ENDOTHELIUM-ENRICHED MICRORNAS AS DIAGNOSTIC BIOMARKERS FOR CARDIAC ALLOGRAFT VASCULOPATHY

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ABSTRACT

Background: Cardiac allograft vasculopathy (CAV) is a limiting factor for the long-term survival of heart transplant recipients. Clinical decisions and care may be improved by the development of prediction models based on circulating biomarkers. The endothelium may play a central pathogenetic role in the development of CAV. We evaluated the hypothesis that endothelium-enriched microRNAs (miRNAs) discriminate between patients with CAV and patients without CAV.

Methods: Fifty-two patients undergoing coronary angiography between 5 and 15 years after heart transplantation were recruited in this cross-sectional study. Circulating levels of endothelium-enriched miRNAs (miR-21-5p, miR-92a-3p, miR-92a-1-5p, miR-126-3p, miR-126-5p) were quantified by real-time RT-PCR. The discriminative ability of logistic regression models was evaluated using the concordance statistic (c-statistic).

Results: Median plasma levels of miR-21-5p, miR-92a-3p, miR-126-3p, and miR-126-5p were 1.82-fold (p=NS), 1.87-fold ($p<0.05$), 1.94-fold (p=0.074), and 1.59-fold (p=0.060) higher, in patients with CAV than in patients without CAV. Recipient age (c-statistic 0.689 (95% CI 0.537-0.842)), serum creatinine (c-statistic 0.703 (95% CI 0.552-0.854)), levels of miR-92a-3p (c-statistic 0.682 (95% CI 0.533-0.831)), and levels of miR-126-5p (c-statistic 0.655 (95% CI 0.502-0.807)) predicted CAV-status in univariable models. In multivariable logistic regression models with recipient age and creatinine as covariates, miR-126-5p (χ^2=4.37; df=1; p=0.037), miR-92a-3p (χ^2=6.01; df=1; p=0.014), and the combination of miR-126-5p and miR-92a-3p (χ^2=8.16; df=2; p=0.017) added significant information. The model with age, creatinine, miR-126-5p, and miR-92a-3p as covariables conferred good discrimination between patients without CAV and patients with CAV (c-statistic 0.800 (95% CI 0.674-0.926)).

Conclusion: Endothelium-enriched miRNAs have diagnostic ability for CAV beyond clinical predictors.

INTRODUCTION

Cardiac allograft vasculopathy (CAV) is a limiting factor for the long-term survival of heart transplant recipients[1, 2]. CAV is characterized by the development of diffuse concentric fibromuscular intimal hyperplasia lesions in epicardial and smaller intramyocardial arteries along with focal, eccentric atherosclerotic plaques in the larger epicardial arteries[3, 4]. The development of these lesions may lead to the progressive narrowing of the lumen[5]. According to the response to injury hypothesis of CAV, these lesions are the result of cumulative endothelial injury induced by alloimmune responses as well as non-immunological risk factors such as ischemia-reperfusion injury, viral infections, and metabolic disorders[3, 6].

Early diagnosis of CAV is essential to implement appropriate prevention and treatment measures. Metabolic parameters like triglycerides to HDL cholesterol ratio[7, 8] and plasma insulin level[9] may discriminate between CAV-positive and CAV-negative patients. Immunological and inflammatory biomarkers of CAV include donor-specific anti-HLA antibodies[10], antibodies against heterogeneous nuclear ribonucleoprotein K[11], C-reactive protein (CRP)[8, 12-14], vascular cell adhesion molecule-1[15], and circulating C-X-C motif chemokine 12 (CXCL12) levels[16]. However, the discriminative ability and the incremental value of these biomarkers beyond clinical risk factors have not been robustly established. Candidate-based approaches using biomarkers of endothelial homeostasis may constitute a solid foundation for the development of prediction models of CAV. The angiogenesis-related proteins vascular endothelial growth factor (VEGF)-C, VEGF-A and platelet factor-4 have been identified as independent biomarkers of CAV[17]. In a recent cross-sectional study[18], we demonstrated that a logistic regression model containing apoptotic circulating endothelial cells (CECs) and apoptotic circulating endothelial microparticles (CEMPs) as independent predictors provided high discrimination between CAV-positive and CAV-negative patients (c-statistic 0.812; 95% CI 0.692-0.932). In several logistic regression models including clinical and biochemical covariates, the introduction of apoptotic CECs and apoptotic CEMPs consistently resulted in added value, indicating that these biomarkers are robust independent predictors.

In line with previous studies demonstrating the ability of biomarkers related to endothelial homeostasis for non-invasive diagnosis of CAV, the aim of this study was to analyze the potential of endothelium-enriched microRNAs (miRNAs) as putative biomarkers for prevalent CAV. MiRNAs are small, non-coding, single-stranded RNA sequences that regulate gene expression at the post-transcriptional level. Because miRNAs circulate in remarkably stable forms in blood[19, 20], they have a significant potential as biomarkers. Several reports indicate that miRNAs may play a role in endothelial homeostasis[21, 22]. In the current cross-sectional study, a candidate-based approach using circulating levels of endothelium-enriched miRNAs (miR-21-5p, miR-92a-3p, miR-92a-1-5p, miR-126-3p, miR-126-5p) for non-invasive diagnosis of CAV was investigated.

METHODS

Study design

Fifty-two clinically stable patients undergoing coronary angiography between 5 and 15 years after heart transplantation and eighty patients with clinically stable native coronary artery disease (CAD) were included in this cross-sectional study. Stable native CAD patients were defined by the presence of at least one stenosis of 50% or more demonstrated by diagnostic coronary angiography. CAV was graded according to the International Society for Heart and Lung Transplantation working formulation of a standardized nomenclature for CAV-2010[23]. Patients with CAV_1, CAV_2, and CAV_3 were pooled and constituted the CAV-positive group. The CAV-negative group comprised patients with CAV_0, defined as no detectable angiographic lesion. The clinical characteristics of CAV-negative and CAV-positive patients have been described in a previous report[18]. Heart transplant patients with prior congenital heart disease and re-transplanted patients were excluded. The study was approved by the Ethics Committee of the University Hospital Gasthuisberg and written informed consent was obtained from all participating subjects. The reference control group included 25 healthy control subjects (12 males and 13 females) with an average age of 43.2 ± 2.0 years[13].

Quantification of circulating levels of endothelium-enriched miRNAs (miR-21, miR-92a, miR-126) in plasma samples

This study was not preceded by a screening phase evaluating a large pool of miRNAs for association with CAV-status. The endothelium-enriched miRNAs (miR-21, miR-92a, miR-126) investigated in this study were *a priori* selected based on an analysis of the literature and no other miRNAs were quantified. Peripheral blood was drawn by venipuncture using Vacutainer® collection tubes (BD Diagnostics, Franklin Lakes, NJ, USA). Plasma derived from EDTA anticoagulated peripheral blood was centrifuged within one hour after collection at 1900 g for 10 min followed by a second centrifugation at 1900 g for 20 min to generate platelet-poor plasma (PPP) that was used for miRNA quantification by real-time PCR. RNA was isolated from 400µl plasma using the Ambion mirVANA RNA extraction kit (AM1560,

Applied Biosciences, Austin, Texas, USA). Subsequently, 300 nanogram of RNA was reverse-transcribed to cDNA using the miScript-II RT PCR kit (218061, Qiagen Benelux NV, Venlo, The Netherlands). Real-time PCR was performed on an ABI-Prism cycler (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) using LNA^{TM}-based miRNA primers (Exiqon A/S, Vedbaek, Denmark) and SYBR Green (Life Technologies, Carlsbad, CA, USA). U6 non-coding small nuclear RNA (snRNA) expression was measured as an endogenous control for data normalization. U6 primers were designed by Eurogentec (Eurogentec, Seraing, Belgium). MiRNA expression levels were compared using the relative threshold cycle (Ct) method ($2^{-\Delta\Delta Ct}$).

Quantification of apoptotic CECs and of apoptotic CEMPS by flow cytometry

Apoptotic CECs and apoptotic CEMPs were defined as Annexin V^+ $CD45^-$ $CD31^{bright}$ VEGFR-2^+ mononuclear cells and Annexin V^+ $CD144^+$ $CD42a^-$ microparticles. Details of the gating strategy and of the flow cytometry analysis have been described before[18].

Statistical analysis

Clinical and biochemical parameters and endothelial biomarkers were compared using Instat 3 (Graphpad software, San Diego, CA, USA). Continuous variables were summarized by means, standard error of the mean, and sample size, and were compared by Student t-test between CAV-negative and CAV-positive patients and between CAV-positive patients and stable native CAD patients. When data were not normally distributed, data are presented as medians and interquartile range (IQR), and were compared by a Mann-Whitney Test. Logistic regression analysis was performed by SAS software, version 9.2 (SAS Institute Inc., Cary, NC, USA). Since the distribution of the concentration of CECs and of CRP is heavily right-skewed, a natural logarithm transformation of the data of these two parameters was applied for logistic regression analysis. The discriminative ability was quantified using the concordance statistic (c-statistic), which is equal to the area under the receiver operating characteristic curve. Evaluation of added value of predictors in multivariable logistic

regression models was based on likelihood-ratio tests comparing two nested models. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Characteristics of heart transplant recipients without and with CAV and of patients with stable native CAD

The clinical characteristics of heart transplant recipients without CAV (n=22) and with CAV (n=30), and of patients with stable native CAD (n=80) are shown in Supplemental Table 1. Patients with CAV were 8.6 years ($p<0.05$) older than patients without CAV and 4.7 years ($p<0.05$) younger than patients with stable native CAD at the time of inclusion in the study. Patients with stable native CAD had a lower prevalence of hypertension ($p<0.0001$) and a higher body mass index ($p<0.05$) compared to patients with CAV. No significant difference of these two parameters was observed between patients without CAV and patients with CAV. Lipoprotein levels were similar among the three patient groups except LDL cholesterol, which was 15.9% ($p<0.05$) lower in patients with CAV than in patients without CAV. Creatinine levels were significantly higher in patients with CAV than in patients without CAV ($p<0.05$) as well as in patients with stable native CAD ($p<0.0001$).

Circulating endothelium-enriched miRNA levels are higher in patients with CAV compared to patients without CAV

All miRNA levels (Figure 1, Figure 2) were normalized against U6 snRNA level. Levels of miR-21-3p were consistently below detection limit (data not shown). As shown in Figure 1A, median plasma miR-21-5p level was 2.34-fold ($p<0.05$) increased in patients with CAV compared to healthy controls whereas no increase was observed in CAV-negative patients. Median plasma miR-21-5p level was 1.82-fold (p=NS) higher in CAV-positive patients compared to CAV-negative patients (Figure 1A). Median plasma miR-92a-3p in patients with CAV was 3.08-fold ($p<0.01$) and 1.87-fold ($p<0.05$) higher, respectively, compared to healthy controls and patients without CAV (Figure 1B). As shown in Figure 1C, median plasma miR-92a-1-5p was 2.11-fold increased ($p=0.075$) in patients with CAV compared to healthy controls but no significant difference was observed between CAV-negative and CAV-

positive patients. Median plasma miR-126-3p in CAV-positive patients was 2.80-fold ($p<0.01$) and 1.94-fold ($p=0.074$) higher, respectively, compared to healthy controls and CAV-negative patients. Finally, median plasma miR-126-5p in patients with CAV was 2.02-fold ($p<0.05$) and 1.59-fold ($p=0.060$) increased, respectively, compared to healthy controls and patients without CAV. Taken together, these results demonstrate that levels of several circulating endothelium-enriched miRNAs are increased in patients with CAV compared to patients without CAV.

Circulating levels of miR-92a-3p and miR-92a-1-5p differ in patients with CAV and in patients with stable native CAD

Median plasma levels of miR-21-5p, miR-92a-3p, miR-92a-1-5p, miR-126-3p, and miR-126-5p were 2.38-fold ($p<0.01$), 1.98-fold ($p<0.01$), 3.16-fold ($p<0.0001$), 3.10-fold ($p<0.0001$), and 1.90-fold ($p<0.0001$) higher, respectively, in patients with stable native CAD (Figure 2) than in healthy controls (Figure 1). Whereas all of these 5 endothelium-enriched miRNAs were elevated in both CAV and native CAD, two distinctions in miRNA levels were observed between these two types of arteriosclerosis. Median plasma level of miR-92a-3p was elevated 1.56-fold ($p=0.051$) in CAV-positive patients compared to patients with stable native CAD (Figure 2B). In contrast, median plasma level of miR-92a-1-5p was 1.50-fold ($p=0.089$) higher in patients with native CAD compared to patients with CAV (Figure 2C).

Strong correlation between plasma levels of four miRNAs in heart transplant recipients

Table 1 shows the Spearman's rank correlation matrix of endothelium-enriched miRNAs and apoptotic CECs and apoptotic CEMPs. With the exception of miR-92a-3p, endothelium-enriched miRNAs were strongly correlated (Spearman's rank correlation coefficient higher than 0.8). Plasma levels of miR-21-5p, miR-92a-1-5p, miR-126-3p, and miR-126-5p were weakly correlated with apoptotic CECs whereas the level of miR-92a-3p was weakly correlated with apoptotic CEMPs. Plasma miRNA levels were not related to clinical parameters (data not shown).

Logistic regression models for discrimination between CAV-positive and CAV-negative transplant recipients

Table 2 summarizes the odds ratio per standard deviation increase and the c-statistic values of univariable logistic regression models for clinical and biochemical parameters and endothelial biomarkers. Plasma levels of miR-126-5p and of miR-92a-3p predicted CAV-status significantly better than chance (Table 3). Since the levels of these two miRNAs were only weakly correlated, miR-126-5p and miR-92a-3p were further analyzed in multivariable logistic regression models. Discrimination between CAV-negative and CAV-positive transplant recipients based on multivariable logistic regression models is shown in Table 3. Data in relation to the previously published model with age, creatinine, apoptotic CECs, and apoptotic CEMPs as predictors[18] are shown as reference values. The model with age, creatinine, miR-126-5p, and miR-92a-3p as covariables conferred good discrimination between patients without CAV and patients with CAV (c-statistic 0.800 (95% CI 0.674-0.926)). In a logistic regression model with recipient age and creatinine as covariates, miR-126-5p (χ^2=4.37; df=1; p=0.037), miR-92a-3p (χ^2=6.01; df=1; p=0.014), and the combination of miR-126-5p and miR-92a-3p (χ^2=8.162; df=2; p=0.017) added significant information. In addition, miR-92a-3p (χ^2=5.45; df=1; p=0.0195) and not miR-126-5p (chi-square=2.04; df=1; p=0.15) added value in a model with apoptotic CECs and apoptotic CEMPs as predictors. The receiver operating characteristic curve for the logistic regression model with apoptotic CECs, apoptotic CEMPs, and miR-92a-3p as predictors is shown in Figure 3.

DISCUSSION

The salient findings of the present study are that 1) plasma levels of miR-126-5p and of miR-92a-3p predict prevalent CAV in univariable models; and 2) these endothelium-enriched miRNAs have diagnostic ability for CAV beyond clinical predictors or other endothelial biomarkers.

Biomarkers that capture key processes in the pathogenesis of CAV, e.g. biomarkers related to endothelial homeostasis[18], may be the cornerstone for adequate prediction models. The five miRNAs analysed in this study (miR-21-5p, miR-92a-3p, miR-92a-1-5p, miR-126-3p, and miR-126-5p) are endothelium-enriched miRNAs and have been shown to play a role in endothelial homeostasis[21, 24]. MiR-21 negatively modulates angiogenesis by targeting RhoB expression[25]. The precursor miRNA miR-21 gives rise to two mature miRNAs: miR-21-3p and miR-21-5p. MiR-21-5p is an important regulator of neointimal hyperplasia development after balloon injury[26, 27]. MiR-92a represses angiogenesis[28, 29] and promotes endothelial activation[30]. MiR-92a-3p and miR-92a-1-5p are two distinct mature miRs produced from the same precursor miRNA pre-miR-92a-1. Finally, miR-126 is one of the most abundant miRNAs in endothelial cells and is involved in the regulation of vascular integrity and angiogenesis[31]. MiR-126-3p and miR-126-5p are two distinct mature miRNAs arising from the same precursor pre-miR-126. MiR-126-3p has been shown to confer anti-inflammatory effects by inhibiting expression of vascular cell adhesion molecule 1 and sprouty-related protein 1[32, 33]. MiR-126-5p enhances endothelial proliferation via inhibition of the Notch1 inhibitor delta-like 1 homolog (Dlk1)[34]. Thus, the two miR-126 strands play a role in endothelial repair mechanisms in response to continuous endothelial inflammation and apoptosis resulting in endothelial damage. Taken together, the specific functions of the different miRNAs investigated in the current study may be biologically relevant for the development of CAV. However, the focus of the current study is the development of diagnostic models for prevalent disease. Therefore, a potential causal role of any of these miRNAs is not under consideration in this report. Interestingly, all investigated miRNAs were

increased both in heart transplants recipients with CAV and in patients with native CAD compared to healthy controls. Nevertheless, whereas plasma levels of miR-92a-3p were elevated in CAV-positive patients compared to patients with stable native CAD, the opposite pattern was observed for miR-92a-1-5p. This distinction in endothelial biology between these two types of arteriosclerosis is also reflected by our previous report demonstrating that markers of endothelial injury are distinct in patients with stable native CAD and with CAV[35]. Whereas miRNAs have been investigated as non-invasive biomarkers for heart transplant rejection[36, 37], this is the first report to demonstrate the discriminative ability of miRNAs in clinical prediction models of prevalent CAV. A strong correlation of plasma levels of 4 of the 5 investigated endothelium-enriched miRNAs was observed in heart transplant recipients. Therefore, consideration of all miRNAs for multivariable modeling was not meaningful and could have led to multicollinearity problems. Moreover, given the number of patients included in the current study, the number of predictors in multivariable models had to be limited. The model with age, creatinine, miR-126-5p, and miR-92a-3p as covariables conferred good discrimination between patients without CAV and patients with CAV and both endothelium-enriched miRNAs had diagnostic ability for CAV beyond clinical predictors. However, this model does not provide better discrimination compared to the previously published model with age, creatinine, apoptotic CECs, and apoptotic CEMPs as predictors[18]. Nevertheless, plasma level of miR-92a-3p added value in a model with apoptotic CECs and apoptotic CEMPs as predictors, which may provide a foundation for a model with improved discrimination. Since the number of subjects in the current study was limited to 22 CAV-negative patients and 30 CAV-positive patients, we cannot test whether plasma level of miR-92a-3p adds information beyond the previously established model with age, creatinine, apoptotic CECs, and apoptotic CEMPs as predictors. Inclusion of too many predictors leads to overfitting of the data and C-indices are overestimated[38]. Specifically, models with more than 3 predictors should be interpreted with extreme caution considering the sample size. A larger study is required to analyse the discriminative ability of a model including clinical predictors, apoptotic CECs, and apoptotic CEMPs, and miR-92a-3p.

There is a biological rational why an endothelium-enriched miRNA has diagnostic ability for CAV beyond clinical predictors or beyond apoptotic CEMPs and apoptotic CECs. Although miRNAs can be released by apoptosis or necrosis, miRNAs can also enter the circulation in exosomes. Exosomes are built by inward budding of the limiting cell membrane of the multivesicular body, a late endosomal compartment[39]. The fusion of the multivesicular body with the plasma membrane leads to the active secretion of exosomes into the blood circulation. Since this active secretion process is fundamentally distinct from apoptosis or necrosis, it is not surprising that no strong correlation is observed between different miRNAs and apoptotic CECs and apoptotic CEMPs. This lack of a strong correlation is a necessary condition to contribute additional information to the prediction of CAV.

Plasma levels of miR-126-5p and miR-92a-3p added value in models with age and creatinine as predictors. The observation that miR-92a-3p has diagnostic ability beyond apoptotic CEMPs and apoptotic CECs may lead to the development of diagnostic models with further improved performance. This hypothesis should be evaluated in the framework of a validation study, which may lead to a clinically applicable tool for improved CAV surveillance. Finally, prospective studies are required to evaluate the potential of endothelial biomarkers in prognostic models predicting incident CAV.

In conclusion, the current study enforces the paradigm that endothelial biomarkers constitute a solid foundation for diagnostic models of CAV.

FINANCIAL CONFLICT OF INTEREST DISCLOSURE

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LEGENDS TO THE FIGURES

Figure 1. Individual value bar graph illustrating a comparison of plasma level of miR-21-5p (panel A), miR-92a-3p (panel B), miR-92a-1-5p (panel C), miR-126-3p (panel D), and miR-126-5p (panel E) in healthy controls (n=25), patients without CAV (n=22), and patients with CAV (n=30). All miRNA levels were normalized against U6 snRNAlevel. Data points show the individual values. Medians are shown by the horizontal lines.

Figure 2. Individual value bar graph showing a comparison of plasma level of miR-21-5p (panel A), miR-92a-3p (panel B), miR-92a-1-5p (panel C), miR-126-3p (panel D) and miR-126-5p (panel E) in patients with CAV (n=30) versus patients with stable native CAD (n=80). All miRNA levels were normalized against U6 snRNAlevel. Data points show the individual values. Medians are shown by the horizontal lines.

Figure 3. Receiver-operating characteristic curve for the logistic regression model with apoptotic CECs, apoptotic CEMPs, and miR-92a-3p as predictors. The area under this curve is equal to the c-statistic.