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Diagnosis of lung cancer using salivary miRNAs expression and clinical characteristics

Negar Alizadeh[1†], Hoda Zahedi[1†], Maryam Koopaie[1*†], Mahnaz Fatahzadeh[2], Reza Mousavi[3] and Sajad Kolahdooz[4]

Abstract

Objective Lung cancer (LC), the primary cause for cancer-related death globally is a diverse illness with various characteristics. Saliva is a readily available biofluid and a rich source of miRNA. It can be collected non-invasively as well as transported and stored easily. The process is also reproducible and cost-effective. The aim of this study was to evaluate the salivary expression of microRNAs *let-7a-2*, *miR-221*, and *miR-20a* in saliva and evaluate their efficacy, using multiple logistic regression (MLR) model, in diagnosis of lung cancer.

Materials Samples of saliva were obtained from 40 lung cancer patients (20 lung adenocarcinoma and 20 lung squamous cell carcinoma) and 20 healthy controls. The levels of *let-7a-2*, *miR-221*, and *miR-20a* expression in saliva were assessed by RT-qPCR. Receiver operating characteristic (ROC) curve was utilized to assess the potential significance of miRNAs in saliva for lung cancer diagnosis with the use of multiple logistic regression (MLR), principal component analysis, and machine learning methods.

Results Diagnostic odds ratio (DOR) of *miR-20a* in lung adenocarcinoma diagnosis versus healthy control was higher than *miR-221*, and DOR of *miR-221* was higher than *let-7a-2*. *miR-20a* demonstrated a higher DOR for small cell lung carcinoma versus healthy control compared to *let-7a-2*, which in turn exhibited a higher DOR than *miR-221*. MLR of *miR-221*, *let-7a-2*, *miR-20a*, and smoking habit using main effects led to accuracy of 0.725 (sensitivity: 0.80, specificity: 0.65) and AUC = 0.795 for differentiation of small-cell lung carcinoma from lung adenocarcinoma. Our results showed that MLR based on salivary miRNAs could diagnose LUAD and SCLC from healthy control using main effects and two-way interactions with the accuracy of 0.90 (sensitivity = 0.95 and specificity = 0.85).

Conclusion A salivary miRNA-based MLR model is a promising diagnostic tool for lung cancer, offering a non-invasive screening option for high-risk asymptomatic individuals.

Keywords Lung cancer, microRNA, Small-cell lung carcinoma, Lung adenocarcinoma, Saliva, Biomarker

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Introduction

Lung carcinoma (LC), the primary reason for cancer-related deaths globally [1], is a diverse illness with various characteristics. Late stage diagnosis is the main cause of poor survival in LC [2]. The early symptoms of LC are usually non-specific resembling those of bronchitis or pneumonia. More typical symptoms include cough, dyspnea, sputum, chest pain, and weight loss. Patients could also present with dysphagia and hoarseness. Unfortunately, lung cancer has often invaded the surrounding structures or metastasized at the time of diagnosis [3, 4].

Based on histology and genetics, LC is categorized into two main types: non-small-cell lung carcinoma (NSCLC) and small-cell lung carcinoma (SCLC). Most lung cancers are histologically classified as NSCLC which includes lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC), the most prevalent subtypes [5, 6]. The most frequently encountered LC is LUAD, a malignancy accounting for nearly 40% of diagnoses and causing 65,000 deaths annually in the United States [7]. Diagnosis of LC is essential, and diagnostic work up should consider age, sex, smoking habit and family history of malignant disease affecting lung or other body sites. In general, lesions detected on imaging as suspicious for metastasis to the mediastinum or outside the chest should be sampled for further examination [4]. The sampling methods include bronchoscopy, mediastinoscopy, needle aspiration, and collection of pleural fluid. Lung cancer is a disease with distinct genetic subtypes and alterations at the molecular level could provide clues regarding utilization and efficacy of targeted therapy [8].

MicroRNAs (miRNAs) are a group of regulatory RNAs that bind to untranslated region of the target mRNAs (3'-U TR) to modulate gene expression by suppressing degradation or translation of mRNA [9]. They also play an essential role in various physiological functions such as cell growth, differentiation as well as apoptosis, and are critically involved in various cancers [10, 11].

MiR-221 is a carcinogenic microRNA involved in development of multiple tumors such as cervical squamous cell carcinoma, pancreatic cancer, and gastric cancer [10, 12]. It also appears to be involved in metastatic invasion of LC and is correlated with poor prognosis among NSCLC patients [13]. Yin et al. investigated the *miR-221* downregulation in lung cancer and its role in promoting metastasis by reducing oxidative stress and apoptosis [10]. Tepebası et al. demonstrated the overexpression of *miR-221* (downregulation) and its role in promotion of cell growth in NSCLC [14].

Tumor-suppressing miRNAs, such as *let-7a-2*, are generally reduced in cancer cells, and their loss leads to improper oncogene expression [15, 16]. Huang et al. found that *miR-20a* is overexpressed in various tumors, including lung cancer, and can be used as a marker for disease diagnosis [17]. Babu et al. also investigated the function of *miR-20a* in controlling the expression of the iron-exporter ferroportin in LC. The group found that elevated *miR-20a* levels in lung cancer may inhibit iron export, lead to iron accumulation within cells and promote cell proliferation [18]. In another study, high expression of circulating *miR-20a* was associated with a poor prognosis in NSCLC patients [19].

Saliva is a readily available oral biofluid which can be collected non-invasively as well as transported and stored easily. The process is also reproducible and cost-effective [20]. Localization of capillaries in the vicinity and surrounding the salivary glands facilitate permeation of biomarkers circulating in blood into saliva [20, 21]. Recent investigations have shown the utility of salivary biomarkers in the diagnosis of malignancies outside the oral cavity [22] such as pancreatic [23], breast [24], and lung cancer [25, 26]. The objective of this study was to evaluate the expression levels of microRNAs *let-7a-2*, *miR-221*, and *miR-20a* in oral biofluid of patients with SCLC and LUAD. By combining these findings with smoking habits and employing a MLR model, we aimed to develop a method for diagnosing lung cancer.

Methods

Ethical statement

Ethical Committee of Tehran University of Medical Sciences approved the conduct of this research project (IR.TUMS.DENTISTRY.REC.1399.106). Before participating in this study, all participants completed a consent form detailing objectives of this investigation. All procedures were conducted in accordance with the standard guidelines for each instrument.

Saliva collection

Forty patients with lung cancer including twenty adenocarcinoma lung cancer and twenty small cell lung cancer patients with central lesions, referred to Masih Daneshvari Hospital's oncology department between 2020 and 2023 (Tehran, Iran) comprised the cases for this study. Twenty healthy individuals referred to the dental school clinic of Tehran University of Medical Sciences (Tehran, Iran) for routine dental checkups in the same year were considered as controls. Inclusion criteria for LC patients was the evidence of LC (SCLC or LUAD) through histopathology. Exclusion criteria for this group included presence of other malignancies, pregnancy, uncontrolled systemic diseases, dental abscesses, and lack of interest to participate. The control group composed of healthy subjects without cancer who were matched in age and gender with subjects in the case group. Exclusion criteria for the control group included the presence or history of stroke, fatty liver, chronic obstructive pulmonary disease (COPD), rheumatoid arthritis, lupus erythematosus,

pregnancy, other chronic diseases, periodontal disease and unwillingness to participate in research (Fig. 1).

All participants were asked to carefully complete a validated and standardized checklist. This allowed disclosure of demographic characteristics and behavioral habits that might influence LC progression. In order to verify the accuracy and thoroughness of responses, completion of each checklists was supervised by one of the trained authors. The supervisor did not provide any directive or clues and, solely provided neutral explanations, when necessary. All individuals were also instructed to disclose history of any diseases, prior radiotherapy, chemotherapy, and cancer surgery as well as habits and lifestyles such as alcohol and tobacco use or addiction to illicit drugs.

Saliva collection

To mitigate the potential impact of circadian rhythm on salivary secretions, oral biofluid samples were collected between 9:00 and 11:00 a.m. To prevent salivary stimulation, patients were instructed not to brush teeth or consume foods, beverages or tobacco within 1 h of sample collection. Unstimulated saliva was collected using spitting method without mechanical or chemical stimulation. Participants were asked to allow collection of saliva in their mouth and to drool gently into a sterile container every 60 s for a total of 5–15 min. Saliva samples were stored in -80˚C until the PCR analysis.

RNA extraction

Trizol reagent was utilized to extract total RNA from saliva (RiboEx Kit; GeneAll Biotechnology, Seoul, Korea). To ascertain the integrity and purity of the isolated total RNAs, gel electrophoresis and the NanoDrop spectrometer (Thermo Scientific, USA) were utilized. The eluted total RNA samples were stored at -80 °C in 30 µL of RNase-free water.

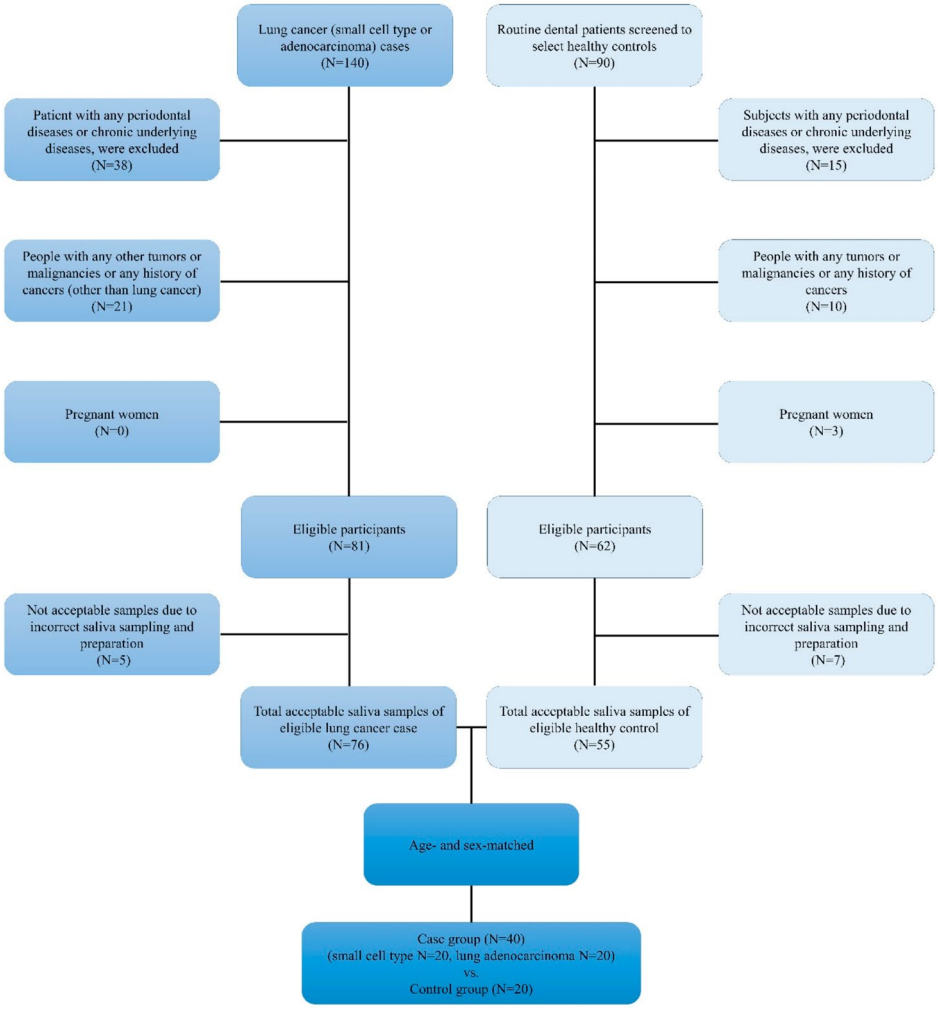

Fig. 1 STARD flow chart for selection of subjects with LC and healthy controls

CDNA synthesis and quantitative real-time PCR (qPCR)

Under the manufacturer's guidelines, synthesis of complementary DNA (cDNA) was executed with the ParsGenome miRNAs kit (ParsGenome, Iran). Reverse transcription-quantative polymerase chain reaction (RT-qPCR) was done with Ampliqon SYBR Green Master Mix (A323402, Ampliqon, Denmark) as well as *miR-20a 5p*, *miR-221 3p* and *let-7a-2 5p* specific primers and housekeeping gene (U6) primers (Table 1). For each sample, all measurements were performed in triplicate, and values averaged for reporting. The expression of U6 snRNA in each salivary sample quantified and served as an endogenous control. The gene expression of U6 snRNA served as a reference for relative expression of miRNA. Raw data are available at GEO database (accession number pending).

Using a Thermal Cycler (Qiagen, Rotor-Gene Q), RT-qPCR was carried out in the following cycles: one cycle lasting 15 min at 95 °C, forty cycles consisting of 15 s at 95 °C, 30 s at 60 °C, and 20 s at 72 °C. Roche software (Roche Group, Basel, Switzerland) was used to calculate the cycle of threshold (Ct). The delta threshold cycle value (ΔCt) for each sample was used to compute the expression of the investigated genes relative to the housekeeping gene. ΔCt_2 and ΔCt_1 represent the ΔCT of case and control group, respectively. The calculation of ΔΔCt involves subtraction of the ΔCT_2 from ΔCT_1. The $2^{-\Delta\Delta Ct}$ technique was used to determine the relative expression levels of miRNAs in relation to U6.

Statistical analysis methods

Statistical analysis was performed using SPSS 18.0.0. (SPSS Inc. Chicago, IL, USA) and Graph Pad Prism software version 9 (GraphPad Software, San Diego, CA, USA). P-values (p) less than 0.05 were considered significant. The study used multiple logistic regression analysis to identify the characteristics with the highest predictive value for salivary *let-7a-2 5p*, *miR-221 3p*, and *miR-20a 5p*. ROC curves were created to evaluate the diagnostic efficacy of salivary *let-7a-2 5p*, *miR-221 3p*, and *miR-20a 5p* for differentiating SCLC and LUAD patients from healthy controls. Youdon's index was used to find optimal cut-off values in ROC curves. In LC patients, MLR with various characteristics differentiated between LUAD and SCLC subtypes of LC. STATA 17.0 utilized to create a forest plot diagram for comparing odds ratios.

Results

Clinical and demographical findings

In the control group, 30.0% of the participants were female and 70.0% were male. In the case group, 22.5% of the participants were female and 77.5% were male. Patients in the case group ranged in age from 38 to 76 years, while control subjects ranged in age from 43 to 76 years (Table 2). The ethnic breakdown of all participants included 65.0% Lurs and Fars, 23.33% Turks, and 11.67% Kurds. 55% of LUAD patients and 95% of SCLC were smokers. Metastases were found in 20% of LUAD participants but not in any SCLC patients. There was no statistically significant difference between healthy controls and LC patients in terms of gender, age, ethnicity, or current alcohol use (Table 2).

Expression analysis of *miR-221 3p*, *miR-20a 5p*, and *let-7a-2 5p*

The expression levels of *miR-221 3p*, *miR-20a 5p*, and *let-7a-2 5p* in saliva samples of forty LC patients (including twenty LUAD participants and twenty SCLC patients) and twenty controls were assessed using qPCR. We found that *let-7a-2 5p* (Fig. 2-D) and *miR-221 3p* (Fig. 2-H) were downregulated (increasing ΔCt in case group and consequently decreasing $2^{-\Delta\Delta Ct}$ and finally downregulation in LC patients) in the saliva of LC patients versus (vs.) controls. We also uncovered significant downregulation of *miR-20a 5p* ($p < 0.001$) in oral biofluid samples from LUAD patients compared to healthy controls (Fig. 2-I). ROC curve analysis was undertaken to assess ΔCt values of salivary *let-7a-2 5p* (Fig. 2:A-C), *miR-221*

Table 1 Primer sequences of *miR-20a 5p*, *miR-221 3p*, *let-7a-2 5p* and housekeeping gene (U6) utilized in RT-qPCR

Genes	Primer sequences
RNU6-1 (U6)	
Backward	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATATGG
Forward	GCAAGGATGACACGCAAATTC
let-7a-2 5p	
Backward	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACTAT
Forward	CACGCTTGAGGTAGTAGGTTGT
miR-221 3p	
Backward	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGAAACC
Forward	CGTGAGGAGCTACATTGTCTGC
miR-20a 5p	
Backward	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTACCT
Forward	GGCTGGTAAAGTGCTTATAGTGC

Table 2 A summary of demographics, clinical features of participants and their laboratory results

Characteristics	LC patients			Healthy controls (*n* = 20)
	SCLC (*n* = 20)	LUAD (*n* = 20)	SCLC & LUAD (*n* = 40)	
Gender				
Female	1 (5.00%)	8 (40.00%)	9 (22.50%)	5 (25.00%)
Male	19 (95.00%) (22.50%)	12 (60.00%)	31 (77.50%)	15 (75.00%)
Age, years	57.85 ± 6.68	56.45 ± 11.83	57.15 ± 9.51	57.25 ± 8.85
Ethnicity				
Lur and Fars	10 (50.00%)	15 (75.00%)	25 (75.00%)	13 (65.00%)
Turk	6 (30.00%)	4 (20.00%)	10 (25.00%)	4 (20.00%)
Kurd	4 (20.00%)	1 (5.00%)	5 (25.00%)	3 (15.00%)
Smoking and alcohol consumption habits				
Current smoking	19 (95.00%)	11 (55.00%)	30 (75.00%)	1 (5.00%)
Current alcohol consumption	5 (25.00%)	2 (10.00%)	7 (17.50%)	0 (0.00%)
Addiction to illicit drugs	12 (60.00%)	3 (15.00%)	15 (37.50%)	0 (0.00%)
Salivary miRNAs (ΔCt value)**				
miR-221 3p	1.32 ± 3.56	0.42 ± 2.56	0.87 ± 3.10	-0.39 ± 2.07
miR-20a 5p	-0.89 ± 2.04	-2.22 ± 1.58	-1.56 ± 1.92	-3.93 ± 1.23
let-7a-2 5p	-5.82 ± 3.46	-7.08 ± 3.58	-6.45 ± 3.53	-7.84 ± 1.75

Values are expressed as mean ± SD or No. (%)

*: P-value (p) for the comparison between patients with LC and healthy control groups

**: ΔCt value: ΔCt for each sample was calculated by subtracting the Ct value of the target miRNA (Ct_{miR}) from the Ct value of the reference miRNA U6 (Ct_{U6})

3p (Fig. 2:E-G), and *miR-20a 5p* (Fig. 2:I-K) from LUAD and SCLC patients vs. controls. The area under the curve (AUC) for *let-7a-2 5p*, *miR-221 3p*, and *miR-20a 5p* were equal to 0.505, 0.578, and 0.815, respectively for LUAD vs. healthy control. The AUC for *let-7a-2 5p*, *miR-221 3p*, and *miR-20a 5p* were equal to 0.645, 0.608, and 0.932, respectively for SCLC vs. healthy control. The AUC for *let-7a-2 5p*, *miR-221 3p*, and *miR-20a 5p* were equal to 0.62, 0.56, and 0.68, respectively for SCLC vs. LUAD.

Multiple logistic regression was used to differentiate LUAD and SCLC from the healthy controls based on combination of *miR-221 3p*, *miR-20a* 5p, and *let-7a-2 5p* (Fig. 3) using main effects. The AUC values of these models were 0.86 (Fig. 3-A), 0.92 (Fig. 3-B), and 0.73 (Fig. 3-C) for differentiate LUAD from healthy, SCLC from healthy control, and SCLC from LUAD.

Multiple logistic regression was performed to differentiate LUAD and SCLC from controls and LUAD from SCLC based on the main effects and tow-way interactions of *miR-221*, *miR-20a*, and *let-7a-2*. The AUC value of the model was 0.93 (Fig. 4-A), 0.965 (Fig. 4-B), and 0.765 (Fig. 4-C) for diagnose LUAD from healthy control, SCLC from healthy control, and LUAD from SCLC, respectively.

To differentiate LUAD and SCLC from controls, and LUAD from SCLC, multiple logistic regression analysis was conducted considering main effects and interactions (two-way and three-way) of *miR-221*, *miR-20a*, and *let-7a-2*. The model achieved an AUC of 0.965 for differentiating SCLC from healthy controls (Fig. 5-A) and 0.765 for distinguishing LUAD from SCLC (Fig. 5-B).

A multiple logistic regression model incorporating miRNAs and smoking habits was used to differentiate LUAD patients from healthy controls. The ROC curve, based solely on the main effects of these predictors, exhibited an AUC value of 0.89 (Fig. 6-A) while Fig. 6-B shows the ROC curve for differentiating SCLC from LUAD (AUC = 0.795).

Table 3 provides a summary result for multiple logistic regression diagnostic models. These findings demonstrated that the fourth model could effectively distinguish between SCLC and LUAD. ROC curve analysis showed that the fourth model had the largest area under the curve (AUC), suggesting it was the most accurate prediction model. These findings show that combination of salivary miRNAs and smoking habits could distinguish between SCLC and LUAD.

Discussion

Numerous investigations have been conducted to assess biomarkers in LC patients. However, most studies have focused on serum and tissues in LC patients rather than salivary biomarkers, especially salivary miRNAs. Utility of biomarkers such as miRNA in oral biofluid for detection of cancer is particularly advantageous because saliva collection in non-invasive and does not require specialized instruments. We, therefore, undertook this investigation to compare salivary expression of miRNA together with clinical and demographic characteristics in LC patients and healthy controls for diagnostic purposes. Our aim was to assess the potential utility of salivary miRNAs as a non-invasive biomarker for diagnosis of

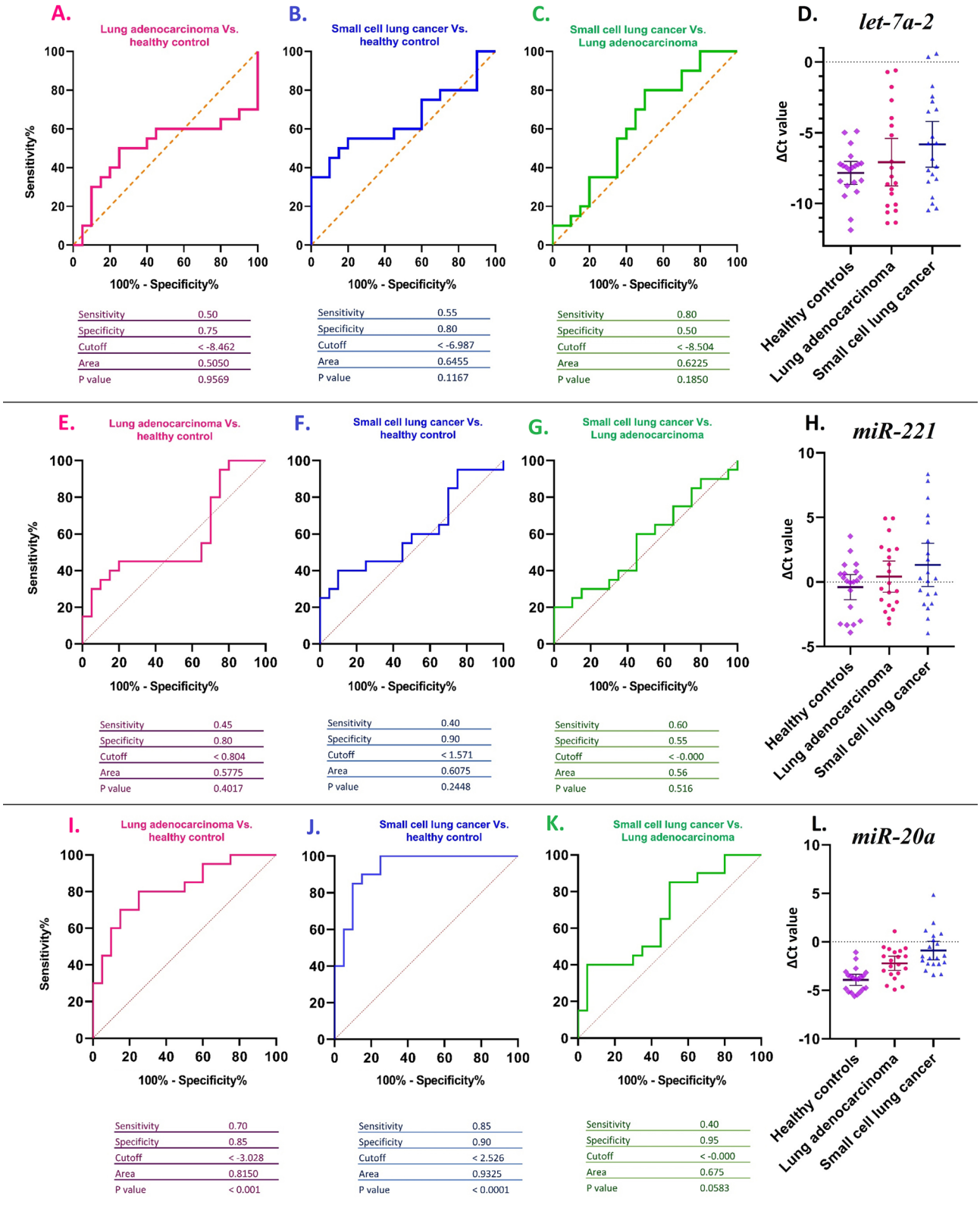

Fig. 2 ROC curves for *let-7a-2* (**A**-**C**), *miR-221* (**E**-**G**), and *miR-20a* (**I**-**K**) comparing LUAD and LUSC groups to healthy controls, and between LUAD and LUSC. Mean ΔCt values (with 95% CI) for *let-7a-2* (**D**), *miR-221* (H), and *miR-20a* (**L**) in saliva for all groups

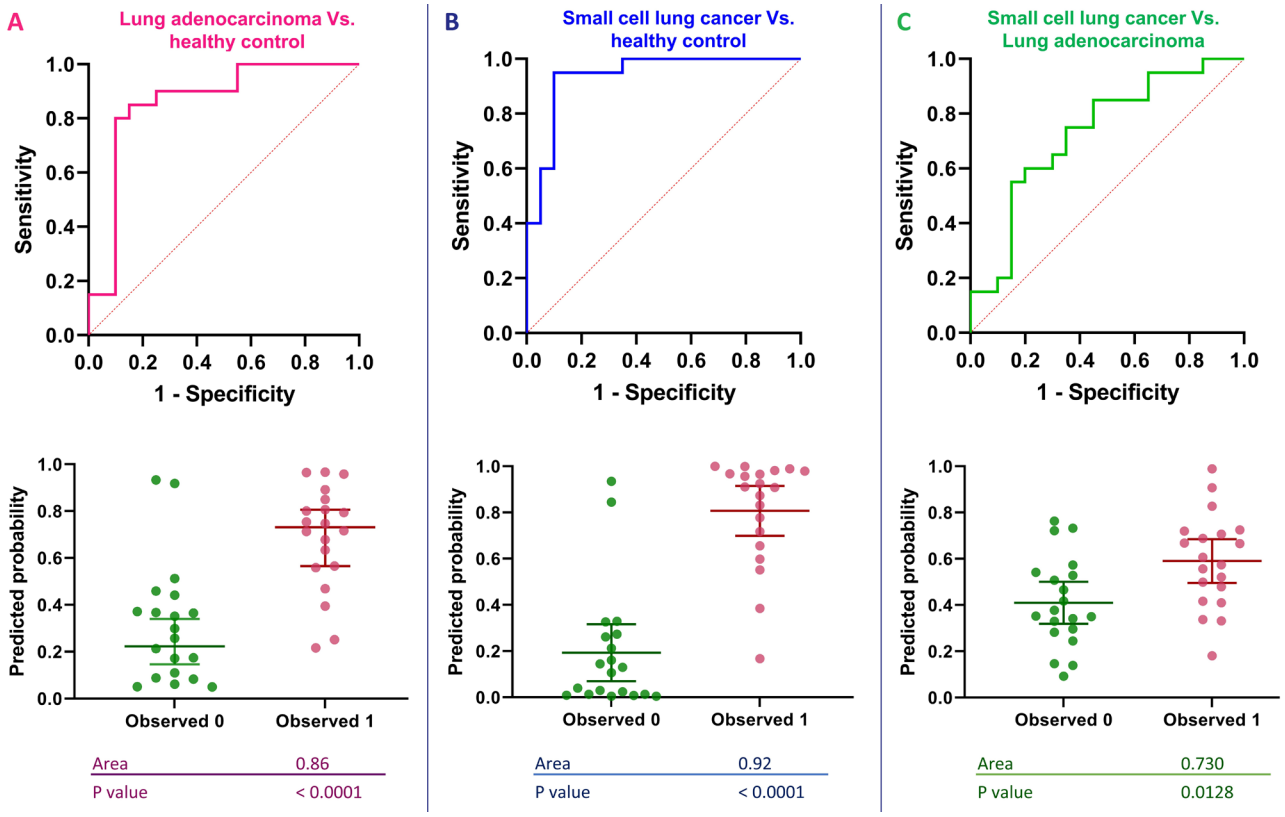

Fig. 3 ROC curve of multiple logistic regression of three miRNAs (*let-7a-2*, *miR-221*, and *miR-20a)* using main effects for **A**) LUAD vs. healthy control, **B**) SCLC vs. healthy control, and **C**) SCLC vs. LUAD

LC and to identify biomarkers in the oral biofluid which could assist with detection of this malignancy. We also used statistical methodology to evaluate the accuracy of these biomarkers in differentiating between different types of LC. Moreover, we generated forest plots to compare various statistical models for diagnosis of LUAD and SCLC from healthy control (Fig. 7). It shows DOR of salivary *miR-20a* is greater than *miR-221* and DOR of *miR-221* is higher than *let-7a-2* for both LUAD and SCLC. Our results showed that MLR based on salivary miRNAs could diagnose both main types of LC (LUAD and SCLC).

Many biomarkers such as proteins, DNAs, and RNAs present in blood could be detected in saliva. Better stability of mRNA and miRNA compared to proteins improves the likelihood of their detection in the oral biofluid [27, 28] particularly because these molecules are carried within exosomes and protected from alteration [29]. Sun and coworkers studied salivary and serum exosomes in LC patients and showed over 80% overlap in exosomal proteins between serum and saliva. They identified eleven biomarkers in both body fluids and suggested their potential utility in diagnosis and monitoring of lung cancer following validation studies [30]. Yang et al. investigated the potential of salivary miRNAs as biomarkers for the diagnosis of malignant pleural effusion (MPE) in LC patients. His group found that two miRNAs, *miR-21* and *miR-486-5p*, were significantly upregulated in oral biofluid of patients with MPE [22]. Zhang et al. investigated salivary transcriptions from patients with SCLC and NSCLC. The group discovered seven different salivary biomarkers and showed a logistic regression model which combines five biomarkers (mRNAs) could differentiate LC patients from the control group (accuracy: 86.46%) [31].

Our study indicates significant downregulation of salivary *let-7a*, *miR-20a*, and *miR-221* (increasing ΔCt and consequently decreasing $2^{-\Delta\Delta Ct}$ and downregulation in LC patients) relative to controls. More specifically, salivary *miR-221* was downregulated significantly in LUAD ($p < 0.001$, Fig. 2-I) and SCLC ($p < 0.0001$, Fig. 2-J) vs. controls. Our observation regarding biomarker *miR-221* in oral biofluid is in line with the majority of studies to date. Xu et al. investigated the role of *miR-221* in regulating NSCLC cell growth and showed increased expression of *miR-221* can promote cell proliferation [32]. Zhang et

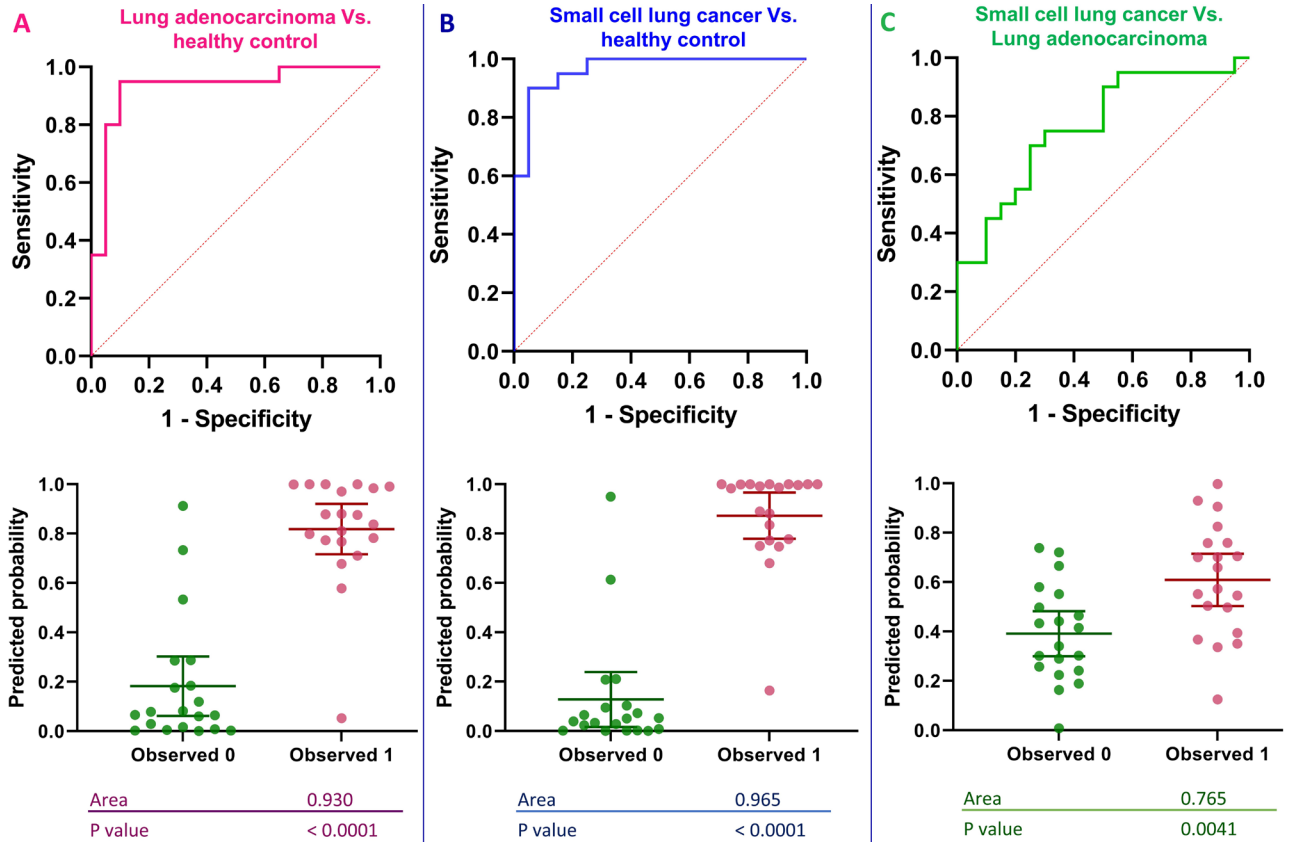

Fig. 4 ROC curve of multiple logistic regression of three miRNAs (*let-7a-2*, *miR-221*, and *miR-20a)* using main effects and tow-way interactions for **A**) LUAD vs. healthy control, **B**) SCLC vs. healthy control, and **C**) SCLC vs. LUAD

al. evaluated the expression level of *miR-221* in NSCLC patients and observed a significant increase in *miR-221* expression compared to healthy tissue samples. Shorter overall survival of patients with higher levels of *miR-221* supports the utilization of *miR-221* as biomarker for NSCLC [33].

Our investigation also showed that saliva samples of patients with LC contained significantly higher levels of *let-7a-2* than saliva samples in the control group. Salivary levels of *let-7a-2* were downregulated in both SCLCs and LUADs compared to healthy controls. Yang et al. demonstrated that exososomal levels of *let-7a* were elevated in patients with chronic inflammatory lung disease and LUAD compared with healthy controls. The group also determined that *miR-21*/*let-7a* was significantly higher in blood samples of patients with LUAD than in healthy individuals, patients with inflammatory lung disease, and patients with benign lung nodules. Their ROC curve analysis showed that *miR21/let-7a* ratio can be a diagnostic tool for LC and benign tumors [34]. Heegaard et al. showed downregulation of the *miR-221* and *let-7a* in serum of cases vs. control group in line with ours [35]. Takamizawa et al. showed that *let-7* are downregulated in lung cancer patients and that *let-7* may also suppress lung cancer cell growth [36]. He at al. state that downregulation of *let-7* plays a critical role in lung cancer [37]. Zhang et al. found that plasma *miR-20a* could serve as a potential biomarker for NSCLC detection with high sensitivity and specificity [38]. Several studies suggested the role *miR-20a* in poor prognosis of lung cancer patients [39, 40].

Our data shows that diagnostic odds ratio (DOR) of *miR20a* is greater than *miR-221*, while DOR of *miR-221* is higher than *let-7a-2* (Fig. 7). Thus, it can be concluded that *miR-20a* and *miR-221* are more reliable biomarkers for diagnosing both LUAD and SCLC. MLR provided most accuracy in differentiating LC from healthy controls by considering salivary miRNAs together in this study. Taken together, the highest DOR was achieved with MLP using main effects and two-way interactions with high sensitivity and specificity. Furthermore, comparison of miRNA expression between LUAD and SCLC revealed significantly higher miRNA levels in SCLC, leading to a greater DOR for this subtype. These findings suggest that

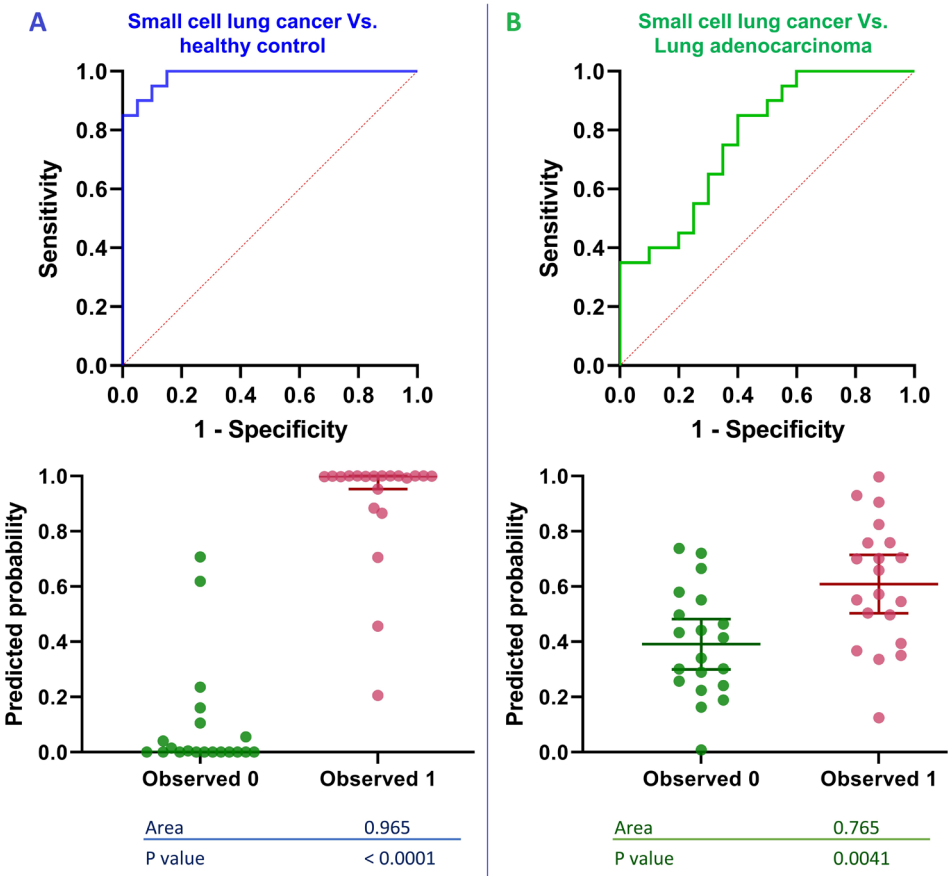

Fig. 5 ROC curve of multiple logistic regression of three miRNAs (*let-7a-2*, *miR-221*, and *miR-20a)* using main effects, tow-way, and three-way interactions for **A**) SCLC vs. healthy control and **B**) SCLC vs. LUAD

Table 3 Results of multiple logistic regression diagnostic models (SCLC vs. LUAD)

Biomarker and method	Sensitivity	Specificity	Accuracy	DOR[#]	AUC[##]
MLR* of α, β, γ (main effects)	0.65	0.65	0.650	3.45	0.730
MLR of α, β, γ (main effects and tow-way interactions)	0.70	0.75	0.725	7.00	0.765
MLR of α, β, γ (main effects, tow-way, and three-way interactions)	0.70	0.65	0.675	4.33	0.772
MLR of α, β, γ, smoking (main effects)	0.80	0.65	0.725	7.43	0.795

DOR[#]: Diagnostic odds ratio

AUC##: Area under the curve

MLR*: Multiple logistic regression analysis

α: *let-7a*, β: *miR-221*, γ: *miR-20a*

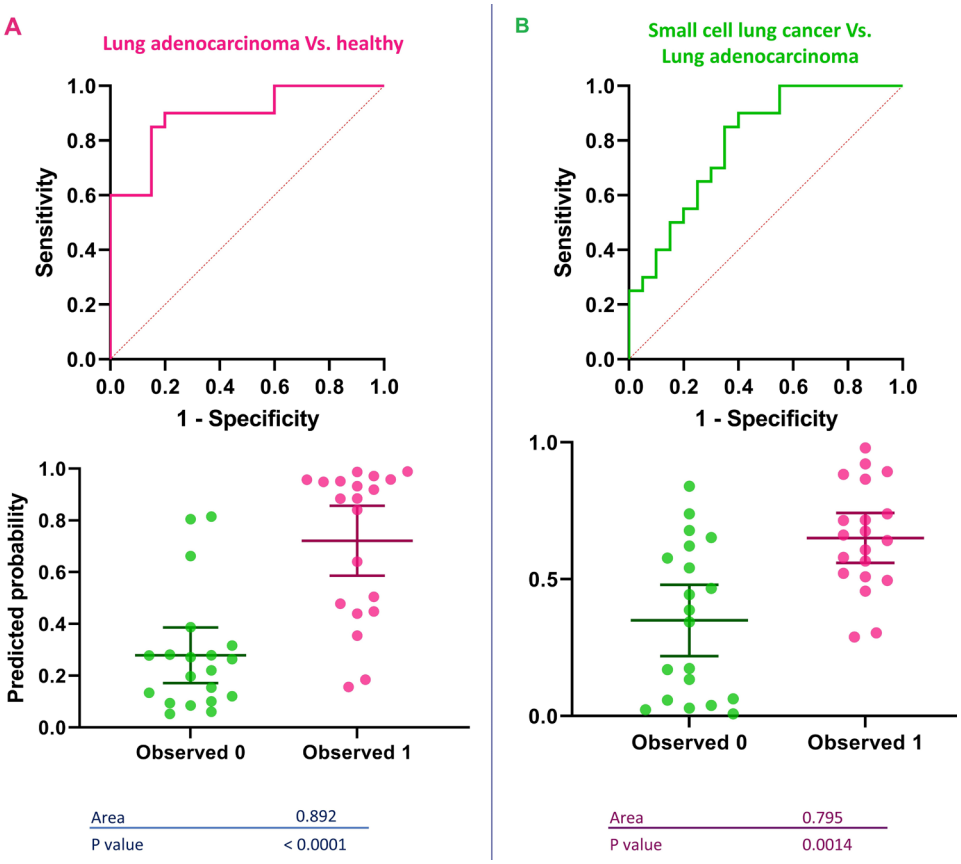

Fig. 6 ROC curve for multiple logistic regression model based on miRNAs and smoking habits using **A**) main effects for differentiating LUAD from healthy controls and **B**) SCLC vs. LUAD

salivary miRNAs may serve as valuable biomarkers for differentiating lung cancer subtypes.

This study has several limitations that should be considered. The sample size may limit the study's statistical power to detect significant differences between groups. Second, the study focuses exclusively on central lesions, which may not generalize the findings to other types of lung tumors. Third, the study uses a specific method for miRNA detection, and any methodological limitations associated with this method, including the reference miRNA (U6), could affect the results. Despite these limitations, this study provides valuable insights into the potential of salivary miRNAs as diagnostic biomarkers for lung cancer. Future studies with larger and more diverse patient samples could further validate the findings of this study and better elucidate the clinical utility of salivary miRNAs. Despite efforts to include ethnic diversity, all participants were from the same country. Multicenter prospective studies with larger sample sizes are recommended to confirm the findings.

Forest plot analysis (Fig. 7) showed that the diagnostic LC using miRNAs (*let-7a*, *miR-221*, and *miR-20a*) in distinguishing between healthy individuals and LUAD patients was lower than SCLC from healthy individuals. Additionally, based on the DOR, *miR-20a*, *miR-221*, and *let-7a* had the highest diagnostic power, respectively. For the differentiation of LUAD from SCLC, the MLR model including *miR-20a*, *miR-221*, and *let-7a* (using main effects, two-way, and three-way interactions) was the most accurate model proposed. Our results strongly support the clinical application of salivary miRNA profiles for lung cancer diagnosis, even in the diagnosis of its subtypes, and encourage further research for clinical applications.

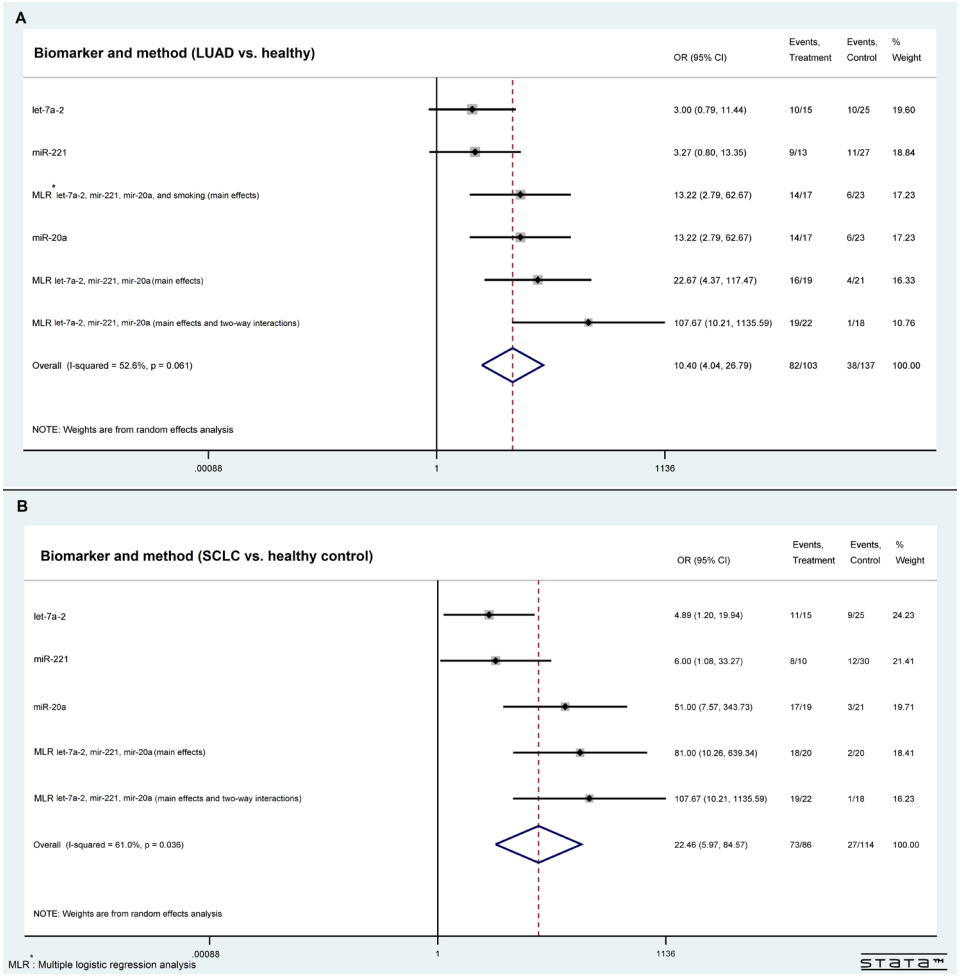

Fig. 7 Forest plots of various model for diagnosis **A**) LUAD from healthy control and **B**) SCLC from healthy control

Conclusion

This study showed that salivary miRNA biomarkers could detect LC with an acceptable accuracy. MLR model using salivary miRNAs was the most accurate for diagnosing LC among the models investigated in this study. This method could serve as a screening test for high risk asymptomatic individuals in clinical settings and help identify those who need additional testing. Considering these findings, salivary miRNA hold the promise of application for lung cancer screening and diagnosis. The simplicity and non-invasive nature of this approach are particularly attractive and likely encourage those at risk to take advantage of it, to avoid unnecessary procedures and to conserve resources.

Abbreviations

AUC	Area under ROC curve
COPD	Chronic obstructive pulmonary disease
CI	Confidence interval
DOR	Diagnostic Odds ratio
LUAD	Lung adenocarcinoma
LUSC	Lung squamous cell carcinoma
MPE	Malignant pleural effusion
miRNAs	MicroRNAs
MLR	Multiple logistic regression analysis
MPE	Malignant pleural effusion
NSCLC	Non-small-cell lung carcinoma
OR	Odds ratio
P	P-value
ROC	Receiver operating characteristic
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction (RNU6-1 (U6))
SCLC	Small-cell lung carcinoma
vs.	Versus

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

Supplementary Material 8

Supplementary Material 9

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Author contributions
MK, HA, and NA led data acquisition and conceived the study concept. The original manuscript of the study protocol was authored by NA, HA, and MK. RM, MK, and SK all made contributions to the data interpretation and analysis, as well as the manuscript preparation. MK, HZ, NA, MF and SK led the writing-review & editing. MK, RM, NA, and HZ interpreted the results. All authors read and approved the final manuscript.

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Data availability
All data generated or analysed during this study are included in this published article and its supplementary information files.

Declarations

Ethical approval
Ethical Committee of Tehran University of Medical Sciences approval was obtained for this research (IR.TUMS.DENTISTRY.REC.1399.106). Before participating in this study, all participants completed a consent form detailing the investigation's objectives. All procedures were conducted in accordance with the applicable guidelines for each instrument. Online version: https://ethics.research.ac.ir/form/osbzthqhbg874rq.pdf.

Consent for publication
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Competing interests
The authors declare no competing interests.

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