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RESEARCH

# Upregulation of a Disintegrin and Metalloprotease 8 Influences Tumor Metastasis and Prognosis in Patients with Osteosarcoma

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**Abstract** To investigate the clinicopathological and prognostic value of a disintegrin and metalloprotease 8 (ADAM8) in osteosarcoma. ADAM8 expression in osteosarcoma tissues was examined by immunohistochemistry in 69 patients. ADAM8 was positively expressed in 61 of 69 (88.4%) osteosarcoma specimens with cytoplasmic staining, and also increased in the specimens with recurrence ($P$=0.008) and metastasis ($P$=0.002). Patients with strong ADAM8 expression had significantly poorer overall survival (OS) and disease-free survival (DFS) (both $P$<0.001) when compared with the patients with the weak expression of ADAM8. On multivariate analysis, ADAM8 expression was found to be an independent prognostic factor for both OS ($P$<0.001) and DFS ($P$<0.001). Our results suggest for the first time that ADAM8 might be applied as a novel marker for the prediction of recurrence and metastasis potency and a significant indicator of poor prognosis for patients with osteosarcoma.

**Keywords** Osteosarcoma · A disintegrin and metalloprotease 8 · Immunohistochemistry · Prognosis · Overall survival · Disease-free survival

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## Introduction

Osteosarcoma is one of the most rapidly growing sarcomas and the most common primary malignant bone tumor [1]. It is characteristically found in the metaphyseal regions of long bones in the appendicular skeleton. The great majority of osteosarcoma patients are represented by high-grade tumors with advanced phenotypes at the time of diagnosis [2]. More than 15% of patients present with clinically detectable pulmonary metastases. It is estimated that 80% or more have micrometastases at presentation [3]. Therefore, cure is rare after surgical treatment alone. The inclusion of aggressive polychemotherapy into an interdisciplinary treatment concept has led to dramatic prognostic improvements in young patients with seemingly localized extremity disease [4]. Despite these advances, patients that present with metastases or those whose tumors are refractory to neoadjuvant chemotherapy continue to have a poor prognosis. Consequently, the studies on the molecular mechanisms leading to the development and progression of osteosarcoma, which remain unclear, may provide novel opportunities for diagnosis, prognosis, and therapeutic interventions.

A disintegrin and metalloprotease (ADAM) family, a group of multidomain proteins consisting of pro-, metalloprotease, disintegrin-like, cysteine rich, EGF-like and transmembrane domains, fulfills different functions, such as spermatogenesis, cell fusion, cell adhesion, myogenesis, proteolysis of membrane proteins, neurogenesis, signaling and leukocyte infiltration [5, 6]. ADAM8 (synonym CD156), one of more than 20 members of the human ADAM family, is a transmembrane protein with a COOH-terminal transmembrane domain and potential extracellular adhesion and protease domains, which are related to soluble snake venom proteins leading to hemorrhage and destruction of the basement membrane [7].

ADAM8 is expressed primarily on immune cells and can be induced with various pro-inflammatory stimuli [8]. In 2009, M.D. Zack et al. [9] identified ADAM8 as a target for osteoarthritis by demonstrating that human osteoarthritis chondrocytes produce ADAM8 which degrades fibronectin to generate neoepitopes seen in diseased cartilage. They also found that mice expressing catalytically inactive ADAM8 are significantly protected in an animal model of rheumatoid arthritis. ADAM8 stimulates osteoclast formation, Ishizuka et al. [10] further found that the loss of ADAM8 did not inhibit basal bone remodeling but only blocks the enhanced osteoclast formation in response to TNF-α, which suggest that ADAM8 may be an attractive therapeutic target for preventing bone destruction associated with inflammatory disease. Additionally, upregulation of ADAM8 has already been demonstrated in various cancer types. Ishikawa et al. [11] found ADAM8 expressed in 47~79% of different cancer types on a tissue microarray of 363 cases. ADAM8 expression was associated with higher tumor stage in adenocarcinomas of the lung and has been proposed as a serum marker for lung cancer. Roemer et al. [12] suggested ADAM8 as a tumor marker for renal cell carcinomas. They found the over-expression of ADAM8 mRNA significantly associated with shorter patient survival and a predictor of distant metastasis. Fritzsche et al. [13] demonstrated that the overexpression of ADAM8 protein in prostate cancer tissues was significantly associated with higher pT status, positive nodal status, higher Gleason score and unfavorable prognosis. However, the expression pattern of ADAM8 in osteosarcoma is still unclear. In the present study, to confirm whether upregulation of ADAM8 has relevance to the progression of osteosarcoma, we investigate the clinicopathological and prognostic value of ADAM8 in this tumor.

**Table 1** Correlation of ADAM8 expression with clinicopathological features of osteosarcoma

| Features | *N* | ADAM8 expression | | | | *P* |
|---|---|---|---|---|---|---|
| | | I | II | III | IV | |
| Age | | | | | | |
| >21 | 20 | 4 | 5 | 3 | 8 | 0.5 |
| ≤21 | 49 | 4 | 15 | 15 | 15 | |
| Gender | | | | | | |
| Male | 38 | 4 | 9 | 10 | 15 | 0.1 |
| Female | 31 | 4 | 11 | 8 | 8 | |
| Tumor size (cm) | | | | | | |
| >8 | 24 | 3 | 2 | 9 | 10 | 0.6 |
| ≤8 | 45 | 5 | 18 | 9 | 13 | |
| Tumor site | | | | | | |
| Femur | 40 | 6 | 11 | 10 | 13 | 0.8 |
| Tibia | 29 | 2 | 9 | 8 | 10 | |
| Histological subtype | | | | | | |
| Osteoblastic | 36 | 4 | 4 | 12 | 16 | 0.1 |
| Chondroblastic | 18 | 4 | 9 | 2 | 3 | |
| Fibroblastic | 8 | 0 | 3 | 3 | 2 | |
| Mixed type | 5 | 0 | 3 | 0 | 2 | |
| Small cell type | 2 | 0 | 1 | 1 | 0 | |
| Recurrence | | | | | | |
| With | 18 | 0 | 2 | 4 | 12 | 0.008 |
| Without | 51 | 8 | 18 | 14 | 11 | |
| Metastasis | | | | | | |
| With | 15 | 0 | 1 | 2 | 12 | 0.002 |
| Without | 54 | 8 | 19 | 16 | 11 | |
| Response to chemotherapy | | | | | | |
| Good | 24 | 2 | 11 | 6 | 5 | 0.3 |
| Poor | 45 | 6 | 9 | 12 | 18 | |

## Materials and Methods

### Patients and Tissue Samples

This study was approved by the Research Ethics Committee of Xiangya Hospital, China. Informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards.

Sixty-nine osteosarcoma patients who received surgical treatment at Xiangya Hospital, China, from August 2000 to August 2006 were included in this study. Of 69 patients, 67 patients had high grade intramedullary osteosarcomas. The clinicopathological information of the patients is shown in Table 1.

Immunostainings were performed on archived paraffin wax embedded biopsy specimens prior to neoadjuvant chemotherapy. All the patients received pre-operative multi-agent chemotherapy following the initial biopsy. The cytotoxic drugs used as pre-operative chemotherapy were cis-Diaminedichloroplatinum (CDDP), Adriamycin (ADR), vincristine, ifosphamid (IFO), and high-dose methotrexate (HD-MTX). The resected specimens were analyzed histologically for response to chemotherapy according to the criteria released previously; corresponding clinical information was obtained from medical records.

### Immunohistochemistry Analysis

ADAM8 expression was detected immunohistochemically for paraffin-embedded specimens from 69 patients with osteosarcoma. The specimens were fixed in 10% neutral buffered formalin and subsequently embedded in paraffin. The paraffin-embedded tissues were cut at 3 μm and stained following being dried on ProbeOn Plus (Fisher Scientific International, Hampton, NH, USA). Staining was done using avidin- biotin complex with a microprobe manual stainer (Fisher Scientific International). The slide to which

a paraffin section was attached went through deparaffinization and hydration, and was then treated with a solution of Peroxidase-blocking reagent (DAKO, Hamburg, Germany) to exhaust endogenous peroxidase activity. It was put in citric acid solution and heated for 10 min in a microwave and then left at room temperature for 20 min to expose antigen hidden inside the tissue due to formalin fixation, and the process was repeated three times. To inhibit non-specific antigen-antibody reactions possible in immunohistochemical staining, reaction was done using a protein blocker (Research Genetics, Huntsville, AL, USA) for 5 min and the slide was washed thoroughly with water. The slides were incubated overnight with the primary ADAM8 antibody [polyclonal rabbit antibody, Cedarlane Laboratories, Hornby, Canada, diluted 1:200 using a background reducing dilution buffer (DAKO, Hamburg, Germany)] at 4°C. Secondary antibody for the detection of primary antibody was reacted for 10 min using anti-rabbit IgG (Sigma, St. Louis, MO, USA) to which biotin was attached, and then washed with buffer solution and reacted with horseradish peroxidase for 10 min. It was washed thoroughly with buffer solution; chromogen AEC (3-amino-9-ethylcarvazole; Zymed, San Francisco, CA, USA) was then applied and reddish brown response was examined. After hematoxylin contrast staining, the slide was enclosed with Universal Mount (Research Genetics) and examined. In each immunohistochemistry run, negative controls were samples that were incubated with normal rabbi serum.

Following a hematoxylin counterstaining, immunostaining was scored by two independent experienced pathologists, who were blinded to the clinicopathological parameters and clinical outcomes of the patients. The scores of the two pathologists were compared and any discrepant scores were trained through re-examining the stainings by both pathologists to achieve a consensus score. The number of positive-staining cells showing immunoreactivity on the cytoplasm (for ADAM8) in ten representative microscopic fields was counted and the percentage of positive cells was calculated. The frequency of ADAM8 immunoreactivity in tissue sections was evaluated as '0' when no positive cells were observed within the tumor, '1' when <25% of the tumor cells were positive, '2' when 25% to 50% of the tumor cells were positive, '3' when 50% to 75% of tumor cells were positive and '4' when >75% of tumor cells were positive. The intensity of staining was evaluated as 0, 1, 2, and 3 for no staining, weak staining, medium staining, and strong staining, respectively. Immunohistochemical scores were determined as the sum of the frequency and intensity score for tumor cells. A total score of 0~12 was finally calculated and graded as: I, score 0~1; II, 2~4; III, 5~8; IV, 9~12. Grade I was considered negative, and grades II, III and IV positive. Grades I and II represented no or weak staining, and grades III and IV represented strong staining. In each case, at least three different areas of tumor were valuated, and the mean of the results was taken as the final expression score.

### Statistical Analysis

The software of SPSS version13.0 for Windows (SPSS Inc, IL, USA) and SAS 9.1 (SAS Institute, Cary, NC) was used for statistical analysis. Continuous variables were expressed as $\overline{X} \pm s$. The Chi-square test was used to show differences of categorical variables. Patient survival and their differences were determined by Kaplan–Meier method and log-rank test. Cox regression (Proportional hazard model) was adopted for multivariate analysis of prognostic factors. Differences were considered statistically significant when $P$ was less than 0.05.

## Results

### ADAM8 Expression and its Correlations with Clinicopathological Features of Osteosarcoma

As the immunohistochemistry analysis, microscopic observations indicated that ADAM8 was intensely expressed in the cytoplasm of tumor cells. In 69 osteosarcoma specimens, ADAM8 staining was positive in 61 samples (88.4%), and strongly expressed in 41 osteosarcoma patients (grades III and IV, 59.4%).

Relationships between clinicopathological features and the ADAM8 staining were shown in Table 1. The ADAM8 protein was significantly over-expressed in osteosarcoma patients with recurrence ($P$=0.008) and metastasis ($P$=0.002), as compared to those without. No significant difference was observed between the expression of ADAM8 and patients' age, gender, tumor size, tumor site, histological subtype and response to chemotherapy.

### Prognostic Values of ADAM8 Expression in Osteosarcomas

Using Kaplan–Meier method and log-rank test, osteosarcoma tissues with higher staining scores of ADAM8 (grade III~IV) were respectively correlated to shorter disease-free survival (DFS, Fig. 1a, $P$<0.001) and overall survival (OS, Fig. 1b, $P$<0.001) of patients. The result showed that the mean DFS for osteosarcoma patients with low ADAM8 expression (grade I~II) was 70.3+6.9 months. In contrast, if the osteosarcoma strongly expressed ADAM8 (grade III~IV), the mean survival was only 50.8+4.1 months. Turning to OS, if the tumors weakly expressed ADAM8, the mean OS time was 71.7+8.2 months. In contrast, when the tumors strongly expressed ADAM8, the mean OS time was 55.0+5.3 months. Besides, the survival benefits were also found in those with smaller tumor size (both $P$=0.02), better

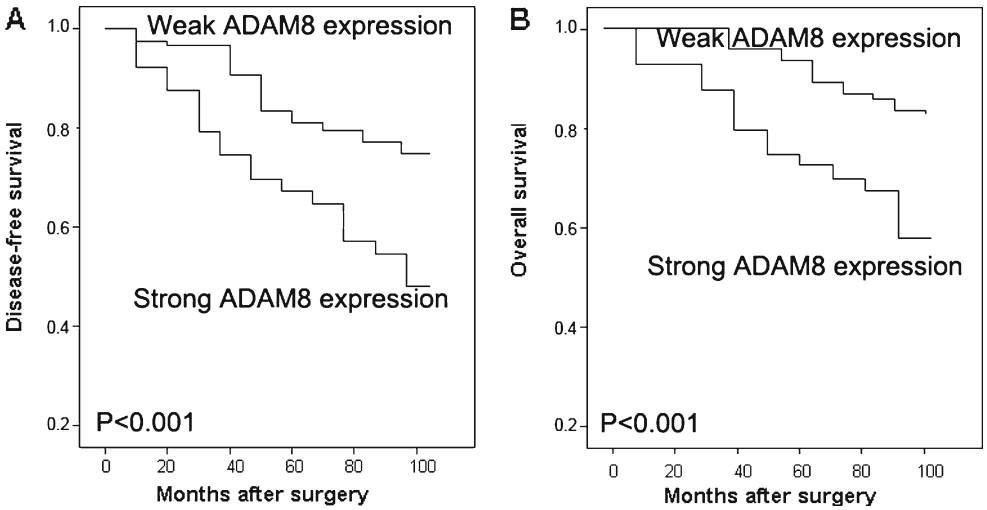


**Fig. 1** Disease-free survival (**a**) and overall survival (**b**) curves for two groups defined by weak (Grade I~II) and strong expression (Grade III~IV) of ADAM8 in patients with osteosarcoma. The patients with strong ADAM8 expression had a significantly worse 5-year overall and disease-free survival rate than those with weak ADAM8 staining (both $P<0.001$)

response to chemotherapy (both $P=0.03$), and without recurrence (both $P=0.01$) and metastasis ($P=0.008$ and 0.006, respectively) for OS and DFS.

Multivariate Cox regression analysis enrolling above-mentioned significant parameters revealed that ADAM8 staining score (RR 5.263, 95%CI, 1.812–10.292, $P<0.001$), tumor size (RR 3.621, 95%CI, 1.635–8.087, $P=0.01$), response to chemotherapy (RR 3.698, 95%CI, 1.828–8.291, $P=0.01$), recurrence status (RR 4.779, 95%CI, 1.923–9.875, $P=0.03$) and metastasis status (RR 4.996, 95%CI, 1.816–10.099, $P=0.02$) were independent prognostic markers for OS of patients with osteosarcoma (Table 2). Meanwhile, ADAM8 (RR 5.618, 95%CI, 1.956–11.518, $P<0.001$), recurrence status (RR 5.935, 95%CI, 1.576–11.817, $P<0.001$) and metastasis status (RR 4.928, 95%CI, 1.382–10.768, $P=0.006$) were independent prognostic markers for disease-free survival DFS of patients with osteosarcoma (Table 2).

## Discussion

ADAM8 was known to play a role in inflammatory processes and has already been found in various human carcinomas. It might be an oncogene operating in prostatic carcinogenesis, renal carcinogenesis and lung carcinogenesis [11–13]. However, until now, no investigation on ADAM8 expression in osteosarcoma patients has been performed. In this study, we investigated ADAM8 expression at protein level in 69 osteosarcoma patients, and found that 61 osteosarcomas had positive ADAM8 staining and 41 osteosarcomas had a high ADAM8 expression (grade III~IV), indicating that ADAM8 is up-regulated in human osteosarcoma. We also found that ADAM8 was significantly over-expressed in osteosarcoma patients with recurrence or metastasis. When regarding the relationship between ADAM8 and clinical outcome in 69 follow-up patients, we found a statistically significant correlation between ADAM8 expression and disease-free survival or overall survival by univariate analysis. Cox multivariate analysis confirmed that ADAM8 over-expression was an important predictor of poor prognosis in both disease-free survival and overall survival.

ADAM family of proteins implicated in cell-cell signaling, cell adhesion and cell migration. Their biological and pathological roles make them candidates for promoting tumor growth and malignancy. In the present study, our special interest is concentrated on ADAM8 because it has been demonstrated to take part in inflammatory bone loss. In addition, ADAM8 plays important roles in cellular processes, such as proliferation, adhesion, migration, and survival. ADAM8 expression is upregulated in particle induced arthritis, and Ainola and colleagues have suggested that ADAM8 may be associated with a variety of inflammatory

**Table 2** Multivariate survival analysis of OS and DFS in 69 patients with osteosarcoma

| Variables | OS | | | DFS | | |
|---|---|---|---|---|---|---|
| | RR | 95%CI | *P* | RR | 95%CI | *P* |
| Tumor size | 3.621 | 1.635–8.087 | 0.01 | 1.667 | 0.818–3.159 | 0.1 |
| Response to chemotherapy | 3.698 | 1.828–8.291 | 0.01 | 1.386 | 0.682–2.816 | 0.3 |
| Recurrence status | 4.779 | 1.923–9.875 | 0.03 | 5.935 | 1.576–11.817 | <0.001 |
| Metastasis status | 4.996 | 1.816–10.099 | 0.02 | 4.928 | 1.382–10.768 | 0.006 |
| ADAM8 expression | 5.263 | 1.812–10.292 | <0.001 | 5.618 | 1.956–11.518 | <0.001 |

arthritides and mediates the bone destruction in these conditions [14]. The structure of ADAM8 suggests that it possesses both proteolytic and adhesive functions. Choi et al. [15] reported that ADAM8 is expressed in osteoclasts and that it stimulates osteoclastogenesis in vitro. They further demonstrated that the disintegrin domain of ADAM8 is responsible for its effects on osteoclastogenesis and that ADAM8 binds to α9β1 on osteoclast precursors to induce osteoclast formation. Recent studies have suggested an important role for ADAM8 in pathologic bone destruction associated with inflammatory conditions such as rheumatoid arthritis or aseptic loosening of hip prostheses [16].

It is essential that the modulation of tissue microenvironment through degradation of the extracellular matrix, processing of growth factors and activation of cell adhesion molecules to cancer cell proliferation and progression [17]. Matrix metalloproteinases, as a family of zinc-dependent endopeptidases, are able to degrade virtually any component of the extracellular matrix. Matrix metalloproteinases are critical for remodeling the extracellular matrix, thereby affecting cell behavior under physiologic and pathophysiologic circumstances, such as embryogenesis and cancer progression. The elevated expression and activation of matrix metalloproteinases have been reported in many human cancer tissues [18]. The ADAM family, a matrix metalloproteinase-related family, has been considered as a new factors associated with the invasiveness of the malignant tumors. In this context, ADAM8 is of our particular interest. Correlation between ADAM8 expression and malignant progression as well as worse prognosis was observed in lung cancer [11], prostate carcinoma [13], renal carcinoma [12] and glioblastoma [19]. Valkovskaya et al. [20] found that ADAM8 was overexpressed in human pancreatic cancer and further reported that ADAM8 mRNA and ADAM8 protein levels were increased in pancreatic cancer cell lines under hypoxia compared to normal conditions of oxygenation, suggesting ADAM8 plays a potential role as a hypoxia-dependent protein in the pathogenesis and evolution of pancreatic cancer that is characterized by high level of intratumoral hypoxia [21]. With the similar results of these previous studies, our data indicated that upregulation of ADAM8 in osteosarcoma tissues is well correlated with tumor recurrence, metastasis and worse prognosis.

In conclusion, our findings suggest for the first time that ADAM8 might be applied as a novel marker for the prediction of recurrence and metastasis potency and a significant indicator of poor prognosis for patients with osteosarcoma.

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