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*Original Article*

# Expression of Zeb1 and Zeb2 indicates metastasis and unfavorable prognosis in osteosarcoma

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**Abstract:** Aim: To explore the expression and clinical significance of Zeb1 and Zeb2 in osteosarcoma (OS). Materials and methods: In our study, we detected the expression of Zeb1 and Zeb2 in 120 cases of OS tissues with immunohistochemistry, and divided the cohort into Zeb1 or Zeb2 high/low expression group according to the cut-off. Moreover, we evaluated the clinical significance of Zeb1/Zeb2 by analyzing the correlation between Zeb1/Zeb2 and clinicopathological parameters with Chi-square test. With univariate analysis and multivariate analysis, we evaluated the prognostic significance of Zeb1/Zeb2. Results: In our study, the percentage of Zeb1 high-expression group was 28.33% (34/120), and percentage of Zeb2 high-expression group was 31.67% (38/120). Both Zeb1 and Zeb2 high-expression were significantly associated with positive metastasis (P=0.006 and 0.008) and advanced Enneking stage (P=0.003 and 0.004). In univariate analysis, both Zeb1 (P=0.007) and Zeb2 high-expression (P=0.017) was proved to be significantly associated with poorer prognosis of OS. In multivariate analysis, Zeb1 high-expression, Zeb2 high-expression and positive metastasis were all identified as independent prognostic factors of OS. Conclusions: Both Zeb1 and Zeb2 expression were positively associated with metastasis, indicating the possibility that Zeb family could promote OS cell invasion and metastasis. Moreover, we identified Zeb1 high-expression and Zeb2 high-expression as independent prognostic factors of OS for the first time, which could trigger the interest of Zeb family as a potential molecular target in OS treatment.

**Keywords:** Zeb1, Zeb2, osteosarcoma, progression, prognosis

## Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor with high prevalence of early metastasis, especially lung [1]. The most vulnerable patients of OS are adolescents, especially from 15-19 years old [2]. The developing surgical methods and adjuvant therapy, especially chemotherapy, could significantly improve the 5-year survival rate of OS to approximately 65%-70% in case of non-metastasis [3, 4]. However, either local recurrence or distant metastasis after resection of OS is still the main threat to patients with OS. Moreover, chemotherapy based on multi-agent strategy is the main strategy to the patients with metastasis [5]. Therefore, effective biomarkers for OS diagnosis or prognosis are still in urgent need to reveal the mechanism of OS progression and indicate potential drug target, even help find new chemotherapy.

As the malignance originated from mesenchyme, the epithelial-to-mesenchymal transition (EMT) has a significant role in OS progression. EMT is a biological process of epithelial cells to gain the property of mesenchyme cells, including losing cell polarity, disassembling the cell-cell junction, increasing cell motility and gaining invasive properties. Lots of evidence proved that EMT could initiate cancer metastasis [6]. The feature of EMT is the loss of E-cadherin. Many factors such as Twist, Snail family and Zinc finger E-box-binding homeobox (ZEB) family are identified as inducers of EMT by suppressing E-cadherin directly or indirectly [7, 8]. The Zeb family (Zeb1 and Zeb2) is a kind of zinc finger transcription factors. As a transcriptional repressor, Zeb family could repress E-cadherin promoter and induce EMT by recruiting SMARCA4/BRG1, which eventually results in cellular polarity loss, remodeling of the basement membrane, cell migration and invasion

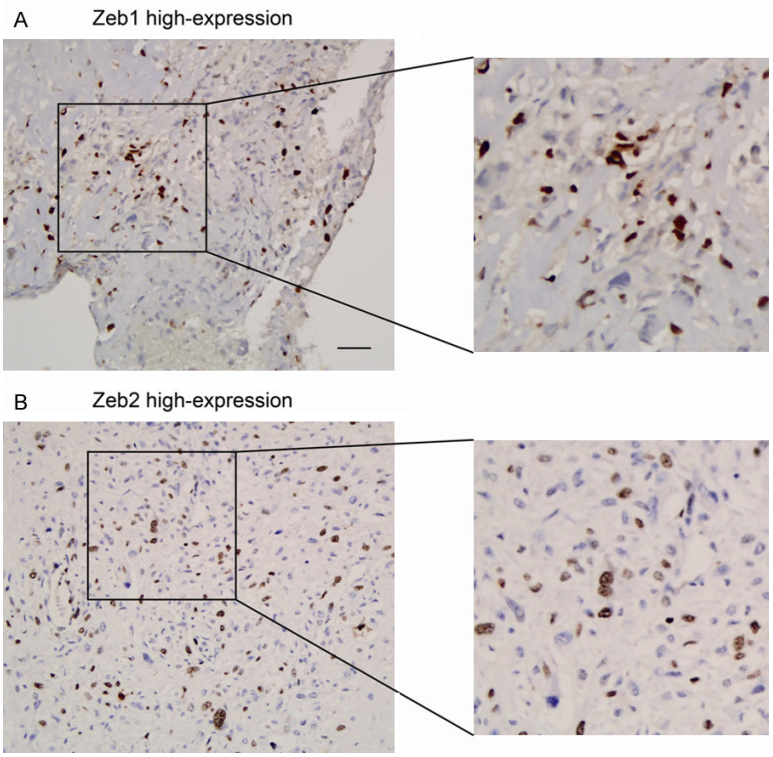


**Figure 1.** A: Representative immunohistochemical figures of Zeb1 high-expression. Scale bar: 50 μm; B: Representative immunohistochemical figure of Zeb2 high-expression.

[9, 10]. Previous study proved that Zeb1 relates to the metastasis and invasion in OS in a small scale of cases, without involving prognosis and underlying molecular mechanism [11]. Although more and more evidence demonstrated the significant role of Zeb family in tumorgenesis and progression as a promoter, the prognostic role of Zeb1/Zeb2 in OS is still unknown. In our study, we for the first time detected the expression of Zeb1/Zeb2 in OS with immunohistochemistry (IHC) and investigated the clinical significance and prognostic role of Zeb1/Zeb2 in patients with OS.

## Materials and methods

### *Patients and follow-ups*

A total of 225 patients underwent surgical treatment and were diagnosed as OS pathologically in Linyi People's Hospital, which consisted of the primary cohort. The validation cohort was selected from the primary cohort, containing 120 patients. The criteria of validation cohort selection were as follows (1) effective follow-ups more than 3 months; (2) standard adjuvant therapy; (3) available specimens for IHC. All tissue specimens were obtained with approval of the Ethics Broad of Linyi People's Hospital, and prior consent of the patients. Clinical stage of OS was according to the Enneking stage criteria [12]. The overall survival rate was calculated from the operation date to the date of death or the last follow-up.

### *Immunohistochemistry and evaluation*

All staining was performed with the streptavidin peroxidase complex method. Briefly, specimens were first incubated in xylene and graded alcohol for de-paraffinization and rehydration, subsequently followed by endogenous peroxidase inactivation with 3% hydrogen peroxide. After unspecific binding blockage with 5% bovine serum albumin, slides were incubated in rabbit polyclonal Zeb1 antibody (Sigma-Aldrich) at 1:200 dilution polyclonal anti-human Zeb2 antibody (Abcam) at 1:150 dilution overnight, then was the secondary antibody labeled with peroxidase. 3,3'-diaminobenzidine(DAB) was finally used for Zeb1/Zeb2 expression visualization.

The results of IHC were evaluated by two independent pathologists unaware of clinical information of patients. The final score of Zeb1/Zeb2 was evaluated by the percentage of positive cells according to the system reported previously [13]. The IHC scores were listed as: 0, no staining or staining in less than 1% of the tumor cells; 1, staining in 1% to 10% of the cells; 2, staining in 10% to 25% of the cells; 3, staining in 25% to 50% of the cells; 4, staining in 50% to 75% of the cells; and 5, staining in more than 75% of tumor cells. The cohort was divided into Zeb1/Zeb2 high-expression and low-expression group according to the cut-off of the IHC score. The cut-off was selected referring to previous study [14].

**Table 1.** Basic information of the cohort

| Characters | Number | Percentage |
|---|---|---|
| Gender | | |
| Female | 36 | 30.00% |
| Male | 84 | 70.00% |
| Age | | |
| <20 | 97 | 80.83% |
| ≥20 | 23 | 19.17% |
| Tumor size (cm) | | |
| <8 | 76 | 63.33% |
| ≥8 | 44 | 36.67% |
| Enneking stage | | |
| I | 13 | 10.83% |
| II | 75 | 62.50% |
| III | 32 | 26.67% |
| Site | | |
| Femur | 53 | 44.17% |
| Tibia | 29 | 24.17% |
| Humerus | 19 | 15.83% |
| Fibula | 11 | 9.17% |
| Others | 9 | 7.50% |
| Histopathology | | |
| Osteoblastic | 49 | 40.83% |
| Fibroblastic | 26 | 21.67% |
| Chondroblastic | 14 | 11.67% |
| Telangiectatic | 14 | 11.67% |
| Others | 17 | 14.17% |
| Metastasis | | |
| No | 87 | 72.50% |
| Yes | 33 | 27.50% |
| Response to chemotherapy | | |
| Good | 66 | 55.00% |
| Poor | 54 | 45.00% |
| Zeb1 | | |
| Low | 86 | 71.67% |
| High | 34 | 28.33% |
| Zeb2 | | |
| Low | 82 | 68.33% |
| High | 38 | 31.67% |

*Statistical analysis*

The correlation between Zeb1/Zeb2 and other clinicopathlogical factors was calculated with Chi-square test. Overall survival curve was displayed by the Kaplan-Meier method and the log-rank test was used to assess the statistical significance. Independent prognostic factor was identified with multivariate analysis using the Cox proportional hazards model. P<0.05 was considered as statistically significant without special instruction. All statistical analysis was carried out with software SPSS.

## Results

*Expression of Zeb1 and Zeb2 in OS tissues*

The expression and location of Zeb1 and Zeb2 were first detected with IHC in paraffin-bedded OS tissues. In our experiment, both Zeb1 and Zeb2 were mainly expressed in cell nucleus, which is corresponding to their function as transcription suppressors (**Figure 1**). As described in Materials and Methods, the cohort was divided into Zeb high-expression group and low-expression group with the cut-off. In our study, the percentage of Zeb1 high-expression group was 28.33% (34/120), and the percentage of Zeb2 high-expression group was 31.67% (38/120) (**Table 1**).

*Clinicopathological factors and their relations with Zeb1 and Zeb2 expression*

The correlation between clinicopathological parameters and Zeb1/Zeb2 expression was analyzed with Chi-square test to screen the possible factor which was involved with Zeb1/Zeb2 expression (**Table 2**). In our cohort, Zeb1 overexpression was significantly associated with positive metastasis (P=0.006), indicating that Zeb1 expression may promote the metastasis of OS cells. Moreover, Zeb1 expression was related to Enneking stage (P=0.003), partially because that metastasis is one important parameter for OS Enneking stage. Similarly, Zeb2 overexpression appeared to be also associated with positive metastasis (P=0.008) and more advanced Enneking stage (P=0.004). These phenomena indicated that Zeb1/Zeb2 may promote the OS progression such as metastasis, which may be related with the function of Zeb1/Zeb2 for inducing EMT.

*Prognostic value of Zeb1/Zeb2 in OS*

The prognostic significance of Zeb2 was evaluated with univariate analysis and confirmed with multivariate analysis. Kaplan-Meier methods and log-rank test were first performed to find the prognostic factors (**Table 3**). Both Zeb1 high-expression (P=0.007) and Zeb2 high-expression (P=0.017) were demonstrated to be significantly related with unfavorable prognosis

**Table 2.** The correlation between Zeb1/Zeb2 expression and clinicopathological factors

| Characters | ZEB1 | | P* | ZEB2 | | P* |
|---|---|---|---|---|---|---|
| | Low | High | | Low | High | |
| Gender | | | | | | |
| Female | 23 | 13 | 0.270 | 23 | 13 | 0.525 |
| Male | 63 | 21 | | 59 | 25 | |
| Age | | | | | | |
| <20 | 68 | 29 | 0.608 | 66 | 31 | 1.000 |
| ≥20 | 18 | 5 | | 16 | 7 | |
| Tumor size (cm) | | | | | | |
| <8 | 56 | 20 | 0.535 | 49 | 27 | 0.309 |
| ≥8 | 30 | 14 | | 33 | 11 | |
| Enneking stage | | | | | | |
| I | 12 | 1 | 0.003 | 12 | 1 | 0.004 |
| II | 58 | 17 | | 55 | 20 | |
| III | 16 | 16 | | 15 | 17 | |
| Site | | | | | | |
| Femur | 38 | 14 | 0.991 | 34 | 18 | 0.434 |
| Tibia | 21 | 8 | | 22 | 7 | |
| Humerus | 13 | 6 | | 11 | 8 | |
| Fibula | 8 | 3 | | 7 | 4 | |
| Others | 6 | 3 | | 8 | 1 | |
| Histopathology | | | | | | |
| Osteoblastic | 31 | 18 | 0.208 | 34 | 15 | 0.929 |
| Fibroblastic | 23 | 3 | | 18 | 8 | |
| Chondroblastic | 9 | 5 | | 10 | 4 | |
| Telangiectatic | 10 | 4 | | 10 | 4 | |
| Others | 13 | 4 | | 10 | 7 | |
| Metastasis | | | | | | |
| No | 69 | 18 | 0.006 | 66 | 21 | 0.008 |
| Yes | 17 | 16 | | 16 | 17 | |
| Response to chemotherapy | | | | | | |
| Poor | 49 | 17 | 0.544 | 45 | 21 | 1.000 |
| Good | 37 | 17 | | 37 | 17 | |
| Zeb2 | | | | | | |
| Low | 61 | 21 | 0.386 | - | - | - |
| High | 25 | 13 | | - | - | |

*means calculated with Chi-square test.

in our test (**Figure 2**). Moreover, the Enneking stage (P=0.003), metastasis status (P<0.001) and response to chemotherapy (P=0.004) were identified as prognostic indicators for poor prognosis.

*Independent prognostic factors in OS*

All the prognostic factors identified in univariate analysis were enrolled in multivariate analysis for further identification of independent prognostic factors in OS survival. Cox-regression model enrolled these factors including Enneking stage, metastasis status, response to chemotherapy and Zeb2 expression (**Table 4**). In our test, both Zeb1 high-expression (P=0.038, HR=2.19, 95% CI= 1.04-4.60) and Zeb2 high-expression (P=0.049, HR=2.10, 95% CI=1.00-4.60) were identified as risk for unfavorable prognosis in OS. Additionally, positive metastasis (P=0.039, HR=9.08, 95% CI=1.11-74.1) was also confirmed as an independent prognostic factor of OS in our study. Poor response to chemotherapy tended to be an independent factor for prognosis, with a statistically insignificant tendency (P=0.058).

## Discussion

The exact mechanism of the carcinogenesis and progression of OS remain unknown. Previous studies proved that the risk of OS includes age, sex, genetic and familial factors [15]. However, it has been well acknowledged that EMT also plays an important role in OS progression. The essential proteins involved in EMT include E-cadherin, N-cadherin, Snail, twist and Zeb family, etc. Among these proteins, the function of Zeb family in EMT was confirmed as a suppressor of E-cadherin, inhibiting the transcription of E-cadherin. The dysfunction or ectopic expression of Zeb1/Zeb2 was observed in many kinds of cancers except OS, such as lung cancer, hepatocellular carcinoma, glioma, etc [16-18]. In OS, Twist overexpression and E-cadherin down-expression were reported before [19]. However, as an important regulator of E-cadherin, the clinical significance of Zeb family was not elucidated in OS.

In our study, we demonstrated that expression of Zeb1/Zeb2 was significantly associated with OS metastasis and Enneking stage. This result indicates the possibility that Zeb family could increase cancer cell invasion/migration

**Table 3.** Univariate analysis of Zeb1/Zeb2

| Characters | Average survival time (months) | 5-year survival rate | P* |
|---|---|---|---|
| Gender | | | |
| Female | 63.3 | 58.0 | 0.660 |
| Male | 66.8 | 50.8 | |
| Age | | | |
| <20 | 68.9 | 56.1 | 0.538 |
| ≥20 | 58.5 | 38.7 | |
| Tumor size (cm) | | | |
| <8 | 73.7 | 62.3 | 0.831 |
| ≥8 | 56.8 | 41.1 | |
| Enneking stage | | | |
| I | 44.7 | 66.7 | 0.003 |
| II | 72.4 | 55.4 | |
| III | 29.6 | 45.6 | |
| Site | | | |
| Femur | 68.5 | 56.3 | 0.759 |
| Tibia | 48.0 | 77.5 | |
| Humerus | 62.8 | 46.0 | |
| Fibula | 35.7 | 31.3 | |
| Others | 35.4 | 0 | |
| Histopathology | | | |
| Osteoblastic | 45.0 | 36.3 | 0.228 |
| Fibroblastic | 53.3 | 65.1 | |
| Chondroblastic | 74.1 | 75.0 | |
| Telangiectatic | 35.4 | 34.5 | |
| Others | 87.9 | 15.1 | |
| Metastasis | | | |
| No | 73.4 | 56.8 | <0.001 |
| Yes | 28.5 | 41.4 | |
| Response to chemotherapy | | | |
| Poor | 53.4 | 34.7 | 0.004 |
| Good | 82.1 | 71.2 | |
| ZEB1 | | | |
| Low | 74.1 | 59.7 | 0.007 |
| High | 37.2 | 25.0 | |
| ZEB2 | | | |
| Low | 74.8 | 60.6 | 0.017 |
| High | 46.1 | 30.7 | |

*means calculated with Log-rank test.

and even promote OS metastasis. To some extent, the function assay of Zeb1 in OS cell lines was investigated in previous study [13]. By regulating Zeb1 expression with siRNA transfection, Shen et al. proved that Zeb1 overexpression could inhibit the E-cadherin expression and promote cell invasion in OS [13]. It is well known that Zeb1 could suppress E-cadherin expression and induce EMT via recruiting the SWI/SNF chromatin-remodeling protein BRG1 [10], and several downstream proteins regulated by Zeb family were identified such as the BMP-inhibitors NOG, FST and CHRDL1 [20]. However, the exact singling pathway and molecular mechanism of Zeb1/Zeb2 promoting progression in OS cell lines still need more experiments to explore. Since there is no available antagonist of Zeb1/Zeb2, more precise underlying signaling pathway could help find the effective drug for blocking Zeb1/Zeb2 function, therefore inhibiting the EMT of OS. Additionally, some micro-RNAs like miR-139-5p may suppress cancer progression via suppressing Zeb1 and Zeb2 in OS [17]. Overall, it is a potential and promising strategy to suppress OS progression via inhibiting Zeb1/Zeb2 signaling with the fact that EMT is essential to OS progression.

Based on our detection of 120 specimens of OS tissues, we demonstrated that both Zeb1 and Zeb2 expression were significantly associated with OS metastasis and Enneking stage. With univariate analysis, Zeb1 and Zeb2 were proved to be related to poorer prognosis of OS. With multivariate analysis, we identified Zeb1 and Zeb2 as independent prognostic factors of OS. We hope our finding of Zeb1/Zeb2 clinical significance may help incite more interest on OS biomarkers, thus finding new and effective chemotherapy for patients with OS.

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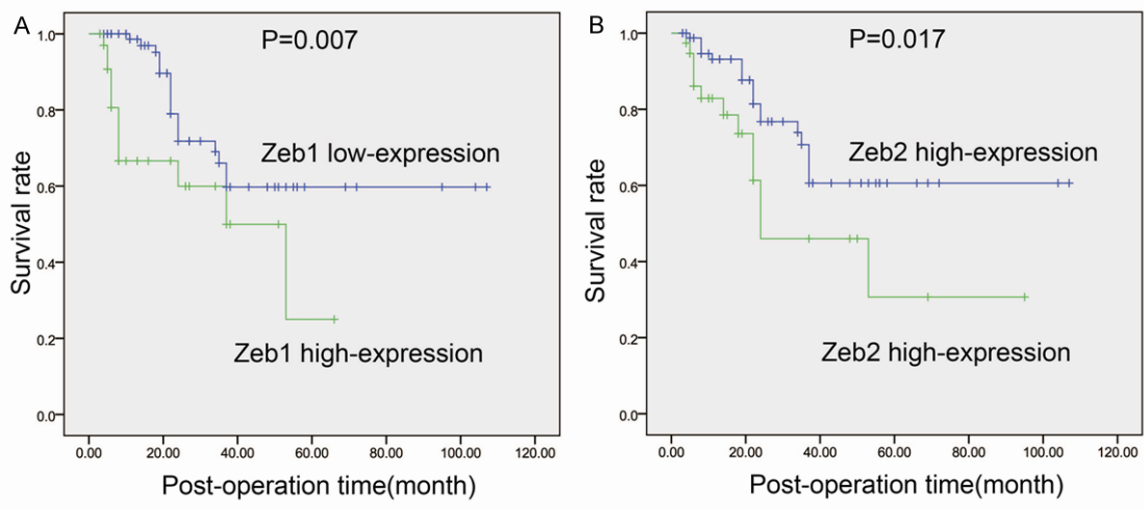


**Figure 2.** A: The overall survival curve of Zeb1 low-expression and high-expression. B: The overall survival curve of Zeb2 low-expression and high-expression.

**Table 4.** Multivariate analysis

| Characters | HR | 95% CI | P* |
|---|---|---|---|
| Enneking stage | | | |
| I st | 1 | | |
| II | 3.84 | 0.44-33.1 | 0.221 |
| Metastasis | | | |
| No | 1 | | |
| Yes | 9.08 | 1.11-74.1 | 0.039 |
| Response to chemotherapy | | | |
| Good | 1 | | |
| Poor | 2.1 | 0.98-4.51 | 0.058 |
| ZEB1 | | | |
| Low | 1 | | |
| High | 2.19 | 1.04-4.6 | 0.038 |
| ZEB2 | | | |
| Low | 1 | | |
| High | 2.1 | 1.0-4.6 | 0.049 |

*means calculated with Cox-regression hazard model.

## Disclosure of conflict of interest

None.

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