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Original Article

Expression of long non-coding RNA ZEB1-AS1 in esophageal squamous cell carcinoma and its correlation with tumor progression and patient survival

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Abstract: Background: LncRNA ZEB1-AS1 has been identified as a tumor oncogene in hepatocellular carcinoma. However, the clinical significance in esophageal squamous cell carcinoma (ESCC) is still unknown. The aim of this study was to explore ZEB1-AS1 expression levels and evaluated its clinical significance in ESCC patients. Methods: LNCRNA ZEB1-AS1 expression was determined by quantitative real-time PCR (QRT-PCR) in 87 pairs of ESCC specimens and adjacent non-tumor tissues. Then, the association of ZEB1-AS1 expression with clinicopathological factors or survival of ESCC patients were determined. Results: LNCRNA ZEB1-AS1 was found up-regulated in ESCC tissues compared to adjacent non-tumor tissues. Increased lncRNA ZEB1-AS1 expression was significantly associated with tumor grade, depth of invasion, and lymph node metastasis. Kaplan-Meier analysis revealed that ESCC patients with high ZEB1-AS1 expression had a poorer overall survival and disease-free survival. Furthermore, multivariate analysis suggested that ZEB1-AS1 expression was identified as an independent prognostic factor in patients with ESCC. Conclusion: These results indicated that lncRNA ZEB1-AS1 was associated with tumor progression and could be an independent prognostic factor for ESCC patients.

Keywords: Esophageal cancer, lncRNA ZEB1-AS1, overall survival, disease-free survival

Introduction

Esophageal cancer is the eighth most common cancer worldwide and the sixth most common cause of death from cancer [1]. Approximately half of the esophageal cancer cases that are newly diagnosed each year occur in China [2]. There are two common types of esophageal cancer: esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC) [3]. Despite the recent advances in ESCC treatment, the prognosis is still unfavorable, and the overall 5-year survival rate is less than 10% [4]. Therefore, it is necessary to search novel biomarkers for ESCC, which can improve therapeutic strategies and predict clinical outcome.

In recent years, genome-wide transcriptional studies found that only approximately 1% of the human genome serves as blueprints for proteins, whereas a much larger proportion of the genome is transcribed into non-coding RNAs (NCRNAs) [5, 6]. Among these ncRNAs are long non-coding RNAs (lncRNAs) which are more than 200 nucleotides in length with little protein-coding potential [7]. In recent years, several lncRNAs have been shown to be involved in carcinogenesis and cancer progression. For example, Zhang et al showed that lncRNA MALAT1 was up-regulated in clear cell renal cell carcinoma and correlated with advanced clinical features and shorter overall survival time [8]. Li et al found that increased expression of the lncRNA ANRIL promoted lung cancer cell metastasis and correlated with poor prognosis [9]. Zhou et al suggested that lncRNA LET was down-regulated in gastric cancer and associated with tumor progression, furthermore, they indicated that lncRNA LET might act as a novel prognostic indicator in gastric cancer [10].

Recently, Li et al found that lncRNA ZEB1 antisense1 (ZEB1-AS1) was up-regulated in hepatocellular carcinoma and correlated with poor prognosis of HCC patients, furthermore, they revealed that ZEB1-AS1 could induce epithelial

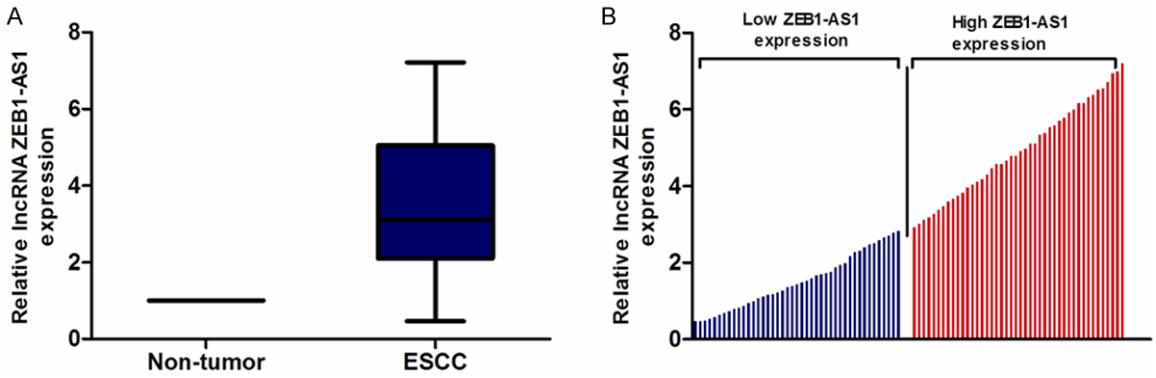

Figure 1. LncRNA ZEB1-AS1 expression were up-regulated in ESCC. The relative ZEB1-AS1 expression levels were determined using qRT-PCR and demonstrated using the comparative ΔCt method. A. Higher relative ZEB1-AS1 levels were detected in ESCC tissues than in adjacent non-tumor tissues. B. ZEB1-AS1 expression was classified into a low ZEB1-AS1 expression group and a high ZEB1-AS1 expression group according to the median value of relative ZEB1-AS1 expression. *P<0.05.

to mesenchymal transition and cancer metastasis [11]. However, to our knowledge, expression of ZEB1-AS1 in ESCC and the relationship between ZEB1-AS1 expression and ESCC remains unclear.

In the present study, we determined the expression patterns of lncRNA ZEB1-AS1 in ESCC tissues and adjacent non-tumor tissues. Moreover, we explored the correlation between ZEB1-AS1 dysregulation and clinical characteristics and prognosis of ESCC patients.

Materials and methods

Tissue specimens

A total of 87 pairs of primary ESCC tissues and adjacent non-tumor tissues were obtained from patients who underwent surgery at the Department of Thoracic Surgery, The First Affiliated Hospital of Xinxiang Medical University between 2006 and 2008. All specimens were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. No patient received chemotherapy or radiotherapy prior to surgery. Written informed consent was obtained fromall patients prior to participation in the study. The medical ethics committee of First Affiliated Hospital of Xinxiang Medical University approved the study.

RNA isolation and quantitative real-time PCR (QRT-PCR)

The total RNA was extracted from tissues with Trizol reagent (Invitrogen) according to the manufacturer's protocol. 1.5 μg total RNA was reverse transcribed in a final volume of 20 μl using random primers under standard conditions using the Prime Script RT Master Mix (Takara). After the RT reaction, the quantitative real-time PCR (QRT-PCR) was performed using the SYBR Select Master Mix (Applied Bio systems) with 0.5 μl complementary DNA (CDNA) on ABI 7900 system (Applied Biosystems) according to the manufacturer's instructions. By using GAPDH as an internal control, ZEB1-AS1 expression level was determined by qRT-PCR using the following primer sequences: forward, 5'-ATTGTTAGGAAAGGTTATAAAATTT-3'; and reverse, 5'-ACCCAAACTATATAAAAAATTAC AC-3'. GAPDH forward primer, 5'-CGCTCTCTGC-TCCTCCTGTTC-3', GAPDH reverse primer, 5'-AT-CCGTTGACTCCGACCTTCAC-3'. All experiments were performed using the $2^{-\Delta\Delta Ct}$ method. Each experiment was performed in triplicate.

Statistical analysis

Statistical analyses were performed using SPSS version 18.0. The chi-square test was used to assess ZEB1-AS1 expression with respect to clinicopathological factors. The survival curves of the patients were determined using the Kaplan-Meier method and the log-rank test was used for statistical evaluations. A Cox proportional hazards model was used for multivariate analysis. All data are presented as the mean ± SD from at least three independent experiments. P<0.05 was considered statistically significant.

Table 1. Correlation between lncRNA ZEB1-AS1 expression and clinicopathologic factors of ESCC patients

Parameters	Group	Total	ZEB1-AS1 expression		*P* value
			Low	High	
Age (years)	<60	41	21	20	0.752
	≥60	46	22	24	
Gender	Male	54	29	25	0.307
	Female	33	14	19	
Tumor size (cm)	<4 cm	51	26	25	0.730
	≥4 cm	36	17	19	
Tumor location	Upper	22	10	12	0.216
	Middle	41	24	17	
	Lower	24	10	14	
Tumor grade	High + Middle	38	26	12	0.002
	Low	49	17	32	
Depth of invasion	T1-T2	36	30	6	0.000
	T3-T4	51	13	38	
Lymph nodes metastasis	Absence	53	33	20	0.003
	Presence	34	10	24	

Results

LncRNA ZEB1-AS1 was up-regulated in ESCC

To determine whether lncRNA ZEB1-AS1 was involved in the tumorigenesis of ESCC, QRT-PCR was performed to detect the differential expression of ZEB1-AS1 in 87 pairs of ESCC tissues and matched adjacent non-tumor tissues. As shown in **Figure 1A**, ZEB1-AS1 expression in ESCC tissues was significantly higher than that in adjacent non-tumor tissues, indicating that ZEB1-AS1 might play an oncogenic role in ESCC progression.

Over expression of lncRNA ZEB1-AS1 was associated with advanced clinicopathologic factors of ESCC patients

The 87 ESCC patients were classified into two groups according to the median expression level of ZEB1-AS1: ESCC patients expressing ZEB1-AS1 less than the median expression level were assigned to the low expression group (n=43), and those samples with expression equal or above the median expression level were assigned to the high expression group (n=44) (**Figure 1B**). The association between clinicopathologic factors and ZEB1-AS1 expression were shown in **Table 1**. Over expression of ZEB1-AS1 was significantly associated with tumor grade, depth of invasion, and lymph node metastasis ($P<0.05$, **Table 1**). In contrast, there was no correlation was detected in the expression level of ZEB1-AS1 with other clinicopathological factors of ESCC patients, including age, gender, tumor size, and tumor location ($P>0.05$, **Table 1**). These data indicated that lncRNA ZEB1-AS1 might play an important role in ESCC progression.

Correlation between lncRNA ZEB1-AS1 expression and ESCC patients' survival

The correlation of lncRNA ZEB1-AS1 expression with prognosis in ESCC patients was further investigated by Kaplan-Meier analysis and log-rank test. Our results showed that 5-year overall survival ($P>0.05$, **Figure 2A**) and disease-free survival ($P>0.05$, **Figure 2B**) of ESCC patients with high ZEB1-AS1 expression were shorter compared to those patients with low ZEB1-AS1 expression. Furthermore, multivariate analysis using the Cox proportional hazard model revealed that tumor grade, depth of invasion, lymph node metastasis and ZEB1-AS1 expression were independent prognostic factors for overall survival (HR=2.371, 95% CI: 1.284-6.115; $P<0.05$), as well as disease-free survival (HR=2.695, 95% CI: 1.379-8.352; $P<0.05$) of ESCC patients after esophagectomy (**Table 2**).

Discussion

LncRNAs are commonly defined as transcribed RNA molecules which are longer than 200 nucleotides, possessing no potential protein-coding capacity [12]. Accumulating evidences suggested that lncRNAs play critical roles in various physiological and pathological processes [13, 14]. Recently, lncRNAs were considered to be novel biomarkers for types of human cancer, including ESCC [15, 16]. For example, Tong et al demonstrated that lncRNA POU3F3 could serve as a potential biomarker for diagnosis of ESCC [17]. Shi et al reported that lncRNA PCAT-1 was correlated with advanced clinical stage and poor prognosis of ESCC patients [18]. Li et

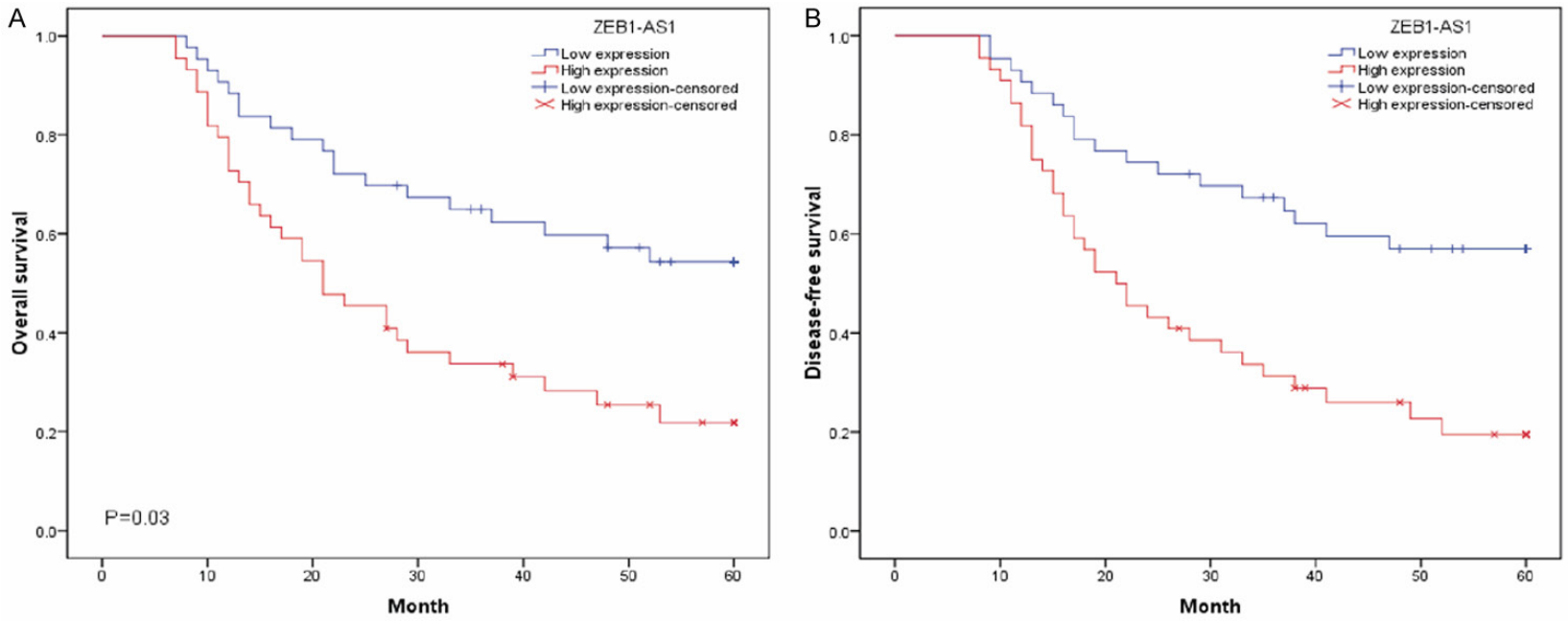

Figure 2. Kaplan-Meier survival analysis of association between lncRNA ZEB1-AS1 expression level and (A) overall survival and (B) disease-free survival of ESCC patients. Patents expressing high level of ZEB1-AS1 have a significantly shorter overall survival and disease-free survival compared with patients with low level of ZEB1-AS1. (P<0.05, log-rank test).

Table 2. Multivariate Cox proportional hazard model analysis of overall survival and disease-free survival inESCC patients

	Overall survival			Disease-free survival		
	HR	95% CI	*P*	HR	95% CI	*P*
Age (years)	0.886	0.528-1.837	0.203	0.966	0.674-2.073	0.261
Gender	1.225	0.413-2.938	0.447	1.336	0.692-3.431	0.387
Tumor size (cm)	1.861	0.557-4.286	0.159	1.525	0.492-4.273	0.118
Tumor location	1.513	0.769-5.115	0.214	1.703	0.783-6.827	0.139
Tumor grade	2.607	1.804-5.312	0.021	2.874	2.016-7.726	0.009
Depth of invasion	2.036	1.413-4.627	0.015	2.412	1.797-6.082	0.011
Lymph nodes metastasis	2.941	1.184-7.153	0.008	3.102	1.263-9.173	0.003
ZEB1-AS1 expression	2.371	1.284-6.115	0.013	2.695	1.379-8.352	0.007

HR: hazard ratio; 95% CI: 95% confidence interval.

al found that lncRNA UCA1 was associated with poor prognosis of ESCC, and in vitro analysis revealed that decreased expression of lncRNA UCA1 inhibited ESCC cell proliferation, migration, and invasion [19]. Hu et al provided that over expression of lncRNA PlncRNA-1 was correlated with advanced tumor stage and lymph node metastasis, and might serve as a potential prognostic marker and therapeutic target for ESCC [20]. However, there were no reports about the clinicopathologic and prognostic significance of lncRNA ZEB1-AS1 expression in human ESCC.

In the present study, we found that lncRNA ZEB1-AS1 expression was up-regulated in ESCC tissues. The relationships of ZEB1-AS1 with clinical features of ESCC were further explored. Our results showed that ZEB1-AS1 expression was associated with tumor grade, depth of invasion, and lymph node metastasis, indicating that ZEB1-AS1 might be involved in the carcinogenesis and metastasis of ESCC. More importantly, we proved that ESCC patients with a high expression of ZEB1-AS1 had a shorter overall survival and disease-free survival than those with low ZEB1-AS1 expression group. In a multivariate Cox model, our data demonstrated that ZEB1-AS1 expression was an independent poor prognostic factor for both overall survival and disease-free survival of ESCC patients, suggesting that lncRNA ZEB1-AS1 could be a promising non-invasive biomarker for prognosis of ESCC patients. However, the precise molecular mechanisms behind the altered expression of ZEB1-AS1 in ESCC and its function is still unclear. Li et al showed that over expression of ZEB1-AS1 could promote HCC cell proliferation and invasion both in vitro and in vivo [11]. Thus, additional studies are needed to more clearly and comprehensively articulate the molecular mechanisms of both the cause and the effects of altered expression of ZEB1-AS1 in the progression of ESCC.

In conclusion, our results revealed that lncRNA ZEB1-AS1 was significantly up-regulated in ESCC and correlated with poorer patients' prognosis and it might be a new and potential prognostic biomarker for ESCC.

Disclosure of conflict of interest

None.

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References

[1] Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D. Global cancer statistics. CA Cancer J Clin 2011; 61: 69-90.

[2] Chen W, He Y, Zheng R, Zhang S, Zeng H, Zou X and He J. Esophageal cancer incidence and mortality in China, 2009. J Thorac Dis 2013; 5: 19-26.

[3] Enzinger PC and Mayer RJ. Esophageal cancer. N Engl J Med 2003; 349: 2241-2252.

[4] van Hagen P, Hulshof MC, van Lanschot JJ, Steyerberg EW, van Berge Henegouwen MI, Wijnhoven BP, Richel DJ, Nieuwenhuijzen GA, Hospers GA, Bonenkamp JJ, Cuesta MA, Blaisse RJ, Busch OR, ten Kate FJ, Creemers GJ, Punt CJ, Plukker JT, Verheul HM, Spillenaar Bilgen EJ, van Dekken H, van der Sangen MJ, Rozema T, Biermann K, Beukema JC, Piet AH, van Rij CM, Reinders JG, Tilanus HW and van

der Gaast A. Preoperative chemoradiotherapy for esophageal or junctional cancer. N Engl J Med 2012; 366: 2074-2084.
[5] Huttenhofer A, Schattner P and Polacek N. Non-coding RNAs: hope or hype? Trends Genet 2005; 21: 289-297.
[6] Esteller M. Non-coding RNAs in human disease. Nat Rev Genet 2011; 12: 861-874.
[7] Mercer TR, Dinger ME and Mattick JS. Long non-coding RNAs: insights into functions. Nat Rev Genet 2009; 10: 155-159.
[8] Zhang HM, Yang FQ, Chen SJ, Che J and Zheng JH. Up regulation of long non-coding RNA MALAT1 correlates with tumor progression and poor prognosis in clear cell renal cell carcinoma. Tumour Biol 2015; 36: 2947-2955.
[9] Lin L, Gu ZT, Chen WH and Cao KJ. Increased expression of the long non-coding RNA ANRIL promotes lung cancer cell metastasis and correlates with poor prognosis. Diagn Pathol 2015; 10: 14.
[10] Zhou B, Jing XY, Wu JQ, Xi HF and Lu GJ. Down-regulation of long non-coding RNA LET is associated with poor prognosis in gastric cancer. Int J Clin Exp Pathol 2014; 7: 8893.
[11] Li T, Xie J, Shen C, Cheng D, Shi Y, Wu Z, Deng X, Chen H, Shen B, Peng C, Li H, Zhan Q and Zhu Z. Upregulation of long noncoding RNA ZEB1-AS1 promotes tumor metastasis and predicts poor prognosis in hepatocellular carcinoma. Oncogene 2015; [Epub ahead of print].
[12] Ponting CP, Oliver PL and Reik W. Evolution and functions of long noncoding RNAs. Cell 2009; 136: 629-641.
[13] Yan B and Wang Z. Long noncoding RNA: its physiological and pathological roles. DNA Cell Biol 2012; 31 Suppl 1: S34-41.
[14] Harries LW. Long non-coding RNAs and human disease. Biochem Soc Trans 2012; 40: 902-906.
[15] Lee JT. Epigenetic regulation by long noncoding RNAs. Science 2012; 338: 1435-1439.
[16] Prensner JR and Chinnaiyan AM. The emergence of lncRNAs in cancer biology. Cancer Discov 2011; 1: 391-407.
[17] Tong YS, Wang XW, Zhou XL, Liu ZH, Yang TX, Shi WH, Xie HW, Lv J, Wu QQ and Cao XF. Identification of the long non-coding RNA POU3F3 in plasma as a novel biomarker for diagnosis of esophageal squamous cell carcinoma. Mol Cancer 2015; 14: 3.
[18] Shi WH, Wu QQ, Li SQ, Yang TX, Liu ZH, Tong YS, Tuo L, Wang S and Cao XF. Up regulation of the long noncoding RNA PCAT-1 correlates with advanced clinical stage and poor prognosis in esophageal squamous carcinoma. Tumour Biol 2015; 36: 2501-2507.
[19] Li JY, Ma X and Zhang CB. Over expression of long non-coding RNA UCA1 predicts a poor prognosis in patients with esophageal squamous cell carcinoma. Int J Clin Exp Pathol 2014; 7: 7938-7944.
[20] Wang CM, Wu QQ, Li SQ, Chen FJ, Tuo L, Xie HW, Tong YS, Ji L, Zhou GZ, Cao G, Wu M, Lv J, Shi WH and Cao XF. Upregulation of the long non-coding RNA PlncRNA-1 promotes esophageal squamous carcinoma cell proliferation and correlates with advanced clinical stage. Dig Dis Sci 2014; 59: 591-597.