Knockdown of the long non-coding RNA CACNA1G-AS1 enhances cytotoxicity and apoptosis of human diffuse large B cell lymphoma by regulating miR-3160-5p

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Abstract. Long non-coding RNAs (lncRNAs) have been confirmed to be connected with tumor proliferation, apoptosis, metastasis and recurrence. Previous studies have indicated that lncRNA calcium voltage-gated channel subunit α1 G (CACNA1G)-antisense 1 (AS1) can function as a pro-oncogene in several types of cancer. However, the specific role and mechanism of CACNA1G-AS1 have not been fully elucidated in human diffuse large B cell lymphoma (DLBCL). In the present study, CACNA1G-AS1 expression was verified in DLBCL tissues and cells by reverse transcription-quantitative PCR, and the relationship between CACNA1G-AS1 and microRNA (miR)-3160-5p was confirmed using luciferase reporter assays. After CACNA1G-AS1-knockdown and miR-3160-5p-overexpression, MTT, colony formation and flow cytometry assays were conducted to assess the changes in the cytotoxicity and apoptosis of OCI-Ly10 and SUDHL-4 cells. In addition, *in vivo* experiments were performed to determine the impact of CACNA1G-AS1-knockdown on tumor growth and apoptosis. It was revealed that CACNA1G-AS1 was highly expressed in DLBCL tissues and cells and that expression of CACNA1G-AS1 was associated with the clinical stage of DLBCL. Functionally, CACNA1G-AS1-knockdown was demonstrated to increase cytotoxicity and expedite apoptosis in DLBCL cells *in vitro* and *in vivo*. In addition, CACNA1G-AS1 could downregulate miR-3160-5p by targeting binding in DLBCL cells. Overexpression of miR-3160-5p had the same effects on the cytotoxicity and apoptosis of DLBCL cells as CACNA1G-AS1-knockdown. Overall, the present study revealed that CACNA1G-AS1-knockdown and miR-3160-5p-overexpression could prevent DLBCL carcinogenesis, which might provide novel therapeutic targets for DLBCL.

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Introduction

Lymphoma is characterized by a malignant tumor of the immune system that originates from lymph nodes and/or lymphoid tissues (1). Among them, Hodgkin's lymphoma and non-Hodgkin's lymphoma (NHL) are the most common malignant lymphomas in adults (2). NHL is a group of lymphatic proliferative diseases with high heterogeneity, mainly occurring in the lymph nodes, spleen, thymus and other lymphatic organs (3). Diffuse large B cell lymphoma (DLBCL) is the most frequent subtype of aggressive NHL (4,5). Statistically, DLBCL accounts for 30-40% of all newly diagnosed cases of B cell NHL (6,7). Even with the extensive application of rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone (R-CHOP) treatments in DLBCL, >30% of patients with DLBCL are still at risk of recurrence (8,9). Therefore, the discovery of new therapeutic targets is still needed to enhance the survival rate of patients with DLBCL.

Long non-coding RNAs (lncRNAs) are RNAs with a length >200 bp that are derived from RNAs catalyzed by RNA polymerase II and cannot be translated into proteins (10). Emerging studies have revealed that lncRNAs are important in biological processes such as gene expression regulation, embryonic development, cellular structural integrity, the cell cycle, stem cell pluripotency and the heat shock response (11-13). Notably, studies have indicated that lncRNAs are associated with the occurrence, development and metastasis of multiple types of cancer, including DLBCL (14-16). According to the literature, the lncRNAs that promote DLBCL progression mainly include lncRNA taurine upregulated gene 1 (17), metastasis associated

lung adenocarcinoma transcript 1 (18), functional intergenic repeating RNA element (19) and small nucleolar RNA host genes 14 (20) and 16 (21); while the lncRNAs that prevent DLBCL progression mainly include SMAD5-antisense 1 (AS1) (22), long intergenic non-coding RNA-p21 (23) and NONHSAG026900 (24).

Calcium voltage-gated channel subunit α1 G (CACNA1G)-AS1 is a member of the lncRNA family and was first discovered in a keloid study (25). An increasing number of studies have also indicated that CACNA1G-AS1 plays notable roles in inducing cancer progression (26-28). However, the role of CACNA1G-AS1 in the pathogenesis and progression of DLBCL has not been previously described. Therefore, it is important to determine the mechanism and function of CACNA1G-AS1 in DLBCL.

MicroRNAs (miRs) are small non-coding RNAs that can regulate gene expression by targeting the 3'-untranslated region of mRNAs (29). miRs have been reported to be associated with cell proliferation, differentiation, metabolism, death and cancer progression (30,31). It has been revealed that lncRNAs can bind to miRs through 'molecular sponges' to prevent the regulatory effect of miRs on downstream target genes (32).

Our preliminary experiments using bioinformatics analysis revealed that miR-3160-5p and miR-4739 have targeted binding sites with CACNA1G-AS1. Thus, it was hypothesized that CACNA1G-AS1 may act on miR-3160-5p and miR-4739. However, it is not clear whether CACNA1G-AS1 can affect DLBCL progression by targeting miR-3160-5p and miR-4739. Therefore, the purpose of the present study was to investigate whether there were direct interactions between CACNA1G-AS1 and miRs (miR-3160-5p and miR-4739) and how these interactions affected CACNA1G-AS1 progression.

Materials and methods

Tissue samples. DLBCL and normal B lymphocyte (normal) samples were collected from 30 patients with DLBCL and 30 healthy control volunteers, respectively, who were admitted to The Affiliated Hospital of Qingdao University (Qingdao, China) from September 2018 to May 2019 (mean age, 65.6 years; age range, 45-78-years; male to female ratio, 1.3:1). Patients were selected if they met the following inclusion criteria: i) Confirmed histopathological diagnosis of DLBCL cases; ii) had not received chemoradiotherapy or other forms of treatment before surgery; and iii) voluntarily provided samples with complete information.

The specimens were immediately frozen in liquid nitrogen and then transferred to a -80˚C freezer for preservation until use. The present study was approved by The Ethics Committee of The Affiliated Hospital of Qingdao University (approval no. bc2020012). All participants provided written informed consent.

Cell lines. GM12878 cells, OCI-Ly7 cells, OCI-Ly10 cells and SUDHL-4 cells were obtained from American Type Culture Collection. GM12878 cells and SUDHL-4 cells were grown in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.); OCI-Ly10 cells were grown in Iscove's modified Dulbecco's medium (IMDM; Gibco; Thermo Fisher Scientific, Inc.); OCI-Ly7 cells were grown in IMDM with 0.3 g/ml glutamine. All media included 10% fetal bovine serum (HyClone; Cytiva), and all cells were cultured at 37˚C in a 5% CO_2 incubator.

Cell transfection. Small interfering RNA (si-RNA) targeting CACNA1G-AS1 siRNA (si-CACNA1G-AS1) and its non-targeting sequence negative control (si-NC), miRNA mimic negative control (miR control), miR-3160-5p mimic, miR-4739 mimic, miR-3160-5p antisense oligonucleotides (ASO) and ASO-NC were purchased from Suzhou GenePharma Co., Ltd. pGL3/CACNA1G-AS1-wild-type (wt; seed sequence, 5'-CGAAAGA-3') and pGL3/CACNA1G-AS1-mutant (mut; seed sequence, 5'-CTCCTAA-3') constructs were synthesized by Suzhou GenePharma Co., Ltd. DLBCL cells, OCI-Ly10 cells and SUDHL-4 cells were seeded into six-well plates at a density of $3x10^5$ cells/well and were transfected with the respective oligonucleotides using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. After 48 h of transfection at 37˚C, the cells were used in follow-up experiments.

Reverse transcription-quantitative PCR (RT-qPCR) analysis. TRIzol® reagent (Thermo Fisher Scientific, Inc.) was utilized to extract total RNA from sample DLBCL tissues or cells, and the purity of RNAs was identified. Then, total RNA was reverse transcribed into cDNA according to the instructions of the Bestar® qPCR RT kit (DBI Bioscience). The target genes were amplified based on the instructions of the SYBR® Green qPCR kit (Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95˚C for 8 min; denaturation at 95˚C for 25 sec, annealing at 60˚C for 30 sec; extension at 72˚C for 30 sec; and final extension at 72˚C for 10 min. The $2^{-\Delta\Delta Cq}$ method was applied to calculate relative gene expression (33). Primer sequences are displayed in Table I.

MTT assay. DLBCL cells from each group were harvested, and the concentration was adjusted to $5x10^4$ cells/ml. After seeding cells in a 96-well plate, 20 μl of MTT solution (5 g/l; Beijing Solarbio Science & Technology Co., Ltd.) was added at 0, 24, 48 and 72 h. After 4 h at 37˚C, the product was dissolved in 200 μl of dimethyl sulfoxide and the absorbance at 490 nm was measured using a microplate reader.

Colony formation assay. Transfected OCI-Ly10 and SUDHL-4 cells ($0.5x10^3$ cells/well) in a six-well plate were incubated for 14 days in an incubator at 37˚C on soft agar. Then, the cells were fixed using methanol at room temperature for 15 min and stained using crystal violet at room temperature for 20 min (Sigma-Aldrich; Merck KGaA). The number of colonies containing >50 cells was counted by a light microscope (Nikon Corporation; magnification, x100).

Flow cytometry. Transfected OCI-Ly10 and SUDHL-4 cells were collected, and the concentration was adjusted to $2x10^6$ cells/ml. After centrifugation, the cell pellet was resuspended in 100 μl of PBS binding buffer (BioVision, Inc.). Subsequently, 5 μl of propidium iodide and 5 μl of Annexin

Table I. Primer sequences used in reverse transcription-quantitative PCR analysis.

Gene	Primer sequences (5'-3')
GAPDH	
Forward	TATGATGATATCAAGAGGGTAGT
Reverse	TGTATCCAAACTCATTGTCATAC
CACNA1G-AS1	
Forward	TGTGCTTCACCATGCTCCAT
Reverse	ATTAGTGCTCCGGCCAACAA
miR-3160-5p	
Forward	ACACTCCAGCTGGGGGCTTTCTAGTCTC
Reverse	CTCAACTGGTGTCGTGGA
U6	
Forward	CGCTTCACGAATTTGCGTGTCAT
Reverse	GCTTCGGCAGCACATATACTAAAAT

CACNA1G-AS1, calcium voltage-gated channel subunit α1 G-antisense 1; miR, microRNA.

V-FITC were added for 20 min in the dark according to the instructions of the FITC Annexin V/PI Apoptosis Detection kit I (Guangzhou RiboBio Co., Ltd.). Flow cytometry (CytoFLEX; Beckman Coulter, Inc.) analysis was performed using FlowJo 10 software (FlowJo LLC).

Western blotting. Total protein was extracted from transfected DLBCL cells using RIPA lysis buffer (cat no. P0013B, Beyotime Institute of Biotechnology). The protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific Inc.). After quantification, the protein (40 μg/lane) in each group was separated by 10% SDS-PAGE and transferred to PVDF membranes (cat. no. 3010040001; Roche Applied Science). After blocking in 5% non-fat milk at room temperature for 2 h, the membranes were incubated with the corresponding primary antibodies at 4˚C overnight, followed by a donkey anti-rabbit IgG HRP-conjugated secondary antibody (1:1,000, cat. no. ab6802; Abcam) at room temperature for 1 h. Then, the results were visualized using an ECL system (Thermo Fisher Scientific, Inc.) and semi-quantitatively analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc.). The following primary antibodies were used: Proliferating cell nuclear antigen (PCNA; 1:1,000; cat. no. ab18197), Bcl-2 (1:1,000; cat. no. ab194583) and Actin (1:2,000; cat. no. ab8227) (all Abcam).

Tumor formation in nude mice. A total of 10 male BALB/c nude mice (20±2 g, 4 weeks old) BALB/c nude mice were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd. All animals were housed in individual air laminar flow chamber with a humidity of 55-60% at 28˚C on a 12 h light/dark cycle, with access to SPF food and water *ad libitum*. The health and behavior of mice were monitored each day in whole study. The mice were divided into the si-NC group (n=5) and si-CACNA1G-AS1 group (n=5). All animal care and experiments were performed according to the guidelines of the National Institutes of Health and approved by The Animal Ethics Committee of The Affiliated Hospital of Qingdao University (approval no. bc20191015-52). OCI-Ly10 cells (200 μl; 1.5x10^6 cells/ml) transfected with si-NC or si-CACNA1G-AS1 were subcutaneously injected into the right dorsal of the mice. The tumor volume was monitored for 21 days. The weights for the mice were measured every 3 days with the electronic balance and tumor volumes were measured every 3 days with the vernier caliper. After that, the mice were sacrificed by cervical dislocation under sodium pentobarbital anesthesia (50 mg/kg; intraperitoneal injection). Sacrifice was confirmed when the heart and spontaneous breathing stopped and nerve reflexes disappeared. There were no mice mortalities before sacrifice. If mice tumor grew >10% of the original body weight of the animal, with an average tumor diameter of >20-mm in mice, or when the tumor metastasized or rapidly grew to ulceration, causing infection or necrosis, the mice should be euthanized for humane endpoints.

Luciferase reporter assay. Wt and mut CACNA1G-AS1 plasmids (pGL3-CACNA1G-AS1-wt and pGL3-CACNA1G-AS1-mut, respectively) were purchased from Hanbio Biotechnology Co., Ltd. OCI-Ly10 and SUDHL-4 cells (1x10^4 cells/well) were co-transfected with either pGL3-CACNA1G-AS1-WT plasmid (0.5 μg) or pGL3-CACNA1G-AS1-Mut plasmid (0.5 μg) and miR-3160-5p mimic (50 nM, forward 5'-GGCUUUCUAGUC UCAGCUCUCC-3', reverse 5'-AGAGCUGAGACUAGAAAG CCGC-3') or miR control (50 nM, 5'-CGAUCGCAUCAG CAUCGAUUGC-3') using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 24 h, the fluorescence activity was determined using a Dual-Luciferase Assay System (Promega Corporation), and *Renilla* luciferase activity was set as a control.

Bioinformatics. The secondary structure of single stranded RNA was predicted using the RNAalifold web server (http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi). The binding sites and the secondary structures were predicted by RegRNA2 (http://regrna2.mbc.nctu.edu.tw/index.html).

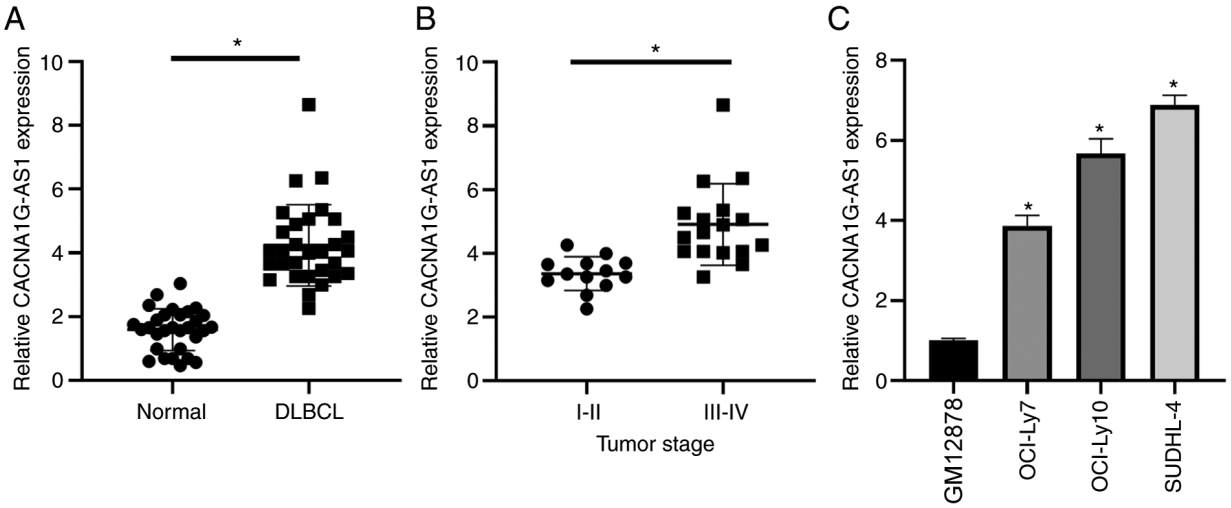

Figure 1. Expression of CACNA1G-AS1 is upregulated in DLBCL. (A) CACNA1G-AS1 expression was assessed in normal and DLBCL tissues using RT-qPCR. (B) Relationship between CACNA1G-AS1 and tumor stage was confirmed using RT-qPCR in I-II stage and III-IV stage DLBCL tissues. (C) Expression levels of CACNA1G-AS1 in B cells and OCI-Ly7, OCI-Ly10 and SUDHL-4 cells were determined by RT-qPCR analysis. *P<0.05 vs. GM12878 or where indicated. CACNA1G-AS1, calcium voltage-gated channel subunit α1 G-antisense 1; DLBCL, diffuse large B cell lymphoma; RT-qPCR, reverse transcription-quantitative PCR.

Statistical analysis. Data were analysed and visualized using SPSS 23.0 (IBM Corp.) and Graphpad Prism 5.0 (GraphPad Software, Inc.). The non-parametric Mann-Whitney U test was used to compare gene expression of lncRNAs between patients with DLBCL and normal controls. A paired t-test was performed to compare the apoptosis rate between two groups. Statistical differences among three groups were tested with one-way analysis of variance test. The Tukey's honest significant difference post hoc test was used for multiple comparisons. A two-tailed P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of CACNA1G-AS1 is upregulated in DLBCL. RT-qPCR analysis revealed that CACNA1G-AS1 expression was significantly elevated in DLBCL tissues compared with normal tissues (P<0.05; Fig. 1A). Simultaneously, CACNA1G-AS1 was revealed to be significantly upregulated in III-IV stage DLBCL tissues compared with I-II stage DLBCL tissues (P<0.05; Fig. 1B). Additionally, the expression levels of CACNA1G-AS1 were significantly increased in OCI-Ly7, OCI-Ly10 and SUDHL-4 cells compared with B cells (P<0.05; Fig. 1C). Thus, these findings confirmed that CACNA1G-AS1 was highly expressed in DLBCL.

Knockdown of CACNA1G-AS1 increases cytotoxicity and induces apoptosis of DLBCL cells. Because CACNA1G-AS1 is highly expressed in DLBCL, it was hypothesized that CACNA1G-AS1-knockdown may prevent DLBCL malignancy. First, CACNA1G-AS1 was silenced using siRNA in OCI-Ly10 and SUDHL-4 cells, and RT-qPCR data demonstrated that CACNA1G-AS1 was significantly downregulated in the si-CACNA1G-AS1 group compared with the si-NC group (P<0.05; Fig. 2A). Next, the cytotoxicity of OCI-Ly10 and SUDHL-4 cells were significantly decreased in the si-CACNA1G-AS1 group compared with the si-NC group (P<0.05; Fig. 2B). Similarly, CACNA1G-AS1-knockdown significantly reduced the colony number of OCI-Ly10 and SUDHL-4 cells compared with the si-NC (P<0.05; Fig. 2C). In addition, CACNA1G-AS1-knockdown significantly increased the apoptosis rates in OCI-Ly10 and SUDHL-4 cells compared with the si-NC groups (P<0.05; Fig. 2D). Overall, CACNA1G-AS1-silencing enhanced cytotoxicity and promoted apoptosis in DLBCL cells.

CACNA1G-AS1 functions as a sponge of miR-3160-5p in DLBCL cells. To identify the potential mechanism of CACNA1G-AS1, its secondary structure was analyzed using the RNAalifold web server (Fig. 3A). Bioinformatics analysis revealed that there were binding sites between miR-3160-5p and -4739 and CACNA1G-AS1; the binding sites in the secondary structures of miR-3160-5p and miR-4739 are also presented (Fig. 3B). To screen the most promising miRs, the changes in the luciferase intensity of CACNA1G-AS1 after miR-3160-5p or -4739-overexpression were monitored. Firstly, transfection success was verified (Fig. 3C). Subsequently, the data confirmed that the luciferase intensity was significantly reduced in the miR-3160-5p group compared with the miRNA control group, indicating that CACNA1G-AS1 could interact with miR-3160-5p (P<0.05; Fig. 3C). However, luciferase intensity in the miR-4739 group had no obvious changes. Furthermore, the interaction between CACNA1G-AS1 and miR-3160-5p was verified by a luciferase reporter assay using CACNA1G-AS1-wt and CACNA1G-AS1-mut. The results indicated that miR-3160-5p significantly reduced the CACNA1G-AS1-wt luciferase intensity in OCI-Ly10 and SUDHL-4 transfected cells (P<0.05; Fig. 3D and E); however, this alteration disappeared in CACNA1G-AS1-mut-transfected cells (P>0.05). The transfection success of miR-3160-5p ASO was verified, as presented in Fig. 3F, and miR-3160-5p ASO decreased the CACNA1G-AS1-wt luciferase intensity in transfected cells. Overall, this indicated that CACNA1G-AS1

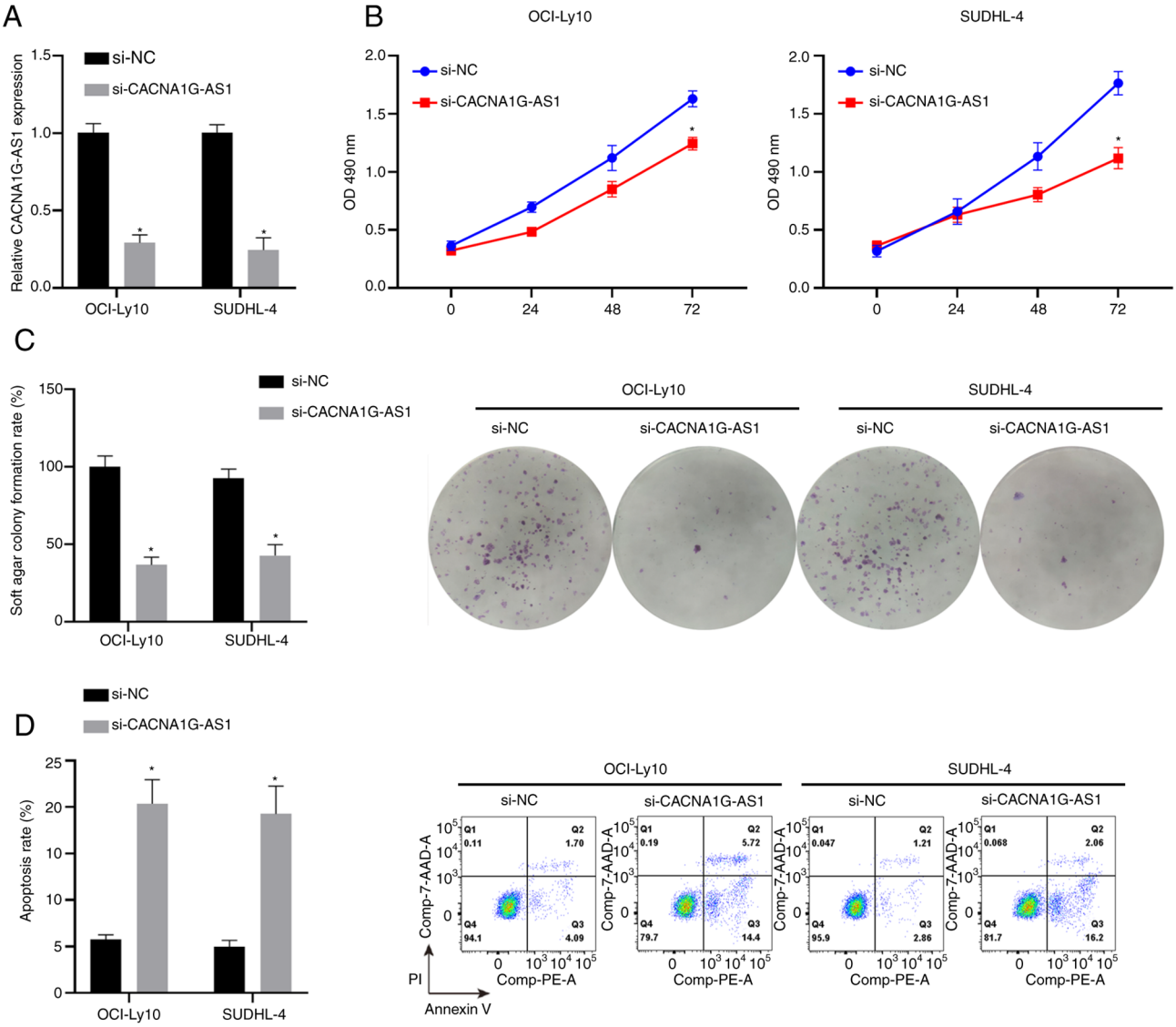

Figure 2. CACNA1G-AS1-knockdown enhances cytotoxicity and induces apoptosis of DLBCL cells. (A) Expression levels of CACNA1G-AS1 were verified by RT-qPCR in OCI-Ly10 and SUDHL-4 cells, which were transfected with si-NC and CACNA1G-AS1 siRNAs, respectively. (B) CACNA1G-AS1-knockdown-mediated cytotoxicity was analyzed by MTT assay in OCI-Ly10 and SUDHL-4 cells. (C) A colony formation assay was utilized to examine the colony number of CACNA1G-AS1-silenced OCI-Ly10 and SUDHL-4 cells. (D) Apoptosis analysis of OCI-Ly10 and SUDHL-4 cells after CACNA1G-AS1-knockdown. *P<0.05 vs. si-NC. CACNA1G-AS1, calcium voltage-gated channel subunit α1 G-antisense 1; DLBCL, diffuse large B cell lymphoma; RT-qPCR, reverse transcription-quantitative PCR; si-, short interfering; NC, negative control; OD, optical density; PI, propidium iodide.

interacted with miR-3160-5p as a competitive endogenous RNA in DLBCL.

miR-3160-5p enhances cytotoxicity and facilitates apoptosis of DLBCL cells. Subsequently, the influence of miR-3160-5p on the cytotoxicity and apoptosis of DLBCL cells was further analyzed. First, miR-3160-5p overexpression was confirmed in OCI-Ly10 and SUDHL-4 cells due to the high level of miR-3160-5p expression in the miR-3160-5p transfection group (P<0.05; Fig. 3C). Secondly, the MTT results revealed that the cytotoxicity of OCI-Ly10 and SUDHL-4 cells was significantly lower in the miR-3160-5p groups compared with the control groups (P<0.05; Fig. 4A). Furthermore, colony formation results revealed that miR-3160-5p also significantly decreased the colony number of transfected OCI-Ly10 and SUDHL-4 cells (P<0.05; Fig. 4B). Next, the apoptosis rates in OCI-Ly10 and SUDHL-4 cells were demonstrated to be significantly elevated in the miR-3160-5p groups compared with the control groups (P<0.05; Fig. 4C). As Fig. 4D depicted, CACNA1G-AS1-silencing reduced the colony formation rate of OCI-Ly10 and SUDHL-4 cells in the si-CACNA1G-AS1 + anti-control group compared with the si-NC + anti-control group; which was then significantly increased by miR-3160-5p overexpression (P<0.05). Compared with si-NC + anti-control, CACNA1G-AS1 depletion markedly elevated the apoptosis rate in both OCI-Ly10 and SUDHL-4 cells; whereas miR-3160-5p overexpression exhibited the opposite impact, evidenced by the significant descrease in apoptosis rate in si-CACNA1G-AS1 + Anti-3160-5p (P<0.05; Fig. 4E). Overall, these data confirmed that CACNA1G-AS1-knockdown enhanced cytotoxicity and induced apoptosis in DLBCL cells via miR-3160-5p.

Silencing CACNA1G-AS1 prevents tumor growth and reduces PCNA and Bcl-2 expression levels in DLBCL tissue. The

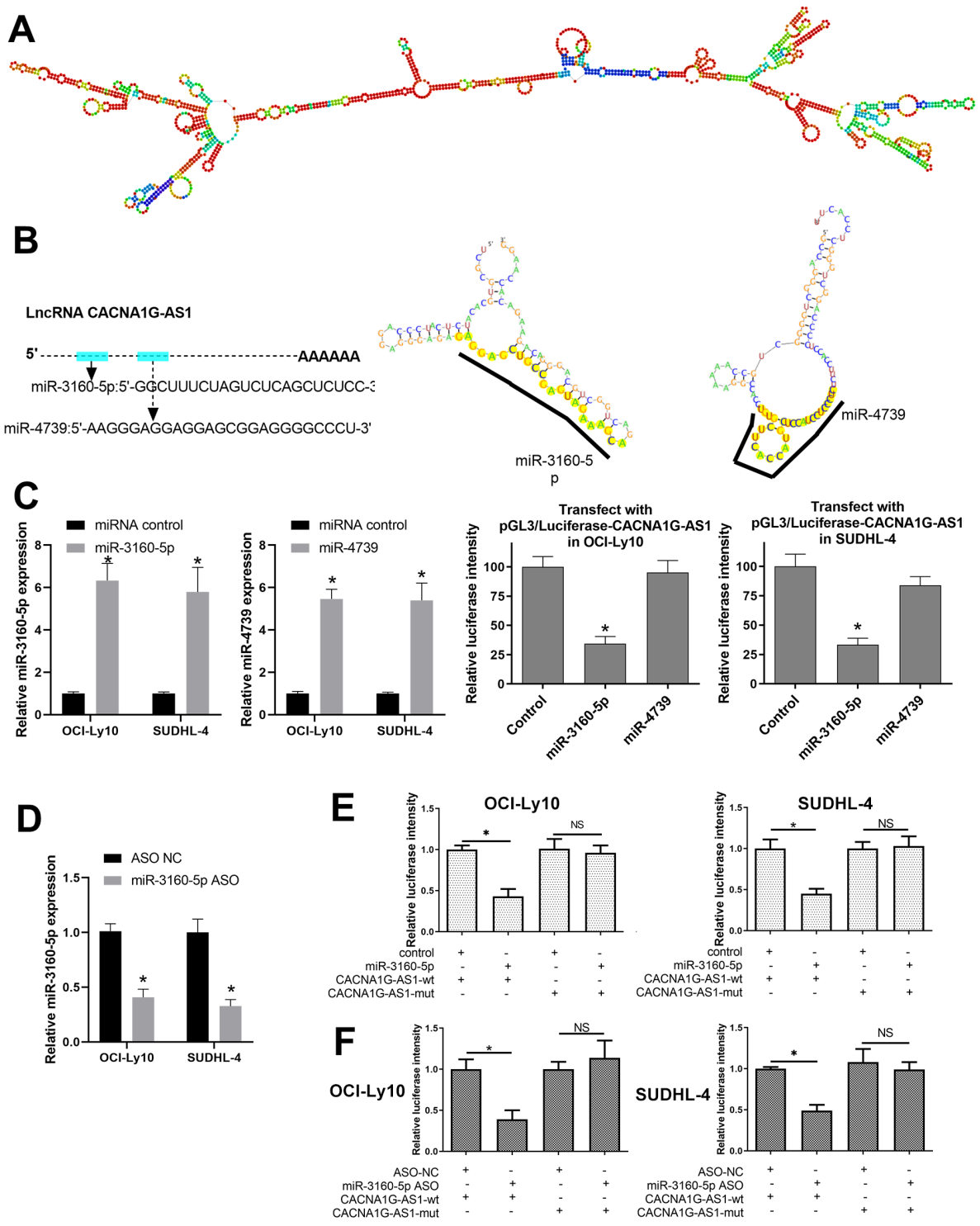

Figure 3. CACNA1G-AS1 functions as a sponge of miR-3160-5p in DLBCL cells. (A) Secondary structure of CACNA1G-AS1 in the RNAalifold web server is shown. (B) Binding sites of CACNA1G-AS1 and miR-3160-5p or miR-4739 are displayed using the secondary structure. (C) Transfection efficiency of miR-3160-5p or miR-4739 were determined using RT-qPCR analysis. The combination of CACNA1G-AS1 and miR-3160-5p or miR-4739 was determined using a luciferase reporter assay. (D) Transfection efficiency of miR-3160-5p ASO was determined using a RT-qPCR analysis. The luciferase intensity of CACNA1G-AS1 was demonstrated in OCI-Ly10 and SUDHL-4 cells after co-transfection with CACNA1G-AS1-wt or CACNA1G-AS1-mut and (E) miR-3160-5p mimic or (F) ASO. *P<0.05 vs. respective control or where indicated. CACNA1G-AS1, calcium voltage-gated channel subunit α1 G-antisense 1; miR, microRNA; DLBCL, diffuse large B cell lymphoma; RT-qPCR, reverse transcription-quantitative PCR; wt, wild-type; mut, mutant; ASO, antisense oligonucleotides; NC, negative control; NS, not significant.

impact of CACNA1G-AS1 on tumor growth and apoptosis *in vivo* was further identified. SUDHL-4 cells transfected with si-NC or si-CACNA1G-AS1 were subcutaneously injected into nude mice, and the tumor volumes were monitored weekly. The data indicated that silencing CACNA1G-AS1 resulted in a significant inhibition of tumor growth (P<0.05; Fig. 5A).

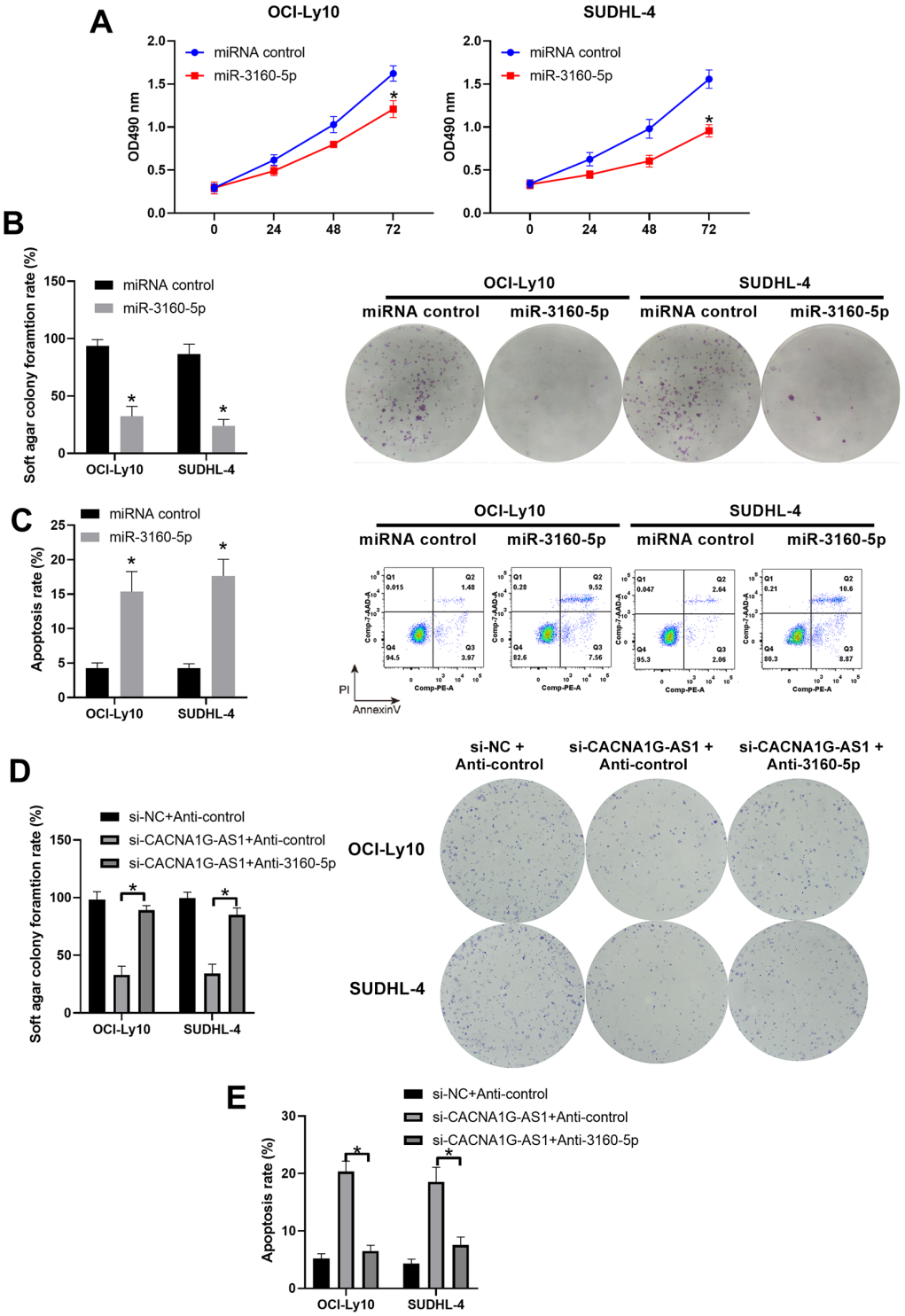

Figure 4. miR-3160-5p enhances cytotoxicity and facilitates apoptosis in DLBCL cells. Changes in the cytotoxicity of OCI-Ly10 and SUDHL-4 cells were confirmed using (A) MTT and (B) colony formation assays after miR-3160-5p-overexpression. (C) Apoptosis rates of miR-3160-5p-overexpressing OCI-Ly10 and SUDHL-4 cells were determined by flow cytometry. (D) Changes in the cytotoxicity of OCI-Ly10 and SUDHL-4 cells were confirmed using colony formation assays after inhibiting miR-3160-5p with CACNA1G-AS1-knockdown. (E) Apoptosis rates of OCI-Ly10 and SUDHL-4 cells were identified by flow cytometry after inhibiting miR-3160-5p with CACNA1G-AS1-knockdown. *P<0.05 vs. miRNA control or where indicated. miR, microRNA; DLBCL, diffuse large B cell lymphoma; CACNA1G-AS1, calcium voltage-gated channel subunit α1 G-antisense 1; si-, short interfering; NC, negative control; OD, optical density; PI, propidium iodide.

The level of CACNA1G-AS1 was also significantly lowered in the si-CACNA1G-AS1 group compared with the si-NC group (P<0.05, Fig. 5B). In addition, the western blotting data revealed that the protein expression levels of PCNA and Bcl-2 were also significantly attenuated in the si-CACNA1G-AS1 groups compared with the si-NC groups (P<0.05; Fig. 5C and D). Overall, this revealed that CACNA1G-AS1-knockdown also prevented DLBCL progression *in vivo*.

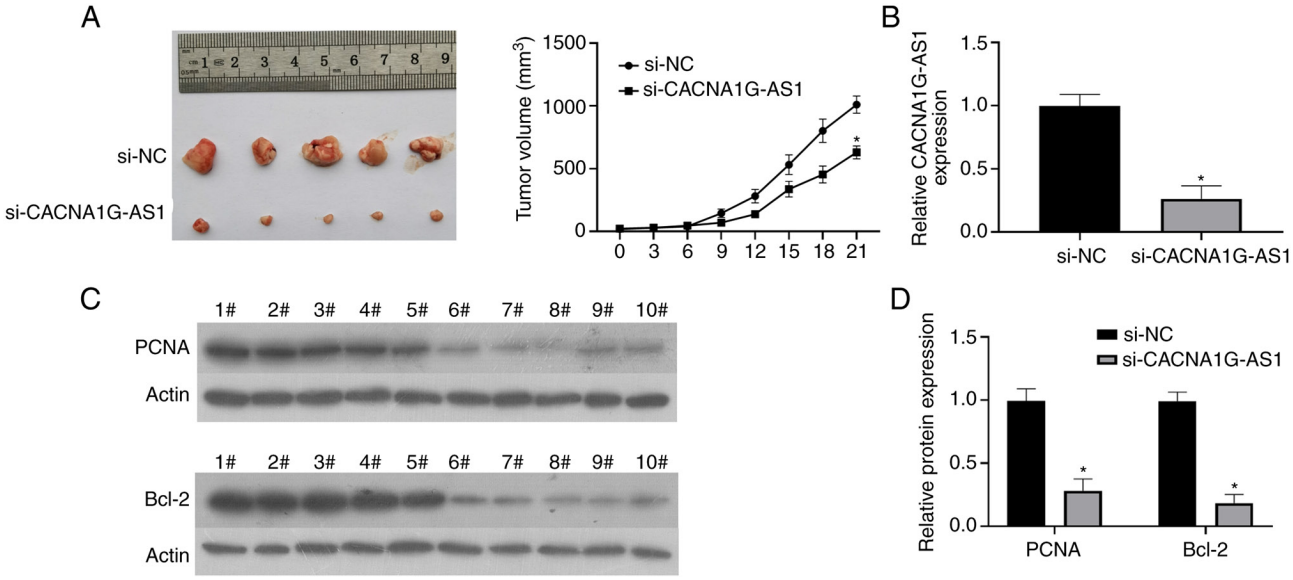

Figure 5. Silencing CACNA1G-AS1 prevents tumor growth and reduces PCNA and Bcl-2 expression levels in DLBCL. (A) Tumor volume of DLBCL tumors was evaluated after CACNA1G-AS1-silencing. (B) CACNA1G-AS1 expression in DLBCL tumors was evaluated by reverse transcription-quantitative PCR analysis after CACNA1G-AS1-knockdown. (C) After CACNA1G-AS1-silencing, western blotting analyses of PCNA and Bcl-2 were performed in DLBCL tumors. (D) Relative levels of PCNA and Bcl-2 proteins were assessed after quantifying western blotting protein band intensities. *P<0.05 vs. si-NC. CACNA1G-AS1, calcium voltage-gated channel subunit α1 G-antisense 1; PCNA, proliferating cell nuclear antigen; DLBCL, diffuse large B cell lymphoma; si-, short interfering; NC, negative control.

Discussion

DLBCL is a type of B cell lymphoma with high heterogeneity and invasion ability (34). The biological characteristics of DLBCL are relatively complex, and a therapeutic strategy has not been developed (35). Consequently, effective biomolecules are needed to be discovered for DLBCL therapy. The functions of numerous lncRNAs in human cancer have been confirmed, including DLBCL (36). Various pathological factors can be involved in DLBCL tumor progression, and lncRNAs have also been demonstrated to regulate several cancer phenotypes, including proliferation, cell cycle and apoptosis (37). Therefore, further exploration of lncRNAs in DLBCL might provide important targets for DLBCL therapy.

CACNA1G-AS1, which is a lncRNA, has been reported to have significant effects in a variety of cancer types (26-28). For instance, CACNA1G-AS1 facilitates colorectal cancer progression via p53 (26); CACNA1G-AS1 aggravates the malignant biological properties of hepatocellular carcinoma via the miR-2392/C1orf61 axis (27); and CACNA1G-AS1 enhances the epithelial-mesenchymal transition and metastasis of non-small cell lung cancer (28). The current study further tested the functions of CACNA1G-AS1 and the regulatory effect on miRs in DLBCL cells through functional and molecular experiments. As hypothesized, the present study revealed that CACNA1G-AS1 affected DLBCL progression by binding to miRs. To the best of our knowledge, the current study is the first to comprehensively demonstrate the expression, function and mechanism of CACNA1G-AS1 in DLBCL. Specifically, CACNA1G-AS1 was confirmed to be highly expressed in DLBCL tissues and cells, and upregulation of CACNA1G-AS1 was associated with a higher tumor stage. In addition, CACNA1G-AS1-knockdown was verified to significantly enhance cytotoxicity and promoted apoptosis in DLBCL cells; simultaneously, silencing CACNA1G-AS1 suppressed DLBCL tumor growth *in vivo*.

PCNA is a cell cycle regulatory protein that can reflect the proliferative activity of cells (38). Currently, research has indicated that PCNA expression is relevant to cell proliferation, and the proliferative activity of cells can reflect the malignant tendency of tumors (39). Therefore, PCNA can be applied as a biomarker to reflect the biological activity and malignant degree of tumors. A previous study revealed that Bcl-2, as one of the vital apoptosis suppressor genes, can effectively regulate the development and differentiation of normal B cells (40). High expression levels of Bcl-2 were reported in ~45% of patients with DLBCL (41). In addition, patients with DLBCL and high expression levels of Bcl-2 have a significantly worse prognosis with R-CHOP treatment (42). Overall, PCNA and Bcl-2 are notable markers that reflect malignant changes in tumors. The results of the present study confirmed that CACNA1G-AS1-knockdown significantly reduced the levels of PCNA and Bcl-2 proteins, which further confirmed the role of CACNA1G-AS1 in promoting DLBCL progression.

Notably, lncRNAs mainly function as miR sponges to reduce the expression of miRs (43). The current study screened predicted miRs by bioinformatics analysis and used a luciferase reporter assay to determine that miR-3160-5p could bind with CACNA1G-AS1 in DLBCL cells. Moreover, miR-3160-5p has been reported to significantly prevent prostate cancer cell proliferation (44). To the best of our knowledge, the present study was the first to fully reveal that miR-3160-5p, as a downstream regulatory mechanism of CACNA1G-AS1, significantly enhanced cytotoxicity and facilitated apoptosis in DLBCL cells. However, there are also limitations in the current study. For instance, rescue experiments need to be conducted to verify the effect of the interaction between CACNA1G-AS1 and miR-3160-5p on

DLBCL progression, and the downstream target genes of miR-3160-5p also need to identified.

In conclusion, the current study might be the first to confirm the role and mechanism of CACNA1G-AS1 in DLBCL progression. The results illustrated that both CACNA1G-AS1-knockdown and miR-3160-5p-overexpression could enhance cytotoxicity and induce apoptosis in DLBCL cells. Moreover, it provided evidence of a novel regulatory network (CACNA1G-AS1/miR-3160-5p) in DLBCL, which might be a therapeutic target for DLBCL.

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Availability of data and materials

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

Authors' contributions

QZ and YZ conceived and designed the study. QZ, MZ, XZ and HX performed the experiments. XZ and HX were major contributors to writing the manuscript. SX and MZ collected the clinical data and analyzed the data. All authors have read and approved the final manuscript. HX and YZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by The Ethics Committee of The Affiliated Hospital of Qingdao University (approval no. bc2020012). All participants provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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