



Down-regulation of circulating microRNA let-7a in Egyptian smokers

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ABSTRACT

Altered miRNAs were associated with cigarette smoking. The study aimed to examine the gene expression level of plasma let-7a among healthy smokers and compared it with the non-smokers. Forty subjects were recruited for the present study and classified into 21 smokers and 19 non-smokers, age, and sex were matched. The software that used to design functional primers was MIRprimer. Quantitative real-time PCR was employed to compare the relative expression of plasma let-7a. Results showed that the level of let-7a was down-regulated in smokers to 0.34fold ($p = 0.006$) that of the non-smokers. Plasma let-7a showed an area under curve (AUC) of 0.749 with sensitivity 43% and specificity 100%. In conclusion, plasma let-7a was significantly down-regulated in the smokers, and it might be considered a candidate biomarker to discriminate between smokers and non-smokers.

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1. Introduction

MicroRNAs (miRNAs) are small family of endogenous and non-coding functional RNA. They are approximately 18–22 nucleotides in length. miRNAs would control regulation of gene expression through binding to mRNA, and hence they either inhibit translation or modify mRNA stability [1]. Furthermore, miRNAs are major key factors in regulation of the different biological processes including: development, differentiation, proliferation, cell death, as well as metabolism [2]. Moreover, unlike the common RNA, miRNAs show a high stability against degradation as they are main inclusions in microvesicles as well as exosomes or they are bound to the high-density lipoproteins or to the argonaute 2 protein complex [3], thus, their expression analysis would suggested to be easily detected and tracked in blood, plasma and other biological tissues. Recently, various findings demonstrated that the miRNAs are good biomarkers not only to evaluate the normal & disease conditions but to assess the pharmacological response as well [4].

Cigarette smoking is a high-risk factor for various cases such as lung cancer [5], chronic obstructive pulmonary disease (COPD) [6] and cardiovascular disease [7]. MicroRNAs are already reported to be deregulated in smoking-related diseases [8] along with their

expression profiles that differ between healthy and diseased tissues. Therefore, some miRNAs such as: plasma miR-21, miR-155 and miR-182 have been reported as potential biomarkers for smoking-related disease and consequently, lung cancer [5,9].

Additionally, miRNAs could show tumor suppressors effect, as a contrasting action to their role as oncogenes. Also, miRNAs have different biological roles for some downstream genes [9]. One of the important tumor suppressors is let-7a, which is one of the let-7 family members. The sequence, function, and misregulation of let-7 and its family members are highly conserved across species, it leads to the less-differentiated cellular state and the development of cell-based diseases [10]. The gene expression level of let-7 is lowered in various types of cancers, such as lung, stomach, and colon [11–13].

The aim of this study is to identify the expression of plasma let-7a among healthy smokers and non-smokers.

2. Subject and method

2.1. Study population

Forty subjects were recruited in this study. They were referred to the outpatient clinics of the National Research Center in Cairo, Egypt. The subjects were divided into two groups based on their smoking status to 19 non-smokers (controls) and 21 current smokers. All of the smokers as well as controls were matched in age and

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sex. All participants were males whose ages ranged from 21 to 68 years old with median 40. Smoking index among smokers group ranged between 0.75–84 pack years which express cumulative cigarette exposure and it was determined by multiplying the number of packs of cigarettes smoked (per day) by the number of years the person has smoked. All subjects who had clinical history of past or present diseases which could have some repercussion on the liver or the kidney and any metabolic symptoms such as hyperglycemia, microvascular diabetic complication and chronic hypertensive patients with blood pressure higher than 140/90 were excluded from this work.

All procedures including human participants were in agreement with the amendments of the Ethical Committees of the National Research Centre, Ain Shams University, Egypt, and with the 1964 Declaration of Helsinki and its later amendments. Informed consent Written informed consent was obtained from all individual participants included in the study.

2.2. Primer design for qRT-PCR

Software miRprimer (<https://sourceforge.net/projects/mirprimer/>), is an automatic and easy method. It was used to design functional primers for mir-specific RT-q PCR. The miRNA-specific primer sequences were designed according to miRNA sequence obtained from miRBase database (<http://microrna.sanger.ac.uk>) as seen in Table 1.

2.3. Isolation of human plasma

Whole blood samples were collected from all subjects into EDTA-tubes, then centrifuged for 10 min at 1900×g at 4 °C degree (Haraeus, Labofuge 400R, Germany), and upper yellow plasma phases were transferred carefully into new RNase-free tubes. After that, plasma samples were centrifuged again for 10 at 12,000×g at 4 °C (Haraeus, Labofuge 400R, Germany) to prevent contamination of cellular nucleic acid and the haemolysed plasma samples were excluded from the study. The resultant plasma samples were separated into aliquots and stored frozen at –80 °C until further need.

2.4. Plasma RNA extraction

Total RNA was extracted from 200 µl plasma using miRNeasy serum/plasma cell lysates kit (Qiagen, Germany), Briefly, QIAzol Lysis Reagent (RNA extraction reagent) was added to the sample, and then vortex was applied. Phase separation step was executed by adding an equal volume of chloroform to the starting sample to the tube containing the lysate, and then vortexing was applied followed by centrifugation at 12,000×g for 15 min (4 °C). The upper aqueous phase was separated, and then transferred to a new collection tube. Volume (1.5) of 100% ethanol were added to and mixed with aqueous phase. The sample was transferred into an RNeasy MinElute spin column in tube (2 ml) and then centrifuged. The RWT buffer was added to the RNeasy MinElute spin column. After centrifugation, the RPE buffer was added and the column was centrifuged again. For drying the membrane, the spin column was placed into a new collection tube and the centrifugation was applied at full speed for 2 min. The RNeasy MinElute spin column was placed in a new 1.5-ml collection tube. RNase-freewater

(30 µl) was added directly to the center of the spin column membrane followed by centrifugation for 1 min at full speed to elute the RNA. The RNA was stored in RNase-free water at –80 °C till use.

2.5. let-7a quantification by qRT-PCR

The reverse transcription reaction was carried out using a miScript HiSpec buffer supplied in miScript II RT Kit (Qiagen, Germany), according to the manufacturer's instructions. Quantitative real-time PCR was carried out in Stratagene Mx3000p, real-time PCR system, Agilent Technologies, Germany. The qRT-PCR was performed in a duplicate for each sample using SYBR Green qPCR Master Mix, (Applied Biosystem, USA). The reaction mixture was performed in a total volume of 20 µl containing 4 µl of cDNA (100 ng/µl), 300 nM of each primer set for let-7a, and 10 µl of SYBR Green Master Mix and completed to 20 µl with nuclease-free water. The thermal cycling consisted of an initial denaturation at 95 °C for 5 min followed by 40 cycle of 94 °C for 15 s, annealing for 55 s according to melting temperature suitable for each primer set, extension at 72 °C for 10 s. Comparative cycle threshold (Ct) was calculated to define the expression level of let-7a. The mean of Ct of mir-16 and let-7a in study population were (Avg_Ct = 30.07, Stdev = 2.2) and (Avg_Ct = 30.7, Stdev = 1.1), respectively. Ct values >35 were considered to represent no expression.

2.6. Normalization of plasma let-7a expression level

MiR-16 was selected as a control according to previous studies [14,15]. miRNA relative expression levels were calculated using the following equation: the $2^{-\Delta\Delta Ct}$ method [16]. The fold difference (RQ) in expression was analyzed using the equation: $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct, \text{let-7a-Ct, mir16}) \text{ ALL smoker sample} - (Ct, \text{let-7a-Ct, mir16}) \text{ nonsmoker as control sample}$.

2.7. Statistical analysis

The Mann-Whitney U test was used for the analyses of the expression of let-7a among groups. Receiver operating characteristic (ROC) the curve was plotted between smoker and controls to detect the sensitivities and the specificities for the let-7a and their diagnostic efficacy. Correlations between let-7a and pack years were performed using Spearman's correlation coefficient. Statistical analyses were performed using SPSS software (version 10.0 for Windows; SPSS INC., Chicago, IL, USA) where *P* values were two-tailed and considered statistically significant when less than 0.05.

3. Results

3.1. Demographic characteristics among non-smoker as control and current smokers

As seen in Table 2, both group (current smoker and non-smoker) were matched in ages. Regarding the pack-years, there was a statistically significant difference in favor of the smokers ($p < 0.001$). The prevalence of shisha (hookah) smoking between current smokers was (9.5%) and no difference was detected between two groups.

Table 1
Primers used in qPCR.

MIRNAs	Sequence	Forward	Reverse
miR-16	TAGCAGCACGTAATATTGGCG	Cgcagtagcagcagcga	cagtttttttttttttcgcaa
let-7a	TGAGGTAGTAGGTTGTATAGTT	gcagtgagtagtaggttg	ggccagtttttttttttaactatac

Table 2

The demographic factors of study population.

	Current smoker	Non-smoker	Statistics
n	21	19	
Age (years)			
Median	43	36	Z = -1.26, p > 0.05
Range	(25–68)	(21–61)	
Pack years			
Median	12	0	Z = -5.7, p < 0.001 ^a
Range	(0.75–84)		
Shisha (hookah) smoking (n, %)	2 (9.5%)	0	$\chi^2 = 1.9$, p > 0.05

^a Statistically significant.

3.2. Level of plasma let-7a in current smoker and non-smoker

Total RNA was extracted from smoker and non-smoker groups, to assess the levels of plasma let-7a of both groups using qRT-PCR. A statistically significant difference in plasma let-7a between two groups was observed ($p = 0.006$). Plasma let-7a was down regulated in smoker with 0.34-fold change than in non-smoker as Fig. 1.

3.3. Diagnostic accuracy of circulating let7a

ROC curve analyses were carried to detect the sensitivity & specificity of plasma let-7a as biomarker among smokers. ROC curve analyses showed that AUC was 0.749 (95%CI: 0.596–0.903), $p = 0.007$ as reported in Fig. 2. Where the cutoff value of let-7a was set at 2.09, the sensitivity and specificity were 43% and 100%, respectively. These findings showed that the let-7a had accurate diagnostic value for smokers.

3.4. Correlation between plasma let-7a expression and other demographic factors

The level of let-7a had no correlation with age ($r = -0.102$, $p > 0.05$), while a negative significant correlation was reported between let-7a expression and pack years ($r = -0.466$, $p = 0.002$).

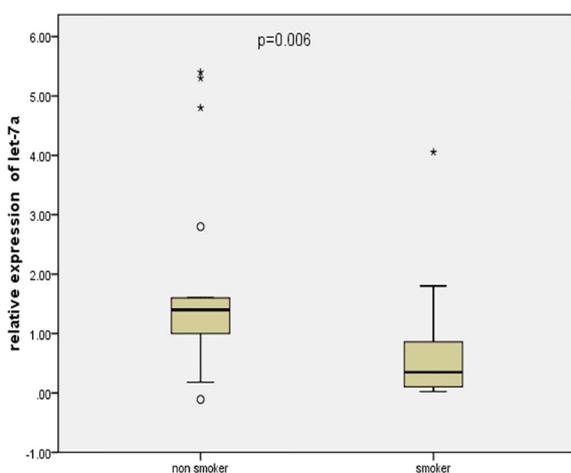


Fig. 1. Relative plasma let-7a levels in smoker and non-smoker population. Expression levels of let-7a were normalized to Mir-16. Data was represent as median value and Mann-Whitney U test was used to define statistical significance.

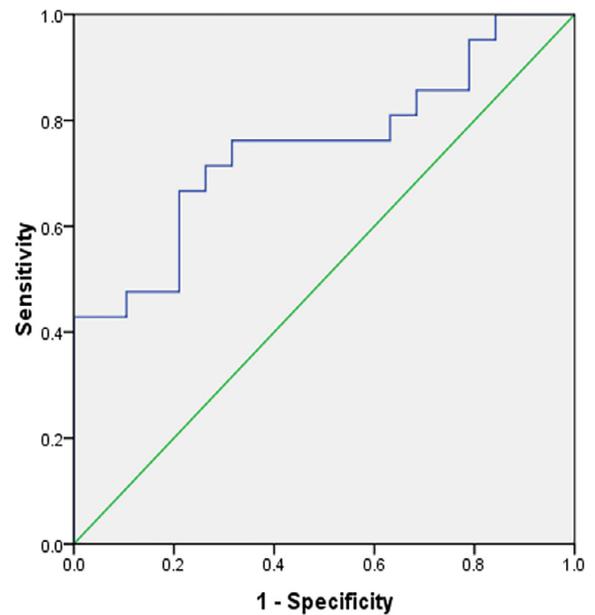
ROC Curve

Fig. 2. Receiver operating characteristics (ROC) curve analysis for investigated plasma let7a.

4. Discussion

The present study showed that plasma let-7a expression was down regulated in the smokers in comparison with the control non-smokers. The previous data confirmed the molecular and genetic variation in the respiratory tract that leads to various diseases, including lung cancer [5], COPD [6], atherosclerosis and asthma [17]. In the literature, altered expression of let-7a has been found in many diseases. The let-7 family was identified firstly in human lung cancers [5]. Lung cancer is responsible for a high percentage of mortality from cancer worldwide, approximately 85% of which are from non-small cell lung cancer (NSCLC) [18]. Takamizawa et al. [11] reported reduced expression of the let-7 microRNA in human NSCLC lung cancers. Furthermore, let-7 family has shown an oncosuppressor activity in NSCLC tumor development in mice xenografts [19]. The let-7 group of miRNAs also interacts with all the Ras protein family, which has oncogenic activity, with reciprocal expression previously being observed in lung tumor samples so that let-7 overexpression has been associated with decreased Ras protein concentrations in human cancer cell lines [20]. This regulation is achieved through direct interaction between let-7 as well as the 3' untranslated region of RAS mRNA [20]. In another study, the reduced expression level of plasma let-7a and its diagnostic value could be used as potential biomarker in diagnosis of esophageal squamous cell carcinoma (ESS) [21].

Moreover, inflammation contributes to the increase of all the smoking-related diseases; one of them diseases is asthma. Asthma is a chronic inflammation disease, characterized by reversible air-flow obstruction and bronchospasm, affecting more than 300 million people of all ages worldwide. Tightly balanced pathways, networks of activators, and suppressors are required for proper regulation of immune responses. MiRNAs have been found to act critical roles in regulating key pathogenic mechanisms in inflammation, involving polarization of adaptive immune responses and activation of T cells such as miR-21 and miR-223, and regulation of IL-13, a key cytokine in allergic lung inflammation as let-7a [22]. Furthermore, Rijavec et al. [23] found that a significantly down-regulation

of let-7a expression in bronchial biopsies from patients who were suffering from severe asthma as compared to patients with mild asthma and those the non-asthmatic controls. These last finding showed that let-7a could be possibly used as a biomarker to discriminate between different asthma Phenotypes.

There is a finding that suggests differences in sample processing and handling would be sources of considerable variation in multiplex assays, For example, plasma processing [24]. There is correlation between the miRNAs which affected by processing and the miRNAs with the highest expression in platelets. One of them is let-7a which is highly expressed in platelets, platelets are not the only source of let-7a, and therefore to minimize the co-founder effects of platelets contamination, a study by Binderup et al. [25] demonstrated that upon collecting fresh samples, it should be added additional centrifugation steps to produce cell-free/platelet-poor plasma.

In the present study plasma let-7a displayed lower expression level in smoker, group compared with their expression levels in non-smoker group ($p = 0.006$). Our finding in line with Izzotti et al. [26] who analyzed the expression level of MiRNAs in the lungs of rats that exposed to environmental tobacco smoke. They revealed that MiRNAs belonged to family let-7 were down-regulation. Small scale studies have reported an association between cigarette smoking and expression level of MiRNAs. One of those studies, Huang et al. [27] has shown a significant down regulation of let-7i-3p in the sera of smokers. Another study showed that let-7c was significantly down regulated in the sputum of currently smoking with chronic obstructive pulmonary disease [28].

In this study plasma let-7a had a significant diagnostic value for the smoker and produced AUC of 0.749 with 43% sensitivity and 100% specificity (95%CI 0.596–0.903) Fig. 2. Thus, the circulating let-7a could be a potential biomarker of biological changes among smokers. Furthermore, we observed a negative significant correlation between expression level of let-7a and pack years ($r = -0.466$, $p = 0.002$). These last findings agree with Banerjee et al. [29] who demonstrated that let-7a was associated with smoking. In the previous study suggesting that the down regulation of let-7a could be reversible in early stages of smoking-related disease but its expression becomes irreversible in cancer [26]. Further, Betsuyaku et al. [30] reported that smoking cessation and age interacted and affected on miRNA profiles.

5. Conclusions

The results suggest that down-regulation of let-7a expression in plasma associated with cigarettes-smoke exposure and it could be used as good biomarker for discriminate between smoker and non-smoker, so that it might detection the smoking-related disease.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interests.

Ethical approval

All procedures including human participants were in agreement with amendments of ethical Committees of the National Research Centre, Ain Shams University, Egypt, and with the 1964 Declaration of Helsinki and its later ethical amendments.

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References

- [1] Carthew RW, Sontheimer EJ. Origins and mechanisms of miRNAs and siRNAs. *Cell* 2009;136:642–55.
- [2] Shen J, Stass SA, Jiang F. MicroRNAs as potential biomarkers in human solid tumors. *Cancer Lett* 2013;329:125–36.
- [3] Sourvinou IS, Markou A, Lianidou ES. Quantification of circulating miRNAs in plasma: effect of preanalytical and analytical parameters on their isolation and stability. *J Mol Diagn* 2013;15:827–34.
- [4] Melo SA, Esteller M. Dysregulation of microRNAs in cancer: playing with fire. *FEBS Lett* 2011;585:2087–99.
- [5] Chen T. The role of MicroRNA in chemical carcinogenesis. *J Environ Sci Health, Part C* 2010;28:89–124.
- [6] Nana-Sinkam SP, Karsies T, Riscili B, Ezzie M, Piper M. Lung microRNA: from development to disease. *Expert Rev Respirat Med* 2009;3:373–85.
- [7] Takahashi K, Yokota S-I, Tatsumi N, Fukami T, Yokoi T, Nakajima M. Cigarette smoking substantially alters plasma microRNA profiles in healthy subjects. *Toxicol Appl Pharmacol* 2013;272:154–60.
- [8] Banerjee A, Luettich K. MicroRNAs as potential biomarkers of smoking-related diseases. *Biomark Med* 2012;6:671–84.
- [9] Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer* 2006;6:857–66.
- [10] Roush S, Slack FJ. The let-7 family of microRNAs. *Trends Cell Biol* 2008;18:505–16.
- [11] Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, et al. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Res* 2004;64:3753–6.
- [12] Zhang H-H, Wang X-J, Li G-X, Yang E, Yang N-M. Detection of let-7a microRNA by real-time PCR in gastric carcinoma. *World J Gastroenterol* 2007;13:2883.
- [13] Akao Y, Nakagawa Y, Naoe T. Let-7 microRNA functions as a potential growth suppressor in human colon cancer cells. *Biol Pharm Bull* 2006;29:903–6.
- [14] Huang Z, Huang D, Ni S, Peng Z, Sheng W, Du X. Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer. *Int J Cancer* 2010;127:118–26.
- [15] Liu CJ, Kao SY, Tu HF, Tsai MM, Chang KW, Lin SC. Increase of microRNA miR-31 level in plasma could be a potential marker of oral cancer. *Oral Dis* 2010;16:360–4.
- [16] Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. *Nat Protoc* 2008;3:1101–8.
- [17] Hancox RJ, Gray AR, Poulton R, Sears MR. The effect of cigarette smoking on lung function in young adults with asthma. *Am J Respir Crit Care Med* 2016.
- [18] Maldonado F, Duan F, Raghunath SM, Rajagopalan S, Karwoski RA, Garg K, et al. Noninvasive computed tomography-based risk stratification of lung adenocarcinomas in the National Lung Screening Trial. *Am J Respir Crit Care Med* 2015;192:737–44.
- [19] Kumar MS, Erkeland SJ, Pester RE, Chen CY, Ebert MS, Sharp PA, et al. Suppression of non-small cell lung tumor development by the let-7 microRNA family. *Proc Natl Acad Sci* 2008;105:3903–8.
- [20] Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, et al. RAS is regulated by the let-7 microRNA family. *Cell* 2005;120:635–47.
- [21] He F-C, Meng W-W, Qu Y-H, Zhou M-X, He J, Lv P, et al. Expression of circulating microRNA-20a and let-7a in esophageal squamous cell carcinoma. *World J Gastroenterol*: WJG 2015;21:4660.
- [22] Kumar M, Ahmad T, Sharma A, Malalirajan U, Kulshreshtha A, Agrawal A, et al. Let-7 microRNA-mediated regulation of IL-13 and allergic airway inflammation. *J Allergy Clin Immunol* 2011;128. 1077–1085. e1010.
- [23] Rijavec M, Korošec P, Žavbi M, Kern I, Malovrh MM. Let-7a is differentially expressed in bronchial biopsies of patients with severe asthma. *Sci Rep* 2014;4.
- [24] McDonald JS, Milosevic D, Reddi HV, Grebe SK, Algeciras-Schimmich A. Analysis of circulating microRNA: preanalytical and analytical challenges. *Clin Chem* 2011;57:833–40.
- [25] Binderup HG, Houliand K, Madsen JS, Brasen CL. Pre-storage centrifugation conditions have significant impact on measured microRNA levels in biobanked EDTA plasma samples. *Biochem Biophys Rep* 2016;7:195–200.
- [26] Izzotti A, Calin GA, Steele VE, Croce CM, De Flora S. Relationships of microRNA expression in mouse lung with age and exposure to cigarette smoke and light. *FASEB J* 2009;23:3243–50.
- [27] Huang J, Wu J, Li Y, Li X, Yang T, Yang Q, Jiang Y. Deregulation of serum microRNA expression is associated with cigarette smoking and lung cancer. *Biomed Res Int* 2014;2014.
- [28] Pottelberge GRV, Mestdagh P, Bracke KR, Thas O, Durme YMV, Joos GF, et al. MicroRNA expression in induced sputum of smokers and patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2011;183:898–906.
- [29] Banerjee A, Waters D, Camacho OM, Minet E. Quantification of plasma microRNAs in a group of healthy smokers, ex-smokers and non-smokers and correlation to biomarkers of tobacco exposure. *Biomarkers* 2015;20:123–31.
- [30] Betsuyaku T, Nishimura M, Moriyama C. Effects of aging and smoking on MicroRNA expression profiling in bronchiolar epithelium. *Am J Respir Crit Care Med* 2010;181:A2025.