Analysis of changes in the expression of Notch1 and HES1 and the prognosis of osteosarcoma patients following surgery

LONG FANG[1], BEI LI[1,2], DAPENG YU[3], BAOLONG WANG[1] and TINGBAO ZHAO[1]

[1]Department of Orthopaedics, Shangdong Provincial Third Hospital, Cheeloo College of Medicine, Shangdong University; [2]Department of Orthopaedics, Shandong Cancer Hospital and Institute, Shandong First Medical University and Shandong Academy of Medical Sciences; [3]Department of Orthopaedics, Shangdong Provincial Western Hospital, Jinan, Shandong 250000, P.R. China

Received September 20, 2019; Accepted June 4, 2020

DOI: 10.3892/ol.2020.11890

Abstract. The present study aimed to analyze the changes in the expression of Notch1 and hairy and enhancer of split-1 (HES1) and the prognosis of patients with osteosarcoma following surgery. Samples from 62 patients with osteosarcoma treated at Shandong Cancer hospital from April, 2011 to June, 2013 were collected as the research group, and those from 52 healthy individuals undergoing physical examination were collected as the control group. The expression levels of Notch1 and HES1 in the serum of patients with osteosarcoma were measured by ELISA before and after surgery. Pearson's correlation analysis was used to analyze the correlation between Notch1 expression and HES1 expression in the osteosarcoma patients. According to the expression levels of Notch1 and HES1, the patients were divided into the high expression group and the low expression group, and the 5-year survival rate of the patients was observed. The expression levels of Notch1 and HES1 in the osteosarcoma patients before surgery were higher than those after surgery ($P<0.05$). The sensitivity, specificity and AUC of Notch1 for osteosarcoma were 93.55%, 58.06% and 0.732 respectively, and those of HES1 were 82.26%, 61.29% and 0.766, respectively. The expression level of Notch1 positively correlated with the expression level of HES1 in the osteosarcoma patients ($r=0.795$, $P<0.001$). According to the expression levels of Notch1 and HES1, the patients were divided into the high and low expression groups. The survival rate of the low expression group was significantly higher than that of the high expression groups ($P=0.045$). Finally, multiple factors were analyzed by logistic regression, and it was found that tumor location, chemotherapy response, tumor size, Notch1 and HES1 were independent risk factors for prognosis. Notch1 and HES1 exhibited a low expression in patients following surgery. ROC curve analysis revealed that the two indicators had good diagnostic efficacy and were expected to become markers for diagnosis and prognosis of osteosarcoma.

Correspondence to: Dr Tingbao Zhao, Department of Orthopaedics, Shangdong Provincial Third Hospital, Cheeloo College of Medicine, Shangdong University, 11 Wuyingshan Road, Jinan, Shandong 250000, P.R. China
E-mail: daos68@163.com; doctorzhaotingbao@163.com

Key words: osteosarcoma, Notch1, HES1, prognosis analysis

Introduction

Osteosarcoma is the most common malignant tumor among children, adolescents and young adults (1). Osteosarcoma originates from primary bone-forming mesenchymal cells, accounting for 20% of all primary osteosarcoma, and is the most common primary bone malignant tumor (2). Osteosarcoma usually occurs in the long bone of limbs near the metaphyseal plate. The most common sites are the femur, tibia and humerus (3). Before 1970, the treatment for osteosarcoma was mainly surgical resection. With the application of multi-drug regimens, chemotherapy has markedly improved the 5-year survival rate of patients with localized osteosarcoma from <20 to 65%; however, its prognosis is still very poor (4). Moreover, the mortality rate of patients with recurrent and metastatic osteosarcoma is still very high. Therefore, it of utmost importance to explore novel prognostic factors for osteosarcoma patients, particularly those diagnosed with metastatic disease.

Notch1 is a type 1 transmembrane receptor protein, which is important for cell fate regulation, the differentiation of various systems and neuronal development, such as neurogenesis and the maintenance of neural stem cells (5). The increased expression of Notch1 is related to the low survival rate of patients with various types of cancer (6-8). The proliferation of cells from these types of cancer can be inhibited by the pharmacological inhibition of Notch1. Therefore, preventing the occurrence of Notch1 is a potential strategy for the treatment of various types of cancers (9). The transcription factor hairy and enhancer of split-1 (HES1) is a member of the basic helix-loop-helix (BHLH) of transcription inhibitor family, and is the downstream target of Notch signal pathway (10). HES1 is overexpressed in a number of tumor types, including colon cancer (11), breast cancer (12), non-small cell lung cancer (13), etc., suggesting that HES1 has carcinogenic activity and is closely associated with cancer.

Therefore, the present study examined the changes in the expression of Notch1 and HES1 in osteosarcoma patients following surgery. The correlation between Notch1 expression and HES1 expression, and its association with prognosis were also investigated, so as to identify novel potential diagnostic and treatment targets that may be used clinical practice.

Patients and methods

General patient information. In the present study, samples from 62 patients with osteosarcoma treated at Shandong Cancer Hospital from April, 2011 to June, 2013 were collected as the research group, and those from 52 healthy individuals undergoing a physical examination were collected as the control group. There were 33 males and 29 females in the research group, with an average age of 18.6±10.1 years, while the control group consisted of 28 males and 24 females with an average age of 19.1±10.3 years. The present study was approved by the Ethics Committee of Shandong Cancer Hospital. Signed written informed consents were obtained from the patients and/or parents or guardians.

Inclusion and exclusion criteria. The inclusion criteria were as follows: Patients who met the ESMO diagnostic criteria (14), or received treatment at Shandong Cancer Hospital after diagnosis; patients who did not receive radiotherapy or chemotherapy prior to surgery; patients who did not receive any treatment within 30 days after surgery; patients aged between 10 to 40 years; patients with complete case data; patients who agreed to cooperate with the work arrangement of the medical staff at Shandong Cancer Hospital; patients or their immediate family members signed informed consents.

The exclusion criteria were as follows: Patients who died during the course of treatment; patients with injury to important organs; patients suffering from other cardiovascular and cerebrovascular diseases, as well as any physical disability; pregnant mothers; patients suffering from other autoimmune diseases and chronic diseases; patients transferred to Shandong Cancer Hospital; patients with contraindications to surgery, mental diseases and language dysfunction.

Surgical treatment plan. The patients were subjected to limb preservation surgery according to the strategies outlined in the study by Ando *et al* (15) and references listed in that study.

Blood sample processing. Before surgery and at 30 days after surgery, early in the morning on an empty stomach, venous blood was drawn and stored at 4˚C for 30 min, and the serum samples were then centrifuged for 10 min at 4˚C (1,500 x g). The supernatant was then extracted and stored in a refrigerator at -80˚C.

Main reagents. Notch1 and HES1 kits were purchased from Wuhan Feien Biotechnology Co., Ltd. (cat. nos. EH0926 and EH3223), and were used strictly in accordance with the operating instructions provided with the kits. The Eppendorf CryoCube F740hi ultra-low temperature refrigerator was purchased from Eppendorf Co., Ltd. (cat. no. ep000000).

Follow-up of patients. The patients were followed-up for 5 years, and their survival rates were recorded via telephone communications and outpatient medical records. The follow-up time points were the 3rd, 6, 9 and 12th month of each year.

Observation indicators. The main observation indicators were as follows: The expression levels of Notch1 and HES1 in osteosarcoma patients before and after surgery were observed, and the diagnostic value of Notch1 and HES1 in osteosarcoma was determined.

The secondary observation indicators were the following: Pearson's correlation analysis was used to analyze the correlation between Notch1 expression and HES1 expression in osteosarcoma patients. According to the expression levels of Notch1 and HES1 (obtained by ELISA), the patients were divided into the high and low expression groups, and the 5-year survival rate of the patients was observed.

Statistical analysis. In the present study, the SPSS20.0 software package was used to perform the statistical analysis on the collected data. The GraphPad 7 software package was used to obtain the required graphs, and the Kolmogorov-Smirnov test was used to analyze the distribution of these data, in which normally distribution data were expressed as the mean ± standard deviation (means ± SD). Inter-group comparisons were conducted using an independent-samples t-test, and intra-group comparisons were conducted using a paired t-test. Count data are expressed as a percentage (%) and analyzed using the Chi-squared (χ^2) test. A receiver operating characteristic (ROC) curve was created to plot the diagnostic value of Notch1 and HES1 in osteosarcoma, which was represented by the χ^2 value. Cut-off values were calculated using Youden's index (YI) calculation formula as follows: YI=[a/(a + c) + d/(b + d)]-1. Pearson's correlation analysis was used to analyze the correlation between Notch1 expression and HES1 expression in the osteosarcoma patients. The 5-year survival of the patients was plotted by the Kaplan-Meier survival curve and data were analyzed using the log-rank test. In addition, univariate and multivariate logistic regression were performed to analyze the independent risk factors affecting the prognosis of the patients. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinical data. No significant differences were observed in the clinical data of the research group and the control group, including age, sex, body mass index (BMI), marital status, nationality, place of residence, smoking, alcohol consumption and exercise, which proved comparability ($P>0.05$), as shown in Table I.

Expression levels of Notch1 and HES1 in osteosarcoma patients before and after surgery. The expression levels of Notch1 and HES1 in the osteosarcoma patients before surgery were 15.03±1.35 and 13.86±1.53, while the expression levels of Notch1 and HES1 in the osteosarcoma patients after surgery were 4.12±1.01 and 5.02±0.99, respectively. Significant differences were observed in the comparisons of Notch1 and HES1

Table I. Clinical basic data of the patients.

Characteristic	Research group (n=62)	Control group (n=52)	χ^2 or t-test value	P-value
Age (years)	18.6±10.1	19.1±10.3	0.261	0.795
Sex, no. (%)			0.004	0.947
Male	33 (53.23)	28 (53.85)		
Female	29 (46.77)	24 (46.15)		
BMI (kg/m^2)	22.26±0.37	22.21±0.25	0.828	0.409
Marital status, no. (%)			0.001	0.981
Married	13 (20.97)	11 (21.15)		
Unmarried	49 (79.03)	41 (78.85)		
Nationality, no. (%)			1.077	0.299
Han	55 (88.71)	49 (94.23)		
Minority	7 (11.29)	3 (5.77)		
Place of residence, no. (%)			0.129	0.719
Cities and towns	32 (51.61)	30 (48.39)		
Countryside	30 (48.39)	32 (51.56)		
Smoking history, no. (%)			0.500	0.480
Yes	11 (17.74)	12 (23.08)		
No	51 (82.26)	40 (76.92)		
Alcohol consumption history, no. (%)			1.273	0.259
Yes	28 (45.16)	29 (55.77)		
No	34 (54.84)	23 (44.23)		
Exercise habits, no. (%)			1.433	0.231
Yes	30 (48.39)	31 (59.62)		
No	32 (51.61)	21 (40.38)		

expression levels before and after surgery (P<0.05), as shown in Fig. 1.

Diagnostic value of Notch1 and HES1 expression in osteosarcoma. ROC curve analysis demonstrated that when the cut-off value was 13.230, the sensitivity, specificity and AUC of Notch1 in the diagnosis of osteosarcoma were 93.55%, 58.06% and 0.732, respectively (P<0.001); when the cut-off value was 12.810, the sensitivity, specificity and AUC of HES1 in the diagnosis of osteosarcoma were 82.26%, 61.29% and 0.766, respectively (P<0.001), as shown in Table II and Fig. 2.

Correlation between Notch1 and HES1 expression in patients with osteosarcoma. Pearson's correlation analysis identified that the expression level of Notch1 positively correlated with that of HES1 in the osteosarcoma patients (r=0.795, P<0.001), 95% CI: 0.681-0.872, as shown in Fig. 3.

High and low expression levels of Notch1 and HES1, and the 5-year survival rate of patients with osteosarcoma. The patients in the present study were then divided into the Notch1 high expression group (≥16.57), the HES1 high expression group (≥15.18) (31 cases), the Notch1 low expression group (<16.57) and the HES1 low expression group (<15.18) (31 cases) according to the median value of the expression levels of Notch1 and HES1. All the patients were interviewed at follow-up. In the Notch1 and HES1 low expression groups, 10 patients died, with a 5-year survival rate of 67.74%; there were 16 patients that died in the high expression group, with a 5-year survival rate of 48.39%. The survival rate of the patients in the low expression group was significantly higher than that of the patients in the high expression group (P=0.045), as shown in Fig. 4.

Table II. ROC curve diagnosis.

Item	Notch1	HES1
AUC	0.732	0.766
Std.Error	0.049	0.043
95% CI	0.636-0.827	0.682-0.850
P-value	0.001	0.001
Cut-off	13.230	12.810
Sensitivity (%)	93.55	82.26
Specificity (%)	58.06	61.29

Univariate logistic regression analysis. The patients were divided into the survival group (36 cases) and the mortality group (26 cases) according to their survival conditions. Univariate analysis based on the clinical data of the survival group and mortality group illustrated that there were no significant differences in age, sex and TNM staging between

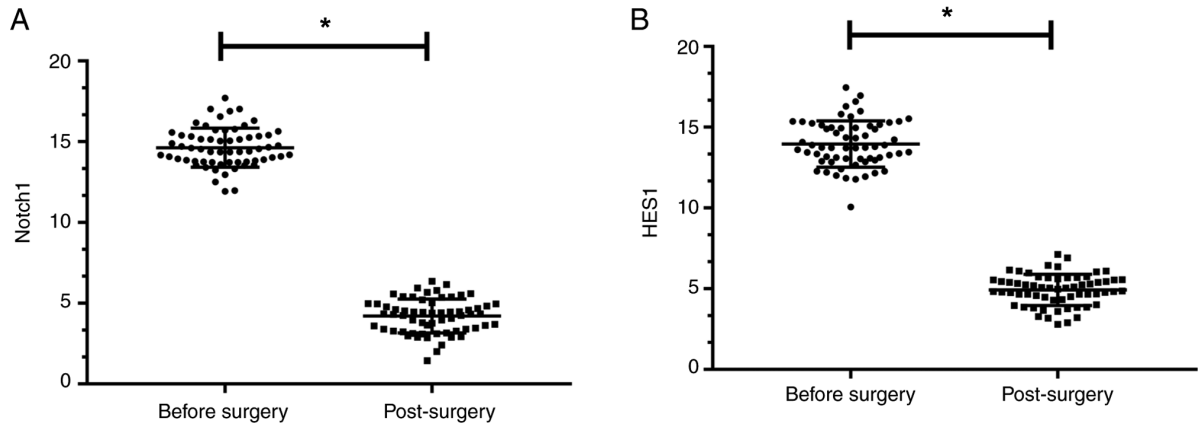

Figure 1. Expression of Notch1 and HES1 in osteosarcoma patients before and after surgery. (A) Expression of Notch1 before and after surgery. (B) Expression of HES1 before and after surgery. The expression levels of Notch1 and HES1 in patients before surgery were significantly higher than those after surgery. *P<0.05.

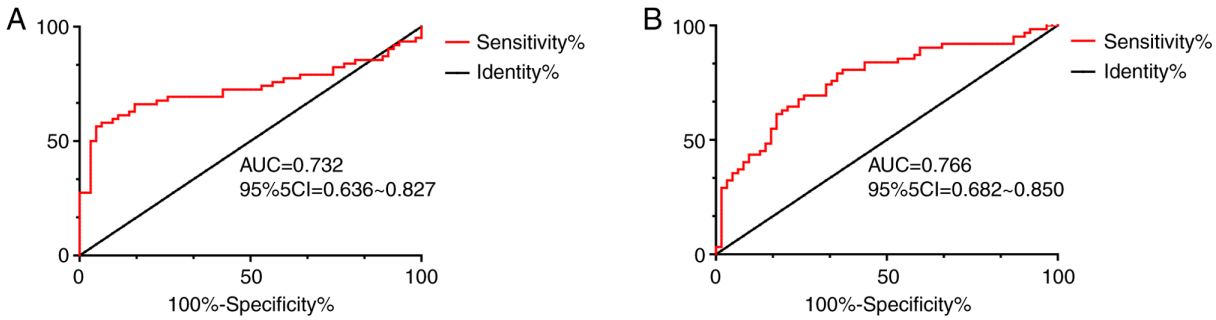

Figure 2. Diagnostic value of Notch1 and HES1 for osteosarcoma. (A) When the cut-off value was 13.230, the sensitivity, specificity and AUC of Notch1 in the diagnosis of osteosarcoma were 93.55%, 58.06% and 0.732, respectively. (B) When the cut-off value as 12.810, the sensitivity, specificity and AUC of HES1 in the diagnosis of osteosarcoma were 82.26%, 61.29% and 0.766, respectively.

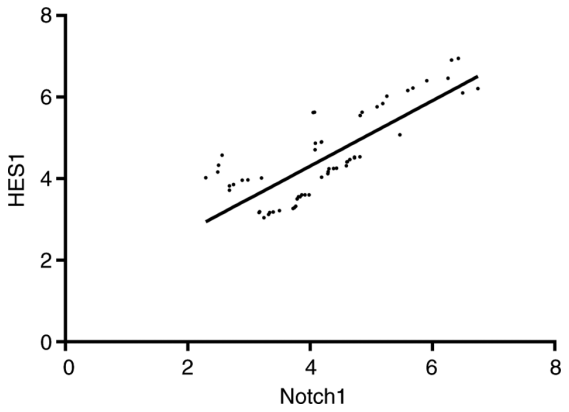

Figure 3. Pearson's correlation analysis. Notch1 expression level positively correlated with the HES1 expression level in osteosarcoma patients (r=0.795, P<0.001).

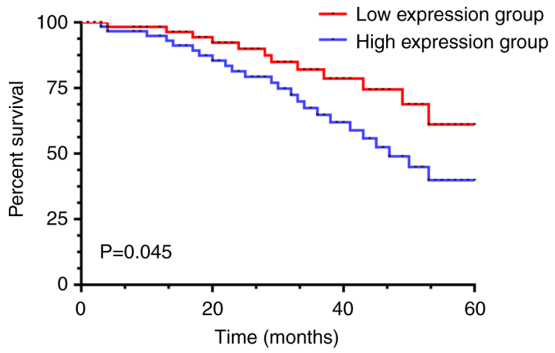

Figure 4. Patients were divided into the high and low expression groups according to the expression levels of Notch1 and HES1, and the 5-year survival rate of the patients was observed. According to the median value of the detection results of the expression levels of Notch1 and HES1, they were divided into Notch1 high expression group (≥16.57), HES1 high expression group (≥15.18) (31 cases), low expression group (<16.57) and low expression group (<15.18) (31 cases). The 5-year survival rate of patients in the Notch1 and HES1 low expression group was 67.74%, while that of patients in the high expression group was 48.39%. The survival rate of patients in the low expression group was significantly higher than that of patients in the high expression group (P=0.045).

the groups (P>0.05). Significant differences were observed for in tumor location, chemotherapy response, tumor size, and Notch1 and HES1 expression (P<0.05), as shown in Table III.

Multivariate logistic regression analysis. Multivariate difference indicators (tumor location, chemotherapy response and tumor size) were assigned, as shown in Table IV. Subsequently, multivariate logistic regression analysis was performed to confirm tumor location (OR, 3.521; 95% CI, 1.061-3.183), chemotherapy response (OR, 5.020; 95% CI, 0.218-0.675),

Table III. Univariate analysis.

Clinicopathological features	Survival group (n=36)	Mortality group (n=26)	χ^2 or t-test value	P-value
Age (years)			0.261	0.610
<20	13 (41.94)	15 (48.39)		
≥20	18 (58.06)	16 (51.61)		
Sex, n (%)			0.272	0.602
Male	20 (64.52)	18 (58.06)		
Female	11 (35.48)	13 (41.94)		
Tumor location, n (%)			5.248	0.022
Limbs	10 (32.26)	19 (61.29)		
Not limbs	21 (67.74)	12 (38.71)		
Chemotherapy response, n (%)			4.239	0.040
Adverse reaction	14 (45.15)	22 (70.97)		
Good reaction	17 (54.84)	9 (29.03)		
TNM staging, n (%)			0.369	0.544
Stages I-II	23 (74.19)	25 (80.65)		
Stages III-IV	8 (25.81)	6 (19.35)		
Tumor size, n (%)			4.133	0.042
≥3 cm	12 (38.71)	20 (64.52)		
<3 cm	19 (61.29)	11 (35.48)		
Notch1 expression	19.03±2.35	16.07±1.55	5.597	0.001
HES1 expression	16.86±1.53	14.02±1.32	7.630	0.001

Table IV. Assignment table.

Factor	Assignment
Tumor location	Limbs,1; not limbs, 0
Chemotherapy response	Good, 1; poor, 0
Tumor size	≥3 cm, 1; <3 cm, 0
Notch1 expression	Data were continuous variables and were analyzed as original data.
HES1 expression	Data were continuous variables and were analyzed as original data.

Table V. Multivariate logistic regression analysis.

						95% CI of Exp (B)	
Factor	B	SE	Wals	Sig.	Exp (B)	Lower limit	Upper limit
Tumor location	0.608	0.280	4.709	0.030	3.521	1.061	3.183
Chemotherapy response	-0.958	0.288	11.056	0.001	5.020	0.218	0.675
Tumor size	0.568	0.742	4.996	0.026	3.227	1.072	2.901
Notch1 expression	0.721	0.239	5.705	0.002	4.019	1.467	4.218
HES1 expression	0.856	0.423	5.512	0.031	4.629	1.353	5.727

SE, standard error; Wals, Chi-squared test value; Sig., significance; Exp (B), odds ratio.

tumor size (OR, 3.227; 95% CI, 1.072-2.901), Notch1 expression (OR, 4.019; 95% CI, 1.467-4.218) and HES1 expression (OR, 4.629; 95% CI, 1.353-5.727). Tumor location, chemotherapy response and tumor size, and Notch1 and HES1 expression were independent risk factors for the prognosis of patients, as shown in Table V.

Discussion

Osteosarcoma is one of the most common primary malignant bone diseases, which severely threatens the health of children and adolescents (16). It has a high tendency of local invasion and early systemic metastasis, such as lung metastasis (17,18). Its morbidity rate is high, mainly among children and adolescents aged between 10 and 25 years, whose skeleton is growing rapidly, accounting for 70% of all osteosarcoma cases (19). Osteosarcoma has a high malignancy and a poor prognosis. According to statistics, approximately 85% of osteosarcoma patients exhibit metastasis (20). Chen *et al* (21) and Shin *et al* (22) demonstrated that the 5-year survival rate of non-metastatic patients increased to 55-70% with the application of high-dose combination chemotherapy. However, the 5-year survival rate of metastatic patients was only 5-20%. Although the survival rate of osteosarcoma patients has improved, there certain serious issues still exist, including severe side-effects and recurrent or metastatic disease (23). Therefore, it is of utmost importance to identify effective indicators for the diagnosis and prognosis of patients with osteosarcoma.

Notch1 is an evolutionarily conserved ligand-receptor signaling system that regulates cell proliferation, survival, apoptosis and differentiation (24,25). The dysfunction of the Notch1 signaling pathway may lead to abnormal differentiation or undifferentiation, and may eventually lead to the malignant transformation of these cells. Of note, it has been revealed that changes in Notch1 signaling are associated with a number of human cancers (26-28); however, the role of Notch1 in osteosarcoma has yet not been elucidated. HES1 is a highly conserved basic helix-loop-helix transcription inhibitor, which mediates its biological effects by binding to N-cassettes (CACNAG) in the entire genome and recruiting chromatin modification factors to these sites (29,30). HES1 is necessary for organogenesis and development of several species as a component of Notch1 (31,32). However, the molecular function of HES1 in adult tissues remains unclear. Therefore, by investigating the clinical diagnostic values of Notch1 and HES1 in osteosarcoma patients and their influence on prognosis, this may provide the basis for the future clinical diagnosis and treatment of osteosarcoma.

In the present study the expression levels of Notch1 and HES1 in osteosarcoma patients before and after surgery, we first observed. It was found that Notch1 and HES1 in osteosarcoma patients after surgery exhibited a low expression, which differed significantly from that before surgery. This indicated that Notch1 and HES1 may become potential diagnostic and therapeutic targets for osteosarcoma. Therefore, a ROC curve was then drawn and it was found that the areas under the Notch1 and HES1 curves were 0.732 and 0.766, respectively, which were not associated with a high specificity, but with a high sensitivity and were clinical diagnostic indicators of osteosarcoma. Zhang *et al* (33) found a new regulatory pathway of invasion and metastasis in osteosarcoma, as well as a novel function of the Notch pathway: The regulation of metastasis. As the Notch pathway can be pharmacologically inhibited, these findings suggest possible novel therapeutic strategies with which to reduce the invasion and metastasis of osteosarcoma. Subsequently, Pearson's correlation analysis demonstrated that the expression level of Notch1 positively correlated with the expression level of HES1 in osteosarcoma patients ($r=0.795$, $P<0.001$). The patients were further divided into the high and low expression groups according to the median value of the expression levels of Notch1 and HES1 in osteosarcoma. Observing the 5-year survival rate of the patients, it was found that the 5-year survival rate of the patients in the Notch1 and HES1 high expression groups was 48.39%, and that of the patients in the Notch1 and HES1 low expression groups was 67.74%. The higher the expression levels of Notch1 and HES1, the lower the survival rate of the osteosarcoma patients, suggesting that Notch1 and HES1 may be used as prognostic survival indicators of osteosarcoma patients. Finally, it was found that tumor location, chemotherapy response, tumor size, and Notch1 and HES1 expression were independent prognostic factors of patients through logistic multivariate analysis, which indicated that tumor location, chemotherapy response, tumor size, Notch1, HES1 and may be used as prognostic indicators for patients with osteosarcoma.

The present study preliminarily proved the clinical value of Notch1 and HES1 through the above-mentioned findings. However, there are still certain limitations to this research. First, tissue samples were not collected and basic cell experiments were not performed. Second, no animal experiments were conducted. Thus, the authors aim to conduct further in-depth experimental analyses as soon to confirm and further broaden the findings of the present study.

In conclusion, the present study demonstrated that Notch1 and HES1 were highly expressed in osteosarcoma patients. Notch1 and HES1 as indicators exhibited a good diagnostic efficacy, as shown by ROC curve analysis, and Notch1 and HES1 expression were strongly associated with the occurrence and development of osteosarcoma. Thus, they may prove to be efficient markers for the diagnosis and prognosis of patients with osteosarcoma. These findings may provide future reference and insight into future studies on osteosarcoma.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

LF, BL, DY and TZ were involved in the conception and design of the study. LF, BL, DY, BW and TZ were responsible for data collection and analysis. LF, BW and TZ were responsible for the interpretation of the data and for drafting the manuscript. LF and TZ made revisions from a critical perspective for important intellectual content. All authors have read and confirmed the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Shandong Cancer Hospital. Signed written informed consents were obtained from the patients and/or parents or guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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