The Effects of Interleukin-33 (IL-33) on Osteosarcoma Cell Viability, Apoptosis, and Epithelial-Mesenchymal Transition are Mediated Through the PI3K/AKT Pathway

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Background: Osteosarcoma is the most common primary tumor of bone. Interleukin-33 (IL-33) is a pro-inflammatory cytokine that also participates in tumor progression. This study aimed to investigate the role of IL-33 in human osteosarcoma cell viability, proliferation, apoptosis, and epithelial-mesenchymal transition (EMT) in vitro and the molecular mechanisms involved.

Material/Methods: The normal osteoblast cell line, hFOB 1.19, and the human osteosarcoma cell lines SOSP-9607, SAOS2, MG63, and U2OS were studied. The expression of IL-33 mRNA and protein in human osteosarcoma cell lines were detected using quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot, respectively. The effects of IL-33 on human osteosarcoma cell viability, apoptosis, EMT, and the signaling pathways were studied using the MTT assay, flow cytometry, qRT-PCR, and Western blot.

Results: IL-33 was upregulated in human osteosarcoma cell lines, including U2OS cells. The use of an IL-33 gene plasmid promoted osteosarcoma cell viability, inhibited cell apoptosis, increased the expression of Bcl-2, and reduced the expression of Bax. IL-33 reduced the level of E-cadherin and increased the levels of N-cadherin and matrix metalloproteinase-9 (MMP-9) in osteosarcoma cells at the mRNA and protein level. The use of the IL-33 plasmid increased the protein expression levels of p-AKT and the p-AKT/AKT ratio in osteosarcoma cells, and IL-33 siRNA reversed these findings.

Conclusions: IL-33 was highly expressed in human osteosarcoma cells. Down-regulation of IL-33 reduced cell viability and EMT of osteosarcoma cells, and induced cell apoptosis through activation of the PI3K/AKT signaling pathway.

MeSH Keywords: Apoptosis • Cell Proliferation • Epithelial-Mesenchymal Transition • Osteosarcoma

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Background

Osteosarcoma is the most common primary tumor of bone and accounts for approximately 60% of cancer-related deaths in children and young adults [1]. Although there have been recent improvements in the treatment of osteosarcoma, including chemotherapy, radiotherapy, and surgery, the survival rates for advanced-stage osteosarcoma remain poor [2]. Therefore, the cellular and molecular mechanisms involved in the pathogenesis and progression of osteosarcoma require further study to identify new approaches to treatment.

Interleukin-33 (IL-33) is a cytokine of the IL-1 superfamily [3], and is considered to be the natural ligand for the orphan IL-1 receptor (IL-1R), ST2 [4]. IL-33 combines with ST2 and IL-1R accessory protein dimers, and IL-33 expression results in the activation of the MyD88-dependent toll-like receptor signaling pathway, the nuclear factor-xB (NF-xB) pathway, and the mitogen-activated protein kinase (MAPK) pathway [5,6]. Recent studies have shown that IL-33 has an important role in driving allergic responses [7], including asthma [8], atopic dermatitis, and anaphylaxis [9,10]. Also, inflammation associated with atherosclerosis and infection-induced tissue damage are associated with the expression of IL-33 [11].

Due to the significance of IL-33 in inflammation, recent studies have identified the role of IL-33 in carcinogenesis. IL-33 is overexpressed in several human cancers, including colorectal cancer (CRC), breast cancer, and non-small cell lung cancer (NSCLC) [12–14]. Recently, a study of breast cancer showed that the IL-33/ST2 signaling pathway suppressed anti-tumor immunity and accelerated the development of breast cancer metastasis [15]. IL-33 has also been reported to play an essential role in glioma and colon cancer cell metastasis [16,17]. These findings indicate that IL-33 may promote tumor progression. However, although IL-33 has previously been reported to be expressed in osteosarcoma [18–20], the role and mechanisms of IL-33 in osteosarcoma cells remain unclear.

Therefore, this study aimed to investigate the role of IL-33 in human osteosarcoma cell viability, proliferation, apoptosis, and epithelial-mesenchymal transition (EMT) in vitro and the molecular mechanisms involved.

Material and Methods

Cell lines and cell culture

The normal osteoblast hFOB1.19 cell line and the four human osteosarcoma cell lines, MG63, SOSP-9607, U2OS, and SAOS2, were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 5 U/ml heparin, 100 U/ml penicillin, and 100 U/ml streptomycin, and incubated at 37°C and 5% CO₂.

Cell transfection and reagents

The IL-33 plasmid (Cat no. sc-417699-ACT), the plasmid control (Cat no. sc-437275), IL-33 siRNA (Cat no. sc75333), and control siRNA (Cat no. sc36869) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For transient transfection, the IL-33 plasmid, the plasmid control, IL-33 siRNA or control siRNA were transfected into U2OS cells using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. At 48 h after cell transfection, the cells were collected for further study. The transfection efficiency was detected by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs were extracted from cell lysates using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. The HiScript™ II QRT SuperMix was used to reverse total RNA (1 µg) into cDNA synthesis following the manufacturer’s instructions. Amplification was performed using qRT-PCR using a Step One Plus system (Roche Molecular Diagnostics, Pleasanton, CA, USA) in 20 µl of reaction mixture including 2 µl of cDNA template, 4 µl of each primer in double-distilled H₂O, and 10 µl of ChamQ™ Universal SYBR qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). The PCR conditions were as follows: 30 cycles of denaturation at 95°C for 60 sec; annealing at 60°C for 60 sec; and PCR extension at 72°C for 1 min; and a final extension step at 72°C for 10 min. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as internal control. The 2^{ΔΔCq} method was used to calculate relative expression levels [21].

The PCR primer sequences used were:

GAPDH, forward: 5'-CTTTGGATCGTGGAAGGACTC-3';
GAPDH, reverse: 5'-GTAAGGCCAGGGATGTTCTC-3';
IL-33, forward: 5'-GCGAAACAACAGGAAACACTCTG-3';
IL-33, reverse: 5'-CCTCAGGATCTCTTGTACT-3';
E-cadherin, forward: 5'-CGAGGCTACAGCGTTACAGG-3';
E-cadherin, reverse: 5'-GGGTTGCGAGGGAATAAGG-3';
N-cadherin, forward: 5'-TTTGGAGGAGCTCCTTACACC-3';
N-cadherin, reverse: 5'-ACGTCCACACCGTTGAAATGTG-3';
MMP-9, forward: 5'-AGCCCACTAGTCCACCTGA-3';
MMP, reverse: 5'-CCATTAGCGAGCACGAC-3'.

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**Western blot**

Total proteins from cells were extracted by RIPA lysis buffer (Beyotime Biotechnology, Inc., Shanghai, China), and centrifuged at 12,000 rpm for 30 min at 4°C. The concentration of protein was quantified with a BCA Protein Assay Kit (Beyotime Biotechnology, Inc., Shanghai, China). Equal amounts of protein samples (40 μg) was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% dried skimmed milk powder at room temperature for 1 h and incubated at 4°C overnight with the primary antibodies. The primary antibodies were obtained from Abcam (Cambridge, MA, USA) and included antibodies to IL-33 (1: 1,000) (Cat no. Ab54385), Bcl-2 (1: 1,000) (Cat no. Ab185002), Bax (1: 1,000) (Cat no. Ab32503), E-cadherin (1: 1,000) (Cat no. Ab1416), N-cadherin (1: 1,000) (Cat no. Ab18203), MMP-9 (1: 1,000) (Cat no. Ab38898), p-AKT (1: 1,000) (Cat no. Ab38449), AKT (1: 1,000) (Cat no. Ab18785), and GAPDH (1: 1,000) (Cat no. Ab181602). The membranes were washed four times in PBST and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1: 2,000) (Cat no. 7074; Cell Signaling Technology, Danvers, MA, USA) at room temperature for 1 h. The protein bands were visualized using enhanced chemiluminescence (ECL) Western blot substrate (Pierce Biotechnology, Rockford, IL, USA) was used. Proteins were quantified by densitometry using Quantity One version 4.5.0 software (Bio-Rad, Hercules, CA, USA).

**Cell viability assay**

Cell viability was measured using the MTT assay according to the manufacturer's protocol. U2OS cells were plated into 96-well plates (10,000 cells per well) in triplicate and incubated overnight. The culture medium was removed, and the IL-33 plasmid, plasmid control, IL-33 siRNA, or control siRNA were added and co-transfected with the cells for 48 h at 37°C. Then, MTT solution (10 μl) was mixed with the cells and incubated at 37°C for another 4 h, according to the manufacturer's instructions. After removing the supernatant, 100 μl of dimethyl sulfoxide (DMSO) (KeyGen Biotech Co. Ltd., Nanjing, China) was added to solubilize the formazan crystals. The optical density (OD) was measured at 490 nm using an automated microplate reader (BioTek, Winooski, VT, USA).

**Flow cytometry for cell apoptosis**

U2OS cells were transfected with the IL-33 plasmid, the plasmid control, IL-33 siRNA, or control siRNA for 48 h. Then, the U2OS cells were trypsinized. An Annexin V, fluorescein isothiocyanate (FITC), and propidium iodide (PI) cell apoptosis detection kit (Sigma-Aldrich, St. Louis, MO, USA) was used to analyze cell apoptosis by flow cytometry using a BD FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer’s protocol. The data were analyzed using FlowJo software version 7.6.1 (FlowJo LLC, Ashland, OR, USA).

**Statistical analysis**

Data were presented as the mean±standard deviation (SD). The experiments were performed in triplicate. Data were analyzed using SPSS version 18.0 software (SPSS Inc, USA). Differences between the groups were analyzed using Student’s t-test or one-way analysis of variance (ANOVA) with Bonferroni’s post hoc test. A P-value <0.05 was considered to be statistically significant.

**Results**

**Interleukin-33 (IL-33) expression was increased in human osteosarcoma cell lines**

The IL-33 level in human osteosarcoma cell lines, SOSP-9607, SAOS2, MG63, and U2OS, and the normal osteoblast cell line, hFOB1.19, was detected using quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot. As shown in Figure 1A and 1B, compared with the normal osteoblast hFOB1.19 cell line, human osteosarcoma cell lines MG63, SOSP-9607, U2OS, SAOS2 expressed increased levels of IL-33. Because the expression of IL-33 was greatest in the U2OS human osteosarcoma cells, the U2OS cell line was used for the subsequent experiments.

**Increased expression of IL-33 increased cell viability and inhibited apoptosis in osteosarcoma cells**

The IL-33 plasmid and the plasmid control were transfected into the osteosarcoma cells to examine the biological effect of IL-33 on osteosarcoma cells. Western blot and qRT-PCR were performed to evaluate the expression levels of IL-33 in U2OS cells. The use of the IL-33 plasmid significantly increased mRNA and protein expression of IL-33 in U2OS cells (Figure 2A, 2B). Cell viability was assessed using the MTT assay to investigate the effect of increased expression of IL-33 on the viability of U2OS cells. As shown in Figure 2C, increased expression of IL-33 significantly increased U2OS cell viability. Flow cytometry was performed to determine the effect of the IL-33 plasmid on cell apoptosis. A significantly reduced cell apoptosis rate was detected in U2OS cells following IL-33 plasmid transfection compared with the plasmid transfection control group (Figure 2D, 2E). Western blot confirmed that the IL-33 plasmid regulated the expression of apoptosis-related proteins in U2OS cells and showed increased Bcl-2 protein expression and decreased Bax protein expression (Figure 2F). These findings...
showed that IL-33 upregulation increased cell viability and reduced cell apoptosis in U2OS cells in vitro.

**Increased expression of IL-33 increased epithelial-mesenchymal transition (EMT) in osteosarcoma cells**

EMT plays an important role in tumor migration. The IL-33 plasmid and the plasmid control were transfected into the U2OS cells for 48 h to investigate the effect of the IL-33 plasmid on the expression of EMT markers, which were detected by qRT-PCR and Western blot. U2OS cells transfected with the IL-33 plasmid showed increased protein and mRNA expression levels of N-cadherin (Figure 3A, 3C) and MMP-9 (Figure 3A, 3D), and lower protein and mRNA expression levels of E-cadherin (Figure 3A, 3B) compared with the plasmid control transfection group. The results showed that increased expression of IL-33 promoted EMT in U2OS cells in vitro.

**Increased expression of IL-33 activated the PI3K/Akt pathway in osteosarcoma cells**

U2OS cells were transfected with the IL-33 plasmid and the plasmid control for 48 h to determine the relationship between increased expression of IL-33 and the PI3K/Akt pathway. The protein levels of p-AKT and AKT were measured by Western blot after transfection. As shown in Figure 3A and 3E, Western blot showed that the transfection of U2OS cells with the IL-33 plasmid significantly increased the protein expression level of AKT and the p-AKT/AKT ratio. The results indicated that overexpressed IL-33 could activate PI3K/Akt pathway in U2OS cells in vitro.

**IL-33 down-regulation inhibited cell viability and induced apoptosis in U2OS osteosarcoma cells**

U2OS cells were transfected with IL-33 siRNA and control siRNA. After cell transfection, the level of IL-33 was detected by using qRT-PCR and Western blot. The mRNA and protein expression of IL-33 observably reduced after transfection with IL-33 siRNA compared with the control siRNA transfection group (Figure 4A, 4B). MTT assay was used to investigate the effects of IL-33 siRNA on osteosarcoma cell viability. The results illustrated that IL-33 down-regulation reduced the cell viability of U2OS cells (Figure 4C). Flow cytometry analysis indicated that IL-33 siRNA induced apoptosis in osteosarcoma cells (Figure 4D, 4E). Also, to clarify the proapoptotic effect of IL-33 siRNA on U2OS cells, expression of apoptosis-related proteins Bax and Bcl-2 were examined by Western blot. Figure 4F shows that IL-33 siRNA reduced the protein expression of Bcl-2 and increased Bax protein levels in U2OS cells in vitro.

**Down-regulation of IL-33 reduced EMT in U2OS osteosarcoma cells**

Transfection with IL-33 siRNA and control siRNA of U2OS cells for 48 h was performed to investigate the effects of IL-33 siRNA on EMT. Western blot and qRT-PCR were performed to investigate the expression levels of markers of EMT. Western blot showed that IL-33 siRNA promoted the protein expression levels of N-cadherin and MMP-9 in U2OS cells (Figure 5A). Similar results were obtained by qRT-PCR (Figure 5B–5D). These findings indicated that the down-regulation of IL-33 reduced EMT in U2OS cells in vitro.

**IL-33 down-regulation inhibited the PI3K/Akt pathway in U2OS human osteosarcoma cells**

The effect of IL-33 siRNA on the PI3K/Akt pathway in osteosarcoma cells was investigated by measuring the levels of p-AKT and AKT in the PI3K/Akt pathway by Western blot after transfection. As shown in Figure 5A and 5E, Western blot showed that IL-33 siRNA significantly reduced the protein expression of AKT and p-AKT in the PI3K/Akt pathway in U2OS cells.
of p-AKT and the p-AKT/AKT ratio in U2OS cells compared with the control siRNA transfection group (Figure 5A, 5E). The results supported that IL-33 down-regulation could inhibit the PI3K/AKT signaling pathway in U2OS human osteosarcoma cells in vitro.

**Discussion**

The expression of interleukin-33 (IL-33) has mainly been studied in inflammatory diseases [22]. Recently, several studies have shown that IL-33 plays a vital role in the development and progression of cancer [23]. IL-33 upregulation has been described in several cancers, including uterine cancer and lung cancer [24,25]. Increased expression levels of IL-33 have
been reported in the growth and metastasis of hepatocellular carcinoma (HCC) [26]. IL-33 has been reported to be associated with tumor cell invasion in head and neck squamous cell carcinoma [27]. The findings on the role of IL-33 from previous studies supported the aims of the present study to investigate the role and mechanisms of IL-33 in human osteosarcoma cells in vitro.

Koster et al. [18] showed that the GLDC/IL33 locus on chromosome 9p24.1 was associated with overall survival (OS) in osteosarcoma.
patients with osteosarcoma. Also, IL-33 gene polymorphisms were reported to be correlated with an increased risk of osteosarcoma [19]. IL-33 has been identified as a potential risk factor for the development and prognosis of osteosarcoma [20]. However, the role of IL-33 in human osteosarcoma cells remains to be determined. The findings from the present study demonstrated that the level of IL-33 in human osteosarcoma cell lines was higher than those in the normal control cells.

Clinical studies have shown that IL-33 may function as an oncogenic cytokine, which resulted in intestinal tumorigenesis in humans and mice [28]. IL-33 may activate tumor stroma to accelerate intestinal polyposis [29]. IL-33 was also shown to increase the growth of gastric cancer cells and ovarian cancer cell growth and invasion [30]. The findings from the present study were consistent with the findings from these previous studies and showed that the upregulation of IL-33 significantly increased cell viability and inhibited the cell apoptosis of human osteosarcoma cells.

Figure 4. Down-regulation of interleukin-33 (IL-33) reduced cell viability, cell apoptosis, and expression of apoptosis-related factors in U2OS human osteosarcoma cells in vitro. IL-33 siRNA and control siRNA were transfected into U2OS cells for 48 h. (A, B) The expression levels of IL-33 were measured by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot. (C) U2OS cell viability was measured by the MTT assay. (D, E) Apoptosis of U2OS cells is shown by double-staining with Annexin V and fluorescein isothiocyanate (FITC) and flow cytometry. (F) Expression levels of Bcl-2 and Bax proteins were measured by Western blot. The data are shown as the mean±standard deviation (SD). ** p<0.01 vs. the control siRNA.
human osteosarcoma cells in vitro. However, the down-regulation of IL-33 significantly inhibited osteosarcoma cell viability and increased osteosarcoma cell apoptosis. IL-33 participated in osteosarcoma cell growth and down-regulation of IL-33 inhibited cell viability and promoted cell apoptosis in vitro.

Epithelial-mesenchymal transition (EMT) is a complex biological process that leads to loss of the cell epithelial phenotype to increase cell motility, cell invasion, and metastasis of cancer cells [31]. EMT is regulated by transcription factors that suppress epithelial genes that express E-cadherin and promote mesenchymal genes that encode N-cadherin and matrix

Figure 5. Down-regulation of interleukin-33 (IL-33) reduced epithelial-mesenchymal transition (EMT) and the PI3K/AKT signaling pathway in U2OS human osteosarcoma cells in vitro. U2OS cells were transfected with IL-33 siRNA and control siRNA for 48 h. (A) The protein levels of the epithelial-mesenchymal transition (EMT) markers, E-cadherin, N-cadherin, matrix metalloproteinase-9 (MMP-9), p-AKT, and AKT in transfected cells were measured by Western blot. (B–D) The mRNA levels of the EMT markers, E-cadherin, N-cadherin, and MMP-9 in transfected cells were detected by quantitative real-time polymerase chain reaction (qRT-PCR). (E) The ratio of p-AKT/AKT was determined. The data are shown as the mean±standard deviation (SD). ** p<0.01 vs. control siRNA.
metalloproteinase-9 (MMP-9) [32]. In the present study, the IL-33 plasmid and IL-33 siRNA were transfected into human osteosarcoma cells to investigate the effects of IL-33 on the level of EMT-associated genes and proteins. The findings showed that over-expression of IL-33 resulted in the down-regulation of E-cadherin and upregulation of N-cadherin and MMP-9 expression in osteosarcoma cells. The down-regulation of IL-33 had the opposite effects. These findings supported that IL-33 down-regulation suppressed EMT in human osteosarcoma cells, indicating that IL-33 knockdown might be a promising therapeutic target for the inhibition of cell invasion in osteosarcoma.

Previously published studies have shown that IL-33 expression resulted in the activation of signaling pathways, including the PI3K/AKT and MAPK pathways [33]. Several studies have shown that the PI3K/AKT pathway is activated in osteosarcoma, and inhibition of this pathway by small-molecule therapeutic compounds is a potential therapeutic approach for osteosarcoma [34–36]. PI3K generates phosphatidylinositol-3,4,5-triphosphate (PIP3) on the plasma membrane, and PIP3 is transferred to the form p-Akt [37]. Then, p-Akt affects cell proliferation and apoptosis by regulating the expression of oncogenic factors [38]. In the present study, the protein expression level of p-AKT and AKT on the PI3K/AKT signaling pathway in osteosarcoma cells was measured by Western blot. The findings showed that the upregulation of IL-33 resulted in increased p-AKT protein levels and an increase in the p-AKT/AKT ratio, and down-regulation of IL-33 resulted in a reduction in the p-AKT level and the p-AKT/AKT ratio in osteosarcoma cells.

This study had several limitations. Although the results showed that IL-33 was significantly upregulated in human osteosarcoma cell lines, one cell line, U2OS, was chosen for most of the experiments. In this in vitro study, down-regulation of IL-33 suppressed osteosarcoma cell viability and EMT, and induced cell apoptosis by inhibiting the PI3K/AKT signaling pathway. However, this in vitro study was preliminary and may not represent the in vivo changes found in human osteosarcoma. Therefore, future in vivo studies are required to validate the preliminary in vitro findings, including the role of the PI3K/AKT pathway.

Conclusions

This study aimed to investigate the role of IL-33 in human osteosarcoma cell viability, proliferation, apoptosis, and epithelial-mesenchymal transition (EMT) in vitro and the molecular mechanisms involved. IL-33 was highly expressed in human osteosarcoma cells. Down-regulation of IL-33 reduced cell viability and EMT of osteosarcoma cells, and induced cell apoptosis through activation of the PI3K/AKT signaling pathway.

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Conflict of interest

None.
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