Original Paper

Bufalin Inhibits the Differentiation and Proliferation of Cancer Stem Cells Derived from Primary Osteosarcoma Cells through Mir-148a

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Key Words

Cancer Stem Cells • Osteosarcoma • MicroRNAs • Bufalin

Abstract

Background/Aims: Osteosarcoma (OS) is the second leading cause of cancer-related death in children and young adults. Chemoresistance is the most important cause of treatment failure in OS, largely resulting from presence of cancer stem cells (CSCs). However, CSCs isolated from cancer cell lines do not necessarily represent those from primary human tumors due to accumulation of genetic aberrations that increase with passage number. Therefore, studies on CSCs from primary OS may be more important for understanding the mechanisms driving the chemoresistance of CSCs in OS. **Methods:** We established a primary culture of OS cells, known as C1OS, from freshly resected tumor tissue. We further isolated CSCs from C1OS cells (C1OS-CSCs). We analyzed the effects of bufalin, a traditional Chinese medicine, on the stemness of C1OS-CSCs. We also analyzed the microRNA (miR) targets of bufalin on the stemness of C1OS-CSCs. Moreover, we examined these findings in the OS specimen. **Results:** Bufalin inhibited the stemness of C1OS-CSCs. Moreover, we found that miR-148a appeared to be a target of bufalin, and miR-148a further regulated DNMT1 and p27 to control the stemness of OS cells. This mechanism was further confirmed in OS specimen. **Conclusion:** Our data suggest that bufalin may be a promising treatment for OS, and its function may be conducted through regulation of miR-148a.

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Introduction

Osteosarcoma (OS) is a type of solid bone cancer mainly arising in young adults. It accounts for approximately 2.4% of all malignancies in young adults and is the second leading cause of cancer-related death of pediatric patients [1-3]. The standard care for OS today is based on a combination of aggressive surgical resection and different pre- and post-operative drugs, but it can provide just 65–70% 5-year survival rate without metastasis for OS patients [4-10]. Therefore, a great need for new treatments for osteosarcoma exists. However, the underlying cellular mechanisms of the development of OS chemoresistance and metastasis remain unclear.

Recently, it has been suggested that within a heterogeneous tumor there was a small subpopulation of cells called cancer stem cells (CSCs) that are responsible for chemoresistance and tumor metastasis [11]. Tumor shrinkage can be achieved by traditional treatment patterns, but most tumors will recur after treatment because of CSCs which could survive and then regenerate tumor growth [12, 13]. Specifically targeting the CSC population may be a more effective therapy strategy. Since the proposal of the CSC hypothesis, a lot of studies have been performed to isolate and identify OS stem cells using various methods [14-17]. In a recent study, we derived CSCs from the human hMG63 osteosarcoma cell line using a new strategy combining a suspension culture system with the chemotherapy drug cisplatin [18]. We also detected the expression of classic stem cell markers and confirm stem cell-like properties of hMG63 derived cancer stem cells.

Bufalin is the major component of the Chinese medicine Chan Su. It is an extract of dried toad venom from the skin glands of *Bufo gargarizans*. Previous studies suggest that bufalin inhibits the proliferation of transplanted human hepatocellular carcinoma in nude mice [19]. In addition, it has been reported that bufalin induces apoptosis in various human cancer cell lines, including leukemia [20], prostate cancer [21], gastric cancer [22], and osteosarcoma [23]. We also reported its potent impact on human osteosarcoma CSCs.

MicroRNAs, a type of noncoding small RNAs of 19–25 nt, can repress translation and induce cleavage of mRNA by binding to the 3'-untranslated region (UTR) of the target genes. MicroRNAs play important roles in multiple physiological and pathological functions, including development, differentiation, cell proliferation, apoptosis, and stress responses [24, 25]. In addition, recent evidence suggests that some microRNAs are involved in OS tumorigenesis and chemoresistance. For example, the inhibition of miR-34c significantly stimulated OS cell invasion and chemoresistance *in vitro*. In contrast, restoring miR-34c inhibited OS cell invasion and chemoresistance [26]. miR-33a promotes OS cell resistance to cisplatin by down-regulating TWIST. Inhibition of miR-33a by antagomir-33a enhances cisplatin-induced apoptosis in OS cells by up-regulating TWIST expression [27]. The expression of miR-21 regulates cellular processes in osteosarcoma, perhaps through regulating RECK [28]. Down-regulation of miR-143 was found in both osteosarcoma cell lines and primary tumor samples. The restoration of miR-143 could reduce cell viability and promote cell apoptosis through repression of Bcl-2 [29]. miR-140 was found to play a role in chemosensitivity to chemicals such as 5-fluorouracil (5-FU) in osteosarcoma. Sensitivity to 5-FU treatment can be obtained by blocking expression of miR-140 in cancer cells; on the other hand, the overexpression of miR-140 made tumor cells more resistant. It suggested miR-140 as a potent target to overcome drug resistance [30]. Given the numerous studies, the mechanism details underlying the interaction of OS CSCs and miRNA are still not well understood.

In this study, we successfully established a primary culture of OS cells, named C10S, from freshly resected tumor tissue. Then, we derived cancer stem cells from C10S cells using methods we described previously [18]. Similar to the cancer stem cells that we derived from the hMG63 cell line in a previous study, the cancer stem cells from C10S cells can respond to bufalin. We also found a significantly upregulated expression of miR-148a in bufalin-treated primary cancer stem cells derived from C10S. Ectopic overexpression of miR148a inhibits the stemness of cancer stem cells by suppressing expression of DNMT1 directly.

Methods and Materials

Establishment of primary osteosarcoma cell lines

This study was approved by ethical committee of Shanghai University of Traditional Chinese Medicine. After receiving signed informed consent forms, human cancer tissues were obtained from patients with primary osteosarcoma. Tumor tissue obtained from patients was washed in medium (DMEM supplemented with penicillin-streptomycin) and then minced into small pieces by scissors. The tissues were then resuspended in culture medium (DMEM medium supplemented with 10% FBS) and explanted to dishes pre-coated with collagen. Explants with epithelial monolayer outgrowth were preserved and transferred to a new dish for subculture. Serial subculture was performed to obtain primary tumor cell lines. All primary tumor cells were maintained at 37°C in a humidified incubator with 5% CO₂.

Animals

This study was approved by the institutional animal care and use committee of Shanghai University of Traditional Chinese Medicine. Nude mice were purchased from Shanghai Laboratory Animal Company. Mice were housed under pathogen-free conditions according to the standard guidelines of the institutional animal care and use committee of Shanghai University of Traditional Chinese Medicine. For xenografts formation assay, the C10S colonies consisting of different number of cells were injected into nude mice. The tumor size was monitored.

Cell culture

hMG-63 cells were obtained from American type culture collection (ATCC) and stored in our laboratory. hMG-63 and C10S were cultured in medium (DMEM supplemented with 10% FBS) at 37°C in a humidified incubator with 5% CO₂. The medium was changed every three days.

Plasmids construction and lentiviral infections

pLemiR-148a was obtained by cloning human pre-miR-148a sequence (amplified from genomic DNA) into pLemiR-tRFP vector. For the production of lentiviruses, specific vectors was transfected into 293T cells by calcium phosphate. 48 hrs after the transfection, supernatants were harvested, filtered with 0.45µm filters. These supernatants were used to infect cells in presence of Polybrene (Sigma-Aldrich).

Realtime PCR

Total RNA was isolated using the TRIzol Reagent (Invitrogen) according to manufacturer's protocol. Realtime PCR was performed on the LightCycle480 system (Roche) using SYBR Green Supermix (Takara). The realtime PCR reaction condition was 45 cycles of 30 s at 94°C, 5 s at 94 °C and 30 s at 60 °C.

Immunohistochemistry

Formalin-fixed samples were washed twice with PBS, followed by incubation with the blocking solution (0.2% Triton-100 and 5% goat serum in PBS) for 1h. Samples were then incubated with primary antibody and secondary antibody for 1h.

Western blotting assay

Whole cell lysates were prepared using pre-chilled RIPA. The samples were then centrifuged at 12000 g for 20min at 4°C and supernatants were collected for protein concentration determination. The total proteins was separated on SDS-PAGE gel, and transferred onto a nitrocellulose membrane. The membrane was then incubated with the blocking solution, followed by incubation overnight with appropriate primary antibodies at

4°C and subsequently with secondary antibodies for 1h at RT. The signals were visualized using LI-COR infrared imaging system (LI-COR) according to the manufacturer's guidelines.

Flow Cytometry

Dissociated cells were incubated with primary antibodies for 20 min on ice, and washed twice with PBS. Cells were then incubated with corresponding secondary antibodies for 20 min. After washing, flow cytometry was carried out using a FACS flow cytometer (BD Immunocytometry Systems). The antibodies used were anti- CD133, anti- CD44, anti- CD90, each at a dilution of 1:40.

Statistical Analysis

All data quantification and statistical analysis were performed with SPSS 13.0 software. Data are presented as the mean ± SEM.

Results

Establishment of primary osteosarcoma cell line C10S

Freshly resected osteosarcoma samples were minced with scissors into small pieces and explanted to pre-coated dishes. One week later, explants with epithelial monolayer outgrowth formed a halo (Fig. 1A). These explants were transferred into a new dish for subculture. After serial cultures, we derived a primary tumor cell line termed C10S (Fig. 1B). To test whether C10S cells may well represent osteosarcoma, an array-based gene

Fig. 1. Isolation and identification of the C10S primary osteosarcoma cell line. (A) Osteosarcoma sample explant in culture for 1 week. Photomicrograph magnification, ×200. (B) The C10S cell line in culture for 4 weeks at the second passage. Photomicrograph magnification, ×200. (C) Self-organizing heat map of differentially expressed genes among the three cell lines. Induced genes are represented in red. Suppressed genes are represented in green. (D) Pathway analysis. For the 20 pathways that are most active in hMG63, there were 17 overlapping pathways in C10S. The other 3 pathways also have significant differences compared with hMG63 (red). (E) Cell-glo assay of hMG63 (control) and C10S in culture. (F) The xenograft tumors formed by different amounts of C10S cells for 4 weeks. Scale bar is 1 cm. (G) Transwell migration assays of C10S cells. (H) Transwell invasion assays of C10S cells. *p<0.05.

Fig. 2. Isolation of cancer stem cells from primary C10S cells. (A) Workflow chart for isolating the cancer stem cells. (B) C10S formed spheres when grown in a suspension culture system for 14 days. (C) Nude mice bearing C10S or C10S-CSCs tumor xenografts were imaged 24 h post-injection of the imaging probe. (D) The expression levels of the cancer stem cell markers in C10S and C10S-CSCs were determined by real time PCR. The data represent the mean $\pm$ SEM. (E) C10S and C10S-CSCs were seeded at equal confluence and allowed to grow for up to 18 days. We found tumor sphere-like colony formation in C10S-CSCs cells but not in C10S cells. (F-G) The expression levels of the surface marker CD133 were determined by immunohistochemistry (F) and flow cytometry (G). Scale bars are 50 μ m.

expression profile analysis was performed. We found that the gene expression profile of C10S was similar to the commercial human OS cell line MG63 but not to skin fibroblast cells (Fig. 1C). In addition, the altered signaling pathways of C10S resembled MG63, as indicated by an analysis on the array data (Fig. 1D). Moreover, the proliferation ability of C10S resembled MG63 (Fig. 1E). The malignant potential of the C10S was evaluated by injection of different amounts (10^6 , 10^5 , 10^4 , 10^3 , respectively) of C10S cells into NOD/SCID mice. All four doses could form xenografts within 4 weeks after the injection, and the volume of the xenografts was correlated to the injection dose (Fig. 1F). The C10S cell lines also displayed a significant ability to migrate and invade when tested using a trans-well system *in vitro* (Fig. 1G-1H). These data suggest that C10S may well represent the original tumor.

Cancer stem cells derived from C10S cells

Cancer stem cells were derived from C10S following a workflow (Fig. 2A). Briefly, we generated a tumor sphere in a suspension culture, which was then injected into nude mice. The CSC populations were enriched under selection stress from cisplatin, and these drug-resistant cells were cultured *in vitro* to form cell colonies (Fig. 2A). The morphology of the acquired cancer stem cells could be seen in culture (Fig. 2B).

When injected into NOD/SCID mice at same amount (10^4) as C10S, C10S-CSC formed a much bigger xenograft than C10S, suggesting that C10S-CSC cells grow faster (Fig. 2C). We thus analyzed the expression patterns of several cell surface markers and confirmed the stem

Fig. 3. Bufalin inhibits the stemness of C10S-CSCs. (A) CSCs were plated on the CSC culture system, and the cells treated with bufalin before and after 4- and 8-day incubations was shown. (B) Cleaved caspase3 was determined by western blotting. (C) Transcript levels of cancer stem cell markers were measured in the C10S-CSCs cultured with bufalin or DMSO by quantitative PCR. (D) PCNA, Ki67 and sox-2 were measured in the C10S and C10S-CSCs cultured with or without bufalin by immunohistochemistry. (E) The xenograft tumor formation of C10S-CSCs cultured with (up) or without bufalin (down). (F) miRNA profiling of C10S-CSCs cultured with or without bufalin was performed using Affymetrix microarray analysis with miRNA array chips. Eight miRNAs were upregulated and ten were down-regulated in the C10S-CSCs cultured with bufalin. * $p < 0.05$.

cell-like properties of the isolated C10S-CSCs (Fig. 2D). Sphere formation and serial passages are hallmarks of CSCs. We found that C10S-CSC was capable of forming spheres while C10S cells failed to form spheres (Fig. 2E). CD133 was a surface marker of OS-CSCs [31]. We found that C10S-csc highly expressed CD133, by immunohistochemistry (Fig. 2F) and by flow cytometry (Fig. 2G). All these data suggest that the C10S-CSC cells are cancer stem cells derived from primary tumor cells.

Bufalin inhibits the proliferation of C10S-CSCs

Recently, we reported that bufalin can inhibit the differentiation, proliferation and sphere formation of hMG63 cancer stem cells [18]. To test whether bufalin affects C10S-CSCs, 10 μ M bufalin was added to the culture of C10S-CSCs cells. We found that bufalin induced shrinkage of C10S-CSC spheres over 8 days of culture (Fig. 3A). This shrinkage of CSC spheres may be a result of the activation of caspase-3, which suggested enhanced apoptosis (Fig. 3B). To further determine of the effects of bufalin on the stemness of C10S-CSCs, we measured the levels of several classic stem cell markers, such as ALDH1, TERT, NANOG, CD133, Notch and Bim1, using quantitative real time PCR. In comparison to C10S cultured with or without bufalin, the levels of stem cell markers were all down-regulated by the addition of bufalin (Fig. 3C). In addition, culturing with 10 μ M bufalin for 4 days was enough to significantly

Fig. 4. Overexpression of miR148a inhibits the stemness of C10S-CSCs. (A-B) The effect of miR-148a on the cell cycle of C10S-CSCs, shown by representative charts (A) and by quantification (B). (C) Quantitative analysis of transwell migration assays of C10S cells transfected/untransfected with miR-148a. (D) Quantitative analysis of transwell invasion assays of C10S cells transfected/untransfected with miR148a. (E) The sphere-like colony formation in C10S-CSC cells was inhibited by transfection with miR-148a. (F) Expression of the stem cell marker SOX-2 was inhibited by transfection with miR-148a. (G) Sequence alignments of miR-148a and its target sites in the 3'-UTR of DNMT1. (H) Expression of DNMT1 was inhibited by transfection with miR-148a. (I) Treatment with bufalin reduced the expression of DNMT1 in C10S-CSCs. * $p < 0.05$.

down-regulate the levels of the proliferation marker ki67 and PCNA and the stem cell marker SOX-2 (Fig. 3D). When injected into NOD/SCID mice, a lower ability of xenograft formation was found in bufalin-treated C10S-CSCs compared to untreated C10S-CSCs (Fig. 4E).

miRNAs were reported to be involved in carcinogenesis and stemness of OS cancer stem cell lines [32]. To identify the differentially expressed miRNAs, miRNA expression levels in C10S-CSC cultured with or without bufalin expression was measured using a commercial microarray from Agilent (<http://www.microrna.sanger.ac.uk/>). miR-148a was found to have the greatest magnitude of increase in the 723 miRNAs tested after treatment with bufalin (Fig. 4F).

Overexpression of miR-148a significantly inhibits the stemness of C10S-CSCs

Based on the above data, we postulated that overexpression of miR-148a may mimic the effects of bufalin on C10S-CSCs. To test this hypothesis, we expressed miR-148a in C10S-CSCs using the pLemiR vector. We found that overexpression of miR-148a inhibited the G2/M transition in C10S-CSCs, which suggests an impaired self-renewing ability (Fig. 4A-B). In addition, miR-148a overexpression also inhibited the migration and invasion abilities of C10S-CSCs (Fig. 4C-D).

Similar to bufalin, which can inhibit sphere formation in hMG63 cancer stem cells and C10S-CSCs cultured in a non-adhesive culture system, overexpression of miR148a also inhibited the sphere formation of C10S-CSCs (Fig. 4E). Moreover, the level of the stem cell

Fig. 5. DNMT1 is the target of miR-148a. (A) Expression levels of miR-148a in osteosarcoma tumor or non-tumor tissue measured by quantitative PCR. (B) The level of DNMT1 in osteosarcoma tumor or non-tumor tissue measured by quantitative PCR. (C) Expression level of p27 in osteosarcoma tumor or non-tumor tissue measured by western blotting. (D) Representative immunohistochemistry images for miR-148a of tumors formed in NOD-SCID mice or untreated C10S-CSCs. * $p < 0.05$.

marker Sox-2 was also down-regulated by ectopically expression of miR-148a (Fig. 4F). To investigate the mechanism of miR-148a on C10S-CSCs, we used a miRNA-target prediction program and found that a putative target gene of miR-148a was DNMT1 (Fig. 4G). We detected a repression of miR-148a on the expression of DNMT1 in C10S-CSCs expressing miR-148a (Fig. 4H). Interestingly, treatment of C10S-CSCs with bufalin also repressed the expression of DNMT1 (Fig. 4I). Taken together, these data suggest that bufalin targets C10S-CSCs by repressing the expression of DNMT1 through inducing the expression of miR-148a.

The association of miR148a with human osteosarcoma

We have identified a critical role for miR-148a in cancer stem cells derived from primary OS cells. Then we further explored the association of miR-148a with human osteosarcoma. We found that the expression level of miR-148a in human osteosarcoma samples was approximately 60% of non-tumor tissues (Fig. 5A). DNMT1, the target of miR148a, was upregulated in these human samples (Fig. 5B). To identify the downstream target of DNMT1, we knocked down the expression of DNMT1 in C10S-CSCs cell lines by siRNA. The expression levels of p27, a cyclin-dependent kinase inhibitor, were higher following the down-regulation of DNMT1 (Fig. 5C). In addition, inverse expression levels of DNMT1 and p27 were detected in the tumor formed in NOD-SCID mice by C10S-CSCs treated with or without miR-148a (Fig. 5D).

Discussion

Over the past several decades, the survival rates of osteosarcoma patients have failed to improve and have reached a plateau, despite accumulating studies that have greatly increased our knowledge of this aggressive malignancy [33]. This is partially because the cellular mechanisms that lead to the development of OS chemoresistance and metastasis

remain unclear. The emerging CSC theory has shed light on the study of the pathobiology of this disease. Based on this understanding, the strategy with which osteosarcoma is treated will also be updated. A new strategy that combines the CSC-targeted therapy and traditional chemotherapy is needed.

The base of developing a CSC-targeted therapy is the isolation of the tumor-type specific CSCs. Various methods, such as tumor spheres, sorting cells according to specific surface markers, side population cell sorting and other methods, were reported to be used in isolation and identification of osteosarcoma stem cells [14-17]. However, all these studies were based on established commercial cancer cell lines, whose ability to represent primary human tumors is limited by the genetic alternations that occur during increasing cultured period [34, 35]. We also need patient-derived primary tumor cells for the development of personalized therapy [36-38]. In this study, we successfully established a primary culture of OS cells, termed C1OS, from freshly resected tumor tissues, and derived a line of cancer stem cells from these primary cells. Because of their similar nature to tumor tissue, the establishment of cancer stem cells from primary tumors will facilitate the screening of new drugs specific to CSCs. However, taking the variability of patients into consideration, it is difficult to cover all the possible conditions by single cancer cell lines. More patient-derived primary tumor cell lines are needed in future studies.

A question arose once we have osteosarcoma CSC in hand. How can these CSCs be targeted and killed? miRNAs may play important roles in development of cancer [39]. However, targeting miRNAs have limitations on the treatment of human cancer because of the lack of appropriate *in vivo* delivery systems. To overcome such limitations, agents that can regulate expression levels of mRNA was used to target the CSCs. Natural agents could be a safer choice to be combined with conventional chemotherapeutics because of their non-toxic nature.

Our group has reported that bufalin can inhibit the proliferation and stemness of cancer stem cells derived from primary OS cells or the hMG63 cell line [18]. We compared the expression profile of miRNA in cancer stem cells cultured with or without bufalin and found miR148a to be the target of bufalin. miRNA expression profiling was performed in osteosarcomas by some researchers, but miR148a was not found to be significantly changed [40, 41]. It suggests that miR148a specifically mediates the effect of bufalin on osteosarcoma cancer stem cells.

DNA methyltransferases (DNMTs) was reported to be upregulated in cisplatin-resistant ovarian cancer [42]. One of the most widely used DNA methyltransferase inhibitors, decitabine (5-aza-2'-deoxycytidine, DAC), was also reported to have effects in targeting the human osteosarcoma HosDXR150 cell line [43]. Here we also found that DNMT1 served as the target of mi-148a. The separated and combined effects of bufalin and DAC on osteosarcoma cancer stem cells are worth exploring in a future study.

In conclusion, we established a new and efficient approach for the establishment of a patient-derived cancer cell line, and we isolated CSCs from the primary cell line. Moreover, we found that bufalin was a potent agent in inhibiting the proliferation and differentiation of C1OS-derived CSCs. By examining the miRNA expression profile of C1OS cultured with or without bufalin, we identified miR-148a as a target that mediates the effects of bufalin. The downstream target of miR148a, DNMT1, was also identified in C1OS derived cancer stem cells. We further identified the function of bufalin, i.e., that it plays a role in the miR148a and DNMT1 pathway in osteosarcoma patients and targets p27 as a downstream signal of DNMT1. Through an in-depth study of biological characteristics and related signal pathways to identify specific targets, our data may offer hope for eradicating osteosarcoma using this compound.

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Disclosure Statement

No potential conflict of interest was declared.

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