Expression and Clinical Implication of Autophagy-Associated Protein p62 in Osteosarcoma

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Keywords

p62 · Osteosarcoma · Autophagy-associated protein

Abstract

Purpose: Recent studies highlight the role of autophagy in cancer tumorigenesis, recurrence, metastasis, and chemoresistance. p62 is an adapter protein that is crucial for the autophagy pathway. In this study, we will describe the expression of p62 and its correlation with clinic prognosis in osteosarcoma. **Methods:** Western blot was used to test the expression of p62 in osteosarcoma cell lines (U2OS, KHOS, MG63, Saos-2, U2OSR2, KHOSR2, and 143B). A tissue microarray (TMA) was analyzed by immunohistochemistry to determine the expression levels of p62 in osteosarcoma patients and evaluate any correlation between p62 and clinical characteristics in osteosarcoma patients. **Results:** p62 was expressed differently in all cell lines. The TMA also showed differential expression in osteosarcoma tissues. Seventy-five of 79 (94.9%) patient tissues exhibited p62 immunostaining, ranging from no staining (4 of 97, 5.1%) to 1+ staining (40 of 79, 50.6%), 2+ staining (17 of 79, 21.5%), and 3+ staining (18 of 79, 22.8%). The low staining (1+) was classified as the p62 weak group (50.6%), the medium staining (2+) and intense

staining (3+) were classified as the p62 strong group (44.3%). Analyzing the clinical data of the osteosarcoma TMA, we found that the 5-year survival rate of patients with weak p62 expression was significantly lower than that of the patients with strong p62 expression ($p = 0.0165$). Furthermore, the decreased p62 expression may be associated with higher metastatic and chemoresistant rates in osteosarcoma patients. **Conclusion:** Our results suggest that p62 may be an effective predictor of prognosis and a potential target for therapy in osteosarcoma.

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Introduction

Osteosarcoma is the most common primary malignant bone tumor affecting both children and adolescents. It is responsible for 20% of all primary bone sarcomas [1]. The treatment strategy for osteosarcoma is preoperative chemotherapy, surgical resection of the tumor(s), and postoperative chemotherapy [2–4]. Although these treatments have improved the 5-year survival rate to 60–70%, osteosarcoma retains a high mortality rate due to recurrence and metastasis, as patients who develop metastasis

survive for less than 1 year on average [5–7]. Treatment and thus survival rates have not improved in almost 3 decades, which highlights the need for more effective therapeutic strategies [7].

Autophagy is a lysosome-mediated intracellular degradation and recycling pathway. Recent studies found that autophagy plays a role in tumorigenesis, recurrence, metastasis, and drug resistance in a variety of cancers, including osteosarcoma [8–10]. Autophagy contributes to the prolonged survival of tumor cells and begins with an autophagosome, a double-membrane vesicle that encloses cellular debris before fusing with a lysosome for degradation and recycling [11]. In addition, autophagy can selectively target and regulate cancer cell signaling proteins. Usually, the function and flux of autophagy are indirectly evaluated by related biomarkers, such as LC3, Beclin-1, and other autophagy-related proteins [9]. Latest studies have shown that autophagy-associated protein p62 plays a critical role in autophagy [12–14]. p62 is an autophagy substrate that accumulates and aggregates in autophagy-deficient cells, and it also has key roles in tumor progression or metastasis. p62 is a cellular protein localized at the autophagosome formation site and directly interacts with LC3, another autophagosome-localizing protein, and it is incorporated subsequently into the autophagosome. Because of the interaction with proteins in different intracellular signaling pathways, p62 plays an important role at the crossroad of autophagy, apoptosis, and cancer [12, 15]. Although expression of p62 has been reported in primary tumor tissues, there is little research on p62 expression in metastatic tumor tissues. The expression of p62 and the relationship between p62 expression and clinic prognosis in osteosarcoma are unknown.

This is the first study that examines the expression of p62 in osteosarcoma cell lines and tumor tissue samples. We also evaluate the association between p62 expression and clinical characteristics in osteosarcoma patients, especially those with metastasis and recurrence.

Materials and Methods

Cell Lines and Cell Culture

The human osteosarcoma cell lines U2OS, MG63, Saos-2, and 143B were obtained from the American Type Culture Collection (Rockville, MD, USA). The human osteosarcoma cell lines KHOS, KHOSR2, and U2OSR2 were provided by Dr. Efstathios Gonos (Institute of Biological Research and Biotechnology, Athens, Greece). Cell lines were maintained in RPMI 1640 supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum in a 5% CO₂, 37 °C cell culture incubator.

Western Blotting Assay

Briefly, total protein was isolated with RIPA Lysis Buffer (Upstate Biotechnology, New York, NY, USA). The concentration of the protein was determined by protein assay reagents (Sigma-Aldrich, St. Louis, MO, USA) and a spectrophotometer (Beckman DU-640, Beckman Instruments, Inc., Indianapolis, IN, USA). Protein extracted from cell lysates was separated by SDS-PAGE electrophoresis and then transferred onto nitrocellulose membranes. Membranes were then blocked with 5% nonfat milk in 1× TBST and then probed with a specific primary antibody for p62 (1:1,000 dilution, Cell Signaling Technology) and mouse monoclonal antibody for human β -actin (Sigma-Aldrich) at 4 °C overnight. Following primary antibody incubation, the membranes were washed with TBST (Cell Signaling Technology) and further probed with secondary antibodies IRDye[®] 800CW and IRDye[®] 680LT which were obtained from LI-COR Biosciences (Lincoln, NE, USA). After incubation at room temperature for 2 h, the relative expression of protein was normalized using Odyssey Infrared Fluorescent Western Blots Imaging System from LI-COR Biosciences. Quantification of western blot results was analyzed with Odyssey software 3.0.

Osteosarcoma Tissue Microarray and Immunohistochemistry

A total of 79 osteosarcoma specimens were taken after informed consent was given from osteosarcoma patients who had surgery in the Orthopaedic Department of Massachusetts General Hospital from 1993 to 2010. Tissue microarrays (TMAs) of osteosarcoma tissues with paraffin-embedded (FFPE) blocks were provided by the Sarcoma Tissue Bank. To ensure that the selection included the core of the tumor, the 3 sites of each FFPE block were selected for assembling the recipient master block. TMA is produced by the Tissue Microarray and Imaging Core at the Dana-Farber/Harvard Cancer Center. Hematoxylin and eosin-stained slides from each tissue block were read by a pathologist to obtain representative triplicate 0.5-mm-diameter core biopsies. All of the patients were treated by preoperative chemotherapy, and the clinical information was collected, including age, gender, tumor location, disease status (primary/metastasis/recurrence), and follow-up time. According to the patients' status, 79 specimens were divided into 3 groups (14 recurrence, 27 metastasis, and 38 primary). The samples were from 49 (62.0%) males and 30 (38.0%) females with a mean age of 31.4 years (range 10–74). All samples were followed up for a mean period of 82.2 months (range 1–273) after surgery.

The expression of p62 was dependent on immunohistochemical staining. First of all, 5- μ m-thick array sections were baked at 60 °C for 1 h, dewaxed 3 times with xylene (each time for 5 min), and then transferred by 100% ethanol twice (each time for 5 min), rehydrated through graded alcohol, and lastly immersed in deionized water for 10 min. Antigen retrieval was processed with Target Retrieval Solution (Dako North America, Inc., Carpinteria, CA, USA). After that, the slide was washed with PBS twice for 5 min. Following the process of antigen retrieval, endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide. After the protein was blocked with blocking solution (Cell Signaling Technology) for 1 h at room temperature, primary antibody (1:50 dilution, in 1% bovine serum albumin PBS) was applied at 4 °C overnight in a humidified chamber. Primary p62 antibody (Santa Cruz Biotechnology, Inc.) was probed at 4 °C overnight. Each step was succeeded by 3 TBS rinses, and the bound antibody

Fig. 1. Expression of protein p62 in osteosarcoma cell lines and osteosarcoma patient tissues. **a** Expression of p62 in osteosarcoma cell lines by western blotting. **b** Western blots of p62 from **a** were analyzed by densitometry, and quantitative results of p62 were presented as relative expression. **c** Representative images of different immunohistochemical staining intensities of p62 or HE stain-

ing are shown in osteosarcoma tissues. The percentage of cells that showed positive cytoplasmic staining for p62 was calculated after analysis of the entire spot. Based on the p62 staining intensities in the tumor samples, the staining patterns were categorized into 4 groups: no staining (0), weak staining (1+), moderate staining (2+), and strong staining (3+). Original magnification, $\times 400$.

on the array was detected using SignalStain[®] Boost Detection Reagent (Cell Signaling Technology) and SignalStain[®] DAB (Cell Signaling Technology). Finally, all the sections were counterstained with Hematoxylin QS (Vector Laboratories), and the slide was mounted with VectaMount AQ (Vector Laboratories) for long-term preservation.

Evaluation of Immunohistochemical Staining

Immunostained slides were evaluated under the microscope. The immunostaining intensity pattern of p62 was assessed on the following semi-quantitative scale: no staining (0); weak staining (1+); moderate staining (2+); and strong staining (3+). The evaluation score of the p62 staining was completed by 2 independent investigators in the Sarcoma Biology Laboratory who were blinded to the clinicopathologic data, and a consensus was reached in all

cases. The tumor necrosis was collected from the clinical data. It was based on the percentage of tumor tissues necrosis of pathological specimens (good response: $\geq 90\%$ necrosis; poor response: $< 90\%$ necrosis). p62 staining images were obtained using a Nikon Eclipse Ti-U fluorescence microscope (Nikon Corp.) with a SPOT RT digital camera (Diagnostic Instruments, Inc.).

Statistical Analysis

GraphPad Prism5 software (GraphPad Software, Inc.) was used for statistical analyses. Statistical significance was assessed using independent two-tailed Student *t* tests for independent data. Kaplan-Meier plots and log-rank tests were used to compare the differences in survival curves. The χ^2 test was used to analyze the relationship between p62 expression and osteosarcoma clinical pathological parameters. Prognostic factors associated with overall

Table 1. p62 staining characteristics of osteosarcoma specimens

p62 staining	No staining, <i>n</i> (%)	1+ staining, <i>n</i> (%)	2+ staining, <i>n</i> (%)	3+ staining, <i>n</i> (%)
Primary	3 (3.8)	11 (13.9)	10 (12.7)	14 (17.7)
Recurrence		11 (13.9)	2 (2.5)	1 (1.3)
Metastasis	1 (1.3)	18 (22.8)	5 (6.3)	3 (3.8)
Total	4 (5.1)	40 (50.6)	17 (21.5)	18 (22.8)

Table 2. Histological response to preoperative chemotherapy and p62 immunostaining

	Good response ^a (% pre-operative chemotherapy)	Poor response ^b (% pre-operative chemotherapy)	N/A (% preoperative chemotherapy)
p62 strong staining	8 (80.0%)	18 (41.9%)	9 (40.9%)
p62 weak staining	2 (20.0%)	25 (58.1%)	13 (59.1%)
Total	10	43	22

N/A, not applicable. ^a ≥90% necrosis. ^b <90% necrosis.

survival or 5-year survival were investigated through the Cox proportional hazards regression model in a stepwise manner. Only those factors that were statistically significant ($p < 0.05$) in the univariate survival analysis were included in the multivariate analyses. All the results were presented as mean $\pm$ SD, and p values <0.05 were regarded as statistically significant.

Results

p62 Expression in Osteosarcoma Cell Lines and Tissues

We first tested the expression of p62 in osteosarcoma cell lines. Western blot analysis revealed that p62 was expressed differently in every cell line (U2OS, KHOS, Saos-2, MG63, U2OSR2, KHOSR2, and 143B). We observed a relatively low expression of p62 in the osteosarcoma cell lines 143B, KHOSR2, and U2OSR2, whereas U2OS, KHOS, and Saos-2 showed a relatively high expression (Fig. 1a, b). We also evaluated the expression of other autophagy-associated proteins, including LC3 and Atg5, in osteosarcoma cell lines by western blotting; we only found the decreased expression of p62, but not LC3 and Atg5, which was correlated with multidrug resistance in these cell lines (see online suppl. Fig. S1; for all online suppl. material, see www.karger.com/doi/10.1159/000487437). We further analyzed the expression of p62 in osteosar-

coma tissues. The immunohistochemical staining showed p62 in the cytoplasm. Among the evaluable specimens, 75 of 79 (94.9%) patient tissues exhibited p62 immunostaining, ranging from no staining (4 of 79, 5.1%) to 1+ staining (40 of 79, 50.6%), 2+ staining (17 of 79, 21.5%), and 3+ staining (18 of 79, 22.8%) (Table 1). The stained specimens were subdivided into 2 categories: the low (1+) staining was classified as the p62 weak group (50.6%), whereas the medium (2+) and intense (3+) staining were classified as the p62 strong group (44.3%) (Fig. 1c).

Correlation between p62 and Clinical Characteristics in Osteosarcoma Patients

After p62 expression in TMA was analyzed, we compared patient prognosis with the expression level of p62. The differences in p62 expression between primary tumor tissues (patients without metastasis/recurrence) and tissues from patients with metastasis/recurrent disease were significant ($p < 0.05$) (Fig. 2a). The expression of p62 in patients with metastatic disease was significantly lower ($p = 0.0164$) (Fig. 2b). In addition, patients with poor response to preoperative chemotherapy displayed weak expression of p62 compared to those who had a good response ($p = 0.0110$) (Fig. 2c; Table 2). Kaplan-Meier analysis demonstrated that patients with poor response to preoperative chemotherapy had a lower overall survival

Fig. 2. Expression of p62 in osteosarcoma tissues of immunohistochemical staining and its clinical significance. **a** Distribution of p62 immunohistochemical staining scores in primary, metastatic, and recurrent osteosarcoma tissues. **b** Comparison of p62 immunostaining density between osteosarcoma tissues of patients with metastasis and nonmetastasis. **c** Comparison of p62 immunostaining density between good-response and poor-response osteosarcoma tissues based on the histological necrosis percentage after preoperative chemotherapy. **d** Kaplan-Meier overall survival curve: patients with osteosarcoma were subgrouped as either good

or poor response to preoperative chemotherapy. **e** Kaplan-Meier overall survival curve: patients with osteosarcoma were subgrouped as either p62 weak staining (staining ≤ 1) or strong staining (staining ≥ 2). **f** Kaplan-Meier 5-year survival curve: patients with osteosarcoma were subgrouped as either p62 weak staining (staining ≤ 1) or strong staining (staining ≥ 2). **g** Kaplan-Meier disease-free survival curve: patients with osteosarcoma were subgrouped as either p62 weak staining (staining ≤ 1) or strong staining (staining ≥ 2).

rate ($p = 0.0202$) (Fig. 2d). However, there was no significant relationship between p62 expression and the other clinical pathological features of the patients, such as age ($p = 0.3850$), gender ($p = 0.1461$), and tumor site ($p = 0.2754$) (Table 3). Compared to the osteosarcoma pa-

tients of the p62 strong expression group, the Kaplan-Meier analysis showed that the weak p62 expression group had a lower overall survival rate ($p = 0.0108$) (Fig. 2e). Meanwhile, Kaplan-Meier analysis also showed that the weak p62 expression patients had a worse 5-year

survival rate than the patients with strong p62 expression ($p = 0.0165$) (Fig. 2f). The 5-year survival rate for patients with weak p62 expression was 37.5%, which was significantly lower than the rate of 65.7% in patients with strong p62 expression. The 5-year overall survival rate for patients with metastasis was 33.8%, which was lower than the rate of 94.1% for patients with absent metastasis. Meanwhile, compared to a rate of 71.1% for patients with absent recurrence, the 5-year overall survival rate for patients with present recurrence was 47.7% (Table 4). Likewise, Kaplan-Meier analysis showed that patients with weak p62 expression had a lower disease-free survival rate than the patients with strong p62 expression ($p = 0.0029$) (Fig. 2g). The above data suggest that there is a close relationship between p62 expression and clinical prognosis as well as the pathological features of osteosarcoma, especially for tumor metastasis or recurrence.

To determine whether p62 expression was an independent prognostic factor for osteosarcoma patients, we used the Cox regression analysis. In the univariate Cox regression analysis, weak expression of p62, present metastasis or recurrence, and the response to preoperative chemotherapy were associated with shorter 5-year survival in osteosarcoma patients, whereas other clinicopathological features showed no prognosis correlations (Table 4). Im-

portantly, multivariate Cox regression indicated that p62 expression was an independent predictor of 5-year survival ($p = 0.0102$) (Table 5). These results demonstrated that the p62 expression level independently predicts osteosarcoma outcomes.

Discussion

Autophagy has been shown to play complex roles in the regulation of cancer development and progression in different stages of tumorigenesis [16–19]. Specifically, after the tumor develops, autophagy is thought to support tumor growth [17, 20]. In this study, we found p62 expressed in osteosarcoma cell lines, and p62 was decreased in recurrence or metastatic tumor tissues in comparison to the primary tumor tissues, which suggests that autophagy activity could be a mechanism associated with metastasis or recurrence in osteosarcoma patients.

Recently, accumulation of p62 has been used as a marker for autophagy inhibition [21–24]. Previous studies have demonstrated aberrant p62 expression to be associated with prognosis in various tumors [12, 13, 25, 26]. In our study, we found differential expression of p62 in osteosarcoma cell lines. Interestingly, we observed a rela-

Table 3. The relationship between p62 expression and clinicopathological features of osteosarcoma

Clinicopathological features	Cases, <i>n</i> (%)	p62 expression N/A, <i>n</i> (%)	p62 expression weak, <i>n</i> (%)	p62 expression strong, <i>n</i> (%)	<i>p</i> value
All patients	79 (100)	4 (5.1)	40 (50.6)	35 (44.3)	
Age					
≤18 years	23 (29.1)	1 (4.3)	10 (43.5)	12 (52.2)	0.3850
>18 years	56 (70.9)	3 (5.4)	30 (53.6)	23 (41.1)	
Gender					
Male	49 (62.0)	2 (4.1)	22 (44.9)	25 (51.0)	0.1461
Female	30 (38.0)	2 (6.7)	18 (60.0)	10 (33.3)	
Tumor site					
Femur	32 (40.5)	1 (3.1)	18 (56.3)	13 (40.6)	0.2754
Tibia	15 (19.0)	1 (6.7)	6 (40.0)	8 (53.3)	
Humeral bone	7 (8.9)		2 (28.6)	5 (71.4)	
Other	25 (31.6)	2 (8.0)	14 (56.0)	9 (36.0)	
Metastasis					
Absent	17 (21.5)	2 (11.8)	3 (17.6)	12 (70.6)	0.0164
Present	62 (78.5)	2 (3.2)	37 (59.7)	23 (37.1)	
Recurrence					
Absent	52 (65.8)	3 (5.8)	21 (40.4)	28 (53.8)	0.0121
Present	27 (34.2)	1 (3.7)	19 (70.4)	7 (25.9)	
N/A, not applicable.					

tively low expression of p62 in the osteosarcoma cell line 143B, which was discovered to be highly metastatic in the lungs of mice after subcutaneous and intramuscular xenotransplantations [27, 28]. Expression of p62 was lower in the multidrug-resistant osteosarcoma cell lines U2OSR2 and KHOSR2. To define the relationship between p62 expression and clinical behavior in osteosarcoma, we analyzed the immunohistochemical staining of p62 in osteosarcoma tissue samples of patients and discovered a close relationship between p62 expression and clinical characteristics and outcomes. Differences in p62 expression from 1+ to 3+ staining were detected in 94.9% of the tumor samples examined, which indicates that p62 expression is frequent in osteosarcoma.

However, expression of p62 is not associated with patients' age, gender, and tumor site of osteosarcoma. In established tumors, autophagy responses usually assist tumor cells with intracellular and environmental stress, thus favoring tumor metastasis [29]. As compared to the primary group, our results showed that the recurrent and metastatic groups of osteosarcoma patients had weaker p62 expression. Consistent with our result, colorectal cancer also showed decreased p62 expression in the recurrent and metastatic groups as compared to the nonrecurrent and nonmetastatic groups [30]. Therefore, under an active autophagy status, decreased p62 expression may have a relationship with tumor metastasis and recurrence. Many studies have implicated that autophagy activities are enhanced in response to treatment, such as chemotherapy, which contributes to treatment resistance in cancer cells [31–33].

Increased autophagy activities have been reported to increase cell growth and promote chemoresistance in osteosarcoma cells [34–36]. In this study, we found decreased p62 expression in osteosarcoma patients with poor response to preoperative chemotherapy. These results suggest that p62 is most likely to be a marker associated with autophagy flux and a prognostic factor in osteo-

sarcoma. This hypothesis is also supported by recent reports showing that Ref(2)P – the *Drosophila* ortholog of p62 – was used to monitor autophagy flux, and p62 was also deemed as the best reporter protein to monitor autophagy, and it was degraded at a basal rate by autophagy activity [37, 38].

Additionally, our study showed that osteosarcoma patients with weak p62 stains had a worse survival rate and disease-free survival rate than patients with tumors that

Table 4. Univariate analysis of prognostic factors in all osteosarcoma patients

Variable	Five-year survival, %	p value
Age		
≤18 years	52.1	0.4150
>18 years	46.4	
Gender		
Male	42.8	0.3310
Female	56.0	
Tumor site		
Femur	59.3	0.4714
Tibia	33.3	
Humeral bone	57.1	
Other	44.0	
Metastasis		
Absent	94.1	<0.0001
Present	33.8	
Recurrence		
Absent	71.1	0.0206
Present	47.7	
p62 expression		
Weak	37.5	0.0165
Strong	65.7	
Response to preoperative chemotherapy		
Good	80.0	0.0182
Poor	52.6	

Table 5. Multivariate survival analysis (5-year survival)

Variable	Hazard ratio	95% CI	p value
p62 expression	2.170	1.152–4.085	0.0102
Metastasis	4.370	2.224–8.585	<0.0001
Recurrence	3.410	2.146–7.026	0.1080
Response to preoperative chemotherapy	4.022	1.744–6.369	0.0136

CI, confidence interval.

had strong p62 stains. Therefore, decreased p62 expression might be a potential biomarker indicative of a mechanism that causes failed treatment in osteosarcoma. Currently, several studies investigating autophagy inhibitors correlated with preclinical and clinical trials are ongoing in different cancers [39–41]. In osteosarcoma, the autophagy inhibitor chloroquine has been noticed to decrease cell proliferation and growth [42]. Moreover, up-regulation of p62 is an effective approach for inhibiting autophagy activity in tumors. In cisplatin-resistant osteosarcoma cells, chloroquine could block autophagy and increase chemosensitivity [43]. Autophagy inhibitor 3-MA combined with doxorubicin or cisplatin could improve the therapeutic response to chemotherapy drugs in U2OS, Sao-2, and MG63 osteosarcoma cell lines [10, 44]. Therefore, enhancing the level of p62 may be an effective therapy for cancer treatment. p62 vaccination has been used to suppress tumor metastasis in mice with sarcoma and in dogs with mammary tumors. It also dramatically decreased the number and sizes of lung or lymphatic metastases [45–47]. For improving the treatment of osteosarcoma, p62 vaccine may also be used as a novel cancer therapy for osteosarcoma in the future.

Conclusion

Our study demonstrated that p62 has an important role in osteosarcoma. The level of p62 expression was directly associated with clinical outcome, especially since, as an autophagy-associated protein, p62 is closely related to tumor recurrence, metastasis, and prognosis in osteosarcoma. Our data suggest that p62 may be a potential novel molecular biomarker in osteosarcoma.

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Disclosure Statement

The authors report no conflicts of interest in relation to this work.

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