis of new molecules rather than the activation of existing molecules [24]. As shown in **Figure 6A** and **6B**, knock down of DNASE2B with shDNASE2B lentivirus vector induced significant S depletion with G_1 and G_2/M accumulation in A549 cells. The cells gated% with shDNASE2B in S-phase was 2.75%, which made significant difference compared to the shCtrl group (17.37%) ($P < 0.001$). Collectively, these data illustrated that suppression of DNASE2B blocked the cell cycle at G_1 and G_2/Mphase in A549 cells.

In summary, we found that DNASE2B exprressing in NSCLC cells. Meanwhile, inhibition of DNASE2B suppressed NSCLC cancer cell proliferation and depleted S-phase with G_1 and G_2/Mphase blocking in A549 cells. This new information of DNASE2B involved in NSCLC cancer cell proliferation and cycle progression will be essential to identify a potential biomarker or treatment target that will have predict value in detection, therapy and predisposition of NSCLC.

Acknowledgements

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Disclosure of conflict of interest

None.

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