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Differential expression of MicroRNA let-7e and 296-5p in plasma of Egyptian patients with essential hypertension

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Abstract

Essential hypertension is a chronic medical condition affecting thousands of people worldwide. Hypertension results from interplay of genetic and environmental factors. MicroRNAs regulate gene expression and can be biomarkers for disease. MicroRNA let-7e and microRNA 296-5p have been linked to different cardiovascular diseases. This study aimed to determine association of serum miRNA let-7e and miRNA 296-5p with essential hypertension in Egyptian patients. MicroRNA let-7e and miRNA-296-5p expression was determined in sera of 25 hypertensive patients and 25 normotensive controls by quantitative real-time polymerase chain reaction. Hypertensive patients showed significantly higher expression of miRNA let-7e (3.23-fold increase, $p = 0.036$) in comparison with normotensive controls. In hypertensive patients, miRNA let-7e expression was positively correlated with increased systolic and diastolic blood pressure. Furthermore, miRNA 296-5p expression was negatively correlated with serum total cholesterol and low-density lipoprotein. Results from this study indicate that miRNA let-7e can potentially be a biomarker for essential hypertension.

Keyword: Cardiology

1. Introduction

Hypertension is a chronic condition caused by environmental and genetic factors. The constant elevation of systemic blood pressure characteristic of hypertension puts patients at risk for coronary artery disease, heart failure, stroke, and chronic renal disease (Romaine et al., 2016). Hypertension can be primary (essential) hypertension, or secondary hypertension. Primary hypertension is constant elevated blood pressure with no underlying medical condition and forms 90–95% of diagnosed cases of hypertension. Secondary hypertension is caused by a diagnosed medical condition that affects the heart, kidney or endocrine system (Batkai and Thum, 2012). The prevalence of hypertension is increasing in both the developed and developing world (Sekar et al., 2017). Several mechanisms are involved in the pathogenesis of hypertension, such as inflammatory reactions (Caillon and Schiffrin, 2016), oxidative stress (Itani et al., 2016), endothelial dysfunction (Kriegel et al., 2015) and impaired angiogenesis (Ferroni et al., 2012). However, the molecular basis of hypertension remains largely unknown (Huang et al., 2017).

MicroRNAs (miRNAs) are short RNA molecules 21–25 nucleotides long and do not encode for any protein. They are post-transcriptional regulators of expression in eukaryotic cells, and are involved in many biological processes, including apoptosis, cell proliferation, stress response, cardiovascular disease and endothelial dysfunction (Batkai and Thum, 2012). A single miRNA can target multiple genes, and conversely, a single gene can be controlled by more than one miRNA. Circulating miRNAs originate from tissues, are stable in plasma, are present in concentrations detectable by available laboratory techniques, and are resistant to endogenous RNases (Mitchell et al., 2008). In addition, plasma levels of miRNAs usually become dysregulated before physical symptoms of disease appear, making them promising biomarkers (Romaine et al., 2016). The potential for using circulating miRNAs as non-invasive, plasma-based biomarkers for detection of disease has been researched and has opened up a field in the monitoring and screening of cardiovascular diseases including hypertension (Nemecz et al., 2016).

Several miRNAs are involved in the development and symptoms of essential hypertension. MicroRNA-126 is essential for maintenance of integrity of the vascular endothelium, suppression of miRNA-126 results in hemorrhage (Levy et al., 2017). MicroRNA-155 was upregulated in plasma of hypertension subjects, where it was found to suppress activity of the angiotensin II type I receptor (Ceolotto et al., 2011). Binding of miRNA-181a to the 3′ untranslated region (UTR) of the renin gene causes decreased renin mRNA levels, and serum levels of miRNA-181a correlates with systolic blood pressure (Romaine et al., 2016). Several other miRNAs were found to be overexpressed in serum of hypertensive patients, but their exact roles have not been identified (Marquez et al., 2015; Levy et al., 2017).

The aim of this study was to assess the expression of miRNA let-7e and 296-5p in serum of Egyptian patients with essential hypertension and normotensive controls. Previous studies have reported alterations in expression of several miRNAs in patients with hypertension (Levy et al., 2017). Of these miRNAs, miRNA let-7e and 296-5p are the focus of our study. MicroRNA let-7e is a regulator of inflammation and endothelial function. Let-7e expression is increased significantly in patients with coronary heart disease (Varga et al., 2014), white coat hypertension (Cengiz et al., 2015), and essential hypertension (Li et al., 2011). Let-7e positively regulates the protein kinase Akt1 in lipopolysaccharide – activated macrophages (Androulidaki et al., 2009). Blood levels of let-7e were higher in patients with ischemic stroke, and a higher level of plasma let-7e was associated with occurrence of ischemic stroke (Huang et al., 2016). Let-7e expression was down-regulated in cardiomyocytes under conditions of ischemic hypoxia (Zhang et al., 2018). Mi-RNA-296-5p plays a role in the regulation of angiogenesis, can enhance apoptosis and inhibit the proliferation of lung cancer cells (Hu et al., 2016). MicroRNA-296-5p expression was found to be down-regulated in hypertensive patients but was up-regulated in white coat hypertension (Cengiz et al., 2015). MicroRNA-296-5p expression was increased in patients with hypertrophic cardiomyopathy with myocardial fibrosis (Fang et al., 2015). Due to the important role of let-7e and miRNA-296-5p in the cardiovascular system, they were selected for investigation in this study. In addition, expression levels of both miRNAs were correlated with biochemical markers of essential hypertension, such as systolic and diastolic blood pressure, serum total cholesterol (TC), high density lipoprotein-cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) and very low-density lipoprotein (VLDL).

2. Materials and methods

2.1. Study population

A case control study was performed on 50 participants from the North Sinai area. Participants were divided into two groups, 25 patients with essential hypertension and 25 normotensive controls. Blood pressure (BP) for all subjects in a sitting position was measured with a standard mercury sphygmomanometer as described (Badawy et al., 2018). Subjects were allowed at least 10 minutes of rest before BP measurement. The BP measurement used in the study was a mean of two independent measurements. Criteria for hypertension were systolic BP greater than or equal to 140 mmHg and/or diastolic BP greater than or equal to 90 mm Hg and/or if the subject was taking anti-hypertensive medication. Subjects with secondary hypertension were excluded. Body mass index (BMI) was calculated as weight (kg)/height (m²). Subjects with BMI \geq 30 kg/m² were considered obese. Written informed consent was obtained from all study participants. The study protocol

was approved by the Research Ethics Committee at the Faculty of Pharmacy, Suez Canal University, and was in the accordance with the principles of the 2013 Helsinki Declaration.

2.2. Sample collection

Venous blood samples (5 mL) were drawn from all subjects after an 8–12 hour fast and added to sterile plain tubes. For serum separation, whole blood was allowed to clot for 30 min at room temperature, then centrifuged at $1200 \times g$ for 10 min at 4 °C. Supernatant was removed to sterile tubes and centrifuged at $4000 \times g$ for 10 minutes at 4 °C. Sera were divided to two aliquots, and frozen at -80 °C. One aliquot was used for lipid analysis. The second aliquot was used for RNA isolation. Triglycerides (TG), TC and HDL-C were measured with an automated chemical analyzer A15 (Biosystem, USA). LDL-C was calculated with the Friedwald's formula (Friedwald et al., 1972).

2.3. RNA isolation and quantitative real time polymerase chain reaction (qRT-PCR)

Prior to RNA extraction, sera were inspected for hemolysis visually and spectrophotometrically by measuring absorbance of hemoglobin at 414 nm using a NanoDrop™ spectrophotometer. An A_{414} reading greater than 0.2 was considered hemolytic (Kirschner et al., 2013) and the sample was excluded. Total RNA was extracted from serum samples with the Qiagen miRNeasy Mini kit (Qiagen, Hilden, Germany), according to manufacturer instructions. RNA concentration and quality were assessed spectrophotometrically with a NanoDrop™ 1000 spectrophotometer (Thermo scientific, USA).

Expression of miRNAs was determined using qRT-PCR. RNA was reverse transcribed to complementary DNA (cDNA) using the miScript II RT Kit (Qiagen, Germany) and oligo-dT primers. Sera were diluted in RNase-free water immediately before use to ensure equal amounts of RNA in the reverse transcriptase (RT) reaction. Each RT reaction (20 μ L final volume) consisted of 4 μ L miScript HiFlex buffer, 2 μ L 10x nucleics mix, 5 μ L (10–20 ng) RNA, and 9 μ L RNase-free water. RT reactions were incubated at 37 °C for 60 min, then 95 °C for 5 min to inactivate reverse transcriptase.

Quantitative PCR was performed in a PikoReal-Time PCR instrument (Applied Biosystems, USA), with miScript SYBR Green PCR Kit (Qiagen, Germany). The qPCR reactions (20 μ L final volume) contained 10 μ L 2X QuantiTect SYBR Green PCR master mix, 2 μ L miRNA specific primers (200 nm final concentration), 4 μ L cDNA and 4 μ L RNase-free water. cDNA was diluted 1:10 immediately before use. U6 was used as an internal control for normalization between samples. Primers used in qPCR

are shown in [Table 1](#). Duplicate reactions and a no-template control were run for all samples. Cycling conditions were as follows: 15 min at 95 °C, 40 cycles of 94 °C for 15 sec, 55 °C for 30 sec and 70 °C for 30 sec. Melt curve analysis confirmed primer specificity. The comparative cycle threshold (ΔC_t) was used to calculate the expression of miRNAs. Mean C_t values were calculated for all samples. The ΔC_t value was calculated by subtracting C_t values of miRNA U6 from the C_t values of the target miRNA. The relative expression of miRNAs was analyzed using the standard $2^{-\Delta\Delta C_t}$ method ([Livak and Schmittgen, 2001](#)).

2.4. Statistical analysis

The data collected from patients and controls was expressed as the mean \pm the standard deviation. The χ^2 test was used to compare the difference of qualitative variables between the two groups. The quantitative variables were compared by Student's t-test. Differences in relative expression of miRNAs were assessed by the one-way analysis of variance (ANOVA). Relationship between miRNA expression and clinical parameters was determined using Pearson's correlation coefficient. The SPSS 17.0 software package (SPSS, Chicago, IL, USA) was used to process data at ($p < 0.05$).

3. Results

Twenty-five patients with essential hypertension and 25 normotensive controls were recruited for the study. The basic characteristics and blood pressure measurements of studied groups are shown in [Table 2](#). Age, sex distribution, BMI, smoking rate, TC and LDL-C were not different among the groups. Systolic and diastolic blood pressure were elevated in the hypertensive group. Hypertensive subjects also had higher serum TG and VLDL-C and lower serum HDL-C.

The internal control U6 was stably and uniformly expressed in all samples, with no significant difference between hypertensive patients and control ([Table 3](#)). Relative expression of miRNA let-7e was increased in hypertensive patients compared with controls ([Table 3](#), [Fig. 1A](#)). The mean let-7e expression in patients increased 3.23-fold compared with control ($p = 0.036$). There was no significant increase in mean miRNA-296-5p expression in hypertensive patients ($p = 0.46$) ([Table 3](#), [Fig. 1B](#)).

Increased systolic and diastolic blood pressure were positively associated with let-7e expression ($p = 0.03$ and 0.04 , respectively). There was no significant correlation between let-7e expression and serum TC, TG, LDL-C, HDL-C and VLDL. There was also no correlation between let-7e expression and age, BMI, sex distribution and smoking.

Table 1. Primers used in the study.

		Primer sequence
miRNA Let-7e	Forward	5'GGG TGAGGTAGGAGGTTGT3'
	Reverse	5'CAGTGC GTGTCGTGGAGT3'
miRNA 296-5p	Forward	5'GAAGGGCCCCCTCA3'
	Reverse	5'GTGCGTGTGTCGTGGAGT3'
U6	Forward	5'GCTTCGGCAGCACATATACT AAAAT3'
	Reverse	5'CGCTTACGAATTTGCGTGCAT3'

Table 2. General characteristics of study population.

Variables	Control (n = 25)	HTN (n = 25)	P
Age (mean ± SD)	48.2 ± 10.5	49.15 ± 11.7	0.197
Sex (F/M); n (%)	(13/12); (52/48)	(12/13); (48/52)	0.381
BMI (mean ± SD)	27 ± 4.4	27.5 ± 3.3	0.096
Smoking (%)	16%	24%	0.382
Systolic BP (mmHg)	116 ± 15	147 ± 26*	<0.001
Diastolic BP (mmHg)	77 ± 8.6	92 ± 9.3*	<0.001
TC (mg/dL)	176.27 ± 43.4	198.9 ± 68.9	0.171
TG (mg/dL)	113.8 ± 48.8	165.2 ± 58.7*	0.002
LDL-C (mg/dL)	115.8 ± 48.7	137.9 ± 66.9	0.188
HDL-C (mg/dL)	37.7 ± 17.9	27.9 ± 13.7*	0.036
VLDL-C (mg/dL)	22.8 ± 9.8	33.1 ± 11.71*	0.001

HTN: hypertension, BMI: body mass index, BP: blood pressure, LDL: low-density lipoprotein, HDL: high-density lipoprotein. Data are presented as mean ± SD, Comparisons between age and BMI were with unpaired student-t test while sex and smoking were compared by χ^2 test. *P-value < 0.05 was considered statistically significant.

Table 3. MicroRNA expression in hypertensive patients (n = 25) and normotensive controls (n = 25).

miRNAs		Mean of C_T	ΔC_T	$\Delta\Delta C_T$	Fold change ($2^{-\Delta\Delta C_T}$)	P value
Mir let-7e	HT group	29.25	-2.76	-1.69	3.23*	0.036
	Control group	31.81	-1.07			
Mir 296-5p	HT group	23.31	-7.49	-0.34	1.26	0.46
	Control group	25.45	-7.15			
U6	HT group	27.94				0.284
	Control group	27.61				

C_T : threshold cycle number. $\Delta\Delta C_T$ and fold change calculated versus control. * Significantly different from normal control at $p < 0.05$.

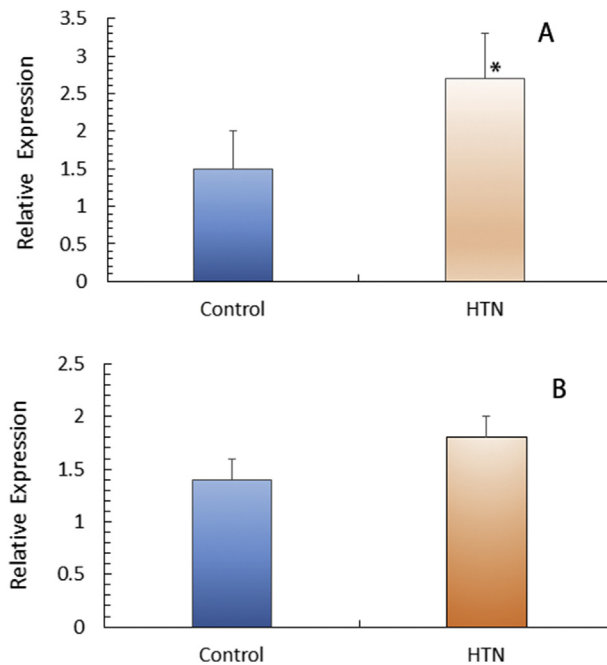


Fig. 1. Relative expression of (A) miRNA let-7e and (B) miRNA 296-5p. * $P < 0.05$. HTN: hypertension.

Table 4. Relationship of miRNA-let7e and miRNA-296-5p expression and blood pressure and lipid profile.

	miRNA let-7e		miRNA-296-5p	
	r	P	r	P
Systolic BP (mmHg)	0.31	0.03	-0.16	0.27
Diastolic BP (mmHg)	0.28	0.04	-0.12	0.38
TC (mg/dL)	0.11	0.52	-0.46	0.02
TG (mg/dL)	0.17	0.46	-0.25	0.08
LDL-C (mg/dL)	0.25	0.08	-0.41	0.003
HDL-C (mg/dL)	-0.15	0.31	0.25	0.07
VLDL-C (mg/dL)	0.11	0.48	-0.13	0.36

BP: blood pressure, HDL: high-density lipoprotein, LDL: low-density lipoprotein. Pearson's correlation coefficient was used for analysis.

MicroRNA 2996-5p expression had a significant negative correlation with serum TC and LDL-C (Table 4). There was no significant correlation between miRNA-296-5p expression and systolic or diastolic blood pressure, serum TC, HDL-C, VLDL-C and age, BMI, sex distribution and smoking.

4. Discussion

Essential hypertension affects greater than 1 billion adults worldwide and is a predisposing factor for congestive heart failure, stroke and myocardial infarction (Romaine et al., 2016). Despite continuous advances in treatment methods, essential hypertension remains a serious condition with significant mortality and morbidity. The molecular basis for development of essential hypertension remains unknown. Dysregulated miRNA expression is related to numerous diseases (Rupaimoole and Slack, 2017). Specific microRNA patterns have been correlated with cardiovascular diseases, including heart failure, ischemic heart disease, atherogenesis, white coat and pulmonary hypertension (Batkai and Thum, 2012; Ferroni et al., 2012; Nemezc et al., 2016; Huang et al., 2017).

MicroRNAs of the let-7 family are highly expressed in cells of the cardiovascular system, such as endothelial cells, vascular smooth muscle cells, cardiomyocytes and arterial smooth muscle cells (Rao et al., 2009). The precise function of let-7e is unknown, but it is thought to play a role in mediation of inflammation. The cellular origin of plasma let-7e is from endothelial cells (Li et al., 2011). A study reported that among 27 differentially expressed miRNAs in plasma of 13 patients with essential hypertension, miRNA let-7e was upregulated (Li et al., 2011). Cengiz et al. (2015) reported upregulation of plasma let-7e in patients with hypertension and white coat hypertension. The results from this study agree with previous reports. MicroRNA let-7e showed a 3.23-fold increase in expression in hypertensive patients compared with normotensive controls. Systolic and diastolic blood pressure were both positively correlated with let-7e expression. These data support a positive relationship between let-7e and essential hypertension, suggesting that let-7e can be a potential regulator and biomarker for hypertension.

Let-7e is involved in the regulation of inflammation through a complex network. Let-7e promotes activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and its translocation to the nucleus by inhibition of expression of its target gene I κ B β , thus increasing expression of adhesion and inflammatory molecules. Let-7e also decreased expression of lnc-MKI67IP-3, further aggravating inflammation (Lin et al., 2017). Both these roles indicate that let-7e plays roles in inflammatory responses of vascular endothelial cells, and therefore can lead to development of hypertension as well as atherosclerosis.

Elevated let-7e expression was not significantly associated with dyslipidemia in this study. This disagrees with Krause et al. who reported that let-7e expression was negatively associated with serum HDL-C, and positively associated with increased serum triglycerides in children with metabolic syndrome. However, the correlation was significant only during the co-existence of at least three traits of the metabolic syndrome, and was most strongly correlated with increased waist circumference

(Krause et al., 2015). Subjects in this study were mostly pre-obese, with BMI between 25 and 30 kg/m² (according to the World Health Organization diagnostic criteria), and thus did not have elevated waist circumference. The dyslipidemia observed in patients in this study could be due that essential hypertension is often complicated by dyslipidemia, regardless of BMI and waist circumference, and is not related to miRNA let-7e expression (Sonawane et al., 2011).

MicroRNA-296 is involved in many biochemical processes such as cell growth, angiogenesis and glucose metabolism (Cazanave et al., 2011). MicroRNA 296-5p expression was not significantly changed in hypertensive patients compared with controls in this study. Previous studies have shown that miRNA 296-5p expression is downregulated in hypertensives (Li et al., 2011). Cengiz et al. reported miRNA 296-5p to be downregulated in patients with hypertension, but upregulated in patients with white coat hypertension, and systolic, diastolic and ambulatory BP were negatively correlated with miRNA 296-5p (Cengiz et al., 2015).

Serum miRNA-296 -5p expression was negatively correlated with serum TC and LDL-C. Literature reports miRNA-296-5p as being involved in regulation of cholesterol and lipid metabolism, but the mechanisms are not known. A previous study reported down regulation of miRNA-296-5p in liver samples from patients with non-alcoholic steatohepatitis (NASH) (Estep et al., 2010). Cholesterol metabolism primarily occurs in the liver, and NASH is commonly associated with obesity, dyslipidemia and hypercholesterolemia (Ferreira et al., 2014).

The small sample size is a major limitation of this study. In addition, the study is observational. This does not allow firm establishment of cause-effect relationships. However, data from the study agree with those reported in the literature and offer preliminary data into the expression of serum miRNA let-7e and miRNA-296-5p in essential hypertension.

To conclude, serum level of miRNA let-7e was significantly higher in hypertensive patients. MicroRNA let-7e was positively correlated with elevated systolic and diastolic blood pressure. Serum miRNA-296-5p was negatively correlated with serum total cholesterol and low-density lipoprotein. While a larger sample size is required to further validate the results obtained in this study, the data indicate that serum miRNA let7e is a potential biomarker for essential hypertension.

Declarations

Author contribution statement

Heba K. Badawy: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Dina M. Abo-Elmatty: Conceived and designed the experiments; Analyzed and interpreted the data.

Noha Mesbah: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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