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# Overexpression of lncRNA AFAP1-AS1 as a diagnostic biomarker in non-small cell lung cancer

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## Abstract

**Background:** Long non-coding RNAs (lncRNAs) play important roles in lung tumorigenesis. Among different lncRNAs, overexpression of the lncRNA actin filament-associated protein 1-antisense RNA 1 (*AFAP1-AS1*) in lung tumors has been reported in different studies. In the current study, we aimed to investigate the potential value of lncRNA *AFAP1-AS1* as a diagnostic biomarker in lung cancer. Ninety samples from patients with lung cancer were collected from Noor-E-Nejat hospital, Tabriz, Iran. The expression of *AFAP1-AS1* was assessed using quantitative reverse transcriptase-PCR (qRT-PCR), followed by the ROC curve analysis to investigate the biomarker potency of AFAP1-AS1.

**Results:** Our results revealed an upregulation of *AFAP1-AS1* in tumor samples as compared to the adjacent non-tumor tissues. We found a significant positive association between *AFAP1-AS1* expression and tumor size, as well as tumor stage.

**Conclusions:** Our results showed overexpression of *AFAP1-AS1* and its capacity as a diagnostic biomarker in lung cancer.

**Keywords:** LncRNAs, AFAP1-AS1, Diagnostic biomarker, Lung cancer, NSCLC

## Background

Cancer is one of the most important health burdens and the second cause of death worldwide [1]. Over 1.8 million new cases and more than 600,000 deaths are estimated by the end of 2020 worldwide. Lung cancer is the second most common malignancy after breast cancer in women and prostate cancer [2, 3]. It has been shown that tobacco smoking is one of the main risk factor of lung cancer susceptibility [4]. However, considering the heterogeneous nature of lung cancer, recent studies demonstrated that lung cancer incident is increasing in East Asia [5]. Lung cancer in never smokers may be associated with genetic and epigenetic profiles and with environmental factors including pollution, second-hand smoking [6]. Lung cancer is classified into two main histological subtypes, i.e., small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC); the latter with 80–85% of all cases is the most prevalent subtype [3, 7]. Also, NSCLC is further subdivided into lung squamous cell carcinoma (LUSC) and lung adenocarcinoma (LUAD), in which the first subtype is more common histology in never smokers and the latter one is more associated with smokers [8]. As a genetic susceptibility factor in lung cancer, non-coding regions of genome play a vital role in initiation and progression [9]. Long non-coding RNAs (lncRNAs) are non-coding endogenous RNAs with longer than 200 nucleotide length [10]. LncRNAs take part in a wide range of cellular processes, and their aberrant expression plays crucial roles in human malignancies [11, 12]. Several lncRNA aberrations are associated with cancer

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progression, invasion and metastasis [13]. For instance, the potential of lncRNAs *MALAT1* (in prostate cancer), *MVIH* (in hepatocellular carcinoma) and *FENDRR* (in gastric cancer) as diagnostic and diagnostic markers has been shown previously [14–16]. Lnc-RNAs exert their functions by affecting expression and post-translational modification of crucial proteins in normal cell development processes [17]. Align with these studies, the role of *AFAP1-AS1* as a diagnostic biomarker in different cancer types has been shown. Thus, further investigations on *AFAP1-AS1* expression can help to verify its potency as a diagnostic biomarker in cancer. Several studies have shown the role of lncRNAs in NSCLC progression and metastasis. Thus, lncRNAs are also of great importance to be used as diagnostic markers in different lung cancer subtypes [18, 19]. For example, overexpression of lncRNA IGFBP4-1 was positively correlated with TNM staging and lung cancer susceptibility [20]. Furthermore, upregulation of other lncRNAs such as *MIR4435-2HG* causing transactivation of *β-catenin*, *MALAT1* via inducing STAT3 activation, *DANCR* through sequestering miR-216a, *UCA1* by targeting ERBB4 and lncRNA *H19* by affecting epithelial to mesenchymal transition (EMT) process has been shown as key players of tumor progression and drug resistance in NSCLC [21–25]. LncRNA *AFAP1-AS1* with 6,810 bp length is mapped to 4p16.1 and was first discovered in 2013 in Barrett's esophagus and esophageal adenocarcinoma [26]. It is transcribed in an antisense fashion and has overlap with AFAP1 gene [27]. Upregulation of *AFAP1-AS1* is involved in lung tumorigenesis and metastasis in vitro [28–30]. *AFAP1-AS1* has a critical function in cancer development, and it has the potential to be used as a diagnostic biomarker and therapeutic target [31]. Overexpression of *AFAP1-AS1* is associated with poor clinical outcome in esophageal adenocarcinoma, pancreatic carcinoma, breast and lung cancer [32]. In addition, *AFAP1-AS1* overexpression is associated with poor prognosis in NSCLC patients [11, 33].

The objective of the current study was to determine *AFAP1-AS1* expression levels in NSCLC tumors compared to non-tumor tissues. We also aimed to study the association between *AFAP1-AS1* expression and clinicopathological characteristics including smoking habits, gender, age, disease stage, tumor size and differentiation. Furthermore, *AFAP1-AS1* value as a feasible and informative diagnostic biomarker was investigated.

## Methods

### Sample collection

Ninety NSCLC tumor and adjacent non-tumor tissues were obtained from Noor-E-Nejat hospital, Tabriz, Iran. Written informed consent was obtained from all participants. The study was approved by the Medical Ethic Committee of Tabriz University. This study was conducted according the Declaration of Helsinki and was in concordance with Good Clinical Practice guidelines. The histopathologic characteristics of the samples were evaluated and characterized by an experienced pathologist. The inclusion criteria for the patients in the current study were set to having NSCLC in patients admitted to the Noor-E-Nejat hospital, Tabriz, Iran. The exclusion criteria were absence of familial history of any cancer and alcohol consumption. Based on these criteria, totally, 90 patients were selected.

### RNA isolation, cDNA synthesis and qRT-PCR

Total RNA was isolated using TRIZOL RNA extraction kit (Invitrogen, Massachusetts, USA) based on the manufacturer's protocol. DNaseI (GeneAll, Seoul, Korea) was applied for the elimination of DNA contamination. NanoDrop (Thermo Fisher scientific Nanodrop 2000, CA, USA) and 2% (v/w) agarose gel electrophoresis were used to assess quantity and quality of RNA samples, respectively.

cDNA Synthesis was carried out using Takara kit (TaKaRa, Kusatsu, Japan) according to the manufacturer's instruction. Approximately, 100 ng of cDNA was used for *AFAP1-AS1* amplification by LightCycler® 96 Real-Time PCR (Roche Molecular Systems, Inc., Pleasanton, CA, USA) using SYBR Green Master Mix (2x) (Amplicon, Odense, Denmark)., following primers were obtained from previously published study [39] for amplification: forward 5′-AGCCTGTTGAATCAGCCAACT-3′ and reverse 5′-GGTTCATACCAGCCCTGTCC-3′. To normalize the expression of target gene, β-actin was amplified as housekeeping gene using following primers: forward 5′-AGAGCTACGAGCTGCCTGAC-3′ and reverse 5′-AGCACTGTCTTGGCGTACAG-3′. The cycle threshold (Ct) was measured and difference between expression of *AFAP1-AS1* and *β-actin* was defined as ΔCt. To determine difference between the expression of *AFAP1-AS1* and *β-actin*, $2^{-\Delta Ct}$ value was calculated for each sample in tumor and corresponding non-tumor samples. All assays were performed in triplicate.

### Receiver operating characteristic (ROC)

The ROC curve analysis was done to assess sensitivity and specificity of *AFAP1-AS1* as a diagnostic biomarker in NSCLC.

### Statistical analysis

Mann–Whitney test was used to compare differences in the expression of *AFAP1-AS1* between tumor and non-tumor samples. Association between *AFAP1-AS1* expression and clinicopathological parameters was assessed

using student's *t* test and one-way ANOVA. The *t* test was applied when the data were normally distributed, and Mann–Whitney test was performed otherwise. Statistical analysis was done using SPSS version 24 and GraphPad Prism 8. *P* values less than 0.05 were considered as significant.

## Results

### Patients

A total number of ninety NSCLC patients were included in the study. The majority of patients (57%) were non-smoker and 43% were smoker. Sixty-two percent (50/90) were below 55 years and 38% (40/90) were older than 55 years. The main proportion of patients had their right side of their lung involved (64%) and the remaining had their left side involved. Thirty-five (72%) out of ninety patients were men and 25 (28/90) were women. Regarding tumor size, 72% (65/90) of patients had a tumor larger than 5 cm and the remaining had a tumor less than 5 cm. Forty-two percent (47/90) of patients had poor and 38% (43/90) had moderate to highly differentiated tumor. The half of patients had stage I/II and the other half had stage III/IV NSCLC.

### *AFAP1-AS1* expression levels

The expression of *AFAP1-AS1* was significantly higher ($p$ value < 0.0001) in tumor samples as compared to the corresponding non-tumor tissues (Fig. 1). We observed a significant positive association between *AFAP1-AS1* expression and tumor size (p = 0.015). In addition, *AFAP1-AS1* mean expression was significantly higher in stage III/IV group as compared to stage I/II group (p = 0.019) (Table 1). However, we did not find any other significant association between *AFAP1-AS1* expression levels and other clinicopathological features including smoking status, age, side of involvement, gender, lymph node metastasis and tumor differentiation.

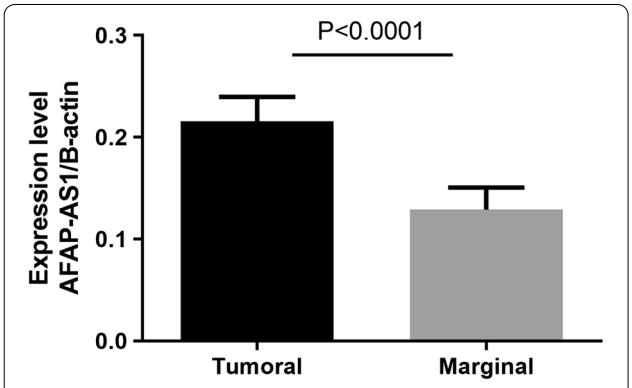


**Fig. 1** The expression level of the lnc-*AFAP1-AS1* between lung tumor and adjacent non-tumor tissues

**Table 1** Association between lncRNA *AFAP1-AS1* expression and clinicopathological characteristics in NSCLC patients

| Clinical parameter | No. of cases (%) | Mean expression ($2^{-\Delta\Delta Ct}$) | *P*-value |
|---|---|---|---|
| Smoking | | | 0.427 |
| Yes | 39 (43) | 14.12 | |
| No | 51 (57) | 13.35 | |
| Age (Year) | | | 0.643 |
| ≤ 55 | 50 (62) | 12.16 | |
| > 55 | 40 (38) | 15.59 | |
| Side of involvement | | | 0.993 |
| Right | 58 (64) | 13.28 | |
| Left | 32 (36) | 14.41 | |
| Gender | | | 0.797 |
| Male | 65 (72) | 14.62 | |
| Female | 25 (28) | 11.26 | |
| Tumor size (cm) | | | **0.015** |
| ≥ 5 | 64 (72) | 15.43 | |
| < 5 | 26 (28) | 9.40 | |
| Lymph metastasis | | | 0.686 |
| No | 42 (47) | 13.57 | |
| Yes | 48 (53) | 13.78 | |
| Differentiation | | | 0.728 |
| Poor | 42 (47) | 12.29 | |
| Moderate/high | 48 (53) | 14.90 | |
| Stage | | | |
| I/II | 45 (50) | 9.98 | **0.019** |
| III/IV | 45 (50) | 17.39 | |

The bold-face values are corresponding to the *P* values < 0.05, therefore statistically significant

### *AFAP1-AS1* expression as diagnostic biomarker

The ROC curve was plotted to assess the sensitivity and specificity of *AFAP1-AS1* as a diagnostic biomarker (Fig. 2). The area under curve (AUC) was 0.6779 (CI 95%, 0.5294 to 0.8263). Sensitivity and specificity of the biomarker were determined as 58% and 73%, respectively, with the cutoff value of 9.909.

## Discussion

In the current study, we investigated the expression of lncRNA *AFAP1-AS1* in lung tumors. We found a significant overexpression in lung tumors as compared to the matched non-tumor tissues. In addition, our analysis revealed a significant association between upregulated *AFAP1-AS1* levels and tumor size, as well as the stage of NSCLC.

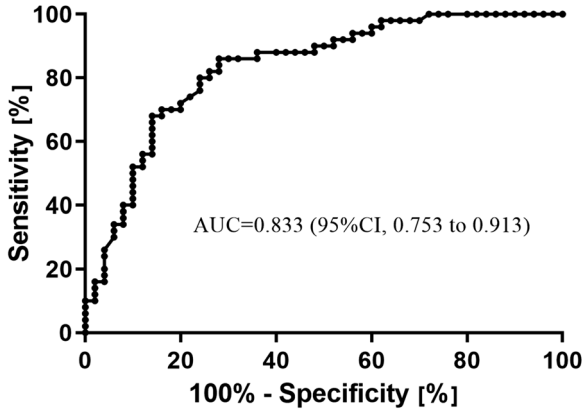


**Fig. 2** The receiver operating characteristic (ROC) curve analysis of AFAP1-AS1. The area under curve (AUC) is 0.6779 with 95% CI = 0.5294–0.8263

Several gene mutations, copy number variations and epigenetic alterations are involved in cancer progression and distant metastasis. Due to the need for identification of novel therapeutic and diagnostic molecules, the role of lncRNAs in tumorigenesis has been highlighted in recent years [34]. For instance, upregulation of lncRNA *H19* in lung cancer cells (A549) contributes to cell migration, invasion and EMT through regulating miR-484 affecting downstream pathways JNK and ROCK2 [25]. LncRNA *UCA1* exerts its oncogenic function via regulating miR-193a-3p by a competitively 'sponging' mechanism which targets *ERBB4* in lung cancer cells [24]. Furthermore, overexpression of other lncRNAs, such as *IGFBP4* and *DANCR*, in lung cancer provides supporting evidence for their oncogenic role [20, 23].

*AFAP1-AS1* is involved in cancer progression via affecting EMT process [35], modulating expression of several small GTPase members and aberrations in actin cytokeratin signaling pathway [36], regulating Rho/Rac pathway, downregulating C-myc and cycling D1 [26, 37], RhoC, p38MAPK, ROCK1 and Twist1 [29, 38]. Zhuang et al. (2017) showed *AFAP1-AS1* upregulation in lung adenocarcinoma as compared to non-tumor tissues. In addition, *AFAP1-AS1* downregulation was significantly associated with higher survival rate; thus, this lncRNA may serve as an effective diagnostic biomarker [28].

We found a positive correlation between *AFAP1-AS1* expression and tumor size, as well as the stage of disease. Furthermore, the ROC curve analysis, by plotting the expression level of *AFAP1-AS1* in stage I/II compared to stage III/IV, revealed *AFAP1-AS1* potential as an acceptable diagnostic biomarker. Our results were concordant with previous studies where *AFAP1-AS1* expression appeared to be a valuable diagnostic marker in patients with NSCLC [11, 30, 33]. Moreover, Li et al. [33] showed the biomarker potency of circulating *AFAP1-AS1* in discriminating between NSCLC patients from healthy people.

## Conclusion

In conclusion, lncRNA *AFAP1-AS1* was upregulated in NSCLC tumors as compared to non-tumor samples. Our analysis revealed that *AFAP1-AS1* might serve as a diagnostic biomarker in NSCLC. As we did not investigate underlying mechanisms by which *AFAP1-AS1* exerts its biological function, more profound studies are warranted.

#### Abbreviations

NSCLC: Non-small cell lung cancer; SCLC: Small cell lung cancer; EMT: Epithelial to mesenchymal transition; ROC: Receiver operating characteristic; AUC: Area under curve; Ct: Cycle threshold.

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Not applicable.

#### Authors' contributions

AS and RS designed the study. AR and SA performed experimental work. AR, SA, MM, STG, AS and RS analyzed the data. AR and SA wrote the manuscript with significant input from AS, MM and RS. AS edited the final draft and provided technical advice. RS supervised the project. All authors have read and approved the manuscript.

#### Funding

Not applicable.

#### Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

#### Ethics approval and consent to participate

The study was approved by the Medical Ethic Committee of Tabriz University (with the approval number of IR.TABRIZU.REC.1398.015). Written consent was obtained from all of the participants. This study was conducted according the Declaration of Helsinki and was in concordance with Good Clinical Practice guidelines.

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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