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Screening of circulating microRNA biomarkers for prevalence of abdominal aortic aneurysm and aneurysm growth

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ABSTRACT

Background and aims: MicroRNA (miR) are important regulators of gene expression and biological processes and have recently been suggested as possible biomarkers for abdominal aortic aneurysm (AAA) disease. The aim of the present study was to assess the role of miR as biomarkers for initiation and progression of AAA disease, through evaluation of a wide range of miRs in a large population-based cohort, with AAA patients with linked clinical data regarding risk factors, AAA size and growth, as well as controls.

Methods: The expression of the 172 most commonly expressed miRs in plasma was analyzed by real-time PCR in samples from 169 screening-detected AAA patients and 48 age-matched controls.

Results: For 103 miRs, there was a significant difference in expression between AAA and controls. Of these, 20 miRs were differently expressed between fast and slow growing aneurysms. These miRs target genes known to be involved in AAA disease as well as novel genes and pathways. By combining the top altered miRs together with clinical variables, strong predictive values, determining growth of AAA, were obtained (area under curve = 0.86, $p < 0.001$).

Conclusions: This large cohort study identified several novel miRs with altered expression in AAA patients when compared to controls. Assessment of miR expression may offer an opportunity to predict disease progression and aneurysm growth.

1. Introduction

Abdominal aortic aneurysm (AAA) is a common and potentially lethal disease. The natural course of an AAA is to gradually expansion and eventually rupture, with significant individual variations [1]. Standard of care for patients with small AAAs (<55 mm) is limited to surveillance. Surgical repair is considered for large (>55 mm), rapidly growing (>10 mm/year) or symptomatic AAAs [2]. Since most AAAs are asymptomatic, ultrasound-based screening programs targeting risk groups (commonly 65-year old men) have been established in some countries [3].

Although the pathophysiological pathways preceding AAA have been extensively studied in animal models, there is still a distinct lack of knowledge regarding what initiates aneurysm formation, and factors affecting disease progression. Far from all, patients with screening-detected small AAA will eventually develop a clinically significant aneurysm that requires repair, or ruptures. Two major limitations in current practice, therefore, include the lack of possibilities to stratify patients to high- or low-risk for disease progression, and lack of specific pharmacological treatment to reduce expansion or rupture risk of an AAA. To be able to find new therapeutic preventive strategies, the molecular mechanisms behind the disease and drug targets need to be elucidated in more detail.

microRNAs (miRs) are 18–22 nucleotide short non-coding RNAs secreted by cells, they can regulate the expression of target genes by interfering with transcription or inhibiting translation, and they

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are stable in plasma for prolonged periods. Recently, a number of investigators reported circulating miR associated with AAA presence, such as miR-155, miR-191-3p, miR-455-3p, miR-1281 and miR-411 [4–7]. Most of these studies are, however, underpowered in their discovery phase and few have investigated the association with AAA progression.

In a recent publication, Maegdefessel and colleagues identified miR-24 in human AAA tissue, and, in functional studies, they also showed that modulation of miR-24 alters AAA progression in animal models by regulating vascular inflammation [8].

The aim of the present study was to evaluate the associations between a wide range of miRs and the presence of AAA, as well as associations with AAA growth, in a large population-based cohort.

2. Materials and methods

2.1. Patient cohort

Since 2008, all patients with an AAA and age- and gender-matched healthy controls in Uppsala are asked to donate blood to explore different pathophysiological biomarkers for expansion. In the present study, blood samples from patients with fast and slow growing AAAs and from subjects with a normal (i.e. non-aneurysmal) aorta were analyzed. The study was approved by the Research Ethics Review Board (EPN) of Uppsala/Örebro region. All subjects gave informed consent prior to the investigation.

The following inclusion criteria were used for AAAs; 1) $\geq$30 mm, 2) follow-up $\geq$6 months, and 3) $\leq$5 mm shrinkage during follow-up. Among 192 AAA patients fulfilling the inclusion criteria, 85 patients with the slowest AAA growth and 85 with the fastest growth, matched for baseline AAA diameter, were selected. In addition, 50 subjects with a normal abdominal aorta at screening were selected as controls. Blood samples from one AAA patient and two controls were not possible to analyze. Thus, the study population consisted of 85 patients with slow AAA growth, 84 with fast growth, and 48 with normal aortas.

The sample size is calculated to observe relative differences from 40% with a power >0.9, $\alpha = 0.05$, when comparing controls with AAA and a power >0.8 $\alpha = 0.05$, when comparing slow and fast growing AAAs.

All participants were asked to complete a standardized health questionnaire on smoking habits and medical history. Coronary artery disease (CAD) was defined as a history of angina pectoris or myocardial infarction, cerebrovascular disease (CVD) as a history of stroke or TIA, hypertension as a history of hypertension or current antihypertensive medication, diabetes mellitus as a history of dietary- or medically-treated diabetes, and renal insufficiency as a history of a clinically relevant renal impairment. A history of smoking was defined as individuals that had been smoking during some periods of their lives.

2.2. Sample preparation

Plasma was thawed on ice and centrifuged at 3000*g* for 5 min in a 4 °C microcentrifuge. Total RNA was extracted from plasma using the miRCURY™ RNA isolation kit – biofluids (Exiqon, Vedbaek, Denmark). Plasma was mixed with Lysis Solution BF containing 1 μg carrier-RNA per 60 μl Lysis Solution BF and RNA spike-in template mixture was added to the sample. The tube was vortexed and incubated for 3 min at room temperature, followed by the addition of 20 μL Protein Precipitation solution BF. The tube was vortexed, incubated for 1 min at room temperature and centrifuged at 11,000*g* for 3 min. The clear supernatant was transferred to a new collection tube and 270 μL isopropanol was added. The solutions were vortexed and transfer to a binding column. The column was incubated for 2 min at room temperature, and emptied using a vacuum-manifold. After washing, the dry columns were transferred to a new collection tube and 50 μL RNase free H_2O was added directly on the membrane of the spin column. The column was incubated for 1 min at room temperature prior to centrifugation at 11,000*g*. The RNA was stored in a −80 °C freezer.

Three different RNA spike-ins (UniSp2, UniSp4 and UniSp5) premixed, each at different concentration in 100-fold increments were added. For the reverse transcription step, one spike-in (UniSp6) was added. Controls (negative and RNA spike-in) indicated good technical performance of the profiling experiment. Each RNA sample was successfully polyadenylated and reverse transcribed into cDNA. Amplification was performed in a Roche Lightcycler 480. Reactions with amplification efficiency below 1.6 were removed. Reactions giving crossing point (Cp) values that are within 5 Cp values of the negative control reaction were removed.

All data were normalized to correct for potential overall differences between samples. For normalization of data, the average of the assays detected in all samples (n = 217 samples), global mean, was found to be the most stable normalizer, using NormFinder. The control assays were evaluated.

2.3. Real-time qPCR analysis of miR

Seven μl RNA was reverse transcribed in 35 μl reactions using the miRCURY LNA™ Universal RT microRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon). cDNA was diluted 50× and assayed in 10 μl PCR reactions, according to the protocol for miRCURY LNA™ Universal RT microRNA PCR; each miR was assayed once by qPCR on the microRNA Ready-to-Use PCR, Human serum/plasma panel using ExiLENT SYBR® Green master mix. The plasma panel includes 172 miRs validated as expressed in plasma, based on our previous experience, screening 752 different miRs in plasma as well as the validation performed by Exiqon. Negative controls, excluding template from the reverse transcription reaction, were performed and profiled like the samples. The amplification was performed in a LightCycler® 480 Real-Time PCR System (Roche) in 384-well plates. The amplification curves were analyzed using the Roche LC software, both for determination of quantitation cycle (Cq) (by the 2nd derivative method) and for melting curve analysis. miR-451 and miR-23a-3p were analyzed to monitor hemolysis.

2.4. Data analysis

The amplification efficiency was calculated using algorithms similar to the LinReg software. All assays were inspected for distinct melting curves and the Tm was checked to be within known specifications for the assay. Furthermore, assays must be detected with 5 Cqs less than the negative control, and with Cq < 37 to be included in the data analysis. Data that did not pass these criteria were omitted from any further analysis. Cq was calculated as the 2nd derivative.

2.5. Statistical analysis

All measurements are shown as average normalized Cq values for each group, and fold change (FC) with standard deviations (SD). Two-group comparisons were performed using Student t-test and false discovery rate (FDR) adjusted using the Benjamini-Hochberg (BH) adjusted *p*-value, correcting for multiple testing. Adjustments of covariates were performed using binary logistic regression analysis. Receiver Operating Characteristics (ROC) was performed using binary analysis of factors, to evaluate the

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