Original Article

Over-expression of HER2 promotes cell metastasis and is associated with the prognosis of osteosarcoma

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Abstract: Purpose: The objective aimed to detect the associations between HER2 expression and osteosarcoma (OS) metastasis as well as its effects on the prognosis of OS. Methods: Immunohistochemistry was adapted to test HER2 expression in 61 OS patients. The metastasis of cells was detected by transwell assays and the expression of HER2 in cell lines was measured by western blot. Results: The positive rate of HER2 expression in OS tissues was 62.1% according to immunohistochemistry. There were significance differences among osteosarcoma tissues, adjacent tissues and healthy tissues (P<0.01). Besides, the expression of HER2 was closely associated with enneking stage. Kaplan-Meier showed the overall survival time of patients with positive-HER2 expression was shorter than those with negative-HER2 expression. Log rank test proved the results was statistical significance. And positive HER2 expression as well as enneking were also important factors in the prognosis of osteosarcoma via Cox regression analysis and they could be independent prognostic markers. Western blot and transwell assays demonstrated that the over-expression of HER2 was promotor for the metastasis of osteosarcoma cells. Conclusion: HER2 was over-expressed in osteosarcoma patients as well as in cell lines and its expression was impacted by enneking. The over-expression of HER2 promoted the metastasis and could be an independent prognostic marker in osteosarcoma.

Keywords: HER2, osteosarcoma, metastasis, prognosis

Introduction

Osteosarcoma (OS), a common primary bone tumor, often occurs in the long bones (inferior femora and superior shinbones) of children and adolescents [1]. Most patients are at II B stages when they are initially diagnosed. It has been reported that 50% patients with osteosarcoma suffer metastasize especially lung metastasis which bringing a low cure rate and 5-years' survival rate [2-4]. As the development of comprehensive therapy, the 5-years survival rate rises from 30% to 60-70% [2, 5]. However, the prognosis of OS is still poor and it is important to study and detect new prognosis factors for OS patients.

HER2, a receptor tyrosine kinase, is a member of the Erbb family which take effect on the regulation of many essential cellular functions such as cell proliferation, differentiation, and apoptosis [6]. In previous studies, the expression of HER2 was up-regulated in 15%-40% breast cancer patients and lead to a poor prognosis [7]. There are two completely opposite opinion about HER2 expression in OS patients. Gorlick et al., found that high HER2 expression was associated with lung metastasis, and insensitive preoperative chemotherapy as well as shortened survival time of OS patients [8]. Nevertheless, high HER2 expression was considered to prolong patients' survival time in OS patients in the study of Akatsuka et al. [9]. So the expression pattern of HER2 was uncertain. Besides, the other function of HER2 in osteosarcoma is not clear.

In this study, our purpose was to further estimate the association between HER2 expression and OS prognosis as well as metastasis. The expression of HER2 was detected in tissues and cell lines by immunohistochemistry and western blot, respectively. Meanwhile, we analyzed the relationship between HER2 expression and clinicopathologic characteristics. Moreover, the function of HER2 in cell
metastasis and prognosis of OS were evaluated.

Materials and methods

Patients and samples

A total of 61 OS patients (33 men and 28 women, aged from 8 to 46 years) were recruited in this study. The study was conducted in Shaanxi Provincial Forestry Hospital and permitted by the Ethical Commission of the hospital. All patients involved hadn’t received any radiotherapy and chemotherapy. Besides, 61 healthy people without osteosarcoma were collected as controls. Written informed consent had been signed by every participator in advance.

The tissues and adjacent tissues from OS patients as well as the healthy tissues from controls were obtained and frozen by liquid nitrogen. Then the samples were stored at -80°C for using. A 5-years’ follow-up was performed to estimate the prognosis of OS. The clinicopathologic characteristics including age, gender, tumor size, enneking, price grading and relapses were recorded in a database. Patients who died from unexpected events or other diseases were excluded from our study.

Immunohistochemistry

Osteosarcoma tissues, adjacent tissues and healthy tissues were initially fixed by formalin, then dehydrated and embedded in paraffin. All samples were cut into a section with thickness of 4 μm. Deparaffinage and rehydration were made to the sections. Then washing the sections with PBS. The sections were hatched for 10 min with 3% H$_2$O$_2$ in room temperature and washed with PBS. Antigen retrieval reaction was implemented and the primary antibody was added and incubated at 4°C for overnight. Rewarming for 45 min at 37°C and washing with PBS. After that, the secondary antibody was put into and incubated for 1 h at 37°C. Then the sections were washed with PBS, colored with DAB coloration for 5-10 min, and redyed with hematoxylin. Finally, the sections were examined by light microscope after routine dehydration and lucency. Positive staining slices were regarded as positive controls and PBS solution were brought in as negative controls. Every section was randomly divided into three fields (100 cells in every field) to calculate the percent of positive stained cells in all of the cells. The positive expression of HER2 was stained as brown and the results were divided as follows: negative (“-”, the percentage of stained cells were less than 10%), weak positive (“+”, the percentage of stained cells were 10%-50%), strong positive (“++”, the percentage of stained cells were more than 50%). For further study, staining results were also grouped into negative and positive (including weak positive, and strong positive).

Cell culture and transfection

Human osteosarcoma cell lines U2OS were obtained from the Type Culture Collection of the Chinese Academy of Sciences and cultured in DMEM medium (Gibco, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C with 5% CO$_2$. Non-silencing (NS) control, and HER2 siRNAs were purchased from Qiagen. When the cells concentration reached to 50-60% (GenePharma, Shanghai, China), U2OS cell lines were transfected with HER2 siRNAs or NS-siRNA using the Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer.

Cell metastasis assay

Transwell migration assays (Millipore, Billerica, MA, USA) were used to detect the metastasis of osteosarcoma cells. Transfected cells were resuspended in serum-free medium. Then, the cell suspensions were seeded into the bottom wells of the chambers filling with complete medium. 24 h later, the cell numbers were counted from five nonoverlapping fields of each membrane. Three independent assays were performed.

Western blot

Protein samples extracted from transfected cells were separated by SDS-PAGE. The brands were transfered into PVDF membrane (Bio-Rad, CA, USA). The membranes were blocked with 5% non-fat milk (Bio-Rad) and incubated with primary antibody at 4°C overnight. Then washing the membrane and adding into horseradish peroxidase-conjugated secondary antibody.
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The expression level of HER2 proteins normalized to β-actin. The bands were visualized using a chemiluminescence reagent (New England Nuclear, Boston, MA, USA).

**Statistic analysis**

All data were stated at Mean ± SD and handled by SPSS13.0 software. The differences of HER2 expression among groups as well as the associations between HER2 and clinicopathologic characteristics were analyzed by chi-square test. The relationship between HER2 and overall survival time was estimated via Kaplan-Meier and Cox regression analysis. It was considered to be significant difference when $P<0.05$.

**Results**

**Immunohistochemistry identification of HER2 in osteosarcoma patients**

The expression of HER2 in the tissues and adjacent tissues of 61 OS patients as well as the tissues of 61 healthy controls were detected by immunohistochemistry. The reaction products are shown as brownish yellow. The positive-stained HER2 was 62.1% (32/61) which indicated that the expression of HER2 was higher in OS tissues than in adjacent tissues and healthy tissues (Figure 1, $P<0.05$).

**Association between HER2 and clinicopathologic characteristics**

The clinicopathologic characteristics were important influential factors for gene expression. In our study, enneking stage ($P<0.05$) were considered to be associated with the expression of HER2 while age, gender, tumor size and price pathology grading had no significant relationship with HER2 expression ($P>0.05$, Table 1). These result showed that HER2 might participate in the development of OS.

**Relationship between HER2 expression and overall survival time of OS patients**

During the follow-up, there were patients died and the follow-up rate was. Kaplan-Meier analysis revealed the overall survival time of patients with positive HER2 expression was shorter than those with negative HER2 expression (Figure 2, log rank test, $P<0.001$). As respect to
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Table 2. The prognostic value of HER2 according to Cox regression analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Risk ratio</th>
<th>95% confidence interval</th>
<th>P value</th>
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<tbody>
<tr>
<td>Ennking</td>
<td>0.110</td>
<td>0.024-0.498</td>
<td>0.014</td>
</tr>
<tr>
<td>High-HER2 expression</td>
<td>0.265</td>
<td>0.097-0.720</td>
<td>0.009</td>
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<tr>
<td>Low-HER2 expression</td>
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the prognostic value of HER2, Cox regression analysis was adopted. The outcome was displayed in Table 2, enneking stage (HR=0.110, 95% CI=0.024-0.498, P=0.014) and HER2 expression (HR=0.265, 95% CI=0.097-0.720, P=0.009) were related to the prognosis of OS and they might be independent prognostic markers in OS (Table 2).

Over-expression of HER2 promoted metastasis of osteosarcoma cells

At the 24 h after gene transfection, the expression of HER2 was increased in HER2-siRNA group compared with in NS-siRNA group (Figure 3). Meanwhile, the cell metastasis was promoted in cells transfected with siRNAs liken to the cell s transfected with NS-siRNA (Figure 4).

Discussion

OS is a differentiation disease caused by genetic changes and accounts for approximately 5% of all pediatric tumors and 8.9% of cancer-related deaths in children [10, 11]. It is characterized as the pain of tumor site which was induced by the erosion and dissolution of bone cortex. Now, many clinical and basic researches about the progress of osteosarcomas have been made [12], but the molecular genetic mechanism involved in osteosarcoma is still unclear. Many genes have been confirmed to be related to the development of OS such as RB1, p53, HER2, RECQL4 genes and so on [13-17].

HER2, also called c-erbB-2 or Her-2, is located in 17q21 chromosome and encodes a kind of transmembrane protein with 185000 relative molecular mass. Its structure is similar with EGFR and its cytoplasmic region possesses with tyrosine protein kinase (TPK) activity. Normally, HER2 is in inactive state and it can regulate the fission, differentiation and proliferation of cells combining with its specific ligands. The structure or expression disorder of HER2 will be activated by some carcinogenic factors which make HER2 owns tumor transformation activity so that it can promote malignant transformation of cells [18]. At present, amounts of studies have discovered that the abnormal expression of HER2 in different kinds of malignant tumors such as ovarian cancer, colorectal cancer, lung cancer and stomach cancer [19-22]. People have confirmed that there were amplification and over expression of HER2 in 20%-40% breast cancers and HER2 could act as the independent factor of prognosis [23]. In our study, according to immunohistochemistry and western blot, the expression of HER2 was verified to be over-expressed both in osteosarcoma tissues and cell lines which revealed HER2 might be an oncogene in OS.

Metastasis often occurs in OS which has become the main reason of death caused by OS [2]. If patients suffered from tumor metastasis before long clinical symptoms occurred, it often suggests typing of cases with higher malignant grade. Owing to the abnormal expression of HER2 in tumor cells, the adhesive force is weakened, mobility is strengthened and the cell differentiation is lowered among cells, then its invasiveness was strengthened. The tumor cells were easy to be broke away from the primary tumors and resulted in lymphatic or even distant metastasis. The metastatic potentials of OS cells might be related to the amount of HER2 expression. Therefore, transwell assay was performed to examine the metastasis of OS cells in current study. The result indicated...
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Figure 3. The expression of HER2 in osteosarcoma cells transfected with HER2-siRNA and NS-siRNA normalized to β-actin.

Figure 4. The cell metastasis was influenced by HER2.

the acceleration effects of HER2 in the metastasis of OS cells. HER2 expression affected the metastasis abilities of OS cells in patients that was to say, HER2 expression could reflect the metastasis potentials of cancer cells and it could be regarded as one of the predicated markers for OS metastasis.

Relevant literatures have pointed out that OS prognosis is influenced by many factors including tumor size, local necrosis rate after chemotherapy, reaction to chemotherapy, and concentration of ALP in serum [24-26]. The prognostic value of HER2 had been reported in various cancers like breast cancer, gastric cancer, ovarian cancer, and bladder cancer [27-30]. Patients with positive HER2 expression lived shorter than those with negative HER2 expression via Kaplan-Meier analysis. The outcome showed that ennkng and HER2 expression were the candidate factors for the prognosis of OS. Besides, this study also tested the role of HER2 and clinicopathologic characteristics in the prognosis of OS using Cox regression analysis which manifested ennkng and HER2 might be independent prognostic marker in OS.

To conclude, HER2 is up-regulated in OS tissues and cell lines. It also plays an important part in the metastasis of OS cells as well as its prognostic value may be good. HER2 may be an independent prognostic marker in OS and it will supply molecular biology references for the treatment options of OS.

Disclosure of conflict of interest

None.

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