Doxorubicin activates the Notch signaling pathway in osteosarcoma

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Abstract. Notch signaling is critical in various biological processes, including cell proliferation, differentiation and apoptosis. Furthermore, accumulating evidence indicated that aberrant Notch signaling has a tumor-promoting function in osteosarcoma. However, the effect of the conventional chemotherapeutic agent, doxorubicin, on Notch signaling remains unclear. In the present study, osteosarcoma cells were treated with various concentrations of doxorubicin and the effect on Notch signaling was analyzed. A cytostatic dose of doxorubicin (<0.5 μM) was identified to significantly activate the Notch signaling pathway in a dose-dependent manner (P<0.01), as demonstrated by the elevated expression levels of Notch target genes. However, a toxic dose of doxorubicin (≥0.5 μM) significantly inhibited the Notch signaling pathway (P<0.01). These results indicated a significant correlation between doxorubicin administration and the Notch signaling pathway. Therefore, the present study supports further investigation into Notch and osteosarcoma chemoresistance.

Introduction

Osteosarcoma is a highly malignant bone cancer that predominantly affects children and adolescents. It presents with aggressive local growth and early metastasis (1). Neo-adjuvant chemotherapy and advanced surgical techniques have improved long-term survival and quality of life for patients with osteosarcoma (2,3). However, 20% of patients will eventually develop recurrence, and patients with metastatic or recurrent disease have a poor prognosis (4,5). Targeting critical molecular signaling pathways involved in osteosarcoma carcinogenesis may be the key to providing novel treatment approaches for patients with recurrent disease.

When systemic chemotherapeutic agents are administered to patients, the response of each cell type is different (6). Various factors contribute to this heterogeneic response, in which cell hierarchy plays an important role. A number of reports have identified the existence of osteosarcoma stem cells, a subpopulation of cells that possess the capacity to self-renew and multi-differentiate (7-9). In addition, cancer stem cells participate in drug resistance, thus contributing to treatment failure (10,11). Therefore, elucidating the effect of conventional chemotherapeutic agents on osteosarcoma is critical for understanding osteosarcoma tumor biology.

The Notch signaling pathway is pivotal in a variety of biological processes, including cell proliferation and apoptosis, as well as stem cell maintenance and differentiation (12-14). The pathway consists of Notch ligands, receptors, negative and positive modifiers, and target transcription factors. The Notch receptor undergoes two successive proteolytic cleavages upon interaction with the ligand. Subsequently, the intracellular domain of Notch is released, translocates to the nucleus and forms a complex that activates the transcription of specific target genes, including hairy/enhancer of split (*Hes*) and *Hes* related with YRPW motif (*Hey*) (15,16). Dysregulated Notch activity has been reported in an increasing number of malignancies, such as colon (17,18), pancreatic (19,20) and cervical (21) cancer. Additionally, dysregulated Notch activity has been reported to contribute to the carcinogenesis of osteosarcoma (22-26). However, the role of Notch in osteosarcoma chemoresistance remains unclear. Therefore, in the present study, the effect of doxorubicin on the activity of the Notch signaling pathway was evaluated in 143B osteosarcoma cell lines.

Materials and methods

Cell culture. The 143B human osteosarcoma cell line was purchased from China Center for Type Culture Collection (Wuhan, China). All the cells were cultured in RPMI-1640 medium containing 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA). The cells were propagated in a humidified atmosphere with 5% CO_2 at 37˚C. Cell viability

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Table I. Primer sequences used for reverse transcription-quantitative polymerase chain reaction.

Gene	Forward primer sequence	Reverse primer sequence
Hes1	5'-CAGATCAATGCCATGACCTACC-3'	5'-AGCCTCCAAACACCTTAGCC-3'
Hes5	5'-AGCCCCAAAGAGAAAAACCGACTG-3'	5'-TGGAGCGTCAGGAACTGCACGG-3'
Hey1	5'-CATGTCCCCAACTACATCTTCC-3'	5'-CCTTGCTCCATTACCTGCTTC-3'
Hey2	5'-ACCTCTCTCTTGTCCCTCTCTG-3'	5'-GGTTTATTGTTTGTTCCACTGC-3'
HeyL	5'-ACCGCATCAACAGTAGCCTTTCT-3'	5'-GCATTTTCAAGTGATCCACCGTC-3'
β-actin	5'-GTCCACCGCAAATGCTTCTA-3'	5'-TGCTGTCACCTTCACCGTTC-3'

Hes, hairy/enhancer of split; *Hey*, *Hes* related with YRPW motif.

was determined by trypan blue staining (Invitrogen Life Technologies).

Cell cytotoxicity assay. Cells were added to 96-well culture plates at a density of 5000 cells/well. The cells were treated with various concentrations of doxorubicin dissolved in DMSO, to a total volume of 100 μl per well; control cells were treated with DMSO only. The cells were cultured (as previously described) for different time periods, as indicated in Fig. 1A. Next, 10 μl Cell Counting Kit-8 (Beyotime Institute of Biotechnology, Shanghai, China) was added to each well and incubated at 37˚C for 2 h. The optical density of each well was measured at a wavelength of 450 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from 143B cells using the RNeasy Plus Mini Kit (Qiagen China Co., Ltd, Shanghai, China) and the concentration and purity determined using an ND-1000 spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific, Wilmington, DE, USA). Reverse transcription was performed using the TaqMan Reverse Transcription Reagents (Applied Biosystems Life Technologies, Foster City, CA, USA). RT-qPCR reactions were set up in triplicate and performed on the 7900 PCR machine (Applied Biosystems Life Technologies) using SYBR Green PCR Master Mix (Applied Biosystems Life Technologies). Conditions used for amplification of cDNA fragments were as follows: 95˚C for 5 min, 40 cycles of amplification (95˚C for 15 sec, 60˚C for 1 min). The expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method as described previously (27) and normalized to β-actin. The gene-specific primers used are listed in Table I.

Western blot analysis. Proteins were extracted with Protein Lysis Buffer (Sigma Aldrich, St. Louis, MO, USA). Lysates were then centrifuged at 10000 x g for 10 min at 4˚C, and supernatants were collected. Protein concentrations were assessed using the Bicinchoninic acid Protein Assay Kit (Sigma-Aldrich). Cell lysates containing 40 μg protein were separated on a 10% SDS-PAGE gel and then were transferred onto polyvinylidene difluoride membranes (Invitrogen Life Technologies) using a Trans Blot Turbo (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Membranes were blocked in a solution of Tris buffered saline with containing 0.05% Tween-20 and 5% skimmed milk for 1 h at room temperature. Primary antibodies were incubated

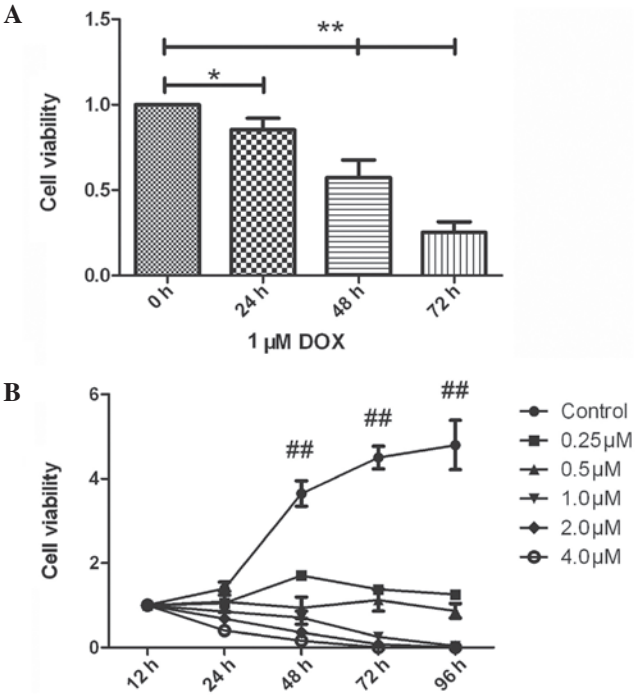

Figure 1. Effect of doxorubicin on osteosarcoma cell viability using a Cell Counting Kit-8 assay. Osteosarcoma 143B cells were treated with various concentrations of doxorubicin for different time periods. Doxorubicin inhibited cell viability in a (A) time- and (B) dose-dependent manner. Results are presented as the mean ± standard deviation of three independent experiments. *P<0.05, **P<0.01 vs. control, $^{\#\#}$P<0.01 vs. DOX. DOX, doxorubicin.

overnight at 4˚C. The following polyclonal rabbit anti-human primary antibodies were used: anti-Hes1 (catalog no. ab71559; Abcam, Cambridge, MA, USA; dilution, 1:500); anti-Hey1 (catalog no. ab22614; Abcam; dilution, 1:500) and anti-β-actin (catalog no. ab8227; Abcam; dilution, 1:2,000). Horseradish peroxidase-conjugated secondary antibodies (Abcam; dilution, 1:5,000) were incubated for 2 h at room temperature. Finally, the membranes were washed again and developed using an enhanced chemiluminescence substrate (Sigma-Aldrich).

Statistical analysis. Statistical analyses were performed using the SPSS 13.0 statistical software package (SPSS Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation of three independent experiments. The Student's *t*-test was used to compare the means of the two groups. When more than three means were compared, a one-way analysis of

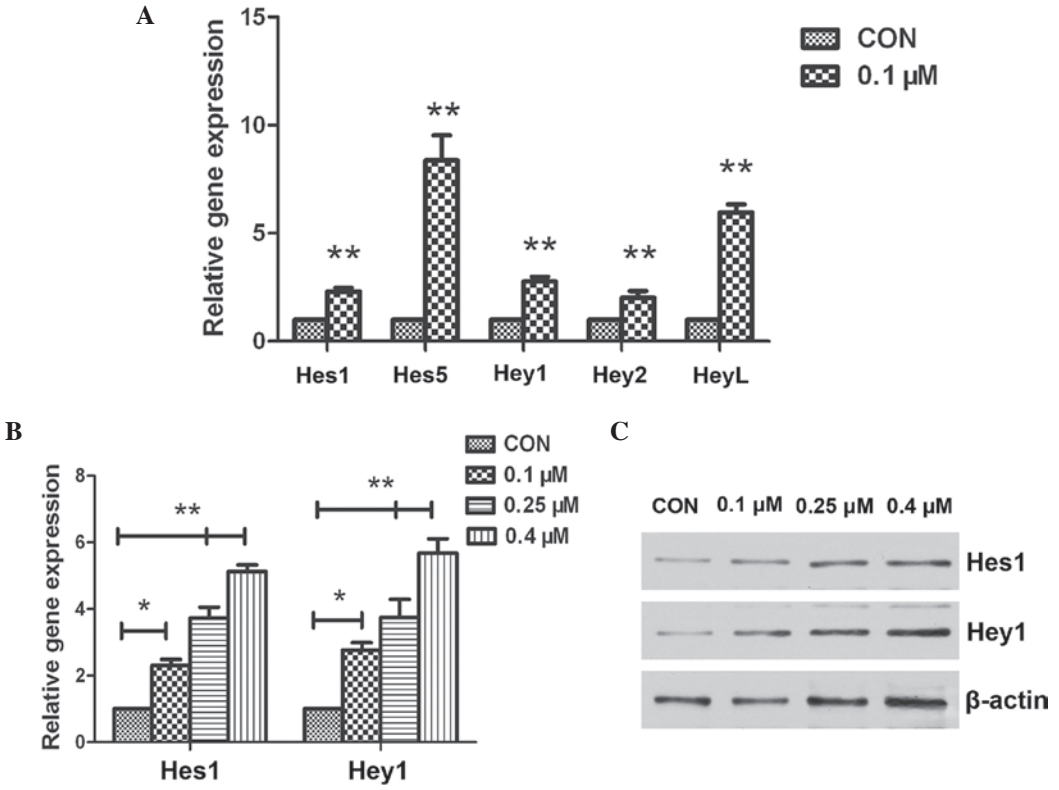

Figure 2. Activation of Notch target genes in osteosarcoma 143B cells by treatment with nontoxic concentration of doxorubicin (<0.05 μM) for 48 h. (A) Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) data indicating that doxorubicin treatment increased the mRNA expression levels of various Notch target genes. (B) RT-qPCR and (C) western blot data demonstrating that doxorubicin treatment increased the mRNA and protein expression levels, respectively, of two Notch target genes (*Hes1* and *Hey1*) in a dose-dependent manner. Results are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01 vs. control. CON, control; *Hes*, hairy/enhancer of split; *Hey*, *Hes* related with YRPW motif.

variance followed by multiple comparisons among the means was used. P<0.05 was considered to indicate a statistically significant difference.

Results

Optimizing the concentration of doxorubicin treatment. To determine an optimum doxorubicin dose range for subsequent studies, time- and dose-dependent cytotoxic assays were performed. The data indicated that the treatment of osteosarcoma cells with doxorubicin exhibited time and dose dependency. Toxicity was significantly enhanced 48 h after exposure to doxorubicin (P<0.01; Fig. 1A). In addition, a concentration of ≥0.5 μM doxorubicin resulted in significantly higher toxicity; however, a doxorubicin concentration of <0.5 μM exhibited a cytostatic effect (Fig. 1B).

Doxorubicin increases Notch target gene expression in osteosarcoma cells. To understand the molecular mechanism involved in doxorubicin-induced stemness, changes in the Notch signaling pathway were investigated. The expression levels of various Notch target genes, including *Hes1*, *Hes5*, *Hey1*, *Hey2* and *HeyL*, were assessed in the 143B cells treated with 0.1 μM doxorubicin for 48 h using RT-qPCR analysis. A significant increase in *Hes1*, *Hes5*, *Hey1*, *Hey2* and *HeyL* mRNA expression levels was detected following doxorubicin treatment (P<0.05; Fig. 2A). Additional analysis was performed to determine whether the increase was dose-dependent. The

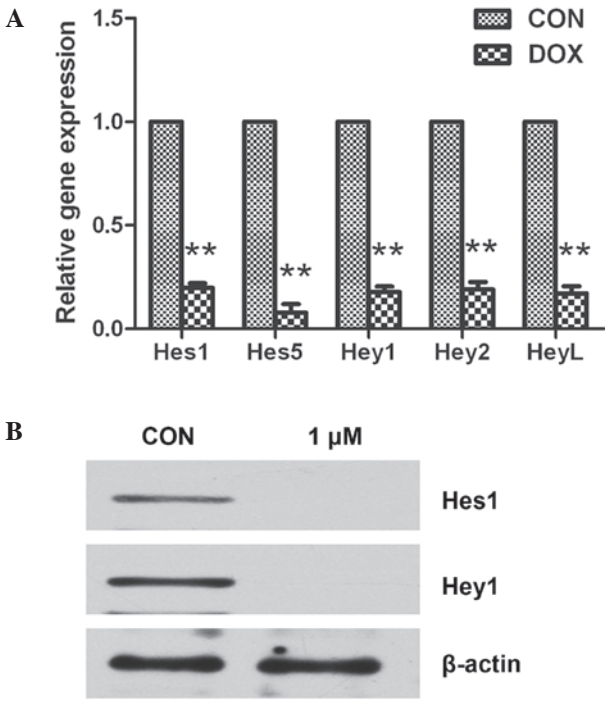

Figure 3. Suppression of Notch target genes in osteosarcoma 143B cells by treatment with a toxic dose of doxorubicin (1 μM) for 48 h. (A) Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and (B) western blot results demonstrated that doxorubicin treatment resulted in a significant decrease in Notch target gene expression levels. Results are presented as the mean ± standard deviation of three independent experiments. **P<0.01 vs. control. CON, control; *Hes*, hairy/enhancer of split; *Hey*, *Hes* related with YRPW motif.

143B cells wre treated with increasing concentrations of doxorubicin (0.1, 0.25 and 0.4 μM) for 48 h and the results demonstrated that *Hes1* and *Hey1* expression levels were upregulated in a dose-dependent manner (Fig. 2B). In order to confirm that Notch signaling was activated by doxorubicin, the expression of Notch target genes were also detected using western blotting. The results demonstrated that the expression levels of *Hes1* and *Hey1* were significantly enhanced by doxorubicin treatment (Fig. 2C).

High-dose doxorubicin decreases the expression of Notch target genes. To examine the effect of toxic doxorubicin on Notch target genes in osteosarcoma, 143B cells were treated with 1 μM doxorubicin. The Notch target genes, including *Hes1*, *Hes5*, *Hey1*, *Hey2* and *HeyL*, were found to be significantly suppressed by doxorubicin treatment ($P<0.05$; Fig. 3A). The results were confirmed using western blot analysis, and the findings were in agreement with the RT-qPCR data, as *Hes1* and *Hey1* were markedly downregulated following treatment with high-dose doxorubicin (Fig. 3B).

Discussion

The acceptance of chemotherapy as an integral and essential component of the treatment of osteosarcoma marked a new era for this disease. Doxorubicin was introduced for the treatment of osteosarcoma in the early 1970s (1). Although it is widely recognized that the agent intercalates into DNA and generates free radicals, the precise effect of doxorubicin on cancer cells requires further investigation (28).

Dysregulated Notch activity has been reported to contribute to the carcinogenesis of osteosarcoma (22), with *Notch1* activity appearing to be crucial for the invasion and metastasis of osteosarcoma (25). Furthermore, inhibition of the Notch signaling pathway suppressed osteosarcoma growth *in vitro* and *in vivo* (26). A number of studies have assessed the effect of conventional chemotherapeutic agents on the Notch signaling pathway. For instance, cisplatin has been reported to activate Notch signaling, as determined by increased expression levels of cleaved *Notch1* (29). However, the effect of doxorubicin on the Notch signaling pathway remains unclear. The present study demonstrated that doxorubicin elicits a dynamic and concentration-dependent effect on the Notch signaling pathway in osteosarcoma cells.

Cells may survive when they are exposed to a sublethal dose of therapeutic agent; however, the specific effects of conventional sublethal agents on osteosarcoma is critically important. Liu *et al* (29) identified that the administration of a low concentration of cisplatin enriched the population of multidrug resistant $CD133^+$ cells in lung adenocarcinoma. By contrast, blocking the Notch signaling pathway sensitizes cancer cells to chemotherapy (29,30). Therefore, the present study aimed to identify an effective but nontoxic optimum dose of doxorubicin for use in subsequent studies. The effect of doxorubicin was found to be dose- and time-dependent, and a period of 48 h was required for the agent to exert its effect. Furthermore, the current data demonstrated that the treatment of osteosarcoma cells with $\geq$0.5 μM doxorubicin for $\geq$48 h resulted in significant toxicity. Thus, subsequent investigations were conducted using concentrations limited to 0.5 μM. It was identified that the expression levels of various Notch target genes, including *Hes1*, *Hes5*, *Hey1*, *Hey2* and *HeyL*, were significantly increased in osteosarcoma cells following treatment with doxorubicin.

The observed enhancement in Notch signaling may be simply explained by the resistance of Notch-active cells to doxorubicin and doxorubicin treatment enriching this resistant cell population. In accordance with this hypothesis, recent studies have demonstrated that Notch appears to be involved in the mechanisms of cisplatin resistance (31-35). *Notch1* expression is negatively correlated with chemosensitivity; therefore, enhanced chemotherapeutic sensitivity may be obtained by blocking Notch signaling, in which case Notch gene expression should not be altered with different concentrations of doxorubicin. In the current study, treatment with a cytostatic concentration of doxorubicin appeared to directly activate the Notch signaling pathway in a dose-dependent manner. However, the underlying mechanism of this process requires further investigation.

In the present study, the expression of Notch genes following exposure to high-dose doxorubicin was significantly inhibited. Considering that the Notch signaling pathway appears to be crucial in the development of osteosarcoma, the authors of the present study propose that a high concentration doxorubicin partially exerts its cytotoxic effect via inhibition of the Notch signaling pathway. Alternatively, this observation may only be a side effect of early apoptosis.

In conclusion, doxorubicin activates the Notch signaling pathway at a sublethal dose and inhibits the Notch signaling pathway at a toxic dose. Along with the results of previous studies observing that cisplatin activates the Notch signaling pathway (29,30), the present study supports the combination treatment of recurrent osteosarcoma with a Notch inhibitor. In addition, the current results support the use of intensive chemotherapy to inhibit chemoresistance, since doxorubicin exerts its chemoresistant effect in a concentration-dependent manner.

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