Int J Clin Exp Pathol 2018;11(8):3876-3883
www.ijcep.com /ISSN:1936-2625/IJCEP0075431

Original Article

Lentinan up-regulates microRNA-340 to promote apoptosis and autophagy of human osteosarcoma cells

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Received March 4, 2018; Accepted April 12, 2018; Epub August 1, 2018; Published August 15, 2018

Abstract: Background: Osteosarcoma (OS) is a common tumor of bone, and the high incidence and poor prognosis of OS call for novel therapeutic strategies. We aimed to explore the functional role of lentinan (LNT) in human OS MG63 cells as well as the underlying mechanisms. Methods: Cell viability of MG63 cells under LNT stimulation was measured by CCK-8 assay to explore the adequate concentration of LNT. Cell proliferation, apoptosis and expression of microRNA (miR)-340 in MG63 cells after LNT treatments were assayed by BrdU incorporation assay, flow cytometry assay and quantitative reverse transcription PCR, respectively. Expression of proteins associated with cell cycle, apoptosis, and autophagy were determined by western blot analysis. Subsequently, whether LNT affected MG63 cells through miR-340 as well as the related signaling pathway was explored. Results: Cell viability was reduced by 5-100 mg/mL of LNT. Percentage of BrdU-positive cells was reduced while that of apoptotic cells was enhanced by LNT treatment. LNT decreased cyclin D1 level but increased levels of active caspase-3 and caspase-9. After treatment, LNT enhanced LC3B-II/LC3B-I and Beclin-1 levels but reduced the p62 level. The miR-340 level was up-regulated by LNT, and further experiments showed LNT promoted apoptosis and autophagy through up-regulating miR-340. Moreover, LNT reduced the phosphorylated levels of MAPK and ERK through up-regulating miR-340. Conclusion: LNT reduced proliferation and induced apoptosis and autophagy by up-regulating miR-340 in MG63 cells, along with inhibition of the MAPK/ERK pathway.

Keywords: Osteosarcoma, anti-tumor, lentinan, miR-340, MAPK/ERK

Introduction

Osteosarcoma (OS) mainly occurs in the metaphyseal region of long bones and is a common malignant bone tumor in children and adolescents [1, 2]. It accounts for 20% of all osseous neoplasms and ranks the fifth in the leading causes of cancer-associated death in young adults [3, 4]. Even though the outcome of OS has been improved due to the developments of surgery and multi-agent chemotherapy, 30-40% of patients succumb to OS [5]. The high incidence and poor prognosis of OS call for identifications of novel therapeutic strategies for OS.

Lentinan (LNT), a polysaccharide that isolated from fruiting body of *Lentinus edodes*, is a β-1,6-glucose branched β-1,3-glucan [6]. The specific triple helical conformation enables the LNT to be a potential anti-tumor agent [7]. In Japan, LNT has been proven to be a biological response modifier for therapy of gastric cancer [8]. As shown in clinical studies, survival of patients who suffer advanced gastric cancer is prolonged by chemo-immunotherapy using LNT, when compared to chemotherapy alone [9]. LNT has also been reported to repress progression of urothelial bladder cancer through augmenting gemcitabine chemotherapy [10]. Nevertheless, the specific role of LNT in OS remains unclear.

Literature focused on regulatory mechanisms of LNT on tumor cells is limited. It has been reported that LNT stimulates T cells and thereby improves the survival of cancer patients [11]. Meanwhile, LNT can enhance peritoneal macrophage cytotoxicity against metastatic tumors [12]. More hypotheses and confirmatory experiments should be proceeded to determine how LNT modulates tumor cells. MicroRNAs (miRNAs/miRs) are short single strand non-coding RNAs that participate in

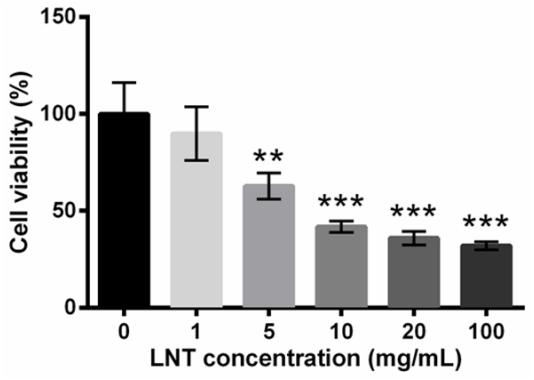

Figure 1. Lentinan (LNT) reduced cell viability of MG63 cells. Cells were incubated in plain medium containing 0, 1, 5, 10, 20 or 100 mg/mL LNT. Cell viability was measured by CCK-8 assay. Data are presented as the mean ± SD of three independent experiments. **, $P < 0.01$; ***, $P < 0.001$.

multiple biological processes, such as cell proliferation, apoptosis, autophagy, and immune cell function [13-15]. Numerous miRNAs are involved in OS pathogenesis [16], thus the alterations of miRNAs after stimulation with LNT are of great importance.

Since aggressive local growth and systemic dissemination are characteristic of OS, cell proliferation and cell death are two key factors for OS progression [17]. Cell apoptosis (Type I cell death) and autophagy (Type II cell death) are the two main biological processes that induce cell death [18]. Herein, we focused on the potential role of LNT in cell proliferation, apoptosis and autophagy of human OS MG63 cells. In addition, we explored the associated miRNAs in the modulation of LNT in MG63 cells as well as downstream signaling cascades. This study is a preliminary study, and the results may provide novel therapeutic strategies for treatment of OS.

Materials and methods

Cell culture and treatments

Human OS cell line MG63 was obtained from Shanghai Institutes for Biological Sciences Cell Resource Center (Shanghai, China). MG63 cells were grown in α-minimal essential medium (α-MEM; Gibco, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco). Cells were grown in a humidified incubator filled with 5% CO_2 and 95% air at 37°C. For LNT stimulation, MG63 cells were incubated in plain α-MEM containing 1-100 mg/mL LNT (Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China) at 37°C.

Cell transfection

MiR-340 inhibitor and its negative control (NC) were purchased from GenePharma (Shanghai, China). miRNAs were transfected into MG63 cells using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the supplier's instructions.

Cell viability assay

Cell viability of MG63 cells was determined using a Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD, USA). Briefly, 5×10^3 cells were plated into each well of 96-well plates and maintained at 37°C overnight. Then, after treatments, 10 μL of CCK-8 solution was added into each well, and the mixture was incubated at 37°C for additional 1 hr. The 96-well plates were subjected to measurements of absorbance at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA, USA).

Cell proliferation assay

Cell proliferation of MG63 cells was determined by a colorimetric immunoassay based on measurements of bromodeoxyuridine (BrdU) incorporation. Briefly, 5×10^3 MG63 cells were plated into 96-well plates and incubated at 37°C overnight. Then, the medium was replaced by plain α-MEM containing 20 mg/mL LNT, and cells were maintained for 48 hr. Then, according to the manufacturer's protocol, BrdU from Cell Proliferation ELISA kit (Roche Diagnostics, Tokyo, Japan) was added into the culture medium, followed by incubation at 37°C for 3 hr. Finally, the BrdU-uptake of LNT-treated cells was analyzed using a Microplate Reader (Bio-Rad) at 450/550 nm.

Cell apoptosis assay

Cell apoptosis of MG63 cells was assayed by using a FITC Annexin V/Dead Cell Apoptosis Kit (Invitrogen). Briefly, after desired treatments, cells were trypsined, washed in phosphate-buffered saline (PBS) and pelleted. Subsequently, the pellets were resuspended in binding buffer, followed by addition of 5 μL FITC-Annexin V and 5 μL PI. The mixture was incubated at room temperature for 15 min. The labeled cells with an amount of 1×10^5 were analyzed using

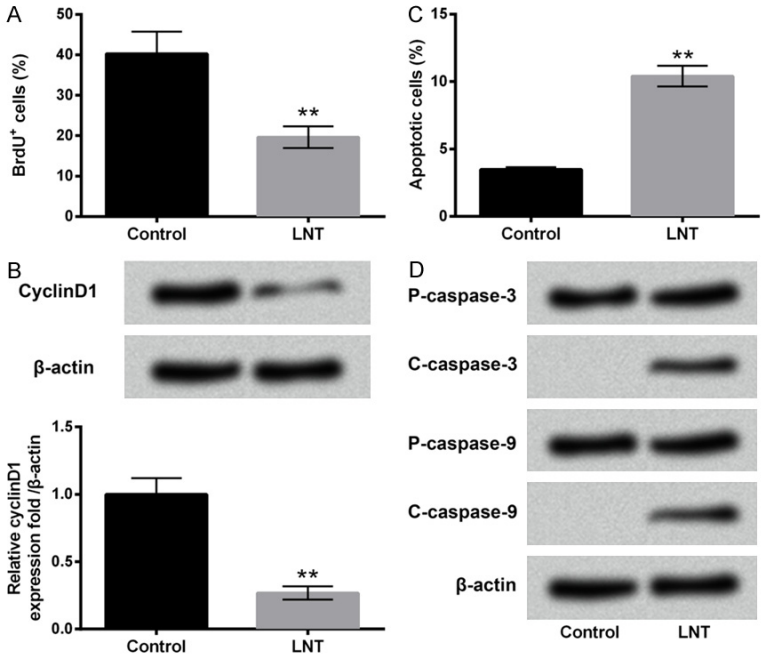

Figure 2. Lentinan (LNT) repressed cell proliferation and induced cell apoptosis in MG63 cells. Cells were stimulated with 20 mg/mL LNT, and non-treated cells acted as control. A. Percentage of BrdU positive cells by BrdU incorporation assay. B. Expression of cyclinD1 by western blot analysis. C. Percentage of apoptotic cells by flow cytometry assay. D. Expression of caspases by western blot analysis. Data are presented as the mean ± SD of three independent experiments. **, $P < 0.01$. P-, pro; C-, cleaved.

a flow cytometer (Beckman Coulter, Miami, FL, USA). Data analysis was performed using FlowJo software (Tree Star, San Carlos, CA, USA).

Quantitative reverse transcription PCR (qRT-PCR)

Total RNAs of MG63 cells were extracted utilizing the RNeasy kit (Qiagen, Hilden, Germany) as suggested by the supplier. Then, after quantification using a Nanodrop 2000 system, 500 ng RNA was converted to cDNA using the Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. The thermocycling program of reverse transcription was 30 min at 16°C, 30 min at 42°C and 5 min at 85°C. After that, 50 ng cDNA was used for semiquantitation of miR-340 level utilizing the Taqman Universal Master Mix II (Applied Biosystems) on the basis of the manufacturer's protocol. The thermal cycling conditions were 1 cycle at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative expression of miR-340 was analyzed following the $2^{-\Delta\Delta Ct}$ method [19]. U6, a housekeeping gene, acted as control.

Western blot analysis

After desired treatments, MG-63 cells were collected and lysed in RIPA lysis buffer (Beyotime, Shanghai, China) containing phenylmethylsulphonyl fluoride (PMSF; 10 mM, Beyotime) and PhosStop phosphatase inhibitors (Roche, Indianapolis, IN, USA). Protein concentration in the supernatant of lysates was determined by using BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA), and approximately 40 μg protein was separated by SDS-PAGE. Afterwards, proteins in the gels were transferred to 0.2 μm nitrocellulose membrane, and the membranes were blocked with 5% non-fat milk, followed by successive incubation with primary antibody and HRP-conjugated secondary antibody (goat anti-rabbit, ab205718, Abcam, Cambridge, UK). Primary antibodies included cyclinD1 (ab134175), pro caspase-3 (ab44976), cleaved caspase-3 (ab2302), pro caspase-9 (ab2013), cleaved caspase-9 (ab2324), microtubule-associated protein 1 light chain 3B (LC3B; ab48394), Beclin-1 (ab62557), p62/sequestosome 1 (p62; ab207305), β-actin (ab8227, all Abcam), total (t)-mitogen-activated protein kinase (t-MAPK; 9212), phospho (p)-MAPK (9211), t-extracellular signal-regulated kinase (t-ERK; 9102) or p-ERK (9101, all Cell Signaling Technology, Beverly, MA, USA). The proteins in the membranes were visualized using an ECL Advanced western blot detection kit (Thermo Fisher Scientific, Waltham, MA, USA). Intensity of the bands was quantified using ImageJ software (National Institutes of Health, Bethesda, MA, USA).

Statistical analysis

Data were presented as the mean ± standard deviation (SD) using sample triplicates. Statistical analysis was performed using Graphpad Prism 5 software (GraphPad, San Diego, CA, USA). The *P*-values were calculated using the one-way analysis of variance (ANOVA) with post

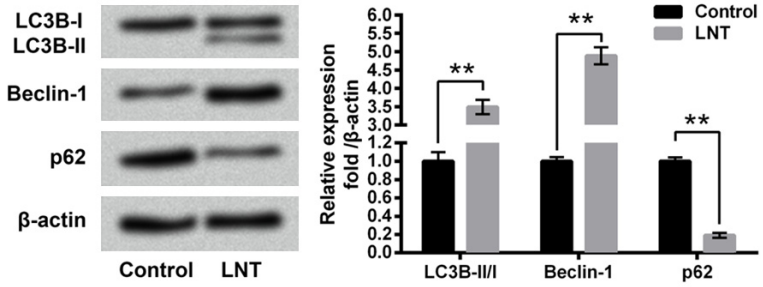

Figure 3. Lentinan (LNT) induced autophagy in MG63 cells. Cells were stimulated with 20 mg/mL LNT, and non-treated cells acted as control. Expression of autophagy marker proteins was measured by western blot analysis. Data are presented as the mean ± SD of three independent experiments. **, $P < 0.01$. LC3B-II/I, LC3B-II/LC3B-I.

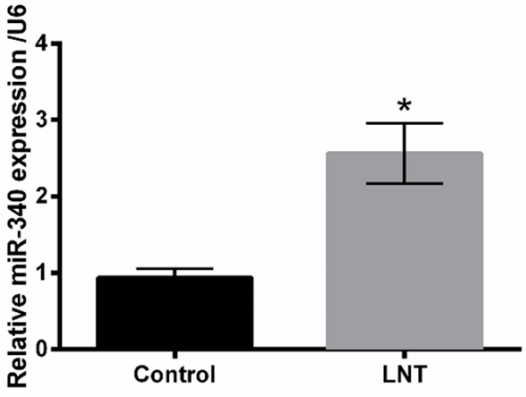

Figure 4. Lentinan (LNT) up-regulated miR-340 expression in MG63 cells. Cells were stimulated with 20 mg/mL LNT, and non-treated cells acted as control. Expression of miR-340 was assessed by quantitative reverse transcription PCR. Data are presented as the mean ± SD of three independent experiments. *, $P < 0.05$.

hoc Tukey's test. A $P < 0.05$ was considered significant.

Results

LNT reduced cell viability of MG63 cells

MG63 cells were stimulated with diverse concentrations of LNT, followed by measurements of cell viability. In **Figure 1**, cell viability was significantly reduced by 5-100 mg/mL LNT when compared with cells incubated in plain medium ($P < 0.01$ or $P < 0.001$), illustrating that LNT could reduce MG63 cell viability.

LNT repressed proliferation and promoted apoptosis in MG63 cells

After stimulation with 20 mg/mL LNT, cell proliferation and apoptosis of MG63 cells were assayed. Cells cultured in plain medium acted as control. Percentage of BrdU positive cells in the LNT group was markedly lower than that in the control group ($P < 0.01$, **Figure 2A**). Western blot results in **Figure 2B** showed protein expression of cyclin-D1 was remarkably down-regulated by LNT treatment as compared to the control group ($P < 0.01$). As evidenced by **Figure 2C**, percentage of apoptotic cells in the LNT group was significantly higher relative to the control group ($P < 0.01$). Western blot results in **Figure 2D** also showed that cleaved caspase-3 and cleaved caspase-9 were both notably up-regulated by LNT stimulation. Those results collectively stated that LNT repressed MG63 cell proliferation and induced cell apoptosis.

LNT promoted autophagy in MG63 cells

After stimulation with 20 mg/mL LNT, expression of autophagy marker proteins in MG63 cells was analyzed. As compared to the control group, ratio of LC3B-II/LC3B-I and expression of Beclin-1 were both dramatically enhanced (both $P < 0.01$) while expression of p62 was significantly reduced ($P < 0.01$) by LNT treatments (**Figure 3**). The alteration in those proteins suggests that LNT can promote autophagy of MG63 cells.

LNT up-regulated miR-340 expression

To determine the associated miRNAs, expression of miR-340 in MG63 cells after stimulation with 20 mg/mL LNT was assessed. As evidenced by **Figure 4**, expression of miR-340 was significantly up-regulated in the LNT group relative to the control group ($P < 0.05$). Data suggested that LNT could up-regulate miR-340 expression in MG63 cells.

LNT enhanced apoptosis and autophagy of MG63 cells through up-regulating miR-340

Subsequent experiments were performed to explore whether LNT affected MG63 cells via regulating miR-340. First, as shown in **Figure 5A**, the miR-340 level was dramatically down-regulated by transfection with miR-340 inhibi-

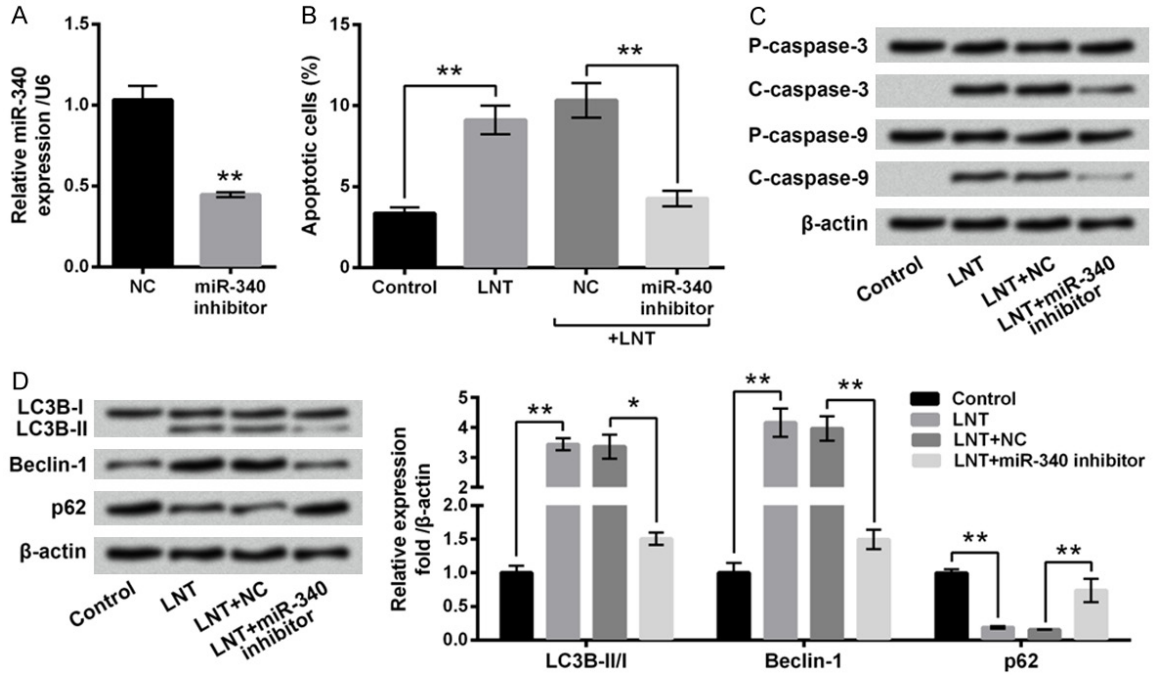

Figure 5. Lentinan (LNT) affected MG63 cells through up-regulating miR-340. Cells were transfected with miR-340 inhibitor or its negative control (NC). A. Expression of miR-340 by quantitative reverse transcription PCR. Transfected and non-transfected cells were stimulated with 20 mg/mL LNT, and non-treated cells acted as control. B. Percentage of apoptotic cells by flow cytometry assay. C. Expression of caspases by western blot analysis. D. Expression of autophagy marker proteins by western blot analysis. Data are presented as the mean ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$. P-, pro; C-, cleaved; LC3B-II/I, LC3B-II/LC3B-I.

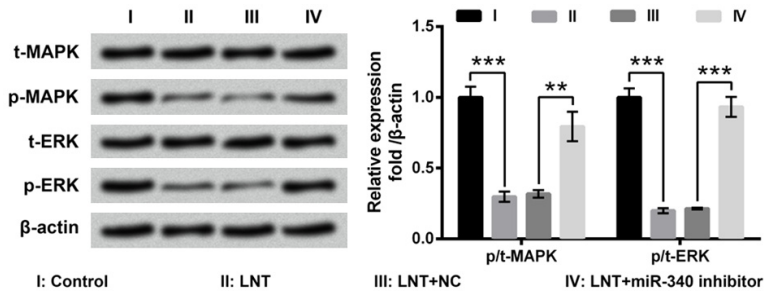

Figure 6. Lentinan (LNT) inhibited the MAPK/ERK pathway through up-regulating miR-340 in MG63 cells. Transfected and non-transfected cells were stimulated with 20 mg/mL LNT, and non-treated cells acted as control. Expression of key proteins in the MAPK/ERK pathway was measured by western blot analysis. Data are presented as the mean ± SD of three independent experiments. **, $P < 0.01$; ***, $P < 0.001$. NC, negative control of miR-340 inhibitor; t-, total; p-, phospho-.

tor compared with the NC group ($P < 0.01$). Then, flow cytometry results showed that LNT-induced stimulation of cell apoptosis (**Figure 5B**), activation of caspase-3 and caspase-9 (**Figure 5C**), and alterations of autophagy maker proteins (**Figure 5D**) were all dramatically reversed by miR-340 inhibition, as compared to the LNT + NC group ($P < 0.05$ or $P < 0.01$). Those results indicated that LNT may affect MG63 cell apoptosis and autophagy by up-regulating miR-340.

LNT inhibited the MAPK/ERK pathway by up-regulating miR-340

The involved signaling pathway was finally investigated. In **Figure 6**, phosphorylated levels of MAPK and ERK were remarkably reduced by LNT treatments (both $P < 0.001$). Meanwhile, the LNT-induced alterations were significantly reversed by miR-340 inhibition relative to the LNT + NC group ($P < 0.01$ or $P < 0.001$). Results illustrated that LNT can inhibit the MAPK/ERK pathway via up-regulation of miR-340 in MG63 cells.

Discussion

Although the outcome of OS has been improved greatly, patients who respond poorly to chemotherapy have a dismal outcome, which urgently

calls for novel therapies for OS. In our study, we found LNT could reduce proliferation and induce apoptosis and autophagy in OS MG63 cells for the first time. Moreover, miR-340 level in LNT-treated cells was elevated, which might be a reason for the alterations of cell apoptosis and autophagy induced by LNT. Finally, we also studied the possible involved signaling cascade, showing that LNT may inhibit the MAPK/ERK pathway through up-regulating miR-340.

The adequate concentration of LNT in MG63 cells was explored by testing the alteration of cell viability. According to the results, cells were stimulated with 20 mg/mL LNT for subsequent experiments. Then, we measured the effects of LNT on proliferation and apoptosis of MG63 cells. BrdU is a synthetic thymidine analog which can be incorporated into the newly replicated DNA during S phase of cells, thus, BrdU is widely utilized for measurements of cell proliferation [20]. In our study, the reduced percentage of BrdU positive cells reflected the inhibited cell proliferation. CyclinD1 is a positive cell cycle regulator that is closely associated with cancer occurrence [21]. Its overexpression leads to transition across the G1/S checkpoint, resulting in rapid cell proliferation [22]. The LNT-induced down-regulation of cyclinD1 consolidated that LNT could repress MG63 cell proliferation. Consistent with the pro-apoptotic role of LNT in S-180 tumor cells [23], MG63 cell apoptosis was enhanced by LNT. A polysaccharide derived from *Ganoderma* lucidum has reported to induce mitochondrial-dependent apoptosis in MG63 cells [24]. LNT is also a polysaccharide, thus we further explored the alteration of caspases after LNT treatments. The activation of caspase-9 and caspase-3, induced by LNT, augmented the pro-apoptotic ability of LNT in MG63 cells.

Autophagy is a conserved degradation process by which damaged or useless cellular components are eliminated [25]. Under stressful conditions, autophagy in cells is facilitated for maintaining of metabolic homeostasis. However, excessive or prolonged autophagy can lead to cell death [26]. Hence, we tested the alteration of autophagy in MG63 cells after LNT treatments. At the initiation of autophagy, LC3B-I is converted to LC3B-phospholipid conjugate (LC3B-II) and LC3B-II accumulation is considered as a marker of autophagy [27]. Beclin-1 is an essential protein that is of great importance for autophagic initiation [28]. p62 is a multifunctional protein that binds ubiquitinated proteins and directs them to the autophagosome, resulting in protein degradation [29]. In our study, enhancements of LC3B-II/LC3B-I and Beclin-1 as well as down-regulation of p62, induced by LNT, indicated the pro-autophagic effects of LNT on MG63 cells.

MiR-340 is widely reported as a tumor suppressor in several cancer types such as breast cancer [30] and non-small cell lung cancer [31]. It has been reported to be down-regulated in OS tissues and cell lines, and can inhibit OS tumor growth [32]. Moreover, miR-340 can induce autophagy in tumor cells through inhibiting anti-autophagy factor [33]. Taken into consideration of all the descriptions above, we hypothesized miR-340 might be involved in the modulation of LNT in MG63 cells. Accordingly, we interestingly found miR-340 level was up-regulated in LNT-treated cells. Following experiments also proved that miR-340 inhibition could reverse the effects of LNT on cell apoptosis and autophagy of MG63 cells, indicating that LNT might affect MG63 cells by up-regulating miR-340.

The MAPK/ERK pathway is associated with cell survival, proliferation, apoptosis, and death [34]. A previous study once reported that autophagy can be selectively changed at the maturation step by activation of the MAPK/ERK pathway, leading to accumulation of defective autolysosomes [35]. Thus, we also explored the alteration of the MAPK/ERK pathway after LNT treatments. Accordingly, LNT repressed this signaling cascade, and the repression was reversed by miR-340 inhibition, suggesting that LNT might inactivate the MPAK/ERK pathway by up-regulating miR-340 in MG63 cells.

In summary, we first found LNT could effectively repress proliferation and induce apoptosis and autophagy in MG63 cells. Moreover, we found miR-340 was involved in the modulation of LNT, showing that LNT affected apoptosis, autophagy, and activation of the MAPK/ERK pathway through up-regulating miR-340. As a preliminary investigation for the effective role of LNT in OS, our study provides innovative therapeutic strategies for the treatments of OS, and more *in vivo* experiments will be performed in the future.

Acknowledgements

This work is supported by Ningbo Natural Science Foundation (2017A610189) and Key Subjects of Ningbo No. 2 Hospital (2016-57).

Disclosure of conflict of interest

None.

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