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Expression of the Nrf2 and Keap1 proteins and their clinical significance in osteosarcoma

Jihong Zhang [a, *], Xiaojuan Wang [b], Wuzhou Wu [a], Hongsheng Dang [a], Bo Wang [a]

[a] Department of Orthopedics, Taihe Hospital, Hubei University of Medicine, Shiyan 442000, PR China
[b] Department of Internal Medicine, Taihe Hospital, Hubei University of Medicine, Shiyan 442000, PR China

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ABSTRACT

Objective: To investigate the expression and clinical significance of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and Kelch-like ECH-associated protein 1 (Keap1) in osteosarcoma tissue.
Methods: The data of 102 osteosarcoma patients who underwent surgical treatment at our hospital from June 2000 to March 2009 were collected. The expression levels of the Nrf2 and Keap1 proteins in osteosarcoma tissue and normal peritumour tissues were detected by immunohistochemistry, and the relationship between the expression level and the clinical and pathological features as well as the prognosis was explored.
Results: The nuclear expression rate of Nrf2 was 77.5% in osteosarcoma tissue, which was significantly higher than the rate in normal peritumour bone tissue (9.8%) ($P < 0.05$). The expression rate of the Keap1 protein in osteosarcoma tissue was 13.7%, which was significantly lower than the rate in normal peritumour tissue (80.4%). In addition, Nrf2/Keap1 expression was unrelated to patient gender and age, tumour site, and histological type and was related to metastasis and patient response to chemotherapy ($P < 0.05$). The five-year survival rate was significantly lower in patients with positive Nrf2 expression than in those with negative Nrf2 expression ($p = 0.023$), and it was significantly higher in patients with positive Keap1 expression than in those with negative Keap1 expression ($P = 0.018$).
Conclusion: The expression of Nrf2-Keap1 is abnormal in osteosarcoma tissue and shows significant clinical relevance for determining the prognosis of osteosarcoma.
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1. Introduction

Osteosarcoma is the most common type of primary malignant bone tumour and is more common in younger individuals, with a high prevalence in the growing metaphyseal section of long bones. The tumour cells originate from osteoblastic cells and are highly malignant and prone to relapse and metastasis, which produces a poor prognosis for the patient. The main clinical treatment presently involves comprehensive therapy that is based on surgery combined with chemotherapy [1]. Currently, despite a deeper understanding of the molecular and pathological mechanisms of the development, progression, and invasion of osteosarcoma, its exact mechanisms are still unclear, thereby posing significant challenges to the clinical treatment of osteosarcoma [2]. Studies have shown that the Kelch-like ECH-associated protein 1 (Keap1) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) proteins play a key role in the development, progression, invasion, and metastasis of various tumours [3]; however, to date, their expression and significance in osteosarcoma tissue have not been studied. In this study, we explored the expression of the Keap1 and Nrf2 proteins in osteosarcoma tissue and the relationship between the expression level and the clinical and pathological features as well as the prognosis to provide new ideas and methods to determine the prognosis and treatment of osteosarcoma.

2. Materials and methods

2.1. General data

A total of 102 patients who underwent surgical treatment at our hospital and were diagnosed with osteosarcoma by post-operative

* Corresponding author. Department of orthopedics, Taihe Hospital, Hubei University of Medicine, No. 32 South Road, Shiyan 442000, PR China.
E-mail addresses: zhangjihong63@163.com (J. Zhang), yangjian142@163.com (X. Wang), jiangchunli68@163.com (W. Wu), luoliping63@163.com (H. Dang), wangmian65@163.com (B. Wang).

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pathological examination from June 2000 to March 2009 were included in this study. A total of 102 specimens of normal peritumour bone tissue were also collected. This study was approved by the Ethics Committee of our hospital. All patients signed an informed consent form before surgery. The following general data were collected from patients: name, age, pre-operative and post-operative lab results, pathological staging, and treatment. Patients who received radiotherapy or chemotherapy before surgery were excluded from this study. The patients were followed up by phone or hospital visit every two months for up to five years.

2.2. Immunohistochemistry and hematoxylin and eosin (HE) staining

All fresh, resected specimens were fixed in 10% formalin, embedded in paraffin, and cut into four-micron sections. The slides were incubated at 60 °C for eight hours for later use. Next, the paraffin sections were deparaffinized and hydrated, followed by three PBS washes for three minutes for each. After rinsing with distilled water, the antigen was incubated in EDTA buffer at 100 °C for 20 min and then cooled to room temperature. After washing with distilled water twice, the slides were washed with PBS twice for three minutes each. Next, the peroxidase blocking solution was added, followed by incubation at room temperature for 10 min to block the activity of endogenous peroxidase. Then, the slides were washed three times with PBS for three minutes each. Next, non-immune serum from an animal source was added and then discarded after incubation at room temperature for 10 min. The primary anti-Nrf2 and anti-Keap1 antibodies (1: 100; purchased from Abcam plc, USA) were added, and the slides were incubated at room temperature for 60 min. PBS was used as a negative control. After washing with PBS (three times, three minutes for each wash), the biotin-labelled secondary antibody (goat anti-rabbit IgG, Zhongshan Jinqiao Biotech Co., Ltd., China) was added, and the slides were incubated at room temperature for 10 min. After washing with PBS (three times, three minutes for each wash), a streptavidin-anti-biotin-peroxidase solution was added, and the slides were incubated at room temperature for 10 min. After washing with PBS (three times, three minutes for each wash), DAB chromogenic reagents were added. Then, the slides were washed with tap water, re-stained with hematoxylin, washed with tap water until a blue colour appeared, dehydrated, dried with ethanol, cleared with xylene, and mounted using a neutral resin. HE staining was performed by following the standard staining procedures.

The interpretation of the immunohistochemical results was described previously [4]. Briefly, two experienced (blinded) pathologists independently interpreted the results. A total of 15 fields on each slide were counted at a high magnification level (x 400). The positive cell rate was calculated as the percentage of cells with Nrf2 or Keap1 expression of every 100 cells. The interpretation of positive immunohistochemical results (brown particles in the cytoplasm) was described previously and conducted as follows: the staining intensity of positive cells: 0 points, negative; 1 point, pale yellow; 2 points, yellow; and 3 points, brown; the interpretation of the final results: negative, 0–2 points and positive, 3–7 points; the percentage of positive cells: negative expression, 0%–5%; weakly positive, 6%–25%; moderately positive, 26%–75%; and strongly positive, > 76%. Weakly positive, moderately positive, and strongly positive results were defined as positive results [3].

2.3. Statistical analysis

The SPSS16.0 software program was used for the statistical analysis. The count data were analysed with a χ^2 test, and the Kaplan–Meier curve was used for the survival analysis. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. HE staining of osteosarcoma tissue and normal peritumour tissue

In this study, all specimens were confirmed by pathological diagnosis. Fig. 1 shows typical HE staining of osteosarcoma tissue in which the tumour was composed primarily of heterogeneous shuttle-shaped or polygonal cells (a large number of cells were in the mitotic phase) with varying nuclear shapes, large and deeply stained nuclei, and prominent nucleoli interspersed with trabecular or sheet-like, bone-like tissue with an irregular shape.

3.2. The expression and clinical significance of the Nrf-2 and Keap1 proteins in osteosarcoma tissue

Nrf2 expression in osteosarcoma tissue was indicated by yellow or brown particles in the cytoplasm and/or nucleus, and the percentage of cells with an Nrf2-positive nucleus was significantly lower in the normal peritumour tissues (Fig. 2). The statistical analysis showed that the Nrf2 positive rate was 77.5% in osteosarcoma tissue and only 9.8% in normal peritumour bone tissue, and the difference was statistically significant (Table 1, $P < 0.05$). In normal bone tissue, Keap1 protein, if expressed, was primarily present in the cytoplasm and observed as brown particles (Fig. 3); its positive expression rate was approximately 80.4%, which was significantly higher than that in osteosarcoma tissue (13.7%) (Table 1, $P < 0.05$).

3.3. The relationship between Nrf2 and Keap1 protein expression and the pathological features and stage of patients with osteosarcoma

After confirming the high levels of Nrf2 expression in prostate cancer tissue, we further investigated the relationship between the expression level and the clinical and pathological features of patients. The results showed that the Nrf2 positive expression rate was unrelated to patient gender and age, tumour site, and the histological type of osteosarcoma ($P > 0.05$) and was closely related to metastasis and the chemosensitivity of the tumour ($P < 0.05$, Table 2).

3.4. The relationship between Nrf2 and Keap1 protein expression and the clinical prognosis

The five-year survival rate was significantly higher in osteosarcoma patients with negative Nrf2 expression than in those with positive Nrf2 expression ($P < 0.05$, Fig. 4), and it was significantly higher in osteosarcoma patients with positive Keap1 expression than in those with negative Keap1expression ($P < 0.05$, Fig. 5).

4. Discussion

Nrf2 contains a basic leucine domain and has a molecular weight of approximately 66 kDa. It is a member of the CNC leucine zipper transcription activator family. The functions and activities of Nrf2 are mainly regulated by the cytosolic protein Keap1. In the resting state, Nrf2 is mainly present in the cytoplasm after binding to the cytoskeleton-associated protein Keap1 [5]. Oxidative stress causes Nrf2-Keap1 uncoupling, and unbound Nrf2 protein is transferred from the cytoplasm to the nucleus, where it binds to macrophage activating factor (Maf) to form heterodimers, which bind to antioxidant response elements (AREs) to initiate the

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