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

Sajjad Ghalib Ibrahim Alnajar1, Ali Rajabi1, Melika Maydanchi2, Samaneh Tayefeh Gholami1, Ali Saber2 and Reza Safaralizadeh1*

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 factors 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
progression, invasion and metastasis [13]. For instance, the potential of IncRNAs 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 IncRNAs in NSCLC progression and metastasis. Thus, IncRNAs are also of great importance to be used as diagnostic markers in different lung cancer subtypes [18, 19]. For example, overexpression of lncRNA IFGBP4-1 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, DANCER through sequestering miR-216a, UCA1 by targeting ERBB4 and IncRNA 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'-AGCCTGTGGAATCAGCCAACT-3' and reverse 5'-GGTTCTACCAGCCTGTC-3'. To normalize the expression of target gene, β-actin was amplified as housekeeping gene using following primers: forward 5'-AGAGCTACGAGCTGCTGAC-3' and reverse 5'-AGCAGTCTCTTTGGC TGAC-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^−ΔCt 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.

**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 IncRNA 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.

| Clinical parameter | No. of cases (%) | Mean expression (2−ΔΔCt) | 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              |                  |                           | 0.019   |
| I/II               | 45 (50)          | 9.98                      |         |
| III/IV             | 45 (50)          | 17.39                     |         |

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

![Fig. 1](image-url) The expression level of the Inc-AFAP1-AS1 between lung tumor and adjacent non-tumor tissues.
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 Rh/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, IncRNA 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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**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.

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**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.

**Author details**

1 Department of Animal Biology, Faculty of Natural Sciences, University of Tabriz, Tabriz, Iran. 2 Zimagene Medical Genetics Laboratory, Avicenna St., Hamedan, Iran.

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