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Clinical significance of long non-coding RNA EWSAT1 as a novel prognostic biomarker in osteosarcoma

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Abstract. – OBJECTIVE: Long non-coding RNA EWSAT1 (EWSAT1) has been identified as a tumor promoter in several tumors, but its prognostic values in osteosarcoma have not been reported. The purpose of this study was to explore the association between EWSAT1 expression and prognosis of osteosarcoma patients.

PATIENTS AND METHODS: EWSAT1 levels were examined in 176 osteosarcoma tissues and matched normal bone tissues by qRT-PCR analysis. The associations of EWSAT1 expression with clinicopathologic variables were analyzed. The survival curves were calculated by the Kaplan-Meier method. The Cox proportional hazards regression model was used to identify independent prognostic factors with independent prognostic for overall survival (OS) and disease-free survival (DFS).

RESULTS: We found that EWSAT1 levels were significantly higher in osteosarcoma tissues compared with matched non-cancerous tissues (p<0.01). The level of EWSAT1 expression was significantly associated with clinical stage (p=0.001) and distant metastasis (p=0.011). Then, Kaplan-Meier analysis showed that high EWSAT1 expression level was associated with poorer OS (p=0.0007) and DFS (p=0.0010). Furthermore, Cox multivariate analyses demonstrated that EWSAT1 expression was an independent prognostic factor for both OS (p<0.001) and DFS (p=0.001) in osteosarcoma patients

CONCLUSIONS: Increased EWSAT1 expression was associated with poor outcomes in osteosarcoma patients, and EWSAT1 could serve as a potential unfavorable prognostic biomarker.

Key Words:
Long non-coding RNA, EWSAT1, Prognosis.

Introduction

Osteosarcoma is the most frequently occurring primary malignant bone tumor and a contributor to the tumor mortality in children and adolescents[1]. It has been confirmed that osteosarcoma arises most often in the metaphysis of long bones[2]. Despite progress in therapeutic technologies, such as surgery, radiotherapy, and adjuvant chemotherapy, the outcome for patients with metastatic osteosarcoma is still unfavorable with a 5-year survival rate at only 5 to 15%[3,4]. Therefore, early diagnosis and prognostic evaluation of osteosarcoma are necessary for more effective therapeutic strategies.

Long non-coding RNAs (LncRNAs) refer to the kind of RNA with over 200 nucleotides in length and lack protein-coding ability[5]. Growing evidence supports that lncRNAs play an important role in regulating diverse biological processes such as differentiation, proliferation, and metastasis[6]. Aberrant lncRNAs have been shown to be involved in the development and progression of various tumor[7,8]. Indeed, previous studies have reported that lncRNAs can function as oncogenes or tumor suppressors during cancer progression. For instance, Wu et al[9] reported that lncRNA HNF1A-AS1, which was significantly up-regulated in lung adenocarcinoma, can promote tumor proliferation and metastasis by regulating EMT. Zhang et al[10] found that up-regulation of lncRNA H19 could promote tumor growth through epigenetically silencing miR-675 and was associated with poor overall survival in glioma patients. Unfortunately, the detail effect of lncRNAs in osteosarcoma remains largely unknown. LncRNA Ewing sarcoma associated transcript 1 (EWSAT1), a newly identified lncRNA, was reported to be highly expressed in osteosarcoma[11]. However, its role in osteosarcoma remains largely unknown. To our best knowledge, this is the first study about the prognostic value of EWSAT1 in osteosarcoma.

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Patients and Methods

Patients and Tissue Samples

This study was approved by the Research Ethics Committee of The 309th Hospital of Chinese PLA and informed written consent was obtained from all patients. The samples of tumor tissue and corresponding normal bone tissues were collected between 2008 and 2012 from 176 patients who were undergoing surgery in our hospital. All the tumors were confirmed pathologically from the specimens obtained from surgery. All patients who had received chemotherapy before surgery were excluded. All the osteosarcoma patients were followed for 60 months. The clinical features of all the patients were presented in Table I. All specimens were handled and made anonymous according to ethical and legal standards.

RNA Extraction and Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. RNA was reversed transcribed into cDNAs using the Primer-Script one step RT-PCR kit (TaKaRa, Dalian, Liaoning, China). RT-PCR was performed using ABI Prism 7900HT (Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. GAPDH was used as reference genes to normalized the expression of EWSAT1. The primers of EWSAT1 and GAPDH were all bought from Invitrogen. Primers used for RT-RCR are presented as follows: EWSAT1-F: GTGTCTGGCAAGGAACACTA, EWSAT1-R:

GGTGGAGAAGAGGGACAATAAG. GAPDH-F:

5'-CGCTCTCTGCTCCTCCTGTTC-3', GAPDH-R:

5'-ATCCGTTGACT-CCGACCTTCAC-3'.

The relative expression of EWSAT1 was calculated by $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

SPSS 17.0 (SPSS Inc., Chicago, IL, USA) was used to analyze the data, which were presented as means ± SD. The difference between means was analyzed with Student's *t*-test. The test for categorical variables was made by x^2-test. Survival curve was constructed by the Kaplan-Meier method and determined with the log-rank test. The Cox proportional hazards model was used to examine independent significance of relevant clinical factors. *p*-value less than 0.05 was considered to be statistically significant.

Results

EWSAT1 is Upregulated in Human Osteosarcoma Tissues

In order to explore the effect of EWSAT1 in osteosarcoma, the expression of EWSAT1 was examined in tissues by qRT-PCR assays. As shown in Figure 1, we observed that EWSAT1 was significantly upregulated in osteosarcoma tissues compared with that in paired adjacent bone tissues ($p<0.01$). These results indicated that EWSAT1 may be involved in osteosarcoma progression.

Association of EWSAT1 Expression with Clinicopathologic Characteristics in Osteosarcoma

For statistical analysis, the patients were divided into the EWSAT1 high expression group (n = 90) and the EWSAT1 low expression group (n = 86) based on the mean value of EWSAT1 expression. Then, we explored the association of EWSAT1 expression with clinicopathological characteristics in patients. As shown in Table I, we found that the level of EWSAT1 expression was significantly associated with clinical stage ($p=0.001$) and distant metastasis ($p=0.011$). However, no associations were observed between EWSAT1 expression and gender, age, tumor size, anatomic location or response to chemotherapy (all $p>0.05$).

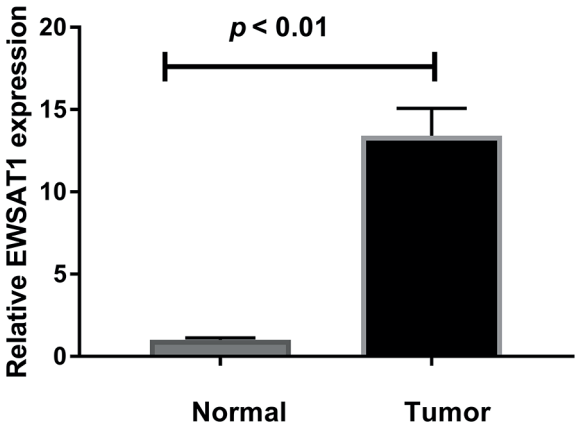

Figure 1. The expression levels of EWSAT1 in osteosarcoma tissues were significantly higher than those in corresponding noncancerous bone tissues ($p<0.01$).

High EWSAT1 Expression is Associated with Poor Prognosis in Osteosarcoma

To explore the prognostic value of EWSAT1 expression in osteosarcoma, we further performed Kaplan-Meier analysis and log-rank test. The results showed that osteosarcoma patients with high EWSAT1 expression have shorter OS (Figure 2, p=0.0007) and DFS (Figure 3, p=0.0010) than those with low EWSAT1 expression. Subsequently, we performed Cox proportional hazards regression analysis. Multivariate analysis indicated that EWSAT1 expression was an independent prognostic factor for both OS (Table II, p<0.001) and DFS (Table III, p = 0.001) of patients with osteosarcoma.

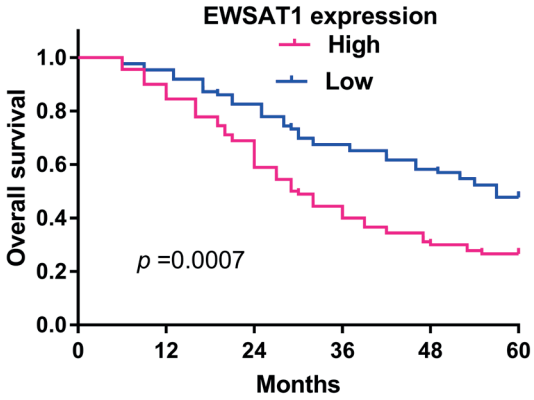

Figure 2. OS rate in patients with high EWSAT1 expression was significantly lower than that in patients with low EWSAT1 expression (log-rank p=0.0007).

Discussion

Screening novel markers for osteosarcoma may help improve therapeutic strategies and predict patients' outcome. Recently, more and more evidence suggests that some lncRNAs can serve as diagnostic or prognostic biomarkers in many cancer types, including osteosarcoma[12-14]. Furthermore, some lncRNAs, such as lncRNA MA-

Table I. Clinicopathological features and the expression of EWSAT1 in OS patients.

Parameters	Group	Total	EWSAT1 expression		*p*-value
			High	Low	
Gender	Male	106	55	51	NS
	Female	70	35	35	
Age (years)	< 60	88	47	41	NS
	≥ 60	88	43	45	
Tumor size(cm)	>8	74	39	35	NS
	≤8	102	51	51	
Anatomic location	Tibia/femur	127	62	65	NS
	Elsewhere	49	28	21	
Clinical stage	I/II	83	31	52	0.001
	III	93	59	34	
Distant metastasis	Present	91	55	36	0.011
	Absent	85	35	50	
Response to chemotherapy	Good	83	43	40	NS
	Poor	93	47	46	

Table II. Univariate and multivariate analyses for overall survival in OS patients.

Variable	Univariate			Multivariate		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Gender	0.931	0.621-1.332	0.388	-	-	-
Age	1.215	0.712-1.884	0.414	-	-	-
Tumor size	1.532	0.637-1.911	0.219	-	-	-
Anatomic location	1.022	0.813-1.554	0.189	-	-	-
Clinical stage	3.672	2.114-6.231	<0.001	3.139	1.462-4.893	0.002
Distant metastasis	3.321	1.562-4.889	0.006	2.637	1.032-3.991	0.009
Response to chemotherapy	1.421	0.552-1.851	0.391	-	-	-
EWSAT1 expression	3.913	1.932-7.441	<0.001	3.078	1.442-5.831	<0.001

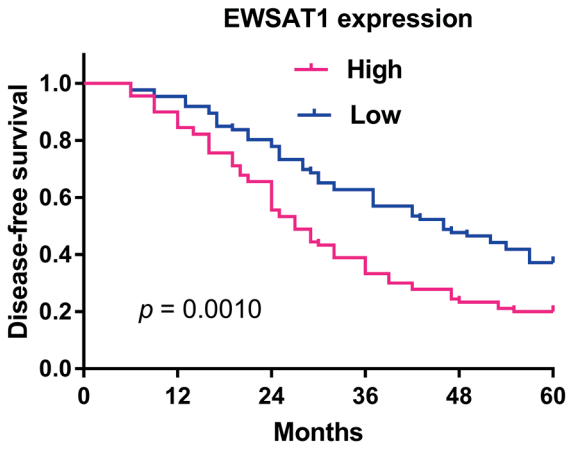

Figure 3. DFS rate in patients with high EWSAT1 expression was significantly lower than that in patients with low EWSAT1 expression (log-rank p=0.0010).

LAT1[15], lncRNA TUG1[16] and lncRNA UCA1[17], have been identified as independent prognostic factors for osteosarcoma. However, the effect of most lncRNAs in osteosarcoma remains unknown. In the present work, we focused on EWSAT1. As a newly identified lncRNA, only a few studies reported the role of EWSAT1 in tumors. For instance, Song et al[18] showed that the expression levels of EWSAT1 were significantly up-regulated in nasopharyngeal carcinoma. Further functional assays indicated that its forced expression could promote human nasopharyngeal carcinoma cell growth by targeting miR-326/330-5p. Michelle et al[19] reported that EWSAT1 dynamically changed in Ewing sarcoma patients, and its expression was increased in Ewing sarcoma tissues compared to normal bone tissues. *In vitro* experiment showed that knockdown of EWSAT1 expression inhibited the proliferation of Ewing sarcoma cells, suggesting that EWSAT1 served as a tumor promoter in this malignancy. The expression pattern of EWSAT1 in osteosarcoma was firstly reported by Li et al[11]. They performed microarray analysis and found up-regulated expression of EWSAT1 in osteosarcoma. Subsequently, Sun et al[20] further explored the detail role of EWSAT1 in osteosarcoma progression. Their functional assay showed that EWSAT1 enhanced osteosarcoma cell proliferation, migration, and invasion through suppression of MEG3 expression. Given the carcinogenic effect of EWSAT1 in osteosarcoma, we wondered whether EWSAT1 could be associated with the prognosis of patients. In the present study, we firstly explore the clinical significance of EWSAT1 in patients with osteosarcoma. In line with previous study, we also found that EWSAT1 was significantly highly expressed in osteosarcoma tissues compared with normal none tissues. Then, statistical assay indicated that the level of EWSAT1 expression was significantly associated with clinical stage and distant metastasis. Kaplan-Meier analysis revealed that osteosarcoma patients with high EWSAT1 expression level had shorter OS and DFS. Further multivariate survival analysis confirmed that EWSAT1 could be used as an independent potential prognostic biomarker for patients with osteosarcoma.

Conclusions

We showed that EWSAT1 is frequently upregulated in osteosarcoma, and it may be a useful poor prognostic biomarker for patients with osteosarcoma. In the future, a well-designed prospective study is necessary to confirm the predictive value of EWSAT1 for prognosis in osteosarcoma patients.

Table III. Univariate and multivariate analyses for disease-free survival in OS patients.

Variable	Univariate			Multivariate		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Gender	0.831	0.571-1.263	0.336	-	-	-
Age	1.361	0.822-1.733	0.518	-	-	-
Tumor size	1.413	0.589-1.838	0.289	-	-	-
Anatomic location	0.956	0.672-1.325	0.254	-	-	-
Clinical stage	3.213	1.832-5.556	0.001	2.779	1.131-4.023	0.003
Distant metastasis	2.452	1.231-4.032	0.008	1.783	1.003-3.131	0.013
Response to chemotherapy	1.218	0.673-1.774	0.318	-	-	-
EWSAT1 expression	3.521	1.642-6.231	<0.001	2.783	1.223-4.781	0.001

Conflict of interest

The authors declare no conflicts of interest.

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