Zebularine enhances apoptosis of human osteosarcoma cells by suppressing methylation of ARHI

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Osteosarcoma is a common primary malignant bone cancer in children and adolescents. Epidemiologic data showed that the annual incidence of osteosarcoma is approximately three cases/million population, accounting for 0.2% of all malignant tumors. The current optimal treatment for osteosarcoma includes neoadjuvant chemotherapy and surgical resection of resectable osteosarcoma. Nonetheless, surgical resection has great limitations for patients with relapsed or metastatic disease, and the effectiveness of postoperative chemotherapy does not satisfy all patients. Moreover, the frequent acquisition of drug-resistant phenotypes and the occurrence of “secondary malignancies” are often associated with chemotherapy. It is difficult to elect appropriate and effective chemotherapeutic drugs for the treatment of osteosarcoma.

Zebularine (1-[(β-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one) is a cytidine analogue that may form a covalent complex with DNA methyltransferase to inhibit DNA methylation. In contrast to other DNA methylation inhibitors, such as 5-aza-2’-deoxycytidine, zebularine has higher stability and lower toxicity detected both in vitro and in vivo. In several studies to date, zebularine has been shown to upregulate tumor suppressor genes by demethylation in carcinoma cells. Furthermore, methylation of tumor suppressor genes causes subsequent interruption of pro-apoptotic pathways, which are deemed to contribute to the improvement of proliferation and/or drug resistance. ARHI, also called DIRAS3 (GTP-binding protein Di-Ras3), is an imprinted tumor suppressor gene; its methylation suppresses ARHI activity. As ARHI is frequently downregulated by methylation, the loss of its expression may contribute to the pathogenesis of the majority of cancers. Therefore, methylation of ARHI may participate in the pathogenesis of malignant tumors. Thus, there may be an association between zebularine and ARHI methylation, which may be applied in tumor therapy.

In this study, we examined the effects of zebularine on viability and apoptosis in human osteosarcoma cells, and investigated the impact of zebularine on ARHI expression. Additionally, we explored the mechanism of zebularine on modulating ARHI methylation in human osteosarcoma cells.

Materials and Methods

Cell culture. Human osteosarcoma cell lines, including those derived from fibroblastic (HOS, MG-63) or osteoblastic (U2OS, Saos-2) high-grade osteosarcoma, and normal human osteoblasts (hFOB 1.19), were obtained from ATCC (Manassas, VA, USA). All cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Invitrogen), 100 U/mL penicillin, and 100 mg/mL streptomycin.
siRNA transfection. The single-stranded DNA methyltransferase 1 (DNMT1) siRNAs, histone methyltransferase G9a siRNAs, ARH1 siRNA, and related negative control siRNAs were respectively transfected into U2OS cells using Lipofectamine 2000 (Invitrogen). The siRNA sequences were designed by Invitrogen Block-iT RNAi Designer (http://rnamender.thermofisher.com/maxygen/). G9a siRNA1, 5'-GCCUCUAUGCACACUGGUU-3'; G9a siRNA2, 5'-CCAUCGUCUGCUACACCAUGG-3'; G9a siRNA3, 5'-UCACGCACUAGGAGGCCAC-3'; DNMT1 siRNA1, 5'-GGAGCUGUUCUGGUAU-3'; DNMT1 siRNA2, 5'-UCAUCUGACGCAAGGCCAC-3'; DNMT1 siRNA3, 5'-ACCATGAGCACCGTTCTCCAC-3'; control siRNA, 5'-UUUAGCGCCGAAAAGAAUCC-3'. ARH1 siRNA, 5'-GCCCACAAUGUAUACGGCGGAU-3'; control siRNA, 5'-UUCUCGAAACGUGCUAGCU-3'.

Cell viability analysis. The hFOB 1.19, U2OS, and MG-63 cells were treated with 50, 100, 200, and 300 μM zebularine for 72 h, or the cells were treated with 200 μM for different times. Cell viability was analyzed by purchased cell counting kits (Sigma-Aldrich, St. Louis, MO, USA). Assays were repeated four times for each sample.

Cell apoptosis assay. The apoptosis cells were measured by flow cytometry and annexin V–FITC/propidium iodide apoptosis detection kit (Abcam, Cambridge, UK) in U2OS cells. The fluorescence intensity was detected at 488 nm using the Gel Imaging System of Bio-Rad. Each band was analyzed by Quantity One 4.62 software (Bio-Rad).

Western blot analysis. Total proteins were extracted using the Tissue or Cell Total Protein Extraction Kit (Amresco, Solon, OH USA) from HOS, MG-63, U2OS, Saos-2, and hFOB 1.19 cell lines. All primary antibodies were purchased from Abcam. The proteins were separated by SDS-PAGE followed by electrotransfer to nitrocellulose membranes. The membranes were probed using antibodies against ARHI (Sigma-Aldrich, St. Louis, MO, USA). The membranes were incubated in blocking buffer (25 mM HEPES [pH 7.5], 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1% NP-40, 0.5 mM MgCl2, 10 mM NaF, 1 mM PMSF, 1 mM Na orthovanadate, 1 mM aprotinin, and leupeptin) for 30 min. Immunoprecipitation was carried out on cleared lysates (13 000 g for 15 min at 4°C) with the DNMT1 or G9a antibodies and protein A/G agarose beads (Merck, Darmstadt, Germany) and treated with bisulfite (Sigma-Aldrich). The proteins were separated by SDS–PAGE and transferred to a nitrocellulose membrane. Blots were incubated with primary antibodies against ARHI (1:1000), DNMT1 (1:2000), and G9a (1:1000) followed by an HRP-conjugated secondary antibody (Abcam). Bands were recorded on X-ray films (Kodak, Rochester, NY, USA). The densitometry of each band was quantified by a gel imaging system and Quantity One 4.62 software (Bio-Rad, Hercules, CA, USA).

Reverse transcription–PCR. Total RNA was extracted using TRIZol reagents (Invitrogen) from HOS, MG-63, U2OS, Saos-2, and hFOB 1.19 cell lines. Isolated RNA was electrophoresed on 1% agarose gel to detect the purity of total RNA. The first-strand cDNA was synthesized using 1 μg total RNA and SuperScript III Reverse Transcriptase (Invitrogen). Polymerase chain reaction amplification was carried out using a PCR amplification kit (Takara Biotechnology, Dalian, China). The specific primers were designed using Primer Premier 6.0 software and synthesized by Sangon Biotech (Shanghai, China). The primers for ARHI were 5'-TCTCTCCCGAGCAGGGCA-3' (forward) and 5'-ATCTTTCCGTGGGCTTGAAGG-3' (reverse). The primers for GAPDH as an internal control were 5'-ACCACAGTGCAAGGTCAT-3' (forward) and 5'-TCCACACCTTGTGGTCT-3' (reverse). The PCR production was electrophoresed on 1% agarose gel and visualized by the Gel Imaging System of Bio-Rad. Each band was analyzed by Quantity One 4.62 software (Bio-Rad).

Combined bisulfite restriction analysis. Combined bisulfite restriction analysis (COBRA) was carried out by referring to Xiong and Laird’s study. Genomic DNA was extracted from cell lines using QIAamp DNA Mini Kit (Qiagen, Dusseldorf, Germany) and treated with bisulfite (Sigma-Aldrich). The modified DNA was amplified using PCR. The specific primers were synthesized by Sangon Biotech. The primers for CpG I (187 bp) were 5'-GAAGGGAGAGGAAGTATGAA-3' (forward) and 5'-TACTATCTACAAAAAACCTC-3' (reverse). The primers for CpG II (207 bp) were 5'-GTTGGGTAGTTTATAGTGTGT-3' (forward) and 5'-AACCCAAACACCAAAAAACAAACATC-3' (reverse). The primers for CpG III (184 bp) were 5'-GTTTTTTAGTTTATAGGAA-3' (forward) and 5'-ATAATATACAAAAACACACACC-3' (reverse). After amplification, PCR products were digested with the restriction enzyme TaqI (New England Biolabs) for CpG I and III or BstUI (New England Biolabs, Ipswich, MA, USA) for CpG II. DNA was then electrophoresed on 2% polyacrylamide gel. The gels were stained with ethidium bromide and visualized by Bio-Rad’s Gel Imaging System. Each band was analyzed by Quantity One 4.62 software (Bio-Rad).

Co-immunoprecipitation. Co-immunoprecipitation, cells were washed with ice-cold PBS and lysed in lysis buffer (25 mM HEPES [pH 7.5], 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1% NP-40, 0.5 mM MgCl2, 10 mM NaF, 1 mM PMSF, 1 mM Na orthovanadate, 1 mM aprotinin, and leupeptin) for 30 min. Immunoprecipitation was carried out on cleared lysates (13 000 g for 15 min at 4°C) with the DNMT1 or G9a antibodies and protein A/G agarose beads (Merck, Darmstadt, Germany) at 4°C overnight. The precipitates were washed in lysis buffer (3×) and boiled in SDS sample buffer containing 100 mM DTT. Samples were subjected to Western blotting analysis using anti-DNMT1 and anti-G9a antibodies and stained using the ECL (Millipore, Boston, MA, USA) system.

Statistical analysis. Data were reported as mean ± SD in at least four replicates per group. Data were analyzed by ssrs 13.0 software (IBM, Almon, NY, USA). Statistical differences between means were calculated using ANOVA, followed by least significant difference multiple comparison tests. Differences were considered significant at P < 0.05.

Results

Expression and methylation status of ARHI in human osteosarcoma cells. Expression of the ARHI protein in human osteosarcoma cells showed a marked reduction compared to normal osteoblast cells (P < 0.01; Fig. 1a,b). Meanwhile, the expression of ARHI mRNA in osteosarcoma cells was also significantly reduced compared with normal osteoblast cells (Fig. 1a, c). To explore the cause of reduction of ARHI expression, we further assessed the methylation status of CpG islands associated with the ARHI gene by COBRA. The sites of CpG I and II, and CpG III were located in the proximate promoters Exon 1 and Exon 2, respectively (Fig. 1d). CpG I and II were hypomethylated, and CpG III was partially methylated in normal human osteoblast cells (Fig. 1e). Conversely, in human osteosarcoma cells, CpG I, II, and III were partially methylated, especially in Saos-2 and U2OS cell lines that were derived from osteoblastic osteosarcoma.

Zebularine suppresses cellular viability in U2OS and MG-63 cells. To discuss the effect of zebularine on the viability of osteosarcoma and normal osteoblast cells, 50, 100, 200, and 300 μM zebularine was used to incubate the cells for 72 h. The Cell Counting Kit-8 (CCK-8) assay showed that the viability of osteosarcoma cells was greatly decreased in a dose-
dependent manner, whereas normal osteoblast cells were less sensitive to zebularine, compared with the osteosarcoma cells (Fig. 2). The cells were then treated with 200 μM zebularine for 0, 12, 24, 48, and 72 h. The viability of osteosarcoma cells and normal osteoblast cells were both suppressed by zebularine treatment, especially following incubation 200 μM zebularine for more than 48 h (Fig. 2). Therefore, in the following experiments, 200 μM and 48 h were the concentration and time applied in the incubation.

Zebularine promotes apoptosis in human osteosarcoma cells. To determine the effect of zebularine on apoptosis in U2OS cells, normal and U2OS cells were respectively treated with 200 μM zebularine for 48 h, apoptosis was monitored by annexin V–FITC/PI double-labeled staining. As shown in Figure 3, the apoptosis rate in normal osteoblast cells did not vary significantly after treatment with 200 μM zebularine for 48 h, but it was in U2OS cells that the apoptosis rate clearly elevated ($P < 0.01$; Fig. 3e).

Zebularine enhances ARHI expression. We then elucidated the effect of zebularine on $ARHI$ expression. The expression of $ARHI$ protein had no significant difference after treatment with zebularine in normal osteoblast cells, but was dramatically enhanced in osteosarcoma cells after treatment with zebularine ($P < 0.01$; Fig. 4a,b). Similar results were obtained with the effect of zebularine on $ARHI$ mRNA expression. Zebularine markedly increased expression of $ARHI$ mRNA in osteosarcoma cells ($P < 0.01$), but not in normal osteoblast cells (Fig. 4a,c). To further identify the effect of zebularine on $ARHI$ methylation, COBRA was used to analyze the methylation status of CpG islands associated with the $ARHI$ gene after treatment with zebularine. In U2OS and MG63 cells, zebularine suppressed the methylation of $ARHI$ gene in a dose-dependent manner (Fig. S1). Moreover, as shown in Figure 4(d), the methylation status of CpG I was reduced by treatment with 200 μM zebularine in osteosarcoma cells.

![Fig. 1. $ARHI$ expression and methylation status. (a) Western blot and RT-PCR analyses of $ARHI$ expression in protein and mRNA levels in human osteosarcoma cells and normal osteoblast cells. (b,c) Semiquantitative histograms of Western blot (b) and RT-PCR (c) analyses. (d) The distribution of CpG islands on the promoter region of $ARHI$ gene. (e) Combined bisulfite restriction analysis was used to analyze the methylation status of CpG islands associated with the $ARHI$ gene. DNA was amplified and digested with restriction enzymes that can distinguish methylated (c) and unmethylated (a or b) fragments. The methylation percentage, show below each lane, was calculated using the formula: %methylation = 100 × (a/b + c). %methylation >85, hypermethylation; %methylation 15–85, partial methylation; %methylation <15, hypomethylation. The values are presented as mean ± SD of four independent experiments. $**P < 0.01$ versus hFOB 1.19 normal osteoblast cells.](image1)

![Fig. 2. Effect of zebularine on cell viability in U2OS and MG-63 osteosarcoma cells. (a) Normal osteoblast hFOB 1.19 cells and U2OS and MG-63 cells were treated with 0, 50, 100, 200, and 300 μM zebularine for 72 h, and Cell Counting Kit-8 (CCK-8) assay was used to detect cell viability. (b) hFOB 1.19, U2OS, and MG-63 cells were incubated with 200 μM zebularine for 0, 12, 24, 48, and 72 h, and CCK-8 assay was used to detect cell viability. The values are presented as mean ± SD of four independent experiments. $*P < 0.05$, $**P < 0.01$ versus 0.](image2)
specifically in HOS and MG-63 cell lines. The methylation status of CpG II was also downregulated by treatment with zebularine, specifically in U2OS and Saos-2 cell lines. Moreover, zebularine triggered weak demethylation of CpG III in all osteosarcoma cell lines.

**Zebularine prevents interaction DNMT1 with G9a to inhibit ARHI methylation.** Zebularine is a DNMT inhibitor, and there was direct cooperation between DNMT1 and G9a. Thus, we intended to estimate the effect of zebularine on interaction between DNMT1 and G9a. As shown in Figure 5(a,b), zebularine markedly downregulated DNMT1 expression ($P < 0.01$), but not G9a. We undertook co-immunoprecipitation of DNMT1 and G9a using anti-DNMT1 or anti-G9a antibodies. Compared with the untreated group, zebularine treatment sharply reduced the binding of DNMT1 and G9a, and the results were consistent when the G9a antibody was used in the immunoprecipitation assay (Fig. 5c). G9a is a novel lysine-preferring mammalian histone methyltransferase with hyperactivity and specific selectivity to lysines 9 and 27 of histone H3.(11) Because the modification of histone methylation frequently has an intimate connection with DNA methylation, we intended to evaluate the effect of silenced DNMT1 or G9a on ARHI methylation. DNMT1 siRNA or G9a siRNA were transfected into U2OS cells using Lipofectamine 2000; the ARHI methylation status was detected by COBRA. Results showed that DNMT1 silencing obviously suppressed methylation of CpG I and CpG II from partial methylation to hypomethylation, and also significantly inhibited the methylation of CpG III from hypermethylation to partial methylation (Figs. 5d,S2). Similar results were observed

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Fig. 3. Effect of zebularine on apoptosis in U2OS osteosarcoma cells. (a) Normal, untreated cells. (b) Normal cells treated with 200 µM zebularine for 48 h. (c) Osteosarcoma cells, untreated. (d) Osteosarcoma cells treated with 200 µM zebularine for 48 h. (e) Sum of quadrant (Q)2 and Q4. Values are presented as mean ± SD of four independent experiments. **$P < 0.01$.**

Fig. 4. Effect of zebularine on ARHI expression and methylation. U2OS osteosarcoma cells were treated with nothing or 200 µM zebularine for 48 h. (a) Analysis of the effect of zebularine on ARHI expression in protein and mRNA levels in both normal and U2OS cells by Western blot and RT-PCR. (b,c) Semiquantitative histograms of Western blot (b) and RT-PCR (c) analyses. (d) Combined bisulfite restriction analysis was used to analyze the effect of zebularine on the methylation status of CpG islands associated with the ARHI gene in osteosarcoma cells. Values are presented as mean ± SD of four independent experiments. **$P < 0.01$.**
when G9a was silenced: CpG II of the ARHI gene was markedly demethylated, and CpG I and CpG III were also feebly demethylated, in U2OS cells (Figs. 5d, S2). These results indicated that ARHI methylation is not only related to DNA methylation, but also related to histone methylation.

Silencing of ARHI rescues cell viability and apoptosis by zebularine treatment. To further validate that zebularine suppressed cell viability and increased cell apoptosis through upregulation of ARHI, negative control siRNA or ARHI siRNA was transfected into U2OS cells with or without zebularine treatment. The CCK-8 analysis showed that knockdown of ARHI rescues the increase of cell viability (Fig. 6a) and resisted the increase of cell apoptosis (Fig. 6b) under zebularine treatment.

Discussion

Drug resistance almost invariably occurs, limiting the treatment effectiveness for osteosarcoma. Chemotherapy shrinks the tumor mass, but may also exert a selective pressure on tumor cells leading to the outgrowth of the fittest surviving clones. Therefore, it is necessary to develop novel drugs with high efficiency to improve the survival of osteosarcoma patients. Our findings indicated that zebularine not only suppressed the viability of osteosarcoma cells in a time- and dose-dependent manner, but also potentiated the dramatic apoptosis of osteosarcoma cells. Moreover, zebularine inhibited ARHI methylation by preventing the interaction of DNMT1 with histone methyltransferase G9a, leading to improvements in ARHI protein and mRNA. As a methylation transferase inhibitor, zebularine has been proven to downregulate the expression level of DNMT1. During this process, zebularine was metabolized to deoxyadenosine triphosphate zebularine (dtZeb) in vivo. The dtZeb and guanine formed base pairs, reducing the required energy of G-C substitutions, so that the DNA and methylation is not only related to DNA and histone methylation.
and apoptosis were tested. (a) Knockdown of zebularine (Zeb) treatment. Negative control siRNA (Ctrl-siRNA) or ARHI inhibitory role of ARHI in growth and its role in promoting apoptosis in osteosarcoma cells. We did not repeat showing the role of ARHI in cell viability and apoptosis in this study. However, we showed that knockdown of ARHI rescues cell viability and apoptosis following zebularine treatment, supporting the proposition that ARHI could inhibit osteosarcoma growth and induce apoptosis.

Recently, the DNA methylation inhibitor zebularine has received increasing attention; studies have reported that continuous treatment with zebularine effectively sustains demethylation in human bladder cancer cells and prevents cell growth of gastric cancer. Besides being an effective inhibitor of DNA methylation, zebularine possesses many properties desirable for a therapeutic agent, such as high stability and low toxicity. Compared with the classic DNA demethylation agent 5-aza-2'-deoxycytidine, zebularine may more effectively suppress the expression of tumor suppressors, such as RASSF1A, ARHI, and BLU, which may be due to a lack of the 4'-amino group and a 5-nitrogen in the structure. ARHI was upregulated 2.5-fold by zebularine in ovarian cancer. However, the mechanism by which zebularine regulates ARHI expression has many different viewpoints. Zebularine not only inhibits DNMT1, but also possibly causes chromatin remodeling and post-transcriptional modification in vitro. We found that zebularine reduced DNMT1 in combination with histone methyltransferase G9a. G9a is a lysine-preferred histone methyltransferase. During carcinogenesis, it usually appears in the form of a Snail/G9a/Dnmt1 triple complex in carcinoma cells. It interacts with DNMT1 and then transfers methyl groups to lysine 9 in histone H3 in vitro with hyperactivity and specific selectivity. Moreover, a recent study showed that DNMT1 could directly bind to the ARHI gene. Therefore, zebularine suppresses G9a and DNMT1 to regulate ARHI methylation.

In summary, we showed that zebularine inhibited the growth and promoted the apoptosis of osteosarcoma cells by suppressing G9a/DNMT1-mediated ARHI methylation. Therefore, zebularine has the prospect of becoming an epigenetic drug in the treatment of osteosarcoma.

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Disclosure Statement
The authors have no conflict of interest.

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Supported Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Zebularine suppresses ARHI methylation in a dose-dependent manner.

Fig. S2. Silencing of DNA methyltransferase 1 (DNMT1) or histone methyltransferase G9a suppresses ARHI methylation.