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Overexpression of long-noncoding RNA ZFAS1 decreases survival in human NSCLC patients

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Abstract. – OBJECTIVE: The purpose of the current study was to characterize the expression of long-noncoding-RNA ZFAS1 (ZFAS1) and assess the clinical significance of ZFAS1 in non-small cell lung cancer (NSCLC).

PATIENTS AND METHODS: A total of 173 patients with NSCLC were addressed in the present retrospective study. Expression levels of ZFAS1 were detected by quantitative real-time PCR. We further analyzed the correlation between ZFAS1 and clinicopathologic features of NSCLC with X^2-test. Survival rate was determined with Kaplan-Meier and statistically analyzed with the log-rank method between groups. Univariate and multivariate analyses were performed to analyze the prognostic significance of ZFAS1 expression.

RESULTS: ZFAS1 was upregulated in NSCLC tissues ($p < 0.01$) and higher expression levels of ZFAS1 were found in more advanced tumor tissues (All $p < 0.05$). ZFAS1 expression levels were significantly associated with tumor differentiation grade ($p = 0.028$), lymph node metastasis ($p = 0.001$) and TMN stage ($p = 0.001$).

Furthermore, we found that patients with higher ZFAS1 expression level are associated with a poorer overall survival. Univariate and multivariate analyses indicated that high ZFAS1 expression was an independent prognostic factor for poor survival of NSCLC patients.

CONCLUSIONS: Our results illustrated the potential role of ZFAS1 as a prognostic marker for NSCLC patients.

Key Words
Long noncoding RNA, ZFAS1, Non-small cell lung cancer, Prognosis.

Introduction

In recent years, primary lung cancer occupies the leading cause of cancer death in the world[1]. Non-small cell lung cancer (NSCLC) accounts for greater than 80% of all lung cancer types[2]. Although the survival rate of patients with NSCLC has been improved due to the advances in surgical techniques and treatment strategies, the 5-year survival rate of NSCLC is still approximately 15% because of the high recurrence rate[3,4]. Therefore, the identification of specific biomarkers, especially for early stage tumors, is important for improving NSCLC prognosis.

Previous studies[5] have found that some non-protein coding RNAs regulate cell differentiation, growth, and metabolism. Long non-coding RNA (lncRNA) is an RNA molecule that is >200 nucleotides long and is not translated into a protein[6]. More and more evidence showed that lncRNAs were emerged as critical regulator and prognostic markers in several tumors. For instance, lncRNA HOTAIR was reported to serve as a tumor suppressor and decreased ovarian tumorigeniesis and metastasis by inhibiting epithelial-mesenchymal transition[7]. Another paper showed that lncRNA UCA1 modulates breast cancer cell growth and apoptosis by targeting miR-143[8]. Additionally, Lin et al[9] reported that knockdown of lncRNA ANRIL expression could inhibit lung cancer cell proliferation, migration and invasion. Previous studies[10] revealed that ZFAS1 expression was significantly upregulated in several tumors including gastric cancer, hepatocellular Carcinoma[11] and colorectal cancer[12]. However, whether ZFAS1 can serve as a prognostic biomarker of NSCLC has not been investigated. Therefore, in the current study, we explored the association of ZFAS1 level with clinicopathological features and prognosis in NSCLC patients.

Patients and Methods

Patients and Tissue Samples

Clinical samples were obtained from 173 patients with NSCLC who were surgically treated at

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The People's Hospital of Pingyi County from June 2007 to July 2010. None of the patients received chemotherapy or radiotherapy before surgery. The histopathological diagnosis of all samples was respectively diagnosed by two Pathologists. Patient information including baseline demographics, clinicopathological characteristics and survival were recorded. Tumor stage was defined according to tumor-node-metastasis (TNM) classification of the American Joint Committee on the International Union against Cancer. Tumor differentiation was assessed according to Edmonson and Steiner grading system. The study was approved by the Ethics Committee of The People's Hospital of Pingyi County. Signed informed consent forms were obtained from all subjects who participated in the study.

Reverse Transcription PCR and Quantitative Real-time PCR

RNAs of cells were extracted by Trizol method (Invitrogen, Carlsbad, CA, USA), and used for first-strand cDNA synthesis using oligo (dT) primers and RT-PCR kit (Takara, Otsu, Shiga, Japan). QRT-PCR reactions were performed using an ABI7500 System (Applied Biosystems, Foster City, CA, USA) and SYBR Green PCR Master Mix (Takara, Otsu, Shiga, Japan). Quantitative analysis was performed using Comparative CT method. The relative expression of each gene was normalized to the expression of GAPDH. The PCR primers for lncRNA HOTTIP or β-actin were as follows:

- lncRNA ZFAS1 forward: 5′-ACGTGCAGACATCTACAACCT-3′;
- lncRNA ZFAS1 reverse: 5′-TACTTCCAACACCCGCAT-3′;
- GAPDH forward: 5′-GGTCTCCTCTGACTTCAACA-3′;
- GAPDH reverse: 5′-GTGAGGGTCTCTCTCTTCCT-3′.

Independent experiments were done in triplicate.

Statistical Analysis

Statistical analyses were performed using SPSS version 16.0 (SPSS, Chicago, IL, USA). An X^2-test was used to analyze the relationship between ZFAS1 expression levels and the clinicopathological characteristics. Survival analyses were performed using the Kaplan-Meier survival curve approach and the Cox regression model. Statistical significance was reached when $p < 0.05$.

Results

ZFAS1 was upregulated in the human NSCLC tissues

To confirm ZFAS1 expression was different between tumor tissues and adjacent noncancerous tissues, we examined 173 pairs of human NSCLC samples. The results showed that the ZFAS1 expression was significantly increased in clinical NSCLC tissues compared to adjacent normal lung tissues ($p < 0.01$; Figure 1A). Furthermore, we evaluated the correlation of ZFAS1 expression with clinicopathological parameters. As shown in Figure 1B, C and D, we found that more advanced tumors had a higher ZFAS1 expression (all $p < 0.01$). However, there was no significant relationship between ZFAS1 expression and tumor size ($p > 0.05$).

ZFAS1 upregulation is associated with aggressive progression in NSCLC

To explore the clinical value of ZFAS1 expression in NSCLC tissues, the association of its expression and clinicopathological characters of 173 patients was statistically analyzed by 2 tests or Fisher's exact test (Table I). We observed that the expression level of ZFAS1 was positively correlated with the TMN stage ($p = 0.001$), lymph node status ($p = 0.001$), and differentiation ($p = 0.028$) in NSCLC patients (shown in Table I). However, neither of the ZFAS1 levels in NSCLC patients correlated with age, gender, tumor size, or smoking history.

Relationship between ZFAS1 expression and NSCLC patients' survival

The association between miR-173 expression and survival of NSCLC patients was investigated by Kaplan-Meier analysis and log-rank test. The five-year overall survival rate of patients in the high ZFAS1 group was 20.1%, which was significantly lower than that of patients (44.2%) in the low serum ZFAS1 group ($p < 0.001$; Figure 2). Univariate and multivariate analyses were carried out using the Cox proportional hazards regression model to explore the prognostic value of ZFAS1 expression, the results indicated that high-level expression of ZFAS1 was considered as an independent prognostic factor of outcomes in patients with NSCLC ($p = 0.007$, Table II).

Discussion

Lung cancer is a leading cause of cancer death worldwide, because most patients are diagnosed at an advanced stage, and treatments are less ef-

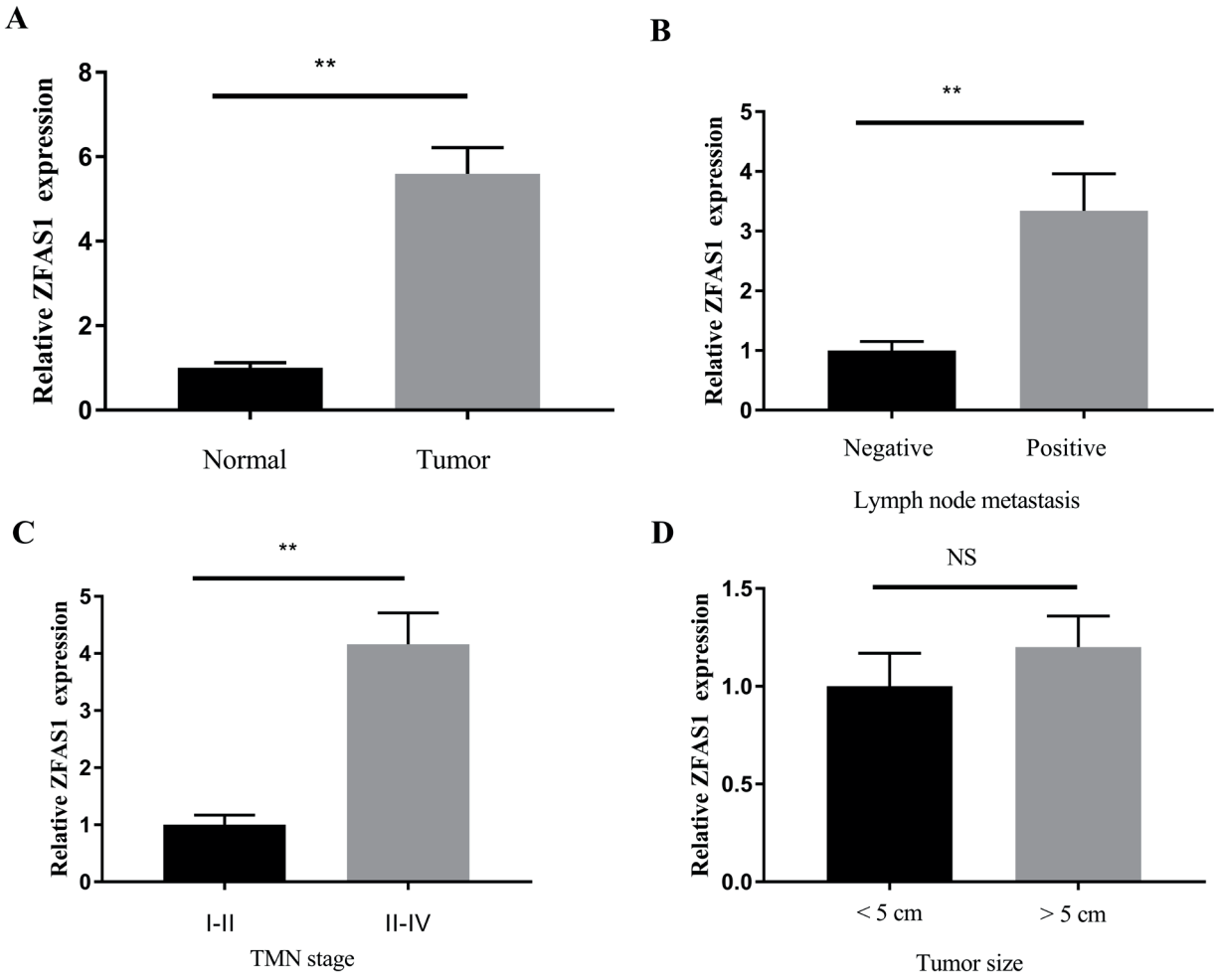

Figure 1. Expression of ZFAS1 in NSCLC samples. The expression of ZFAS1 was detected using qRT-PCR. A. ZFAS1 expression was decreased in NSCLC tissues compared with normal lung tissues. B. ZFAS1 increased in tissues with lymph node metastasis. C. ZFAS1 expression was significantly lower in patients with early clinical stage than those with an advanced clinical stage. D. Here was no significant relationship between the ZFAS1 expression and tumor size. *$p < 0.05$, **$p < 0.01$.

fective, the prognosis of NSCLC patients is still quite poor. Many studies[13-15] and systematic empirical research specific on NSCLC have identified certain prognostic factors and several biomarkers for NSCLC. However, only a few effective predictive makers were used for clinical application. In recent years, it has been shown that the aberrant level of lncRNAs is closely related to the occurrence, development, and prognosis of cancer[16,17]. However, lncRNAs that are involved in the progression of NSCLC and their clinical application values remain largely unknown.

ZFAS1, a newly identified lncRNA, was shown to be dysregulated in several tumors. Increasing evidence showed that ZFAS1 play an important regulation marker in the progression of tumors[18]. For instance, Li et al[11] showed that the relative expression of ZFAS1 in NSCLC tissues was significantly higher than those in adjacent normal tissues. Further mechanism experiment indicated that ZFAS1 repressed hepatocellular carcinoma cell invasion and migration by targeting ZEB1 and the matrix metalloproteinase MMP14 and MMP16. Nie et al[10] found that ZFAS1 may act as a tumor promoter in gastric cancer by downregulating KLF2 and NKD2 expression. Another study by Wang et al[12] showed that high ZFAS1 expression was associated with worse survival time in patients with colorectal cancer. They also demonstrated that the downregulation of ZFAS1 inhibited the *in vivo* metastasis of colorectal cancer cells[12]. These findings demonstrated that the dysregulation of ZFAS1 may participate in human malignancy and carcinogenesis.

Table I. Correlation between ZFAS1 expression and clinicopathological characteristics of NSCLC patients.

Characteristics	Patients		ZFAS1 expression		
	n	%	Low	High	*p*-value
Age (years)					0.203
≤65	75	43.4	34	41	
>65	98	56.6	54	44	
Gender					0.411
Male	93	54	50	43	
Female	80	46	38	42	
Smoking history					0.234
Smokers	100	57.8	47	53	
Never smokers	73	46.2	41	32	
Differentiation					0.028
Well, moderate	102	46.2	47	53	
Poor	73	53.8	41	32	
Tumor size					0.908
≤ 5 cm	101	58.4	51	50	
> 5cm	71	41.6	37	35	
Lymph node metastasis					0.001
Positive	92	53.8	34	58	
Negative	81	46.8	54	27	
TMN stage					0.001
I-II	92	53.2	58	34	
II-IV	81	46.8	30	51	

In the present study, to our best knowledge, we firstly reported that expression of ZFAS1 was significantly higher in NSCLC tissues than in adjacent normal lung tissues. In addition, we found that higher expression levels of ZFAS1 were found in more advanced tumor tissues. Based on the calculation of relative expression, we analyzed the association of ZFAS1 with the clinicopathological characteristics as well as the prognosis of the patients. Our results showed that patients with NSCLC with high ZFAS1 expression had a better prognosis than those with low expression. Finally, we performed univariate and multivariate analysis; the results revealed that ZFAS1 expression might be an independent prognostic indicator of survival in NSCLC patients. Altogether, our data suggest that ZFAS1 expression could be a valuable marker of prognosis and malignant progression of NSCLC.

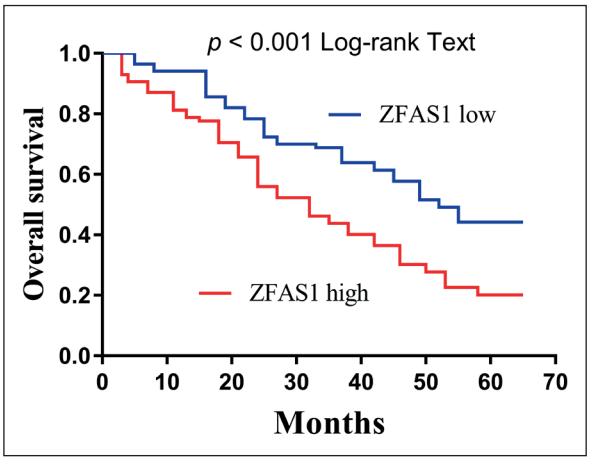

Figure 2. Log-rank test showed that patients with high ZFAS1 expression had significantly poorer overall survival vs. patients with low ZFAS1 expression ($p < 0.001$).

Conclusions

Our study demonstrated that ZFAS1 expression was associated with tumor grade and histological type in NSCLC. Also, overexpression of ZFAS1 associates with poor clinical survival outcome. Therefore, ZFAS1 may be a valuable biomarker for poor prognosis in human NSCLC. However, further studies are needed to more precisely characterize the functional significance of ZFAS1 in NSCLC progression.

Conflict of interest

The authors declare that no conflicts of interest exist.

Table II. Univariate and multivariate Cox logistic regression analyses for the prediction of NSCLC patients' OS course.

Variable	HR	95% CI	*p*-value	HR	95% CI	*p*-value
	Univariate			Multivariate		
ZFAS1						
Low	1.00			1.00		
High	2.76	1.24-4.12	0.003	1.83	1.04-3.83	0.007
Differentiation						
Well, moderate	1.00					
Poor	2.45	1.33-4.87	0.005	1.92	1.12-4.32	0.009
Tumor size						
≤5 cm	1.00					
> cm	1.65	0.83-2.77	0.137	2.14	1.18-3.11	0.144
Lymph node metastasis						
Positive	1.00					
Negative	2.45	1.17-5.13	0.002	2.11	0.93-4.66	0.003
TMN stage						
I-II	1.00			1.00		
II-IV	3.13	1.66-6.38	0.001	2.98	1.37-5.73	0.002
Age (years)						
≤65	1.00					
>65	0.733	0.347-1.66	0.473	1.13	0.46-1.93	0.326
Gender						
Male	1.00					
Female	0.91	0.45-1.42	0.321	1.32	0.67-1.73	0.266
Smoking history						
Smokers	1.00			1.00		
Never smokers	1.76	0.91-2.44	0.219	1.44	0.72-2.15	0.373

References

1) Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin 2012; 62: 10-29.
2) DeSantis CE, Lin CC, Mariotto AB, Siegel RL, Stein KD, Kramer JL, Alteri R, Robbins AS, Jemal A. Cancer treatment and survivorship statistics, 2014. CA Cancer J Clin 2014; 64: 252-271.
3) Lemjabbar-Alaoui H, Hassan OU, Yang YW, Buchanan P. Lung cancer: biology and treatment options. Biochim Biophys Acta 2015; 1856: 189-210.
4) Minna JD, Roth JA, Gazdar AF. Focus on lung cancer. Cancer Cell 2002; 1: 49-52.
5) Gokhale S1, Nyayanit D, Gadgil C. A systems view of the protein expression process. Syst Synth Biol 2011; 5: 139-150.
6) Guttman M, Rinn JL. Modular regulatory principles of large non-coding RNAs. Nature 2012; 482: 339-346.
7) Wang J, Chen D, He X, Zhang Y, Shi F, Wu D, Chen J, Zhang Y, Zhao F, Dou J. Downregulated lincRNA HOTAIR expression in ovarian cancer stem cells decreases its tumor genesis and metastasis by inhibiting epithelial-mesenchymal transition. Cancer Cell Int 2015; 15: 24.
8) Tuo YL, Li XM, Luo J. Long noncoding RNA UCA1 modulates breast cancer cell growth and apoptosis through decreasing tumor suppressive miR-143. Eur Rev Med Pharmacol Sci 2015; 19: 3403-3411.
9) Lin L, Gu ZT, Chen WH, 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) Nie F, Yu X, Huang M, Wang Y, Xie M, Ma H, Wang Z, De W, Sun M. Long noncoding RNA ZFAS1 promotes gastric cancer cells proliferation by epigenetically repressing KLF2 and NKD2 expression. Oncotarget 2016; doi: 10.18632/oncotarget.9611. [Epub ahead of print].
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, Zhu Z. Amplification of long noncoding RNA ZFAS1 promotes metastasis in hepatocellular carcinoma. Cancer Res 2015; 75: 3181-3191.
12) Wang W, Xing C. Upregulation of long noncoding RNA ZFAS1 predicts poor prognosis and prompts invasion and metastasis in colorectal cancer. Pathol Res Pract 2016; 212: 690-695.
13) Wang CZ, Yuan P, Xu B, Yuan L, Yang HZ, Liu X. RLIP76 expression as a prognostic marker of breast cancer. Eur Rev Med Pharmacol Sci 2015; 19: 2105-2111.
14) Wang LP, Niu H, Xia YF, Han YL, Niu P, Wang HY, Zhou QL. Prognostic significance of serum sMICA levels in non-small cell lung cancer. Eur Rev Med Pharmacol Sci 2015; 19: 2226-2230.
15) Guo F, Guo L, Li Y, Zhou Q, Li Z. MALAT1 is an oncogenic long non-coding RNA associated with

tumor invasion in non-small cell lung cancer regulated by DNA methylation. Int J Clin Exp Pathol 2015; 8: 15903-15910.

16) Jiang W, Zhang D, Xu B, Wu Z, Liu S, Zhang L, Tian Y, Han X, Tian D. Long non-coding RNA BANCR promotes proliferation and migration of lung carcinoma via MAPK pathways. Biomed Pharmacother 2015; 69: 90-95

17) Chai X, Ye X, Song Y. Long noncoding RNA ENST00000434223 suppressed tumor progression in non-small cell lung cancer. Tumour Biol 2016; 37: 10851-10860.

18) Thorenoor N, Faltejskova-Vychytilova P, Hombach S, Mlcochova J, Kretz M, Svoboda M, Slaby O. Long non-coding RNA ZFAS1 interacts with CDK1 and is involved in p53-dependent cell cycle control and apoptosis in colorectal cancer. Oncotarget 2016; 7: 622-637.