

MiR-335 functions as a tumor suppressor and regulates survivin expression in osteosarcoma

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Abstract. – **OBJECTIVE:** Previous studies have shown that miR-335 plays an anti-tumor role in several types of cancer. However, whether it is able to regulate the tumorigenesis of osteosarcoma (OS) has not been fully investigated. The present study was designed to study its potential role in regulating apoptosis of OS cells.

MATERIALS AND METHODS: The expression of miR-335 in a total of 18 paired OS tumor tissues and adjacent non-cancerous tissues was measured by Real-time PCR, and its different expression in OS cell lines was also measured. The effect of miR-335 on apoptosis was measured by MTT assay, caspase-3 activity assay and TUNEL assay. The effect of survivin inhibition on apoptosis of OS cells was determined by MTT assay and western blot. Luciferase reporter assay and western blot were conducted to confirm the relationship between miR-335 and the 3'UTR of survivin mRNA.

RESULTS: MiR-335 expression was found to be significantly downregulated in OS tumor tissues and OS cell lines. Overexpression of miR-335 led to decreased cell viability and increased apoptosis. MiR-335 directly targeted the 3'UTR of survivin mRNA and suppressed survivin gene expression, and inhibition of survivin exhibited similar effects to miR-335 overexpression.

CONCLUSIONS: MiR-335 might function as a tumor suppressor in OS, and downregulation of miR-335 in OS cells contributes to the decreased apoptotic potential of OS cells through derepression of survivin.

Key Words:

Osteosarcoma, miR-335, Survivin, Apoptosis.

Introduction

Osteosarcoma (OS) is one of the most frequently diagnosed malignancies among teenagers all over the world¹. The treatment of OS is largely dependent on surgery and chemotherapy; however,

the therapeutic efficacy varies among patients because of metastasis and chemotherapeutic drug resistance. Therefore, elucidating the molecular mechanisms for the pathogenesis of OS is of vital importance in identifying new effective drugable biological molecules.

Recent reports have shown that microRNAs (miRs), a family of small non-coding RNAs that are often conserved among species, play crucial roles in the development and progression of multiple cancers. MiRs are endogenously expressed, and it suppresses critical genes through its binding with the complementary sequences locate in the 3'UTR of its potentially targeted mRNAs². In OS, several miRs are differentially expressed, and most of which are reported to play essential roles in regulating cell death, proliferation and differentiation, etc³⁻⁶. Previous studies have demonstrated the antitumor role of miR-335 in several cancer types and downregulation of miR-335 was detected in gastric cancer, breast cancer and hepatocellular carcinoma⁷⁻⁹. However, there are evidences showing that, opposite to the findings in the above-mentioned cancers, miR-335 is upregulated and promotes tumorigenic activity in meningiomas and glioma^{10,11}. Therefore, the actual function of miR-335 in OS is still enigmatic. Although Wang et al¹² recently have revealed that miR-335 is also downregulated in osteosarcoma and it suppressed the migrative and invasive capacity of OS cells by targeting ROCK1, it is still unclarified whether miR-335 is involved in other critical cellular processes associated with the OS tumorigenicity such as apoptosis and cell cycle regulation.

Herein, we showed that miR-335 expression was significantly downregulated in human OS tumor tissues compared with that in adjacent non-cancerous tissues. The gain of function by transfection of miR-335 mimics in OS cell line

MG-63 significantly altered the phenotype of cell proliferation and apoptosis. Survivin, an oncogene which is likewise, associated with the prognosis of OS, was then established as the functional target of miR-335. Our study may, thus, reveal a novel pro-apoptotic role of miR-335 in OS and give a hint for the clinical treatment of patients with OS.

Materials and Methods

Tumor Sample Collection

A total of 18 OS tumor tissues and the matched adjacent non-cancerous tissues were collected from surgeries. All the patients did not undergo chemotherapy or radiotherapy before surgeries. The diagnosis of each tumor sample was approved by an independent, experienced pathologists. The tissues were snap-frozen with liquid nitrogen and transferred to -80°C before use. Each patient was asked to sign an informed consent about the use of the tissues for scientific research purpose. The study protocol was approved by the ethics committee of Xinjiang Uygur Autonomous Region Chinese Medicine Hospital.

Cell Culture, Drug Treatment and Transfection

The two human OS cell lines (MG63 and Saos2) were purchased from the cell bank of Peking Union Medical College (Beijing, China), and the normal human osteoblast cell line hFOB 1.19 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM/F12 medium supplemented with 10% FBS (HyClone, Logan, UT, USA). To avoid bacterial contamination, penicillin/streptomycin solution (Gibco, Grand Island, NY, USA) was supplemented to culture medium according to the dose recommended by the manufacturer. Cells were maintained in a CO₂ incubator with humidified atmosphere at 37°C. In order to overexpress or inhibit miR-335 in OS cells, miR-335 mimics or the antisense inhibitor (anti-miR-335) was transfected. MiR-335 mimics, anti-miR-335 and their corresponding negative controls (NC) were all synthesized by Genepharma Biotechnology Inc. (Shanghai, China). The small nucleotides were transfected into OS cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To inhibit survivin expression, both pharmacologic inhibitor YM-

155 (Selleck Chemicals, Houston, TX, USA) and small interference RNA (siRNA) were used. YM-155 was used at the final concentration of 50 nM. SiRNA for survivin and the negative control were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). In the same manner, the siRNAs were transfected with the microRNA transfection protocol described above.

Cell Viability Assay

In order to determine the cell viability after experimental treatment, an MTT assay was employed. Briefly, cells were seeded in 96-well plates. After transfection with miR-335 mimics or the negative control, cells were allowed to incubate for 48h or 72h. After the desired incubation, 20 µl MTT (5 mg/ml) was added to each well. Cells were incubated with MTT reagent for 4h to develop formazan. 200 µl DMSO was then pipetted to each well to resolve the formazan, and the purple color was visualized as a consequence. The optical density (OD) at the wave length of 490nm was acquired using a spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

Caspase-3 Activity Assay

Caspase-3 activity was determined by a kit purchased from Beyotime Biotechnology, Inc. (Shanghai, China). Cells grown in 6-well plates were transfected with miR-335 mimics or miR-NC and further incubated for 72h. Cells were then treated with trypsin (0.25%) and collected in culture medium followed by centrifuge at 600 rpm for 5 min. After washing with PBS, cells were treated with the kit-supplied lysis buffer. The cell debris was discarded after centrifuging at 13500 rpm, 4°C for 10 min. The supernatant was then incubated with caspase-3 substrate Ac-DEVD-pNA at 37°C for 1h. The reaction mixture with Ac-DEVD-pNA was then transferred into 96-well plates and detected for the OD value at 405 nm wave length using a spectrophotometer. Caspase-3 activity was converted from the OD value using the standard curve prepared in each assay. For the relative caspase-3 activity, Caspase-3 activity of miR-335 transfected cells was then normalized to that of miR-NC transfected cells.

TUNEL (TdT-mediated dUTP Nick-End Labeling) Assay

In order to detect the apoptosis rate, TUNEL assay was employed to monitor the DNA fragmentation. Cells were plated on coverslips and subsequently transfected with miR-335

mimics or miR-NC at 80% confluence. Fixation was performed by incubating cells with 4% paraformaldehyde, and cells were then treated with 0.1% Triton X100 for 5 min. 50 µl TUNEL reaction mix ((Roche, Mannheim, Germany) was applied to each coverslip. The coverslips were incubated at 37°C for 1h and then stained with DAPI. The images were taken under an Olympus fluorescent microscopy (Tokyo, Japan).

Luciferase Activity Assay

A segment of BIRC5 (survivin) 3'UTR containing the putative binding site of miR-335 was amplified by PCR and cloned into the pGL3 plasmid (Promega, Madison, WI, USA) to construct a firefly expressing reporter. pGL3, pRL (coding for internal control luciferase) and miR-335 mimics or anti-miR-335 were co-transfected into MG-63 cells. 48h after transfection, luciferase activity was assayed using the Dual-Luciferase Report Assay System (Promega) as per manufacturer's protocol.

Real-time PCR

RNA from the tumor specimens or the cells was extracted by a miRNeasy Mini kit (QIAGEN, Hilden, Germany) according to the instructions provided by the manufacturer. The RNA samples were transcribed using the Bulge-loop RT primer (RiboBIO, Guangzhou, China), and the PCR amplification was conducted with an ABI PRISM 7700 real-time PCR system (Applied Biosystems, Foster City, MA, USA). The difference in the expression of miR-335 between samples was compared after normalization to the expression of U6.

Western Blot

To analyze the protein level of survivin, whole cell lysates were obtained using the western and IP lysis buffer (Beyotime, Nanjing, Jiangsu, China). The cell lysates were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis. Proteins in the SDS-PAGE gel were then transferred onto polyvinylidene fluoride (PVDF) membranes and blocked with 5% bovine serum albumin (BSA) at room temperature for 1.5h. The membranes were incubated with primary antibodies against cleaved-caspase-3, survivin and beta-actin (Santa Cruz) overnight at 4°C. After washes with PBST (PBS+0.5% Tween 20), HRP secondary antibodies were applied to the membrane. Finally, the protein bands were visualized by an ECL detection system (Beyotime).

Statistical Analysis

The difference of miR-335 between normal tumor tissues and the adjacent non-cancerous tissues was compared by *t*-test. For comparisons of three or more groups, one-way ANOVA was used, pairwise comparisons were conducted with Bonferroni post-hoc test. $p < 0.05$ was set as the criteria of statistical significance. All data were expressed as mean $\pm$ SD, every experiment was repeated for 3 times.

Results

MiR-335 is Downregulated in OS Tissues and Cell Lines

Firstly, we tested whether there exists a differential expression of miR-335 in OS tissues and cell lines. We collected 18 paired OS tumor samples and the adjacent non-cancerous tissues. RNA from these samples was extracted and subjected to real-time PCR analysis. We observed a remarkable decrease of miR-335 expression in OS tissues (Figure 1A). For *in vitro* analysis, we compared miR-335 expression in normal human osteoblast cell line (hFOB1.19) and two human OS cell lines (Saos-2 and MG-63). MiR-335 expressed at lower levels in both OS cell lines compared with that in hFOB1.19 (Figure 1B). Moreover, the expression level of miR-335 in MG-63 was the lowest; therefore, MG-63 was chosen for further studies.

MiR-335 Induces Apoptosis in OS Cells

To address the importance of miR-335 downregulation in OS cells, we transfected MG-63 cells with miR-335 mimics or the negative control miR (miR-NC). MTT assay revealed that cells overexpressing miR-335 showed a decreased growth rate and viability at the time points of 48h and 72h (Figure 2A). We performed caspase-3 activity assay and found that caspase-3 activity was significantly increased 72h after miR-335 transfection (Figure 2B). Furthermore, the TUNEL assay revealed that the TUNEL positive cells were significantly increased in miR-335 overexpressing cells (Figure 3C). These results suggested that miR-335 induced apoptosis in OS cells.

Survivin is a Target of miR-335

To understand the molecular mechanism underlying the pro-apoptotic effect of miR-335, we used bioinformatical approach to identify the

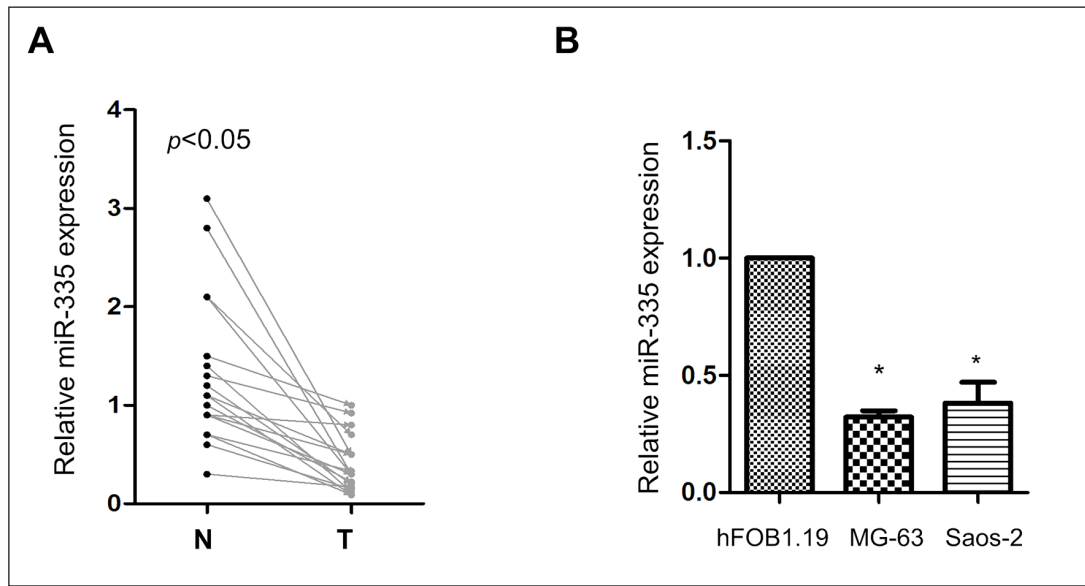


Figure 1. Downregulation of miR-335 in human OS tissues and cell lines. **(A)** The RNA of a total of 18 paired tumor tissues (T) and the adjacent normal tissues (N) were analyzed by real-time PCR. **(B)** The expression of miR-335 in human osteoblast cell line (hFOB1.19) and OS cell lines (MG-63 and Saos-2). * $p < 0.05$ versus hFOB1.19, $n = 3$.

potential target of miR-335. We found that the gene encoding for survivin, BIRC5, contains a putative miR-335 binding site in the 3'UTR of its mRNA (Figure 3A). We constructed a luciferase reporter, and co-transfection of this reporter with miR-335 mimics or the miR-335 inhibitor (anti-miR-335) was performed. As expected,

miR-335 decreased the luciferase activity while anti-miR-335 increased the luciferase activity (Figure 3B). Transfection of miR-335 into MG-63 cells resulted in a significantly decreased level of survivin expression (Figure 3C). These data indicated that survivin is targeted by miR-335 in OS cells.

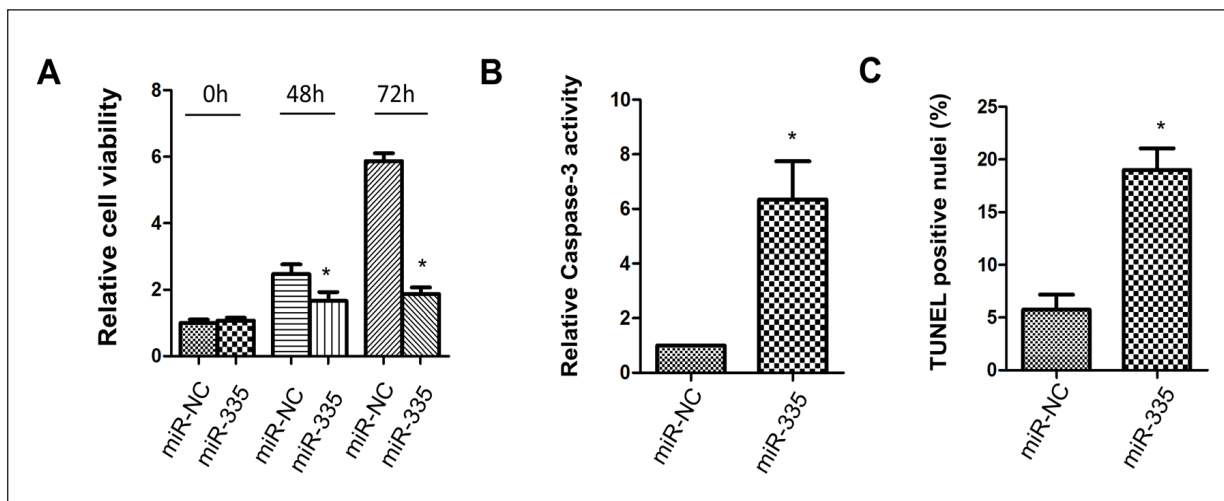


Figure 2. Increased apoptosis after miR-335 overexpression. **(A)** The relative cell viability after miR-335 transfection. Cells were transfected with negative control (miR-NC) or miR-335 mimics, the cell viability was detected at the indicated time points. Cells were transfected with miR-335 for 72h, **(B)** the relative caspase-3 activity and **(C)** the TUNEL positive cells were analyzed. * $p < 0.05$ versus miR-NC, $n = 3$.

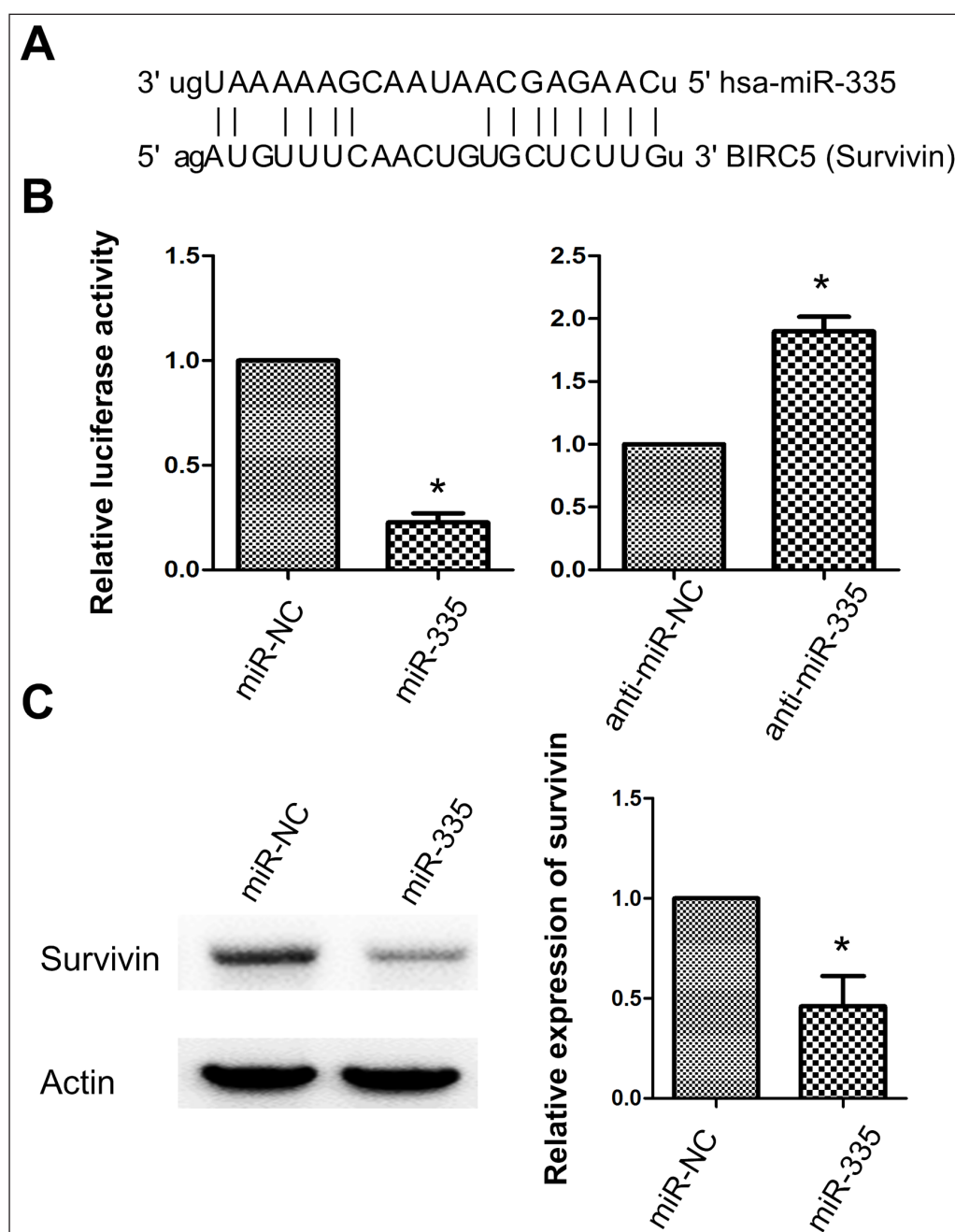


Figure 3. Identification of survivin (BIRC5) as a target of miR-335. **(A)** A schematic showing the predicted binding between miR-335 and survivin gene 3'UTR. **(B)** The relative luciferase activity after miR-335 overexpression or knockdown. **(C)** The levels of survivin protein after miR-335 overexpression. * $p < 0.05$ versus miR-NC, $n = 3$.

Inhibition of Survivin Exhibited Similar Effects to miR-335 Overexpression

We next confirmed the involvement of survivin in the pro-apoptotic effect of miR-335. We inhibited survivin in both pharmacological and biological ways. Pharmacological inhibitor of survivin (YM-155) and siRNA-mediated

knockdown of survivin both resulted in significantly decreased cell viability (Figure 4A). Moreover, the molecular marker of apoptosis, cleaved-caspase-3, was found to be significantly up-regulated after survivin inhibition (Figure 4B), suggesting an increased apoptosis. These effects mimicked that of miR-335 overexpression,

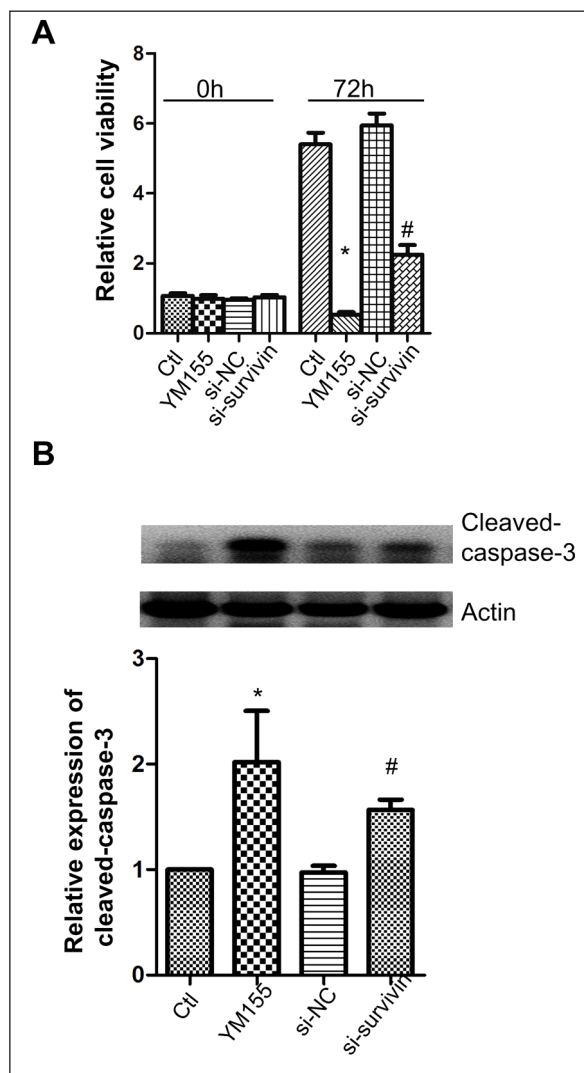


Figure 4. Inhibition of survivin exhibited similar effects to miR-335 overexpression. **(A)** The relative cell viability and **(B)** the expression of cleaved-caspase-3 after survivin inhibitor, cells were incubated with survivin inhibitor YM155 or transfected with survivin siRNA, 72h after treatment, cell viability was determined by MTT assay, and cleaved-caspase-3 protein was determined by Western blot. * $p < 0.05$ versus control (Ctl), # $p < 0.05$ versus si-NC, $n = 3$.

indicating that miR-335 contributes to OS cell apoptosis at least partly by inhibition of survivin.

Discussion

OS is one of the most frequently happened malignancies in children and adolescents¹. The low survival rate of advanced OS emphasized the urgency of meeting the unmet medical needs of patients who receive standard chemotherapy and

surgery. Elucidating the molecular pathology of OS should be the preliminary issue to support the development of new drugs.

Previous studies have highlighted a group of small non-coding RNA molecules, microRNAs, in the neoplasia of OS³, which benefited greatly to the identification of therapeutic targets and biomarkers for diagnosis and prognosis. Up to now, a great number of microRNAs have been demonstrated to be dysregulated in OS, and both the oncogenic and tumor suppressive role of a group of microRNAs have been uncovered⁶. For instances, miR-449 and miR-29 have been reported to participate in the regulation of apoptosis by targeting BCL2 in OS^{13,14}, and miR-23a was reported to repress the expression of the transcriptional factor RUNX2 that is frequently amplified and functions as an oncogene in OS¹⁵. The role of miR-335 in the regulation of tumor cell activity has been previously studied. It has recently been proposed that miR-335 promotes tumor growth and invasion in brain tumors but to exert an opposite role in other cancers such as gastric cancer and breast cancer^{7,10,11,16}. These reports suggested that the function of miR-335 varies in different pathological context.

In the present study, downregulation of miR-335 in OS tissues has firstly been identified, which is consistent with the report by Wang et al¹². In their study, they showed that miR-335 was downregulated in OS tissues, and ROCK1 was a functional target of miR-335 to confer the tumor suppressive role of miR-335 by inhibiting cell migration and invasion. The apoptotic machinery plays an important role in the maintenance of tissue homeostasis. We thus asked whether miR-335 was able to regulate apoptosis in addition to its previously reported roles in OS. To our expectation, forced overexpression of miR-335 decreased the growth activity and increased the apoptotic potential *in vitro*, as exemplified by the pronounced increases in caspase-3 activity and the rate of TUNEL positive cells. Our data suggested an involvement of miR-335 in apoptosis regulation in OS. Computational prediction further revealed that the 3'UTR of survivin mRNA contains a putative binding site of miR-335, considering that survivin is a potent negative regulator of apoptosis. We, therefore, hypothesized that miR-335 can regulate apoptosis through inhibition of survivin. Both Western blot and luciferase assay confirmed our presumption. These evidences extended the role of miR-335 in regulating tumor behaviors by modulating apoptosis in OS cells.

Recently, evidence has shown that the expression of survivin was upregulated in canine OS and inhibition of survivin might represent a novel therapeutic approach in several cancers^{17,18}. In agreement with the data shown by Zhang et al²⁰ and Gao et al¹⁹, our findings demonstrated that survivin inhibition using both the pharmacological inhibitor and survivin siRNA yielded powerful pro-apoptotic action towards OS cells. Particularly, a recent meta-analysis by Liu Y et al²¹ proposed that survivin can be used to predict the prognosis in OS, which further highlighted the therapeutic value of survivin inhibition by miR-335.

Conclusions

We propose a novel model of the apoptosis regulation by miR-335 in OS cells. Downregulation of miR-335 might contribute to a derepression of survivin gene in OS cells, which further leads to its retardation of the apoptotic process. Our data indicates that normalization of miR-335 may be utilized as an effective therapeutic approach for treating OS.

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Conflict of Interests

The Authors declare that they have no conflict of interests

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