ORIGINAL PAPER

Endokrynologia Polska
DOI: 10.5603/EP.a2019.0061
Volume/Tom 70; Number/Numer 6/2019
ISSN 0423–104X

Expression of selected angiogenesis-related small microRNAs in patients with abnormally increased secretion of glucocorticoids

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Abstract

Introduction: Higher cortisol levels are associated with cardiovascular morbidity and mortality in the elderly, partially resulting from biologic effects of glucocorticoids (GCs) on endothelial cells observed in an experimental setting. These features are replicated in patients with endogenous GC excess (Cushing's syndrome) or with exogenous hypercortisolism due to excessive pharmacological application of GCs. Both groups present also an increased cardiovascular disease event rate. GCs may also adversely influence recovery after myocardial infarction. Recently it was proposed that microRNAs (miRNAs) — small noncoding RNAs functioning as antisense regulators of gene expression by targeting mRNA — may have a central role in regulating endothelial function through multiple mechanisms. Thus, the purpose of this study was to evaluate the effects of chronic GC excess on the expression of selected endothelium-controlling miRNAs expressed in nucleated cells circulating in peripheral blood (PBNCs) of patients with endogenous hypercortisolism either due to corticotrophin independent or corticotrophin dependent Cushing's syndrome (CS).

Material and methods: Peripheral blood nuclear cells were collected from 35 healthy subjects and 31 patients with endogenous hypercortisolism as a source of miRNAs. A self-validated individual quantitative RT-PCR study was then performed to evaluate the expression levels of selected miRNAs in PBNCs. Additionally, endothelin-1 (ET-1) expression in peripheral blood was assessed with respect to endothelial dysfunction using Western blotting.

Results: The ET-1 expression levels in CS were higher than in controls, confirming endothelial dysfunction in the CS group. Furthermore, miRNA analysis revealed a significantly decreased intracellular expression of selected endothelium-related miRNAs in patients with endogenous hypercortisolism, including miRNA-17-5p, miRNA-126-3p, and miRNA-126-5p, compared to controls. In contrast, two other angiogenic miRNAs, miRNA-150-5p and miRNA-223-3p, were significantly upregulated compared to controls.

Conclusions: Cardiovascular events related to hypercortisolism remain a challenging problem in medical practice. This study has demonstrated that the chronic excess of GCs in endogenous CS might induce significant dysregulation of selected miRNAs involved in the control of endothelium biology. However, the lack of knowledge about specific miRNA expression postpones the full understanding of the biological roles of such miRNAs in hypercortisolism. Moreover, dysregulated miRNAs seem to be promising targets for further research, especially to search for potential therapies for several GC-induced cardiovascular complications. **(Endokrynol Pol 2019; 70 (6): 489–495)**

Key words: *endocrine diseases; Cushing's syndrome; cardiovascular abnormalities; microRNA*

Introduction

Glucocorticoids (GCs) are used extensively to cure many diseases due to their anti-proliferative, immunosuppressive, and anti-inflammatory effects [1]. Thus, synthetic GCs are widely used with multiple indications and many administration forms used for both systemic and local disorders, including inflammatory and autoimmune diseases or haematological neoplasms. About 1% of the general population are using long-term GC therapy at any given time, due to the increased prevalence of diseases requiring such therapy [2–3]. There is growing concern that markedly increased use of GCs can lead to supraphysiological GC exposure and may induce specific adverse cardio-metabolic complications, such as dyslipidaemia, adiposity and central obesity, decreased insulin sensitivity, hyperglycaemia and diabetes, inflammation, immune dysregulation and platelet activation, and finally hypertension and atherosclerosis followed by cardio-vascular derangements, such as increased risk of coronary artery disease, heart insufficiency, or stroke [4–5]. These exogenous

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GC-related complications have been strictly linked to increased cardio-vascular morbidity or mortality [4–5]. Nevertheless, there is no medical consensus on the best strategies for the prevention and detection of GC-induced complications, and available data on these adverse events are rather poor, with many of the clinical studies being inconclusive, resulting in the lack of optimal treatment strategies for patients affected with glucocorticoid-induced adverse cardio-metabolic disorders.

Nonetheless, some of these questions can be answered by analysing available data from patients with hypercortisolism in the course of endogenous Cushing's syndrome (CS), which is a model of the pathological state with extremely elevated cortisol levels. This is a condition characterised by excessive and continuous elevation of cortisol, usually caused by a pituitary or adrenal adenoma. It is known that ACTH and cortisol play a complex role in the regulation of carbohydrate, lipid, and protein metabolism, energy homeostasis, and the regulation of body fluids and body composition, all of them strongly related to vascular functions [6]. Thus, the excess of endogenous cortisol can lead to a variety of clinical manifestations, including cardio-metabolic disorders. Although long-term elevated cortisol is an important cardiovascular risk factor and higher cortisol levels were associated with a higher incidence of cardiovascular diseases (CVD), the molecular mechanisms responsible for GC-induced cardiovascular complications are not well established. Several studies have suggested the crucial role of nitric oxide (NO), eicosanoids, and oxidative stress together with renin-angiotensin system alterations in haemodynamic changes in the cardiovascular system of patients with hypercortisolism [7]. Glucocorticoid overexposure leads to alterations in the cardiovascular system structure and function also via vasoconstrictor factor endothelin-1 (ET-1). It was demonstrated that GCs induce ET-1 release, and suppress the activity of the NO system and thus cause vasoconstriction resulting in endothelial dysfunction [8]. Related studies have also shown that endothelial dysfunction is involved in other GC-induced vascular pathologies including hypertension [9], myocardial failure [10], dyslipidaemia [11], and atherosclerosis [12]. The coexisting persistent inflammatory environment further contributes to elevation of ET-1 [13], endothelial dysfunction, and impaired endothelium-dependent relaxation, all contributing to the pathogenesis of cardiovascular complications [14].

Importantly, the mechanisms for the development and progression of cardio-metabolic complications and the markers for remission and recurrence have not been sufficiently investigated yet. Several studies suggested that epigenetic regulation based on microRNA (miRNA) would have some importance in the development and progression of GC-related cardiovascular complications. These small non-coding RNAs control gene expression by targeting messenger RNA (mRNA) and disturb translation, and thus represent a major class of molecular regulators. An increasing number of specific miRNA expression signatures have been identified in CVD, and they have gained importance in recent years by helping diagnosis as biomarkers. It has also been demonstrated that miRNAs influence various genes known to be associated with angiogenesis. "AngioMIRs" are a set of miRNAs that play important roles in the regulation of angiogenic responses and endothelial cell (EC) functions. For instance, the miRNA-126 family, composed of two strands: miRNA-126-3p and miRNA-126-5p, is enriched in endothelial cells and is recognised as a leading regulator of angiogenesis and vascular integrity. The miRNA-126-5p strand promotes EC proliferation, improving re-endothelialisation after vascular injury [15]. In contrast, miRNA-126-3p interferes with the process of EC activation by inhibiting the expression of VCAM-1 molecules and impeding leukocyte adhesion to ECs and their transmigration [16]. The miRNA-126 cluster is a master regulator in endothelial dysfunction as well. The level of both miRNA-126 (miRNA-126-3p and miRNA-126-5p) were lowered in diabetic patients, and their expression was reduced in EC exposed to high glucose in vitro [17]. Low levels of miRNA-126 in diabetic EC are associated with decreased proliferation, migration, and NO production [18]. These observations suggest a strong role of the miRNA-126 cluster in the modulation of atherosclerosis development and vascular complications in diabetes. Other miRNAs shown to regulate angiogenesis, such as miRNA-17 cluster, are regulated by vascular shear stress and promote the growth of blood vessels [19]. Of note, blood levels of miRNA-17 and miRNA-126 were decreased in stable coronary artery disease. Regulation of EC function has also been demonstrated in the case of miRNA-150, which, secreted from activated monocytic cells, was able to enhance EC migration [20]. Other studies showed that levels of miRNA-150 were higher in patients with myocardial infarct and increased in response to inflammatory stimuli [21]. Likewise, miRNA-223, the expression of which is dependent on intracellular cholesterol levels, has also been reported to play a role in endothelium, especially due to the fact that miRNA-223 negatively regulates the inflammatory response by blocking the NLRP3 inflammasome and IL-1β production [22]. Altogether, it seems that the involvement of miRNAs in angiogenesis and endothelial cell function in cardiovascular system is substantial, making miRNAs presumably major governing forces in the pathogenesis of endothelial dysfunction developing

in different CVD and cardio-metabolic complications found in other diseases.

The purpose of the study was to evaluate intracellular PBNC expression levels of five selected candidate "angioMIRs" that regulate various angiogenesis signalling pathways and endothelial dysfunction potentially involved in the pathogenesis of GC-induced cardiovascular complications in patients with Cushing's syndrome. We advocate that CS, usually affecting young and presumably healthy patients, is a good in vivo model to examine the effects of GC excess on the expression of angiogenesis-related microRNAs known to affect endothelial cell homeostasis in human vasculature, minimising other confounding factors including the disease itself, the dose and type of GC, as well as the compliance of the patients.

Material and methods

Characteristics of the study group

Thirty-one patients with hypercortisolism due to newly diagnosed endogenous Cushing's syndrome (CS), as a result of either adrenocortical or pituitary tumours [23], were recruited in the Department of Endocrinology of the Pomeranian Medical University in Szczecin, Poland. The control group consisted of 35 age-matched participants with no symptoms or signs of CS (defined as the absence of typical clinical features of CS). All of the enrolled subjects underwent a complete medical examination for statistical analyses. Data regarding medical history, current drug use, and smoking status were collected based on laboratory data, pathology tests, and other information, with a particular focus on heart and vascular conditions and existing arterial hypertension. The study adhered to the tenets of the Declaration of Helsinki, and approval was obtained from the Local Research Ethics Committee. Moreover, each patient provided written, informed consent for involvement in the study.

Blood sample collection

Venous blood samples (~7.5 mL) collected in EDTA tubes were centrifuged (2000 rpm, 4°C, 10 min), and the plasma was stored at −20°C to −80°C until assayed. Next, the red blood cells were lysed using BD Pharm Lyse lysing buffer (BD Biosciences, San Jose, CA, USA) for 15 min at room temperature to isolate peripheral blood nuclear cells (PBNCs).

Western blot analysis

The total protein concentrations were determined using the Bradford protein assay (Sigma-Aldrich). Subsequently, the extracted proteins (20 μg/well) were separated on 14% gel (SDS-PAGE; Mini Protean Tetra Cell System; Bio-Rad). Fractionated proteins were transferred onto a 0.2-μm PVDF membrane (Bio-Rad), and the membranes were blocked with 3% bovine serum albumin (BSA) in buffer for one hour at RT. Endothelin-1 protein was detected using rabbit polyclonal IgG antibody (Santa Cruz Biotechnology) and goat anti-rabbit IgG with horseradish peroxidase as a secondary antibody (Santa Cruz Biotechnology). The membranes were developed with Western blot analysis (ECL Advance Western Blotting Detection Kit; Amersham Life Sciences, Buckinghamshire, UK), and bands were subsequently visualised (Gel DOC-It Imaging system; Bio-Rad, Hercules, CA). Equal loading in the lanes was evaluated by stripping the blots for two hours at 37°C and then overnight at RT (IgG Elution Buffer; Thermo Scientific, Rockford, IL) and re-probing them with GAPDH (goat polyclonal IgG) HRP-conjugated antibody. Protein levels were analysed densitometrically by ImageJ software.

MicroRNA analysis

MicroRNA for molecular analysis was obtained from peripheral blood nuclear cells (PBNCs). Cellular RNA was isolated from 3×10^6 nucleated cells using the mirVana Isolation Kit with organic phenol extraction (Thermo Fisher Scientific, MA, USA) according to the manufacturer's protocol. The RNA concentration was measured using an Epoch Microplate Spectrophotometer (BioTek, VT, USA), and 100 ng was used for first-strand cDNA synthesis. For cDNA synthesis, 4 μL of each sample was used. First-strand cDNA synthesis was performed in all samples using a qScript microRNA cDNA Synthesis Kit (Quantabio, MA, USA). qPCR for the assessment of miRNA expression was performed with a Bio-Rad CFX96 Real-Time Detection System (Bio-Rad, CA, USA). The reaction solution consisted of 1 μL of cDNA sample, iQ SYBR Green Supermix (Bio-Rad, CA, USA), Universal Primer provided in qScript microRNA Synthesis Kit, and a forward primer specific to miRNA analysis. Quantification of the target microRNA expression value was expressed as 2^{Ct}. To find the best reference gene, the NormFinder algorithm was used; miRNA-93 was set as a reference miRNA.

Statistical analysis

The arithmetic means and S.D.s were calculated on an IBM computer using Statistica version 5.0 software (Chicago, IL, USA). Data are given mostly as the mean ± S.D., and were analysed using the Mann–Whitney U test. Statistical significance was defined as $p < 0.05$.

Results

In total, 66 subjects were evaluated. The Cushing's syndrome (CS) and healthy control groups were matched for age and gender as well as well-known cardiovascular risk factors, including hypertension, history of ischaemic heart disease, myocardial infarct, cerebral stroke, peripheral artery disease, and aortic aneurysm. There were no significant differences in clinical characteristics between the study groups except for the existence of hypercortisolism in the former group.

Endothelial dysfunction marker analysis

Hypercortisolaemia is associated with increased risk of endothelial dysfunction. Endothelin-1 is a powerful vasoconstrictor peptide of small and large vessels, which also acts as a modulator of vasomotor tone. It was reported that in most CVD the circulating levels of ET-1 were increased, and ET-1 at elevated pathological concentrations acts also as proinflammatory and promotes cell proliferation, and thus attributes to the development of atherosclerosis and hypertension. Many cardiovascular complications are associated with endothelial dysfunction, which is particularly defined as dysregulation of the vascular function associated with an imbalance in the close interdependence of NO and ET-1 [24]. Due to difficulties in NO measurement, the analysis of ET-1 is presumably a good marker of vascular endothelial dysfunction. Thus, taking into consideration its biological and pathophysiological significance, we first analysed the expression profile of ET-1 using anti-ET-1 antibody in plasma of peripheral

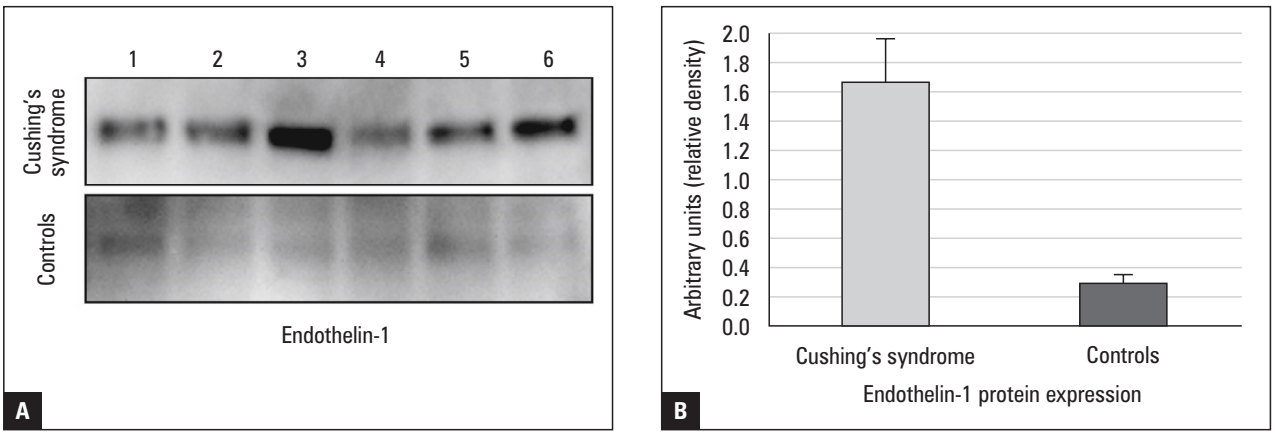

Figure 1. *The expression profile of endothelium dysfunction-related molecule, ET-1, in peripheral blood from patients with Cushing's syndrome and healthy controls. The protein expression of ET-1 was determined by Western blot (**A**). Protein bands obtained were analysed by densitometry (**B**). Bars represent mean ± standard deviation (SD) of selected protein to GAPDH ratios calculated in both examined groups. Representative images of the performed analyses are shown*

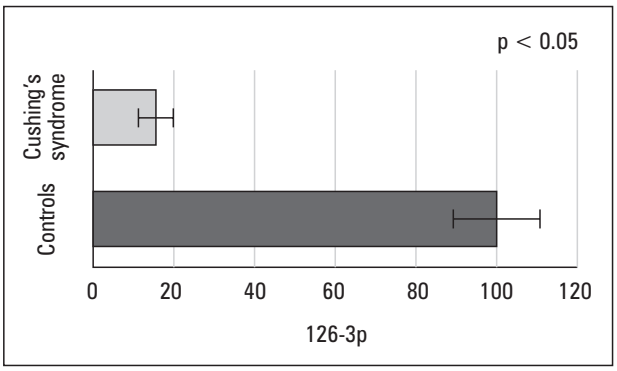

Figure 2. *Cellular miRNA-126-3p profile in Cushing's syndrome (CS) patients and controls. MiRNA-126-3p was significantly down-regulated in peripheral blood nuclear cells (PBNCs) collected from patients with CS compared to healthy volunteers. The results are expressed as the percentage of the control value taken as 100%. *p < 0.05 vs. control group*

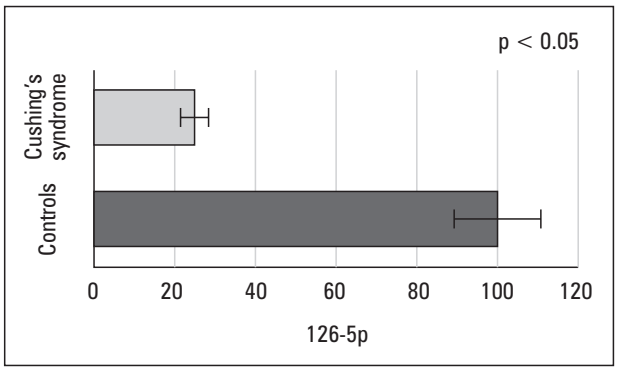

Figure 3. *Cellular miRNA-126-5p profile in Cushing's syndrome (CS) patients and controls. MiRNA-126-5p was significantly down-regulated in peripheral blood nuclear cells (PBNCs) collected from patients with CS compared to healthy volunteers. The results are expressed as the percentage of the control value taken as 100%. *p < 0.05 vs. control group*

blood collected from all examined subjects. As shown in Figure 1, we observed significantly increased expression of ET-1 protein in CS patients compared to healthy controls. This result indicates that patients with CS recruited to this study could develop endothelial dysfunction, and thus potentially suffer from GC-induced cardiovascular complications.

MicroRNA expression profiles

Next, we performed a quantitative analysis of the expression of the selected miRNAs in peripheral blood nucleated cells (PBNCs) of Cushing's syndrome patients and controls using qRT-PCR. We chose a panel of five miRNAs (miRNA-17-5p, -126-3p, -126-5p, -150-5p, and -223-3p). Of the five analysed miRNAs, three microRNAs (miRNA-17-5p, -126-3p, and -126-5p) showed significantly lower expression, whereas two microRNAs (miRNA-150-5p and -223-3p) showed significantly higher expression in PBNCs circulating in the cardiovascular system of CS patients compared with the expression in these cells collected from control subjects (Fig. 2–6). These results indicate that selected "angioMIRs" are differently expressed in CS patients and healthy subjects, which may be related to the developed endothelial dysfunction present in the former group.

Discussion

This is the first study evaluating both plasma ET-1 expression profile and selected angiogenesis-related microRNA transcript quantification in cells circulating in PB from patients with active Cushing's syndrome.

The relationship between glucocorticoids and cardiovascular disorders has been extensively studied,

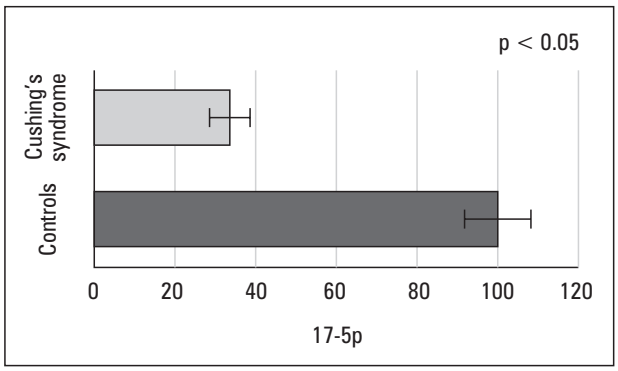

Figure 4. *Cellular miRNA-17-5p profile in Cushing's syndrome (CS) patients and controls. MiRNA-17-5p was significantly downregulated in peripheral blood nuclear cells (PBNCs) collected from patients with CS compared to healthy volunteers. The results are expressed as the percentage of the control value taken as 100%. *p < 0.05 vs. control group*

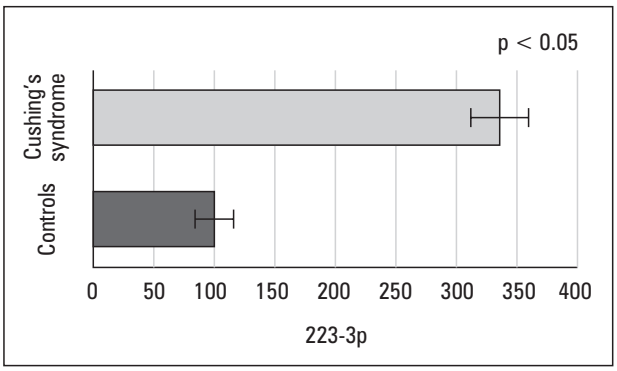

Figure 6. *Cellular miRNA-223-3p profile in Cushing's syndrome (CS) patients and controls. MiRNA-223-3p was significantly upregulated in peripheral blood nuclear cells (PBNCs) collected from patients with CS compared to healthy volunteers. The results are expressed as the percentage of the control value taken as 100%. *p < 0.05 vs. control group*

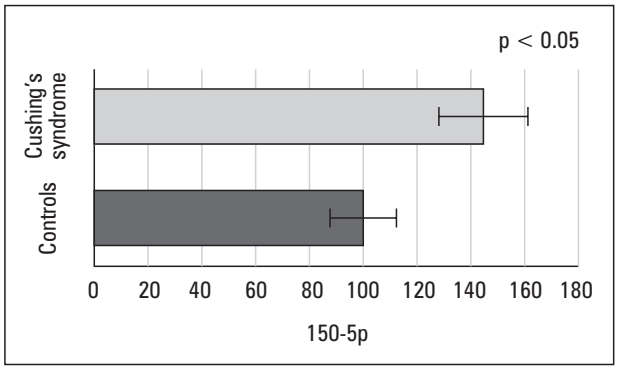

Figure 5. *Cellular miRNA-150-5p profile in Cushing's syndrome (CS) patients and controls. MiRNA-150-5p was significantly upregulated in peripheral blood nuclear cells (PBNCs) collected from patients with CS compared to healthy volunteers. The results are expressed as the percentage of the control value taken as 100%. *p < 0.05 vs. control group*

but with conflicting results. Some studies have shown that hypercortisolaemia is associated with increased cardiovascular morbidity and mortality, whereas other researchers cannot confirm this observation. Consistent with the previous report [8], we found significant over-expression of ET-1 activity in the PB of CS patients, which presumably indicates an overall tendency for the development of endothelial dysfunction in the cardiovascular system of subjects with CS. Patients with endogenous CS are typically exposed to high cortisol levels for longer period of time than the usual duration of laboratory experiments and interventional studies in humans, which suggests that CS could be an appropriate disease model in vivo that permits analysis of the influence of an excessive amount of GCs on the cardiovascular system and may potentially link it with GC-induced cardio-metabolic complications; thus, they could be adequately investigated. Moreover, it was reported that there were no major differences between endogenous hypercortisolism in Cushing's syndrome or diet-induced cardiometabolic disorders in terms of genetic cardiometabolic risk factors and regarding the pathogenesis of their complications [25].

We found that important "angioMIRs", both miRNA-126-3p and miRNA-126-5p, were significantly downregulated in PBNC circulating in CS patients, which, according to the literature, may indicate defective angiogenesis and reduced neovascularisation as miRNA-126 stimulates angiogenesis via VEGF signalling [26]. In animal models, areas of disturbed blood flow typically present the reduced miRNA-126 expression associated with diminished endothelial cell proliferation [15]. Apart from endothelial cells, the miRNA-126 cluster plays additional roles in distinct cell types such as macrophages or endothelial progenitor cells circulating also in blood. In the latter, proangiogenic activity is associated with increased expression levels of miRNA-126, and inhibition of this expression resulted in the restriction of their proangiogenic activity [27]. Moreover, it was reported that CD34+ cells, after high glucose treatment or isolated from DM2 patients, expressed lower miRNA-126 and impaired angiogenic properties that were recovered with miRNA-126 treatment [27]. Besides, CD34+ cells from patients with cardiac insufficiency showed significantly lower expression of miRNA-126 [28]. Finally, the reduced miRNA-126 levels in monocytes from DM2 patients were associated with increased tissue factor expression and higher pro-thrombotic activity [29]. It was also found that intracellular miRNA-126 expressed in circulating platelets has also the potential to influence endothelial cells, stimulating their proliferation and inhibiting apoptosis

via AKT signalling pathway [30]. Consequently, the intracellular downregulation of both miRNA-126-5p and miRNA-126-5p in circulating PBNCs from CS patients observed in our study are likely to contribute to the dysregulation of angiogenesis in this disease.

Consistent with previous findings in individuals with coronary artery disease and in patients with myocardial infarct [21], we determined that levels of miRNA-17 were significantly reduced and levels of miRNA-150 were upregulated in circulating PBNCs from CS patients compared to healthy subjects. The MiRNA-17 cluster controls the expression of VCAM-1, ICAM-1, and E-selectin, being involved in the coordinated manner of regulation of the inflammatory response by the complex miRNA network in endothelial cells that maintain intercellular communication with monocytic cells in the course of atherosclerosis [31]. Likewise, monocytes secrete miRNA-150, and its levels in microvesicles targeted towards endothelium increase in response to inflammatory stimuli present in atherosclerotic arteries [32]. The latter miRNA also showed the greatest discriminatory power for the diagnosis of unstable angina compared to patients with non-coronary chest pain or healthy subjects [20].

MiRNA-223 levels were also increased in circulating PBNCs from CS patients in our study. This finding is in accordance with previous studies demonstrating the significant role of intracellular miRNA-223 that can be transferred from platelets to endothelial cells. It was shown that platelet miRNA-223 has the ability to target several genes, including B1-integrin, IGF-IR, and Fbxw7, which can regulate migration and angiogenesis [30]. Furthermore, miRNA-223 was shown to have a suppressive effect on macrophage proinflammatory activation, and miRNA-223-deficient macrophages exhibited increases in M1 and decreases in M2 polarisation biomarkers [33]. Our results are in line with another study by Zampetaki et al., who showed that miRNA-223, together with miRNA-126, has a high predictive value for risk of developing acute myocardial infarct, as a result of a 10-year follow-up prospective trial [34]. Consequently, our results in GC patients could be interpreted such that the increased level of miRNA-223 might represent attempts to compensate for the above-mentioned negative effects of GC excess on endothelium-driven vascular homeostasis in a long-term exposure period in CS patients.

Conclusions

This is the first study documenting the intracellular expression of "angioMIRs" in CS patients. There were significant differences in selected miRNA expression levels in nucleated cells circulating in the peripheral blood of CS patients compared to healthy subjects. This study enriches our understanding of differential expression of miRNA related to the control of the endothelium functions in the cardiovascular system of CS patients with typical cardio-metabolic complications, and may guide towards future therapeutic interventions. Nevertheless, it requires further investigation.

Acknowledgement

This work was supported by Polish NCN grant: UMO-2011/01/B/NZ5/04224.

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