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

Sanaa A. Rizk [a], Fateheya M. Metwally [a], Asmaa M. Elfiky [a,*], Asmaa A. Mahmoud [b], Nadia A. Badawi [a], Nevin E. Sharaf [a], Mahmoud M. Elhefnawi [c]

[a] Occupational and Environmental Medicine Department, Environmental Research Division, National Research Centre, Cairo, Egypt
[b] Department of Zoology, Faculty of Science, Ain Shams University, Cairo, Egypt
[c] Informatics and Systems Department, Engineering Research division, National Research Centre, Cairo, Egypt

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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.

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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* Corresponding author.
E-mail address: asmaaelfiky@yahoo.com (A.M. Elfiky).

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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 ΔΔCT = (Ct, let-7a-Ct, mir16) ALL smoker sample−(Ct, let-7a-Ct, mir16) 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	TAGCAGCACGTAAATATTGGCG	Cgcagtagcagcacgta	cagttttttttttttttcgccaa
let-7a	TGAGGTAGTAGGTTGTATAGTT	gcagtgaggtagtaggttg	ggtccagttttttttttttttaactatac

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