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Regular paper

MicroRNA-196-5p targets Derlin-1 to induce autophagy in human osteosarcoma cells

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Osteosarcoma is a highly prevalent type of primary bone tissues in children and young adolescents. Micro-RNA (miR) dysregulation has been linked to osteosarcoma tumorigenesis. The role of miR-196-5p was investigated in modulating the growth and metastatic behaviour of human osteosarcoma cells, along with exploring its mechanism of action. As shown by RT-qPCR expression analysis, osteosarcoma cell lines exhibited prominent ($P<0.05$) transcriptional repression of miR-196-5p. The latter was thus transiently overexpressed in osteosarcoma cells, which resulted in the loss of cell viability and colony formation via induction of autophagy. The western blot analysis of the autophagy marker proteins revealed that the expression of Beclin 1 and LC3B II proteins was induced by miR-196-5p, whereas that of p62 and LC3BI was repressed. Moreover, osteosarcoma cells overexpressing miR-196-5p showed significantly ($P<0.05$) lower migration and invasion concerning the control osteosarcoma cells. According to the results of the in-silico analysis, Derlin-1 participates in the regulation of miR-196-5p in osteosarcoma, and this prediction has been validated using a dual luciferase assay. The results indicated that miR-196-5p exerted its molecular role by targeting Derlin-1 at the post-transcriptional level. Summing up, the study revealed the modulatory potential of miR-196-5p/Derlin-1 on osteosarcoma cells and provided insights into the possible implications for the treatment and prognosis of the disease.

Keywords: metastasis, autophagy, miR-196-5p, Derlin-1, osteosarcoma

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Abbreviations: AO, Acridine orange; DERL1, Derlin 1; DMEM, Dulbecco's Modified Eagle Medium; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; HCC, hepatocellular carcinoma; MUT, Mutant type; RIN, RNA integrity number; TER94,Transitional endoplasmic reticulum ATPase; TLR4,Toll-like receptor 4; UTR, untranscribed region; WT, Wild type

INTRODUCTION

Osteosarcoma is the most prevalent bone cancer, commonly affecting adolescents and young people (Ferguson & Turner, 2018; Sadykova *et al.*, 2020). This disease has been reported to exhibit a very poor prognosis, and in a considerable proportion of the diagnosed cases, it metastasizes to the pulmonary tissues (Li *et al.*, 2020). Together, these factors make osteosarcoma very lethal and therefore require the formulation of novel and efficient therapeutic measures against osteosarcoma. Pertinently, elucidation of the tumorigeneses of osteosarcoma on a molecular basis is essential to recognise the potential prognostic and therapeutic targets against this devastating malignancy. The vast majority of localised osteosarcomas are treated with surgery, but with the introduction of multiagent chemotherapy regimens there has been an increase in survival rate to 70% in patients with osteosarcoma (Miwa *et al.*, 2019). Several patients and their families have been reported to have undergone limb salvage surgery. Most patients, more than 85%, choose to salvage a limb rather than have it amputated (Rougraff *et al.*, 1994). It is also possible to employ radiation therapy to aid in the removal of microscopic or marginal residual tumours (DeLaney *et al.*, 2005). It has been estimated that approximately 70% of cancer patients with localized tumors will receive chemotherapy treatment (Chou *et al.*, 2005). There is a compound known as Mifamurtide, which is a liposomal TLR4 agonist that triggers macrophages and monocytes to channel the antitumor immune response against cancerous cells and inhibit malignant metastasis.

Micro-RNAs (miRs) belong to endogenous non-coding RNAs ranging in average size from 20–25 nucleotides (Donlic & Hargrove, 2018) that regulate specific protein-coding genes, mainly at the post-transcriptional level (Loukas *et al.*, 2021). These genes encode proteins that are modulated by miRs that bind to 3'-UTRs (untranscribed regions) of mRNAs post-transcriptionally *via* the mRNAs' 3'-UTRs (Abdalla *et al.*, 2020). The miRs displayed a vital role in human biology and physiology (Catalucci *et al.*, 2009). Interestingly, several microRNAs' deviations from the normal expression levels were linked to cancer development, among other disorders (Olson *et al.*, 2009). MiRs regulate the proliferation, cell division, apoptosis, autophagy, migratory potential, and invasiveness of human cancer cells (Gao *et al.*, 2020; Lin *et al.*, 2017; Ashrafizadeh *et al.*, 2020). Human osteosarcoma reportedly also exhibits an aberrant expression of several miRs' and regulates the growth, metastasis, and proliferation of osteosarcoma cells (Zhou *et al.*, 2018; Salah *et al.*, 2015).

Currently, miR-196-5p is known to be one of the most important functional molecules in immunity and cancer development in humans (Huang *et al.*, 2014). According to recent evidence, miR-196-5p may suppress the growth of breast cancer and melanoma (Li *et al.*, 2010; Braig *et al.*, 2010). Additionally, in several studies in human cancers like oral and colorectal cancer, miR-196-5p expression is believed to be involved in oncogenesis or tumour suppression, regulating their growth and progression (Xin *et al.*, 2019a; Stiegelbauer *et al.*, 2017; Maruyama *et al.*, 2018). Evidence suggests that the miR-196-5p gene appears to be downregulated in certain hu-

man tumours and acts as a tumour suppressor in regulating tumorigenesis (Zheng *et al*., 2019b; Lu *et al*., 2016). Although downregulation of miR-196-5p resulting from long noncoding RNA sponging has been shown in surface osteosarcoma, its regulatory role has not yet been reported (Chen *et al*., 2019).

Derlin-1, one of the ATPases in the p97 complex, is believed to be involved in ER-associated degradation. The function of this enzyme is to retrotranslocate misfolded or unfolded proteins into the cytosol for degradation by proteasomes (Klopfleisch *et al*., 2010). Many tumors have overexpressed Derlin-1, which is involved in the progression of tumors (Wang *et al*., 2008; Dong *et al*., 2017). There is evidence that Derlin-1 is expressed in several varieties of human carcinomas, and antibodies that target Derlin-1 have been shown to inhibit the growth of colon tumours in mice (Tan *et al*., 2015). In a study published by Wang and others in 2008 (Wang *et al*., 2008) it was found that Derlin-1 expression is correlated with tumor grade and lymph node metastasis in breast cancer. There is, however, little information about the biological significance of Derlin-1 in osteosarcoma cancer, and the exact relationship between microRNA196-5p, which targets Derlin-1 to induce autophagy in human osteosarcoma cells, remains unknown. Therefore, a recent study aimed to investigate the effects of miR-196-5p/Derlin-1 pathway at the molecular level involved in osteosarcoma.

MATERIALS AND METHODS

Tissues samples

Fifty five paired osteosarcoma and normal matching human tissue samples were obtained from osteosarcoma subjects during surgical resection in the Department of Orthopedics, Puren Hospital Affiliated to Wuhan University of Science and Technology, Wuhan, China. Two pathologists, blind to the study, conducted the pathological survey of the excised tissue specimens. The ethics committee of our institute approved the study (approval No. OR14PUR256/22). Liquid nitrogen was used to snap-freeze the specimens, and the latter were stored at –80°C until experimentation.

Cell lines

Three different humans (MG-63; ATCC Catalogue no.: CRL-1427), (Saos2; ATCC Catalog no.: HTB-85) and (HOS; ATCC Catalog no.: CRL-1543) osteosarcoma cell lines were obtained from the ATCC (American Type Culture Collection, USA) and the hFOB1.19 (normal osteoblast cell line; Catalog No.: CL-0353) were obtained from the Elabscience Biotechnology Inc, USA. The culturing of the cell lines was carried out using DMEM (Dulbecco's modified Eagle's medium) added with 10% FBS (fetal bovine serum), penicillin (100 U/ml) and streptomycin (10 μg/L) in an incubator (5% CO_2) at room temperature. The lowest passage numbers for the cell lines were between 3–5, respectively. Colorimetric mycoplasma detection assay using PlasmoTest™ (Catalog code: rep-pt1, InvivoGen USA) for the visual, colorimetric detection of mycoplasma contamination in cell cultures was performed. A positive result indicates the presence of a cell culture contaminant. The results of our tests for mycoplasma contamination in cell cultures were negative.

Cell line transfection

As a result of comparing miR-196-5p expression levels in Saos2, MG-63, and HOS cell lines with the normal osteoblast cell line, we selected the Saos2 cell line due to their relatively significant miR-16-5p expression levels ($P<0.01$).

After reaching 80% confluence, cells were seeded at a density of 1×10^4 cells/cm^2 into six-well plates. We carried out transient transfection of miRNAs as previously reported (Xin *et al*., 2019a). Twenty-four hours after seeding, transfection with miR-196-5p mimic (50 nM), miR-NC (50 nM), si-NC (50 nM), pcDNA-derlin1 (50 nM) or si-DERL1 (50 nM) was carried out using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions. The presynthesised miR-196-5p mimics, miR-NC or si-NC (negative control) oligos were obtained from the Gene-Pharma Company (Suzhou, Jiangsu, China). To construct the Derlin1 expression plasmid, the mammalian overexpression vector, pcDNA3.1, was cloned with the full-length ORF of derlin1 (pcDNA-derlin1) to obtain Derlin1 whereas control vector-transfected cells were used as the negative control. As a result of using siRNA Derlin-1 sequences (Oligobio, Beijing, China) and pcDNA3.1-Derlin-1 sequences (Oligobio, Beijing, China), we were able to knock down or upregulate the expression of Derlin-1.

A Lipofectamine 2000 (5 μl) solution was placed in DMEM (250 μl) in the absence of serum and incubated for 10 minutes at 37°C. Each 5 μl of miR-196-5p mimic, miR-NC, pcDNA-derlin1, si-NC, and si-DERL1 was placed in DMEM (250 μl) in the absence of serum. Following incubation for 10 minutes, the diluted miR-196-5p mimic, miR-NC, pcDNA-derlin1, si-NC, and si-DERL1 was gently mixed with diluted Lipofectamine 2000, and then incubated at room temperature for 30 minutes. The cells were washed twice in PBS (phosphate buffered saline) after the old medium was removed from the 6-well plate. To each well containing cells and medium, 500 μl of the complexes were added before the plate was gently rocked to mix the solution. A 24-hour incubation period was conducted at 37°C in a CO_2 incubator to test for the expression of transgenes in the cells.

Extraction and quantitative RT-qPCR of RNA

Microfluidics-based Agilent Bioanalyzers (Agilent Technologies Co. Ltd., Beijing, China) classify RNA samples according to their integrity using RNA Integrity Numbers (RIN) ranging from 1 to 10. The RIN value above 8.0 indicates intact, high-quality RNA samples, the value between 5.0 and 8.0 indicates moderately degraded samples, and the value below 5.0 indicates degraded samples (Padhi *et al*., 2018). It is typically recommended to use RNA samples that have RIN values above 5.0 in order to ensure a reliable measurement of gene expression by RT-qPCR.

Table 1. Primers used in real-time polymerase chain reaction

Primers	Sequence (5′-3′)	Size (bp)
miR196-5p	5′-TAGGTAGTTTCCTGTTGTTGGG-3′	178
	5′-GCGAGCACAGAATTAATACGAC-3′	
derlin 1	5′-TCGGACATCGGAGACTGGTT-3′	186
	5′-AGCCAGTAATCACGATGCAAA-3′	
GADPH	5′-CTTCTACAATGAGCTGCGTG-3′	156
	5′-TCATGATTGAGTCAGTCAGG-3′	

TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used according to the manufacturer's instructions to isolate total RNA from the cells. The expression of mRNA of a cell was detected by a real-time PCR system, which used SYBR Green (Roche Diagnostics, Mannheim, Germany), with two micrograms of total RNA. The cDNA was synthesized with a Transcriptor cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). In order to normalize the expression of the target gene mRNA, GAPDH expression was used as an endogenous control. The inverse logarithms of the ΔΔCt levels are used to calculate the relative levels of miR-196-5p. The primer sequence information is listed in Table 1. The specific method is described in the earlier study (Jhan *et al*., 2022).

CCK-8 assays

A 96-well plate was used to plate the cells (3×10^4 cells) into each of the 96 wells after they had been transfected. After culture of cells for 0, 12, 24, 48, or 96 h at 37°C, the addition of CCK-8 reagent (10 μl, Solarbio Life Science, Beijing, China) was transferred to each well in accordance with the manufacturer's guidelines. The incubation was extended at 37°C for 3 h, and finally, in order to measure the absorbance of the wells at 470 nm, the microplate reader (Agilent Bioscience Co. Ltd, Hangzhou China) was utilized to measure the absorbance of each well at 0 h and at intervals of 24 h until 96 h post-transfection (Xin *et al*., 2019b).

Colony formation assay

Proliferation of cells was also determined by colony formation assays (Wang *et al*., 2018). The transfected Saos2 cells (2.5×10^4 cells in each well) were plated in a six-well plate. During the 17 days of culture, fresh medium was changed every day during incubation and cells were incubated at room temperature after 96 hours of culture. The colonies formed were washed with PBS and paraformaldehyde (4%) fixed at 37°C for 15 min. A crystal violet stain of 0.25% was applied to the colonies at 37°C for 15 minutes. The percent colony number (for colonies with > 50 cells) was analysed by two independent researchers (blind) counted under a bright-field microscope with a low-resolution bright field microscope (Olympus, Tokyo, Japan). Image of each plate was scanned using colony counting software (ImageJ, USA)

Analysis of apoptosis in cells by flow cytometry

The FITC-Annexin V Apoptosis Detection Kit (4A Biotech, Nanjing, China) was used to measure cell apoptosis according to the instructions provided by the manufacturer (Badhai *et al*., 2009). Briefly, 24 h after transfection with 50 nM miR-NC, miR-196-5p mimic and pcDNA-derlin1, the medium was then removed, and the cells were incubated in serum-free medium for 24 hours after removal of the medium. After digestion with trypsin, the cells were washed twice in cold phosphate buffer saline, centrifuged for five minutes at 1200 rpm, and resuspended in phosphate buffer saline (100 μl). The cells were then resuspended in phosphate buffer saline. Incubation was carried out in the dark at room temperature in the presence of 5 μl of FITC-labelled Annexin V and PI. We used flow cytometry to analyze apoptosis five minutes after adding 400 μl of phosphate buffer saline. FlowJo software (Tree Star, Ashland, USA) was used to analyse the data in this study.

The Annexin V/PI assay was developed to differentiate between live and apoptotic cells, but it can also be used to differentiate between apoptotic subpopulations of cells which are in an early or late stage of apoptosis (i.e., healthy cells: (Annexin-/PI-), early apoptotic cells: Annexin V+/PI-), late apoptotic cells: (Annexin V+/PI+), necrotic/dead cells: Annexin V-/PI+). Hence, for the same miR-196-5p treated Saos2 cells, we first removed any cells having a negative value before performing further analyses. We analyzed a total of 10 000 cells per measurement.

Acridine orange (AO) autophagy staining

The acidic vesicles formed during autophagy were visualized with the help of the acridine orange staining protocol (Pierzyńska-Mach *et al*., 2014). In brief, the transfected Saos2 cancer cells were placed at 5×10^4 in each well of 12-well plate. A 24-hour culture at 37°C was carried out on the cells. After that, the cells were collected, washed with phosphate buffer saline and stained with 0.5% acridine orange (AO). We then removed the acridine orange from the cells, washed them rapidly with fresh medium, and examined them under a fluorescence microscope (Leica, Germany). The imaging conditions were AO-excitation 448 nm, emission 470–550 nm (monomers)and 580–650 nm (stacks); the scanning speed was 7 000 Hz. The cover slip with the live cells were mounted on custom stainless-steel holders. The sample temperature was maintained at 37°C. ImageJ software (Rasband, WS. ImageJ) was used to perform basic image processing and analysis.

The accumulation of acrylidine orange in acidic autophagosomes was detected by fluorescence microscope equipped with a digital camera. The presence of cells with intense red staining indicated that autophagy occurred in these cells.

Western blot

To lyse the cells, ice-cold RIPA lysis buffer (CW-BIO, Beijing) was used after the cells were transfected for 48 hours and the total protein concentration of the transfected Saos2 cells was detected using a BCA Protein Assay Kit (Beyotime, Shanghai, China). Subsequently, 20 μg of protein of each sample was separated by 10% SDS-PAGE gel (Bio-Rad Laboratories Co., Ltd. Shanghai, China) and then transferred onto the PVDF membranes (Millipore, USA). Primary antibodies anti-Beclin1 (Cat#11306-1-AP, Proteintech Group Inc., Rosemont, IL, USA; dilution 1:1000), anti-p62 (Cat#18420-1-AP, Proteintech Group Inc. USA; dilution 1:6000), anti-LC3BI/II (Cat# ABC929, Sigma Aldrich, USA; dilution 1:800), anti-Derlin-1 (Cat#SAB4200148, Sigma Aldrich, USA; dilution 1:2000) were added to the PVDF membrane in blocking solution (10% fat-free milk in TBST (Tris buffered saline/Tween-20), followed by washing three times with TBST and incubated for 3 h at 37°C. Next, PVDF membranes were incubated for two hours with goat anti-rabbit HRP-conjugated secondary antibodies (Proteintech Group Inc., dilution 1:7 000) at room temperature. Chemiluminescent fluid (GE Healthcare Life Sciences, UK) was applied to cover the membranes after they were washed. A chemiluminescence analyzer (Biotech Co., Ltd., Beijing) was used to detect and photograph the bands. Quantity One software was used to quantify protein bands by densitometry. In order to correct for the differences in loading between the groups, the volume per group was divided by the volume of normalized β-actin for each group. A normalization pro-

cedure was then conducted to determine the differences between each group and the control group. Based on the control data, the results were presented as a percentage (Xin *et al*., 2019a).

Assays of Migration and invasion

It was examined whether miR-196-5p mimics or miR-NC transfected into Saos2 cells could affect migrating and invading cells within the chambers using the Transwell chamber and wound healing assays, respectively. Approximately 2×10^5 transfected cells were placed in each of six wells of a six-well plate to perform the migration assay. Post 24 h cell culturing, the cell surface was scraped by a 200 µl pipette tip. The wound was imaged with the aid of a light microscope. Upon incubation at room temperature for 24 hours, the wound was again visualised and the percent wound closure was analysed using Image J software. For the Transwell chamber invasion assay, 5×10^4 transfected cells suspended in 250 µl of serum-free culture medium were added to the Matrigel-coated upper chamber of a 24-well Transwell plate. DMEM with 10% FBS (650 µl) was placed in the bottom of the cell culture chamber. We fixed and stained the invaded cells in methanol with 0.2% crystal violet after 24 hours of incubation at 37°C. An inverted microscope (Olympus, Tokyo, Japan) was used to visualize the cells. Based on the number of cells counted, two independent researchers (blind) were able to calculate the percentage of cells that invaded seven random fields under the microscope (Xin *et al*., 2019b).

miR-196-5p target analysis and dual luciferase assay

In silico analysis was conducted to predict the interaction between miR-196-5p and Derlin-1 3'-UTR using TargetScan online (http://www.targetscan.org/vert_72/). The prediction was validated using a dual luciferase assay (Promega, USA) and Microplate Luminometer (BioTek, USA). In an earlier study, the specific method was described (Jhan *et al*., 2022). Briefly, Derlin-1 3'-UTR with wild-type miR binding site (derlin1-WT) or mutant miR binding site (Derlin-1-MUT) was first cloned in the pmir-GLO luciferase reporter vector. Next, Saos2 cells were co-transfected with 1 ng/µl concentrations of reporter vectors (WT or MUT) and 2.5 µg of miR-196-5p mimics or miR-NC with the help of Lipofectamine 3000. To quantify the luciferase activity of the cells, host cells were isolated, washed, and trypsinized before being assayed with the dual luciferase assay to determine their luciferase activity 48 hours after transfection as directed by the manufacturer following the instructions.

Statistical analysis

Each experiment was presented at least three times independently in order to obtain the best results. Data were shown as means ± S.D. GraphPad version 9.0 was run to accomplish the statistical analysis. A Student's t test was used to assess the significance of differences between the two treatment groups, assuming that the data were normally distributed. One-way ANOVA was used to compare the values of the experimental and control groups. A multiple comparison test was performed between the groups using Tukey's multiple comparison test. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

MiR-196-5p expression is repressed in osteosarcoma

To gain insight into the miR-196-5p expression pattern in osteosarcoma, quantitative RT-qPCR was performed. Interestingly, osteosarcoma tissue specimens displayed a remarkably lower ($P<0.05$) miR-196-5p transcript ($P<0.05$) compared to the normal tissues (Fig. 1A). Expression analysis was also performed from three different osteosarcoma cell lines (MG-63, Saos2, and HOS) with reference to normal osteoblast cell lines. The miR-196-5p expression was shown to be substantially ($P<0.05$) reduced compared to that of normal cells (Fig. 1B). Additionally, miR-196-5p expression was considerably ($P<0.01$) low in Saos2 cells compared to MG-63 and HOS cell lines. Therefore, we selected Saos2 cell lines for transfection. Thus, the results indicate that miR-196-5p is markedly reduced in osteosarcoma, suggesting its possible involvement in its growth and pro-

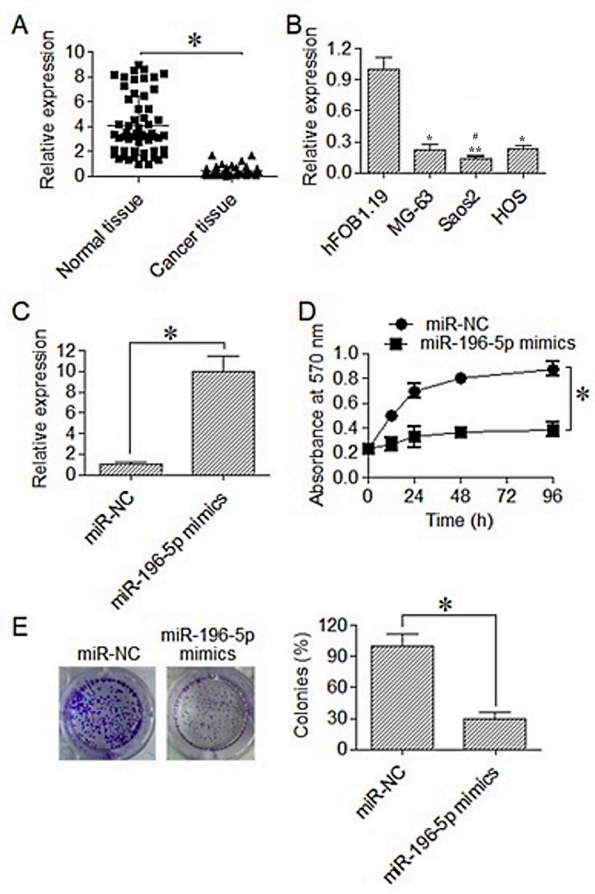

Figure 1. Over-expression of miR-196-5p in osteosarcoma cells declined their viability, *in vitro*.
(**A**) Relative transcript levels of miR-196-5p in osteosarcoma and normal matching human tissues. (**B**) Relative expression analysis of miR-196-5p from Saos2, HOS, MG-63 cell lines and with respect to hFOB1 normal osteoblast cell line. (**C**) qRT-PCR based confirmation of miR-196-5p over-expression in miR-196-5p mimics transfected Saos2 osteosarcoma cells with reference to respective miR-NC transfected negative control cells. (**D**) Viability analysis at various intervals of miR-196-5p mimics and miR-NC transfection in Saos2 cancer cells of *in vitro* culturing. (**E**) Colony formation assay of Saos2 cells transfected with miR-196-5p mimics or miR-NC. Each experiment was conducted using three independent biological replicates (*$P<0.05$ *vs*. control group, #$P<0.01$ *vs*. MG-63 and HOS cells)

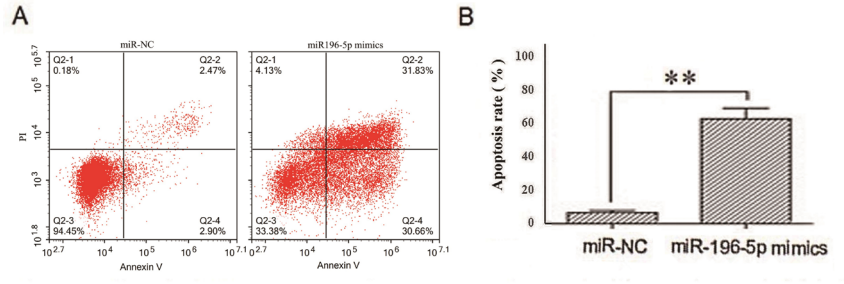

Figure 2. Effect of miR-196-5p overexpression on the rate of apoptosis.
(**A**) Analysis of flow cytometry results of Saos2 cells transfected with miR-196-5p mimics or miR-NC. (**B**) Rate of apoptosis (%) in Saos2 cells transfected with miR-196-5p mimics or miR-NC. Significant change in the percentage rate of apoptotic cells (**P<0.01) observed from the representative images of apoptotic cells. Cell percentages were calculated based on three independent flow cytometry experiments. Q1 represents death cells, Q2 represents the late apoptosis cells, Q3 represents the normal or live cells, Q4 represents the early apoptosis cells. The statistical result of apoptosis rate (B), which equals to the rate of late apoptosis cells (Q2) plus the rate of early apoptosis cells (Q4) plus the rate of necrotic or dead cells (Q1) respectively.

gression. The Saos2 cell line was therefore used to determine whether miR-196-5p acts in the development of osteosarcoma.

miR-196-5p inhibited osteosarcoma cell growth

To induce miR-196-5p overexpression in Saos2 cancer cells, miR-196-5p mimics were transfected into the cells. The results of RT-qPCR displayed that miR-196-5p mimics transfected Saos2 cancer cells expressed ~8-fold miR-196-5p transcript levels relative to miR-NC transfected Saos2 cells (Fig. 1C). To analyze the effect of miR-196-5p overexpression on Saos2 host cell viability, a CCK-8 assay was carried out. The findings revealed that miR-196-5p overexpressing Saos2 cells showed markedly lower (P<0.05) *in vitro* viability (P<0.05) compared to negative control cells in different cultures (Fig. 1D). Again, colony formation of Saos2 cells overexpressing miR-196-5p was remarkably lower (P<0.05) comparatively to normal cells (Fig. 1E). Colony formation of Saos2 cancer cells overexpressing miR-196-5p was only 32% of that of control cells. As a result of these findings, it might be speculated that miR-196-5p has a growth-inhibitory function in osteosarcomas.

miR196-59-induced apoptosis in Saos2 cells

We evaluated the changes in apoptosis using Annexin/PI staining. The results showed that miR-196-5p transfection significantly (P<0.01) increased in the percentage

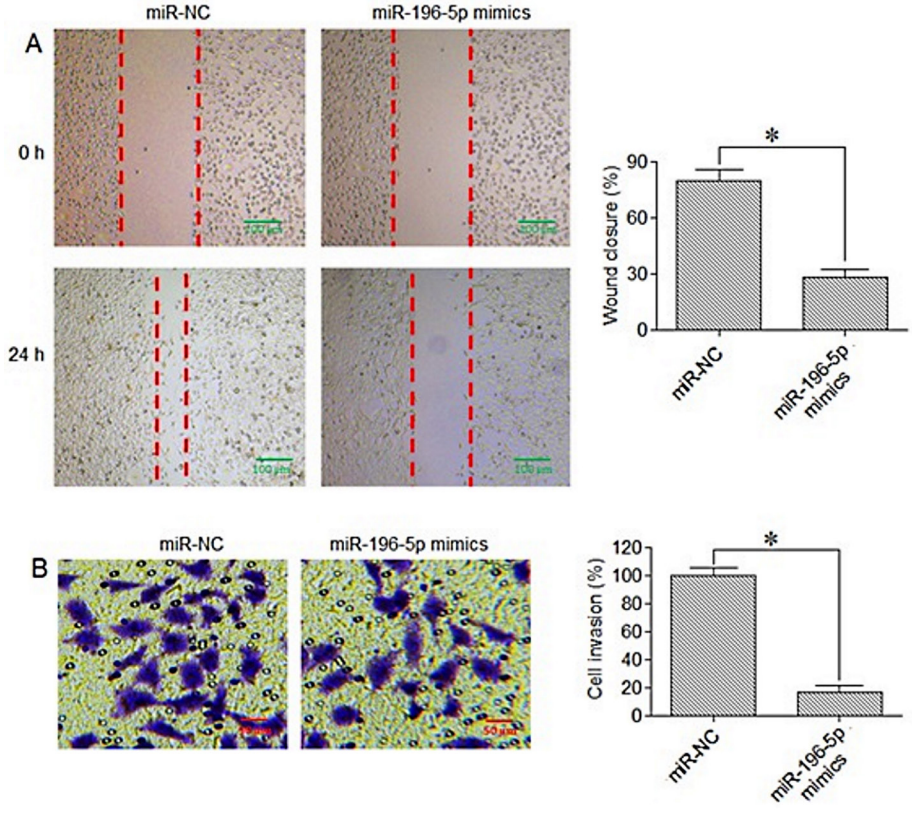

Figure 3. Osteosarcoma cells exhibited restrained motility *in vitro* by miR-196-5p up-regulation.
(**A**) The wound-healing assay was used to investigate the migration patterns of Saos2 osteosarcoma cells that had been overexpressed with miR-196-5p mimics or miR-NC. (**B**) Transwell chamber invasion assay of Saos2 osteosarcoma cells transfected with miR-196-5p mimics or miR-NC. Each experiment was conducted using three independent biological replicates (*P<0.05).

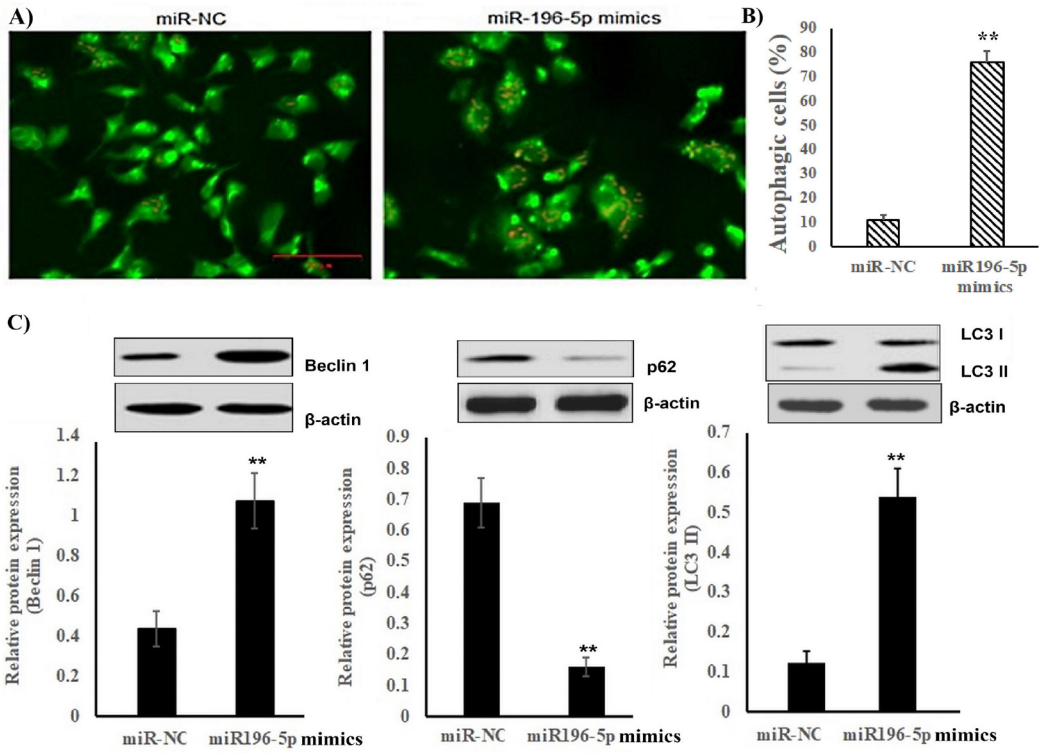

Figure 4. miR-196-5p over-expression promoted the ability of osteosarcoma cells to undergo autophagy.
(**A**) Detection of autophagosomes by acridine orange staining in Saos2 cancer cells transfected with miR-196-5p mimics or miR-NC. (**B**) Quantification of autophagosomes (percent of autophagic cells) with miR-196-5p mimics or miR-NC. (**C**) Western blot analysis of LC3B I & II, p62 and Beclin 1 proteins from Saos2 osteosarcoma cells transfected with miR-196-5p mimics or miR-NC. The experiments were performed using three independent biological replicates.

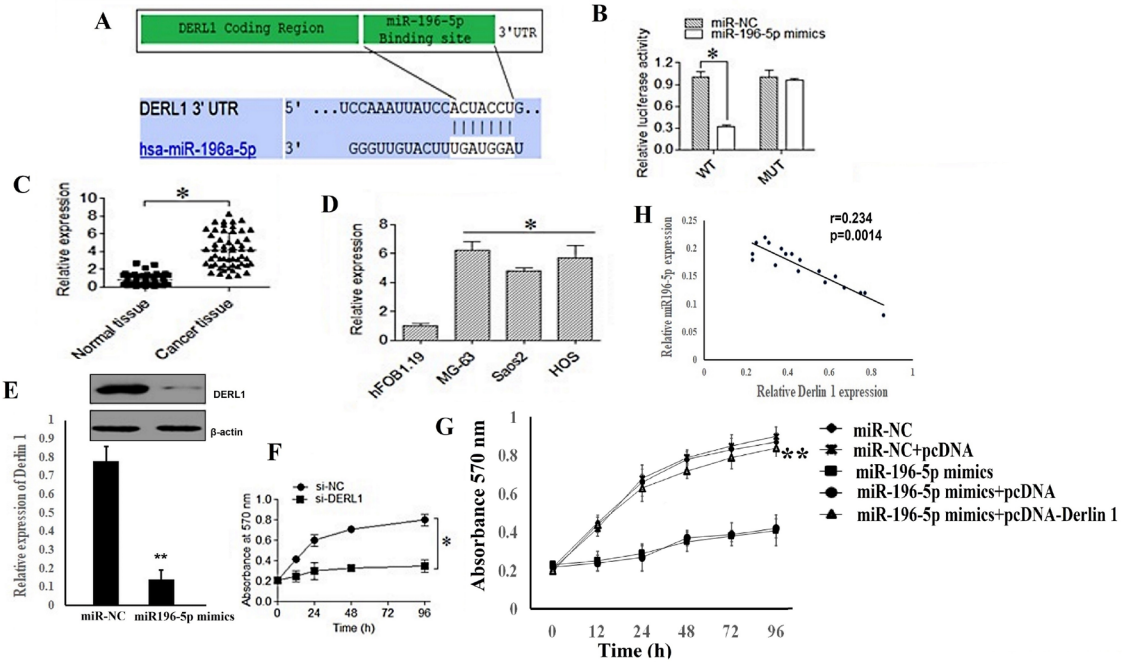

Figure 5. Derlin-1 is functionally targeted by miR-196-5p in osteosarcoma.
(**A**) In silico analysis of miR-196-5p with Derlin-1 3′-UTR. (**B**) miR-196-5p site-specific interaction with Derlin-1 3′-UTR was assessed using a dual luciferase assay. (**C**) qRT-PCR expression analysis of Derlin-1 from normal human and osteosarcoma cells. (**D**) Relative transcript levels analyses of Derlin-1 from Saos2, HOS and MG-63 cell lines relative to hFOB1 normal osteoblast cell line. (**E**) Western blotting of Derlin-1 miR-196-5p mimics or miR-NC expressing Saos2 cancer cells. (**F**) CCK-8 assay for the viability analysis of Saos2 cells transfected with si-Derlin-1 or si-NC at different intervals of in vitro culturing. (**G**) Viability analysis of Saos2 cells transfected with miR-NC, miR-NC+pcDNA, miR-196-5p mimics or miR-196-5p mimics + pcDNA or miR-196-5p mimics+pcDNA-Derlin-1 at different intervals of *in vitro* culturing. (**H**) A Pearson correlation analysis was performed on OS tissues to determine the correlation between miR-196-5p and Derlin-1. Each experiment was conducted using three independent biological replicates (*P<0.05; **P<0.01).

rate of apoptosis in Saos2 cells (Fig. 2A) compared to negative control (miR-NC). These findings indicate that the miR-196-5p overexpression showed substantial increase in the rate of apoptosis in Saos2 cells (Fig. 2B). Collectively, these results suggest that miR-196-5p overexpression induces triggering cell death in Saos2 cells. In total, 9989 cells were recovered in miR-NC control group, which contained 94.45% live or normal cells (Q3), 2.9% early apoptotic cells (Q4), 2.47% late apoptotic cells (Q2) and 0.18% necrotic or dead cells (Q1). Out of 9972 total cells recovered in miR-196-5p treated Saos2 cells, 33.38% live cells (Q3), 30.66% early apoptotic cells (Q4), 31.83% late apoptotic cells (Q2) and 4.13% necrotic or dead cells (Q1).

miR-196-5p restrained migration and invasion of osteosarcoma cells

Whether miR-196-5p also regulates the migratory ability and invasiveness of osteosarcoma cells, the effect of miR-196-5p overexpression was visualized on the migration and invasion of Saos2 sarcoma cells. The results showed that Saos2 cells over-expressing miR-196-5p migrated at remarkably ($P<0.05$) reduced rates *(*$P<0.05$*),* as evidenced by the percent wound closure being markedly lower compared to control cells (Fig. 3A). Furthermore, miR-196-5p overexpression in osteosarcoma cells significantly inhibited *in vitro* invasion considerably ($P<0.05$), and it was shown that percent cell invasion was reduced to less than 20% relative to that of negative control osteosarcoma cells. Thus, the results suggest that miR-196-5p has an antimetastatic regulatory role in osteosarcoma (Fig. 3B).

Osteosarcoma cells overexpressing miR-196-5p exhibited induction of autophagy

To assess whether the reduction in the replication of the Saos2 cancer cell overexpressing miR-196-5p resulted from the induction of cell autophagy, acridine orange (AO) staining was performed, followed by fluorescent microscopy. Cancer cells overexpressing miR-196-5p were shown to accumulate AO stain indicative of the possession of acidic autophagosomes (Fig. 4A). The western blot analysis of the autophagy marker proteins revealed that LC3B II and Beclin 1 expression levels were significantly elevated ($P<0.01$), whereas p62 levels were significantly decreased ($P<0.01$), indicating that miR-196-5p stimulated autophagy (Fig. 4B). Taken together, our findings demonstrated that miR-196-5p overexpression induces apoptosis through autophagy-related proteins (Beclin 1, p62, LC3B) in osteosarcoma cells.

miR-196-5p functionally targets Derlin-1 in osteosarcoma

A comprehensive in silico analysis of miR-196-5p was performed to predict the specific molecular target of miR-196-5p in osteosarcoma. Derlin-1 was recognized as the probable target of miR-196-5p in osteosarcoma, and this miRNA binds to a precise site within the untranscribed region (3'-UTR) of Derlin-1 mRNA (Fig. 5A) corresponding to the seed region of miR-196-5p. TargetScan identified Derlin-1 as a possible target of miR-196-5p with the indicated target sites. Consequently, we assumed that miR-196-5p was regulating Derlin-1 in Saso2 cells.

Further study results revealed that overexpression of miR196-3p could significantly reduce the level of 3'-UTR activity of Derlin-1, which was in contrast to that detected for mutant Derlin-1 3'-UTR activity (Fig. 5B). Furthermore, the results of the luciferase reporter assay revealed that aberrant cells were identified, which showed that the activity of luciferase in Saos2 cells decreased significantly ($P<0.01$) only after the miR-196-5p mimics were co-transfected with luciferase plasmids containing the 3'-UTR of Derlin-1 with native binding sites. Moreover, the gene expression of Derlin-1 was shown to be considerably increased ($P<0.05$; Fig. 5C) in osteosarcoma cell lines, negatively correlating with that of miR-196-5p expression (Fig. 1A), thus substantially linked with tumor progression. A significant increase in Derlin-1 mRNA and protein levels was also observed in Saos2 cells (Fig. D, $P<0.05$). According to our findings, miR-196-5p targets Derlin-1 by direct binding to predicted sites in the 3'-UTR of Derlin-1 mRNA. The results of our luciferase reporter assay support the hypothesis that miR-196-5p targets the Derlin-1 gene. Again, the expression of the Derlin-1 protein was shown to be repressed in miR-196-5p overexpressing Saos2 cancer cells, which provides further insight into post-transcriptional down-regulation of Derlin1 by miR-196-5p in osteosarcoma (Fig. 5E).

Furthermore, it was shown that the effect of miR-196-5p on Saos2 cell migration, invasion, and proliferation was modulated by Derlin-1. The CCK-8 assay showed that Derlin-1 silencing mimicked the antiproliferative effects of miR-196-5p overexpression, suggesting that the latter could be exerting its role in osteosarcoma through post-transcriptional repression of Derlin-1 (Fig. 5F). Besides, Saos2 cancer cells over-expressing Derlin-1

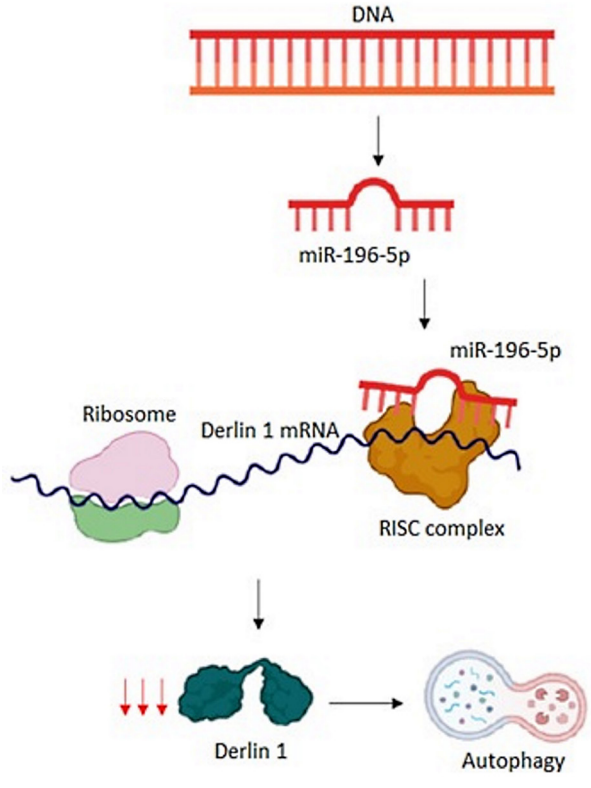

Figure 6. Model showing the molecular mechanism of miR-196-5p/derlin1 axis in osteosarcoma.
miR-196-5p is synthesized and binds to the 3'-UTR of Derlin-1 through the RISC complex. This binding blocks Derlin-1 translation eventually leading to autophagy mediated inhibition of cell proliferation.

did not show proliferative decline when miR-196-5p was overexpressed (Fig. 5G). However, these findings were not noticed between the groups: miR-NC *vs.* miR-NC+pcDNA & miR-196-5p mimics vs. miR-196-5p mimics+pcDNA ($P>0.05$). Nevertheless, we observed significant difference in the effects of miR-196-5p overexpression suppressed by Derlin-1 between the groups ($P<0.01$): miR-196-5p mimics *vs.* miR-196-5p+pcDNA – Derlin 1 and miR-196-5p mimics+pcDNA *vs.* miR-196-5p mimics+pcDNA-Derlin 1. The results reveal that miR-196-5p targets Derlin-1 for its post-transcriptional repression to exert its functional role in osteosarcoma (Fig. 6). These findings are consistent with our earlier demonstration about the results of the luciferase reporter assay and the published results on pcDNA (Zhang *et al.*, 2021). The molecular basis of the antiproliferative effects of miR-196-5p overexpression that mediates through the decrease in the expression of Derlin-1 in Saos2 osteosarcoma cells remains unclear and needs further investigation. However, our findings confirmed that miRNA (si-Derlin-1) was silenced in Saos2 cells, thus decreasing the expression levels of Derlin-1. Based on the results of these experiments, we were able to confirm our predictions and suggest that miR-196-5p might regulate Derlin-1 to offer its antiproliferative effects. Consequently, miR-196-5p and Derlin-1 showed a significant correlation in OS tissues, demonstrating the effects of miR-196-5p on cell growth, metastasis, and apoptosis (Fig. 5H).

DISCUSSION

Numerous studies have suggested that miRs might emerge as potential molecular markers in cancer diagnosis and therapy (Iacona & Lutz, 2019). The exact mechanisms by which miRNA regulate tumorigenesis remain unclear, despite several miRNAs found to be downregulated in tissues and cells (Wang *et al.*, 2018). It is possible, however, that miR-196 suppresses tumour growth in breast cancer and melanoma (Braig *et al.*, 2010; Li *et al.*, 2010). Additionally, in several studies in human cancers like oral and colorectal cancer, miR-196-5p expression is believed to be involved in oncogenesis or tumor suppression, regulating their growth and progression (Xin *et al.*, 2019a; Stiegelbauer *et al.*, 2017; Maruyama *et al.*, 2018). Therefore, the present study was planned to show that miR-196-5p could be useful for treating osteosarcoma due to its potential efficacy. Our findings showed that miR-196-5p overexpression inhibited OS cell proliferation, colony formation, and invasion by inducing autophagy, signifying that miR-196-5p functions as a tumour suppressor of osteosarcoma progression (Fig. 1). Similar results from a study were reported, but using a different miRNA in osteosarcoma, showing that overexpression of miR-423-5p suppressed osteosarcoma invasion, colony formation, and proliferation, decreasing STMN1 expression. In osteosarcoma, miR-423-5p appears to act as a tumour suppressor gene (Wang *et al.*, 2018). Another study supporting our findings found that miR-196-5p overexpression worsened HCC (hepatocellular carcinoma) cell proliferation and metastasis *in vitro* and *in vivo* (Zheng *et al.*, 2019a). Similarly to the results of our study, recent research demonstrated that overexpression of miR-196b-5p inhibited proliferation, migration and invasion (Wharton's umbilical cord) by targeting Cdkn1b (Meyer *et al.*, 2018).

Autophagy and apoptosis are interrelated processes whose outcomes depend on both cell type and environmental factors. It has been described that autophagy can antagonize apoptosis, but can also act as an agonist (Fan & Zong, 2012). Despite this, autophagy can induce cell death regardless of apoptosis. Consequently, we found a statistically significant difference in the percentage rate of apoptosis of Saos2 cells overexpressing miR-196-5p measured using the FITC-Annexin V Apoptosis Detection Method (Fig. 2). Therefore, we believe that miR-196-5p overexpression increases the percentage rate of apoptosis by down-regulation of Derlin-1 in Saos2 osteosarcoma cells. However, the exact molecular mechanisms underlying the cytotoxic mechanism of miR-196-5p–induced apoptosis remained unclear and warrants further investigation. However, miR-196-5p expression has been reported to be markedly reduced markedly under hypoxic conditions, and overexpression of this miRNA reversed HCC effects of hypoxia on the proliferation and invasion (hepatocellular carcinoma) (Zheng *et al.*, 2019b). Consequently, additional studies are needed to understand the molecular mechanisms of overexpression of miR-196-5p and hypoxic conditions in Saos2 cell lines.

Derlin-1 is upregulated in various human cancers, such as lung, colon, and breast cancer, and activates oncogenic pathways in the body (Dong *et al.*, 2017; Wang *et al.*, 2008; Dong *et al.*, 2013). Derlin-1 overexpression has been linked to aggressive phenotypes in breast, pancreatic, and lung cancers (Dong *et al.*, 2017). There is, however, no evidence of its involvement in human osteosarcoma. The results of our study demonstrated that osteosarcoma cells and tissues expressed low levels of miR-196-5p. Similar to our study findings, a report showed that miR-423-5p overexpression was decreased in osteosarcoma tissues and osteosarcoma (MG-63, SAOS-2, U2OS and SOSP-9607) cell lines (Wang *et al.*, 2018).

However, the effect of the overexpression of miR-196-5p targeting Derlin-1 is still unknown in osteosarcoma. Our results found that the expression of Derlin-1 was upregulated in osteosarcoma tissues and cell lines. The results of the present study revealed that Derlin-1 is a direct target gene of miR-196-5p in osteosarcoma cells. According to this report, miR-196-5p directly regulates Derlin-1 expression in Saos2 cells, and modifying Derlin-1 expression reverses miR-196-5p overexpression/inhibition of Saos2 cell proliferation and metastasis. Based on the results of this study, it appears that the miR-196-5p/Derlin-1 axis is a key pathway that regulates the progression of osteosarcomas (Zheng *et al.*, 2019a). It is in agreement with a study which demonstrated that miR-196-5p targets HMGA2 thus regulating hepatocellular carcinoma progression involving miR-196-5p/HMGA2 axis as a key pathway (Zheng *et al.*, 2019a). Additionally, a study found that miR-30b inhibited breast cancer progression and metastasis by targeting Derlin-1 *in vitro*, similar to our results.

The autophagy and apoptosis pathways are usually impaired in malignant cells, resulting in the lengthening of their survival (Jain *et al.*, 2013). Autophagy is an intrinsic process which clears off cell debris and assists in maintaining normal cell behaviour (Chua *et al.*, 2022). Our findings clearly indicated that miR-196-5p targets the expression of Derlin-1 and significantly altered the autophagy-related proteins (Beclin 1, p62, and LC3B II; Fig. 3) that demonstrate an increase of autophagosomes formation through increased levels of LC3-II, confirming the increased autophagy formation in Saos2 cells (Murugan & Amaravadi, 2016; Ji *et al.*, 2015). The results were further confirmed by the decreased expression of the autophagic flux-related protein p62 with increa-

sed autophagy (Murugan & Amaravadi, 2016). In light of these results, we hypothesized that miR196-p targets Derlin-1 to induce autophagosome formation in Saos2 cells. Our results demonstrated that the anticancer effects resulting from miR-196-5p overexpression are rising from the promotion of autophagy in osteosarcoma cells which suggests the beneficial activity of miR-196-5p against human osteosarcoma. Furthermore, our findings were supported by a study demonstrating that Derlin-1 expression was elevated in most nonsmall lung cancer cell lines, and silencing Derlin-1 mRNA suppressed autophagic flux in A549 cells that induce autophagy (Cai *et al*., 2022). Thus, targeting autophagy will be an important consideration in developing novel strategies for cancer therapy (Fan & Zong, 2012).

In the current study, Derlin-1 was post-transcriptionally regulated (repressed) by miR-196-5p, and the former was confirmed to act as a mediator of miR-196-5p role in osteosarcoma. Down-regulation of miR-196-5p would mitigate Derlin-1 silencing at the post-transcriptional level, culminating in Derlin-1 upregulation. The latter, with its established oncogenic function, could be one of the crucial elicitors for osteosarcoma tumorigenesis. Our findings further clarified the involvement of the interactional interplay of miR-196-5p with Derlin-1 to control the progress and subsequent progression of human osteosarcoma. As a consequence, it is speculated that Derlin-1 is involved in miR-196-5p-mediated proliferation, invasion, and colony formation in osteosarcoma (Saos2) cell lines. The results of our research showed that miR-196-5p inhibited the expression of Derlin-1 in osteosarcoma, thus acting as a tumor suppressor gene. However, further studies are warranted to investigate the potential molecular mechanisms underlying its biological effects involving the miR-196-5p/Derlin-1 axis in the regulation of osteosarcoma progression.

However, it is speculated that the cause of the markedly overexpression of miR-196-5p with a decrease in Derlin-1 expression in Saos2 cells in the present study emphasises inhibition of the unfolded protein response (UPR). In addition, the removal of misfolded proteins is crucial for normal cellular functioning. Derlin-1 is a component of the p97 ATPase complex and has been shown to play a deciding role in the elimination of misfolded / unfolded proteins by regulating the ER-mediated degradation response and retrotranslocation of abnormal cellular proteins from the lumen of the endoplasmic reticulum (ER) towards the cytosol for degradation by the proteasome complex (Katiyar *et al*., 2005).

The accumulation of misfolded proteins in the ER, a sign of disrupted ERAD (endoplasmic reticulum-associated degradation), supports the notion that Derlin-1 acts in the retro-translocation process. It is clear from the fact that loss of Derlin-1 results in lethality that ER homeostasis is crucial for the survival of animals. The results of these studies suggest that Derlin-1 and its associated retrotranslocation machinery (such as the TER94/Derlin-1 complex) are limited without ER stress. ER homeostasis is restored by synthesising additional Derlin-1 and complexing it with existing retro-translocation machinery (Liang *et al*., 2014).

Overexpression of Derlin-1 causes increased stress in the ER, with the activation of apoptosis-triggering signals. When moderate ER stress is present, Derlin-1 levels that were induced by low levels of ER stress are likely to retrotranslocate misfolded proteins. Severe or chronic ER stress induces Derlin-1 expression, resulting in a population of Derlin-1. It is well recognised that Derlin-1 binds to retrotranslocation components to restore homeostasis to the ER. However, an unbound Derlin-1 would lead to a worsening of ER stress, ensuring the triggering of pro-apoptotic signals (Liang *et al*., 2014).

A cell with irreparable ER stress may exhibit a high level of unbound Derlin-1 due to the need to balance proapoptotic and cytoprotective functions during UPR induced by ER stress-induced UPR. It is possible that Derlin-1 serves as a sensor for irreparable ER stress in cells. A prolonged ER stress appears to increase the levels of unbound Derlin-1 in cells, suggesting that UPR upregulates Derlin-1 (Liang *et al*., 2014). Although there is still a lack of knowledge about the exact mechanism by which excessive Derlin-1 induces UPR and apoptosis in cells, but we provide the evidence that comprises the effects of miR-196-5p overexpression targeting Derlin-1 involving autophagic processes. On the other hand, it is possible that there is an additional microenvironmental factor that induces cellular stress in tumor cells, such as a lack of interaction with the extracellular matrix, or diminishing contact between the cells, in addition to ER-stress (Klopfleisch *et al*., 2010). However, further detailed molecular investigations will be needed identifying factors that interact with miR-196-5p overexpression to define the mechanism that involves Derlin-1-dependent cytotoxicity for the treatment and subsequent progression of human osteosarcoma.

LIMITATIONS OF THE STUDY

However, interpretation of the results described above should take into account some potential limitations.

First, we used a single Saos2 cell line for the functional experiments. It is because Saso2 showed a higher invasive ability employing miR-196-5p than other used cell lines (MG-63, HOS) in our preliminary study.

Second, although our data found that miR-196-5p overexpression could be beneficial in the treatment of osteosarcoma by inhibiting Derlin-1 expression through autophagic processes, but to validate the specificity of miR-196-5p, it is imperative to investigate the use of miR-196-5p inhibitors to reverse the anticancer effects of miR-196-5p/Derlin-1 axis on cell growth and osteosarcoma metastasis.

Third, our data presented and confirmed the autophagic mechanisms involved in the effects of the miR-196-5p/Derlin-1 pathways on osteosarcoma, but it is fundamental to investigate further the effects of the miR-196-5p/Derlin-1 axis using autophagic inhibitors and different cell lines.

Fourth, although our findings showed the effects of miR-196-5p overexpression on apoptosis and autophagic processes, but further studies are warranted to investigate the cell cycle proteins involved in the processes of apoptosis and autophagy.

A fifth reason is that we were only able to examine Derlin-1 and miR-196-5p expression levels in a limited number of tissue samples to compare the differences between the different tumor specimens.

The sixth aspect is that further studies are required to clarify the molecular mechanisms underlying the relationship between the miR196/Derlin-1 axis and ER stress associated with UPR.

Lastly, *in-vitro* results were not supported by *in-vivo* experiments.

CONCLUSION

The current findings revealed that miR-196-5p overexpression leads to suppression of cell migration and invasion by direct targeting of Derlin-1 in Saos2 cells. Transient overexpression of miR-196-5p in Saos2 cancer cells inhibited cell growth by inducing autophagy *in vitro*. In Sao2 cells, miR-196-5p overexpression could inversely regulate Derlin-1 expression, suggesting that miR-196-5p acts as a tumor suppressor by binding to Derlin-1. Derlin-1 showed a functional post-transcriptional modulatory effect of miR-196-5p overexpression in osteosarcoma. Thus, the findings explored the regulatory miR-196-5p/Derlin-1 axis in controlling the proliferation, movement, and invasion of osteosarcoma cells. Our findings suggested that Derlin-1 may be useful in predicting the prognosis and identifying potential therapeutic targets in osteosarcoma.

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