CLINICAL REPORT

Expression of Ezrin correlates with lung metastasis in Chinese patients with osteosarcoma

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Abstract

Purpose: To determine the prognostic value of the expression of Ezrin, CD44 and Six1 genes in osteosarcoma tissues of Chinese patients.

Methods: Fluorescent quantitative real-time PCR was applied to study the mRNA levels of Ezrin, CD44 and Six1 genes in 32 osteosarcoma patient samples and 10 adjacent normal tissues and MG63 osteosarcoma cell lines. The analysis of relationships between pulmonary metastasis and overall survival time were carried out based on the clinical data.

Results: mRNA levels of Ezrin and Six1 genes in osteosarcoma tissues were higher than those in adjacent normal tissues ($P=0.015$, 0.025). The mRNA levels of Ezrin, CD44 and Six1 genes were closely correlated with Enneking GTM clinical staging, while no correlations were demonstrated between the mRNA level of these genes with sex, age, location or pathological types. In addition, we demonstrated that the high mRNA level of Ezrin gene was related to shorter lung metastasis-free and overall survival time of the Chinese patients with osteosarcoma ($P<0.001$).

Conclusion: Our data suggest that Ezrin, but not CD44 and Six1, could be a prognostic factor and a predictor of potential lung metastasis in osteosarcoma. Further large sample studies need to be done to confirm the potential value of Ezrin as a new therapeutic target.

Osteosarcoma is the most common malignant primary bone tumour with high capacity of distant metastasis.

Lungs and bones are the common sites for metastases. About 20% of patients present visible metastases with imaging at diagnosis and about 25% patients have metastases during the course of treatment.¹ Once the metastases, especially pulmonary metastases, occur it is difficult to treat them due to their poor prognosis. Currently, the characteristics of high metastasis in osteosarcoma cell lines remain unclear. Therefore, it is necessary to discover new indicators to assess the metastasis and prognosis in clinic.

Recent studies have shown that ERM protein family members (Ezrin, Radixin and Moesin) play vital roles in the occurrence and progression of tumours. They can regulate cell activities in adhesion, migration and penetration and act as the link of cell membranes to cytoskeleton.²⁻⁴ CD44, a cell adhesion molecule, was shown to play an important role in the process of invasion and metastasis of tumour cells, while the investigators found that Ezrin might have a synergistic effect with CD44 on the progression of tumours.³ Thus, the prognosis of sub-group with CD44 and Ezrin high expression should be analyzed and discussed. Homology domain transcription factor Six1, a material needed in the development of normal cells is often abnormal in the process of tumour occur-

rence and may have a synergistic effect with Ezrin proteins in the process of tumour metastasis.⁵

The objectives of this study were to find the differences of mRNA expression levels in Ezrin, CD44 and Six1 genes in Chinese patients with osteosarcoma and to identify the relationships between the expression levels of the three genes and lung metastasis-free and overall survival time, hopefully providing the basis for clinical assessment of metastasis and prognosis.

Materials and methods

Samples Collection and Clinical Data

All patients involved in this study had signed the informed consent. All osteosarcoma samples were collected from patients undergoing surgical resection in the Orthopaedic Department of the Sixth People's Hospital affiliated to Shanghai Jiao Tong University between February 2003 and March 2007. Thirty-two cases of tumour tissues and 10 normal tissues (above 5cm from the surgical margin of tumour tissues) were collected. Resection samples were placed into a liquid nitrogen jar immediately after they were washed and then transferred to an ultra-low-temperature freezer of -80°C for long-term preservation. All samples were confirmed to have a pathological diagnosis of osteosarcoma. MG63 osteosarcoma cell lines were purchased from Shanghai Institute of Biological Cells, CAS. Patients' medical charts were reviewed retrospectively to collect the clinical information relevant for the analysis.

All patients had received the adjuvant chemotherapy with the improved Ferrari protocol⁵ including methotrexate (MTX), ifosfamide (IFO), doxorubicin (ADM) and cisplatin (CDP). Each cycle of chemotherapy was done as follows: MTX was administered as a 4-hour infusion (10 g/m²) with leucovorin (folinic acid) rescue (8 mg/m²) every 6 hr, beginning 12 hr from the start of the MTX infusion. CDP was delivered at a dose of 100 mg/m² during 48 hr as a continuous intravenous infusion, and was followed by ADM

TABLE 1. Clinical characteristics of the osteosarcoma patients

Age at diagnosis (median), y	18 (7-67)
Sex	
Female/male	20/12
Duration of follow-up (median), mo	22 (11-51)
Site of primary disease [n]	
Tibia	9
Femur	14
Humerus	6
Other	3
Histologic subtype [n]	
General	28
Special	4
Enneking GTM	
IIB	29
III	3
Lung metastatic status at diagnosis [n]	
Nonmetastatic	29
Metastatic	3
Survival [n]	
Alive	20
Dead	12

Total number of patients: 32

75 mg/m² administered as a 24-hr continuous infusion. IFO, in combination with an equimolar dose of mesna, was delivered as a continuous intravenous infusion at a dose of 3 g/m²/d for 3 consecutive days.

All drugs were administered at the same preoperative dose during postoperative chemotherapy. Two cycles of preoperative neoadjuvant chemotherapy together with 3-5 cycles of routine postoperative adjuvant chemotherapy were given. Follow-up time was 11-51 months, with an average of 25.75 months and the median of 22 months. (Table 1 for details).

RNA extraction and reverse transcriptase

Total RNA was extracted from the homogenate of 50 mg fresh tissues in strict accordance with the instructions on RNA Extraction Kit (Invitrogen). The D260nm / D280nm measured with Spectrophotometer should be between 1.8 and 2.1. Then, SuperScript III First-Strand Synthesis System Kit (TaKaRa) was used

TABLE 2. Primers

Gene	Primer	Tm (°C)
Ezrin-F	5'TAGAGGCTGACCGTATGGCT 3'	57.4
Ezrin-R	5'TGTGCTGCCACTCTTCAACTT 3'	58.2
CD44-F	5'AATGACAACGCAGCAGAGTAA 3'	56.1
CD44-R	5'CCTTCGTGTGTGGGTAATGA 3'	56.3
SIX1-F	5'CACCACCACCCAGGTCAG 3'	56.5
SIX1-R	5'GCAGCAGAAGGACCGAGTT 3'	57.5
βactin-F	5'CCTGGCACCCAGCACAAAT 3'	56.5
βactinR	5'GCTGATCCACATCTGCTGGAA 3'	56.5

to synthesize cDNA. Parallel pipe without addition of RT enzyme could be used as the control. The prepared cDNA was preserved in an ultra-low-temperature freezer at -80 °C.

Real-time quantitative fluorescence PCR

The primers were designed according to the sequences of Ezrin, CD44, Six1 and housekeeping gene, β-actin in GeneBank (Table 2 for gene primers). The primers were synthesized by the Shanghai Invitrogen company. ABI 7500 fluorescence quantitative PCR was used. The standard plasmid Ezrin, CD44, Six1 and standard housekeeping genes β-actin were diluted in four concentrations. The reaction system was added with 12.5 μ L SYBR Premix Ex Taq (2 ×), 0.5 μ L of 10 μmol/L downstream and upstream primers, 0.5 μ L Rox reference dye, 1 μ L Template DNA and 10 μ L sterile water. Reaction process: polymerase was activated at 50 °C, 94 °C for 2 min, respectively. Amplified with PCR for 40 cycles (10 sec at 94 °C, 40sec 60 °C), Then the reaction completed. Each sample was made with three holes and then the average value was adopted. Blank parallel pipe was used for negative control.

Real-time quantitative fluorescence PCR data processing

Data (CT value: the number of cycles) was processed using 2-ΔΔCT method.⁶ Statistical analysis was carried out after the relative expression of target genes

compared with housekeeping gene (β-actin) was obtained.

Immunohistochemistry (IHC)

The primary antibodies and dilutions used in our studies were: anti-Ezrin (Ab-1, Oncogene), anti-Six 1 (Ab-1,). Both antibodies were diluted at 1:5000. Five-μm -thick sections were cut and mounted on Super-Frost Plus slides. A case was considered positive if >10% of cells were stained. Immunostaining was analyzed with an Olympus Corp. BX-50 microscope, and pictures were taken with an Olympus Corp. Oly 760 video camera. The staining results were read by a group of senior pathologists. In each case, 2,000 tumour cells were counted from 10 randomly selected areas, ensuring that the whole section was scanned.

Statistical Analysis

Both real-time RT-PCR and IHC were used for investigate the Ezrin and Six 1 genes expression. MG63 cell was set as control. One case was considered as high expression of Ezrin (or Six 1) when the arbitrary expression unit of this gene was higher than that in MG63 cell. The log-rank test was used to analyze the relationship between the target gene expression, lung metastasis-free time and overall survival time. The correlation between gene expression and clinical data was analyzed with t-test. The correlation of target genes was tested by Pearson Correlation. Kaplan-Meier survival curves were used to compare lung metastasis-free time and overall survival time. The above analyses were conducted with SPSS 10.0 statistical software. $P < 0.05$ was considered statistically significant.

Results

The expression of Ezrin gene in osteosarcoma and normal tissues

Samples of 32 patients with osteosarcoma were studied for Ezrin gene expression, including 29 cases in

TABLE 3. Relationship between expression of Ezrin, CD44 and that of Six1

Ezrin	N	CD44		Six1	
		Low expression	High expression	Low expression	High expression
Low expression	25	19	6	20	5
High expression	7	0	7	0	7

Phase IIB and 3 in Phase III. Both real-time RT-PCR and IHC were used for investigate the gene expression. There were 6 cases without expression of Ezrin gene (Table 3). Accordingly, the Ezrin gene-positive expression rate was 81.3% (M= 0.0085, SD =0.0075). High expression of Ezrin gene was found in all 3 patients in Stage III. Low expression of Ezrin gene was found in all normal tissues. Ezrin gene expression level in osteosarcoma tissues was significantly higher than that in normal tissues. The difference showed statistically significance. ($P = 0.015$, Figure 1, Figure 2A, 2B)

Expression of Six1 gene in osteosarcoma and normal tissue.s

Among the 32 cases in phase IIB, 8 cases did not express Six1 gene. The Six1 gene positive expression rate was 75.0% (M=0.0071, SD=0.0053). High

expression of Six 1 gene was found in all 3 patients in Stage III. Low expression of Six 1 gene was found in all normal tissues. Six1 gene expression level in osteosarcoma tissues was significantly higher than that in normal tissues. The difference showed statistically significance. ($P = 0.025$, Figure 2C, 2D and Figure 3).

Expression of CD44 gene in osteosarcoma and normal tissues

Among the 32 cases in phase IIB, 7 cases did not express CD44 gene (Table 3). The CD44 gene positive expression rate was 78.1% (M=0.0067, SD=0.0056). The CD44 gene expression values of the 3 patients in Stage III were 0.0122, 0.0169 and 0.0217. The MG63 CD44 gene expression was 0.00692. CD44 gene expression level in osteosarcoma tissues was higher than that in normal tissues, but the difference was not statistically significance ($P = 0.068$, Figure 4).

Relationship between Ezrin, Six1 and CD44 gene expressions and clinical data of patients with osteosarcoma

The expressions of Ezrin, Six1 and CD44 genes was not related to the patients data in terms of sex, age, tumour primary site. The expression of Ezrin, Six1 and CD44 genes in patients in phase III were 3.11, 2.55 and 3.10 times higher than those in patients in Phase IIB.

Relationship between expressions of Ezrin, Six1 and CD44 genes and lung metastasis-free time

The 32 patients with osteosarcoma were divided into two groups according to the expression of Ezrin (compared with MG63), high expression group (> 0.01024) and low expression group(≤ 0.01024). In high expression group, there were 7 patients with median lung metastasis-free time of 6.0 months, while in 25 lower expression group, data was 18.0 months. The results showed that the lung metastasis-free time in the group with high expression of Ezrin was shorter

FIGURE 1. Ezrin in osteosarcoma patient samples without lung metastasis (A); with lung metastases (B) and Normal (C) tissue compared with control (MG63; median, range).

FIGURE 2. Low (A) and High (B) Ezrin and Low (C) and High (D) Six1 expression in osteosarcoma tissue.

than that in lower expression group (hazard ratio: 3.52, Fig.5). Among the 32 patients with osteosarcoma, Six1 gene was highly expressed in 8 cases and low expressed in 24 cases. The median lung metastasis-free time of the two groups with high and low expression of Six1 gene were 14.5 and 17.5 months, which presented no statistically significant difference (hazard ratio: 0.974,). According to the expression of CD44 gene in MG63 cell line, among the 32 cases with osteosarcoma, there were 14 cases of high expression and 18 of low expression with the median lung metastasis-free time of 14.0 and 18.0 months respectively. The lung metastasis-free time of low expression group was longer than that of high expression group (hazard ratio: 2.68, Fig. 5 A). There were 7 cases with both Ezrin and CD44 high expression whose median lung metastasis-free time was 8.0 months. 18 cases with low expression had median lung metastasis-free time of 26.0 months. The lung metastasis-free time of patients with Ezrin and CD44 high expression was shorter than those with low Ezrin and CD44 expression (hazard ratio: 4.21, Fig. 5 B). The median lung metastasis-free time of patients with both Ezrin and Six1 high expression was 8.0 months, while those with low Ezrin and Six1

FIGURE 3. Relative Expression of six1 in osteosarcoma patient samples without lung metastasis (A); with lung metastasis (B) and normal tissue (C) compared with control.

FIGURE 4. Relative expression of CD44 in in osteosarcoma patient samples without lung metastasis (A); with lung metastasis (B) and normal tissue (C) compared with control.

expression was 26.0 months. The lung metastasis-free time of patients with high expression of Ezrin and Six1 was shorter than those with low Ezrin and Six1 expression (hazard ratio: 5.37, Fig. 5C)

Relationship between expressions of Ezrin, Six1 and CD44 genes and overall survival (OS) time of the patients with osteosarcoma

Six patients died in the Ezrin highly expressed group when the follow-up ended. The median survival time

FIGURE 5. Kaplan-Meier Lung metastasis-free curve according to Ezrin expression (A); both Ezrin and CD44 expression(B) and both Ezrin and Six1 expression(C).

FIGURE 6. Kaplan-Meier overall survival curve according to Ezrin expression (A); both Ezrin and CD44 expression(B). and both Ezrin and Six1 expression(C).

was 15 months. Also, 6 patients died in low Ezrin expression group with the median survival time of 27 months, which showed that the OS survival time in low Ezrin expression group was longer than that in high Ezrin expression group ($P < 0.001$, Fig 6A).

However, the expression of Six1 and CD44 genes was not significantly related with OS time. The median OS time of patients with both Ezrin and CD44 high expression was 15.0 months, while those with low Ezrin and CD44 expression was 31.0 months ($P < 0.001$, Fig. 6 B). The median OS time of patients with both Ezrin and Six1 high expression was 15.0 months, while those with low expression of Ezrin and Six1 was 31.0 months. The OS time of high expression group was also shorter than that of low expression group ($P < 0.001$, Fig. 6 C).

Discussion

In this study, the Ezrin gene expression level in 32 Chinese patients with osteosarcoma was studied. Only 6 cases did not express the Ezrin gene. The positive expression rate of Ezrin gene reached 81.3%. The expression level of Ezrin gene in phase III patients was about 3 times higher than that in phase IIB. Ezrin expression in osteosarcoma was higher than that in the adjacent normal tissues ($P = 0.015$). Osteosarcoma cell line MG63 is very invasive and has high carcinogenicity. It is, therefore, usually applied to establish metastatic osteosarcoma animal model.^{7,8} Ezrin gene expression level of MG63 cell lines as the baseline, 32 patients with osteosarcoma in this study were divided into high expression group and low expression group (six cases without expression of the Ezrin gene were included into low expression group).⁹ The results showed that lung metastasis-free time and overall survival time in the low expression group were longer than those in the high expression group. However, larger samples are needed. Due to inadequate follow-up time, extension of follow-up time might be needed to ensure more reliable conclusions.

There is considerable current research interest in the roles of ERM protein family members, especially Ezrin protein, in the occurrence and development of tumours. In this experiment, expression of Ezrin, Six1 and CD44 genes in tumour tissues of Chinese patients with osteosarcoma was studied for the first time and the results showed that Ezrin gene was highly expressed. The expression level was correlated with the pulmonary metastases-free time and the overall survival time. Ezrin is a member of the ERM (Ezrin–radixin–moesin) cytoskeleton-associated protein family.¹⁰ ERM proteins have been implicated in a wide variety of important cellular complexes and processes¹¹, such as the formation of specialized cell surface domains and contribution in the localization of molecules into these structures.¹² Ezrin is known to be a component of cell-surface structures involved in cell adhesion to the extracellular matrix.^{3,13} Ezrin interacts directly with the cytoplasmic tail of the CD44 gene product, hyaluronic-acid receptor. The latter is thought to play a role in the cell migration of invasive tumours.³ In addition, Ezrin had been shown to be associated with the product of the Met gene¹⁴, which has previously been implicated in the progression of many cancers.¹⁵ Previous studies showed that Ezrin interacted with signal transduction mechanisms.^{16,17} With over expression of Ezrin, the normal balance of the cellular signaling network is disrupted. The importance of Ezrin in the metastasis of cancers, however, is seldom mentioned. Some studies showed that high expression of Ezrin might be correlated with the invasion of different tumours.¹⁸⁻²² However, some investigators suggested that Ezrin gene might be a tumour suppressor, inhibiting tumour development. Others proposed that the location of Ezrin protein in cells migrated with the occurrence and development of tumour, but the total amount of Ezrin protein showed no change. The positioning of different cells might be correlated with tumour differentiation and patient prognosis.^{23,24} Therefore, Ezrin might play different roles in different tumours, but the mechanism and their roles in the tumour remained unknown

The expression of Ezrin protein in patients with osteosarcoma at phase IIB is closely correlated with prognosis and overall survival time.²⁵ Park²⁶ found that Ezrin in advanced osteosarcoma samples was higher than that in low-phase. Another investigation by Ferrari²⁷ indicated that patients with Ezrin expressed in membrane and cytoplasm might have lower event free survival (EFS) than that in those patients with Ezrin only expressed in cytoplasm. However, as osteosarcoma paraffin-embedded specimens had relatively higher calcium content, decalcification would be required when immunohistochemistry experiments were conducted and, during decalcification, some antigens might be lost. The results of the experiments might be less reliable than those by fresh tissues.

CD44 adheres to the surface of cell membranes. Osteosarcoma patients with high expression of CD44 gene were more prone to have metastases than those with low expression, but CD44 gene expression level was not related with patient overall survival time. The investigators believed that both Ezrin protein and CD44 molecules were involved in tumour metastasis, and had synergies. Ezrin protein can have a direct effect on the cytoplasm of cell matrix hyaluronic acid receptor CD44 molecules, while over-expression of CD44 molecules can activate the function of the connecting protein between membrane and cytoskeleton, thereby enhancing the tumour cell metastasis.³

Homology domain transcription factor Six1, was considered to be a factor playing an important role in the process of muscle stem cell migration and promotes metastasis in the mice model of children with rhabdomyosarcoma.²⁸ In 2004, the cDNA microarray method was used by Yu et. al²⁹ to screen the Six1 and Ezrin genes that were closely correlated with rhabdomyosarcoma metastasis from the rhabdomyosarcoma cells with high and low metastatic potential. Subsequently, they found that Six1 could activate cyclin D1, c-Myc and Ezrin proteins, as well as regulating cellular proliferation, migration, apoptosis etc. Ezrin protein was an essential element for the Six1 transcription factor to induce the migration of tumour cells. So they

considered Six1 was a key factor that regulated Ezrin expression upstream. In our study, the expression of Six1 gene in osteosarcoma was higher than that in adjacent normal tissues.

Although Six1 and CD44 genes have less influence on lung metastasis-free time and overall survival than Ezrin a high correlation with the Ezrin gene was demonstrated in this experiment.. The lung metastasis-free and OS time of the high expression group was shorter than that of the low expression group. Our results are consistent with those by Martin and Yu, which once again indicated that Ezrin gene might have synergic effect with Six1 or CD44 gene in the course of tumour metastasis. The detection of these three genes may provide more valuable references for clinical practitioners to assess the prognosis of patients.

In conclusion, these data suggest that Ezrin is a good indicator to predict pulmonary metastases of the patients with osteosarcoma. Simultaneous determination of Six1, Ezrin and CD44 genes may provide greater clinical significance. In our study, postoperative samples were detected with IHC and Real-time PCR. The latter might be a simple and fast way to assess the lung metastasis and prognosis of the patients with osteosarcoma if it could be used to detect the Ezrin mRNA expression level in biopsy tissues rapidly. Ezrin gene may become the new target for future treatment of osteosarcoma, but a large and more uniform group of osteosarcoma samples are needed.

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