CYC1 Silencing Sensitizes Osteosarcoma Cells to TRAIL-Induced Apoptosis

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Key Words
Osteosarcoma • Cytochrome c1 • Cytochrome c • Apoptosis • TRAIL

Abstract
Aims: Osteosarcoma (OS) is an aggressive bone malignancy with poor prognosis. Many OS cells are resistant to apoptotic induction by tumor necrosis factor-related apoptosis inducing ligand (TRAIL). In our previous study, we found that the serum level of cytochrome c1 (CYC1) is significantly higher in OS patients than in healthy subjects. Our aim was to investigate the effects of CYC1 silencing on TRAIL-induced apoptosis in human OS in vitro and in vivo along with the underlying mechanisms. Methods: First, we determined the expression of CYC1 in human OS tumors and cell lines versus normal adjacent tissues and cell line. We then studied the effects of CYC1 silencing alone or in combination with TRAIL on OS cell growth and apoptosis in vitro and OS tumorigenesis in vivo. Results: We found that CYC1 is overexpressed in human OS tissues and cell lines. CYC1 silencing by shRNA transfection inhibits proliferation, slightly induces apoptosis in human OS cells in vitro, and suppresses human OS tumor growth in a mouse xenograft model in vivo. Additionally, CYC1 silencing sensitizes OS to TRAIL-induced apoptosis in vitro and in vivo. Our results also showed that CYC1 silencing significantly reduces complex III activity and potentiates TRAIL-induced cytochrome c release and caspase-9 activation in OS cells, suggesting that CYC1 silencing acts via the mitochondria-dependent apoptotic pathway. Conclusion: Taken together, our results provide evidence that CYC1 plays an important role in OS tumorigenesis, and modulation of CYC1 may be an effective strategy to potentiate OS to apoptotic induction by TRAIL.

G. Li, D. Fu and W. Liang contributed equally to this work.

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Introduction

Osteosarcoma (OS) is the most common malignant bone tumor affecting children and adolescents [1]. Current standard treatment is to use neoadjuvant chemotherapy (chemotherapy given before surgery) followed by surgical resection [2]. However, owing to resistant to commonly used chemotherapeutic agents [3], OS has one of the lowest survival rates for pediatric cancer, with five-year survival ranging from 65% to 75% for localized disease and <30% for patients with metastases [4]. Therefore, new approaches need to be considered to overcome chemotherapy resistance in the treatment of OS in order to improve the clinical outcome in patients.

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) is a member of the tumor necrosis factor (TNF) family of cytokines. TRAIL activates the cell surface death receptors (DRs) DR4 and DR5 (also referred to as TRAIL-R1 and TRAIL-R2, respectively), which in turn initiate a cascade of events resulting in stimulation of the extrinsic apoptotic pathway. TRAIL exhibits selective cytotoxicity towards cancer cells; and recombinant TRAIL and agonistic TRAIL-R1/R2 monoclonal antibodies are being developed as novel anti-cancer agents for a variety of malignancies [5]. However, many osteosarcomas are resistant to TRAIL, and induction of apoptosis requires combination treatment with other chemotherapeutic agents [6, 7].

Cytochrome c1 (CYC1) is a heme-containing subunit of complex III (also referred to as cytochrome bc1 complex), which forms the third proton pump in the mitochondrial electron transport chain. CYC1 directly interacts with cytochrome c (Cyt c) and mediates electron transport from cytochrome B to Cyt c during oxidative phosphorylation [8]. In our previous study, we identified CYC1 as a promising biomarker for early diagnosis of OS [9]. We found that the serum level of CYC1 in OS patients is significantly higher than that in healthy subjects. Additionally, the serum level of CYC1 in OS patients exhibits a significant decrease after surgical resection. These findings suggest that CYC1 might play a role in OS tumorigenesis. In addition to OS, CYC1 has also been found to be overexpressed in nasopharyngeal carcinoma (NPC) tissues and cell lines [10].

Interestingly, a recent study reported that CYC1 cleavage by caspase-3 disrupts mitochondrial function and enhances Cyt c release and apoptosis [11]. Thus, we speculated that CYC1 downregulation in OS might sensitize OS cells to apoptotic induction by TRAIL through promoting Cyt c release. In this study, we investigated the effects of CYC1 silencing on OS cell growth and apoptosis in vitro and OS tumorigenesis in vivo, either alone or in combination with TRAIL.

Materials and Methods

Clinical specimens

Human OS and matched adjacent normal tissues (n = 30) were obtained from patients at Shanghai Tenth People’s Hospital affiliated with Tongji University. All patients provided written informed consent. The study protocol was approved by the Ethics Committee of Tongji University.

Cell culture and lentiviral transfection

Human fetal osteoblastic cell line (hFOB) and the human OS cell lines HOS, 143B, and MG-63 were provided by the Chinese Academy of Medical Sciences. Cells were grown in RPMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated at 37°C, 5% CO₂ in a humidified incubator [12].

For CYC1 silencing, we constructed a lentiviral small hairpin RNA (shRNA)-encoding system using the pHBLV-U6-Puro lentiviral RNAi vector (Hanbio, Shanghai, China). The targeting sequence of CYC1 shRNA was: 5′-GCTCTGGAGCATGGTCTGATCT-3′. The recombinant lentivirus of CYC1 shRNA was produced by co-transfection of 293T cells with plasmids PSPAX2 and PMD2G using LipoFiter (Hanbio, Shanghai, China). The lentivirus-containing supernatant was harvested 48 h after transfection and filtered through 0.22-μm
Table 1. Primers used for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
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<tbody>
<tr>
<td>CYC1 (Cytochrome c1)</td>
<td>5′-CTCCCTGCTCATGGCTACT-3′ (forward)</td>
</tr>
<tr>
<td></td>
<td>5′-GGGTGCGATCTGAAGCAG-3′ (reverse)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-GCCACGTAAGACTCCAGAC-3′ (forward)</td>
</tr>
<tr>
<td></td>
<td>5′-CGGCAATGTTGACAGCACG-3′ (reverse)</td>
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To establish stable CYC1-knockdown cell lines, MG63 and 143B cells were transduced with the lentiviral RNAI vector at an MOI of approximately 10–50 in the presence of 5 μg/ml polybrene. After 24 h, culture medium was removed and fresh medium was added. At 72 h after transduction, puromycin (5 mg/ml) was added to the medium for stable cell line selection. The empty lentivirus lentivirus was used as negative control. After antibiotic selection for 3 weeks, stable CYC1-knockdown cells were obtained.

**RNA isolation and quantitative real-time PCR**

Total RNA was extracted using Trizol reagent (Invitrogen, USA) following manufacturer’s instructions. cDNA was synthesized using PrimerScript RT reagent Kit with gDNA Eraser (Takara, Japan). Quantitative real-time PCR was performed using SYBR-Green PCR kit (Takara) on a Step one plus System (Applied biosystems).

The specific primers used for PCR are shown in Table 1. The relative mRNA expression was normalized to GAPDH. All reactions were performed in triplicate.

**Protein extraction and western blot analysis**

Protein from enriched fractions of mitochondria and cytosol was extracted using isotonic mitochondrial buffer (MB: 210 mM mannitol/70 mM sucrose/1 mM EDTA/10 mM Hapes, pH 7.5) as described elsewhere [13]. Protein concentration was determined using a BCA protein assay kit (Keygen, China). Protein samples (50 μg) were separated by SDS-PAGE and transferred to NC membranes (Millipore, USA). After incubation in 5% BSA/TBST for 1 h at room temperature to block non-specific binding, membranes were incubated with antibodies against CYC1 (Protein Tech Group, Chicago, IL, USA), PARP, cytochrome c, cytochrome c oxidase subunit 4 (COXIV), and β-actin (all from Santa Cruz) in Tris-buffered saline at 4°C overnight. After washing, membranes were incubated with secondary antibody for 1 h at room temperature. Protein bands were visualized with enhanced chemiluminescence system (Amer sham, Arlington Heights, IL, USA). β-actin or COXIV was used as an internal control.

**Cell proliferation assay**

143B and MG-63 cells transfected with control, Lv-NC, or Lv-CYC1 shRNA were seeded in 96-well plates at 2×10^3/well and cultured at 37°C, 5% CO_2_ in a humidified incubator. At specific time points, medium was removed, and cells were incubated with MTT (Sigma, USA) at a final concentration of 5 mg/ml for 4 h. The reaction was stopped by addition of 150 μl DMSO and the optical density at 570 nm was determined on a microplate reader (Molecular Devices, CA, USA).

**Cell apoptosis assay**

Cell apoptosis was determined using the ApoAlert Annexin V kit (Clontech, Mountain View, CA, USA) following manufacturer’s instructions. 143B and MG-63 cells transfected with control, Lv-NC, or Lv-CYC1 shRNA were seeded in six-well plates at 2 × 10^5/well and cultured for 24 h. Cells were subsequently incubated with TRAIL (1, 10, 50, 100 ng/ml) for 72 h. After treatment was completed, cells were harvested, stained with annexin V-FITC and propidium iodide (PI), and analyzed on a FACS flow cytometer (San Diego, CA).

**Complex III activity**

143B and MG-63 cells transfected with control, Lv-NC, or Lv-CYC1 shRNA were seeded in six-well plates at 1 × 10^5/well for 3 d. The activity of Complex III was measured by following increase in absorbance due to the reduction of cytochrome c at 550 nm using the Complex III activity quantitative assay kit (Genmed, Shanghai, China) following manufacturer’s instructions.
Caspase 9 activity

Activity of Caspase 9 was measured using Caspase 9 activity assay kit (Beyotime, China) following the vendor’s protocol. Caspase activity assay was based on the ability of the active enzyme to cleave the chromophore from caspase-9 substrate Ac-LEHD-pNA to p-nitroanilide (pNA) [14]. The release of pNA was measured at 405 nm in a microplate reader. Results are presented as the relative change in activity compared to the control.

In vivo tumorigenesis

6-Week-old male nude mice were housed under pathogen-free conditions. 143B cells transfected with control, Lv-NC, or Lv-CYC1 shRNA were cultured to near confluence, harvested, and resuspended in PBS at 1×10^6 cells/ml. Cells (0.1 ml) were then injected subcutaneously into the flank of nude mice. Mice received a single intravenous injection of 10 mg/kg TRAIL (six in each treatment group) every three days for six weeks. The tumor size was measured every week using a caliper. The tumor volume was calculated using the formula 1/6 πab2 (p = 3.14; a, long-axis diameter of the tumor; b, short-axis diameter of the tumor). Growth curves were plotted from the tumor volume (means ± SD) in each treatment group. At the end of the 6-week treatment, the animals were sacrificed under anesthesia and tumors were immediately harvested, measured, and weighed.

Tumor tissue analysis

After the tumors (n = 6) were harvested, tumor tissues were immediately fixed in paraformaldehyde at room temperature for 48 h and embedded in paraffin. The nuclei with fragmented DNA were detected by the terminal deoxynucleotidyl transferase (TDT)-mediated dUTP-digoxigenin nick end labeling (TUNEL) assay using an in situ cell death detection kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer’s protocol.

Statistical analysis

Data are expressed as means ± SD. Multiple comparisons were made using one-way analysis of variance followed by Fisher’s tests. Differences with a p <0.05 were considered statistically significant.

Results

CYC1 is overexpressed in human OS tissues and cell lines

It has been reported that the serum CYC1 level in OS patients is significantly higher than that in healthy subjects [9]. In this study, we assessed the mRNA expression of CYC1 in 30 human OS tissues vs. matched adjacent normal tissues using quantitative real-time PCR. We found that the CYC1 mRNA level in OS tissues was significantly higher than that in adjacent normal tissues (Fig. 1A). We then determined the mRNA and protein expression of CYC1 in the human fetal osteoblastic cell line hFOB and the human OS cell lines HOS, 143B, and MG-63, using quantitative real-time PCR and western blot analysis, respectively. Our results showed that the mRNA and protein levels of CYC1 were significantly higher in the OS cell lines than in the normal osteoblastic cell line (Fig. 1B, 1C). Therefore, CYC1 is overexpressed in human OS tissues and cell lines in vivo and in vitro. These findings, along with previous reports [9] suggest that CYC1 might play a role in OS tumorigenesis.

CYC1 silencing inhibits growth and slightly induces apoptosis in OS cells

We then investigated the effects of CYC1 silencing on 143B and MG-63 cell growth and apoptosis. First, we confirmed that Lv-CYC1 shRNA transfection effectively reduced the mRNA and protein expression of CYC1 in 143B and MG-63 cells (Fig. 2A). We then studied cell proliferation using the MTT assay, and cell apoptosis using annexin V-FITC/PI-double staining flow cytometry. Our data showed that Lv-CYC1 shRNA transfection significantly inhibited growth in 143B and MG-63 cells compared with control or Lv-NC transfection (Fig. 2B). Although the increase of apoptosis in CYC1-silenced cells was statistical significant compared with control (Fig. 2C), it was actually slight (1.2±0.6% to 2.9±0.7% for 143B cells...
CYC1 silencing caused severe mitochondrial distress. Collectively, these results suggested that CYC1 silencing inhibited growth in OS cells via increasing apoptosis and damaging energy metabolism.

**CYC1 silencing enhances TRAIL-induced Cytc release, caspase-9 activation, and apoptosis in OS cells**

To find out whether CYC1 silencing sensitizes OS cells to TRAIL-induced apoptosis, we studied the effects of TRAIL alone or in combination with CYC1 silencing. Our results showed that TRAIL (0 – 100 ng/ml) alone dose-dependently induced apoptosis in 143B and MG-63 cells. Importantly, the percent of apoptotic cells increased further in 143B cells transfected with Lv-CYC1 shRNA (Fig. 3A). Similar effects were observed in MG-63 cells. In addition, treatment with TRAIL at 10 ng/ml triggered greater PARP cleavage in Lv-CYC1 shRNA-transfected 143B and MG-63 cells than control with TRAIL alone (Fig. 3B). Therefore, our results demonstrated that CYC1 silencing potentiates TRAIL-induced caspase activation and apoptosis in OS cells.

**CYC1 silencing enhances TRAIL-induced Cytc release and caspase-9 activation in OS cells**

A key step in mitochondria-dependent apoptotic pathway involves the release of Cytc into the cytosol and subsequent activation of caspase-9 [15]. To look into the mechanisms by which CYC1 silencing enhances TRAIL-induced apoptosis in OS cells, we assessed Cytc release and caspase-9 activity. We found that CYC1 silencing alone slightly increased Cytc release but had no effect on the activity of caspase-9 in 143B and MG-63 cells (Fig. 4A, 4B). Meanwhile,
Fig. 2. CYC1 silencing inhibits growth and induces apoptosis in OS cells. 143B and MG-63 cells were transfected with control, Lv-NC, or Lv-CYC1 shRNA as described in materials and methods. (A) The protein and mRNA expression of CYC1 in transfected cells by western blot and real-time PCR, respectively. B) Proliferation of transfected cells by the MTT assay. (C) Apoptosis of transfected cells by annexin V-FITC/PI double-staining flow cytometry. (D) Levels of PARP and cleaved PARP in transfected cells by western blot analysis. (E) Complex III activity in transfected cells. Data are expressed as means ± SD. **p < 0.01 compared with control.

treatment with 10 ng/ml TRAIL for 72 h resulted in moderate increases in Cytc release and caspase-9 activity. Importantly, the combination of CYC1 silencing and TRAIL triggered significantly greater Cytc release and caspase-9 activation in OS cells compared with either
Fig. 3. CYC1 silencing sensitizes OS cells to apoptotic induction by TRAIL. 143B and MG-63 cells transfected with control, Lv-NC, or Lv-CYC1 shRNA were treated with TRAIL (1, 10, 50, 100 ng/ml) for 72 h. (A) The percent of apoptotic cells by annexin V-FITC/PI double-staining flow cytometry. (B) Levels of PARP and cleaved PARP by western blot analysis. Data are expressed as means ± SD. **p < 0.01 compared with control.

CYC1 silencing or TRAIL alone. These effects of CYC1 silencing were blocked by the caspase 3 inhibitor Z-DEVD-FMK (Fig. 4A and 4B). It has been reported that caspase-3 amplifies CytC release and mitochondria fragmentation by cleaving CYC1 during mitochondria-dependent apoptosis [11]. In the mitochondrial electron transport chain, CYC1 directly interacts and stabilizes CytC. It is believed that CYC1 cleavage by caspase-3 disrupts its physical interaction with CytC, resulting in increased concentration of free CytC and consequent CytC release. We
Think that CYC1 silencing likely promotes CytC release via similar mechanisms. Our results also suggested that blocking CYC1 cleavage with caspase-3 inhibitors might compensate for the loss of CYC1 function caused by reduced expression.


cytochrome C silencing sensitizes OS tumors to growth inhibition and apoptotic induction by TRAIL in vivo

To investigate the relevance of our in vitro findings to OS tumorigenesis in vivo, we monitored the growth of tumors derived from 143B cells transfected with control, Lv-NC, or Lv-CYC1 shRNA in nu/nu nude mice. Consistent with our in vitro findings, tumors derived from Lv-CYC1 shRNA-transfected 143B cells grew at a much slower rate than those derived from control- or Lv-NC-transfected cells, as reflected in significantly smaller tumor volume (p < 0.05) (Fig. 5A, 5B). Meanwhile, treatment with 10 mg/kg TRAIL every three days for six weeks only slightly inhibited growth of tumors derived from control- or Lv-NC-transfected 143B cells, indicating that these tumors were resistant to TRAIL in vivo. Importantly, we found that tumors derived from Lv-CYC1 shRNA-transfected 143B cells were extremely sensitive to TRAIL. Growth of these tumors was almost completely inhibited by TRAIL administration throughout the six-week treatment period (Fig. 5A, 5B). After the treatment was completed, we assessed the level of apoptosis in tumor tissues based on detection of fragmented DNA by the TUNEL assay. We found that tumors derived from Lv-CYC1 shRNA-transfected 143B cells had higher levels of apoptosis compared with those derived from control- or Lv-NC-transfected cells (p < 0.05) (Fig. 5C). TRAIL treatment alone did not induce apoptosis in tumor tissues; however, the combination of CYC1 silencing and TRAIL administration resulted in greatly increased apoptosis (p < 0.01) (Fig. 5C). Thus, the level of apoptosis in tumor tissues was in alignment with the rate of tumor growth.
Discussion

Diverse chemotherapeutic agents kill cancer cells by activating common apoptotic pathways. Inherent resistance to apoptosis underpins both tumorigenesis and drug resistance of many malignancies. Thus, new therapeutic strategies to overcome cancer cell's resistance to apoptosis are required for effective cancer treatment. TRAIL is a promising strategy since it induces targeted apoptosis in malignant cells without affecting normal cells [16]. However, a considerable number of cancer cells, especially some highly malignant tumors, are resistant to apoptotic induction by TRAIL, and require combination treatment with other chemotherapeutic agents [17]. Apoptosis can be induced by distinct but overlapping pathways. TRAIL induces cancer cell apoptosis via an “extrinsic”, death-receptor-mediated pathway. Numerous studies have shown that compounds stimulating the “intrinsic”, mitochondria-dependent apoptotic pathway potentiate tumors to apoptotic induction by TRAIL [18-21]. Thus, mitochondrion is proposed as a direct target for the development of effective TRAIL sensitizers [19].
Osteosarcoma (OS) is an aggressive malignancy with poor prognosis. It has been reported that inherent resistance to apoptosis is one of the mechanisms by which OS cells escape therapeutic control [22, 23]. Most tumor cells are sensitive to apoptosis induced by TRAIL; however, many osteosarcomas are resistant to TRAIL [24, 25]. Intriguingly, our previous study showed that cytochrome c1 (CYC1), an indispensable protein component of the mitochondrial electron transport chain, is found at elevated concentration in the serum of OS patients, and the concentration drops after surgical resection [9]. CYC1 has also been found to be overexpressed in nasopharyngeal carcinoma (NPC) tissues and cell lines [10], implying that CYC1 might play a role in the development of specific types of cancer. Interestingly, a recent study identified CYC1 as a potential gate-keeper against mitochondria-dependent apoptosis. Its cleavage by caspase-3 amplifies death stimuli-induced mitochondria dysfunction, CytC release, and apoptosis [11]. Therefore, we speculated that suppression of CYC1 expression in OS cells might stimulate the “intrinsic”, mitochondria-dependent apoptosis pathway, and thereby potentiate OS cells to apoptotic induction by TRAIL.

In the present study, we first demonstrated that CYC1 is overexpressed in human OS tumor tissues and cell lines, which agrees with previous reports [9]. Our subsequent in vitro studies demonstrated that CYC1 silencing by shRNA transfection inhibited growth in human OS cell lines. Further studies showed that CYC1 silencing had a slight effect on apoptosis, but significantly reduced mitochondrial complex III activity in OS cell lines. Similar to our in vitro findings, CYC1 silencing curbed OS tumor growth in vivo. We subsequently tested the effects of TRAIL, either alone or in combination with CYC1 silencing. The OS cell lines 143B and MG-63 showed moderate sensitivity to apoptotic induction by TRAIL in vitro; however, tumors derived from 143B cells were resistant to TRAIL in vivo. We found that CYC1 silencing significantly enhanced TRAIL-induced CytC release, caspase activation, and apoptosis in OS cells in vitro, and sensitized OS tumors to apoptotic induction by TRAIL in vivo, resulting in almost complete inhibition of tumor growth through a six-week treatment period.

A variety of agents to sensitize OS to TRAIL-induced apoptosis have been proposed [26]. In this study, we showed that CYC1 downregulation effectively potentiates OS to apoptotic induction by TRAIL in vitro and in vivo, and these effects are mediated by activation of the mitochondria-dependent apoptotic pathway. Since CYC1 physically interacts with CytC, CYC1 silencing may destabilize its interaction with CytC. A small amount of activated caspase-3 may further disrupt the interaction and lead to an increase in free CytC concentration and consequent CytC release. Although the mechanisms are not fully elucidated, our results demonstrate that modulation of CYC1 may be an effective strategy to potentiate OS to apoptotic induction by TRAIL.

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References


