Circulating miR-21 and TGF- β 1 expression levels in patients with cardiovascular diseases

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ABSTRACT

Recent works focus on the role microRNAs (miRNAs) play in regulation of cardiovascular pathology. One of the miRNAs highly expressed in cardiovascular system is miR-21.

The aim of this study was to investigate the relationship between the expression level of miR-21 in cardiac disease and correlate with TGF- β 1, one of its targets.

Methods: Plasma samples from patients with CVD (cardiovascular diseases) established according to clinical and paraclinical exams were subjected to total RNAs isolation. miR-21 expression levels in patients with CVD and in healthy control subjects were quantified on ABI 7300 real-time PCR (Applied BioSystems) and normalized to RNU-43. TGF- β 1 was predicted as potential target of miR-21 using MicroCosm TARGETS and quantified in TaqMan system (normalized to GAPDH). Statistical analysis was performed with GraphPad Prism.

Results: Significant lower levels of miR-21 were found in CVD patients vs. controls ($p < 0.0001$). An inverse correlation between miR-21 and TGF- β 1 expression levels was noted in CVD patients. miR-21 levels vs. control were significantly different for ATHE, CAD and ATH groups ($p = 0.0016$, 0.0058 and 0.0032 respectively) and correlated with TGF- β 1 expression and cardiac overload.

Conclusions: The results of this study suggest a good correlation between miR-21 and TGF- β 1 in cardiovascular diseases.

Key words: miR-21, TGF- β 1, cardiovascular disease

INTRODUCTION

Cardiovascular diseases represent the major cause of morbidity and mortality in developed countries. Their development involve genetic and epigenetic alterations that were object of many studies in the past decades.

miRNAs have emerged as novel gene regulators including pathways associated with cardiovascular pathology (1). MicroRNAs (miRNAs) are a class of naturally occurring small noncoding RNAs. They bind to the complementary target sites in 3'-untranslated regions and suppress targeted mRNA through translational inhibition or transcriptional degradation (2,3). Each mRNA can be

targeted by several miRNAs and each miRNA might target different mRNAs. As more than 30% of genes are regulated by this epigenetic mechanism, miRNAs are implied in normal development and pathogenesis (4). miRNAs might modulate various pathophysiological mechanisms of CVD, the most important being atherosclerosis. In atherosclerosis, miRNAs exert a dual role, this making them potential diagnostic and prognostic biomarkers, especially in coronary pathology. Targeting miRNAs might become a new strategy for the treatment of CVD.

Recent studies show that miR-21 is highly expressed in cardiovascular system, its levels being deregulated under CVD conditions (5). In animal model, it was reported down-regulated expression levels of miR-21 in infarcted hearts while an upregulated expression in border areas of the infarct (5) correlated to a protective effect against ischemia-induced cardiac cell death. Also, Yin et al. demonstrated that myocardial infarct size was significantly reduced after treatment with heat-shock- or preconditioning-induced miRNAs including miR-21 (6,7). Still, there are controversial data about miR-21 levels in cardiac diseases, probably because different types of samples (tissue, blood) were used or depending on the time point at which samples are taken (8).

Hemodynamic overload caused by chronic hypertension, atheromatosis or chronic myocardial infarction leads to adaptative remodeling of the heart, a complex process that can include cardiomyocyte hypertrophy, fibrosis, EMC deposition. At molecular level, TGF- β 1 is one of the key mediators of these processes. In cardiac tissue repair, new connective tissue needs to be formed, this being achieved by fibroblasts migration and proliferation in the lesion. TGF- β 1 induces fibroblasts to produce and remodel ECM (extracellular matrix) resulting in healing the wound (9). This cytokine is released especially by platelets (10) and is expressed in high concentrations in inflammatory and pro-thrombotic states. Increased expression of TGF- β 1 was found in human atherosclerotic plaques (11) and in heart failure (12), while in the overloaded human heart its up-regulation is directly dependent by the fibrosis degree (13). Studies conducted on transgenic mice had shown that TGF- β 1 overexpression leads to interstitial fibrosis and hypertrophic growth of cardiac myocytes (14).

Multiple miRNA target prediction tools are now available. Using MicroCosm TARGETS software, ver. 5, (TargetScan, ver. 7) TGF- β 1 (transforming growth factor- β 1) was proposed as a predicted target of miR-21.

The aim of this study was to investigate miR-21 circulating levels in patients with cardiovascular diseases and correlate with TGF- β 1 expression.

MATERIALS AND METHODS

Study group

59 subjects, aged 23 - 83 years old (mean = 53.6) were selected from patients with CVD treated in "Coltea" Clinical Hospital and Institute of Geriatric and Gerontology "Ana Aslan" during 2011-2013. The patients were selected based on clinical and paraclinical exams (EKG, coronarography, TTE - transthoracic echocardiogram, Holter EKG, MATA, ABI and metabolic profile). Also, risk factors like smoking, blood pressure, lipid and glycemic profiles were analyzed. A control group consisting in subjects without CVD (n=21, aged 18 - 69 years old, mean = 47.9) was included.

From each enrolled subject blood samples were collected in EDTA sprayed tubes after written informed consent was obtained. Plasma samples were obtained following centrifugation at 700 rpm for 6 minutes.

Total RNA isolation

Total RNA was isolated from plasma samples using Trizol reagent (Invitrogen) according to manufacturer instructions. After on-column DNase I treatment with RNase-free DNase (Qiagen), RNA quantity and quality were determined using a NanoDrop Technologies ND-1000 spectrophotometer and Experion Analyser (BioRad) (RIN>7.5). Samples were stored at -80°C and used to generate cDNAs.

miR-21 quantification

The expression levels of miR-21 in patients with CVD and healthy control subjects were quantified using TaqMan MicroRNA assays (Applied Biosystems). 10 ng total RNA were used for miRNA specific reverse transcription according to manufacturer protocol: each sample was incubated 30 minutes at 16°C, 30 minutes at 42°C, than 5 minutes at 85°C. The reverse transcription reaction was performed at 37°C for 1 hour followed by 5 min at 95°C for enzyme denaturation.

miR-21 expression levels were quantified on ABI 7300 real-time PCR (Applied BioSystems) and normalized to RNU43. RNU43 has demonstrated both stable and abundant expression across different human tissues and organs; it is regarded as one of the controls with the least variability for miRNAs assays and has been widely used in different fields (15). Each reaction was performed in a final volume of 25 μ l containing 5 μ l cDNA (10ng), 12.5 μ l TaqMan Universal PCR Master Mix

(No AmpErase) and 1.25 µl TaqMan miRNA probe (Applied BioSystems).

The thermal cycle was set as follows: 10 min template denaturation at 95°C, 40 cycles of denaturation at 95°C for 15 sec and primer annealing/elongation at 60°C for 1 min.

Bioinformatic tools

MicroCosm TARGETS software, version 5 (16) was used to find miR-21 mRNA predictive targets. This program uses miRanda algorithm to find potential binding sites for a given miRNA and verifies the complementarity at the 5' end of the miRNA throwing away alignments with more than one base not complementary with a target site. Targets are passed through Vienna RNA folding routines to estimate the thermodynamic stability. TGF-β1 was found to be a potential target of miR-21 having a Pct = 0.83 (probability of conserved targeting – according to Friedman et al 2009 (17).

TGF-β1 quantification

For estimating TGF-β1 (predicted target gene) expression, total RNA was reverse transcribed using oligo-dT primers (Promega). Briefly, 2.5 µg RNA, 1 µl oligo-dT (0.5 µg/µl) and 1 µl dNTPs (10 mM) were incubated 5 minutes at 65°C. After cooling on ice, 4 µl RT buffer (5x), 2 µl DTT (200 mM) and 1 µl RNase inhibitor (40U) were added. After 2 minutes at 37°C, 1 µl of M-MLV (200U) reverse transcriptase was added and the samples were incubated for 60 minutes at 37°C and afterwards 15 minutes at 70°C. cDNAs were stored at -20°C until use.

TGF-β1 levels were quantified on ABI 7300 real-time PCR (Applied BioSystems) and normalised to GAPDH. Each reaction was performed in a final volume of 25 µl containing 5 µl cDNA (100 ng), 12.5 µl TaqMan Universal PCR Master Mix (No AmpErase) and 1.25 µl

TaqMan TGF-β1 probe (Applied BioSystems).

To check for amplicon contamination, every run contained a negative control in which nuclease-free water was a substitute for template. Samples were tested in duplicate.

Statistical analysis

Data collected were analysed using Graph Pad Prism software (Graph Pad Software Inc., San Diego, CA, USA) for the statistical analyses. For each group, the values were expressed as mean, median, minimum and maximum, and standard error. One-way ANOVA (and non-parametric) test was used for analysing the groups and distinguish the significant differences and Mann-Whitney test (non-parametric) values for comparing each case group with controls. P values <0.05 were considered significant. Linear regression and Spearman correlation were used to investigate the correlation degree between the expression levels of miR-21 and TGF-β1.

RESULTS

The physical and physiological characteristics of the patients and controls are presented in *table 1*. No significant differences ($p>0.05$) between cases and controls were observed for sex, age, smoking, HDL and diastolic blood pressure.

Higher values were obtained for fasting glucose, total cholesterol and LDL ($p=0.0112$, 0.0057 and 0.0186 respectively), while values for HDL were similar ($p=0.1301$).

Analyzing the circulating levels of miR-21 we found significant differences between patients and controls ($p=0.0003$). Assessing TGF-β1 levels, differences between groups were significant ($p=0.0079$).

Linear regression test between TGF-β1 and miR-21 n-fold values was performed (*figure 2*) in order to

Table 1. Patients' characterization. * = significant p

	CASE	CONTROL	P (case / control)
number of cases	59	21	-
men / women	27/32	12/9	0.3767
age, [years]	53.6 (23 – 83)	47.9 (18 – 69)	0.1014
smokers [y/n]	21/38	11/10	0.1823
Fasting glucose, [mg/dl]	108.1 (78 – 259)	89.6 (81 – 102)	0.0112*
CHOL [mg/dl]	195.9 (114 – 293)	150.6 (80 – 188)	0.0057*
HDL [mg/dl]	42.9 (33.0 – 68.0)	47.3 (26.8 – 57.1)	0.1301
LDL [mg/dl]	125.1 (83.5 – 168.7)	92.7 (87.0 – 99.1)	0.0186*
Systolic blood pressure (mmHg)	138.1 (100.0 – 190.0)	116.6 (95.0 – 135.0)	$P<0.0001^*$
Diastolic blood pressure (mmHg)	75.16 (55.0 – 100.0)	70.1 (55.0 – 90.0)	0.1307

Figure 1. The expression levels of miR-21 (a) and TGF- β 1 (b) in patients and controls

verify the cytokine as a potential target of this miRNA.

An inverse correlation between the investigated parameters was observed as the test returned a good correlation coefficient ($r^2 = 0.3753$).

Based on cardiovascular diagnosis and according with ESC guides, the patients were divided into 4 distinct groups: (1) atheromatosis (ATHE) (2) coronary artery disease - CAD (3) cardiomyopathy (CM) and (4) arterial hypertension (AHT). The patients in ATHE group presented Ankle-Brachial Index (ABI) < 0.7, those with CAD displayed > 50% coronary stenosis and the subjects with CM had < 40% LVEF.

ANOVA test was performed to compare the expression levels of miR-21 and TGF- β 1 in the four groups of patients. miR-21 and TGF- β 1 significantly differences among the studied groups were noted ($p < 0.0001$ and $p = 0.0002$ respectively) (figure 3).

Figure 2. Correlation between miR21 and TGF- β 1

Figure 3. miR-21 (a) and TGF- β 1 (b) expression levels in cases and controls

miR-21 expression levels vs. control were significantly different for ATHE, CAD and ATH groups ($p=0.0016$, 0.0058 and 0.0032 respectively).

For TGF- β 1, increased values were found in ATHE, CAD and AHT groups, this sustaining that TGF- β 1 over-expression is correlated with cardiac overload. Significant differences were noticed for ATHE ($p<0.0001$), CAD ($p=0.0017$) and AHT ($p=0.0021$) groups when compared with controls (table 2).

Aging is accompanied by remodeling of the regulatory pathways, including miRNAs. The levels of the circulating miRNAs are suggested to display an age-dependent pattern that can modulate either the longevity of processes they control, or age-related diseases (18). On the other hand, TGF- β levels also correlates with aging and age-related diseases (CVD). Therefore, in our study we investigated the correlation between subjects' age vs. miR-21 and TGF- β 1 respectively (figure 4).

We found lower miR-21 levels in aged patients, and, as a consequence, TGF- β presented higher values, regardless the cardiovascular conditions. Spearman

correlation returned -0.5058 for miR-21 and 0.5501 for TGF- β 1.

DISCUSSION AND CONCLUSIONS

Most miRNAs linked to CVD display altered expressions as a consequence of the complex miRNAs network that regulate the cardiac physiologic processes. Among these miRNAs, miR-21 has been shown to play important role in development of heart disease. Several studies reported a protective effect of miR-21 shortly post AMI (acute myocardial infarct) (19, 20) and in dilated, ischemic and hypertrophic cardiomyopathy with heart failure (21). In our study, CM patients presented higher levels of miR-21 compared with CAD, ATHE and AHT groups and lower levels comparing with controls.

The rupture of atheroma fibrous cap leads to abnormal miR-21 and miR-24 levels that together regulate the matrix metalloproteinases components of the fibrous head. On the other hand, in vitro TGF- β 1 activates endothelial matrix metalloproteinase expres-

Table 2. miR-21 and TGF- β 1 expression levels (n-fold reported to house-keeping RNU-43 and GAPDH respectively)

		ATHE	CAD	CM	AHT	CONTROL
miR-21 expression level (n-fold)	Mean	1,096	1,167	2,274	1,540	2,561
	Median	1,050	1,195	2,082	1,167	2,564
	Minimum	0,05867	0,4963	0,9053	0,4262	0,8979
	Maximum	2,408	1,641	4,633	3,181	4,778
	Std. Error	0,1714	0,1397	0,1837	0,2320	0,2923
	P	0.0016	0.0058	0.7081	0.0032	n/a
TGF- β expression level (n-fold)	Mean	1,526	1,610	0,5974	1,613	0,4859
	Median	1,388	1,858	0,5116	2,085	0,5735
	Minimum	0,4413	-0,5307	-0,3727	0,09121	-0,4537
	Maximum	3,227	2,381	1,956	2,926	1,573
	Std. Error	0,2638	0,3683	0,1175	0,2592	0,1156
	P	<0.0001	0.0017	0.1208	0.0021	n/a

*To compare each group with the controls, MANN-WHITNEY test was performed

Figure 4. Correlation between miR21 (a) and TGF- β 1 (b) expression levels and age

sion and activity via miR-21, this confirming interconnectivity between them (22). Diehl et al noted that CAD patients without AMI display low levels of miR-21 (23). The results are similar to Lacatta et al who noted miR-21 downregulation in CAD cases with unstable angina and AMI (24).

We found miR-21 decreased expression levels of miR-21 in ATHE, CAD and AHT groups, as compared with controls. For CAD, the results confirm data of Diehl et al. On the other hand, our data are not concordant with study of Li et al who found increased miR-21 levels in patients with peripheral arterial disease (atherosclerosis obliterans), but their study was performed on tissue samples (25).

In subclinical atherosclerosis miR-21 was found increased (26). In our study, patients enrolled in ATHE group display lower levels of miR-21 vs control.

miR-21 values were correlated with the expression levels of TGF- β 1. Down-regulation of miR-21 was found in aged patients. F. Olivieri et al reported higher miR-21 values in aging, but on healthy subjects (18). Several studies performed on both human and animal model showed that TGF- β 1 expression levels increase in stressed cardiac myocytes (27). Our data demonstrate that TGF- β 1 has increased expression in blood of patients having different heart and circulation conditions, that may stress the myocardium (atheromatosis, coronary artery disease, arterial hypertension), but the values were found to be similar with the controls in the case of patients with cardiomyopathy. In several studies TGF- β 1 was found particularly overexpressed in ventricular myocardium during transition from stable hypertrophy to heart failure (28,29). The authors considered that TGF- β 1 mediates the adaptation of heart muscle to overload.

A good correlation was found between expression levels of miR-21 and those of TGF- β 1 in concordance with the bioinformatics prediction that TGF- β 1 could be a target of miR-21.

During aging the heart suffers changes like hypertrophy, decreased in left ventricular diastolic and systolic function, arterial rigidity and impaired of endothelial function. Also, apoptotic and necrotic processes decreased cardiomyocytes and increased oxidative stress, leading to proinflammatory status.

The results of this study suggest a good correlation between miR-21 and TGF- β 1 in cardiovascular diseases.

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