High expression of Dll4 and CD44V6 is associated with clinicopathological characteristics and poor prognosis in osteosarcoma patients

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Background: Osteosarcoma is a malignant tumor with a poor prognosis. Dll4 and CD44V6 are involved in the regulation of tumor development. This study aims to analyze the expression of Dll4 and CD44V6 in osteosarcoma and the correlation with clinicopathological characteristics and prognosis.

Methods: Clinical data of 47 patients with osteosarcoma admitted to our hospital from April 2016 to May 2018 and confirmed by surgery or pathological examination were retrospectively analyzed. Osteochondroma and normal bone tissues from these cases were taken as controls. The protein levels of Dll4 and CD44V6 were detected by immunohistochemistry (IHC). The relationship between the expression of Dll4 and CD44V6 and the clinicopathological characteristics of patients with osteosarcoma was analyzed. An unconditional multivariate logistic regression model was used to analyze the risk factors affecting the prognosis of patients with osteosarcoma.

Results: The positive expression rates of Dll4 and CD44V6 in osteosarcoma were 85.11% (Dll4) and 91.49% (CD44V6), which were significantly higher than those in normal bone tissues of 18.52% (Dll4) and 7.41% (CD44V6), and in osteochondroma of 22.22% (Dll4) and 7.41% (CD44V6). The expression of CD44V6 and Dll4 were related to the Enneking stage and metastasis (P<0.05), and the expression of Dll4 was closely related to the degree of tumor differentiation (P<0.05). The 2-year survival rates of patients with Dll4 or CD44V6 positive expression were 25.00% and 21.88%, respectively, which were significantly lower than those with Dll4 or CD44V6 negative expression (85.71% and 93.33%) (P<0.05). The results of the unconditional multivariate logistic regression analysis showed that low tumor differentiation, high Enneking stage, metastasis, and positive expression of Dll4 and CD44V6 were risk factors affecting the mortality rate and prognosis of patients with osteosarcoma (P<0.05).

Conclusions: The positive expression rate of Dll4 and CD44V6 in osteosarcoma is high, and the expression level is closely related to the degree of tumor differentiation, Enneking stage, and metastasis. Dll4 and CD44V6 are risk factors affecting the survival prognosis for patients. Therefore, in clinical settings, the prognosis for patients could be evaluated by detecting the expression of Dll4 and CD44V6.

Keywords: Osteosarcoma; Dll4 protein; CD44V6; clinicopathological characteristics; prognosis

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Introduction

As a malignant tumor, osteosarcoma originates in mesenchymal tissue and is characterized by the production of spindle-shaped stromal cells of bone-like tissue, which can be divided into primary and secondary osteosarcomas (1). Osteosarcoma is common in young people aged 10 to 20 years old, and mostly appears in the long tubular metaphyseal end, distal femur, tibia and proximal humerus. The prognosis of osteosarcoma is poor because it is highly malignant, aggressive, prone to lung metastasis in the early stage, and has a high recurrence rate after surgical treatment. Therefore, it is of great importance to study the invasion and metastasis of osteosarcoma (2,3). However, there are few studies on the correlation between the expression of DLL4 protein and CD44v6 and the prognosis of patients with osteosarcoma. CD44 is a type of adhesion molecule that has not yet been categorized, while CD44V6 is a subtype of the CD44 molecule containing a variant exon V6 coding sequence. The abnormal expression of CD44V6 is reported to be closely related to the occurrence and development of osteosarcoma (4). The DLL4 protein is a ligand of Notch receptors and has been reported to be involved in the regulation of tumor angiogenesis and growth (5). Therefore, this retrospective study analyzed the expression of DLL4 and CD44V6 proteins in osteosarcoma and the correlation with clinicopathological characteristics and prognosis. We present the following article in accordance with the REMARK reporting checklist (available at http://dx.doi.org/10.21037/tcr-21-31).

Methods

General information

Clinical data from 47 osteosarcoma patients admitted to our hospital from April 2016 to May 2018 were analyzed retrospectively, with all cases confirmed by surgery or pathological examination. Inclusion criteria were as follows: (I) no chemotherapy, radiotherapy or anti-angiogenesis drugs had been used prior to surgery; (II) the clinical data records were complete; and (III) no history of drug allergies. Patients were excluded based on the following criteria: (I) patients with poor blood coagulation function; (II) patients with abnormal heart, liver, kidney or other functions; (III) patients who were unconscious; (IV) patients with immune dysfunction; (V) pregnant women during lactation or pregnancy.

Among the 47 patients included in the study, 27 cases were male, and 20 cases were female. Patients were aged from 8 to 61 years, with an average age of 24.5 (±13.05) years. According to the Enneking surgical staging system, 13 cases were in stage I, 19 cases were in stage II, and 15 cases were in stage III. The degree of differentiation amongst cases was as follows: 10 cases had high differentiation, 13 cases had moderate differentiation, and 24 cases had low differentiation. In regard to pathological type, 28 cases had osteogenic osteosarcoma, 13 cases had chondroid osteosarcoma and 6 cases had fibroblastic osteosarcoma. Osteochondroma and normal bone tissues from each of the 27 cases were taken as controls. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by institutional committee of Affiliated Hospital of North Sichuan Medical College (2019ER(R)108-1) and informed consent was taken from all the patients.

S-P immunohistochemical staining

S-P immunohistochemical staining was used. Firstly, specimens were prepared by dewaxing and hydrating the tissue sections. Then the expression of DLL4 and CD44V6 proteins was detected according to the S-P immunohistochemical staining methods. The main reagents were mouse anti-human CD44V6 monoclonal antibody (Santa Cruz, USA) and rabbit anti-human DLL4 polyclonal antibody (Abcam, UK). The testing process was strictly in accordance with the manufacturer's instructions. The negative control was the use of PBS (phosphate buffered saline) to replace the primary antibodies.

The protocol for judging the staining result was as follows: the positive expression of DLL4 and CD44V6 proteins was located in tumor cells whose cytoplasm or cell membrane was brownish yellow, and percentages of positive cells were scored as 0 points for no positive cells, 1 point for positive cells <10%, 2 points for positive cells between 10–50%, 3 points for positive cells between 50–75%, and 4 points for positive cells >75%. The degree of staining was scored as 0 points for colorless, 1 point for light yellow staining, 2 points for brown staining and 3 points for tan staining. The total scores were defined as the product of the above 2 types of scores, and positive expression was classified as a total score >4 points.

Observation indicators

In order to observe the expression of DLL4 and CD44V6
proteins in various bone tissues and analyze their relationship with the clinicopathological characteristics of osteosarcoma, patients were followed up for two years. By analyzing the 2-year survival rate after surgery and the expression level of the Dll4 and CD44V6 proteins, we could further analyze the relationship between Dll4 and CD44V6 expression and the risk factors affecting prognosis.

**Statistical methods**

The data were statistically analyzed using SPSS 18.0 software (IBM, USA). The measurement data were described as the mean ± standard deviation (X±s) and analyzed using t-tests. The pass rate or composition ratio of the count data was analyzed by the χ² test. An unconditional multi-factor logistic regression was used to analyze the risk factors affecting the prognosis of osteosarcoma patients. Results with a P value <0.05 were considered to be statistically significant.

**Results**

**The expression of Dll4 and CD44V6 proteins in various bone tissues**

The positive expression of Dll4 and CD44V6 proteins was located in tumor cell cytoplasm or cell membranes that were brownish yellow (Figures 1, 2). The positive expression rates of Dll4 and CD44V6 in osteosarcoma were 85.11% and 91.49%, respectively, compared with 18.52% (Dll4) and 7.41% (CD44V6) in normal bone tissues, and 22.22% (Dll4) and 7.41% (CD44V6) in osteochondroma tissues, indicating that the positive expression rates of Dll4 and CD44V6 in osteosarcoma were significantly higher than those in normal bone tissues and osteochondroma tissues (P<0.05, Table 1).

**The relationship between the expression levels of Dll4, CD44V6 and the clinicopathological characteristics of osteosarcoma**

The results showed that the expression of CD44V6 was closely related to the Enneking stage and metastasis (P<0.05) but did not show a significant relationship with gender, age, tumor diameter, degree of differentiation, or pathological type of osteosarcoma (P>0.05). Similarly, the expression of Dll4 was significantly related to the degree of tumor differentiation, Enneking stage, and metastasis (P<0.05) but was not related to gender, age, tumor diameter, or pathological type of osteosarcoma (P>0.05, Table 2).

**The relationship between Dll4 and CD44V6 expression and postoperative survival time**

The patients were followed up for two years after surgery, and in that time 22 cases died, resulting in an overall survival rate of 53.19%. The 2-year survival rates of patients with Dll4 or CD44V6 positive expression were 25.00% and 21.88%, respectively. The 2-year survival rates of patients with Dll4 or CD44V6 negative expression were 85.71% and 93.33%, respectively, which were significantly higher than those with a Dll4 or CD44V6 positive expression (P<0.05, Table 3). Results of the survival analysis showed that patients
Table 1 Expression of Dll4 and CD44V6 proteins in various bone tissues [n (%)]

| Expression of protein | Normal bone tissue (n=27) | Osteochondroma (n=27) | Osteosarcoma (n=47) | χ² | P |
|-----------------------|---------------------------|-----------------------|---------------------|----|---|
| CD44V6                |                           |                       |                     |    |   |
| Negative (−)          | 25 (92.59)                | 25 (92.59)            | 15 (31.91)          | 71.405 | 0.001 |
| Positive (+)          | 2 (7.41)                  | 2 (7.41)              | 32 (68.09)*         |    |   |
| Dll4                  |                           |                       |                     | 42.201 | 0.001 |
| Negative (−)          | 22 (81.48)                | 21 (77.78)            | 7 (14.89)           |    |   |
| Positive (+)          | 5 (18.52)                 | 6 (22.22)             | 40 (85.11)*         |    |   |

*, P<0.05, compared with normal bone tissue or osteochondroma.

Table 2 Relationship between the expression levels of Dll4 and CD44V6 and the clinicopathological features of osteosarcoma [n (%)]

| Pathological characteristics | n | CD44V6 (+) | χ² | P | Dll4 (+) | χ² | P |
|-----------------------------|---|-----------|----|---|----------|----|---|
| Gender                      |   |           |    |   |          |    |   |
| Male                        | 27| 20 (62.50)| 23 (57.50) | 0.050 | 0.822 |
| Female                      | 20| 12 (37.50)| 17 (42.50) |    |   |
| Age (years)                 |   |           |    |   |          |    |   |
| ≥25                         | 28| 19 (59.38)| 23 (57.50) | 0.069 | 0.793 |
| <25                         | 19| 13 (40.63)| 17 (42.50) |    |   |
| Diameter of tumor (cm)     |   |           |    |   |          |    |   |
| ≥5                          | 22| 18 (56.25)| 21 (52.50) | 3.590 | 0.058 |
| <5                          | 25| 14 (43.75)| 19 (47.50) | 3.494 | 0.062 |
| Differentiation degree     |   |           |    |   |          |    |   |
| High                        | 10| 6 (18.75)| 7 (17.50) | 2.870 | 0.238 |
| Moderate                    | 13| 7 (21.88)| 8 (20.50) | 7.372 | 0.025 |
| Low                         | 24| 19 (59.38)| 23 (57.50) |    |   |
| Pathological type           |   |           |    |   |          |    |   |
| Osteogenic osteosarcoma     | 28| 21 (65.63)| 24 (60.00) | 1.835 | 0.400 |
| Chondroid osteosarcoma      | 13| 7 (21.88)| 11 (27.50) |    |   |
| Fibroblast type             | 6 | 4 (12.50)| 5 (12.50) |    |   |
| Enneking staging            |   |           |    |   |          |    |   |
| Stage I                     | 13| 4 (12.50)| 8 (20.00) | 11.746 | 0.003 |
| Stage II                    | 19| 15 (46.88)| 17 (42.50) | 8.608 | 0.014 |
| Stage III                   | 15| 13 (40.63)| 15 (37.50) |    |   |
| Metastasis                  |   |           |    |   |          |    |   |
| Yes                         | 23| 20 (62.50)| 22 (55.00) | 7.382 | 0.007 |
| No                          | 24| 12 (37.50)| 18 (45.00) | 3.952 | 0.047 |
Table 3 Relationship between Dll4 and CD44V6 expression and postoperative survival time

| Death within 2 years | CD44V6 | | Dll4 | |
|----------------------|--------|--------|--------|--------|
|                      | Negative (−) | Positive (+) | Negative (−) | Positive (+) |
| Yes                  | 1      | 25     | 1      | 30     |
| No                   | 14     | 7      | 6      | 10     |
| Survival rate (%)    | 93.33  | 21.88  | 85.71  | 25.00  |

Figure 3 Survival rate analysis of the positive and negative expression of CD44V6.

Figure 4 Survival rate analysis of the positive and negative expression of Dll4 protein.

with Dll4 and CD44V6 negative expressions had a longer survival period, and the Kaplan-Meier survival curves were significantly different from those of patients with Dll4 and CD44V6 positive expressions (Figures 3, 4, P<0.05).

The analysis of risk factors affecting the prognosis of osteosarcoma patients

The results showed that tumors with a low differentiation, a high Enneking stage, metastasis, and a positive expression of Dll4 and CD44V6 were risk factors that affected the prognosis of osteosarcoma patients (P<0.05, Table 4).

Discussion

CD44 is a very widely distributed cell surface transmembrane glycoprotein that can bind to ligands such as hyaluronic acid, mediating the adhesion of cells to cells, and cells to matrix and which displays a broad range of biological functions (6,7). In recent years, a large number of clinical researchers have found that CD44 plays an important role in the occurrence and development of tumors, and lately CD44V6 has attracted more attention due to its role in tumor invasion, metastasis, and prognosis. It has been reported that the overexpression of CD44V6 in various tumor tissues such as colorectal cancer, ovarian cancer, endometrial cancer, and other tumor tissues is closely related to tumor metastasis and poor prognosis (8,9). In ovarian cancer, CD44V6 is not only related to the prognosis and recurrence of ovarian cancer but also the soluble CD44V6 in serum can be used as an effective indicator to evaluate prognosis and recurrence.

Some researchers have suggested that CD44V6 may also participate in tumor metastasis and invasion in osteosarcoma tissues (10), which is consistent with the results in this study. CD44V6 can bind with a variety of extracellular matrix components to mediate the adhesion of tumor cells to the extracellular matrix, which helps tumor cells to survive and develop in the primary lesion (11). In addition, CD44V6 also participates in a variety of transmembrane
signal transmission processes, which affects intracellular cytoskeletal proteins and promotes neovascularization, adhesion to extracellular matrix, and invasion of tumors (12,13). In this study, the results showed that the positive expression rate of CD44V6 in osteosarcoma tissue is higher than in normal bone tissue or osteochondroma, suggesting that the expression of CD44V6 is related to the occurrence, development, and malignant behavior of osteosarcoma. Consequently, the expression of CD44V6 might be used as an assistant index to assess the degree of tumor malignancy. In addition, results of this study also showed that the positive expression rate of CD44V6 of patients with tumor metastasis and Enneking stage II + III was significantly higher than those without metastasis, and at Enneking I. Furthermore, the survival rate of patients with a CD44V6 positive expression was significantly lower than those with a negative expression (P<0.05). Because CD44V6 is a key factor in the invasion and metastasis of osteosarcoma, its positive expression indicates a poor prognosis for patients, and a significantly reduced 2-year survival rate (14). It is suggested that CD44v6 is involved in the occurrence and metastasis of osteosarcoma, and can be used as a potential therapeutic target for malignant tumors.

The Dll4 protein contains 658 amino acid residues, eight EGF-like repeats, and Delta/Serrate/Lag-2 (DSL) domains outside the cell membrane (15). At present, it has been found that the blocking of Dll4-Notch1 signaling pathway, thus inducing angiogenesis (16). Pitulescu et al. have detected that the Dll4 protein is significantly overexpressed in liver cancer and bladder cancer, and this abnormal expression may be involved in tumor angiogenesis (17). Tumor angiogenesis is a key step in tumor growth and metastasis, because not only the growth of primary tumor depends on angiogenesis, but also the occurrence of metastasis and the progress of metastatic foci. The results in this study found that the positive expression rate of Dll4 in osteosarcoma tissue was significantly higher than that in normal bone tissue or osteochondroma (P<0.05), indicating that the occurrence of osteosarcoma is related to an abnormal increase in Dll4 expression. The expression of Dll4 was also found to be related to tumor differentiation, the Enneking stage, and metastasis (P<0.05), which further demonstrates that Dll4 plays an important role in the occurrence and development of osteosarcoma, and the tumor growth could be inhibited by blocking the activity of DLL4. In addition, the results of this study showed that the survival rate of patients with a Dll4 positive expression was also significantly lower than those with a negative expression (P<0.05), indicating that Dll4 might also be used as an effective indicator for evaluating the prognosis of patients. Many studies have shown that Dll4 and CD44V6 play important roles in the invasion and metastasis of a variety of malignant tumors. They are also one of several indicators that reflect the invasion and metastasis of tumor cells, and as such could be important indicators in evaluating the prognosis of patients with malignant tumors, especially osteosarcoma (18,19). In the future treatment of osteosarcoma, we suggest that the expression levels of Dll4 and CD44V6 could be

| Variate                                      | Single factor analysis | Multiple-factor analysis |
|----------------------------------------------|-----------------------|-------------------------|
|                                              | OR        | 95% CI     | P     | OR        | 95% CI     | P     |
| Gender: male (control: female)               | 1.239     | 0.989–1.552 | 0.724 | –         | –         | –     |
| Age: ≥25 years (control <25 years)           | 1.250     | 0.871–1.793 | 0.697 | –         | –         | –     |
| Diameter of tumor: ≥5 cm (control <5 cm)     | 1.260     | 0.850–1.868 | 0.514 | –         | –         | –     |
| Differentiation degree: low (control: moderate and high) | 1.644 | 1.312–2.059 | 0.014 | 1.730     | 1.254–2.386 | 0.001 |
| Enneking staging: stage II + III (control stage I) | 1.650 | 1.289–2.113 | 0.023 | 1.749 | 1.261–2.426 | 0.015 |
| Metastasis: yes (control: no)                | 1.672     | 1.273–2.196 | 0.006 | 1.763     | 1.278–2.431 | 0.004 |
| Dll4 positive (control: negative)            | 1.692     | 1.269–2.527 | 0.002 | 1.824     | 1.137–2.925 | 0.012 |
| CD44V6 positive (control: negative)          | 1.709     | 1.254–2.330 | 0.007 | 1.842     | 1.111–3.055 | 0.005 |
routinely tested so as to comprehensively assess the prognosis of the disease and provide reliable guidance for clinical treatment (20).

However, there are also some limitations in this study, such as a small sample size, a retrospective study, and inevitable deviation of the results. In addition, further prospective and large-sample studies on the research of DLL4 and CD44V6 in malignant tumors, clinical stages of osteosarcoma, and latest treatment methods need to be conducted.

In summary, DLL4 and CD44V6 show high positive expression rates in osteosarcoma, and these are closely related to the degree of tumor differentiation, Enneking stage, and metastasis. Furthermore, DLL4 and CD44V6 are risk factors that affect the prognosis and survival rate of patients. Therefore, in clinical settings, we suggest that the expression of DLL4 and CD44V6 could be tested to evaluate the prognosis of osteosarcoma patients.

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Footnote

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