

circKMT2D contributes to H₂O₂-attenuated osteosarcoma progression via the miR-210/autophagy pathway

JUN ZHANG, XUBIN CHOU, MING ZHUANG, CHENLEI ZHU, YONG HU, DONG CHENG and ZHIWEI LIU

Department of Spine Surgery, The Third Affiliated Hospital of Soochow University, Changzhou 213001, P.R. China

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Abstract. Circular RNAs (circRNAs) have been demonstrated to be involved in osteosarcoma (OS) development; however, the underlying mechanism of circKMT2D in OS progression remains unclear. The present study aimed to elucidate how circKMT2D could affect hydrogen peroxide (H₂O₂)-induced OS progression. H₂O₂ (100 μmol/l) was used to treat MG63 and U2OS cells. The cell viability, invasive ability, apoptosis and circKMT2D expression were detected using Cell Counting Kit-8 assay, Transwell assay, flow cytometry and reverse transcription-quantitative PCR, respectively. Furthermore, MG63 and U2OS cells transfected with circKMT2D short hairpin RNA and negative control were treated with H₂O₂, and circKMT2D expression and cell phenotype were determined. Dual-luciferase reporter assay was conducted to determine the association between circKMT2D and miR-210 expression level. Rescue experiments were conducted to examine the mechanisms through which circKMT2D and miR-210 could affect H₂O₂-treated MG63 cells. In addition, the effects of miR-210 on the expression of the autophagy-related proteins Beclin1 and p62 in H₂O₂-treated MG63 cells were detected by western blotting. An autophagy inhibitor was used to treat the MG63 cells, and whether miR-210 could affect the H₂O₂-treated MG63 cell phenotype through autophagy was investigated. The results demonstrated that H₂O₂ treatment promoted cell apoptosis and decreased cell viability, invasive ability and circKMT2D expression in MG63 and U2OS cells. Furthermore, circKMT2D knockdown decreased the cell viability and invasive ability and enhanced the apoptosis of H₂O₂-treated MG63 and U2OS cells. circKMT2D possessed binding sites for miR-210 and inhibited miR-210 expression. In H₂O₂-treated MG63 cells, miR-210 silencing partially reversed the circKMT2D knockdown-induced cell viability inhibition and apoptosis promotion. In addition, miR-210

elevated Beclin1 expression and decreased p62 expression in H₂O₂-treated MG63 cells. The use of the autophagy inhibitor partially reversed the miR-210 overexpression-induced promotion of apoptosis and inhibition of the viability and invasive ability of H₂O₂-treated MG63 cells. Taken together, these findings indicated that circKMT2D knockdown may contribute to the inhibition of H₂O₂-attenuated OS progression via miR-210/autophagy pathway.

Introduction

Osteosarcoma (OS) is a type of malignant tumor that often occurs in children and adolescents (1). As previously reported, the prevalence rate of OS increases by 0.3% per year (2). Similar to several other human malignancies, a high mortality rate, recurrence and metastasis are key factors leading to the poor prognosis of patients with OS (3). Surgical resection combined with adjuvant therapy, including chemotherapy and radiotherapy, has been reported to improve the prognosis of patients with OS to a certain degree (4). However, over the past two decades, no evident improvements have been observed in the survival rates of these patients (5).

Oxidative stress refers to an increased cell production of free radicals, including reactive oxygen species, ultimately resulting in damage to cells and tissues (6). A previous study demonstrated that tumor cells can be destroyed by increased oxidative stress (7). Inducing tumor cell apoptosis via increased oxidative stress may therefore be considered as a promising therapeutic strategy for malignancies (7). Hydrogen peroxide (H₂O₂) is a main intermediate of oxidative stress and is considered as a potent oxidant that induces cell senescence (8). In OS, it has been reported that treatment with 100 μM H₂O₂ decreases the viability and enhances the apoptosis of MG63 cells (9). However, the underlying mechanisms of H₂O₂ interference with OS cell phenotype remains unclear.

Circular RNA (circRNA) is a type of non-coding RNA that was discovered decades ago (10,11). Increasing evidence indicates that circRNAs play an important role in the development of human tumors (12). Salmena *et al* (13) proposed the competitive endogenous RNA mechanism (ceRNA mechanism) theory that gene transcripts, such as long-chain non-coding RNA, can influence mRNA expression by binding microRNAs (miRs) through competitive sponge attachment. In recent years, circRNAs have also been found to regulate mRNA expression through miRNA adsorption (14). A previous study has

Correspondence to: Dr Zhiwei Liu, Department of Spine Surgery, The Third Affiliated Hospital of Soochow University, 185 Juqian Street, Changzhou 213001, P.R. China
E-mail: zhiweiliu1213@163.com

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demonstrated that circ_0002052 inhibits OS progression by sponging miR-1205 (15). Huang *et al.* (16) reported that circ-NASP upregulates Forkhead box F1 expression by sponging miR-1253 in OS cells, thereby regulating the development of OS. These findings suggest that circRNAs could play a crucial role in the development of OS.

circKMT2D (also known as circRNA9920) is a recently discovered circRNA (17); however, its role in human tumors, including OS, remains unknown. In particular, we previously determined that circKMT2D expression is abnormally upregulated in H₂O₂-treated OS cells *in vitro* and that circKMT2D knockdown in H₂O₂-treated OS cells markedly decreased OS cell viability. Subsequently, it was hypothesized that circKMT2D could serve a regulatory role in the progression of OS. The effect of circKMT2D on H₂O₂-treated OS cell phenotype was therefore investigated in the present study. Furthermore, the role of miRNAs in regulating key pathways related to tumorigenesis may provide a powerful novel tool for diagnosis and targeted therapy. miR-210 has been reported to be involved in the occurrence and development of various types of cancer, including bladder cancer, glioblastoma and hepatocellular carcinoma (18-20). The clinical treatment of human tumors is in urgent need for the development of novel strategies to provide novel and reliable biomarkers and therapeutic targets (21). Therefore, the mechanisms through which circKMT2D may affect H₂O₂-attenuated OS cell progression were investigated by exploring the miR-210/autophagy pathway in the present study.

To the best of our knowledge, the present study was the first to investigate the role of circKMT2D in the progression of H₂O₂-attenuated OS cells. The findings from the present study may provide new insights into OS progression and may therefore provide a novel theoretical basis and a potential effective target for OS therapy.

Materials and methods

Cell lines. MG63 and U2OS cell lines were provided by The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin (Thermo Fisher Scientific, Inc.) and 100 µg/ml streptomycin (Thermo Fisher Scientific, Inc.) and placed at 37°C in a humidified incubator containing 5% CO₂.

Cell treatment with H₂O₂. MG63 and U2OS cells in the logarithmic phase were collected and prepared as dispersed cell suspensions in DMEM complete medium containing 100 µmol/l H₂O₂ (Beijing Solarbio Science & Technology Co., Ltd.) for 24 h at 37°C (22). The density of each cell suspension was 2x10⁶ cells/ml and each cell suspension was seeded into 6-well plates at a volume of 1 ml. All plates were maintained in an incubator at 37°C and 5% CO₂.

Transfection. MG63 and U2OS cells in the logarithmic phase were collected and seeded in 6-well plates at a density of 5x10⁵ cells/well. A total of 1 ml serum-free DMEM medium was then added into each well. When the cell confluence rate reached ~70%, MG63 and U2OS cells were transfected with

10 nM circKMT2D short hairpin (sh)RNA (shcircKMT2D group; 5'-UGAUGAAUGCCGGCUCUAG-3') and negative control (shNC group; 5'-UGACGGAUGCACGCUCUA G-3') using Lipofectamine™ 2000 transfection reagent (Thermo Fisher Scientific, Inc.). In addition, MG63 cells were subjected to transfection with 10 nM miR-210 mimic (miR-210 mimic group; 5'-UCAGUCCAUGGUAGAACU UCG-3'), miR-210 inhibitor (miR-210 inhibitor group; 5'-UCG CUUGUGUCAGGUCCGCAA-3') and miR-210 NC (NC group; 5'-GGACCGUAGCCACUGUGAGUU-3'), respectively. Furthermore, pcDNA3.1-circKMT2D vector (10 nM) was used to transfect the MG63 cells (pcDNA3.1-circKMT2D group). Co-transfection was also performed on the MG63 cells using 10 nM circKMT2D shRNA and 10 nM miR-210 inhibitor simultaneously (shcircKMT2D + miR-210 inhibitor group). All plates were maintained in an incubator at 37°C and 5% CO₂ for 6 h of transfection. Transfection was conducted using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The transfection efficiency was measured by reverse transcription-quantitative (RT-q)PCR. Subsequently, transfected cells were cultured in DMEM containing 10% FBS at a volume of 1 ml and were placed at 37°C and 5% CO₂ for 48 h. circKMT2D shRNA, shRNA NC and pcDNA3.1-circKMT2D vector were purchased from Genechem, Inc. miR-210 mimic, miR-210 inhibitor and miR-210 NC were provided by Shanghai GenePharma Co., Ltd..

Autophagy inhibitor treatment. MG63 cells in the miR-210 mimic group were incubated with DMEM complete medium containing 12.5 µg/ml 3-methyladenine (3-MA; Sigma-Aldrich; Merck KGaA) for 12 h at 37°C and 5% CO₂. These cells constituted the miR-210 mimic + 3-MA group.

Cell counting kit-8 (CCK-8) assay. Cells cultured at 48 h post-transfection were harvested and seeded in 96-well plates at a density of 2x10³ cells/well. Cells were incubated with 100 µl DMEM complete medium containing or not 100 µmol/l H₂O₂ for 48 h at 37°C. Subsequently, 10 µl CCK-8 solution (Dojindo Molecular Technologies, Inc.) was added to each well at 37°C for 2 h before reading the absorbance at 450 nm using a microplate reader. Each sample was assessed in triplicate and the experiments were repeated three times.

Transwell assay. Transwell assay was performed using an 8-µm polycarbonate filter culture chamber (96-well plates; EMD Millipore) pre-plated with Matrigel for 1 h at room temperature. At 48 h post-transfection, cells were harvested and were seeded in the upper chamber of the chamber at the density of 2x10⁵ cells per well. Serum-free DMEM (100 µl, with or without 100 µM H₂O₂) was also added to the upper chamber. The lower chamber was filled with 500 µl DMEM complete medium with three replicate wells per group. Cells were cultured for 36 h at 37°C and 5% CO₂. After the non-invasive cells were removed, the invading cells were washed twice with PBS, fixed for 10 min with 4% paraformaldehyde at room temperature for 20 min and stained with 0.1% crystal violet for 5 min. The invading cells were placed under a light microscope (Nikon Corporation; magnification, x200) and photographed.

Apoptosis detection. Cells cultured for 48 h were washed with PBS, harvested and centrifuged at room temperature for 5 min at 1,000 x g. A total of 500 μ l binding buffer was used to resuspend the cells, and 5 μ l AnnexinV-fluorescein isothiocyanate (FITC; cat. no. 40302ES20; Shanghai Yeasen Biotechnology Co., Ltd.) and 10 μ l propidium iodide (PI) were then added to the cells. Cells were evenly mixed with the reagent and were stored in the dark for 15 min at room temperature. The apoptosis rate of each cell sample was measured by flow cytometry (BD Biosciences) and analyzed using FlowJo software (version 7.6.1; FlowJo LLC).

RT-qPCR. Total RNA was extracted from the cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed into a cDNA template using the reverse transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturers' instructions. The PCR amplification reaction was carried out using cDNA as template with the following conditions: 95°C for 10 min, 95°C for 15 sec, 62°C for 30 sec, and 72°C for 30 sec. This process was cycled 40 times, followed by an extension at 72°C for 10 min. The relative expression levels of circKMT2D, miR-210, Beclin1 and p62 were calculated using the $2^{-\Delta\Delta Cq}$ method (23). U6 was used as the internal control for circKMT2D and miR-210, while GAPDH served as the internal control for Beclin1 and p62. The primer (Shanghai GeneChem) sequences used in the present study were as follows: circKMT2D forward, 5'-GAT TTAATATTAAGTACG-3', reverse, 5'-GTTATGGATCCC GGCATGGC-3'; miR-210 forward, 5'-ATGCCTGTGCGT GTGA-3', reverse, 5'-GTGCGTGTCTGGAGTC-3'; U6 forward, 5'-CGCTTCGGCAGCACATATACTA-3', reverse, 5'-CGCTTCACGAATTTGCGTGTCA-3'; Beclin1 forward, 5'-GATGGAAGGGTCTAAGACGTCCAA-3', reverse, 5'-TTT CGCCTGGGCTGTGGTAAG-3'; p62 forward, 5'-CCAGCA CCAAGAGCACGGACAGCG-3', reverse, 5'-TGGGGAGAA GAAGGGGACCACGAA-3'; and GAPDH forward, 5'-AAG GTCATCCCTGAGCTGAAC-3' and reverse, 5'-ACGCCT GCTTACCACCTTCT-3'.

Bioinformatic prediction and dual luciferase reporter assay. StarBase 2.0 (<http://starbase.sysu.edu.cn>) was used to predict whether the miRNAs could bind to circKMT2D. MG63 cells (1.5×10^4 cells/well) in the NC group, miR-210 mimic group and miR-210 inhibitor group were seeded into 6-well plates at logarithmic growth phase. Following 24 h culture, pmirGLO-circKMT2D-Mutant-type (Promega Corporation) and pmirGLO-circKMT2D-Wild-type (Promega Corporation) luciferase reporter vectors were used to transfect these cells. Transfection was performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Three replicate wells were set in each group. Following 24 h of incubation at 37°C and 5% CO₂, the relative firefly and *Renilla* luciferase activities were detected using a Dual-Luciferase Reporter assay system (Promega Corporation). Firefly luciferase activity was normalized to that of *Renilla* luciferase.

Western blotting. Cells were harvested and lysed using RIPA buffer (Thermo Fisher Scientific, Inc.). Protein concentration was detected using a BCA kit (Beyotime Institute of Biotechnology). Proteins (20 μ g) were separated by 10%

SDS-PAGE and transferred to PVDF membranes. Membranes were washed three times with TBST (0.1%) and blocked with 5% non-fat milk for 2 h at room temperature. Membranes were incubated with rabbit anti-mouse Beclin1 (1:1,000; Cell Signaling Technology, Inc.) and p62 (1:1,000; Cell Signaling Technology, Inc.) primary antibodies for 12 h at 4°C. Membranes were washed with TBST and were incubated with goat anti-rabbit secondary antibody (1:2,000, Beijing Solarbio) for 2 h at room temperature. Enhanced chemiluminescence chromogenic solution (Beijing Solarbio Science & Technology Co., Ltd.) was used to detect the signal on the membrane. The data were analyzed via densitometry and normalized to expression of the internal control GAPDH. Protein expression was evaluated using Image-Pro® Plus software (version 6.0; Media Cybernetics, Inc.).

Statistical analysis. The data were expressed as the means \pm standard deviation. SPSS 19.0 software (IBM Corp.) was used for statistical analysis. Comparison between two groups was carried out using Student's t-test. Comparison between three groups was performed using ANOVA followed by Tukey's test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

H₂O₂ treatment inhibits OS progression and decreases circKMT2D expression in OS cells. MG63 and U2OS cells were treated with H₂O₂, and cell viability, invasive ability and apoptosis were detected. The results demonstrated that MG63 and U2OS cells treated with H₂O₂ exhibited a significantly lower cell viability and invasive ability, and a significantly increased apoptotic rate compared with untreated cells (Fig. 1A-C). In addition, circKMT2D expression level was significantly decreased in H₂O₂-treated MG63 and U2OS cells compared with control cells (Fig. 1D).

circKMT2D knockdown accentuates H₂O₂-treated OS cell viability and invasion inhibition. Compared with the shNC group, MG63 and U2OS cells in the shcircKMT2D group exhibited a significantly lower circKMT2D expression level (Fig. 2A), indicating that circKMT2D was successfully knocked down in MG63 and U2OS cells. The results of CCK-8 assay, Transwell assay and apoptosis detection indicated that MG63 and U2OS cells in the shcircKMT2D group exhibited a significantly lower cell viability and invasion ability, and a significantly higher apoptotic rate compared with cells in the shNC group (Fig. 2B-D).

circKMT2D possesses binding sites for miR-210 and directly inhibits miR-210 expression. The binding site between circKMT2D and miR-210 was predicted by Starbase V2.0 and is illustrated in Fig. 3A. The results from dual luciferase reporter assay reported that, compared with the luciferase activity of wild-type circKMT2D in MG63 cells of the NC group, a significantly decreased luciferase activity was observed in the miR-210 mimic group, and a significantly increased luciferase activity was demonstrated in the miR-210 inhibitor group. However, compared with the luciferase activity of mutant circKMT2D in the NC group, no changes were observed in the

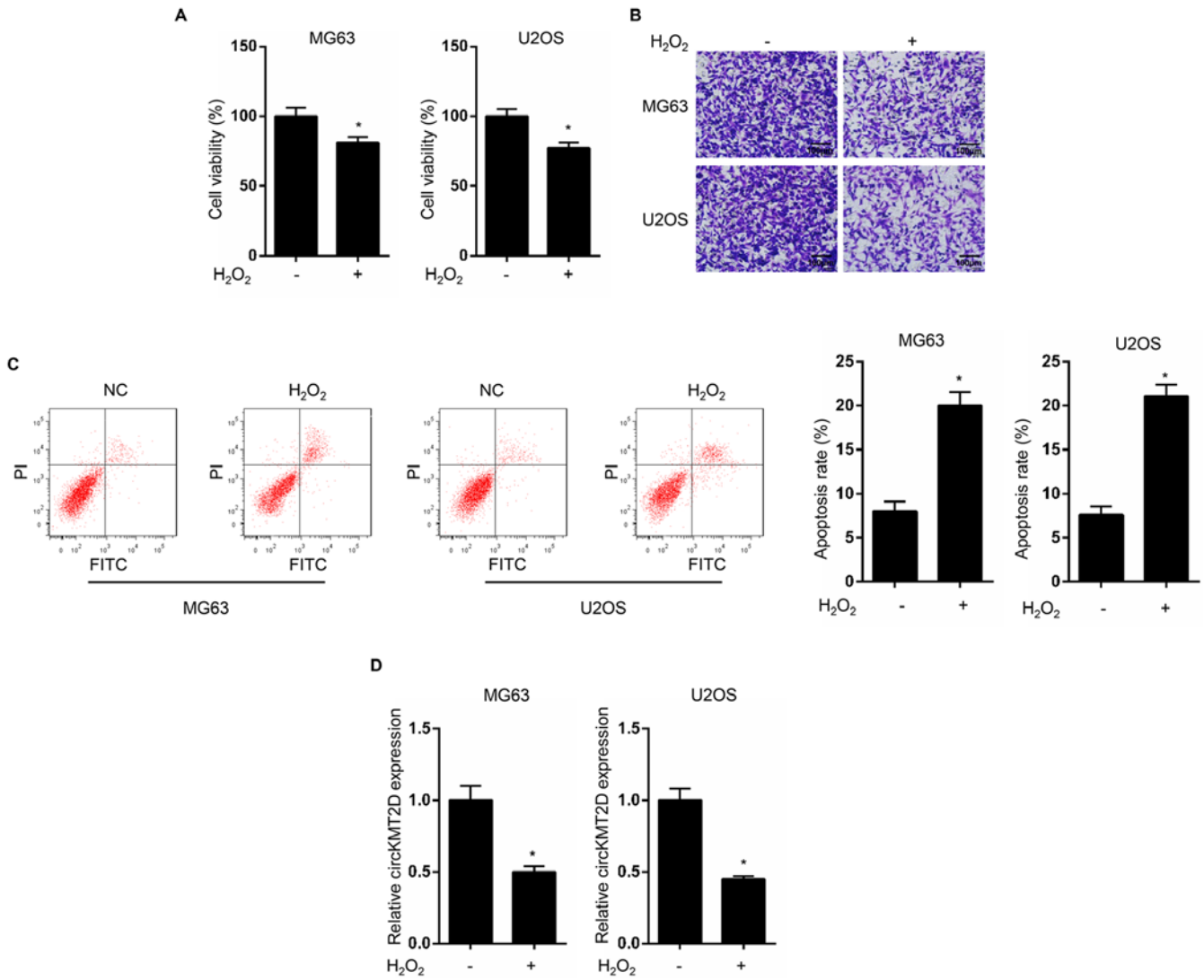


Figure 1. H₂O₂ treatment inhibited OS progression and decreased circKMT2D expression in OS cells. (A) MG63 and U2OS cells were treated with 100 μM H₂O₂ and cell viability was detected using Cell Counting Kit-8 assay. (B) After treatment with 100 μM H₂O₂, MG63 and U2OS cells invasive ability was measured by Transwell experiment. (Magnification, x100; scale bar, 100 μm). (C) MG63 and U2OS cell apoptosis rate was detected by flow cytometry following treatment with 100 μM H₂O₂. (D) circKMT2D expression in MG63 and U2OS cells was detected by reverse transcription quantitative PCR following treatment with 100 μM H₂O₂. *P<0.05. NC, negative control; FITC, fluorescein isothiocyanate; PI, propidium iodide; H₂O₂, hydrogen peroxide.

miR-210 mimic and miR-210 inhibitor groups (Fig. 3B). Thus, miR-210 is a target gene of circKMT2D, and its expression is directly inhibited by circKMT2D.

To further verify the regulatory association between the two genes, MG63 cells were transfected with NC, miR-210 mimic and miR-210 inhibitor and the expression of circKMT2D and miR-210 was detected. The results demonstrated that, compared with the NC group, a significantly higher miR-210 expression and a significantly lower circKMT2D expression were found in the miR-210 mimic group. However, when compared with the NC group, MG63 cells transfected with miR-210 inhibitor exhibited a significantly lower miR-210 expression and a higher circKMT2D expression (Fig. 3C). In addition, MG63 cells in the pcDNA3.1-circKMT2D group exhibited a significantly higher circKMT2D expression and a significantly lower miR-210 expression compared with those in the shNC group; however, cells in the shcircKMT2D group exhibited a lower circKMT2D expression and a significantly

increased miR-210 expression compared with those in the shNC group (Fig. 3D). These findings further confirmed that miR-210 expression may directly be inhibited by circKMT2D.

miR-210 silencing partially reverses the OS cell phenotype induced by circKMT2D knockdown. miR-210 expression in shNC, shcircKMT2D and shcircKMT2D + miR-210 inhibitor groups was assessed. A significantly higher miR-210 expression was observed in the shcircKMT2D group compared with the shNC group. However, compared with the shcircKMT2D group, the expression of miR-210 was significantly decreased in the shcircKMT2D + miR-210 inhibitor group (Fig. 4A). Furthermore, compared with the shNC group, MG63 cells in the shcircKMT2D group exhibited a significantly lower cell viability and invasive ability, and a significantly higher apoptotic rate. However, MG63 cells in the shcircKMT2D + miR-210 inhibitor group exhibited a significantly higher cell viability and invasive ability, and a significantly lower

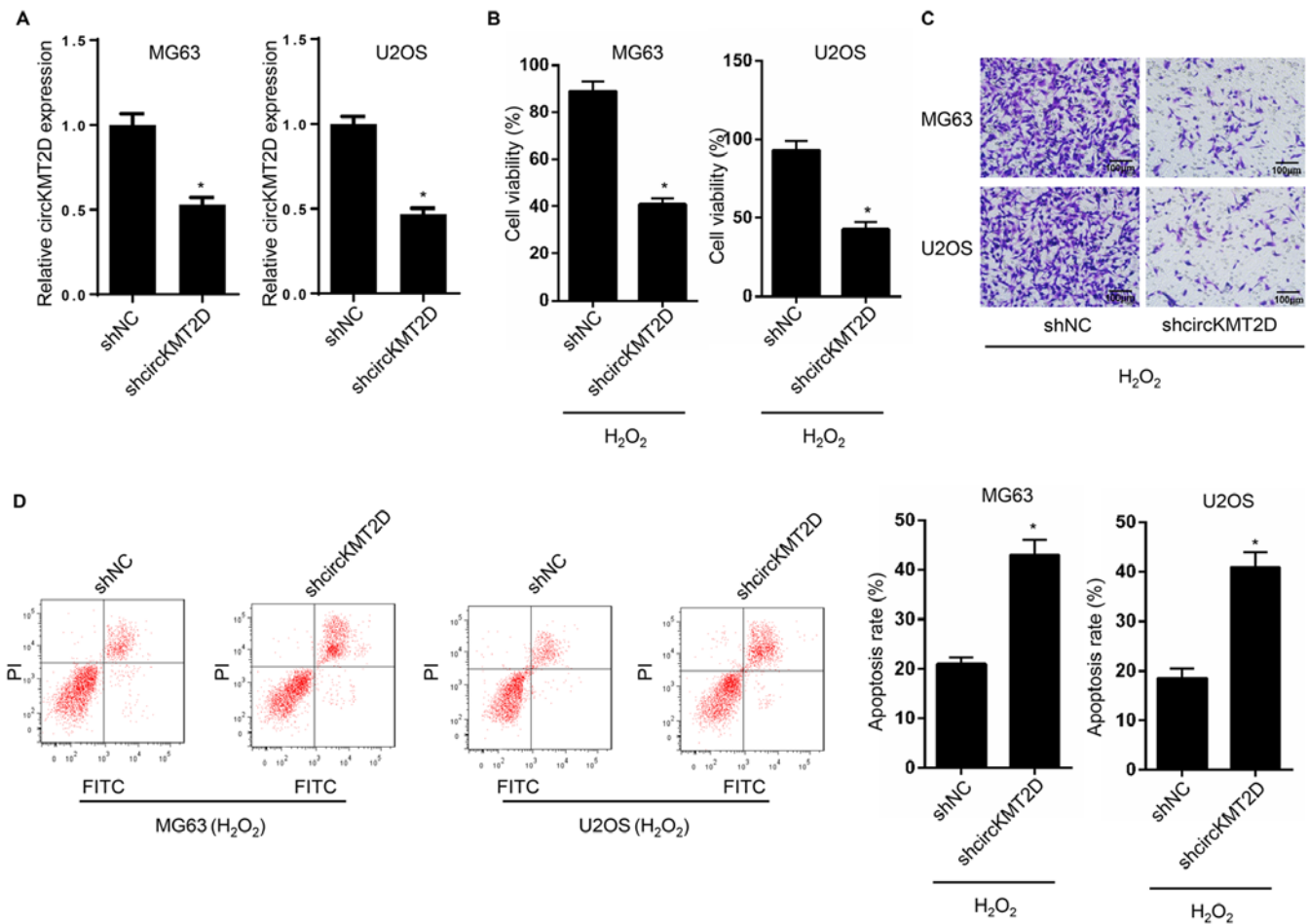


Figure 2. Knockdown of circKMT2D contributed to H_2O_2 -induced OS cells viability and invasion inhibition. (A) MG63 and U2OS cells were transfected and circKMT2D expression was detected by reverse transcription quantitative PCR. (B) After transfection, MG63 and U2OS cell viability was evaluated using the Cell Counting Kit-8 assay. (C) After transfection, MG63 and U2OS cell invasion ability was detected by Transwell experiment (Magnification, $\times 100$; scale bar, $100 \mu m$). (D) Following transfection, MG63 and U2OS cell apoptosis was detected by flow cytometry. * $P < 0.05$. NC, negative control; FITC, fluorescein isothiocyanate; PI, propidium iodide; H_2O_2 , hydrogen peroxide; sh, short hairpin.

apoptotic rate compared with those in the shcircKMT2D group (Fig. 4C and D).

miR-210 promotes OS cell autophagy. Beclin1 and p62 are genes associated with autophagy. In the present study, the expression of Beclin1 and p62 in MG63 cells was determined. The results from RT-qPCR demonstrated that MG63 cells in the miR-210 mimic group exhibited a significantly higher Beclin1 expression level and a significantly decreased p62 expression level compared with those in the NC group. However, compared with the NC group, a significantly lower Beclin1 expression level and a significantly decreased p62 expression level were observed in the miR-210 inhibitor group (Fig. 5A). These results were similar to those observed following western blotting analysis (Fig. 5B).

Autophagy inhibitor reverses the OS cell phenotype induced by miR-210 overexpression. The autophagy inhibitor 3-MA was used to treat MG63 cells transfected with miR-210 mimic. MG63 cells in the miR-210 mimic group exhibited a significantly decreased cell viability and invasive ability, and a significantly higher apoptotic rate compared with those in the NC group. However, compared with the miR-210 mimic

group, a significantly elevated viability and invasive ability, and a significantly decreased apoptotic rate were observed in the miR-210 mimic + 3-MA group (Fig. 6A-C). These findings suggested that circKMT2D knockdown may contribute to H_2O_2 -attenuated OS progression inhibition via the miR-210/autophagy pathway (Fig. 6D).

Discussion

OS is the most common primary malignant bone tumor. It mostly occurs in individuals under 20 years of age and is characterized by a rapid speed of deterioration (24,25). The prognosis of patients with OS is poor due to invasion and metastasis occurring in the early stages of the disease (26). Currently, OS treatment with chemotherapy has limited therapeutic efficacy as well as unsatisfactory side effects, in particular for relapsed and advanced OS (27). H_2O_2 is one main contributor of oxidative damage (28). Enhanced metabolic activity is one of the key features of tumor cells, and overproduction of H_2O_2 induces increased oxidative stress (29). Continuously elevated H_2O_2 levels can promote the survival of tumor cells and may therefore promote tumor cell ability to adapt to the tumor microenvironment. However, high levels of H_2O_2 can also

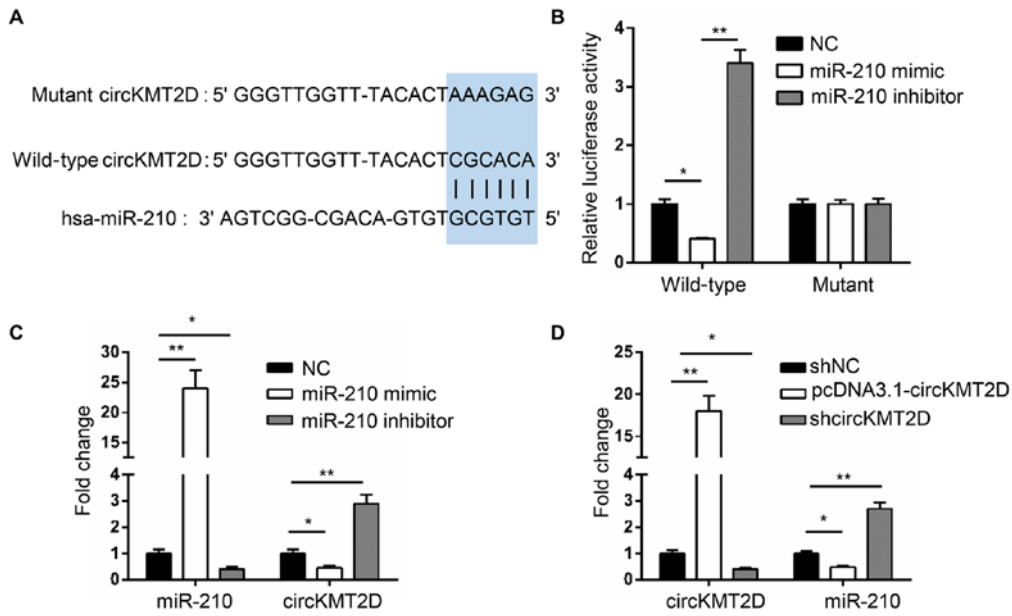


Figure 3. Expression of miR-210 and circKMT2D was mutually inhibited. (A) Binding site between circKMT2D and miR-210 was predicted by Starbase V2.0. (B) Dual luciferase reporter gene assay was used to analyze the regulatory relationship between circKMT2D and miR-210. (C and D) After transfection, circKMT2D and miR-210 expression in MG63 cells was detected by reverse transcription quantitative PCR. *P<0.05 and **P<0.01. NC, negative control; FITC, fluorescein isothiocyanate; PI, propidium iodide; H₂O₂, hydrogen peroxide; sh, short hairpin; miR, microRNA.

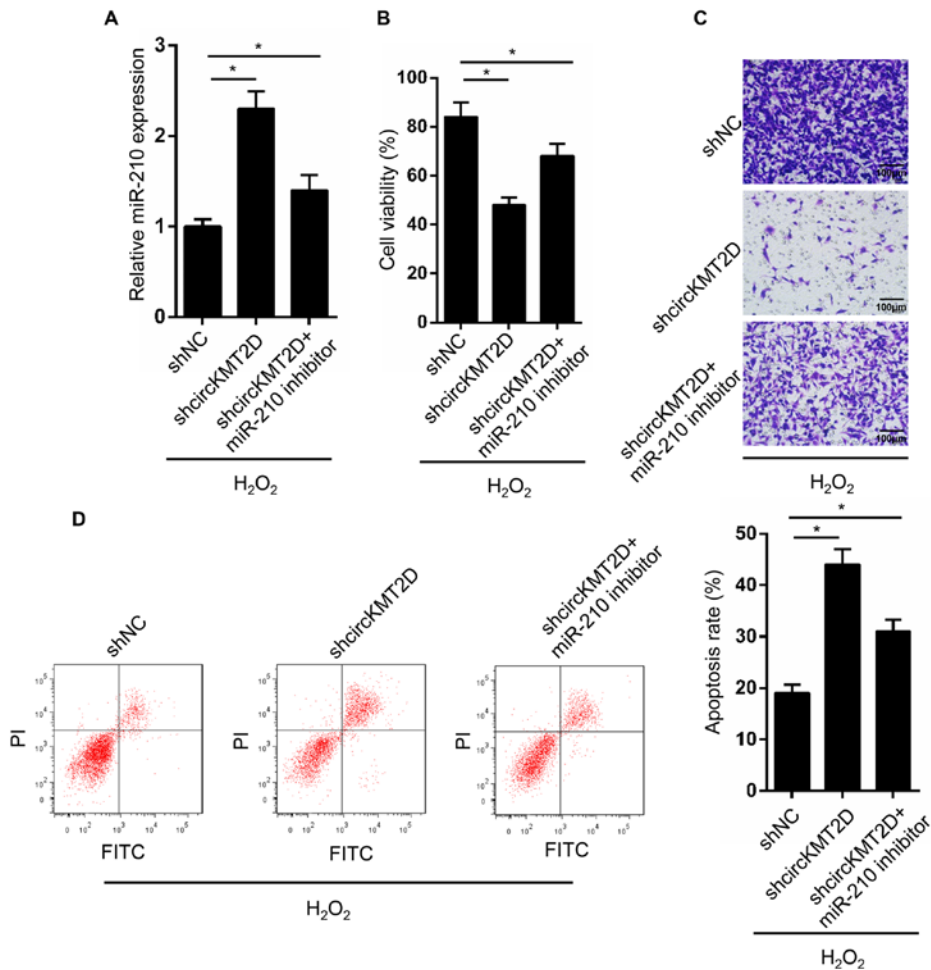


Figure 4. Silencing of miR-210 partially reversed OS cells phenotype induced by circKMT2D knockdown. (A) MG63 cells were transfected and miR-210 expression in MG63 cells of each group was detected by reverse transcription quantitative PCR. (B) After transfection, MG63 cell viability was evaluated using the Cell Counting Kit-8 assay. (C) After transfection, MG63 cell invasive ability was detected by Transwell experiment (Magnification, x100; scale bar, 100 μm). (D) After transfection, MG63 cell apoptosis was detected by flow cytometry. *P<0.05. NC, negative control; FITC, fluorescein isothiocyanate; PI, propidium iodide; H₂O₂, hydrogen peroxide; sh, short hairpin; miR, microRNA.

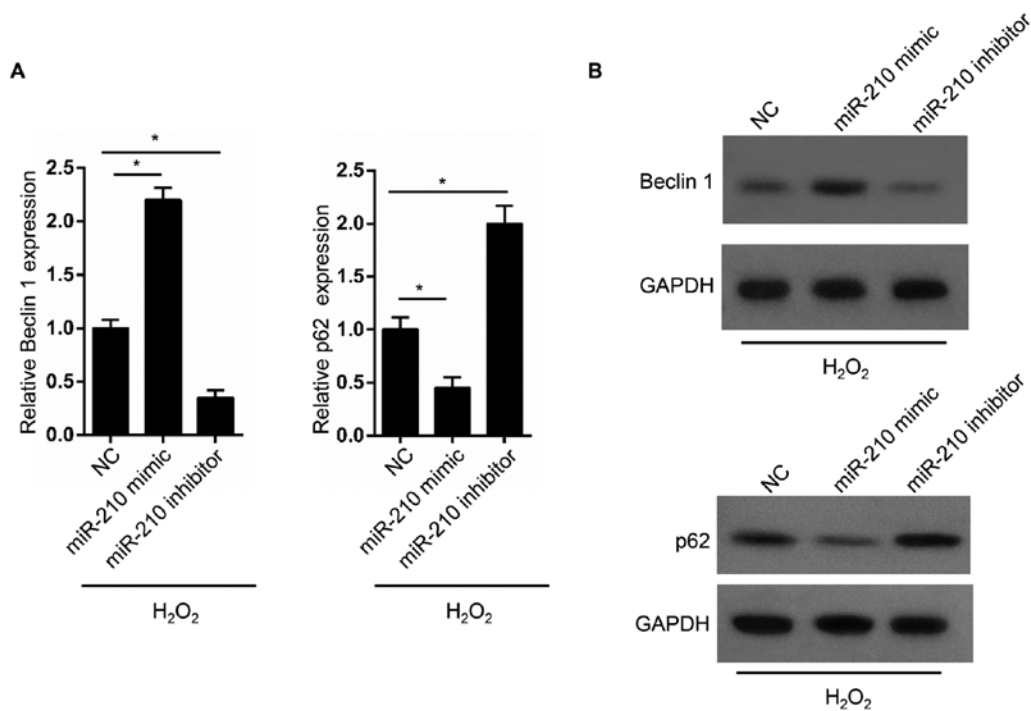


Figure 5. miR-210 promoted autophagy in OS cells. (A) MG63 cells were transfected and Beclin1 mRNA and p62 mRNA expression was detected by reverse transcription quantitative PCR. (B) After transfection, Beclin1 and p62 protein expression in was detected by western blotting. * $P < 0.05$. NC, negative control; H_2O_2 , hydrogen peroxide; miR, microRNA.

induce cell cycle arrest and cell death by apoptosis, thereby inhibiting the progression of tumors (30). Park (31) reported that $100 \mu M H_2O_2$ suppressed the proliferation of lung cancer cells by arresting the cell cycle and inducing cells necrosis or apoptosis. In 2003, Ogawa *et al* (32) reported that potent oxidative stress caused by exogenous H_2O_2 induces the apoptosis of human OS cells. A recent study also demonstrated that treatment with H_2O_2 at a concentration of $100 \mu M$ resulted in a significant decrease in OS cell viability and an increase in apoptosis (9). The present study also confirmed that $100 \mu M H_2O_2$ inhibited OS cell viability and invasive ability and promoted OS cell apoptosis. The underlying mechanism may involve the inhibition of circKMT2D expression.

To the best of our knowledge, the effects of circKMT2D on human tumors have not yet been reported. The present study demonstrated for the first time that circKMT2D may function as a cancer-promoting gene in OS. The regulatory effects of circRNAs on OS progression have been previously reported. Zhang *et al* (33) indicated that circUBAP2 enhanced the progression of OS by acting as a sponge of miR-143. Liu *et al* (34) reported that circNT5C2 promoted the metastasis of OS by targeting miR-448. However, circ0002052 can inhibit OS development by inhibiting OS cell proliferation and invasion, and by promoting apoptosis via targeting miR-1205 (15). In the present study, the cancer-promoting effects of circKMT2D in OS were discovered for the first time, and circKMT2D knockdown contributed to the inhibition of H_2O_2 -attenuated OS progression by targeting miR-210.

Accumulating evidence indicates that miRNAs, as new types of diagnostic molecules and markers, have great prospects in targeted therapy of tumors, including OS (35,36). Fan *et al* (37) screened some key genes involved in the

development of OS, including miRNAs, which may be related to the metastasis of OS. In several human malignancies, such as breast cancer, malignant peripheral nerve sheath tumors and renal cell carcinoma, miR-210 has been found to promote tumor development due to its upregulation and the enhancement of tumor cell proliferation or invasive ability (38-40). In OS, it has been reported that miR-210 functions as an oncogene. It promotes the migration and invasion of OS cells and is closely related to the aggressive development of OS (41,42). miR-210 has also been demonstrated to promote the dedifferentiation of OS cells (43). However, in the present study, rescue experiments revealed that the silencing of miR-210 partially reversed the H_2O_2 -induced OS cell phenotypes induced by circKMT2D knockdown. miR-210 may therefore function as a tumor suppressor in OS and may serve as a potential target for targeted therapy and diagnosis of OS. Further experiments indicated that miR-210 may regulate the development of OS by interfering with autophagy in OS cells.

Autophagy is present in eukaryotes and plays an important role in cellular homeostasis, which is usually activated during adverse microenvironmental stress (44,45). During the autophagy process, organelles with damaged or aged structures and unwanted biomacromolecules are relocated to lysosomes for digestion and degradation, and autophagic products can be utilized by cell reconstruction to provide material basis and energy for cell survival (46,47). Numerous studies have demonstrated that the loss or inhibition of autophagy may lead to the development of a variety of tumors, including OS (48-50). A previous study has reported that the damage of lysosomal degradation function can lead to the accumulation of autophagosomes, whereas excess autophagosome accumulation and autophagy degradation blocking play important

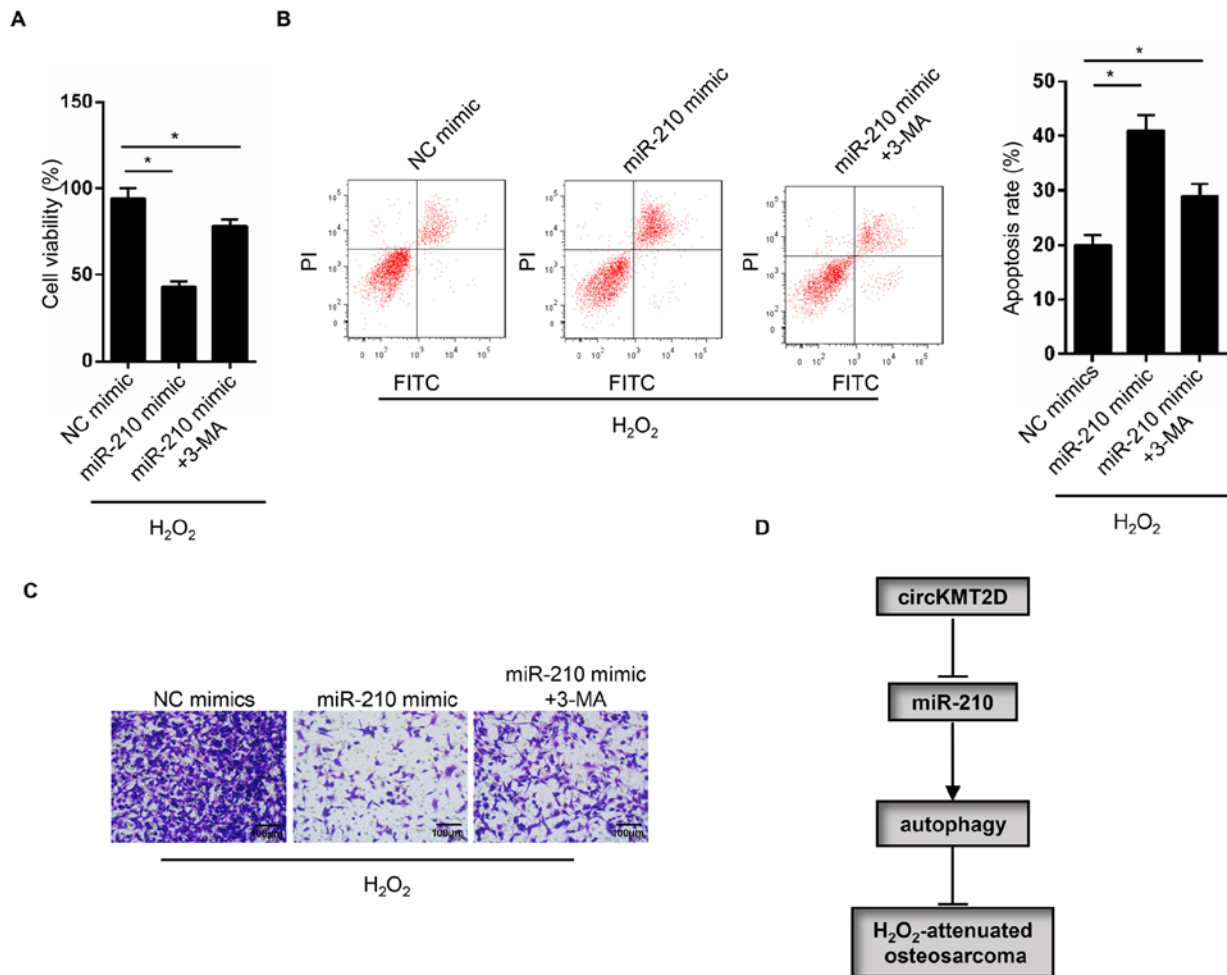


Figure 6. Autophagy inhibitor promoted the suppressed OS cell phenotype induced by miR-210 overexpression. (A) MG63 cells were transfected and treated with 3-MA. MG63 cell viability was evaluated using the Cell Counting Kit-8 assay. (B) MG63 cells were transfected and treated with 3-MA. MG63 cell apoptosis was detected by flow cytometry. (C) MG63 cells were transfected and treated with 3-MA. MG63 cell invasive ability was evaluated by Transwell experiment (Magnification, x100; scale bar, 100 μ m). (D) Schematic diagram of findings from the present study. * P <0.05. NC, negative control; FITC, fluorescein isothiocyanate; PI, propidium iodide; H₂O₂, hydrogen peroxide; miR, microRNA; 3-MA, 3-methyladenine.

roles in OS cell death (51). Beclin-1 is a key regulatory protein in autophagy and its downregulation has been confirmed to promote the progression of several human cancers, including OS (52,53). Under normal conditions, the p62 level is very low in cells, since it is degraded in selective autophagy. However, p62 will accumulate in cells in the state of autophagy inhibition and autophagy defects (54). The accumulation of p62 can lead to the activation of NF- κ B and stabilize Nrf2, whereas activated NF- κ B can promote tumorigenesis and Nrf2 can enhance the tolerance of tumor cells to hypoxia-induced stress (55,56). In the present study, miR-210 overexpression elevated Beclin1 expression and decreased p62 expression in OS cells. miR-210 may therefore function by promoting autophagy to hinder the development of OS.

The PI3K/AKT/mTOR pathway has been demonstrated to be transcriptionally activated in OS, and the silencing of Wilms tumor gene 1 can inhibit the proliferation of OS cells by inactivating the PI3K/AKT pathway (57,58). Researchers have discovered that the enhancement of the PI3K/AKT/mTOR pathway inhibits doxorubicin-induced autophagy in OS cells and the diallyl disulfide-induced autophagic death of OS cells by inhibiting the PI3K/Akt/mTOR signaling pathway (59-61).

It is therefore hypothesized that circKMT2D and miR-210 may affect autophagy through the PI3K/AKT/mTOR signaling pathway, thereby regulating the development of OS. This hypothesis will be further investigated.

In conclusion, the present study reported the potential molecular mechanisms of circKMT2D in H₂O₂-attenuated OS development. To the best of our knowledge, the present study was the first to demonstrate that circKMT2D knock-down contributed to the inhibition of H₂O₂-attenuated OS progression via the miR-210/autophagy pathway. These findings provided a theoretical basis for the treatment of OS with H₂O₂. In addition, circKMT2D may be considered as a novel target for the targeted therapy of OS, which will be further investigated in future studies.

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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

JZ, ZL and XC designed the present study. MZ, CZ and DC performed the experiments. YH and JZ analysed the data and prepared the figures. JZ and XC drafted the initial manuscript. ZL reviewed and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by Ethics Committee of The Third Affiliated Hospital of Soochow University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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