

SENEX gene promotes cell proliferation by activating RB/E2F pathway in diffuse large B-cell lymphoma cells

JiYU WANG[#]; YING PAN[#]; YANG WAN; ZHIXIANG WANYAN; ZHITAO WANG; QIANSHAN TAO; ZHIMIN ZHAI*

Department of Hematology, The Second Hospital of Anhui Medical University, Hefei, 230601, China

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Abstract: The present study aimed to clarify the role of SENEX in malignant cell proliferation in diffuse large B-cell lymphoma (DLBCL). 22 DLBCL patients (6 newly diagnosed cases, 7 cases at complete remission, and 9 relapsed cases) were included in the study. Our results indicated that both SENEX gene and protein were significantly increased in peripheral blood mononuclear cells (PBMCs) and tumor cells of relapsed DLBCL patients, accompanied by overexpression of p21, p16, and phosphorylated retinoblastoma (Rb). Silencing the SENEX gene in a DLBCL cell line caused a significant decrease in cell proliferation and inhibited cell cycle progression in the G1 phase. Phosphorylated Rb and E2F1 were also decreased, and activation of the Rb/E2F1 pathway was obviously suppressed. To conclude, the SENEX gene promotes proliferation in PBMC and tumor cells of DLBCL patients by activating the Rb/E2F1 pathway, in a manner suggesting that increased SENEX expression affects the relapse of DLBCL and may serve as an important target for DLBCL therapy.

Introduction

As the most frequent subtype of non-Hodgkin's lymphoma (NHL), diffuse large B-cell lymphoma (DLBCL) is often aggressive and fatal (Miao *et al.*, 2019). Although some clinical advances, including monoclonal antibodies targeting CD20 and hematopoietic stem cell transplantation (HSCT), have been applied over the past years, the malignancy outcome remains a significant challenge, as ~30% of patients are not cured and succumb to their disease (Miao *et al.*, 2019; Quan *et al.*, 2018; Rovira *et al.*, 2015). Clearly, therapeutic intervention for these patients needs further improvement. Cell immunotherapy, especially Chimeric Antigen Receptor T (CAR-T) therapy, has recently advanced rapidly for clinical treatment; however, the efficacy of CAR-T therapy on DLBCL is not as good as in acute lymphoblastic leukemia due to the tumor immune micro-environment barrier (Batlevi *et al.*, 2016). Further understanding the pathogenesis of DLBCL and how malignant lymphoma cells escape the immune surveillance might provide a basis for future rational combinatorial treatment approaches.

SENEX, based on the Latin for "old man", is a new gene associated with cellular senescence that was identified and cloned successfully in 2004. It is located at 4q31.23, with a total length of 2901 bp, and encodes a protein (Named ARHGAP18 in the RefSeq system) of approximately 75 kD. ARHGAP18 (encoded by SENEX gene) has RhoGAP determinants and belongs to the GTPase activating protein (GAP) family (Calvisi *et al.*, 2011; Coleman *et al.*, 2010; Dransart *et al.*, 2005; Katoh and Katoh, 2004; Sahai and Marshall, 2002). Analysis of Panel expression profile shows that SENEX gene expresses in different kinds of tissues such as kidney, spleen, brain, and even in peripheral blood leukocytes (Sahai and Marshall, 2002). The gene functions as an important regulator of cell shape, spreading, migration and angiogenesis, and has been demonstrated to be involved in cell senescence, cell cycle, and even in cancer (Humphries *et al.*, 2017; Li *et al.*, 2018; Sahai and Marshall, 2002). A previous study has shown the expression level of the SENEX gene in gastric tumor tissue was lower than that of adjacent normal tissue, and SENEX acted as a tumor suppressor by restraining the over-activation of MAPK signaling pathways in gastric cancer (Li *et al.*, 2018). While in breast cancer and mammary tumors, the SENEX gene was over-expressed (Kim *et al.*, 2011). In hematological malignancies, research on SENEX were very few. Our previous study found ARHGAP18 was significantly increased in hydrogen peroxide-induced senescent DLBCL cells, and the p16

*Address correspondence to: Zhimin Zhai, zzzm889@163.com

[#]These two authors contributed equally to this study

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pathway was activated in senescent DLBCL cells (Wang *et al.*, 2019a). However, *SENEX* gene expression, role, and related molecular mechanism in DLBCL tumorigenesis and development are unknown.

Retinoblastoma (Rb) is the first identified tumor suppressor. The loss of it generally promotes tumor development and progression (Hutcheson *et al.*, 2015). It is a regulator of cellular proliferation, and the G1-to-S cell cycle progression is regulated by the Rb/E2F pathway. Phosphorylation (inactivation) of the Rb protein results in the release of the transcription factor E2F1 and will activate S-phase entry (Yaswen *et al.*, 2015). Decreased expression of Rb has been found in DLBCL (Monti *et al.*, 2012), and *SENEX* gene can activate the p16/pRb pathway in endothelial cells (Coleman *et al.*, 2010). However, the role of *SENEX* in DLBCL and the related molecular pathway are unclear and need to be elucidated. In this study, clinical specimens and DLBCL cell line were used to detect *SENEX* expression and the possible molecular pathways.

Materials and Methods

Patients

22 patients diagnosed with DLBCL and 7 healthy volunteers from April 2017 to April 2019 at the Second Hospital of Anhui Medical University, Anhui, China, were enrolled in this study. The detailed criteria for exclusion of patients are listed as follows: (1) Less than 18 years old; (2) Pregnant or lactating women; (3) Congenital/acquired immunodeficiency; (4) Human immunodeficiency virus (HIV) or syphilis; (5) Other hematological or non-hematological tumors; (6) Autoimmune diseases such as systemic lupus erythematosus. According to Chinese guidelines for diagnosis and treatment of DLBCL (2013) (Chinese Society of Hematology CMA and Chinese Society of Lymphoma Ca-CA, 2013), patients were diagnosed and divided into the newly diagnosed group, complete remission group, and relapsed group. The detailed clinical data of the patients were shown in Tab. 1. This study was approved by the Institutional Review Board (IRB) Institutional of the Second Hospital of Anhui Medical University. All patients and healthy volunteers enrolled in the study have signed informed consent.

Cell culture

The Human DLBCL cell line, OCI-LY8 (abbreviation in this article: LY8), was cultured in RPMI-1640 supplemented with 10% FBS. Cell cultures were maintained and incubated at 37°C in humidified air with 5% CO₂.

Immunohistochemistry

6 newly diagnosed and 3 relapsed DLBCL patients were collected and embedded in paraffin for HE-staining and Immunohistochemistry (IHC) analysis. 4 μm thick paraffin sections and monoclonal antibodies against human ARHGAP18 (1:100 dilution) (Tab. 2) were used in the study. The sample sections were deparaffinized in xylene and rehydrated in a series of grades of alcohol. Then pre-treat them in citric acid antigen retrieval solution (pH 6.0). After inhibiting the internal peroxidase activity with 3% hydrogen peroxide, the sections were incubated with an anti-ARHGAP18 antibody at 4°C overnight. Then the slides were incubated with goat anti-rabbit IgG secondary antibody at 37°C for 10 min. Finally, the sections were visualized with DAB solution (DAKO, Carpinteria, USA) and counterstained with hematoxylin (DAKO). The yellow-brown positive staining was mainly located in the nucleus, cytoplasm, and cell membrane. Semi-quantitative methods were used for IHC positive staining scoring: (1) Cell staining intensity: 0 for negative, 1 score for light brown stained cells, 2 scores for brown, 3 scores for dark brown; (2) Selected 10 high-power fields, 400×, and counted the percentage of positive-stained lymphoma cells to the total number of tumor cells: 0 ≤ 5%, 1 score for 5–33%, 2 scores for 34–67%, 3 scores for 68–100%; (3) Assessment: ≤1 was Negative, >1 was Positive. 2–3 scores as Low expression; 4–6 as high expression.

SiRNA synthesis and transfection

The scramble negative control siRNA (NC) and individual small interfering RNA target *SENEX* gene (*SENEX*-siRNA) were synthesized by Sangon (Sangon, Shanghai, China). The final siRNA concentration was 33 nM (Chen *et al.*, 2014). LY8 cells (4×10^5 /well) were transfected with *SENEX*-SiRNA or NC for 12 and 48 h by using Lipofectamine 2000 (Invitrogen, CA, USA). Sequences used for SiRNA transfection have been previously published (Wang *et al.*, 2019a).

Enzyme linked immunosorbent assay (ELISA)

LY8 cells were transfected with *SENEX*-SiRNA or NC for 24 h and then removed precipitation by centrifugation (1500 rpm for 5 min) and store samples at –20°C. Avoid repeated freeze-thaw cycles. The concentration of Lecithin-Cholesterol Acyltransferase (E2F1) was measured using Human E2F Transcription Factor 1 (E2F1) ELISA Kit (OmnimAbs, USA). Use the stock solution (8000 pg/mL) to produce a 2-fold dilution series (including 4000 pg/mL, 2000 pg/mL, 1000 pg/mL, 500 pg/mL and 250 pg/mL).

TABLE 1

Characteristics of healthy control and DLBCL patients

State of disease at sample draw	No. of patients	Average age (range)	Gender distribution (Male/Female)
Healthy Control	7	37.6 (23–63)	2/5
Newly Diagnosed	6	59.3 (47–79)	4/2
Complete Response	7	35.9 (31–66)	5/2
Relapsed	9	60.1 (46–85)	5/4

TABLE 2

Primary antibodies used for Western-blot and Immunohistochemistry

Name	Company	Item No.	Dilution ratio
Rabbit polyclonal to ARHGAP18	Abcam	ab175970	1:1000
Rabbit polyclonal to ARHGAP18	Abcam	ab118585	1:500
P16 INK4A(D7C1M) Rabbit mAb	Cell signaling technology	#80772	1:1000
P21 Waf1/Cip1(12D1) Rabbit mAb	Cell signaling technology	#2947	1:1000
Rb(D20) Rabbit mAb	Cell signaling technology	#9313	1:1000
Phospho-Rb(Ser780)(D59B7) Rabbit mAb	Cell signaling technology	#8180	1:1000
Mouse anti-Actin mAb	ZSGB-BIO	TA-09	1:2000

All Standards and Samples were added in duplicate to the microtiter plate according to standard procedures. And take blank well as zero, measured the optical density (OD) at 450 nm after Adding Stop Solution and within 15 min.

RNA extraction and quantitative real time polymerase chain reaction (qRT-PCR) analysis

Gene expression levels were detected by qRT-PCR. Peripheral blood was collected from DLBCL patients for evaluation of SENEX gene levels via qRT-PCR. Total RNA in peripheral blood was extracted by TRIzol (Invitrogen, Carlsbad, CA, USA). RNA was reverse-transcribed using a Transcript RT Kit (Sangon, Shanghai, China) according to the manufacturer’s protocol. Real-time PCR was performed on the ABI 7500 Real-Time PCR System (Life Technologies, Grand Island, NY, USA) by SYBR Green PCR Master Mix (TaKaRa, Dalian, China). All primers were synthesized by Sangon (Shanghai, China). The relative SENEX expression level was calculated using the $2^{-\Delta\Delta Ct}$ method. Sequences used for qRT-PCR primers were listed in a previous study (Wang et al., 2019a).

Western blot

Protein expression levels were detected by Western blot. Total proteins from Peripheral blood mononuclear cells (PBMCs) and LY8 cells were extracted by Western blot with IP cell lysis liquid (Beyotime, Shanghai, China) in accordance with standard procedures. Primary antibodies used for Western blot are shown in Tab. 2. Proteins were detected with the SuperSignal West Femto Trial Kit (Thermo Fisher Scientific, Shanghai, China) as previously described (Wang et al., 2017).

Proliferation analysis

To further investigate the effect of the SENEX gene on DLBCL cell proliferation, we performed proliferation analysis. LY8 cells transfected with SENEX-SiRNA or NC were plated at a density of 5000 cells/well.

At 12 or 48 h after transfection, cell proliferation was measured with the CCK-8 Kit (BestBio, Shanghai, China). Each assay was performed with 5 replicates in 3 independent experiments.

Cell cycle analysis

The cell cycle distribution was detected at 48 h after transfection with COULTER DNA PREP Reagents kit

(Beckman). Cells were washed with PBS twice, stained with DNA Pre Stain and DNA Prep LPR according to standard procedures. And the cell cycle was detected by flow cytometer FC-500 (Beckman Coulter, Miami, FL).

Statistical analysis

The Student’s *t*-test was employed for analysis of two-sample and two-tailed comparisons by SPSS 16.0 (SPSS Inc., Chicago, IL). Pearson correlation was used to measure the degree of dependence between variables by SPSS 16.0. *p*-values were calculated, and *p* < 0.05 was considered statistically significant.

Results

SENEX expression is significantly increased in relapsed DLBCL patients

To investigate the expression level of SENEX in DLBCL patients, we extracted peripheral blood mononuclear cells (PBMCs) from 7 healthy volunteers, 6 newly diagnosed, 7 in complete remission, and 9 relapsed DLBCL patients. As tumor cells were not available in healthy volunteers and complete response patients, we chose PBMCs for analysis. In addition, the lymph node tissues of 6 newly diagnosed and 3 relapsed DLBCL patients were collected and embedded in paraffin for HE-staining and immunohistochemical analysis. The detailed clinical data of all enrolled patients are shown in Tab. 1.

Total RNA and protein extracted from PBMCs were detected. As shown in Fig. 1A, SENEX mRNA expression level was significantly increased in the PBMCs of relapsed patients with lymphoma cell infiltration in peripheral blood compared to healthy control, newly diagnosed, and complete response patients. Consistent with this, ARHGAP18 expression in the PBMCs of relapsed patients also increased significantly (Fig. 1B). Then, we detected the expression of ARHGAP18 in the lymph node tissues of newly diagnosed and relapsed DLBCL patients. ARHGAP18 expression was significantly increased in the lymphoma cells of relapsed DLBCL patients compared with newly diagnosed patients (Figs. 1C and 1D and Tab. 3).

Furthermore, we found that expressions of p21 and p16 were also significantly increased in PBMCs of relapsed DLBCL patients compared to healthy control, newly diagnosed, and complete response patients (Fig. 1E). On the other hand, the expression level of phosphorylated-RB had an increasing

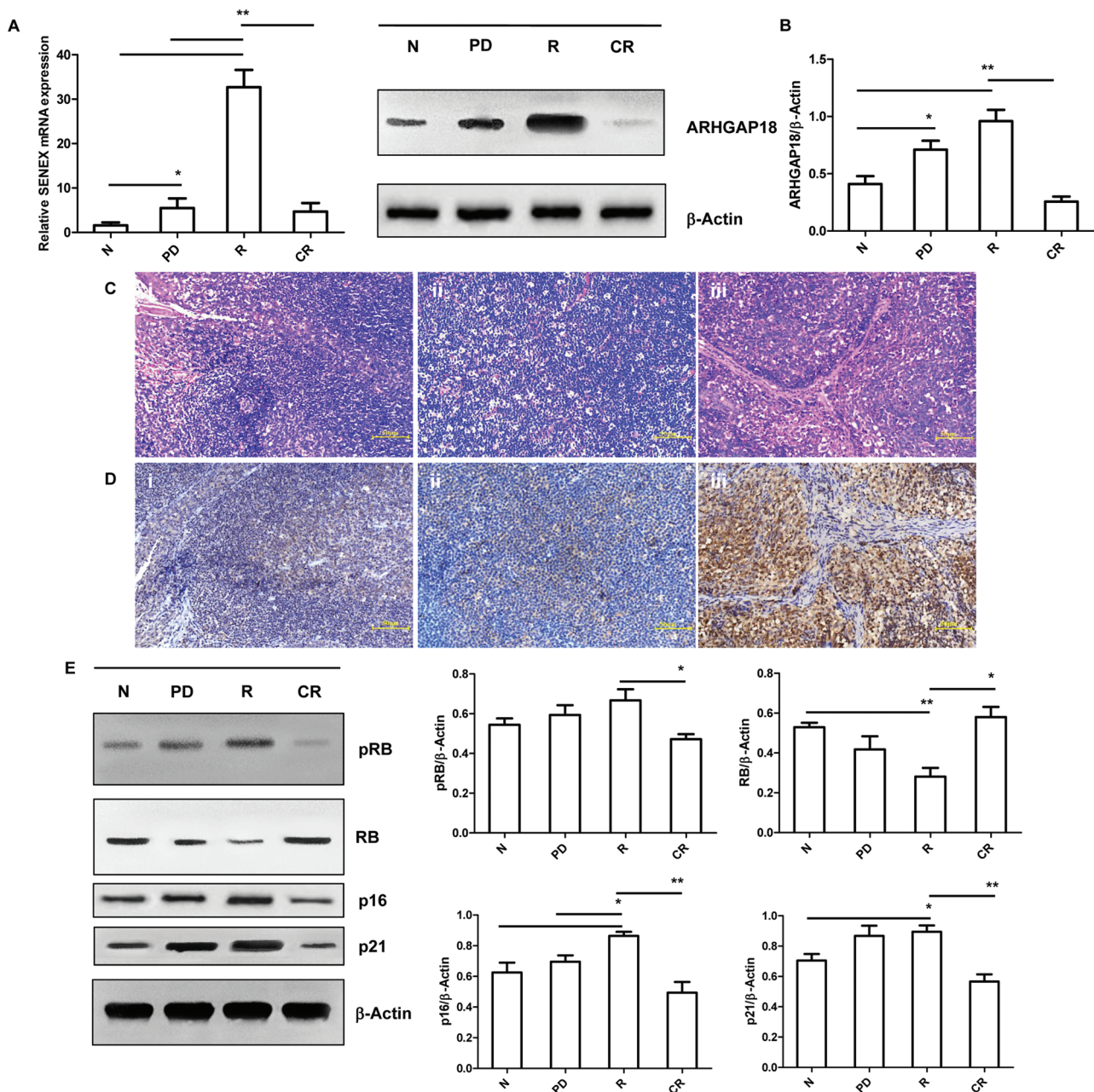


FIGURE 1. *SENEX* expression is significantly increased in relapsed DLBCL patients. Peripheral blood mononuclear cells (PBMCs) were extracted from normal persons (N) (N = 7) and DLBCL patients including Newly Diagnosed (ND) (N = 6), recurrent patients (R) (N = 7) and complete remission patients (CR) (N = 9). Total RNA and protein were extracted from PBMCs for qRT-PCR and Western blot analysis. (A) The *SENEX* mRNA expression was measured by qRT-PCR. (B) The expression of ARHGAP18 protein was measured with Western blot. (C) HE-staining of tonsils in patients with chronic tonsillitis (i) and lymph node tissue in ND (ii) and R (iii) DLBCL patients, 400×, scale bar = 50 μm. (D) Typical immunohistochemical staining images of tonsils in patients with chronic tonsillitis (i) and lymph node tissue in ND (ii) and R (iii) DLBCL patients, 400×, scale bar = 50 μm. (E) The expression of pRB/Rb and p16/p21 in PBMCs of DLBCL patients and healthy volunteers were measured with Western-blot. * $p < 0.05$, ** $p < 0.01$.

trend, but the expression of Rb was significantly decreased in relapsed DLBCL patients compared to healthy control, newly diagnosed, and complete response patients (Fig. 1E). These results indicate that *SENEX* expression is significantly increased in relapsed DLBCL patients, and p21, p16, and Rb, were also activated. The overexpressed *SENEX* gene is closely related to the relapse of DLBCL.

Silencing of the *SENEX* gene

In this study, LY8 cells were transfected with the individual small interfering RNA (*SENEX*-siRNA, SS-RNA) to silence

SENEX gene, and the scramble negative siRNA (NC) group was set as control. Next, the efficiency of transfection both in genetic and protein levels was verified. Expression of *SENEX* mRNA was significantly decreased in the *SENEX*-siRNA group compared with the control group (LY8 cells without any treatment) and the NC group (Fig. 2A). And ARHGAP18 expression level was also obviously reduced in the *SENEX*-siRNA group compared to the control and NC groups (Fig. 2B). These results suggested that transfection with *SENEX*-siRNA can inhibit the expression *SENEX* gene in DLBCL cells. On this basis, we investigated the effect of

TABLE 3

Immunohistochemical score of 9 DLBCL patients

Pathology No.	Group	Expression of ARHGAP18	Staining strength	Percentage of positive cells in total tumor cells	Grade
1907651	ND	50% Cytoplasmic weakly Positive	1	2	Low
1911050	ND	50% Cytoplasmic/membranous weakly Positive	1	2	Low
1917432	ND	55% Cytoplasmic/Nuclear Moderate Positive	2	2	High
1935055	ND	Negative	0	0	Negative
G200244	ND	50% Cytoplasmic/membranous Moderate Positive	2	2	High
G200254	ND	30% Cytoplasmic weakly Positive	1	1	Low
1912253	r/r	80% Cytoplasmic weakly Positive	1	3	High
1917203	r/r	50% Cytoplasmic Moderate Positive	2	2	High
HT20002	r/r	95% Nuclear strongly Positive	3	3	High
Tonsil	Chronic tonsillitis	40% Nuclear weakly Positive	1	1	Low
Tonsil	Chronic tonsillitis	40% Nuclear weakly Positive	1	1	Low
Tonsil	Chronic tonsillitis	30% Cytoplasmic/membranous Moderate Positive	1	1	Low

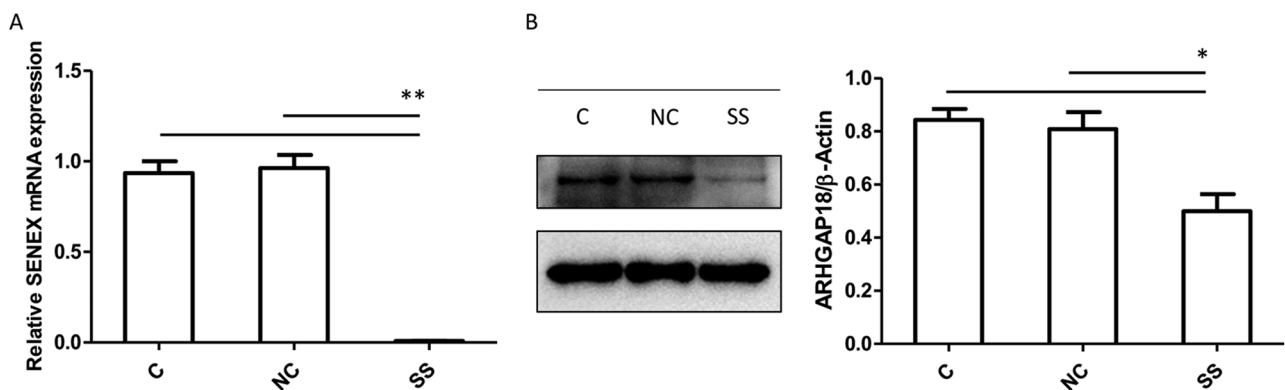


FIGURE 2. Transfection with *SENEX*-SiRNA significantly suppresses the expression of *SENEX* in LY8 cells. LY8 cells were transfected with *SENEX*-SiRNA or NC, and then total RNA and protein were extracted for qRT-PCR and Western blot analysis. (A) The expression of *SENEX* mRNA was measured by qRT-PCR. (B) The expression of ARHGAP18 was measured by Western blot. C represented unprocessed LY8 cells; NC represented LY8 cells transfected with NC; SS represented LY8 cells transfected with *SENEX*-siRNA. * $p < 0.05$, ** $p < 0.01$.

intervening *SENEX* gene on proliferation and related pathways in DLBCL cells.

SENEX promotes the proliferation of DLBCL cells in vitro

To further investigate the function of *SENEX* gene in DLBCL, we transfected the LY8 cell line with *SENEX*-SiRNA or NC (as a control), and then analyzed the cell morphology and proliferation. Compared with the control and NC groups, the number of LY8 cells after transfection was reduced (Figs. 3A–3C).

Cell proliferation was detected at 12 and 48 h after transfection. Consistent with the above result, cell proliferation was decreased in the *SENEX*-SiRNA group when compared with the unprocessed group and the NC group (Fig. 3D). In addition, cell proliferation in the *SENEX*-SiRNA group was more markedly decreased than in the unprocessed group and the NC group at 48 h after transfection (Fig. 3E). Thus, these results suggest that *SENEX* promotes the proliferation of DLBCL cells *in vitro*.

As compared with the control and NC group cells, cells transfected with *SENEX*-SiRNA were arrested at the G1 phase, and the percentage of cells in the G2 phase was decreased in the SS group (C: 28.32%, NC: 28.17%, SS: 21.28%) ($p < 0.01$) (Fig. 4). The percentage of cells in the G2/M phase was also significantly decreased in the SS group compared to the control and NC groups ($p < 0.01$) (Fig. 4). These results indicated that inhibiting the expression of *SENEX* arrested the G1 phase, resulting in inhibition of cell growth.

SENEX promotes RB/E2F1 activation in DLBCL cells

The E2F transcription factor is thought to regulate the transition of the cell cycle from G to S by interacting with various cell cycle-dependent proteins and kinases such as RB/pRB (van den Heuvel and Dyson, 2008). RB/pRB protein is one of the major constituent proteins of human E2F regulatory protein, and E2F regulatory protein can regulate the activity of the E2F transcription factor (van den Heuvel and Dyson, 2008). To further explore the effect of

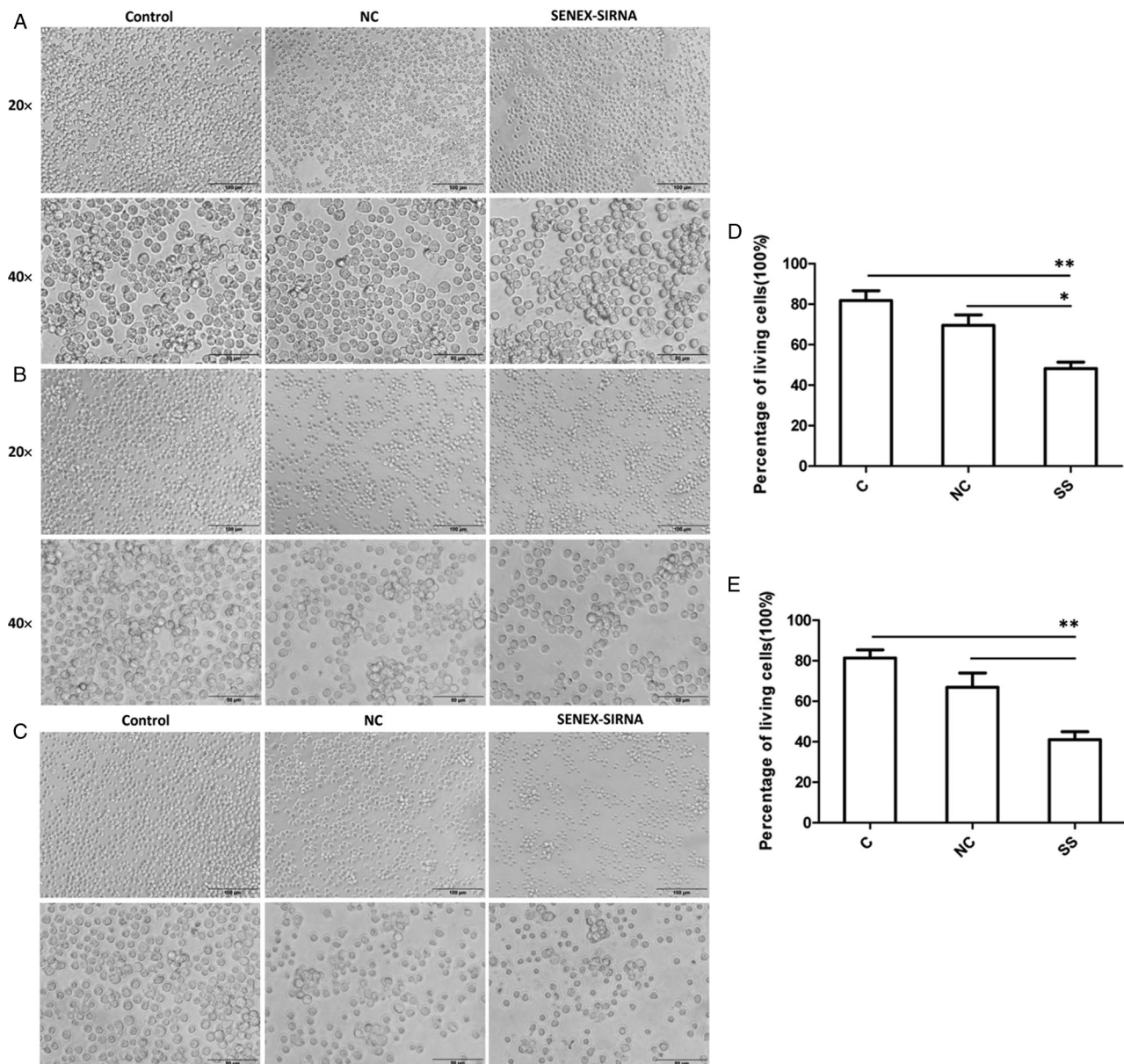


FIGURE 3. Transfection with *SENEX*-SiRNA significantly decreases cell proliferation rates in LY8 cells at 12 and 48 h. LY8 cells were transfected with *SENEX*-SiRNA or NC. (A) Cell morphology before transfection was observed by a microscope at the amplification of 10 \times , 25 \times , and 40 \times . (B) Cell morphology at 12 h after transfection was observed by a microscope at the amplification of 10 \times , 25 \times , and 40 \times . (C) Cell morphology at 48 h after transfection was observed by a microscope at the amplification of 10 \times , 25 \times , and 40 \times . (D) Cell proliferation rate at 12 h after transfection was measured by CCK8 proliferation analysis. (E) Cell proliferation rate at 48 h after transfection was measured by CCK8 proliferation analysis. Each assay was performed with 5 replicates in 3 independent experiments. C represented unprocessed LY8 cells; NC represented LY8 cells transfected with NC; SS represented LY8 cells transfected with *SENEX*-siRNA. * $p < 0.05$, ** $p < 0.01$.

SENEX in DLBCL cell proliferation, RB/E2F1 pathway was analyzed. The concentration of E2F1 was significantly decreased in the *SENEX*-SiRNA group as compared to the NC group (Fig. 5A). Expressions of p21, p16, and phosphorylated-RB were significantly reduced in the *SENEX*-SiRNA group compared to the control and NC groups (Fig. 5B). These results suggest that *SENEX* promotes RB/E2F1 activation in DLBCL cells.

Discussion

Tumor immune escape is a key factor in tumorigenesis and progression of malignant tumors (Pan *et al.*, 2014; Tao *et al.*, 2015; Wang *et al.*, 2019b). DLBCL is a genetically and clinically heterogeneous disorder, and the efficacy of CAR-T

immunotherapy in DLBCL is not as powerful as in acute leukemia, suggesting that DLBCL has a more rigorous and complex ability to evade immune destruction (Quan *et al.*, 2018). DLBCL lymphoma cells escaping the immune system is a pivotal problem and needs to be clarified and solved urgently (Li *et al.*, 2014). Multiple genes and molecular pathways may involve in this complicated progress. *SENEX*, a new gene associated with cellular senescence, was found to regulate cell spreading, migration in the life cycle of a cell and may be involved in the growth and metastasis of tumors (Humphries *et al.*, 2017; Li *et al.*, 2018). Our previous study indicated that increased *SENEX* expression promoted tumorigenesis and metastasis of urinary bladder

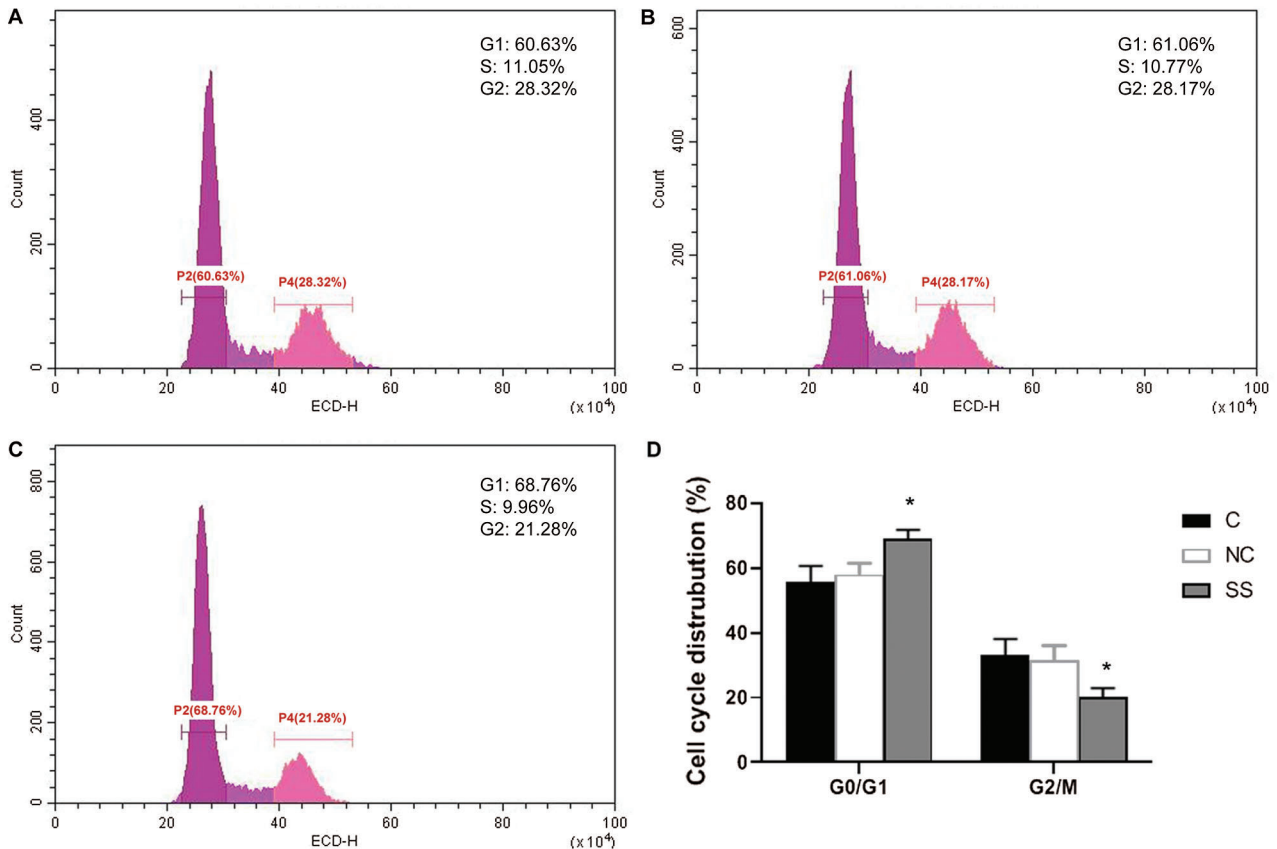


FIGURE 4. Effect of *SENEX*-SiRNA on the cell cycle distribution of LY8 cells. (A) Cell cycle distribution of the control group was measured with FCM analysis. (B) Cell cycle distribution of the NC group was measured with FCM analysis. (C) Cell cycle distribution of SS group was measured with FCM analysis. (D) Effect of *SENEX*-SiRNA on the cell cycle distribution of LY8 cells. Each assay was performed in 3 independent experiments. C represented unprocessed LY8 cells; NC represented LY8 cells transfected with NC; SS represented LY8 cells transfected with *SENEX*-siRNA. **p* < 0.05, compared with C and NC.

cancer (Chen *et al.*, 2014). Moreover, we found that *SENEX* activates the p16 pathway in hydrogen peroxide-induced senescent DLBCL cells (Wang *et al.*, 2019a). However, the expression, role, and related molecular mechanisms of *SENEX* in DLBCL tumorigenesis and development have not been precisely described to date.

In the present study, we found that the expression level of *SENEX* gene and protein in the PBMC of newly diagnostic DLBCL patients was higher than that of normal controls, it decreased when the patients reached complete remission. However, while the tumor relapsed, *SENEX* expression increased significantly. Besides, ARHGAP18 expression in lymphoma cells in lymph nodes of relapsed DLBCL patients was increased than that of newly diagnosed patients, indicating that *SENEX* might play a role during this process, especially at relapse of DLBCL. Therefore, the function of *SENEX* needed to be further explored in DLBCL at the cellular level. OCI-LY8 cells, a human diffuse large B-cell lymphoma cell line was used for further studies. Following transfection of OCI-LY8 cells with *SENEX*-SiRNA, RT-PCR, and Western blot detections found that *SENEX* expression was highly decreased than that of the control and NC groups. Moreover, cellular proliferative activity and the percentage of cells in the G2/M phase were apparently decreased in the *SENEX*-siRNA group compared with another two groups at different times after transfection, indicating *SENEX* can also promote the proliferation of

DLBCL at the cellular level. The related role and possible molecular pathway in this process at this point need to be further investigated.

Studies (Hutcheson *et al.*, 2015; Shats *et al.*, 2017) have shown that Rb plays an important role in controlling cell cycle progression as well as cell fate decisions. It was the first identified tumor suppressor in view of germline predisposition to the pediatric eye tumor (Hutcheson *et al.*, 2015). Although Rb can act as a transcriptional activator or repressor, the function of Rb is usually attributed to its ability to inhibit transcription or regulate cell cycle progression. In this regard, the interaction between Rb and the E2F transcription factor family is a typical example of Rb function. Generally, in the process of normal cell differentiation and proliferation, cyclin D1 and CDK4/6 form a complex, which makes Rb protein phosphorylation, and then relieves the effect of Rb protein. Phosphorylated Rb can dissociate the crucial transcription factor E2F1, then activate important cyclin E and cyclin A, and last initiate DNA replication and proliferation. However, dephosphorylated Rb can directly mask the transcriptional activation domain of E2F by interacting with these proteins and indirectly by recruiting additional co-repressors and lead to cell growth arrest. Dysfunction of the Rb/E2F network is a common event in many human malignancies (Hutcheson *et al.*, 2015; Shats *et al.*, 2017). In our study, we found the expression level of pRb was significantly increased

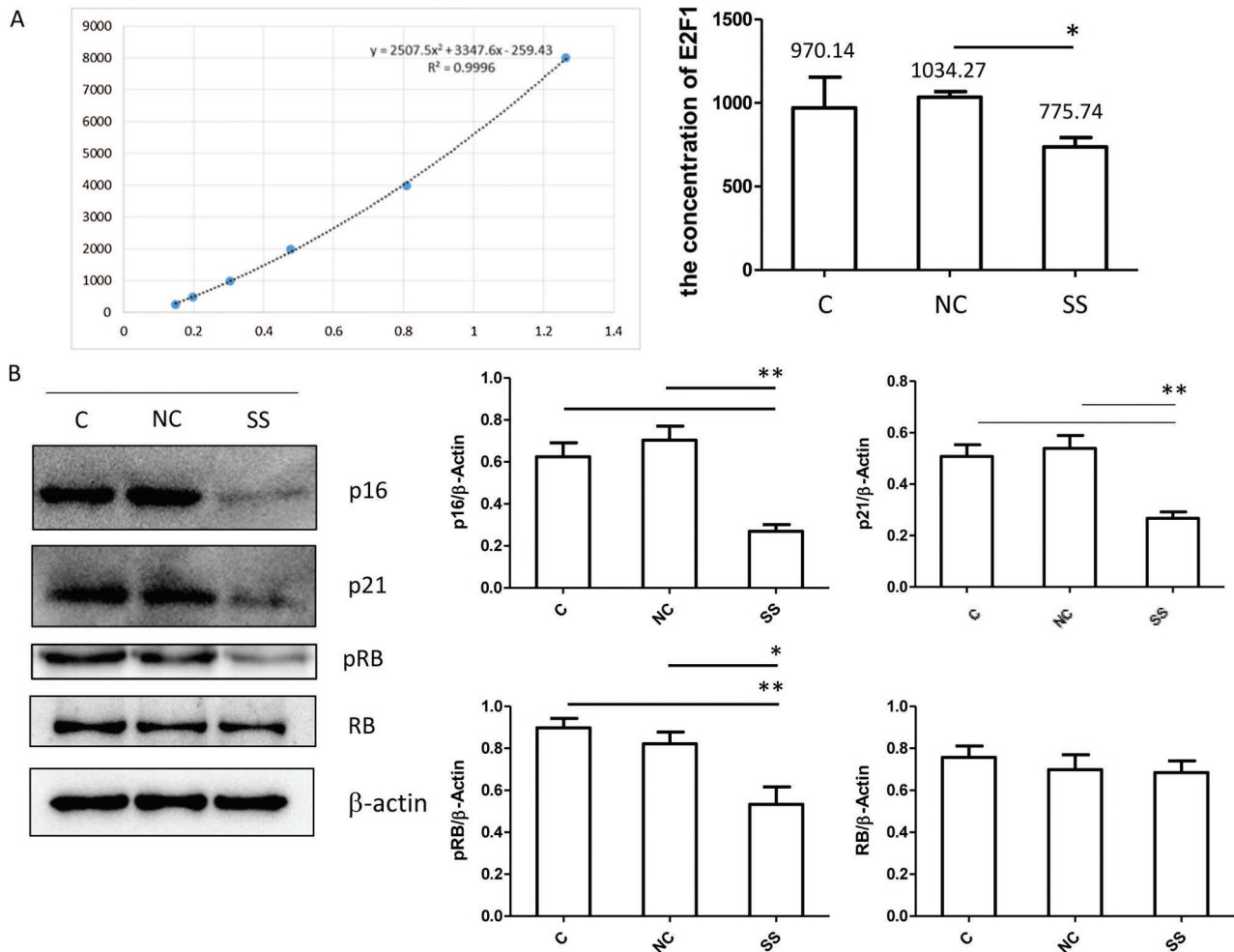


FIGURE 5. Transfection with *SENEX*-siRNA significantly inhibits RB/E2F1 activation in LY8 cells. LY8 cells were transfected with *SENEX*-SiRNA or NC.

(A) The concentration of E2F1 was measured by ELISA. Standard curve of ELISA, Y-axis represented mean absorbance for each standard; X-axis represented the concentration. (B) Expressions of the p16/Rb pathway at 48 h after transfection were measured with Western blot. C represented unprocessed LY8 cells; NC represented LY8 cells transfected with NC; SS represented LY8 cells transfected with *SENEX*-siRNA. * $p < 0.05$, ** $p < 0.01$.

in relapsed DLBCL patients as compared with healthy volunteers, newly diagnosed, and complete response DLBCL patients. However, Rb expression showed opposite trends. Next, we investigated the effect of *SENEX* on pRb/Rb and E2F1 expressions in DLBCL cells. After transfection with *SENEX*-SiRNA in LY8 cells, there is a significantly reduced trend of pRb level in the SS group than in the control. The concentration of free E2F1 was significantly decreased in the *SENEX*-SiRNA group compared to controls. These results indicate that *SENEX*-induced Rb/E2F1 activation may play a vital role in the proliferation and relapse of DLBCL. Besides, the role of CDK in cell proliferation is regulated by CDK inhibitors (CKIs), while p16 and p21 are two of the CKIs. In endothelial cells, overexpression of *SENEX* resulted in an increase of p16, but no significant change of p21 (Coleman *et al.*, 2010). In this study, we also detected p16 and p21 expression levels, and we found p16 and p21 were also activated in relapsed DLBCL patients.

There are still some limitations of this study. First, in *in vitro* assays, we used the LY8 cell, and this type of lymphoma cell is derived from germinal cancer and may not represent the

nongerminal center source lymphoma. Next, the sample size of patients and lymph nodes in this study was limited. Last, how *SENEX* regulates Rb/E2F1 pathway in DLBCL was not included in this study. A previous study has shown that *SENEX* can induce stress-induced senescence and specific senescence-associated secretory phenotype (SASP), making endothelial cells escape from immune attack and obtain the ability to resist apoptosis (Coleman *et al.*, 2010). Whether this mechanism also exists in DLBCL is unknown. In our future research, we will also try to clarify the mechanism, and we will also expand the sample size and use more DLBCL cell lines to validate our findings in this study.

In conclusion, our study provides the first evidence that *SENEX* is essential for the proliferation and relapse of DLBCL. Clinically, overexpression of *SENEX* is associated with the relapse of DLBCL. At the cellular level, our study demonstrates that the *SENEX* gene promotes cell proliferation in DLBCL cells via activating the Rb/E2F1 pathway. Increased *SENEX* expression level affects the relapse of DLBCL and may serve as an important target for DLBCL therapy.

Ethics Statement: This studies was conducted ethically in accordance with the World Medical Association Declaration of Helsinki, and approved by the Ethics Committee of the Second Hospital of Anhui Medical University. All patients enrolled in the study have signed informed consent.

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author's Contributions: Z Zhai designed this research; J Wang, Z Wanyan, and Y Wan performed experiments; J Wang and Y Pan contributed to the writing of the manuscript; Y Wan analyzed all the immunohistochemistry results. Z Wang and Q Tao collected clinical specimens; J Wang analyzed the data.

Ethics Approval: This study was approved by the Institutional Review Board (IRB) Institutional of the Second Hospital of Anhui Medical University (LLSC20160082). All patients enrolled in the study have signed informed consent.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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