European Review for Medical and Pharmacological Sciences 2019; 23: 7929-7934

Upregulation of long noncoding RNA LEF1-AS1 predicts a poor prognosis in patients with esophageal squamous cell carcinoma

M.-Z. ZONG, W.-T. FENG, N. DU, X.-J. YU, W.-Y. YU

Department of Oncology, the Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University, Huai'an, Jiangsu, China

Abstract. – OBJECTIVE: Recent studies have suggested that long noncoding RNAs (lncRNAs) are involved in various tumors. The present research was designed to examine the prognostic values of a newly identified lncRNA, lncRNA LEF1-AS1 (LEF1-AS1), in esophageal squamous cell carcinoma (ESCC).

PATIENTS AND METHODS: The relative levels of LEF1-AS1 in ESCC tissues and normal esophageal tissues were examined by applying quantitative Real Time- Polymerase Chain Reaction (qRT-PCR). The relations between LEF1-AS1 expressions and clinical factors were analyzed by conducting Chi-square test. The Kaplan-Meier assay was used for assays of the overall survival (OS) and disease-free survival (DFS) dates. Univariate and multivariate analyses were applied for the identification of the independent prognostic factors for ESCC.

RESULTS: We showed that LEF1-AS1 was distinctly upregulated in ESCC tissues compared with the matched normal tissues ($p < 0.01$). Higher levels of LEF1-AS1 were associated with lymph nodes metastasis ($p = 0.009$) and clinical stage ($p = 0.008$). Clinical investigation revealed that ESCC patients with high LEF1-AS1 level showed a significant shorter 5-year OS ($p = 0.0028$) and DFS ($p = 0.0025$). Multivariate analyses confirmed LEF1-AS1 as an independent prognostic parameter indicating unfavorable clinical prognosis for ESCC patients.

CONCLUSIONS: The present study suggested that LEF1-AS1 could be a novel ESCC-related lncRNA involved in the clinical progression of ESCC, which may be used as a potential predictor.

Key Words:
LncRNA, LEF1-AS1, Esophageal squamous cell carcinoma, Prognosis.

Introduction

Esophageal cancer is the eighth most common neoplasm and the sixth leading cause of tumor-associated mortality worldwide[1]. In China, the main histologic type of esophageal cancer is esophageal squamous cell carcinoma (ESCC). Many patients with ESCC attempt to ask for medical attention when they undergo a period of involuntary weight loss and progressive dysphagia[2,3]. In recent years, the incidences of ESCC have increased in many regions of China[4]. Although clinical therapies and perioperative managements have germinated in the past ten years with impressive advances in diagnosis, operation procedures, and combined chemoradiotherapies, the long-term survivals of ESCC patients remain poor and are approximately 12-30%[5,6]. Up to date, sensitive molecular biomarkers for prediction of the clinical outcome and therapies responses are extremely limited. Therefore, the exploration of novel markers for ESCC is necessary.

Long non-coding RNAs (lncRNAs), typically transcribed by RNA polymerase II, are defined as one of the largest and differing classes of transcripts containing more than 200 nucleotides[7]. Unlike mRNAs, lncRNAs lack open reading frames, meaning their limited protein-coding capabilities[8]. LncRNAs have gained extensive attention as the novel and crucial regulators of cellular progress due to their important effects in the regulation of various gene expressions at the transcriptional levels[9,10]. In recent years, increasing lncRNAs are confirmed to act as a critical role in the cellular development of various diseases *via* a variety of unclear mechanisms[11,12]. Of note, many dysregulated lncRNAs attract growing attention for the clarification of interconnected pathways in the modulation of oncogenesis and progression of various neoplasm, including ESCC[13,14]. In addition, several tumor-associated lncRNAs are reported to act as potential biomarkers for diagnosing tumors and predicting clinical outcome of patients due to their abnormal expression in

Corresponding Author: Wei-yong Yu, MD; e-mail: yyyy99898@163.com

plasma and tissues[15-17]. Unfortunately, the emerging functional role and clinical significance of lncRNAs in ESCC remain to be further clarified.

LncRNA lymphoid enhancer-binding factor-1 antisense RNA 1 (LEF1-AS1), a new type of tumor-associated lncRNA, was firstly identified by Wang et al[18]. They observed LEF1-AS1 as a highly expressed lncRNA in glioblastoma using bioinformatics analyses and RT-PCR in tumor tissues. Then, overexpression of LEF1-AS1 was also demonstrated in colorectal cancer and prostate cancer[19,20]. However, the roles of LEF1-AS1 in ESCC have not been reported. In this study, we identified LEF1-AS1 as a novel ESCC-associated regulator whose overexpression was correlated with the clinical development of ESCC patients. Subsequently, we explored its prognostic values in ESCC patients.

Patients and Methods

Patients and Tissue Samples

Between 2011 and 2014, ESCC tissues and matched normal tissues were obtained from 185 patients suffering from ESCC undergoing clinical surgeries at the Affiliated Huaian No.1 People's Hospital of Nanjing Medical University. To avoid various gene changes involved in treatment, all patients who were aged between 33.7 and 66.4 years did not receive adjuvant treatments when these specimens were collected. The histopathological detection was used for confirming the diagnosis of all tissues with ESCC according to the World Health Organization (WHO) criteria. The clinicopathological data of all patients are presented in Table I. For the legal application of the related clinical tissues, the Institutional Research Ethics Committee of our hospital approved our research methods. All patients with ESCC provided informed consent prior to their inclusion.

RNA Isolation and Quantitative Real Time PCR

TRIzol reagent, which was purchased from Invitrogen (Haidian, Biejing, China) was used for the isolation of total RNA from tumor and normal tissues. For the cDNA syntheses, 2 mg of total RNA was collected, and the miScript II RT Kit (Qiagen, Pudong, Shanghai, China) was used. Reverse transcription was carried out by the use of the M-MLVReverse Transcriptase (Invitrogen, Carlsbad, CA, USA). QPCR for the detection of lncRNAs was carried out *via* the use of the FastStart Universal SYBR-Green Master (#4913915; Roche, Xuhui, Shanghai, China) on an ABI QuantStudio 6 Flex system (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was employed to be the control. The collected data from RT-PCR were examined and expressed relative to the threshold cycle (CT) values. The primers sequences are as follows: LEF1-AS1, 5'- TTTGTGTGGCCTGGACTCTC-3'(forward) and 5'-AACCCCTGGGACACAAACTG-3' (reverse); GAPDH, 5'- ACCACAGTCCATGCCATCAC-3' (forward) and 5'- TCCACCACCCTGTTGCTGTA -3' (reverse);

Table I. LEF1-AS1 expression and clinicopathologic features in ESCC patients.

			LEF1-AS1 expression		
Parameters	**Group**	**Total**	**High 92**	**Low 93**	***p***
Age (years)	< 55	91	43	48	0.507
	≥ 55	94	49	45	
Gender	Male	112	55	57	0.834
	Female	73	37	36	
Tumor size	< 4 cm	114	52	62	0.156
	≥ 4 cm	71	40	31	
Histological grade	G1	118	54	64	0.152
	G2+G3	67	38	29	
Lymph nodes metastasis	Absence	140	62	78	0.009
	Presence	45	30	15	
Clinical stage	I-II	133	58	75	0.008
	III-IV	52	34	18	

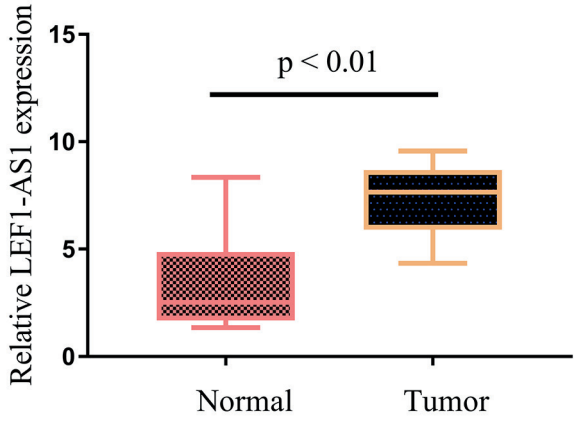

Figure 1. The relative levels of LEF1-AS1 in human ESCC tissues (n = 185) and matched normal esophageal tissues (n = 185) by the use of RT-PCR.

Statistical Analysis

Statistical assays were performed using the SPSS statistical software package (18.0, SPSS Inc., Chicago, IL, USA). The levels of LEF1-AS1 in ESCC tissues and normal tissues were compared by Mann-Whitney U tests. The Chi-square tests were applied for the assessment of LEF1-AS1 expression with respect to clinical parameters. Disease-free survival (DFS) and overall survival (OS) rates were calculated by the Kaplan-Meier method. Further survival assays of univariate and multivariate were conducted using the Cox regression model. A p-value of < 0.05 was statistically significant.

Results

High Expressions of LEF1-AS1 in ESCC Tissues

To explore whether LEF1-AS1 acted as a functional regulator in ESCC, we performed RT-PCR for the examination of LEF1-AS1 in ESCC tissues. As shown in Figure 1, the results indicated that up-regulation of LEF1-AS1 was observed in 136 (73.5%) cases of ESCC specimens, which was distinctly higher than that in matched normal tissues (p <0.01).

Clinicopathological Significance of LEF1-AS1 Expressions in ESCC

Through statistical assays, we determined the median value (6.24) of LEF1-AS1 expressions in ESCC specimens. Then, we assigned ESCC to LEF1-AS1 low-expressing group (n = 92) and high-expressing group (n = 93) based on the above median value. Subsequently, Chi-square test was conducted for the examination of the clinical significance of LEF1-AS1. As shown in Table I, the data suggested that LEF1-AS1 expression was associated with lymph nodes metastasis (p = 0.009) and clinical stage (p = 0.008). However, no significant difference in LEF1-AS1 expression was observed with other factors (p >0.05). Our findings suggested LEF1-AS1 as a functional modulator in clinical progress of ESCC.

Higher LEF1-AS1 Expressions Correlated with Poor Prognosis in ESCC Patients

The frequent up-regulation of LEF1-AS1 and its positive roles in ESCC progression encouraged us to further explore the clinical prognostic values of LEF1-AS1 in ESCC patients. As shown in Figures 2 and 3, the results of Kaplan-Meier analysis suggested that patients with higher levels of LEF1-AS1 have a shorter OS (p = 0.0028) and DFS (p = 0.0025). For a further demonstration of the prognostic effect of LEF1-AS1 expressions in ESCC, the univariate and multivariate assays were conducted. As presented in Table II and Table III, three possible factors (lymph nodes metastasis, clinical stage, and LEF1-AS1 expression) were shown to be associated with both OS and DFS of ESCC patients. Moreover, the results of multivariate assays detected that LEF1-AS1 expression was an independent poor prognostic factor for both OS (p = 0.0014; Table II) and DFS (p =0.0017; Table III) in ESCC.

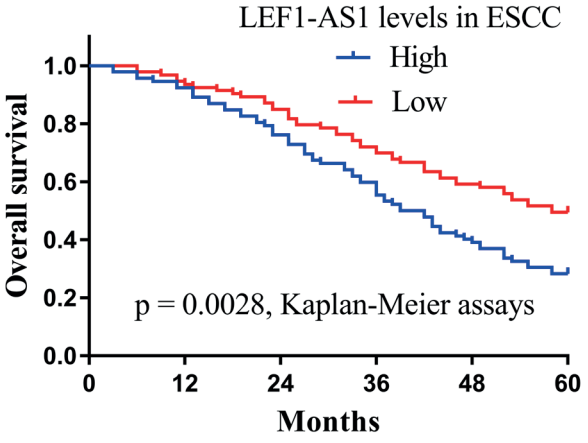

Figure 2. Kaplan-Meier analysis for overall survival based on the levels of LEF1-AS1.

Table II. Univariate and multivariate analysis of prognostic factors in for overall survival.

	Univariate analysis		Multivariate analysis	
Variables	**HR (95% CI)**	***p*-value**	**HR (95% CI)**	***p*-value**
Age (years)	1.687 (0.682-2.318)	0.244	-	-
Gender	1.446 (0.782-1.994)	0.129	-	-
Tumor size	1.285 (0.895-1.827)	0.114	-	-
Histological grade	1.192 (0.572-2.442)	0.092	-	-
Lymph nodes metastasis	3.261 (1.337-5.018)	0.002	2.885 (1.124-4.572)	0.006
Clinical stage	3.032 (1.248-4.869)	0.007	2.857 (1.123-4.362)	0.013
LEF1-AS1 expression	3.162 (1.225-4.654)	0.008	2.942 (1.169-4.156)	0.014

Discussion

ESCC has been frustrating to treat, with sluggish progresses made on extending the long-term survival time[21]. The early diagnosis and prediction of prognosis are very imperative for the clinical treatment of ESCC. However, it has been proved that the development of ESCC screening programs are particularly challenging because the molecular biomarkers with exceptionally high specificity were not easy to identify[22,23]. Recently, growing studies[24,25] suggested that lncRNAs may be suitable as prognostic diagnostic biomarkers for tumors due to their high stability and potential function on regulating tumor progression. Several lncRNAs have confirmed to be potential prognostic regulators in ESCC. For instance, lncRNA HOTAIR, a well-studied lncRNA in various tumors, has been demonstrated, by Kaplan-Meier assays, as a sufficient prognostic biomarker for the survival of ESCC patients[26]. LncRNA SNHG16 and lncRNA CASC9 were also identified as prognostic biomarkers in ESCC patients[27,28]. However, a large number of lncRNAs remained to be functionally elucidated. LEF1-AS1 was a recently discovered lncRNA. Wang et al[18] firstly reported its differential expressions in glioblastoma and reported its potential as an unfavorable biomarker. Functionally, LEF1-AS1 was found to suppress the proliferation and invasion of tumor cells by regulating Akt/mTOR signal-

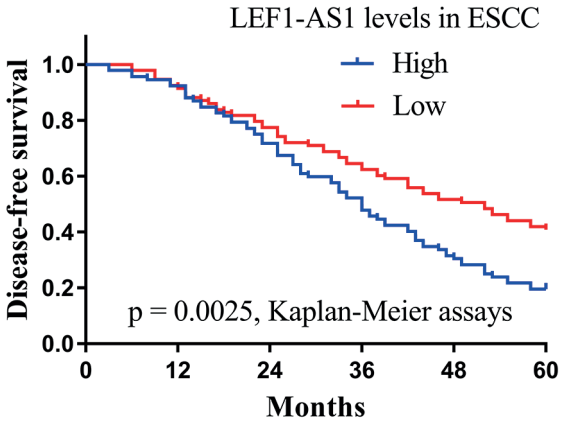

Figure 3. Kaplan-Meier analysis for disease-free survival based on the levels of LEF1-AS1.

Table III. Univariate and multivariate analysis of prognostic factors in for disease-free survival.

	Univariate analysis		Multivariate analysis	
Variables	**HR (95% CI)**	***p*-value**	**HR (95% CI)**	***p*-value**
Age (years)	1.145 (0.782-1.889)	0.149	-	-
Gender	1.546 (0.952-2.331)	0.215	-	-
Tumor size	1.722 (0.772-2.518)	0.134	-	-
Histological grade	1.482 (0.885-2.145)	0.188	-	-
Lymph nodes metastasis	3.172 (1.355-5.236)	0.001	2.896 (1.148-4.664)	0.008
Clinical stage	2.996 (1.365-4.987)	0.005	2.758 (1.186-4.572)	0.021
LEF1-AS1 expression	3.056 (1.218-4.664)	0.008	2.856 (1.123-4.327)	0.017

ing when it was silenced using si-LEF1-AS1. Then, Liu et al[20] showed that LEF1-AS1 levels were distinctly down-regulated in prostate cancer and predicted a shorter overall survival of patients. Further functional investigations confirmed its tumor-promotive function by performing loss-of-function assays. In addition, the results of biological information also revealed that LEF1-AS1 was up-regulated and may be a metastasis-related lncRNA in colorectal cancer[19]. These findings indicated that LEF1-AS1 may be a tumor promoter in tumors. However, the conclusion was inaccurate since its effects on other tumors had not been investigated.

In this study, our group firstly reported the frequent overexpression of LEF1-AS1 in ESCC tissues by performing RT-PCR. Then, the clinical significance of LEF1-AS1 was analyzed, and the results showed that high LEF1-AS1 expression was distinctly associated with lymph nodes metastasis and clinical stage, which suggested that LEF1-AS1 may act as a positive regulator in the clinical progress of ESCC. Moreover, we further performed Kaplan-Meier assays, finding that the patients with high LEF1-AS1 levels had worse OS and DFS than those with low LEF1-AS1 levels. Finally, the results of the multivariate analysis confirmed that LEF1-AS1 were independently associated with both OS and DFS, which highlighted the important clinical application of LEF1-AS1 in ESCC. Some limitations of our present research should be noted. First, the sample size used for the clinical assays is relatively small, large clinical trials are necessary for further confirmation of our conclusion. Second, the cutoff levels of LEF1-AS in ESCC tissue to predict clinical outcome remain to be established due to the arbitrary LEF1-AS1 levels in our work. Third, the functional assays for the exploration of the effects of LEF1-AS1 in ESCC have not been conducted and further cells researches are needed to supply a gap.

Conclusions

LEF1-AS1 might serve as a tumor promoter in the initiation and progression of ESCC, and would be a novel prognostic biomarker for this tumor.

Conflict of Interests

The Authors declare that they have no conflict of interests.

References

1) Siegel RL, Miller KD, Jemal A. Cancer statistics, 2017. CA Cancer J Clin 2017; 67: 7-30.
2) Almhanna K, Shridhar R, Meredith KL. Neoadjuvant or adjuvant therapy for resectable esophageal cancer: is there a standard of care? Cancer Control 2013; 20: 89-96.
3) Paul S, Altorki N. Outcomes in the management of esophageal cancer. J Surg Oncol 2014; 110: 599-610.
4) Lin Y, Totsuka Y, Shan B, Wang C, Wei W, Qiao Y, Kikuchi S, Inoue M, Tanaka H, He Y. Esophageal cancer in high-risk areas of China: research progress and challenges. Ann Epidemiol 2017; 27: 215-221.
5) Ikebe M, Morita M, Yamamoto M, Toh Y. Neoadjuvant therapy for advanced esophageal cancer: the impact on surgical management. Gen Thorac Cardiovasc Surg 2016; 64: 386-394.
6) Hoppo T, Jobe BA. Personalizing therapy for esophageal cancer patients. Thorac Surg Clin 2013; 23: 471-478.
7) Jarroux J, Morillon A, Pinskaya M. History, discovery, and classification of lncRNAs. Adv Exp Med Biol 2017; 1008: 1-46.
8) Nicolas FE. Role of ncRNAs in development, diagnosis and treatment of human cancer. Recent Pat Anticancer Drug Discov 2017; 12: 128-135.
9) Audas TE, Lee S. Stressing out over long noncoding RNA. Biochim Biophys Acta 2016; 1859: 184-191.
10) Gloss BS, Dinger ME. The specificity of long noncoding RNA expression. Biochim Biophys Acta 2016; 1859: 16-22.
11) Kanduri C. Long noncoding RNAs: lessons from genomic imprinting. Biochim Biophys Acta 2016; 1859: 102-111.
12) Leone S, Santoro R. Challenges in the analysis of long noncoding RNA functionality. FEBS Lett 2016; 590: 2342-2353.
13) Tang Y, Cheung BB, Atmadibrata B, Marshall GM, Dinger ME, Liu PY, Liu T. The regulatory role of long noncoding RNAs in cancer. Cancer Lett 2017; 391: 12-19.
14) Saha P, Verma S, Pathak RU, Mishra RK. Long noncoding RNAs in mammalian development and diseases. Adv Exp Med Biol 2017; 1008: 155-198.
15) Zhou DD, Liu XF, Lu CW, Pant OP, Liu XD. Long non-coding RNA PVT1: emerging biomarker in digestive system cancer. Cell Prolif 2017; 50:
16) Ma W, Zhang CQ, Li HL, Gu J, Miao GY, Cai HY, Wang JK, Zhang LJ, Song YM, Tian YH, Song YH. LncRNA FER1L4 suppressed cancer cell growth and invasion in esophageal squamous cell carcinoma. Eur Rev Med Pharmacol Sci 2018; 22: 2638-2645.
17) Gao GD, Liu XY, Lin Y, Liu HF, Zhang GJ. LncRNA CASC9 promotes tumorigenesis by affecting EMT and predicts poor prognosis in esophageal squamous cell cancer. Eur Rev Med Pharmacol Sci 2018; 22: 422-429.

18) Wang J, Liu X, Yan C, Liu J, Wang S, Hong Y, Gu A, Zhao P. LEF1-AS1, a long-noncoding RNA, promotes malignancy in glioblastoma. Onco Targets Ther 2017; 10: 4251-4260.

19) Chen Y, Yu X, Xu Y, Shen H. Identification of dysregulated lncRNAs profiling and metastasis-associated lncRNAs in colorectal cancer by genome-wide analysis. Cancer Med 2017; 6: 2321-2330.

20) Liu DC, Song LL, Liang Q, Hao L, Zhang ZG, Han CH. Long noncoding RNA LEF1-AS1 silencing suppresses the initiation and development of prostate cancer by acting as a molecular sponge of miR-330-5p via LEF1 repression. J Cell Physiol 2019; 234: 12727-12744.

21) Sasaki Y, Kato K. Chemoradiotherapy for esophageal squamous cell cancer. Jpn J Clin Oncol 2016; 46: 805-810.

22) Mimura K, Yamada L, Ujiie D, Hayase S, Tada T, Hanayama H, Thar Min AK, Shibata M, Momma T, Saze Z, Ohki S, Kono K. Immunotherapy for esophageal squamous cell carcinoma: a review. Fukushima J Med Sci 2018; 64: 46-53.

23) Sato Y, Motoyama S, Saito H, Minamiya Y. Novel candidate biomarkers of chemoradiosensitivity in esophageal squamous cell carcinoma: a systematic review. Eur Surg Res 2016; 56: 141-153.

24) Wu T, Du Y. LncRNAs: from basic research to medical application. Int J Biol Sci 2017; 13: 295-307.

25) Bolha L, Ravnik-Glavac M, Glavac D. Long noncoding RNAs as biomarkers in cancer. Dis Markers 2017; 2017: 7243968.

26) Song W, Zou SB. Prognostic role of lncRNA HOTAIR in esophageal squamous cell carcinoma. Clin Chim Acta 2016; 463: 169-173.

27) Han GH, Lu KJ, Wang P, Ye J, Ye YY, Huang JX. LncRNA SNHG16 predicts poor prognosis in ESCC and promotes cell proliferation and invasion by regulating Wnt/beta-catenin signaling pathway. Eur Rev Med Pharmacol Sci 2018; 22: 3795-3803.

28) Wu Y, Hu L, Liang Y, Li J, Wang K, Chen X, Meng H, Guan X, Yang K, Bai Y. Up-regulation of lncRNA CASC9 promotes esophageal squamous cell carcinoma growth by negatively regulating PDCD4 expression through EZH2. Mol Cancer 2017; 16: 150.