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Original Article

HES1 is a positive prognostic factor and inhibits osteosarcoma tumorigenesis

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Abstract: HES1 is the target of Notch signaling which is reported to affect cell differentiation and maintain the cells in G0 phase in various tissues. HES1 expression appears to be a positive prognostic factor for survival of osteosarcoma patients. To better assess its significance, we analyzed HES1 expression in osteosarcoma patients and correlated its expression with the OS and RFS of osteosarcoma patients. First, we compared the expression of HES1 in human osteosarcoma tissue with non-tumor adjacent tissue by realtime PCR. According to the expression of HES1, the patients were divided into two groups: HES1-high group and HES1-low group. OS was calculated from the date of diagnosis until the date of death from any cause or until the date of final follow-up. Relapse-free survival (RFS) was determined for responders from the time of diagnosis until relapse or death from any cause. Kaplan-Meier analysis revealed that expression of HES1 predicted good prognosis for osteosarcoma patients. Then we up-reglated the expression of HES1 in osteosarcoma cell lines MNNG/HOS and U2OS. Results showed that induced activation of HES1 by retrovirus in osteosarcoma cell lines consistently led to osteosarcoma cell lines growth arrest and apoptosis induction. Our findings suggest that HES1 functions as a tumor suppressor and has an important role in inhibiting human osteosarcoma cell proliferation. Consequently, HES1 may have potential as a novel diagnostic and therapeutic target of osteosarcoma.

Keywords: Osteosarcoma, HES1, prognostic factor, proliferation

Introduction

Osteosarcoma is the most common type of malignant bone tumor, and it frequently originates in the metaphysis of the long bones [1]. In the past, the most common treatment for osteosarcoma was amputation. While the 5-year survival rate for patients with osteosarcoma has significantly improved, many patients are insensitive to available chemotherapeutics and have poor prognoses [2].

It is well known that HES1 is the target of Notch signaling which is reported to affect cell differentiation and maintain the cells in G0 phase in various tissues [3-5]. All of these confirmed that HES1 functioned as a suppressor of cell cycle. In this study, we found that the expression of HES1 in osteosarcoma tissue was down-regulated compared to that in non-tumor adjacent tissue which suggested that HES1 may be a prognostic factor of osteosarcoma. Kaplan-Meier analysis revealed that expression of HES1 predicted good prognosis for osteosarcoma patients. And induced activation of HES1 by retrovirus in osteosarcoma cell lines consistently led to osteosarcoma cell lines growth arrest and apoptosis induction.

Materials and methods

Patient samples

A total of 40 human osteosarcoma samples and the adjacent non-tumor tissue were collected between August 2008 and January 2014 after approval of the ethics committee and informed consent. ALL of these patients completed follow-up. The characteristics of patients are shown in **Table 1**.

Table 1. Clinical characteristics of the HES1 high-expression and low-expression groups

	Low-expression group	High-expression group
No. of patients	20	20
Male	13	11
Female	7	9
Age	17 (10-23)	28 (16-33)
Tumor location		
Femur	10	8
Tibia	4	5
Humerus	2	3
Fibula	2	2
Others	2	2
Histological classification		
Osteoblastic	15	13
Chondroblastic	3	4
Others	2	3

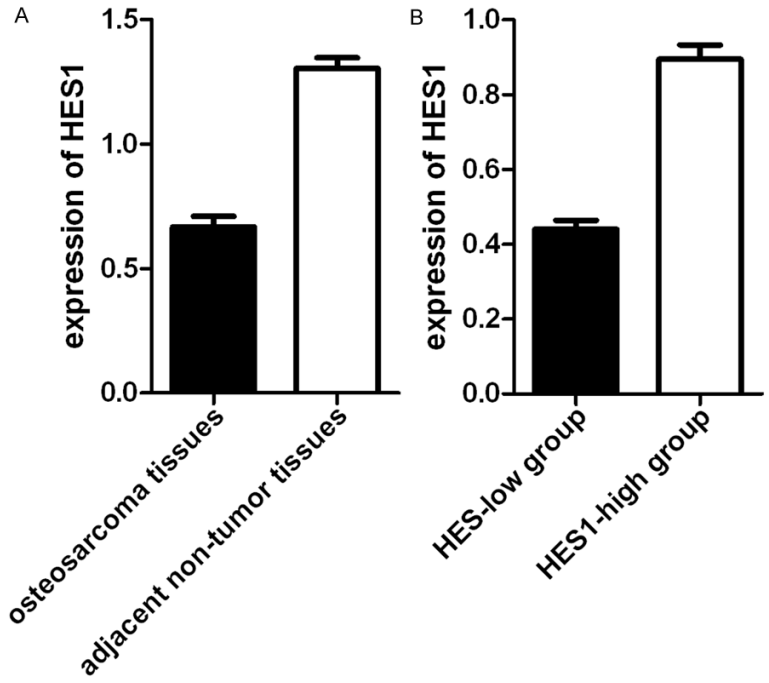

Figure 1. Expression of HES1 in osteosarcoma was analyzed by real-time PCR and wester. A. Relative expression of HES1 in osteosarcoma tissues and adjacent non-tumor tissues by real-time PCR. B. Different expression of HES1 in HES1-high and low group respectively.

Cell lines and cell culture

Two osteosarcoma cell lines were used: MNNG/HOS and U2OS. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (MNNG/HOS cells) or RPMI-1640 (U2OS cells) and supplemented with 10% fetal bovine serum (FBS).

Real-time RT-PCR analyses

Total RNA of osteosarcoma cells was extracted with Trizol (Invitrogen). Reverse transcription was achieved using QuantiTect Reverse Transcription Kit (Qiagen). Real-time PCR was performed using ABI-Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA) and Power SYBR Green PCR Master Mix (Applied Biosystems, #4367-659). The parameters for the thermal cycling of PCR were as follows: 15 seconds at 95°C and 60 seconds at 60°C, 45 cycles. The sequences of HES1 primers were upper: 5-GCAGATGACGGCTGCGCTGA-3, lower: 5-AAGCGGGTCACCTCGTTCATGC-3. GAPDH was used as housekeeper. The sequences of GAPDH primers were upper: 5-CGGAGTCAACGGATTTGGTCGTAT-3, lower: 5-AGCCTTCTCCATGGTGGTGAAGAC-3.

Cell proliferation assays and cell cycle analysis

Transduced cell lines were analyzed with MTT assay. Transduced cell lines were stained with annexin V and PI (BD Pharmingen) for 20 min at room temperature. The ratio of apoptosis was assessed by FACS.

Statistical analysis

Statistical analysis was performed using SSPS. For Kaplan-Meier estimates graphs, GraphPad Prism version 3.0 (GraphPad Software, San Diego, CA) software package for Windows was used. OS was calculated from the date of diagnosis until the date of death from any cause or until the date of final follow-up. Relapse-free

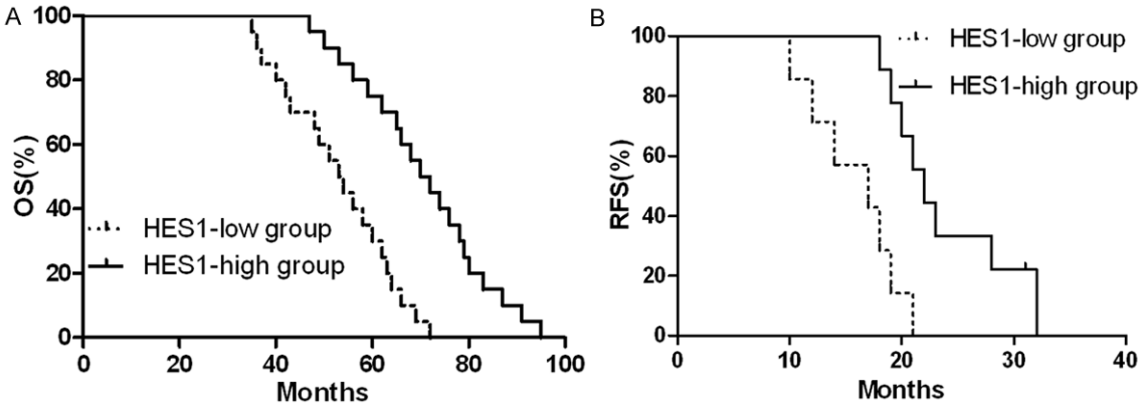

Figure 2. OS and RFS analysis of HES1-high and low groups. A. Effect of HES1 expression on overall survival (OS) in osteosarcoma patients. The OS time of the high-expression group is significantly longer than that of the low-expression group. B. Effect of HES1 expression on relapse-free survival (RFS) in osteosarcoma patients. The RFS time of the high-expression group was longer than that of the low-expression group.

Table 2. *P* values of all prognostic makers for overall survival as determined by the log-rank test and Gehan-Breslow-Wilcoxon test

Prognostic maker	p, log-rank	p, Gehan-Breslow-Wilcoxon
HES1	< 0.0001	< 0.0001
Sex	> 0.05	> 0.05
Age	> 0.05	> 0.05
Lung metastasis	< 0.05	< 0.05

survival (RFS) was determined for responders from the time of diagnosis until relapse or death from any cause. The significance of difference between survival curves was calculated by the log-rank test. Groupwise comparisons of the distributions of variables were performed with the generalized Wilcoxon test. A *P* value < 0.05 was considered significant.

Results

HES1 expression was down-regulated in osteosarcoma

To investigate whether HES1 expression levels were associated with the prognosis of osteosarcoma patients, we correlated results from real-time PCR data with clinical outcome of 40 patients with osteosarcoma. First, we compared the expression of HES1 between osteosarcoma tissues and the adjacent non-tumor tissues. Results showed that the average expression of HES1 in osteosarcoma tissues was 0.65±0.02, which was lower than that in adjacent non-tumor tissues (1.3±0.08, $P < 0.05$, **Figure 1A**). The top 50% of osteosarcoma cases with the high HES1 expression (> 0.66, n=20) were compared with the rest of the osteosarcoma cohort (< 0.65, n=20, **Figure 1B**).

Low HES1 expression was a poor prognostic factor for OS and RFS

The OS time in the low-expression group was significantly shorter than that in the high-expression group. The median survival of HES1-low group was 54±1.3 months, while that of HES1-high group was 70±1.5 months (P < 0.05, **Figure 2A**). The RFS time in the low-expression group was also significantly shorter than that in the high-expression group (17±0.002 months vs. 22±0.002 months, P < 0.005, **Figure 2B**). The possible predictive factors of OS are summarized in **Table 2**. Lung metastasis and HES1 expression were correlated with a poor OS.

HES1 inhibited the proliferation of osteosarcoma cells in vitro

MNNG/HOS and U2OS cells were transfected with a HES1-overexpression retrovirus to increase expression of HES1. The relative expression of HES1 after transfection was shown in **Figure 3A**. MTT assay was used to measure cell proliferation. The results showed that upregulation of HES1 inhibited osteosarcoma cell proliferation (**Figure 3B**). Cell cycle changes were analyzed by flow cytometry after transfection. Results of cell cycle analysis

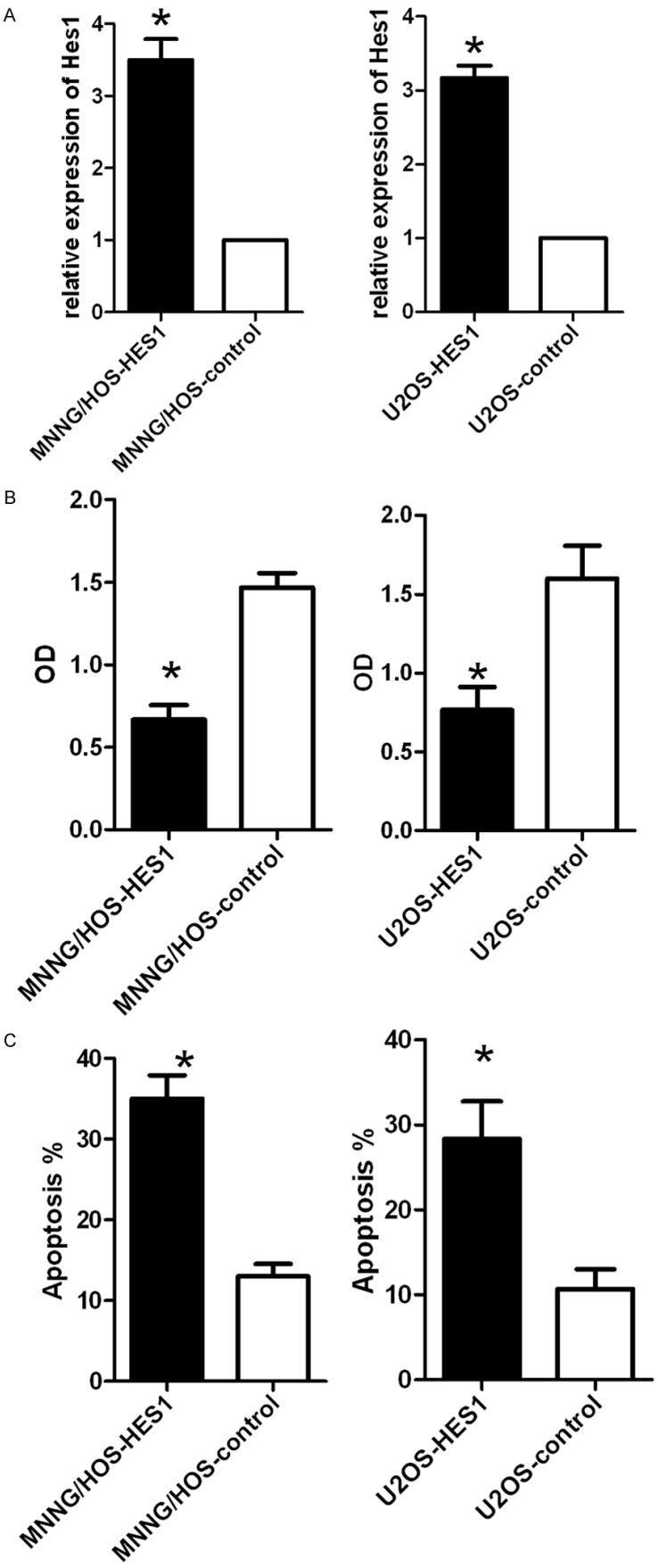

Figure 3. HES1 Activation induced growth arrest and apoptosis. A. RT-PCR was done to test the expression of HES1 after transfection. B. MTT assay of GFP+ cells on day 2 after transfection. C. Annexin V staining of GFP+ cells on day 2 after transfection. The data in the bar graphs were means with standard deviations. *$P < 0.05$ (t-test).

revealed that overexpression of HES1 inhibited cell cycle progression, with increased numbers of cells in G0 phase (**Figure 3C**).

HES1 inhibits tumor formation in vivo

MNNG/HOS cells stably expressing the HES1 or the control were subcutaneously injected into the left scapulas of nude mice, and the animals were closely monitored for tumor growth for 6 weeks. The results demonstrated that HES1-overexpressing tumors were significantly smaller in size and volume compared with control tumors (**Figure 4**).

Discussion

Osteosarcoma (OS) is one of the most common types of primary sarcoma of bone in children and young adults. To date, tumor excision, adjuvant chemotherapy, and radiotherapy have provided effective treatment strategies for osteosarcoma patients, significantly increasing their survival rate [6, 7]. But the long-term prognosis for OS patients still remains dismal due to the lack of effective early diagnostic biomarkers [8]. Identifying sensitive and specific biomarkers in carcinogenesis may improve diagnostic and therapeutic strategies for this malignancy.

HES1, the downstream effector of Notch pathway, is a member of basic helix-loop-helix tran-

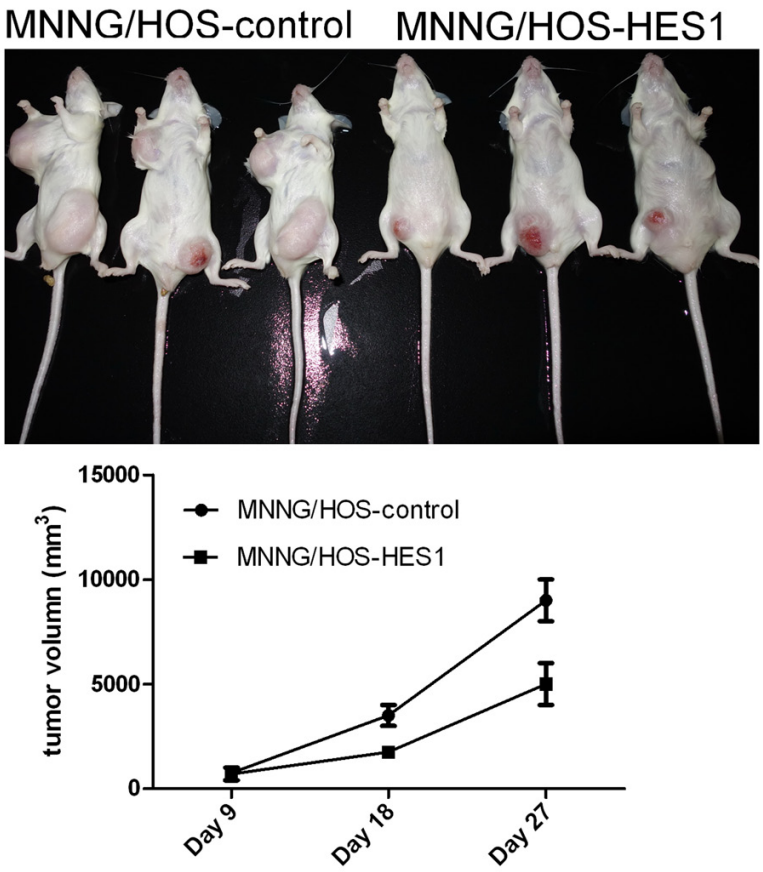

Figure 4. HES1 overexpression inhibited osteosarcoma cell growth *in vivo*. HES1+ osteosarcoma cell lines (10^6) were subcutaneously injected into NOD/SCID mice. The graph showed tumor size measured at the indicated days after cell injection (*$P < 0.05$).

scription factors which belongs to the Hes family [9]. Interestingly, HES1 expression can be used as a marker for poor prognosis for medulloblastoma and T-ALL [10, 11]. However, the role of HES1 in the prognosis of osteosarcoma has not well demonstrated. To investigate the clinical significance, we analyzed the HES1 expression in 40 patients with osteosarcoma by quantitative real-time reverse-transcription polymerase chain reaction.

According to the PCR results, these patients were then divided into the high-expression group and low-expression group. We then evaluated the expression of HES1 as a prognostic factor for osteosarcoma patients by Kaplan-Meier analysis. We showed that the high-expression group had a longer OS time and RFS time compared with those of the low-expression group. Of course, it was the limitation that we couldn't be sure whether HES1 was a reliable predictor for OS and RFS in other or larger cohorts. Till now, the prognosis of lung metastasis for osteosarcoma has been elucidated. HES1 is a newly epigenetically-regulated gene found to be prognostic factor for osteosarcoma.

HES proteins generally act as repressors of transcription. It has been reported that HES1 was involved in cell cycle, and maintained multipotent precursor cells in an undifferentiated state in several tissues during development and adulthood [12, 13]. However, the function and regulatory mechanism of SOX7 in OS remains unclear. In our study, we confirmed that HES1 could inhibit cell proliferation and promote apoptosis of human osteosarcoma through in vitro and in vivo experiments.

In summary, we observed that HES1 was downregulated in clinical osteosarcoma tumor samples and our results indicated that the expression of HES1 can be used as a poor prognostic factor for patients with osteosarcoma. We provided the antitumor activity of HES1 activation by inhibiting proliferation and inducing apoptosis of osteosarcoma cells and suggested that HES1 could be a potential approach for osteosarcoma therapy.

Disclosure of conflict of interest

None.

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